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Original Paper

Knockdown of PRKAR2B Results in the Failure of Oocyte Maturation

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Key Words

Prkar2b • Oocyte maturation • MI arrest • Pentose phosphate pathway • cAMP-dependent protein kinase A

Abstract

Background/Aims: Cyclic adenosine monophosphate (cAMP)-dependent type 2 regulatory subunit beta (*Prkar2b*) is a regulatory isoform of cAMP-dependent protein kinase (PKA), which is the primary target for cAMP actions. In oocytes, PKA and the pentose phosphate pathway (PPP) have important roles during the germinal vesicle (GV) stage arrest of development. Although the roles of the PKA signal pathway have been studied in the development of oocyte, there has been no report on the function of PRKAR2B, a key regulator of PKA. ***Methods:*** Using reverse transcription polymerase chain reaction (RT-PCR), quantitative real-time PCR (qRT-PCR), immunohistochemistry, and immunofluorescence, we determined the relative expression of *Prkar2b* in various tissues, including ovarian follicles, during oocyte maturation. *Prkar2b*-interfering RNA (RNAi) microinjection was conducted to confirm the effect of *Prkar2b* knockdown, and immunofluorescence, qRT-PCR, and time-lapse video microscopy were used to analyze *Prkar2b*-deficient oocytes. ***Results:*** *Prkar2b* is strongly expressed in the ovarian tissues, particularly in the growing follicle. During oocyte maturation, the highest expression of *Prkar2b* was during metaphase I (MI), with a significant decrease at metaphase II (MII). RNAi-mediated *Prkar2b* suppression resulted in MI-stage arrest during oocyte development, and these oocytes exhibited abnormal spindle formation and chromosome aggregation. Expression of other members of the PKA family (except for *Prkaca*) were decreased, and the majority of the PPP factors were also reduced in *Prkar2b*-deficient oocytes. ***Conclusion:*** These results suggest that *Prkar2b* is closely involved in the maturation of oocytes by controlling spindle formation and PPP-mediated metabolism.

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Introduction

Cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) is the chief mediator of cAMP in a variety of mammalian cells [1, 2]. PKA is a well-studied holoenzyme of the protein kinase family [3, 4], which functions as a hetero-tetramer form consisting of two catalytic (C) and two regulatory (R) subunits [5]. Murine PKA is comprised of four R subunits (RIα, RIβ, RIIα, and RIIβ) and two C subunits (Cα and Cβ) [3, 4]. When cAMP is bound to two sites of each R subunit, the C subunits separate, inducing phosphorylation of the Ser/Thr residues of the target protein [3, 6]. Included in the PKA family is cAMP-dependent type 2 regulatory subunit beta (*Prkar2b*), a form of RIIβ; these genes are primarily expressed in brain tissue, brown adipose tissue, and white adipose tissue [7, 8]. *Prkar2b* has the ability to regulate leukemia cell differentiation [9], plays a role in motoneurons [10] and alternative signaling of cAMP pathways [11], suppresses the effect of cAMP responsive element binding protein (CREB) activity in T cells [12], regulates adipocyte differentiation [13], and plays an oncogenic role in prostate cancer [14]. A recent study found that *Prkar2b*-deficient mice experience a decrease in white adipose tissue and an increase in resting metabolic activity, body temperature, and lipid hydrolysis [7]. However, only a small number of studies have investigated PRKAR2B signaling; the role of PRKAR2B in oocyte development has not yet been elucidated.

Various cellular processes are controlled by cAMP, including cell growth, differentiation, ion channel conductivity, synaptic release of neurotransmitters, and gene transcription [15], and is used as a second messenger in cells and for activation of different cAMP receptor molecules, which consist of ion channels and several PKA holoenzymes [15]. During oocyte maturation, cAMP-PKA signaling plays an important role in the regulation of meiosis [16]. A high concentration of cAMP activates PKA and arrests the oocyte in the GV stage, whereas decreased cAMP results in inactivation of PKA and resumption of meiosis [17]. Although PRKAR2B is part of the PKA family of proteins and is highly expressed in ovarian follicles, its role in oocyte maturation has not yet been determined.

Oocyte developmental processes are highly complex and dynamically regulated by various factors such as luteinizing hormone, anti-Müllerian hormone, and follicle stimulating hormone during folliculogenesis [18-21]. In ovarian follicles, the oocyte is present in the diplotene dictyate stage until development of the pre-ovulatory follicle [22-24]. At ovulation, the oocyte is in a state of germinal vesicle (GV) arrest. During oocyte maturation, the oocyte enters the GV breakdown (GVBD) stage and then serially undergoes further meiotic divisions (metaphase I [MI] and metaphase II [MII]) until secondary meiotic arrest at fertilization. As meiotic division of the oocyte progresses, the chromosomes and spindle migrate to the cortex, establishing asymmetric positioning and resulting in polarity of the oocyte, which is induced by signal pathway proteins including CDC42, RAC, ARP2/3 complex, and RASD1 [25-28]. Although several studies regarding significant processes during oocyte maturation have been performed, the detailed mechanisms of these processes, including the role of Prkar2b, have not yet been investigated. In this study, we determined the role of Prkar2b in mouse oocyte maturation.

Materials and Methods

Animals

All animal studies were carried out on 3- or 6-week-old ICR female mice and the total number of mice used in the study was approximately 100. Mice were housed in the Animal Care Facility of CHA University, maintained in a controlled environment, and supplied with a normal diet and water. Animal care was in compliance with the Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC NO.150047) of CHA University.

Table 1. Primer sequences and RT-PCR conditions

Genes	Accession No.	Primer sequences	Annealing temperature (°C)	Product size (bp)
Prkar2b (1) RT-PCR	NM_011158.3	For - GACGATGCAGAGTCCAGGAT Rev - TCTCCTGAAGGTCACCCTGT	60	384
Prkar2b (2) dsRNA	NM_011158.3	For - CAGGAGAACGAGCGCAAG Rev – GGCCAGTTCTCCAAAACTCC	60	549
PrKar 2b (3) confirm	NM_011158.3	For – GACGATGCAGAGTCCAGGAT Rev - CTCCTGAAGGTCACCCTGTC	60	384
Prkaca	NM_008854.5	For - GCAGATCGAGCACACTCTG Rev - GTAGGTGGGAGAACATCTCG	60	149
Prkacb	NM_011100.4	For - GGAGATCATCCTCAGCAAGG Rev - GCAGAAGGTCCTTGAGATCG	60	188
Prkar1a	NM_001313975.1	For - CAGAGCAGCCACTGTCAAAG Rev – TGCATCGGCTACTGTGAGAC	60	181
Prkar1b	NM_001253890.1	For - CGAGATGTACGTGCAGAAAC Rev – CACTGGGAGTTTGACTTCTG	60	171
Prkar2a	NM_008924.2	For - GCCAGGAATCAGACACGTTC Rev –CGTTATCCTCCTCTTCTTCATC	60	204
Gpi1	NM_008155.4	For - GTGGTCAGCCATTGGACTTT Rev – CTGGAAATAGGCAGCAAAGC	60	232
G6pd2	NM_019468.3	For - GCCTGGCATGTTCTTTAACC Rev – CAATCTTGTGCAGCAGTGGT	60	194
Rpe	NM_001310642.1	For - GGGCAGAAATTCATGGAAGA Rev – GTACTGCCAGACACAATCAT	60	152
Rpia	NM_009075.2	For - AAGATCGTGGCTGGTTATGC Rev – ACAGCCATTCGAAGTTCCAC	60	182
Tkt	NM_009388.6	For – TCCACCGTCTTTTACCCAAG Rev – CAAGGCCTCATGCAGAGTTA	60	222
Taldo1	NM_011528.4	For – TGACGCTCATCTCTCCCTTT Rev – GGAGATGGTGAGGAAGTCAC	60	221
Rbks	NM_153196.1	For - AGTGGCTGGAGCAAATCTGT Rev – GCGTGGCCTGTTAAAATCTC	60	261
Prps1	NM_021463.4	For - TTGATATCCCGGTGGACAAT Rev – ATAGCCACACGGTCCTTCAC	60	238
Gapdh	NM_008854.5	For - AGGTCGGTGTGAACGGATTTG Rev –TGTAGACCATGTAGTTGAGGT	60	123
H1foo	BC092294	For - GTTGCCGCAGAATCCAAGC Rev - GCTACAACTGATGTGCCCTGG	60	103

RT-PCR and qRT-PCR

Mouse tissues were isolated from 6-week-old mice and total RNA prepared using Trizol reagent (Invitrogen, USA) according to the manufacturer's manual. The complementary DNA (cDNA) was synthesized using the TOPscript™ cDNA Synthesis Kit (Enzynomics, Korea) according to the manufacturer's instruction. Reverse transcription polymerase chain reaction (RT-PCR) was carried out using Solg™ Taq DNA Polymerase (SolGent, Korea). PCR conditions and primer sequences are provided in Table 1. Quantitative real-time PCR (qRT-PCR) was performed using iQ™ SYBR Green Supermix (Bio-Rad Laboratories, USA). Experiments were normalized with H1 histone family member O, oocyte specific (*H1foo*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*).

Histology and immunostaining

Six-week-old mouse ovaries were fixed with 4% formaldehyde for 2 days and embedded in paraffin. Sectioned samples (5 μm thickness) were applied to slides (Fisher Scientific, USA) and dried overnight. Following deparaffinization with xylene, immunohistochemistry was performed as previously described using rabbit anti-PRKAR2B antibody (1:100 dilution, Abcam, UK, cat# ab75993) [29].

Immunofluorescence was performed on mouse oocytes. The oocytes were washed 3 times with 0.1% polyvinyl alcohol in phosphate-buffered saline (PVA-PBS) at room temperature. Oocytes were then fixed in fixation solution (4% paraformaldehyde in PVA-PBS) for 2 min and treated with permeabilization solution (0.5% Triton X-100 in PVA-PBS) for 20 min at room temperature. Oocytes were washed 3 times with PVA-PBS and incubated in 1% bovine serum albumin in PVA-PBS (1% BSA-PVA-PBS) at 4°C overnight. The oocytes were then transferred to blocking solution (3% BSA in PVA-PBS) for 1 h at room temperature. After blocking, oocytes were treated with anti-PRKAR2B (1:100 dilution, Abcam, cat# ab75993) and anti-α-tubulin (1:100 dilution, Santa Cruz Biotechnology, USA, cat# sc-8035) in 1% BSA-PVA-PBS at 4°C overnight. After washing 3 times in PVA-PBS, oocytes were incubated in secondary antibody conjugated to Alexa Fluor 546 immunoglobulin (IgG) (1:100 dilution, Invitrogen, cat# A11001 and A11010) in 1% BSA-PVA-PBS at 4°C for 1 h. After washing, oocyte nuclei and F-actin were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1:200 dilution, D3571, Invitrogen) and phalloidin (1:200 dilution, Invitrogen, cat# A12379), respectively, for 10 min at room temperature. Samples were then mounted with Malinol mounting medium (Muto, Japan).

Preparation of dsRNA

Partial sequences of the *Prkar2b* coding region were amplified using *Prkar2b*-specific primers (Table 1), and then cloned into pGEM-T Easy Vector (Promega, USA) for sense and anti-sense strands. *Prkar2b* clones were linearized with SpeI and sense or anti-sense RNAs were *in vitro* transcribed by T7 polymerase using the MEGAscript Kit (Ambion, USA). Sense and anti-sense strands were mixed, hybridized at 75°C for 5 min, and then slowly cooled to room temperature. The remaining nucleic acids were removed with DNase I and RNase A. After ethanol precipitation, double-stranded RNA (dsRNA) was dissolved in Tris-EDTA (TE) buffer (3.3 μg/μl) and stored at -80°C.

Collection and microinjection of oocytes and in vitro culture

Three-week-old ICR female mice were intraperitoneally injected with 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich, USA). After 43-45 h, mice were sacrificed and their ovaries isolated

and maintained in M2 medium (Sigma-Aldrich) with 0.2 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich). Ovaries were pierced with a 26-gauge needle and cumulus-enclosed oocyte complexes (COCs) collected. Cumulus cells (CCs) were then removed from COCs and GV oocytes obtained using a fine-bore pipette. CCs and granulosa cells (GCs) were collected and stored at -80°C. *Prkar2b* dsRNA (10 pl of 3.3 μg/μl) was microinjected into the cytoplasm of the GV stage oocytes (in M2 medium with 0.2 mM IMBX). A constant-flow system (FemtoJet; Eppendorf, Germany) was used for microinjection. Green fluorescent protein (*GFP*) dsRNA was used as a control. Microinjected GV oocytes were cultured in 5% CO_2 at 37°C in M16 medium (Sigma-Aldrich) with 0.2 mM IMBX in order to degrade the target transcript. After 8 h, culture medium was replaced with M16 medium containing 3 mg/ml BSA (Sigma-Aldrich) and oocytes were incubated in 5% CO_2 at 37°C for 16 h, after which the maturation rate was determined.

Oocyte mRNA isolation and cDNA synthesis

Oocyte messenger RNA (mRNA) preparation was accomplished using the Dynabeads mRNA DIRECT Kit (Dynal, Norway). Dynabeads oligo$(dT)_{25}$ beads (10 μl) were prewashed by combining them with lysis/binding buffer (100 mM Tris-HCl [pH 7.5], 500 mM LiCl, 10 mM EDTA, 1% LiDS, and 5 mM dithiothreitol [DTT]) (300 μl). Oocytes were resuspended in the lysis/binding buffer, and then the prewashed Dynabeads oligo$(dT)_{25}$ were added to the oocyte lysate and incubated for 3 min at room temperature. The samples were then subjected to a Dynal magnetic particle concentrator (MPC)-S, the RNA-binding beads washed with buffer A (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 1 M EDTA, 0.1% LiDS) followed by buffer B (10 mM Tris-HCl [pH 7.5], 0.15 M LiCl, 1 M EDTA) for 1 min each at room temperature, and the beads were then pelleted under a magnetic field for 1 min. The RNA was then eluted in 13 μl 10 mM Tris-HCl [pH 7.5] at 70°C for 3 min. Eluted mRNA and 1 μl oligo(dT) were combined and annealed at 70°C for 10 min using the TOPscript™ cDNA Synthesis Kit (Enzynomics). After annealing, TOPscript™ Reverse Transcriptase (200 U/ul), 10× TOPscript™ RT Buffer, dNTP mixture (2 mM each nucleotide), and RNase inhibitor (40 U/ul) were added and cDNA was synthesized at 42°C for 60 min. The reaction was halted by incubating at 95°C for 5 min.

Time lapse video microscopy

Time lapse video microscopy (JuLITM, Digital Bio, Korea) was used to determine the phenotype of the oocytes. Images of the oocytes in a 37°C CO_2 incubator were captured every 5 min for 36 h.

Statistical analyses

The experimental data were presented as the mean standard error of the mean (SEM). Real-time PCR data were analyzed using a one-way analysis of variance (ANOVA) for statistical evaluation. P-values were considered statistically significant.

Results

Expression of Prkar2b mRNA in mouse tissues

Because the dramatically changed genes during primordial follicle activation to primary follicles means that which play critical roles in ovarian follicle development, the expression levels of the whole genes in dormant follicles and growing follicles are analyzed using microarray analysis. Then, we selected the *Prkar2b*, a novel and less studied regulator, among the 947 up-regulated genes in activated follicles. To compare the expression level of *Prkar2b* mRNA in mouse tissues, RT-PCR and qRT-PCR were performed using various tissues such as small intestine, stomach, kidney, spleen, heart, liver, lung, brain, ovary, uterus, and testis from 6-week old mice. The results showed that *Prkar2b* was highly expressed in the ovary (Fig. 1A and 1B). Additionally, to examine the expression levels of other PKA families in these tissues, alternative isoforms of PKA such as *Prkar2a*, *Prkar1a*, *Prkar1b*, *Prkaca*, and *Prkacb* were measured using RT-PCR, and the results indicated that only *Prkar2b* was highly expressed in mice ovary (Fig. 1A). To further study of Prkar2b, we tested the anti-Prkar2b antibody by western blotting using flag-conjugated Prkar2b cloning vector in Hek-293T cells. The results showed that both anti-flag and anti-Prkar2b detected the proteins (Fig. 1D). To

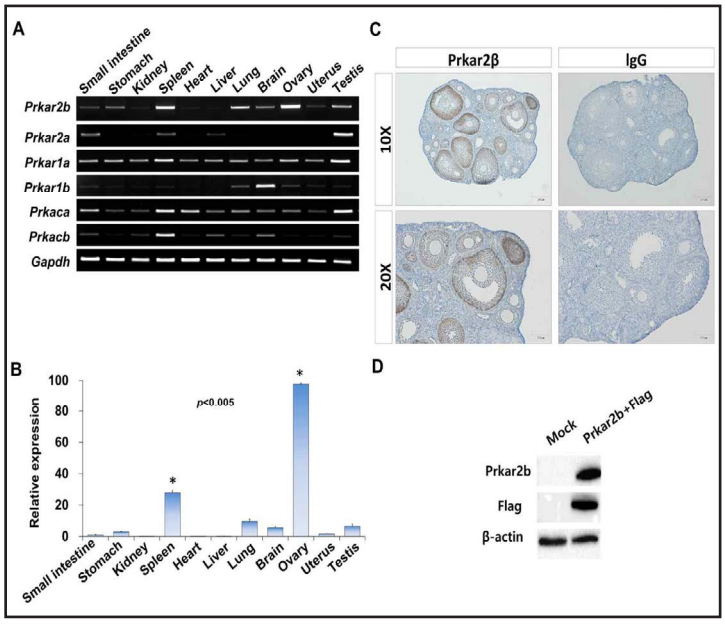

Fig. 1. Expression of protein kinase A family members mRNA and protein in mouse tissues. The mRNA was isolated from the tissues of 6-week-old mice. (A-B) RT-PCR and qRT-PCR analysis of protein kinase A family members transcript in mouse tissues (small intestine, stomach, kidney, spleen, heart, liver, lung, brain, ovary, uterus, and testis). Expression levels were normalized with Gapdh mRNA. Data are expressed as mean±SEM. (C) Immunohistochemical analysis of PRKAR2B in 6-week-old mouse ovary. Hematoxylin was used for nuclei staining. (D) Antibody test of Prkar2b for the experiment. Mouse Prkar2b gene was cloned into Flag-tagged expression vector, and then transfected to hek293T cells for 48h. Western blot analysis showed that both anti-Prkar2b antibody and anti-flag antibody detected the protein product of cloned Prkar2b-flag.

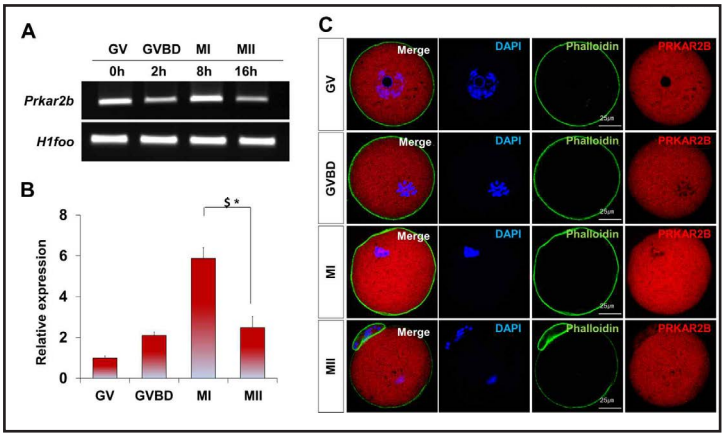

Fig. 2. Expression of Prkar2b mRNA and protein during in vitro maturation of the oocyte. Oocytes were collected at 0 h (germinal vesicle, GV), 2 h (germinal vesicle break down, GVBD), 8 h (metaphase I, MI), and 16 h (metaphase II, MII) after incubation in M16 medium in 5% CO2 at 37°C. (A-B) RT-PCR and qRT-PCR analysis of Prkar2b at GV, GVBD, MI, and MII stages of oocyte development. H1foo was used as an oocyte-specific internal control to normalize expression levels. Data are expressed as mean±SEM. The Student t-test was applied to calculate p-value. *p<0.05, $p<0.05. (C) Immunofluorescence staining of each stage of oocyte development using anti-PRKAR2B antibody. DAPI and phalloidin were used to detect chromosomes and F-actin-mediated oocyte membrane, respectively. Images were analyzed using a confocal microscope.

identify whether Prkar2b is expressed in stage of follicles of ovary, immunohistochemistry was performed. The results indicated that Prkar2b was specifically expressed in grown follicles as graffian and pre-ovulatory follicles in mouse ovary (Fig. 1C). These results suggest that Prkar2b may play an important role in the maturation process of oocyte.

Expression of Prkar2b during oocyte maturation

To examine whether *Prkar2b* has a critical role during oocyte maturation, *Prkar2b* expression levels were analyzed in the stages of oocyte maturation as GV, GVBD, MI, and MII by *in vitro* maturation experiment. RT-PCR and qRT-PCR showed that *Prkar2b* was significantly increased in the MI stage, whereas recovered during MII stage (Fig. 2A and 2B).

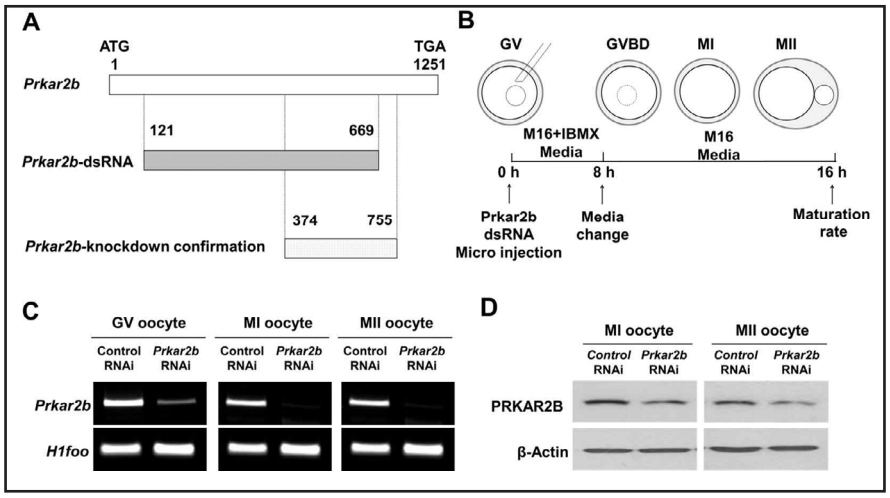

Fig. 3. Construction of a dsRNA-mediated Prkar2b knockdown system. (A) Schematic diagram of the mouse Prkar2b coding region. The dsRNA (Prkar2b-dsRNA; location 121-669) was used for knockdown of the Prkar2b gene. A pair of primers was used to confirm knockdown of Prkar2b (location 374-755). (B) The oocyte microinjection processes. The dsPrkar2b was injected into the GV stage of the oocyte, then incubated with IBMX containing M16 media for 8 h. After incubation, the media was replaced with M16 media to induce in vitro maturation of oocytes for 16 h. (C) RT-PCR analysis of Prkar2b expression in control or Prkar2b RNAi-injected GV, MI, and MII oocytes. H1foo was used as an oocyte-specific internal control. (D) Western blot analysis of PRKAR2B expression in MI and MII stage oocytes. Approximately 500 microinjected oocytes were loaded for each group. β-actin was used as an internal control.

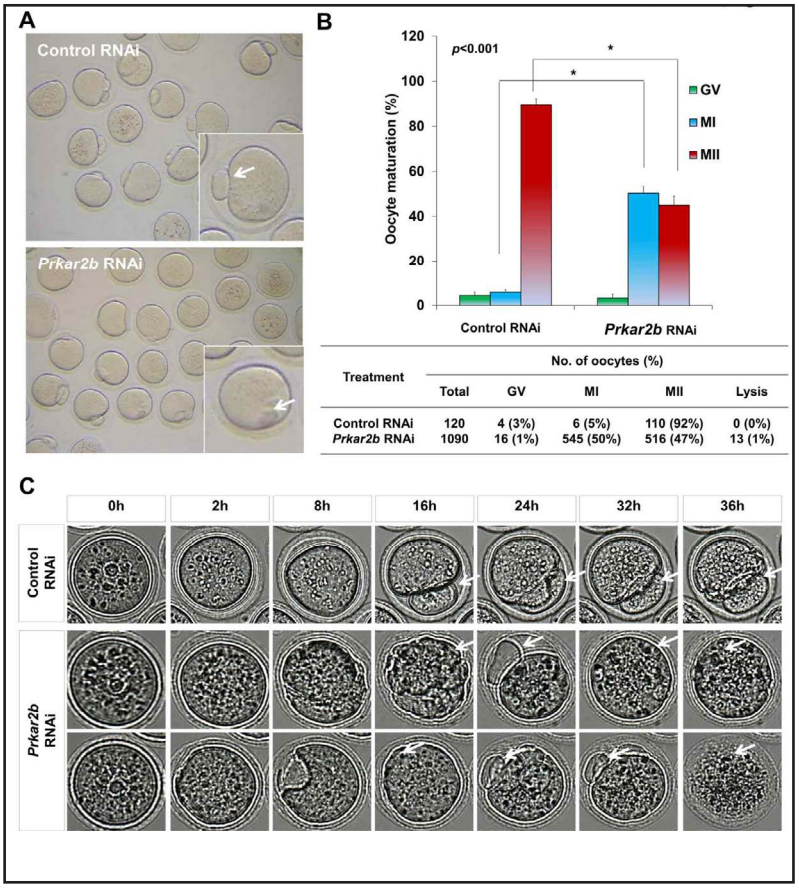

Fig. 4. Failure of meiotic division in Prkar2b-deficient oocytes. After microinjection of dsPrakr2b and ds-GFP RNA, oocytes were incubated for 16 h to induced in vitro maturation. (A) Microphotographs of control and dsPrkar2b-injected oocytes after 16 h. The polar body asymmetrically formed at oocytes in the control group. (B) Oocyte maturation rate of control and Prkar2b RNAi-injected oocytes. The number of Prkar2b RNAi oocytes significantly increased at MI and decreased at MII, compared to the control. (C) Time lapse video analysis of oocyte meiotic division and formation of the polar body during MII of an oocyte in a time-dependent manner.

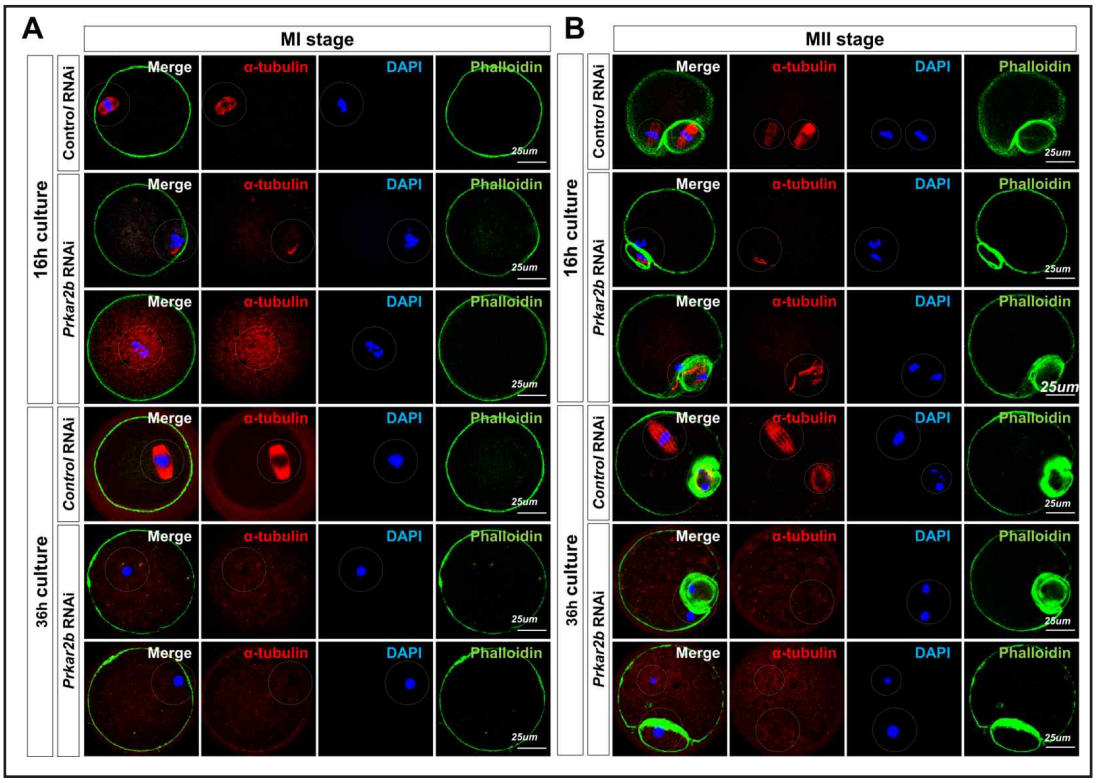

Fig. 5. Spindle formation in oocytes during MI and MII following injection of control or Prkar2b RNAi. (A) Immunofluorescence of control (dsGfp) and dsPrkar2b-injected oocytes during MI. (B) Immunofluorescence of control (dsGfp) and dsPrkar2b-injected oocytes during MII. Each stage of metaphase was divided into 16 and 36 h. The α-tubulin (red) is a marker of the spindle for chromosome division, and phalloidin (green) is a marker of F-actin, a structural component of the oocyte membrane. DAPI was used to detect chromosomes. The image was analyzed by confocal microscope.

To further confirm the expression of PRKAR2B protein in the stage of oocyte maturation, immunofluorescence was performed using anti-PRKAR2B antibody. As same above, the PRKAR2B expression was high in the MI stage of oocytes (Fig. 2C). These results indicate that Prkar2b may be essential to work on MI stage of oocyte.

Effects of Prkar2b knockdown on oocyte maturation

To identify the effect of inhibition of *Prkar2b* on oocyte maturation, double strand RNA-mediated *Prkar2b* (*dsPrkar2b*) knockdown system was constructed for microinjection of oocyte. *Prkar2b* coding sequences were selected and the primers confirming knockdown were designed (Fig. 3A) and the process of the dsRNA microinjection was performed with control (*dsGFP*) and *dsPrkar2b* (Fig. 3B). After incubation for 16 hours, *Prkar2b* knockdown was confirmed during stage of oocyte maturation by RT-PCR and western blot analysis (Fig. 3C and 3D). Following dsRNA-injected oocytes maturation for 16 h, the morphology and polar body formation were analyzed. Most of control RNAi-injected oocytes were fully matured to MII stage, whereas about half of *Prkar2b* RNAi-injected oocytes were arrested at MI stage (Fig. 4A and 4B). Time lapse video also showed that Prkar2b deficient oocytes were not developed and were no polar body formation compared with the control oocytes (Fig. 4C). Taken together, these results mean that deficiency of Prkar2b suppresses development of MII stage of oocyte, and Prkar2b has a critical role during polar body formation on oocyte meiotic metaphase.

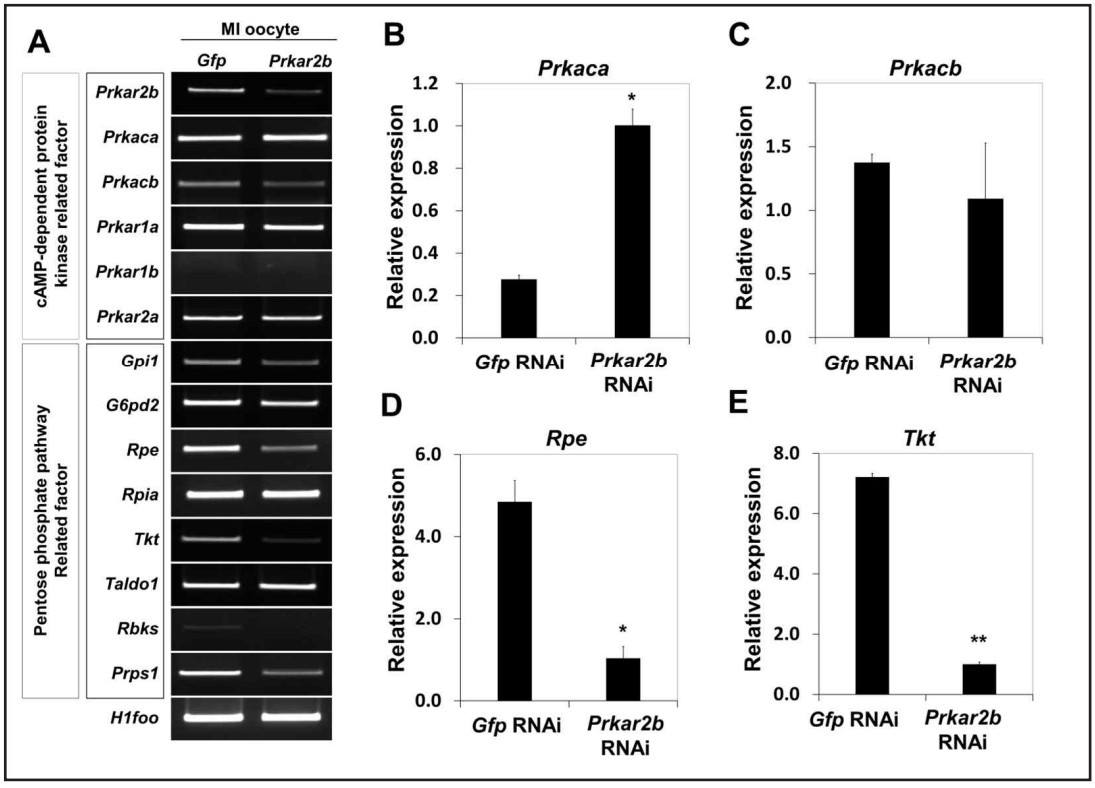

Fig. 6. Expression of PKA and the PPP-related factors in Prkar2b-knockdown oocytes. (A) RT-PCR using control and Prkar2b dsRNA-injected MI oocytes. PKA family genes, including Prkaca, Prkacb, Prkar1a, Prkar1b, Prkar2a, and Prkar2b, and PPP-related factors, including Gpi1, G6pd2, Rpe, Rpia, Tkt, Taldo1, Rbks, and Prps1 were investigated. (B-C) qRT-PCR analysis to confirm the RT-PCR of Prkaca and Prkacb. (D-E) qRT-PCR analysis of Rpe and Tkt expression in Prkar2b-deficient cells. Data are expressed as mean±SEM. The Student t-test was applied to calculate p-value. *p<0.05, **p<0.05.

Role of Prkar2b on spindle and polar body formation during oocytes maturation

To further confirm how *Prkar2b* affects polar body formation of oocyte meiosis, immunofluorescence was performed using anti-alpha tubulin antibody, a marker of spindle fiber, at MI and MII arrested oocytes after *Prkar2b* RNAi microinjection. In the MI stage for 16h to 36h, the spindle was formed and the chromosomes were aligned to split, whereas in the Prkar2b suppressed oocyte, there was no spindle formation and the chromosomes were aggregated (Fig. 5A). In the MII stage, the polar body was clearly formed in the control group, while normal division by forming polar body did not occur in Prkar2b-deficient oocytes (Fig. 5B). These results suggest that *Prkar2b* is essential for spindle formation during oocyte maturation.

Effects of Prkar2b knockdown on the expression of cAMP-dependent protein kinase related genes and oocyte metabolism related genes.

To examine the effect of *Prkar2b* in the expression of PKA subfamilies, RT-PCR and qRT-PCR were performed. *Prkar1b* and *Prkar2a* were showed no significant changes between control and *Prkar2b* RNAi treated oocytes. While *Prkacb* and *Prkar1a* were decreased, *Prkaca* expression was significantly increased in *Prkar2b*-deficient oocytes (Fig. 6A-C). Because pentose phosphate pathway (PPP) was known to control maturation and development [30], expression level of PPP-related factors, such as *Gpi1*, *G6pd2*, *Rpe*, *Rpia*, *Tkt*, *Taldo1*, *Rbks*, and *Prps1*. The expression levels of the majority of PPP-related genes were reduced in *Prkar2b*-deficient oocytes, especially in *Rpe* and *Tkt* cases (Fig. 6A, 6D-E). These results indicated that Prkar2b regulate oocyte maturation through controlling PPP.

Discussion

Oocyte development depends on a variety of signal pathways, such as the dynamic cellular process of asymmetric meiosis. Although there are several studies regarding this signaling pathway mechanism, the oocyte maturation process has not been thoroughly described. The role of PRKAR2B (RIIβ), a regulatory isoform of PKA, in oocyte maturation, has not been studied, even though PKA signaling is known to play an important role in oocyte maturation [31-34]. Here, we investigated the expression of PRKAR2B in ovarian follicles and its critical role in oocyte development.

The few studies that explore the effects of Prkar2b signaling do so using immune cells [9, 12], nerve cells [10], and fat cells [13]. As our microarray analysis indicated that expression of *Prkar2b* was dramatically increased during dormant follicle maturation (*data not shown*), we hypothesized that PRKAR2B, a novel isoform of PKA, would be a critical regulator of follicle maturation and oocyte dormancy. As expected, we found that *Prkar2b* was more strongly expressed in large follicles, such as antral and pre-ovulatory follicles, than in small, growing follicles, such as primary and primordial follicles, and is also highly expressed in follicle GCs (Fig. 1C). These results suggest that PRKAR2B may be essential to follicle maturation, as well as play a role in hormonal regulation of GCs. However, further studies are needed to confirm the involvement of PRKAR2B in hormonal regulation of the reproductive system through activating ovarian GCs.

In this study, we focused on the role of PRKAR2B in oocyte maturation, as the PKA family of proteins is known to regulate maturation of oocytes [34]. Oocyte maturation involves a variety of complex procedures that may include specific factors such as PRKAR2B signaling, as well as the immune and nervous systems. *Masui* cited that cytostatic factor and maturation-promoting factor, major regulators of the cell cycle in oocyte maturation, are closely related to protein kinases, and the phosphorylated proteins are critical for oocyte maturation through interaction with various factors [28, 35]. The novel protein kinase, PRKAR2B, is a possible candidate for a major regulator of the cell cycle of oocyte meiosis.

Newborn female ovaries contain their total number of oocytes, which are arrested at the diplotene stage of meiosis I until puberty, when luteinizing hormone surges induce the continuation of meiosis. In meiosis I, a diploid cell divides into two haploid cells; one becomes the secondary oocyte and the other forms the first polar body. These processes are MI to MII. We found that PRKAR2B expression peaked at MI stage of oocyte development, and then dropped to basal levels at MII stage (Fig. 2). Thus, it can be inferred that PRKAR2B plays an important role in oocyte division. These results are consistent with previous reports that various factors, including PKA family proteins, play an important role in cell division of the oocyte meiosis stage [36, 37]. We also confirmed that PRKAR2B deficiency [38] results in division failure of the oocyte, as determined using time lapse video recording for 36 h (Fig. 4). Furthermore, *Prkar2b* RNAi-injected oocytes could not form the polar body through abnormal division, thus we focused on spindle formation to determine the mechanism by which this phenomenon was triggered. Immunofluorescence staining of PRKAR2B-deficient oocytes showed that the α-tubulin assembly involved in formation of the spindle did not proceed in the MI stage. In addition, PRKAR2B deficiency also triggered abnormal formation of the secondary polar body (Fig. 5). These results indicate that one of the roles of Prkar2b is to regulate spindle formation in oocyte meiosis.

In most organisms, the PPP is essential for the synthesis of nicotinamide adenine dinucleotide phosphate (NADPH), as well as five-carbon sugars [39], nucleotides, fatty acids, and neurotransmitters [30]. Recently, several studies reported that the PPP is involved in the metabolic pathways of various cells, including cancer cells and oocytes [30, 40]. TKT, an enzyme in the PPP, plays a critical role in energy production and nucleic acid synthesis during embryonic development [41]. TKT is involved in several diseases, including Alzheimer's disease, diabetes, eye transparency, and cancer [42-44]. We found that *Prkar2b* deficiency results in down-regulation of *Tkt* mRNA expression in the MI stage of oocytes, suggesting that PRKAR2B is involved in the PPP. Furthermore, the PPP-related genes, such as *Gpi1*, *Rpe*,

and *Prps1*, were significantly decreased when an oocyte is deficient in *Prkar2b* (Fig. 6A, 6D-E). Further studies are needed to understand the correlation between the PPP-mediated metabolism and PRKAR2B signaling pathway.

Conclusion

We demonstrated that *Prkar2b* is highly expressed in large follicles, which increased during the MI stage of the oocyte. *Prkar2b* deficiency triggers failure of oocyte meiotic division by suppressing formation of the spindle. In addition, PRKAR2B plays important roles in oocyte maturation through regulation of the PPP. It is the first evidence showing one of isoforms of PKA is important for oocyte maturation. The limitation of this study is that there are other isoforms expressed in the ovary. In fact, we didn't examine other's expression and location in the ovary. Also, the phenotype by knockdown of target gene will be leaky or compensated by other isoforms during oocyte maturation. We observed maturation of some oocytes under knockdown system. Therefore, we need further studies using several combinatory knockdown with *Prkar2b* and other members to investigate any cooperation and specificity among them during oocyte maturation. It will be give us better understanding for clinical application during *in vitro* fertilization in the future.

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Disclosure Statement

The authors declare no competing financial interests.

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