

# PKC $\delta$ promotes fertilization of mouse embryos in early development via the Cdc25B signaling pathway

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**Abstract.** Protein kinase C type  $\delta$  (PKC $\delta$ ) is involved in B-cell signaling and the regulation of growth, apoptosis and differentiation of a variety of cell types. Cell division cycle 25 (Cdc25) is a key mediator of cell cycle progression that activates cyclin-dependent kinase complexes that drive the cell cycle and participates in the regulation of DNA damage checkpoints. Cdc25B is a member of the Cdc25 family of phosphatases. The present study investigated the role and mechanism of PKC $\delta$  in regulating the fertilization of mouse embryos in early development. The expression and subcellular localization of PKC $\delta$  and Cdc25B were detected using reverse transcription-quantitative polymerase chain reaction, western blotting and immunofluorescence in one-cell stage mouse embryos. Specific small interfering RNAs targeting PKC $\delta$  were used to knockdown the expression of PKC $\delta$ . Subsequently, Scansite software was used to predict the target of phosphorylated Cdc25B. Western blotting was used to measure the effects of phosphorylation and dephosphorylation in one-cell stage mouse embryos at different cell cycle phases. PKC $\delta$  was expressed during M phase and served a positive role in one-cell stage mouse embryos. Immunofluorescence data revealed that PKC $\delta$  and Cdc25B were expressed during G<sub>1</sub>, S, G<sub>2</sub> and M phases of the cell cycle. Furthermore, phosphorylated levels of Cdc25B-Ser96 were observed during G<sub>2</sub> and M phases. Microinjection with mimics of phosphorylated Cdc25B-Ser96 mRNA promoted the development of one-cell stage mouse embryos. When PKC $\delta$  was suppressed, microinjection with mimics of phosphorylated Cdc25B-Ser96 mRNA reversed the inhibition of PKC $\delta$ . To conclude, PKC $\delta$  serves a positive role in the first cell cycle of mouse embryos by

phosphorylating Cdc25B-Ser96, and provides novel insights for the regulation of early embryonic development.

## Introduction

Protein kinase C (PKC) is a major intracellular kinase implicated in transducing extracellular signals into intracellular events (1). The PKC family has numerous isozymes, including a number of PKC isotypes that are expressed in mouse oocytes. PKC $\delta$  and  $\lambda$  have been detected at mRNA and protein levels in prophase I and MII stage oocytes, whereas PKC $\alpha$ ,  $\beta$  and  $\zeta$  have been detected only at protein level (2). Downs *et al* (3) reported that PKC  $\gamma$ ,  $\lambda$ ,  $\mu$  and  $\zeta$  are expressed at mRNA and protein levels in early embryos, whereas PKC  $\alpha$  and  $\delta$  are expressed only at protein level. Oocyte maturation involves the activation of various signal transduction pathways. PKC regulates meiosis I and regulates the progression of meiosis I in LTXBO oocytes (4). Therefore, PKC serves an integral role in directing the transformation from egg to embryo, participating in genome activation in mouse one-cell stage fertilized embryos. The activity of PKC has also been demonstrated to increase at fertilization (5). In addition, the cell cycle resumes in embryos treated with PKC (6). PKC is thus involved in the progression of meiosis, oocyte maturation, fertilization and early embryo development. Evidence suggests that PKC is also a key regulator of critical cell-cycle transitions during mitosis, including G<sub>1</sub>/S and G<sub>2</sub>/M transitions (7-9). In different cell types, PKC may promote or inhibit the regulation of G<sub>1</sub>/S and G<sub>2</sub>/M transitions depending on the timing of PKC activation during the cell cycle and the specific PKC isoforms involved (10).

The mitosis promoting factor (MPF), consists of the cell division cycle (Cdc)2/cyclin B complex, and is crucial for G<sub>2</sub>/M transition of the cell cycle (11). In addition, it functions as the key molecule in regulating cell cycle progression during mitosis and meiosis (12). Prior to mitosis, the activity of MPF is suppressed by the inhibitory phosphorylation of Tyr15 and Thr14 residues of Cdc2, the Cdc25 phosphatases and Myt1 kinase (13). The inactive pre-MPF phosphatase is acted on by the dual specificity of Cdc25 and can be modified to an active dephosphorylated form (13). Notably, Cdc25B has a central role in regulating the re-initiation of meiosis in mammalian oocytes (14). A previous study indicated that oocytes from mice lacking the Cdc25B gene were unable to

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activate MPF and therefore could not resume mitosis, which suggests the regulatory role of Cdc25B in G<sub>2</sub>/M transition of the mammalian cell cycle (15).

A series of experiments have provided evidence that PKC promotes the maturation in oocytes of *Chaetopterus pergamentaceus* by inducing the activity of MPF (16). Previous studies demonstrated that PKC participates in activating MPF and in developing fertilized mouse embryos (17,18). The entry into the first mitotic M phase at the end of the first embryonic cell cycle (one-cell stage mouse embryo) requires the activation of MPF (19). PKC $\delta$  has been observed in the cytoplasm of zygotes, and the activities of PKC and MPF are high during M phase (20). Furthermore, it has been implicated that the major contributor of PKC activity in mouse embryos derives from that of PKC $\delta$ , and MPF is a possible target substrate for PKC $\delta$  (21). Thus, it was hypothesized that PKC $\delta$  controls the cell cycle by regulating the activity of MPF. Therefore, in the present study, the role of PKC $\delta$  in the regulation of one-cell stage mouse embryos was explored.

## Materials and methods

**Animals and reagents.** A total of 32 female (age, 4 weeks; weight, 20–24 g) and 28 male (age, 8–9 weeks; weight, 30–40 g) Kunming genealogy-specific pathogen-free mice were obtained from the Department of Laboratory Animals, China Medical University (Shenyang, China). Mice were housed in environmentally controlled conditions (20±1°C, 60% relative humidity, with a 12-h light/dark cycle). All mice had access to food and water *ad libitum*. All experiments were performed at China Medical University in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Reagents, unless otherwise specified, were from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The current study was approved by the Ethics Committee of the China Medical University (Shenyang, China).

**Collection and culture of one-cell stage mouse embryos.** Female mice were injected with 10 IU of pregnant mare's serum gonadotropin (PMSG) and then with 10 IU of human chorionic gonadotropin (hCG) 48 h later. The following day, one-cell embryos were collected and placed in M2 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) from the oviducts of females with a vaginal plug at 20 h post-hCG injection. Following collection, embryos were maintained in M16 medium (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a 5% CO<sub>2</sub>-humidified incubator. Cell cycle stages (G<sub>1</sub>, S, G<sub>2</sub> and M phases) were defined as described previously (22).

**RNA extraction, cDNA synthesis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from one-cell mouse embryos at different cell-cycle stages (G<sub>1</sub>, S, G<sub>2</sub> and M phase) using the QuickPrep MicromRNA Purification kit (GE Healthcare Life Sciences, Little Chalfont, UK), according to the manufacturer's protocol. At each stage 100 embryos were collected and total RNA was reverse transcribed into cDNA using the RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher

Scientific, Inc., Waltham, MA, USA) using random primers, according to the manufacturer's protocol. A total of 2  $\mu$ l ( $\leq 100$  ng) cDNA was used in a 20- $\mu$ l qPCR using 2X SYBR Premix Ex TaqII (Takara Biotechnology Co., Ltd., Dalian, China) with 0.4  $\mu$ M of each primer. The following primers were used for qPCR: PKC $\delta$  forward, 5'-TCATCTGCGGAC TGCAGTTTCTA-3' and reverse, 5'-CAAAGTCAGCGATCT TGATGTGG-3'; and  $\beta$ -actin forward, 5'-CAACGAGCGGTT CCGATG-3' and reverse, 5'-GCCACAGGATTCCATACC CA-3'. The following thermocycling conditions were used for the qPCR: Initial denaturation at 95°C for 30 sec; 40 cycles of 95°C for 5 sec and 60°C for 20 sec using the Applied Biosystems 7900HT Fast Real-Time PCR System. PKC $\delta$  mRNA levels were quantified using the 2<sup>- $\Delta\Delta C_q$</sup>  method and normalized to the internal reference gene  $\beta$ -actin (23). All experiments were repeated at least three times.

**Treatment with rottlerin and observation of mouse embryos.** Rottlerin is a natural product derived from *Mallotus philippinesis* (24). This compound has been demonstrated to inhibit PKC $\delta$  with greater selectivity for PKC $\delta$  (IC<sub>50</sub>=3–6  $\mu$ M) over other PKC isoforms (25). Rottlerin was dissolved in dimethyl sulfoxide (DMSO) and diluted with M16 medium at different concentrations (0, 0.5, 1 and 1.5  $\mu$ M). Each Zona pellucida-free one-cell stage embryos (removed with Tyrode's buffer, pH 2.5) at G<sub>1</sub> phase were seeded into 24-well plates at a density of 25 embryos/well and incubated with various concentrations (0, 0.5, 1 and 1.5  $\mu$ M) of rottlerin. Fertilized embryos in the control group (0  $\mu$ M rottlerin) were incubated with the DMSO only in M16 medium. The percentage of egg cleavage was determined by counting the number of cleaved embryos under a dissecting microscope, and the activity of MPF was measured 28–35 h post-hCG injection. Each experiment was repeated at least three times and the results were statistically analyzed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). Images were captured using a phase-contrast microscope (magnification, x600; DP70; Olympus Corporation, Tokyo, Japan).

**RNA interference.** To examine the possible function of PKC $\delta$ , small interfering RNAs (siRNAs) specific to PKC $\delta$  were used to knockdown transcript levels in embryos. Control groups included: Non-injected embryos, and embryos injected with negative control (NC) siRNA. The fertilized embryos at G<sub>1</sub> phase were divided into three groups: Blank, NC and siRNA group, to which 20  $\mu$ M siRNAs (10 pl; GenPharma, Shanghai, China) were microinjected directly into the cytoplasm of fertilized embryos at G<sub>1</sub> phase (20 h post-hCG injection). Following microinjection, embryos were cultured in M16 medium for 48 h at 37°C in a 5% CO<sub>2</sub>-humidified incubator. Initial control experiments were undertaken to determine the specificity and efficacy of PKC $\delta$  siRNA to knockdown endogenous PKC $\delta$  expression. A total of 4 h post-microinjection, each group of embryos was processed for western blotting to assess overall PKC $\delta$  protein expression levels. Transfection efficiency was confirmed using western blot analysis. The following siRNA duplexes were used: PKC $\delta$  siRNA sense, 5'-CCAUGUAUC CUGAGUGGAATT-3' and antisense, 5'-UUCCACUCAGGA UACAUGGTT-3'; negative control siRNA sense, 5'-UUCUCC GAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACAC

GUUCGGAGAATT-3'. Western blot analysis was performed following 4 h incubation with siRNA.

In addition, cleavage stage embryos were counted 30-35 h post-hCG injection and MPF activity was measured at different time points (26-29.5 h).

**Plasmid construction and site-directed mutagenesis.** Mouse Cdc25B cDNA was provided by Dr Tony Hunter (Laboratory of Molecular Biology, The Salk Institute for Biological Studies, La Jolla, CA, USA). The pBluescript II/SK-Cdc25B-S96Alanine (pBSK-Cdc25B96A) was obtained by mutating Ser96 to alanine of Cdc25B by using the site-directed mutagenesis kit (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA). The aspartic acid residue at position 96 in the pBSK-Cdc25B-S96 (pBSK-Cdc25B96D) was replaced with alanine to form pBSK-Cdc25B96A, were used as templates for primer design. Primers were designed using the 96A and 96D templates were as follows: 96A forward, 5'-CACCTCTGAGTGCGCCCTGTCATCTGAG-3' and reverse, 5'-CTCAGATGACAGGGCGCACTCAGAGGTG-3' and 96D forward, 5'-ACCTCTGAGTGCGACCTGTCTGAGTTCCTCA-3' and reverse, 5'-TGAGGACTCAGATGACAGGTCGCACTCAGAGGT-3'. The above recombinant plasmids were sequenced to verify correct gene insertion and successful mutation, and were used as templates for *in vitro* transcription.

One-cell stage mouse embryos were divided into five groups: Blank (only mouse embryos), TE (mouse embryos injected with TE buffer), Cdc25B-WT (mouse embryos injected with Cdc25B-WT), Cdc25B-S96A (mouse embryos injected with Cdc25B-S96A) and Cdc25B-S96D (mouse embryos injected with Cdc25B-S96d).

**In vitro transcription.** As previously described (26), all pBluescript II/SK constructs were linearized with *Xba*I and transcribed *in vitro* into 5'mRNA for microinjection using the mMESSAGE mMACHINE kit (Ambion; Thermo Fisher Scientific, Inc.).

**Microinjection and morphology analysis.** Various Cdc25B mRNAs were microinjected into the cytoplasm of one-cell embryos at the G<sub>1</sub> or S phase, as previously described (26). The typical injection volumes were 5% (10 pl, cytoplasm) and 1% (2 pl, nuclear) of the total cell volume per egg. mRNAs were diluted to various concentrations with TE buffer (5 mmol/l Tris-HCL and 0.5 mmol/l EDTA, pH 7.4).

The fertilized embryos at the S phase (22 h post-hCG injection) were incubated in M16 medium in the presence of rottlerin (0.5  $\mu$ M) for 1 h. Subsequently, embryos microinjected with various Cdc25B mRNAs were cultured in M16 medium with 0.5  $\mu$ M/l rottlerin. The rate of embryo cleavage was counted in three independent experiments under a phase contrast microscope at 30 and 35 h post-hCG injection in the absence or presence of rottlerin. Morphological analysis was performed by using Image J software (version 1.46; National Institutes of Health, Bethesda, MD, USA) and the Sholl Analysis plugin (version 3.4.5; fiji.sc/Sholl), as previously described (27).

**Western blot analysis.** Total protein was extracted from treated embryos using 250  $\mu$ l radioimmunoprecipitation assay

buffer (cat. no. P0013; Beyotime Institute of Biotechnology, Haimen, China) for 30 min at 4°C. Total protein was quantified using a bicinchoninic acid assay kit (cat. no. 23225; Thermo Fisher Scientific, Inc.) and an average 200 embryos (40  $\mu$ g protein/lane) were separated via SDS-PAGE on a 10% gel. The separated proteins were subsequently transferred onto nitrocellulose membranes and blocked for 1 h at room temperature with 5% skimmed milk (Blotto; cat. no. sc-2325; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Membranes were incubated with primary antibodies against PKC $\delta$  (1:1,000; cat. no. 610108; BD Biosciences, San Jose, CA, USA), phospho-PKC $\delta$  (Thr505; 1:800; cat. no. 9374; Cell Signaling Technology, Inc., Danvers, MA, USA), Cdc25B (1:200; cat. no. sc-5619), Tyr15 of Cdc2 (1:500; cat. no. sc-24579; both Santa Cruz Biotechnology, Inc.), phospho-Cdc25B-pSer96 (1:200; cat. no. 11503; Signalway Antibody LLC, College Park, MD, USA) and  $\beta$ -actin (1:500; cat. no. AF0003; Beyotime Institute of Biotechnology) overnight at 4°C. Membranes were washed with PBS for 30 min at room temperature. Following primary incubation, membranes were incubated with horseradish peroxidase-conjugated anti-mouse (1:5,000; cat. no. ZB-2305), anti-goat (1:5,000; cat. no. ZB-2306) or anti-rabbit (1:5,000; cat. no. ZB-2301; all OriGene Technologies, Inc., Beijing, China) secondary antibodies. Protein bands were subsequently visualized using the enhanced chemiluminescence substrate kit (Thermo Fisher Scientific, Inc.). Protein expression was quantified using Image Lab analysis software (version 4.1; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The phospho-Cdc25B-pSer96 antibody was raised in New Zealand white rabbits against the keyhole limpet hemocyanin-conjugated phosphopeptide.

**Immunofluorescence.** Immunostaining was performed as previously described (28). PKC $\delta$  was detected using anti-PKC $\delta$  (1:200; cat. no. 610398; BD Biosciences) at 4°C overnight. Subsequently, the embryos were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG secondary antibody (1:100; cat. no. AP130F; Chemicon International, Inc., Temecula, CA, USA) at room temperature for 2 h. Chromosomes were labeled with 10 g/ml DAPI (cat. no. P36931; Invitrogen; Thermo Fisher Scientific, Inc.). For PKC $\delta$  and Cdc25B double staining, PKC $\delta$  was detected using anti-PKC $\delta$  and FITC-conjugated goat anti-mouse IgG secondary antibody. Following PKC $\delta$  immunostaining, embryos were washed three times with PBS and Cdc25B was detected using anti-Cdc25B (1:200; cat. no. sc-326; Santa Cruz Biotechnology, Inc.) at room temperature for 2 h, followed by tetra-rhodamine isothiocyanate-conjugated rabbit anti-goat IgG secondary antibody (1:200; cat. no. ab7087; Abcam.) at room temperature for 2 h. Following secondary incubation, the embryos were stained with DAPI (10 g/ml) for 10 min at room temperature and observed under a confocal laser-scanning microscope (magnification, x400; Leica Microsystems GmbH, Wetzlar, Germany).

**MPF activity assay.** MPF kinase activity was measured using a histone H1 kinase assay, as previously described (29). Embryos from each time-point (26-35 h) post-hCG injection were collected and added to 5  $\mu$ l collection buffer (PBS) containing 1 mg/ml polyvinyl alcohol, 5 mM EDTA, 10 mM



Na<sub>3</sub>VO<sub>4</sub>, and 10 mM NaF. In total, 25  $\mu$ l MPF buffer (54 mM  $\beta$ -glycerophosphate; 14.5 mM p-nitrophenylphosphate; 24 mM 3-(N-morpholino)-propanesulfonic acid, pH 7.2; 14.5 mM MgCl<sub>2</sub>; 14.5 mM EGTA; 0.12 mM EDTA; 1 mM dithiothreitol; 2.4  $\mu$ M PKI; 75 mM genistein (a tyrosine kinase inhibitor); 10  $\mu$ M ML-9 (a myosin light chain kinase inhibitor); 1 mg/ml histone H1 (type III-s); and 1  $\mu$ g/ml each of leupeptin, aprotinin, pepstatin, chymostatin, and trypsin-chymotrypsin inhibitor.) was subsequently added to the disrupted cells following three freezing and thawing steps. The histone H1 kinase reaction was initiated by adding 25  $\mu$ l of 20  $\mu$ Ci/ml ( $\gamma$ -<sup>32</sup>P) ATP (Peking YaHui Biotechnology Co., Beijing, China) to the sample, which was incubated at 30°C for 10 min. Following this, 25- $\mu$ l aliquots were spotted onto Whatman p81 paper, and the reaction was stopped with 5% H<sub>3</sub>PO<sub>4</sub> solution. Following thorough washing, the radioactivity on the filter paper was counted with a Beckman scintillation counter (Beckman Coulter, Inc., Brea, CA, USA).

**Statistical analysis.** Data are presented as the mean  $\pm$  SD. Statistical analyses were performed using SPSS statistical software (version 16.0). All experiments were repeated independently at least three times. One-way analysis of variance with Tukey's post hoc test was used to evaluate the difference between groups, and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression and subcellular localization of PKC $\delta$  and Cdc25B, and phosphorylation status of Cdc25B-Ser96 *in vivo* in one-cell stage mouse embryos.** Samples of one-cell stage mouse embryos were obtained from all four cell cycle phases (G<sub>1</sub>, S, G<sub>2</sub> and M), and RT-qPCR and western blotting were performed to identify the mRNA and protein expression levels of PKC $\delta$ . It was observed that PKC $\delta$  mRNA was expressed during the development of one-cell stage embryos. No significant difference in PKC $\delta$  expression was observed among the G<sub>1</sub>, S and G<sub>2</sub> phases; however, a significant increase in PKC $\delta$  expression was indicated during the M-phase (Fig. 1A). Similar results in protein expression were also identified with western blotting (Fig. 1B). The activity of PKC $\delta$  is promoted by phosphorylating Thr505 in the activation loop (30). In the present study, anti-p-PKC $\delta$ Thr505 antibody was used to explore PKC $\delta$  kinase activity (Fig. 1B). Notably, PKC $\delta$  phosphorylated Thr505 (active form of PKC $\delta$ ) and increased the expression during M phase. To identify whether Cdc25B-Ser96 was phosphorylated *in vivo*, fertilized mouse embryos were collected at different phases and the phosphorylation status of Cdc25B-Ser96 was measured using the phosphor-specific antibody. As indicated in Fig. 1C, higher expression levels of phosphorylated Cdc25B-Ser96 were observed during the G<sub>2</sub> and M phases compared with expression in G<sub>1</sub> and S phases. Taken together, these results demonstrated that Cdc25B-Ser96 was phosphorylated during G<sub>2</sub> and M phases *in vivo*.

Immunofluorescence analysis by laser scanning confocal microscopy detected a uniform distribution of PKC $\delta$  in the cytoplasm during G<sub>1</sub>, S, G<sub>2</sub> and M phases (Fig. 1D), with slightly higher levels in the cortex region during G<sub>2</sub> and early M phase. In addition, PKC $\delta$  protein was also detected in the male and

the female pronuclei during the G<sub>1</sub> and S phases. These results provide indirect evidence to support an association between PKC $\delta$  and Cdc25B.

**Rotterlin and knockdown of PKC $\delta$  suppresses G<sub>2</sub>/M transition and inhibits the activity of MPF in one-cell mouse embryos.** To investigate the role of PKC $\delta$  on the G<sub>2</sub>/M transition, rottlerin, a selective inhibitor of PKC $\delta$ , was used to pretreat one-cell stage mouse embryos (Fig. 2). Embryo cleavage and MPF activity at different time points were examined. Notably, embryo cleavage was significantly inhibited when the concentration of rottlerin was  $>0.5$   $\mu$ M (Fig. 2A). However, when the concentration of rottlerin was  $>0.5$   $\mu$ M, it was considered toxic to embryos (data not shown). The present results demonstrated that peak MPF activity was observed 28.5 h post-hCG injection and MPF activity was significantly increased in the rottlerin-treated group compared with the control group (Fig. 2B). These data suggest that rottlerin suppresses G<sub>2</sub>/M transition in one-cell mouse embryos.

PKC $\delta$  expression was knocked down in G<sub>1</sub> phase mouse embryos by microinjecting PKC $\delta$ -specific siRNA. The embryo cleavage was counted 30-35 h post-hCG injection and the activity of MPF was measured at different time points (Fig. 2C and D). Knockdown of PKC $\delta$  expression significantly inhibited MPF activity 29 h post-hCG injection ( $P < 0.05$ ; Fig. 2C). In addition, the rate of embryo cleavage in the siRNA group significantly decreased compared with NC control group ( $P < 0.05$ ; Fig. 2D). Western blot analysis revealed a decrease in expression of PKC $\delta$  in the siRNA microinjected group compared with the control groups, indicating that endogenous PKC $\delta$  was effectively inhibited by PKC $\delta$ -specific siRNA (Fig. 2E). Additionally, PKC $\delta$  siRNA also suppressed Cdc25B expression (Fig. 2E). The findings concluded that PKC $\delta$  serves a role in G<sub>2</sub>/M transition in one-cell stage mouse embryos through phosphorylating Cdc25B on Ser96.

**Effects of phosphorylation and dephosphorylation in one-cell stage mouse embryos.** PKC $\delta$  phosphorylation sites were predicted using the Scansite software (scansite.mit.edu). It was revealed that Ser96 on Cdc25B may be a potential site (Fig. 3A). Thus, a series of Cdc25B mutants were created, in which alanine and aspartic acid were substituted for a serine residue at positions 96 (S96A and S96D). The three types of Cdc25B mRNA were microinjected into mouse embryos and their effects on the development of early mouse embryos were observed. The results demonstrated that wild-type and both mutant Cdc25B elevated Cdc25B expression in mouse embryos (Fig. 3B). In addition, MPF activity in the Cdc25B-S96D was induced earlier compared with the other groups, reach its peak at 27 h post-hCG injection and was the lowest at 29 h post-hCG injection (Fig. 3C).

As shown in Fig. 3D, a similar pattern was also observed in the Cdc25B-S96A mRNA-injected group, and the rate of embryo cleavage was 61.46% at 31 h, which was nearly the same as that in the control groups. These results suggested that the Cdc25B-96S/D mutant could trigger one-cell stage mouse embryo division. The cleavage of one-cell cycle mouse embryos was counted at 26-31 h post-hCG injection, and the

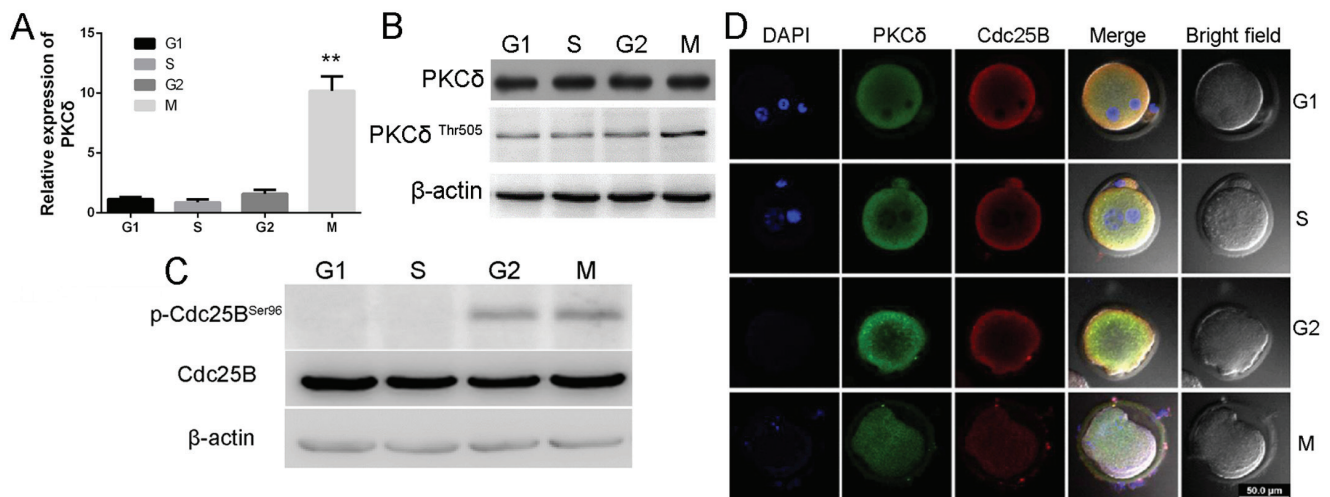


Figure 1. Expression and subcellular localization of PKCδ and Cdc25B, and phosphorylation status of Cdc25B-Ser96 *in vivo* in mouse one-cell stage embryos. (A) mRNA expression levels of PKCδ detected by reverse transcription-quantitative polymerase chain reaction analysis, which indicated that the expression level of PKCδ was significantly elevated in M phase. \*\*P<0.01 vs. G1 group. (B and C) Expression levels of PKCδ, p-PKCδ-Thr505 and p-Cdc25B-Ser96 were detected in one-cell mouse embryos using western blotting. The data revealed that the expression levels of PKCδ and p-PKCδ-Thr505 in M phase were increased. p-Cdc25B-Ser96 expression in G<sub>2</sub> and M phases were increased. (D) Laser scanning confocal microscope images of PKCδ at G<sub>1</sub>, S, G<sub>2</sub> and M phases; embryos were double labeled with PKCδ (green), Cdc25B (red) and DAPI (blue) staining of the DNA. Scale bar, 50 μm. Slightly higher levels of PKCδ in the cytoplasm of the cortex at G<sub>2</sub> and early M stage were indicated. PKCδ, protein kinase C type δ; Cdc25, cell division cycle 25; DAPI, 4',6-diamidino-2-phenylindole.

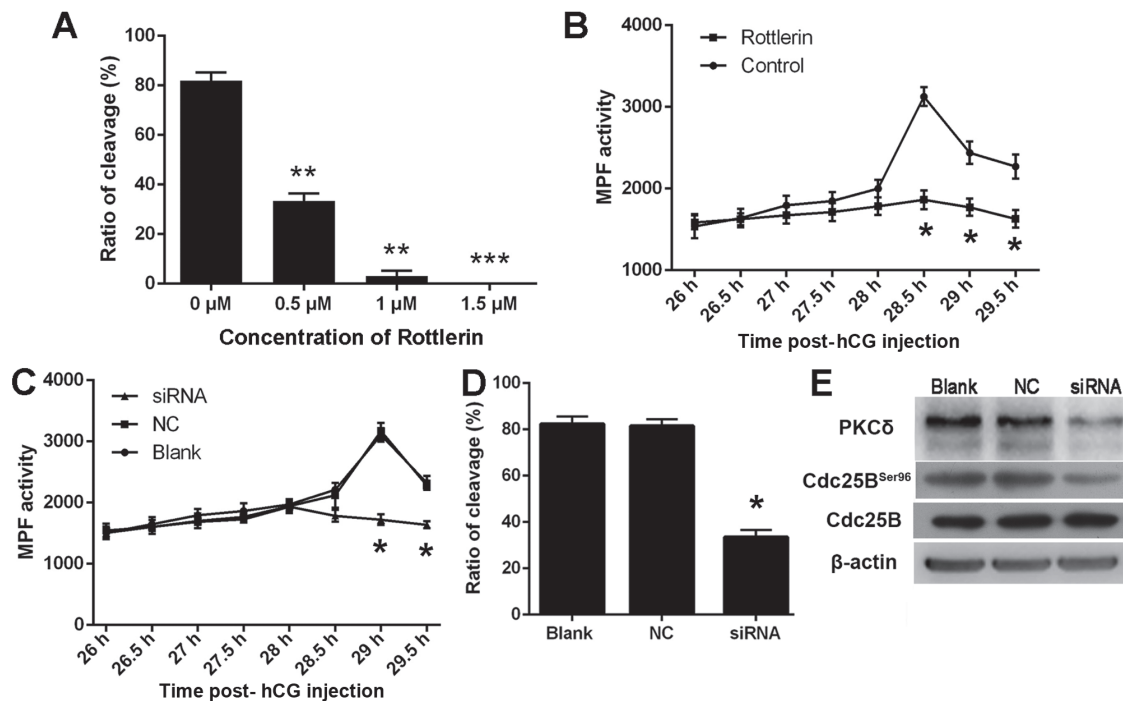


Figure 2. Ratio of embryo cleavage and activity of MPF with the treatment of rottlerin and knockdown of PKCδ. (A) The embryo cleavage rate of mouse embryos was examined in the presence of rottlerin at various concentrations (0, 0.5, 1 and 1.5 μM/l). The decreasing rate of embryo cleavage was presented in a dose dependent manner. \*\*P<0.01, \*\*\*P<0.001 vs. control group (0 μM rottlerin). (B) MPF activity was detected following treatment with rottlerin. \*P<0.05 vs. control group (C) MPF activity was detected post-siRNA microinjection. The use of rottlerin or treatment of PKCδ-specific siRNA reduced MPF activity at 28.5-29 h post-hCG injection. \*P<0.05 vs. NC group. (D) Ratio of embryo cleavage in the siRNA group was inhibited compared with the two control groups. \*P<0.05 vs. NC group. (E) Western blotting demonstrated that the expression levels of PKCδ and Cdc25B in the siRNA-microinjected group were downregulated compared with that of the control groups. MPF, mitosis promoting factor; PKCδ, protein kinase C type δ; Cdc25, cell division cycle 25; siRNA, small interfering RNA.

rate of embryo cleavage was calculated at 31 h. therefore, a significantly higher rate of embryo cleavage was identified in embryos treated with Cdc25B-WT and Cdc25B-S96D.

Western blotting was used to examine the phosphorylation status of Cdc2-Tyr15 (Fig. 3E). In the control groups, strong Cdc2-Tyr15 phosphorylation signals at 26.5-28 h post-hCG

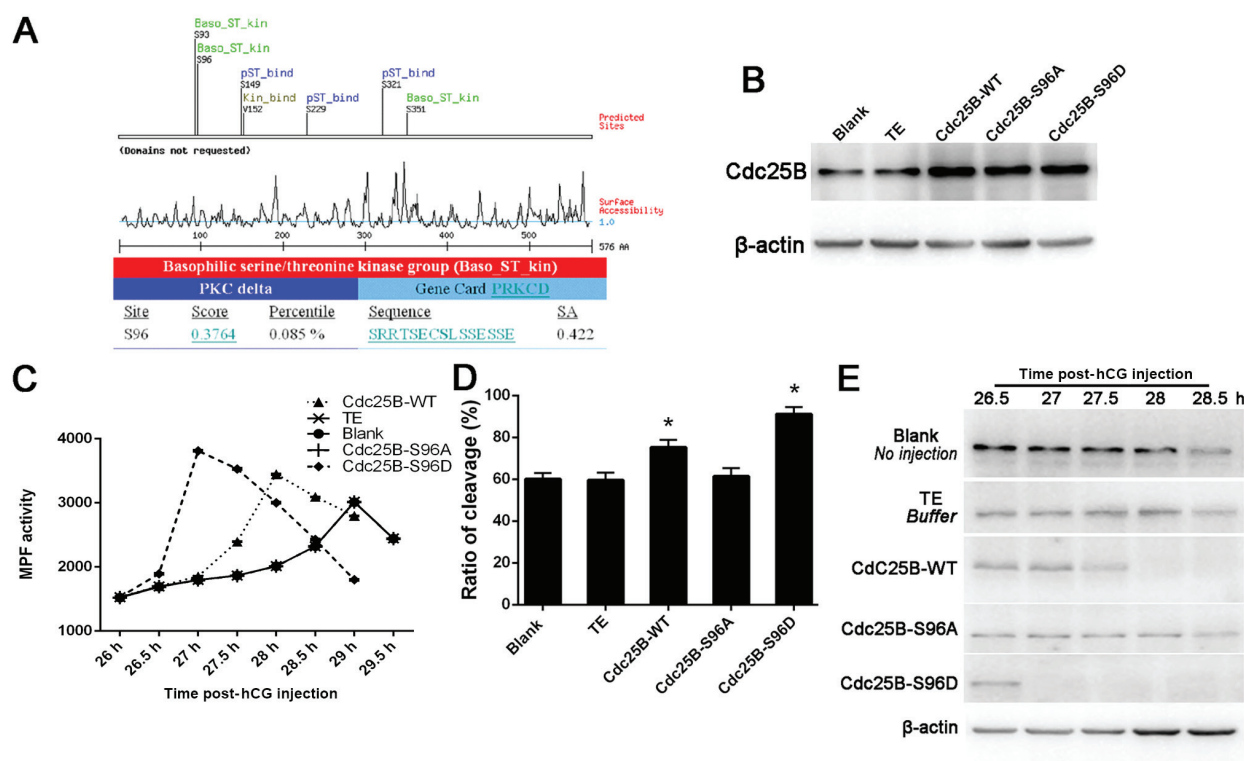


Figure 3. Effects of phosphorylation and dephosphorylation in one-cell stage mouse embryos. (A) The phosphorylation site on Cdc25B was predicted by using Scansite software. (B) Western blotting was performed to demonstrate the Cdc25B protein expression at 4 h post-microinjection of Cdc25B mRNA and the three exogenous Cdc25B mRNAs. (C) MPF activity in various mRNA-injected groups. Cdc25B-96D and Cdc25B-WT reduced the time required for MPF activity elevation. (D) Ratios of cleavage in mouse embryos were significantly increased after either Cdc25B-WT or Cdc25B-S96D mRNA injections. (E) Western blotting of the phosphorylation status of Cdc2-Tyr15 in the five groups. Embryos were collected at 26.5, 27, 27.5, 28, 28.5 h post-hCG injection. The phosphorylation of Cdc2-Tyr15 was inhibited with Cdc25B-WT or Cdc25B-S96D mRNA injections compared with the control group. \* $P < 0.05$  vs. TE group. Cdc25, cell division cycle 25; MPF, mitosis promoting factor; hCG, human chorionic gonadotropin; WT, wild-type; TE, Tris-EDTA.

injection were detected, with a weak signal appearing at 28.5 h post-hCG injection. In the WT group, a weak Cdc2-Tyr15 phosphorylation signal was observed at 27.5 h and no signal at 28–29 h was indicated post-hCG injection. In the Cdc25B-S96D group, a weak Cdc2-Tyr15 phosphorylation signal was detected at 26.5 h, but there was no signal identified at 27–29 h post-hCG injection. By contrast, in the Cdc25B-S96A group, weak Cdc2-Tyr15 phosphorylation signals were detected 26.5–27.5 h post-hCG injection, but no signal was detected after 28 h. This data suggests that Cdc25B-S96D can activate MPF prior to Cdc25B-WT and that Cdc25B-S96D directly affects the phosphorylation of CDC2-Tyr15 in mouse.

**Microinjection of Cdc25B-S96D and Cdc25B-WT mutants speed mitotic resumption in rottlerin arrested embryos.** To observe the association of PKC $\delta$  with Cdc25B, Cdc25B-WT, two mutant mRNAs (Cdc25B-S96A and Cdc25B-S96D) were microinjected into fertilized mouse embryos at S stage. Samples were pre-incubated in M16 medium with 0.5  $\mu$ M rottlerin for 1 h. All three microinjected Cdc25B mRNAs were efficiently in rottlerin-treated mouse fertilized embryos and the level of Cdc25B expression was subsequently examined. As demonstrated in Fig. 4A, Cdc25B expression was increased in embryos microinjected with all three mRNAs. MPF activity was measured between 28–35 h post-hCG injection. MPF activity was higher in the Cdc25B-S96D and Cdc25B-WT groups between 29–35 h post-hCG injection compared with the

blank and TE groups (Fig. 4B), suggesting that PKC $\delta$  inhibition is responsible for the inhibition of MPF activity. Notably, MPF activity in the Cdc25B-WT mRNA-injected embryos increased at 31 h post-hCG injection and gradually decreased thereafter. In the TE group, MPF activity peaked at 30 h and then declined slowly. Conversely, MPF activity remained low at 29–35 h post-hCG in the Cdc25B-S96A group, which was similar to the control groups.

The cleavage of fertilized mouse embryos was observed at 29–35 h post-hCG injection and the embryo cleavage rate was calculated at 35 h. The embryo cleavage rate induced by rottlerin in each group decreased (Fig. 4C). The rate of embryo cleavage was inhibited in the control and Cdc25B-S96A groups (Fig. 4C) compared with Cdc25B-WT. The embryo cleavage rates were 32.59% (blank group), 31.70% (TE-injected group) and 32.84% (Cdc25B-S96A mRNA-injected group) with no significant differences observed among the three groups. Furthermore, embryos microinjected with Cdc25B-WT mRNA entered M phase at 31 h post-hCG injection (data not shown). In addition, the embryo cleavage rate was significantly increased (45.14%) compared with the control group. By contrast, embryos microinjected with Cdc25B-S96D mRNA entered M phase at 29.5–30 h post-hCG injection, and ~61.69% of embryos had entered the M phase at 35 h (data not shown). Overall, this data suggests that microinjection of either Cdc25B-S96D or Cdc25B-WT mRNA could effectively resume G<sub>2</sub> phase



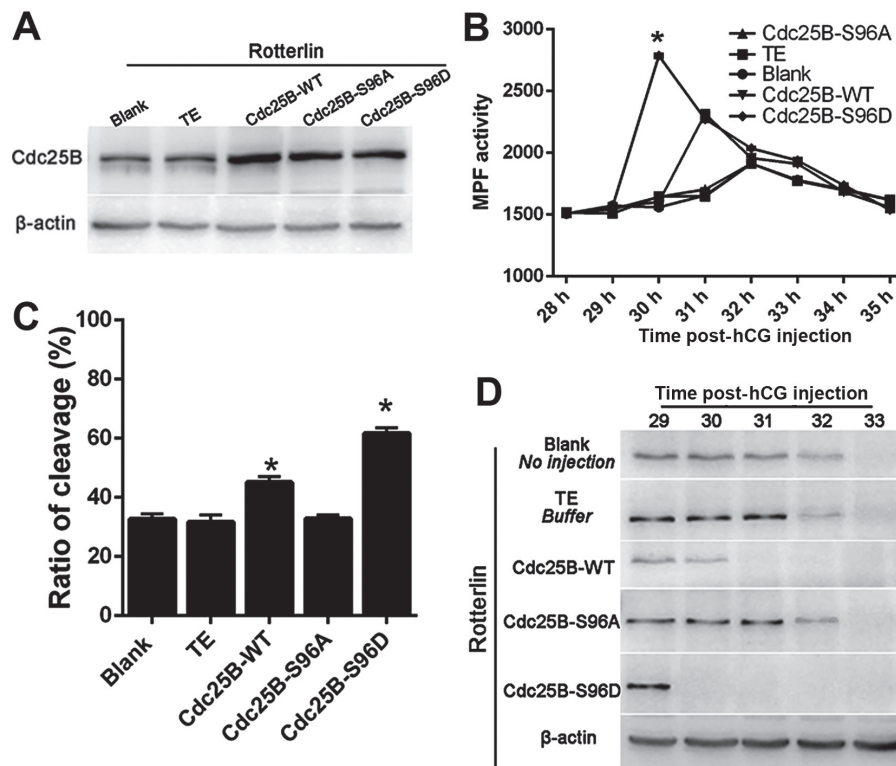


Figure 4. Cdc25B mRNA microinjections reverse the inhibitory effect on embryos that were treated with rottlerin. (A) Western blotting indicated that Cdc25B protein expression levels were elevated at 4 h post-Cdc25B mRNA microinjections. (B) Activity of MPF 28–35 h post-hCG injection. Data suggested that Cdc25B-S96D and Cdc25B-WT served inducible roles in the activity of MPF. (C) The embryo cleavage rate in cultured mouse embryos injected with various Cdc25B mRNA at 35 h post-hCG injection. (D) Western blotting of the phosphorylation status of Cdc2-Tyr15 in the five groups. Embryos were collected at 29, 30, 31, 32 and 33 h post-hCG injection. Results demonstrated that Cdc25B-S96D and Cdc25B-WT suppressed the phosphorylation of Cdc2-Tyr1 under rottlerin treatment compared with the control, although the overall reaction time increased. \* $P < 0.05$  vs. TE group. Cdc25, cell division cycle 25; hCG, human chorionic gonadotropin; MPF, mitosis promoting factor; WT, wild-type; TE, Tris-EDTA.

arrest induced by rottlerin, particularly the microinjection of Cdc25B-S96D mRNA.

The phosphorylation status of Cdc2-Tyr15 at 29, 30, 31, 32 and 33 h post-hCG injection was detected following treatment with rottlerin (Fig. 4D). In the control groups, strong bands of phosphorylated Cdc2-Tyr15 were identified at 29–32 h post-hCG injection, demonstrating that MPF activity was inhibited. In the Cdc25B-WT group, Cdc2-Tyr15 phosphorylation was weakly detected at 30 h post-hCG injection, which was consistent with the results regarding MPF activity. However, in the Cdc25B-S96D group, almost no signal was observed at 30–33 h post-hCG injection, which implied that MPF was completely activated. Notably, the inhibitory phosphorylation signals of Cdc2-Tyr15 were still observed at 32 h post-hCG injection in the Cdc25B-S96A mutant group, which was similarly observed in the control groups. These data suggest that overexpressing either Cdc25B-S96D or Cdc25B-WT can override the inhibition of rottlerin in regulating the activity of MPF, particularly the microinjection of Cdc25B-S96D mRNA. Overall, these data suggested that microinjection of Cdc25B-S96D and Cdc25B-WT mRNAs could resume the arrest of mitosis by rottlerin; however, the effects of Cdc25B-S96D mRNA were more prominent.

*Microinjection of Cdc25B-S96D and Cdc25B-WT mRNAs can resume mitosis following knockdown of PKC $\delta$  in one-cell cycle mouse embryos.* Results in Fig. 5A revealed that the

three different microinjected Cdc25B mRNAs were translated efficiently in mouse fertilized embryos following PKC $\delta$  knock-down. As revealed in Fig. 5B, MPF activity was low and stable at 29–35 h, and increased slowly at 32 h in the control groups. However, MPF activity in the Cdc25B-WT mRNA-injected group increased slowly, reached a peak at 31 h post-hCG injection and then decreased gradually. In the Cdc25B-S96D mRNA-injected group, MPF activity exhibited a rapid increase at 29 h, peaked at 30 h and then declined slowly. In the Cdc25B-S96A mRNA-injected group, MPF activity was similar to the levels detected in the control groups. Data in Fig. 5C suggested that the rate of embryo cleavage was 31.52% in the non-injected group, 31.88% in the TE-injected group and 31.01% in the Cdc25B-S96A-injected group, and there were no significant differences among these groups. However, 50.73% of embryos in the WT mRNA-injected group and 65.31% in the Cdc25B-S96D group, respectively, entered M phase at 32 and 31 h post-hCG injection.

The phosphorylation status of Cdc2-Tyr15 at 29, 30, 31, 32 and 33 h post-hCG injection was determined (Fig. 5D). In the control groups, phosphorylated Cdc2-Tyr15 signals were detected at 29–32 h post-hCG injection and MPF activity was inhibited. In the Cdc25B-WT group, Cdc2-Tyr15 phosphorylation was weakly detected at 30 h post-hCG injection, which was consistent with the results regarding MPF activity. However, in the Cdc25B-S96D groups, almost no signal was detected at 30–33 h post-hCG injection, which implied that

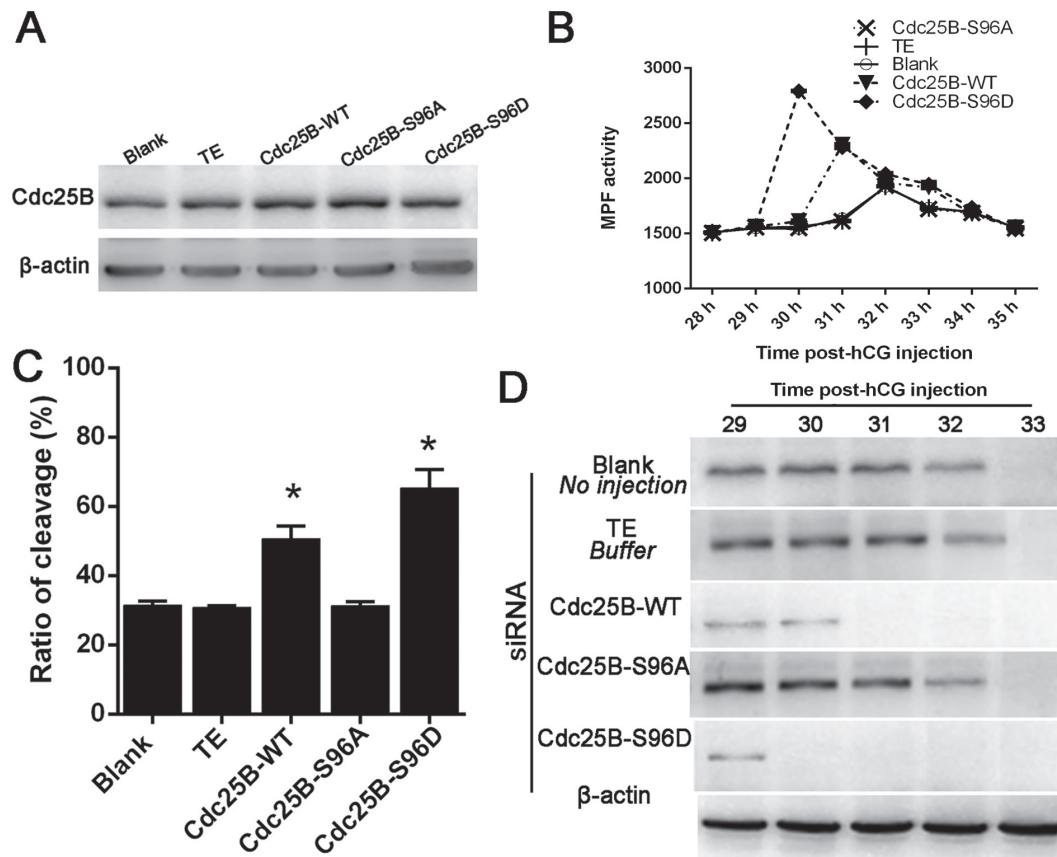


Figure 5. Cdc25B mRNA microinjection reverses the inhibitory effect on embryos that were treated with Cdc25B mRNAs. (A) Western blotting revealed no significant differences in Cdc25B protein expression levels at 4 h in the PKC $\delta$ -silenced groups following Cdc25B mRNA microinjections, indicating that all of the microinjected Cdc25B mRNAs were translated efficiently in fertilized mouse embryos. (B) Activity of MPF 28–35 h post-hCG injection. Extra time was required for the induction of MPF activity as the level of PKC $\delta$  was limited. (C) Embryo cleavage rate in cultured mouse embryos injected with various Cdc25B mRNAs at 35 h post-hCG injection. The ratios of cleavage in mouse embryos were significantly increased following Cdc25B-WT or Cdc25B-96D mRNA injections. (D) Western blotting of the phosphorylation status of Cdc2-Tyr15 in the five groups. Embryos were collected at 29, 30, 31, 32 and 33 h post-hCG injection. Results revealed that Cdc25B-S96D and Cdc25B-WT suppressed the phosphorylation of Cdc2-Tyr1 after PKC $\delta$  siRNA transfection when compared with the TE group, although the overall reaction time increased. \* $P < 0.05$  vs. TE group. Cdc25, cell division cycle 25; hCG, human chorionic gonadotropin; MPF, mitosis promoting factor; PKC $\delta$ , protein kinase C type  $\delta$ ; WT, wild-type; TE, Tris-EDTA.

MPF was completely activated. By contrast, the inhibitory phosphorylation signals of Cdc2-Tyr15 were still observed at 32 h post-hCG injection in the Cdc25B-S96A mutant group, which was similarly observed in the control groups. Overall, the data suggested that microinjection of Cdc25B-S96D and Cdc25B-WT mRNAs could override the arrest of PKC $\delta$  knockdown; however, the effects of Cdc25B-S96D mRNA were more prominent.

## Discussion

Our group has already reported the significant role of PKC in the development of embryos (17,18). The PKC family participates in pre-implantation development (3), but the relative importance of each isozyme still requires further exploration. It is important to study the functions of specific PKC isotypes and not just the role of PKC in general since specific PKC isotypes have been demonstrated to have opposing functions within the same cell (31). As a member of the PKC super family, PKC $\delta$  is implicated in a significant proportion of the biochemically measurable PKC increases occurring at fertilization (32). Notably, Cdc25B is involved in cell cycle regulation in vascular endothelial cells and the PKC/Cdc25B

signaling pathway negatively regulates G<sub>2</sub>/M transition (33). To the best of our knowledge, the present study explored for the first time the specific function of the PKC $\delta$ /Cdc25B signaling pathway and its underlying mechanism in one-cell stage mouse embryos.

The present study focused on the expression and subcellular localization of PKC $\delta$  during G<sub>1</sub>, S, G<sub>2</sub> and M phases in one-cell stage mouse embryos. PKC $\delta$  expression was detected in all four cell cycle phases at mRNA and protein levels; however, increased mRNA and protein expression levels were observed in the M phase. The present study extends on earlier reports by Gangeswaran and Jones (34). In their study, PKC $\delta$  expression was detected in mouse embryos at mRNA and protein levels, but only PKC $\delta$  expression at protein level was demonstrated in mouse embryos. Location is typically associated with function. Notably, analysis with confocal microscopy revealed that PKC $\delta$  protein not only persisted in the cytoplasm but also concentrated in the maternal and paternal pronuclei of early post fertilization zygotes (G<sub>1</sub> and S phase). Using selective inhibitors and siRNAs, the activity of MPF was inhibited and the effects on the cell cycle were observed. The present results suggested that PKC $\delta$  may be involved in regulating the development of fertilized mouse embryos.



Scansite software predicted Cdc25B as a potential substrate of PKC $\delta$ . Scansite predicts whether a candidate site is a phosphorylation site for a specific kinase or a specific group of kinases (35). Characterization of substrates for PKC $\delta$  revealed a single basic amino acid close to the phosphorylation site essential for specific recognition and phosphorylation (36). Ser96 was marked among the amino acid sequence of Cdc25B from site 91-100. Based on the selectivity of PKC $\delta$  substrates, an association between PKC $\delta$  and Cdc25B was considered. When either Cdc25B-WT or Cdc25B-S96D mRNA was overexpressed in early-fertilized mouse embryos, the mitotic G<sub>2</sub>/M transition was accelerated. Specific inhibition of endogenous PKC $\delta$  activity was attempted and the function of Cdc25B was examined. The embryo cleavage rate induced by rottlerin in each group decreased, and a large number of embryos microinjected with either Cdc25B-S96D or Cdc25B-WT mRNAs entered the M phase at 32 h post-hCG injection. Thus, it was concluded that PKC $\delta$  directly phosphorylates Cdc25B Ser96 and promotes its function during the first cell cycle of mouse embryos.

Dephosphorylation of specific tyrosine/threonine residues on Cdc2 is associated with G<sub>2</sub>/M transition (37). Hence, in the present study, the phosphorylation of Tyr15 of Cdc2 was examined during the early development of mouse fertilized embryos employing anti-phospho-Tyr15 antibody. In addition, the present study aimed to elucidate the effects of the Ser96 residue of Cdc25B on MPF activity. Data revealed that the phosphorylation status of Cdc2-Tyr15 at different time points in each group was consistent with MPF activity, whether rottlerin was added or not. The data suggested that the phosphorylated Cdc25B at Ser96 activates MPF by directly dephosphorylating Cdc2-pTyr15 in the first mitotic cell cycle of fertilized mouse embryos. The MPF activity in the TE group peaked in the Cdc25B-S96D mutant group earlier than in the Cdc25B-WT and Cdc25B-S96A groups in the presence of rottlerin. MPF activity remained low at 29-31 h post-hCG injection in the control groups. However, MPF activity peaked in the Cdc25B-S96D mutant group, which was 3 h later than in the Cdc25B-S96D mutant group without rottlerin, suggesting that overexpression of Cdc25B-S96D mutants may largely overcome G<sub>2</sub> arrest induced by rottlerin.

In the present study, western blotting revealed that Cdc25B-Ser96 is phosphorylated during G<sub>2</sub> and M phases and dephosphorylated during the G<sub>1</sub> and S phases *in vivo*. Different phosphorylated sites on Cdc25B give rise to different functions. Notably, activation of MPF is associated with Cdc25B (38). In our previous report (39), when Ser351 was phosphorylated by PKB MPF was activated, which in turn dephosphorylated Cdc2-Tyr15. By contrast, phosphorylation on Ser321 by 14-3-3 epsilon caused germinal vesicle breakdown in mouse oocytes. When 149,321 sites were phosphorylated by PKA, the MPF activity was inhibited and Cdc2-Tyr15 remained phosphorylated (40). Taken together, when this site (Cdc2-Tyr15) is mutated to the phosphorylated residue Asp, the Cdc25B mutants were activated and the subsequent increase in MPF activity was indicated.

Ma *et al* (41) indicated that the active form of PKC $\delta$  (p-PKC $\delta$  Thr505) localizes to the microtubule organizing center that serves a role in meiotic spindle organization in mammalian oocytes. Following fertilization, PKC $\delta$  is associated with spindle microtubules (42). PKC $\delta$  serves a role in

spindle integrity and/or function in embryos (43). Cdc25B accumulation in the cytoplasm has been correlated with spindle formation, and it has been suggested that this phosphatase pool located in close vicinity of the centrosome is responsible for the activation of the cytoplasmic pool of MPF (44). The colocalization of PKC $\delta$  and Cdc25B produced the same diffuse signal in one-cell cycle mouse embryos according to immunofluorescence analysis. Previous results have suggested that active MPF first appears at centrosomes in prophase, and Cdc25B specifically activates MPF on centrosomes (45). Thus, it was speculated that PKC $\delta$  is post-translationally modified in the present study, which was consistent with observations from Ma *et al* (41). The phosphorylation on Cdc25B caused by PKC $\delta$  also participates in the activation of MPF in the centrosomes; however, the underlying mechanisms are not known.

In conclusion, the present study demonstrated that PKC $\delta$  serves an efficient role in the development of the first cell stage of mouse embryos by phosphorylating Cdc25B on Ser96. The results provide novel perspectives for the understanding of the function of the PKC $\delta$ /Cdc25B signaling pathway in the regulation of early embryo development.

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## Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

## Authors' contributions

YCL and BZY designed the experiments. YCL performed the experiments. YCL, XD and BZY collected the data. DDW and MLJ analyzed the data and YCL validated the analysis. YCL prepared the manuscript and BZY revised the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The current study was approved by the Ethics Committee of the China Medical University (Shenyang, China).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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