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Original article

Effects of CDK inhibitors on the maturation, transcription, and MPF activity of porcine oocytes

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ABSTRACT

In mammals, cyclin-dependent kinases (CDKs) are involved in regulating both the cell cycle and transcription. Although CDK1 is known to act as the kinase subunit of maturation-promoting factor (MPF), the roles of the other CDKs in mammalian oocyte maturation are not yet understood. Here, we show that inhibition of various CDKs by small molecule inhibitors has different effects on the maturation and transcriptional activity of pig oocytes in vitro. Inhibition of CDK1 did not significantly affect cumulus cell expansion, but its kinase activity was necessary for germinal vesicle breakdown (GVBD). The inhibitions of CDK2, CDK4, or CDK6 had no effect on cumulus expansion or GVBD. The catalytic activity of CDK7 was crucial for GVBD but less important for cumulus expansion, whereas inhibition of CDK9 severely blocked both cumulus cell expansion and GVBD. CDK1, -2, -4, and -6 appeared to be dispensable for nuclear transcription, as their inhibitions did not affect nascent RNA production in oocytes. However, inhibition of CDK7 or CDK9 dramatically decreased the transcriptional activity in oocytes. Finally, we found that the GVBD arrest triggered by CDK9 inhibition was not due to altered MPF activity, but rather the inhibition of transcription. Overall, our results show that CDK7 and CDK9 are important for the nuclear maturation and transcriptional activity of pig oocytes.

1. Introduction

Mammalian oocytes are transcriptionally active, producing and storing maternal factors (mainly mRNAs and proteins) that support the early stages of embryonic development. Studies have shown that treating mammalian oocytes with transcription inhibitors triggers defects in their subsequent growth and maturation. For example, treatment of mouse oocytes with the positive transcription elongation factor b (P-TEFb) inhibitor, DRB (5,6-dichloro-1-beta-D-ribofuranosy-benzimidazol), leads to failure of germinal vesicle breakdown (GVBD) [1]. The oocytes of large animals have also been found to arrest at the GV stage when treated with transcription inhibitors. Bovine oocytes, for instance, arrest at the GV stage in the presence of α-amanitin [2] or DRB [3], while porcine oocytes show GV arrest when they are exposed to α-amanitin in vitro [4]. Moreover, protein synthesis inhibitors (e.g., cycloheximide) cause GV arrest in both bovine and porcine oocytes [2,5,6]. Therefore, it seems that both mRNA transcription and protein synthesis are needed for oocyte maturation in mammals.

The numerous maternal proteins that are produced and stored during oocyte growth include various protein kinases. Among them are the cyclin-dependent kinases (CDKs), which are predominantly involved in cell cycle regulation [7]. The meiotic maturation of an oocyte involves dynamic waves of protein phosphorylation that are partially regulated by the activation of CDKs. The first and best-characterized oocyte-maturation-associated CDK is CDK1. This catalytic subunit of maturation-promoting factor (MPF) was initially obtained from frog egg extracts [8,9] and is activated by its binding to a cyclin subunit; this activation triggers GVBD and the transition from GV stage to metaphase I. CDK1 is activated by the phosphorylation of its Thr161 residue by CDK-activating kinase (CAK) [10,11]. For meiotic prophase I arrest to occur, CDK1 must be inactivated; this occurs mainly via the phosphorylations of the Thr14/Tyr15 residues of CDK1 by the membrane associated tyrosine/threonine 1 (MYT1) and Wee1B kinase, respectively [12,13].

Among the other CDK family members, CDK2 is known to form a complex with its regulatory partners, cyclin E or cyclin A, to regulate several cell cycle events [14], such as the phosphorylation of retinoblastoma protein (Rbp), which supports the transition from G1 to S phase. Cdk2-knockout mice are viable, however, and it seems that CDK1 compensates for their lack of CDK2 activity [15,16]. Both CDK4 and CDK6 bind to cyclin Ds and act in the G1 phase of the cell cycle. These two CDKs also phosphorylate Rbp and induce DNA synthesis and

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entry into S phase [17]. CDK7 or CDK9, in contrast, are mainly responsible for regulating transcription via their abilities to phosphorylate the carboxyl-terminal domain of the largest subunit of RNA polymerase II (Pol II CTD). CDK7 acts as the catalytic subunit of general transcription factor IIH (TFIIH); it binds to cyclin H and phosphorylates the Ser5 residue of the heptapeptide, YSPTSPS, of the Pol II CTD, to promote transcription initiation [18]. CDK9, which acts as the kinase subunit of the positive transcription elongation factor, P-TEFb, binds to cyclin Ts and phosphorylates the Ser2 residue of the above mentioned heptapeptide to drive transcription elongation [19].

Development of a bidirectional communication between the oocyte and its surrounding cells has been shown to have a crucial role in regulation of the oocyte nuclear maturation [20,21]. This communication is directed via transmembrane protein structures called gap junctions. Studies using radioisotopes have revealed that a diversity of macromolecules including amino acids, carbohydrates and nucleotides can be transferred via gap junctions from cumulus cells to oocytes in mouse [22]. Also, oocyte-secreted factors (OSF) can traverse through gap junctions from oocytes to cumulus cells and regulate their function [23]. The main regulatory factor introduced by cumulus cells to oocytes is cyclic adenosine mono phosphate (cAMP). This nucleotide plays a crucial role in regulation of oocyte maturation. Before ovulation, it prevents the spontaneous enter of the GV oocyte to meiosis, and after ovulation, it triggers the resumption of meiotic events. Removal of the oocytes from the surrounding somatic cell/follicles disrupts the cAMP transfer and reduces the internal level of this nucleotide in the oocyte which in turn induces CDK1/cyclin B activity and eventually leads to resumption of meiosis in GV arrested oocytes [24,25]. Despite the fact that the role of CDK1 and its relation to cAMP is clearly understood in oocyte meiosis, the potential roles of this CDK and other CDKs are not investigated in cumulus cell function and regulation.

Although CDK1 is known to contribute to regulating oocytes, the oocyte-related roles of the other CDKs have not yet been explored in detail. Very limited studies have shown that CDK2 [26,15] and CDK4 [26,27] are important in mammalian oocytes, and we recently showed that CDK9 activity is required for the proper maturation of mouse [28] and pig [29] oocytes in vitro. Here, we used immunocytochemical analyses to explore the effects of various CDK small molecule inhibitors on the maturation and transcriptional activity of pig oocytes. We show that although CDK1 activity is crucial for GVBD, it is dispensable for cumulus expansion and the transcriptional activity of oocytes. Our results demonstrate CDK7 and CDK9 as crucial kinases for nuclear transcription and cumulus expansion, and highlight the key roles of these CDKs in the processes that regulate pig oocyte maturation.

2. Materials and methods

2.1. Ethics

All animal care and use procedures were approved by the Institutional Animal Care and Use Committee of Chungnam National University.

2.2. Reagents and antibodies

Reagents and antibodies have been described previously [29]. RO-3306 (SML0569), palbociclib/PD-0332991 isethionate (PZ0199) and 5-fluorouridine (FU; F5130) were purchased from Sigma and dissolved in DMSO or sterile double-distilled water to form stock solutions. CDK2 inhibitor II (sc-221409) was purchased from Santa Cruz Biotechnology and dissolved in DMSO. LDC000067 (S7461), BS-181 (S1572), dinaciclib (S2768), and JNJ-7706621 (S1249) were purchased from Selleck Chemicals and dissolved in DMSO to form 0.5-mM stock solutions. The antibodies against Cdc2 p34 (F-5), lamin A/C (636), and p-Cdc2 p34 (Thr14/Tyr 15) were purchased from Santa Cruz and diluted 1:50. The monoclonal antibody anti-mitotic proteins, MPM-2 (16-220), was purchased from Millipore. The monoclonal antibody against BrdU (Sigma, B2531) was diluted 1:200. The utilized secondary antibodies were conjugated with FITC or Texas Red, and were purchased from Santa Cruz.

2.3. Porcine oocyte collection, culture, and treatment

About three hundred porcine ovaries (in total) were collected from prepubertal commercial gilts at a local abattoir (NH Livestock Cooperation Association, Nonsan City, Chungnam Province, Korea) and transported to the laboratory at 30–35 °C. As previously described [29] the ovaries were washed three times with warm PBS containing 100 IU/mL penicillin and 50 μg/mL streptomycin, and then stored in a water bath at 37 °C until use. COCs were aspirated from ovarian follicles (2–6 mm in diameter) and washed three times with TL-HEPES containing 0.1% (w/v) PVA. The COCs were then subjected to either experimentation or in vitro maturation. For maturation, the COCs were transferred to 500 μL of mineral-oil-covered maturation medium in a four-well multidish (Nunc, Roskilde, Denmark) with about 20 COCs per well and incubated for 44 h at 38.5 °C in an atmosphere of 5% CO2 at maximum humidity. The medium used for the in vitro maturation of COCs comprised bovine serum albumin (BSA)-free M199 supplemented with 10% (v/v) porcine follicular fluid (FF), 0.57 mM L-cysteine, 2% (v/v) basal medium Eagle amino acids, 1% (v/v) minimum essential medium non-essential amino acids, 0.5 μg/mL LH, 0.5 μg/mL FSH, 10 ng/mL epidermal growth factor, 75 μg/mL penicillin G, and 50 μg/mL streptomycin. After 22 h in maturation culture, the COCs were washed three times and transferred to 500 μL of hormone-free basic medium for an additional 22 h of culture. For all experiments, COCs were treated with 10 μM of RO-3306, 20 μM of CDK2 inhibitor II, 5 μM of palbociclib, 10 μM of BS-181, 10 μM LDC67, 250 nM of dinaciclib, or 250 nM of JNJ-7706621.

2.4. Experimental design

2.4.1. Experiment 1: the effects of CDK inhibitors on the cumulus expansion

The objective of this experiment was to evaluate the effect of various CDK inhibitors on cumulus cell expansion after 22 h and 44 h of in vitro maturation. COCs were obtained from medium-to-large size antral follicles, but only fully grown COCs (~250 μm) were chosen for the experiment and were randomly placed into the culture media with or without the inhibitors. After 22 h of culture the COCs were examined for their diameter. The experiment was repeated in the same conditions but this time the COCs were cultured for 44 h (two sequential 22 h with and without gonadotropins respectively) and again were measured for their diameter.

2.4.2. Experiment 2: the effects of CDK inhibitors on nuclear maturation

The aim of this experiment was to examine the effect of CDK inhibitors on progression of GV oocytes to metaphase I as well as to understand the reversibility of the inhibitors. COCs were collected from large antral follicles. Only fully grown COCs were chosen because they show highest competency to undergo GVBD and progress to metaphase I. The COCs were randomly placed in media with or without the inhibitors for 22 h and then were examined for their nuclear situation by confocal microscopy. The experiment was repeated in the same condition but this time after 22 h, the COCs were released from the inhibitors and cultured for more 22 h and then were again examined by confocal microscopy.

2.4.3. Experiment 3: the effect of CDK inhibitors on nuclear transcription

The aim of this experiment was to understand the effect of CDK inhibitor on the production of nascent RNAs in transcriptionally active oocytes. Because fully grown oocyte with surrounded nucleolus (SN) chromatin configuration show virtually no transcriptional activity, we chose growing oocytes from small antral follicle in which the non-

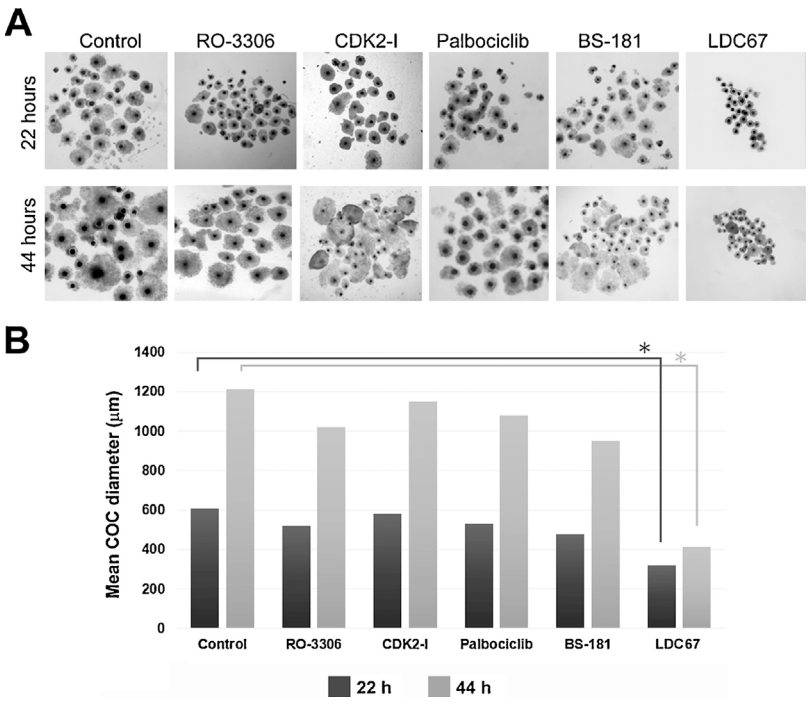

Fig. 1. The effects of various CDK inhibitors on the expansion of cumulus cells in vitro. Pig COCs were collected from large antral follicles and placed in maturation medium containing small molecule inhibitors against various CDKs. After 22 h and 44 h, the diameters of the COCs were analyzed using a graded light microscope. (a) Light micrograph of the COCs. (b) The experiment was repeated three times. The values indicate the mean numbers. A p value less than 0.05 was taken as indicating a statistically significant difference, and is indicated by an asterisk.

surrounded nucleolus (NSN) configuration occurs in higher proportion. The COCs were placed randomly in media with or without the inhibitors for 6 h and then were exposed to fluorouridine and subjected to immunostaining to label nascent RNAs.

2.4.4. Experiment 4: the effect of CDK inhibitors in MPF activity

The objective of this experiment was to understand the relationship between the inhibitory effect of CDK inhibitors and the kinase activity of CDK1. CDK1 phosphorylates numerous proteins that share the peptide motifs, Leu-Thr-Pro-Leu-Lys (LTPLK) and Phe-Thr-Pro-Leu-Gln (FTPLQ) [30], and MPM-2 specifically recognizes these motifs when they are phosphorylated on their Thr residues. To this, COCs were obtained from large antral follicles in which MPF activity has been elevated and the oocyte are competent to undergo GVBD. The COCs were placed randomly in media with or without the inhibitors for 6 h and then were examined for their MPF activity or for the level of inhibitory phosphorylation of CDK1.

2.5. Immunostaining and nascent RNA labeling

For immunofluorescence staining, oocytes were washed twice with 0.1% (w/v) PVA in PBS and fixed with 2% (v/v) formaldehyde in PBS for 15 min at room temperature. The oocytes were then permeabilized for 30 min with 0.5% (v/v) Triton X-100 in PBS, and washed for 10 min with 100 mM glycine in PBS (to inactivate free aldehyde groups). Nonspecific binding sites were blocked with 3% (w/v) BSA for 20 min, followed by a 5-min exposure to PBG [PBS containing 0.5% (w/v) BSA and 0.1% (w/v) gelatin from the skin of a cold-water fish (Sigma)]. The primary antibodies were applied in PBG for 16 h at 4 °C. The cells were then washed four times (5 min per wash) in PBG, and incubated with the appropriate secondary antibodies for 1 h in PBG at room temperature. Finally, the cells were washed (5 min per wash) twice in PBG and twice in PBS. For microscopic observation, oocytes were deposited on slides and mounted under coverslips using Vectashield mounting medium containing DAPI (Vector Laboratories). In situ run-on transcription was performed as previously described [29]. Briefly, oocytes were rinsed with PBS/PVA and incubated in 5 mM 5-FU for the indicated times. For immunolabeling, cells were incubated overnight with mouse monoclonal anti-BrdU, washed, and further incubated for 1 h with Texas Red-conjugated mouse IgG.

2.6. Confocal microscopy

Images were captured using a Zeiss scanning laser confocal microscope running the Zeiss LSM Image Browser software [28]. Serial optical sections (Z-series) were collected at 0.5-μm intervals. The Z-stacks were captured, and images depicting the staining patterns and intensities of all nuclear areas were generated. All oocyte and embryo samples were prepared and processed simultaneously prior to fluorescence intensity measurements. The laser power was adjusted to ensure that the signal intensity was below saturation for the specimen that displayed the highest intensity, and all images were scanned at this laser power.

2.7. Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) followed by the Fisher's protected least significant difference (LSD) test using the SPSS. At least three replicates were performed for each experiment.

3. Results

3.1. Effects of CDK inhibitors on cumulus cell expansion

To investigate the potential roles of CDKs in porcine oocyte maturation, we first collected COCs from medium-to-large antral follicles and treated them with various CDK small molecule inhibitors for 44 h. We analyzed cumulus cell expansion at 22 h and 44 h using a graded-lens light microscope (Fig. 1A). We found that the cumulus cells of non-treated COCs reached average diameters of 600 μm and 1200 μm after 22 h and 44 h of in vitro culture, respectively. The average diameters of COCs treated with the CDK1 inhibitor, RO-3306, were slightly decreased to 520 μm and 1020 μm, respectively. CDK2 Inhibitor II (CDK2-I) had no clear effect on cumulus cell expansion at 22 h or 44 h of in vitro culture, as the average diameters of the COCs were 582 μm and 1150 μm respectively after 22 h and 44 h of culture. The CDK4/6 inhibitor, palbociclib (PD-0332991), slightly decreased the average COCs

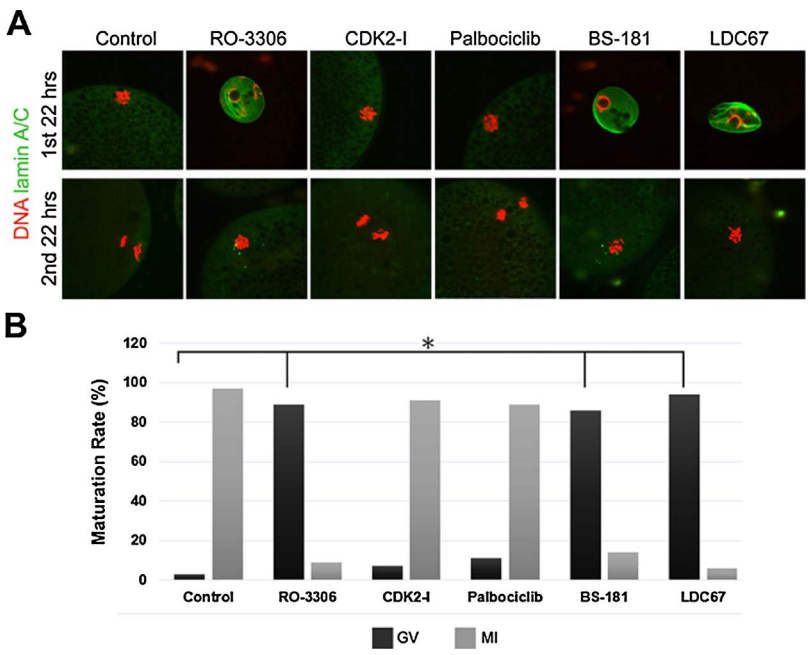

Fig. 2. The effects of CDK inhibitors on the nuclear maturation of pig oocytes. (a) COCs were collected from large antral follicles and treated with CDK inhibitors for 22 h. The COCs were then released from the inhibitors, cultured under normal conditions, denuded, and subjected to immunostaining with an anti-lamin A/C antibody (green) to detect the germinal vesicle (GV) situation. DNA was counterstained with DAPI and is pseudo-colored red for better visualization. (b) The percentages of GVs and prometaphase chromosomes were counted under a confocal microscope. The experiment was repeated three times. The graph depicts the mean values; a p value less than 0.05 was taken as indicating a statistically significant difference (*). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

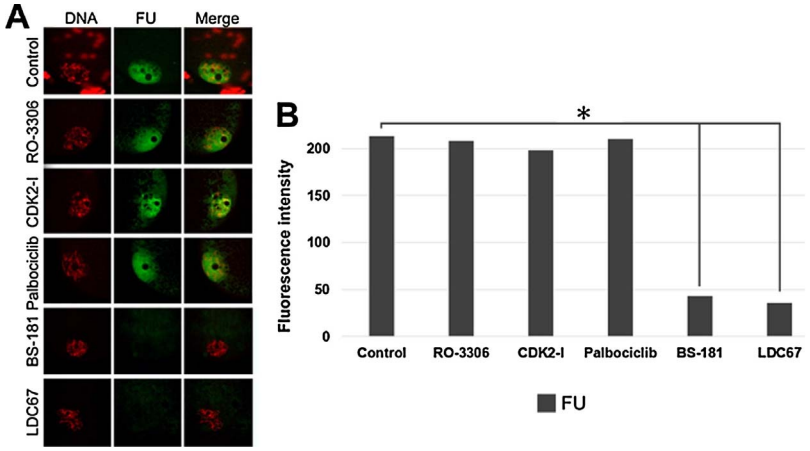

Fig. 3. The effect of CDK inhibitors on the nuclear transcription of oocytes. COCs were collected from small antral follicles for analysis of transcriptional activity and treated with CDK inhibitors for 6 h. (a) For the last hour of treatment, the COCs were transferred to the same medium containing 5 mM FU. At the end of the experimental period, the COCs were denuded, fixed, and immunostained with an anti-BrdU antibody. DNA was counterstained with DAPI, and is pseudo-colored red for better visualization. (b) The fluorescence intensities corresponding to FU labeling were measured in at least fifteen NSN oocytes in either untreated or each treated group. The mean intensity of each group was measured and depicted in the graphs. A p value less than 0.05 was taken as indicating a statistically significant difference and shown by asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

diameters seen after 22 h and 44 h of culture. The CDK7 inhibitor, BS-181, also slightly decreased the average COC diameter, to 480 μm and 950 μm after 22 h and 44 h, respectively. Notably, the CDK9 inhibitor, LDC67, significantly decreased the average diameter of the COCs to 320 μm and 413 μm respectively. Fig. 1B summarizes the results of the COC diameter measurements.

3.2. Effects of CDK inhibitors on nuclear maturation and meiosis progression

Next, we treated the COCs with the various CDK inhibitors for 22 h in vitro, denuded the oocytes, and used immunostaining with an anti-lamin A/C antibody to examine GVBD (Fig. 2A). As expected, treatment with the CDK1 inhibitor, RO-3306, significantly blocked GVBD in the majority of treated oocytes; and 9% of treated oocytes were at prometaphase of meiosis I (Pro-MI), compared to 97% of the untreated control oocytes. The oocytes could progress to Pro-MI when they were released from the inhibitor and cultured for an additional 22-h period, indicating that the inhibitory effect of RO-3306 was reversible. The inhibitions of CDK2 or CDK4/6 did not affect GVBD, and treated oocytes progressed normally to Pro-MI after release. In contrast, the inhibition of CDK7 reversibly blocked GVBD significantly in about 86% (102/120) of the oocytes. Additionally, the CDK9 inhibitor, LDC67, severely blocked GVBD with very few treated oocytes (6%, 8/132) undergoing chromosome condensation or progression to Pro-MI. Most of the treated oocytes reached Pro-MI following release from the compound. Fig. 2B depicts the average number of oocytes arrested at GV or progressed to Pro-MI after 22 h in the absence or presence of each CDK inhibitor.

3.3. Effects of CDK inhibitors on the transcriptional activity of oocytes

We next examined the effects of CDK inhibitors on the in vitro production of nascent RNAs in treated and non-treated oocytes. COCs were obtained from small or medium-size antral follicles, and treated with or without each CDK inhibitor for 6 h. In the final hour of treatment, 5-fluorouridine (FU) was added to culture medium for incorporation into the nascent RNAs. The COCs were denuded and subjected to immunostaining against FU. Only oocytes exhibiting an open NSN chromatin configuration were analyzed. As expected, non-treated oocytes exhibited a clearly defined nuclear signal upon FU labeling, which is consistent with the high transcriptional activity of NSN oocytes (Fig. 3A). The inhibitors against CDK1, CDK2, and CDK4/6 had no clear effect on the signal intensity of FU labeling, whereas those against CDK7 or CDK9 dramatically decreased the incorporation of FU into nascent RNAs. Fig. 3B summarizes the mean values of signal intensities

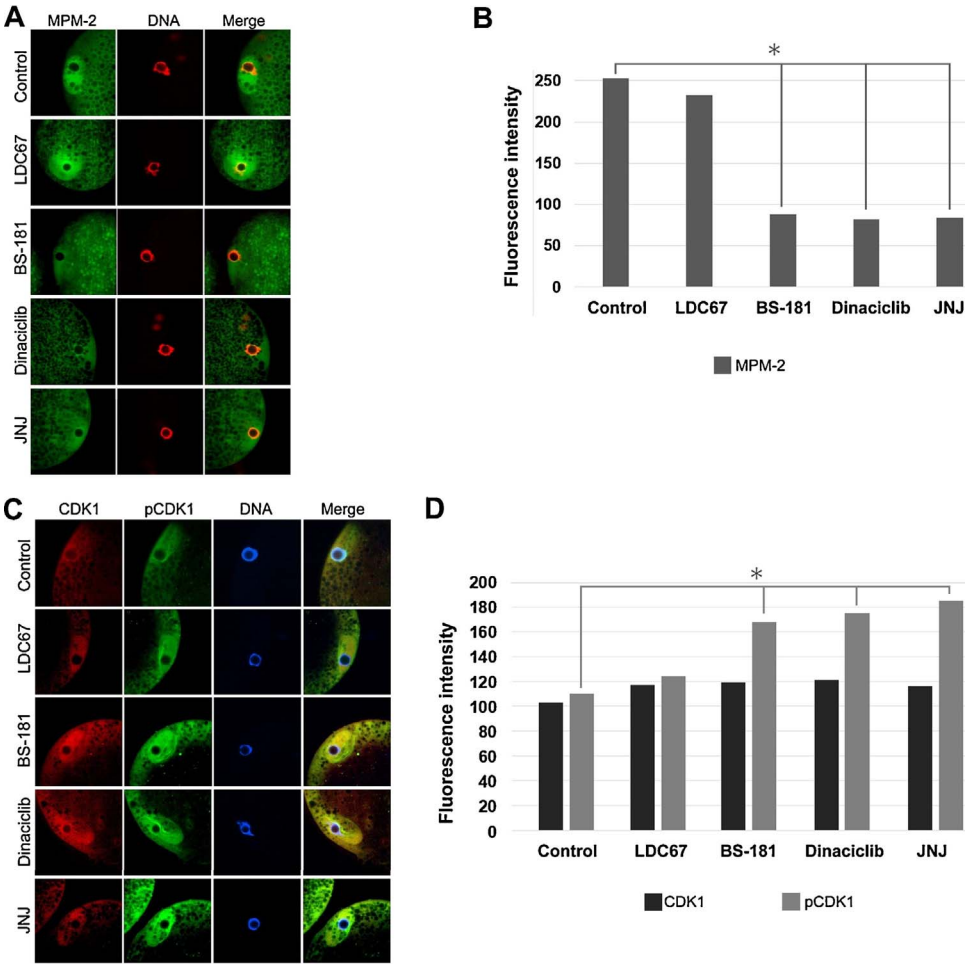

Fig. 4. The effects of CDK inhibition on CDK1 kinase activity and its phosphorylation. COCs were collected from large antral follicles for analysis of MPF activity and treated with CDK inhibitors for 6 h. (a) COCs treated with CDK inhibitors were denuded and immunostained with the MPM-2 antibody (green), which recognizes CDK1 target motifs only when they are phosphorylated. (b) The graph depicts the mean fluorescence signal intensity of each group. At least fifteen SN oocytes were analyzed. A *p* value less than 0.05 was taken as indicating a statistically significant difference and shown by asterisk. (c) The effects of CDK inhibitors on the inhibitory phosphorylation of CDK1 were analyzed by immunostaining against inhibitory pCDK1 (T14/Y15). The results were normalized with respect to the intensity of staining for pan CDK1 (red) in each specimen. More than fifteen SN oocytes were analyzed. (d). The mean fluorescence signal intensity of both CDK1 and pCDK1 in each group were measured and depicted in the graph. A *p* value less than 0.05 was taken as indicating a statistically significant difference and shown by asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of FU labeling in each experimental groups.

3.4. Effects of CDK9 inhibitors on CDK1 activity of oocytes

To elucidate the effect of CDK9 inhibition on CDK1 activity, we measured nuclear transcription and MPF kinase activity in the presence or absence of the CDK9 inhibitor, and compared our results with those obtained from cells treated with two other CDK inhibitors: one that inhibited CDK1, -2, -5 and -9, and one that inhibited CDK1 and 2 (Fig. 4A). For this set of experiments, COCs were obtained only from large antral follicles. We first analyzed the effects of the selected CDK inhibitors on CDK1 activity, using the monoclonal antibody, MPM-2. In non-treated oocytes, intense MPM-2 signals inside the GVs were observed. In LDC67-treated oocytes, the MPM-2 signal intensity was comparable with that of the control group. However, the MPM-2 signals were decreased in oocytes treated with BS-181, dinaciclib or JNJ-7706621 (Fig. 4B), indicating that the specific inhibition of CDK9 by LDC67 did not affect CDK1 kinase activity. Next the effects of the selected inhibitors on the inhibitory Thr14/Tyr15 phosphorylation of CDK1 were analyzed using immunocytochemistry and an antibody that recognized these residues only when both are phosphorylated (pCDK1). For experiments, the signal intensity of pCDK1 and that obtained from a pan-CDK1 antibody in the same oocyte were compared (Fig. 4C). In the non-treated control group, the pCDK1 signal was lower than the CDK1 signal, indicating that there was a relatively low level of CDK1 subject to inhibitory phosphorylation under basal conditions. Inhibition of CDK9 did not alter the level of inhibited CDK1. However, treatment with BS-181, dinaciclib and (to some extent) JNJ-7706621 increased the signal intensity of pCDK1 compared to the control group. These data collectively show that the inhibitory effect of CDK9 activity on GVBD was not due to inhibition of CDK1, but rather reflected an inhibition of transcription.

4. Discussion

No previous report has examined the effect of a specific CDK1 inhibitor on cumulus cell expansion. Roscovitine, which dose-dependently inhibits CDK1, CDK2, and CDK5, was previously shown to decrease the cumulus cell expansion of bovine COCs in vitro [31]. We propose that this can be attributed to the inhibition of CDK1 because CDK2 inhibition had no significant effect on cumulus cell expansion in our present experiments, and CDK5 is mainly expressed in the brain [32], and is thus unlikely to play any role in the cumulus cell expansion of COCs. The present work is the first to examine the involvement of CDK4 and CDK6 in pig oocyte maturation. We found that, at least in terms of cumulus cell propagation, pig COCs do not solely depend on these cell-cycle-regulating kinases. Instead, it seems very likely that other CDKs, such as CDK1, can compensate for the inactivation of CDK4 and CDK6. Inhibition of CDK7, which is a kinase involved in the initiation of Pol II-mediated transcription [33], had a mild effect on cumulus expansion. CDK7 can also act as a catalytic subunit of the CDK-activating kinase (CDK7-cyclin H-MAT1 complex), which phosphorylates Thr160 of CDK1 (MPF). This activating phosphorylation is essential for the activity of MPF in mitosis. This kinase was previously shown to be essential for oocyte maturation in pig [34]. However, our current results suggest that in cumulus cells, mitotic division is less dependent on CDK7 activity and/or may be regulated by another CDK. Notably, inhibition of CDK9 had a very severe effect on cumulus cell

proliferation. CDK9 is a kinase subunit of the positive transcription elongation factor, P-TEFb, which is involved in regulating Pol II-mediated transcription elongation [35]. CDK9 is also known to be involved in the DNA damage response and histone modification [36,37]. We previously showed that the flavopiridol-mediated inhibition of P-TEFb blocks the expansion of cumulus cells [29]. Here, another CDK9 inhibitor, LDC000067 (LDC67) was used. Our findings confirmed that CDK9 activity is essential for cumulus expansion, and that other CDKs are unable to compensate for the lack of CDK9 activity. Together, the results obtained from our experiments show that CDK1 and CDK9 are important for pig cumulus cell expansion in vitro.

CDK1 activity is known to be necessary for the resumption of meiosis in mammalian oocytes. Oocytes from Cdk1-null mice permanently arrest at GV stage, and injection of Cdk1 mRNA into these oocytes triggers the resumption of meiosis [38], indicating that CDK1 is necessary and sufficient to trigger meiotic progression in mouse oocytes. In pig, we herein found that treatment of COCs with the CDK1 inhibitor, RO-3306, reversibly blocks GVBD. A previous study showed that CDK2 did not appear to affect the resumption of meiosis in mouse [38], but its antibody-mediated inhibition in pig oocytes decreased their entry into second meiosis [39]. Although we did not culture the COCs continuously for 44 h in presence of the CDK2 inhibitor, however, under our experimental conditions, GVBD and the progression to Pro-MI did not appear to be affected by the inhibitor after 22 h of treatment.

Although the roles of CDK4 or CDK6 in mammalian oocyte meiosis have not previously been reported, knockout studies revealed that mice lacking the Cdk4 or Cdk6 genes are viable [40]. Here, we found that the simultaneous inhibitions of both CDK4 and CDK6 had no significant effect on GVBD or the further progression to Pro-MI. Inhibition of CDK7, on the other hand, dramatically blocked GVBD. In both somatic cells and oocytes, the Thr161 residue of CDK1 must be phosphorylated by the CAK (CDK7:cyclin H) complex. This phosphorylation activates CDK1 (MPF) and triggers a cascade of MPF-mediated protein phosphorylation that eventually leads to GVBD and chromosome condensation. It was recently reported that knockdown of either CDK7 or its regulatory partner, cyclin H, prevents GVBD in about half of pig oocytes in vitro [34]. Under our experimental conditions, more than 86% of the CDK7 inhibitor-treated oocytes arrested at the GV stage. This apparent difference could reflect that oocytes contain a considerable amount of maternally stored CDK7 enzyme that is not eliminated by genetic knockdown but could be inactivated by inhibitor treatment. We previously showed that inhibition of CDK9 by flavopiridol or C-AN508 blocks GVBD in porcine oocytes [29]. Here, using another CDK9 inhibitor, it was confirmed that the kinase activity of CDK9 is crucial for pig oocyte nuclear maturation and chromosome condensation. CDK9 kinase activity is known to be necessary for most mRNA transcription [41]. CDK9 may also be involved in mRNA translation, as its knockdown or chemical inhibition was found to decrease the Ser209 phosphorylation of eIF4E, which reduces the translation of mRNAs [42]. Thus, the elimination of CDK9 activity may block GVBD by inhibiting both mRNA transcription and protein synthesis.

The novel results presented herein show that the CDKs known to be involved in cell cycle regulation (CDK1, -2, -4, and -6) do not participate in the transcriptional activity of maturing oocytes. Our findings also reveal that the RO-3306-mediated inhibition of GVBD does not directly affect nuclear transcription in oocytes, and confirm that the BS-181- or LDC67-induced inhibitions of GVBD can be attributed to inhibition of transcription rather than inhibition of MPF activity. LDC67 did not inhibit CDK1 kinase activity in our system, but both dinaciclib and JNJ-7706621 decreased the phosphorylation of CDK1 targets. Finally, our experiments with JNJ-7706621 confirm that CDK1 inhibition blocks GVBD in a transcription-independent manner. In conclusion, our results indicate that, in addition to the need for CDK1 activity, CDK7 and CDK9 are important for the maturation of pig oocytes. CDK9 seems to be crucial for both cumulus cell expansion and nuclear maturation in porcine oocytes, and these functions appear to be transcription-dependent.

The present research can provide, in the near future, an insightful interpretation of topical and coming trends in the assisted reproductive technologies (ARTs) involving the efficient generation of high-quality in vitro fertilization (IVF)- or SCNT-derived pig embryos as a result of initial treatment of ex vivo maturing oocytes with desirable (highly specific or largely selective) inhibitors of the adequate CDK family members. For this reason, it appears to have a relatively great potential to make a valuable contribution to elucidation of the underlying causes of failures in the epigenetic reprogramming of transcriptional activity of somatic cell-inherited nuclear DNA, which can give rise to decrease in both developmental competences and cytological/molecular quality of SCNT-derived oocytes and resultant cloned embryos. This could be helpful for improvement in the effectiveness of somatic cell cloning in pigs.

Conflicts of interest

The authors declare that there is no conflict of interest.

Authors' contribution

RKO and DIJ designed the experiments and wrote the manuscript. RKO and TL performed the immunochemical experiments and analyzed the data. JEL, SYK and JWK carried out oocyte collection and culture experiment. DIJ provided the financial support.

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