Received: 22 November 2019 | Accepted: 13 February 2020

DOI: 10.1002/jcp.29655

ORIGINAL RESEARCH ARTICLE

Journal of Cellular Physiology WILEY

Meiotic block with roscovitine improves competence of porcine oocytes by fine-tuning activities of different cyclin-dependent kinases

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Abstract

Successful use of oocytes from small follicles (SFs) is of great importance for animal embryo production and human in vitro fertilization with reduced hormone-related side effects. How in vitro meiotic arrest maintenance (MAM) increases the competence of oocytes is not clear. In this study, pig oocytes recovered from SF of 1–2 mm and medium-follicles (MF) of 3–6 mm in diameter from abattoir ovaries were treated by various MAM treatments to improve their competence. The results showed that 25 μM roscovitine or 1 mM db-cAMP efficiently blocked germinal vesicle breakdown in both SF and MF oocytes suggesting a similar cyclin-dependent kinase (CDK) 1 level between the two oocyte groups. MAM with 15- and 25-μM roscovitine alone or with 1-mM db-cAMP improved competence of SF and MF oocytes, respectively, with a promoted chromatin configuration transition from surrounded nucleoli (SN) to re-decondensation (RDC) pattern that supported substantial gene transcription. However, MAM with db-cAMP alone or with higher concentrations of roscovitine did not improve oocyte competence, could not support an SN-to-RDC transition, and/or evoked a premature chromatin condensation (PMC) that suppressed gene transcription. Both CDK2 and CDK5 contents were higher ($p < .05$) in MF than in SF oocytes. It is concluded that the competence of pig oocytes, particularly that of SF oocytes can be improved by MAM using a proper roscovitine concentration that promotes gene transcription by inhibiting CDK5 while letting CDK2 off to prevent PMC.

KEYWORDS

cyclin-dependent kinase, meiosis, oocyte competence, pig, roscovitine

1 | INTRODUCTION

Many studies conducted in various species including the pig have shown that oocytes from in vitro maturation (IVM) show significantly lower competence than their counterparts from in vivo maturation (Abeydeera et al., 1998; Gil et al., 2010; Nagai, Funahashi, Yoshioka, & Kikuchi, 2006). We all know that mammalian oocytes must complete both nuclear maturation and cytoplasmic maturation to be capable of sustaining fertilization and embryo development (Hyttel, Fair, Callesen, & Greve, 1997). During in vivo maturation, oocytes achieve ooplasmic maturation following a long preparation including messenger RNA (mRNA) transcription and subsequent translation before germinal vesicle breakdown (GVBD; Gosden, Krapez, & Briggs, 1997; Hyttel et al., 1997). During IVR, however, oocytes undergo a sudden

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GVBD with no sufficient ooplasmic maturation when they are aspirated from follicles and placed in a culture medium. Therefore, studies have been conducted to improve cytoplasmic maturation of IVM oocytes through in vitro meiotic arrest maintenance (MAM) before maturation culture.

For example, Wu et al. (2002) and Bilodeau-Goeseels (2012) conducted MAM in porcine and bovine oocytes, respectively, and they obtained blastula rates as high as those in control oocytes. Funahashi, T. C. Cantley, and Day (1997) obtained significantly increased blastula rates after they performed MAM with dibutyryl cyclic adenosine monophosphate (db-cAMP) and in vitro fertilization (IVF) in porcine oocytes. Hashimoto, Minami, Takakura, and Imai (2002) reported higher blastocyst rates after MAM of bovine oocytes with butyrolactone I compared with direct maturation without MAM. In recent years, several laboratories significantly improved oocyte developmental potential by increasing cAMP levels in culture medium before IVM in different species (Ezoe et al., 2015; Zeng et al., 2013, 2014). Furthermore, MAM of juvenile mouse oocytes with C-type natriuretic peptide significantly increased the blastocyst rates (Romero, Sánchez, Lolicato, Van Ranst, & Smitz, 2016). However, MAM with roscovitine produced adverse effects on the developmental potential in bovine (Adona, Lima Verde, & Leal, 2004), goat, (Jimenez-Macedo, Izquierdo, Urdaneta, Anguita, & Paramio, 2006) and cat oocytes (Sananmuang, Techakumphu, & Tharasanit, 2010). Thus, the mechanisms by which MAM increases oocyte competence must be further explored.

Although the techniques to produce embryos through IVM-IVF of oocytes from medium-follicles (MFs) with diameter of 3–6 mm are mature in the pig (Day & Funahashi, 1996; Funahashi & Day, 1997), the number of MFs are very limited in pig ovary as small follicles (SFs) with a 1–2 mm diameter are in a large number (Morbeck, Esbenshade, Flowers, & Britt, 1992). Thus, in vitro pig embryo production efficiency would be significantly increased if oocytes from SFs can be successfully used. Furthermore, IVM of human oocytes from SFs would reduce hormone stimulation and related side effects for patients (Nastri, Ferriani, Rocha, & Martins, 2010; Sánchez et al., 2017). However, we all know that the competence of SF oocytes is significantly lower than the competence of MF oocytes (Kohata, Izquierdo-Rico, Romar, & Funahashi, 2013; Marchal, Vigneron, Perreau, Bali-Papp, & Mermillod, 2002; Pandey et al., 2017; Yoon et al., 2000). It was suggested that MAM in vitro might be a way out to increase SF oocyte competence. For example, nuclear maturation and embryo development of the metaphase II (MII)-incompetent goat oocytes from SFs were significantly improved after MAM with roscovitine, although that in oocytes from MFs was not improved at all following the same treatment (Han et al., 2006). Similarly, Pavlok, Kanka, Motlík, and Vodicka (2000) reported that MAM with butyrolactone I significantly increased the meiotic competence of bovine oocytes from SFs although they did not observe embryo development. Furthermore, Pandey et al. (2018) pre-maturation incubation of buffalo oocytes from SFs with roscovitine significantly improved their developmental competence.

It is known that cyclin-dependent kinases (CDKs), such as CDK1, CDK2, and CDK5 regulate cell cycling and gene transcription, and CDK1 is the sole CDK that is essential and sufficient to launch meiosis resumption (Adhikari et al., 2012). While CDK2 promotes a large-scale chromatin decondensation (Alexandrow & Hamlin, 2005), CDK5 suppresses gene transcription (Li et al., 2004). We all know that different concentrations of roscovitine are required to inhibit different CDKs in somatic cells (Meijer et al., 1997). However, although studies suggested that roscovitine could be used for MAM to increase oocyte competence (Han et al., 2006; Pavlok et al. 2000), studies on MAM with different concentrations of roscovitine to regulate different CDKs are limited (Chen et al., 2017). Thus, the objective of this study was to test whether the developmental competence of porcine oocytes, particularly SF oocytes could be improved by fine-tuning roscovitine concentrations during MAM to differentially regulate CDK5 and CDK2 activities.

2 | MATERIALS AND METHODS

If not noted otherwise, we bought all chemicals from Sigma-Aldrich Corp. (St. Louis, MO).

2.1 | Oocyte recovery

Pig ovaries were collected at a local abattoir of Yinbao Food Corporation Ltd at Shandong province and were transported to a lab in 3 hr at 30°C to 35°C in a thermos containing 0.9% physiological saline to which penicillin (100 IU/ml) and streptomycin (0.05 mg/ml) were added. SF and MF oocytes were collected from 1 to 2 mm and 3–6 mm follicles, respectively, using a 10-ml syringe with an 18-gauge needle. Oocytes showing a uniform cytoplasm and over three layers of cumulus cells were collected under a stereoscopic microscope for experiments.

2.2 | Oocyte culture

Both the MAM medium and the maturation medium were prepared using a basic 199 medium (Zhang et al., 2017). To prepare the maturation medium, LH (0.05 IU/ml) and porcine follicular fluid (10%) were added to the basic 199 medium. To prepare the MAM medium, 10% porcine follicular fluid and 5, 10, 12.5, 15, 25, or 50 μM roscovitine (R7772-1MG; Sigma-Aldrich); 0.5, 1, or 2 mM db-cAMP (D0260-25MG; Sigma-Aldrich); or both roscovitine and db-cAMP were added to the basic 199 medium. To inhibit transcription, some oocytes were treated in MAM medium containing 50, 100, or 200 nm α-amanitin. Porcine follicular fluid and stock solutions of roscovitine and db-cAMP were prepared as previously reported by Zhang et al. (2017). We dissolved α-amanitin (28563-1MG; MedChemExpress) in distilled water to make a 10-mM stock solution, and stored it at minus 20°C and diluted to 50, 100, or 200 nM in MAM medium immediately before use. We transferred approximately 20 oocytes into

150 μl of mineral-oil-covered medium and cultured them at 38.5°C in an incubator with 5% CO_2 in the saturated humid air. We cultured control oocytes in the maturation medium continuously for 48 hr, but cultured MAM oocytes for 24 hr in the MAM medium before a further 24-hr culture in the maturation medium.

2.3 | Observation of GVBD and GV chromatin configurations

Both GVBD and GV chromatin configuration was observed immediately after MAM culture for 24 hr, and GV chromatin configuration was also observed in freshly collected oocytes for controls. We removed cumulus cells from the MAM oocytes by pipetting them in Dulbecco's phosphate-buffered saline (D-PBS; SH0013.09; HyClone, Logan, UT) containing 0.1% hyaluronidase and from the freshly recovered oocytes by pipetting them in D-PBS with 0.25% trypsin. To observe GVBD, we fixed cumulus-denuded oocytes (DOs) for over 30 min in paraformaldehyde (4%) before 10-min staining in D-PBS containing 10 μg/ml Hoechst 33342. To examine GV chromatin configuration, we stained DOs for 10 min in D-PBS containing 10 μg/ml of Hoechst 33342 without fixation. After washing in D-PBS, the DOs were placed on glass slides, compressed with coverslips, and then, observed within 0.5 hr under a fluorescence microscope (Leica-DMLB), with Hoechst fluorescence excited at 220–360 nm using a mercury lamp (50 W) attenuated with neutral filters. Oocytes were judged as undergoing GVBD when the nuclear envelop and nucleoli disappeared. The GV chromatin configurations were classified by the degree of chromatin condensation as Pan et al. (2018) described.

2.4 | Activation of oocytes and culture of embryos

At the end of the maturation culture, cumulus cells were removed as described above. After washing in D-PBS, the MII oocytes showing PB1 were picked out and activated first for 5 min in D-PBS containing ionomycin (5 μM, 407953-1MG; Sigma-Aldrich), and then for 5 hr in porcine zygote medium-3 (PZM-3) with 6-DMAP (2 mM, D2629-1G; Sigma-Aldrich) at 38.5°C in an incubator with 5% CO_2 in saturated humid air. Then, we washed oocytes in PZM-3, transferred them into 150-μl mineral-oil-covered PZM-3 medium, and cultured them for 7 days. We observed the rates of 2- and 4-cell embryos and blastulae after embryo culture for 48 and 168 hr, respectively. Cell numbers per blastula were counted under a fluorescence microscope after 10-min staining with Hoechst 33342 (10 μg/ml, in D-PBS). The PZM-3 medium was prepared as described previously by Zhang et al. (2017).

2.5 | Examination for global gene transcription

Oocyte labeling with 5-ethynyluridine (EU) and the EU detection steps was conducted exactly as reported previously by Pan et al. (2018).

2.6 | One-step real-time-polymerase chain reaction

Zona-free oocytes were lysed to extract RNA using a commercial cell lysis kit (CellAmp Direct Prep Kit for real-time-polymerase chain reaction (RT-PCR) and Protein Analysis, code no. 3733Q; TaKaRa). The lysate obtained was preserved at −80°C before use. Quantification of mRNA was performed with a One-Step TB Green PrimeScript PLUS RT-PCR Kit (Perfect Real-Time, code no. RR096A; TaKaRa). We used a 10-μl reaction volume for the amplification reaction (1-μl template, 2.2-μl RNase Free dH_2O, 5-μl 2× One Step TB Green RT-PCR Buffer 4, 0.6-μl TaKaRa Ex Taq HS Mix, 0.2-μl PrimeScript PLUS RTase Mix, 0.2-μl ROX Reference Dye II, and 0.8-μl each of forward and reverse primers (10 μM). Gene-specific primers used are as follows. For *ACTB*, F: CGTGCGGGACATCAAGGA, R: AGGAAGGAGGGCTGGAAGA; for *NFE2I2*, F: CCCATTCACAAAAGACAAACATTC, R: GCTTTTG CCCTTAGCTCATCTC; and for *MATER*, F: AGCATCTCACC TCCCTCTTG, R: AATCAATCCCTTCCACCTCA. The cycle amplification facilities: (a) reverse transcription reaction of one cycle at 42°C for 5 min and 95°C for 10 s; (b) polymerase chain reaction of 40 cycles at 95°C for 5 s and annealing temperature for 30 s; and (c) dissociation protocol at 95°C for 15 s, 65°C for 1 min, and 95°C for 15 s. We conducted sequencing, dissociation curve analysis, and gel electrophoresis on the PCR products to confirm the reaction specificity. The gene expression level was determined using the expression level of internal control (*ACTB*). All resultant values were expressed relative to control samples by using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001).

2.7 | Immunofluorescence detection of CDK2 and CDK5

Cumulus-DOs were subjected to (a) a 40-min fixation in 4% paraformaldehyde, (b) a 30-min permeabilization with 0.5% Triton X-100, (c) an 1-hr block in PBS with 3% bovine serum albumin, (d) an overnight incubation at 4°C using rabbit Anti-CDK2 (ab32147; 1:200 dilution; Abcam) or rabbit Anti-CDK5 (ab40773; 1:200 dilution; Abcam), (e) an 1-hr incubation with CyTM3-conjugated AffiniPure Goat Anti-Rabbit immunoglobulin (H+L; 1:800 dilution; 111-165-144; Jackson ImmunoRerearsh), (f) a 10-min Hoechst 33342 (10 μg/ml) staining. Negative control oocytes were incubated with only secondary antibody without primary antibody treatment. Oocytes were then observed under a laser confocal microscope exactly as Chen et al. (2017) reported.

To quantify the relative expression level, we measured fluorescence intensities of each protein on raw images using the Image-Pro Plus software (Media Cybernetics Inc., Silver Spring, MD). All the images were acquired with identical settings for each experimental series. We measured both the fluorescence density and the area under which an object gives fluorescence, and we calculated the mean relative intensity of fluorescence for each oocyte.

3 | DATA ANALYSIS

Each treatment was repeated at least three times. We used the software of Statistics Package for Social Sciences (SPSS 11.5; SPSS Inc., Chicago, IL) to analyze the data. One-way analysis of variance was adopted when each measure had more than two groups, and *t* test was used when each measure had only two groups. We always expressed the data as mean ± standard error of mean, and considered the difference significant only when $p < .05$.

4 | RESULTS

4.1 | The optimal concentrations of roscovitine and db-cAMP for MAM of SF and MF oocytes

To select an optimal concentration of roscovitine or db-cAMP for MAM of oocytes from differently sized follicles, SF or MF oocytes were incubated in the MAM medium with 12.5, 25, or 50 μM of roscovitine or 0.5, 1, or 2 mM of db-cAMP before the examination for GVBD. We tested these roscovitine and db-cAMP concentrations because Zhang et al. (2017) had found that 25-μM of roscovitine and 1-mM db-cAMP was optimal concentrations for GVBD inhibition of pig MF oocytes. In this study, oocytes at the pMI, MI, A/TI, and MII stage were considered undergoing GVBD (Figure 1a). The results showed that GVBD was efficiently blocked at 25-μM roscovitine or 1-mM db-cAMP in both SF and MF oocytes, and the difference in percentages of GVBD oocytes was not significant between the two oocyte groups at any concentration of roscovitine or db-cAMP tested (Figure 1b,c). Thus, 25-μM roscovitine and 1-mM db-cAMP was selected to inhibit GVBD in the following experiments.

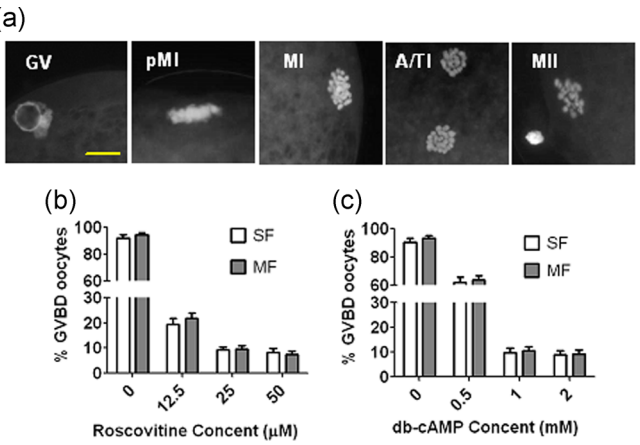

FIGURE 1 Meiotic maintenance effects of MAM with different concentrations of roscovitine. Panel (a) shows micrographs of oocytes at GV, pro-metaphase I (pMI), metaphase I (MI), anaphase/telophase I (A/T1), and metaphase II stages. The photographs were taken under a fluorescence microscope following Hoechst 33342 staining. Original magnification: ×400. Bar is 25 μm and applies to all images. Graphs (b) and (c) show percentages of oocytes undergoing GVBD after SF or MF oocytes were cultured for 24 hr in the MAM medium with different concentrations of roscovitine or db-cAMP. Each treatment was repeated 5 times with each replicate containing about 30 oocytes. No significant difference ($p > .05$) in GVBD percentages was observed between SF and MF oocytes at any concentration of roscovitine or db-cAMP. db-cAMP, dibutyryl cyclic adenosine monophosphate; GV, germinal vesicle; GVBD, germinal vesicle breakdown; MAM, meiotic arrest maintenance; MF, medium-follicles; SF, small follicles

4.2 | Maturation and embryo development of SF and MF oocytes following MAM with roscovitine or db-cAMP

We blocked oocytes for 24 hr in the MAM medium with various concentrations of roscovitine and/or 1 mM db-cAMP before culture for maturation. We then activated the matured oocytes to observe embryonic development. No significant difference in percentages of MII oocytes (86–93%) and 2- (84–91%) and 4-cell embryos (79–87%) were observed between treatments except for the treatment of MF oocytes with 50 μM roscovitine, where percentages of MII oocytes (42.9 ± 1.2%) and 2-cell embryos (46.0 ± 3.2%) decreased significantly ($p < .05$). MAM with db-cAMP alone had no effects on blastula rates and cell counts per blastocyst in either SF or MF oocytes (Figure 2). MAM with 15 and 25 μM roscovitine significantly increased blastocyst rates in SF and MF oocytes, respectively. MAM of MF oocytes with 25 μM roscovitine also increased cell number per blastocyst significantly. However, blastocyst rates decreased significantly when roscovitine increased to 25 in SF oocytes or to 50 μM in MF oocytes. Thus, MAM for MF and SF oocytes should be conducted with 25 and 15 μM roscovitine, respectively, to enhance their developmental potential.

4.3 | Chromatin configurations in SF and MF oocytes following MAM with roscovitine or db-cAMP

We observed chromatin configurations in SF and MF oocytes after MAM with various concentrations of roscovitine and/or 1 mM db-cAMP, to find out why different MAM protocols differentially affected the competence of differently sized oocytes. For controls, freshly recovered oocytes were examined without MAM. Similar to those observed by Zhang et al. (2017), six chromatin configurations were observed including the non-surrounded nucleolus (NSN), intermediate (IN), surrounded nucleolus (SN), prematurely condensed (PMC), re-decondensed (RDC), and early diakinesis (ED) patterns (Figure 3a). A major difference between the SN and PMC configuration was that in the former, a complete heterochromatin sheath enclosed the nucleolus and the nucleoplasm contained little heterochromatin, but in the latter, there was only an incomplete heterochromatin sheath surrounding the nucleolus, and heterochromatin formed clumps and strands in the germinal vesicle.

For SF oocytes, while MAM with 25-μM roscovitine with or without db-cAMP significantly promoted the transition of

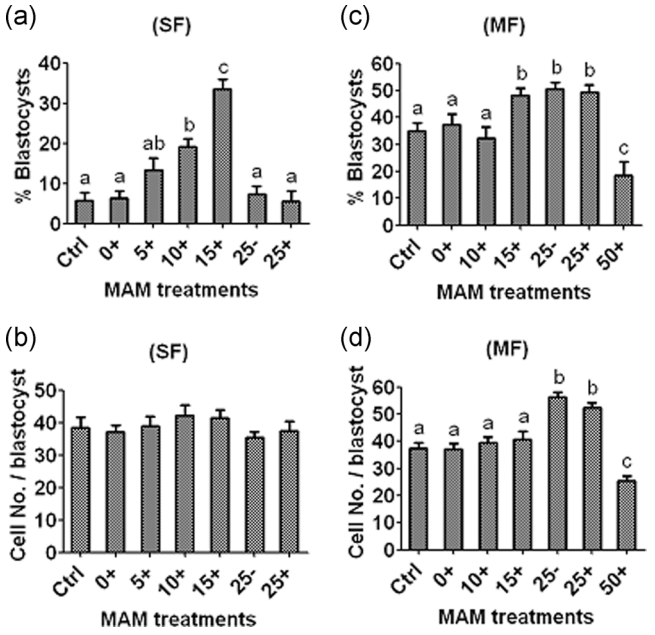

FIGURE 2 Effects of MAM with roscovitine and/or db-cAMP on maturation and embryo development of SF and MF oocytes. Oocytes were blocked for 24 hr in the MAM medium containing 0, 5, 10, 15, 25, or 50 μM roscovitine with (+) or without (−) 1 mM db-cAMP before culture for 24 hr in maturation medium. Control (Ctrl) oocytes were cultured continuously for 48 hr in the maturation medium. Mature oocytes were activated for embryo development. Graphs (a) or (c) and (b) or (d) show percentages of blastocysts (% blast)/cell number per blastocyst (cell no) in SF and MF oocytes, respectively. Each treatment was repeated five to six times with each replicate including about 40 oocytes. Percentages of MII oocytes, 2-cell embryos, 4-cell embryos, and blastocysts were calculated from oocytes put in maturation culture, MII oocytes, 2-, and 4-cell embryos, respectively. (a–c) Values with a different letter above bars differ significantly ($p < .05$). db-cAMP, dibutyryl cyclic adenosine monophosphate; MAM, meiotic arrest maintenance; MF, medium follicle; MII, metaphase II; SF, small follicle

NSN or IN to PMC pattern, MAM using db-cAMP alone or using 15 μM roscovitine inhibited such a transition (Figure 3b). Notably, MAM with 15 μM roscovitine plus db-cAMP promoted all the SN oocytes to take on the RDC configuration. In MF oocytes, no NSN pattern was observed. While MAM with 25 μM roscovitine with or without db-cAMP promoted a significant transition from SN to RDC, MAM with db-cAMP alone forced most of the IN oocytes to take on the PMC pattern, with only about 5% of the SN oocytes assuming the RDC configuration (Figure 3c). Furthermore, MAM with 50 μM roscovitine and db-cAMP forced all the IN oocytes into PMC configuration. Thus, the results suggested that MAM with 15 and 25 μM roscovitine alone or with db-cAMP improved oocyte competence with a promoted transition from SN to RDC in SF and MF oocytes, respectively, whereas MAM with db-cAMP alone or with higher concentrations of roscovitine did not improve oocyte competence because it could not support such a transition and/or they evoked a PMC configuration.

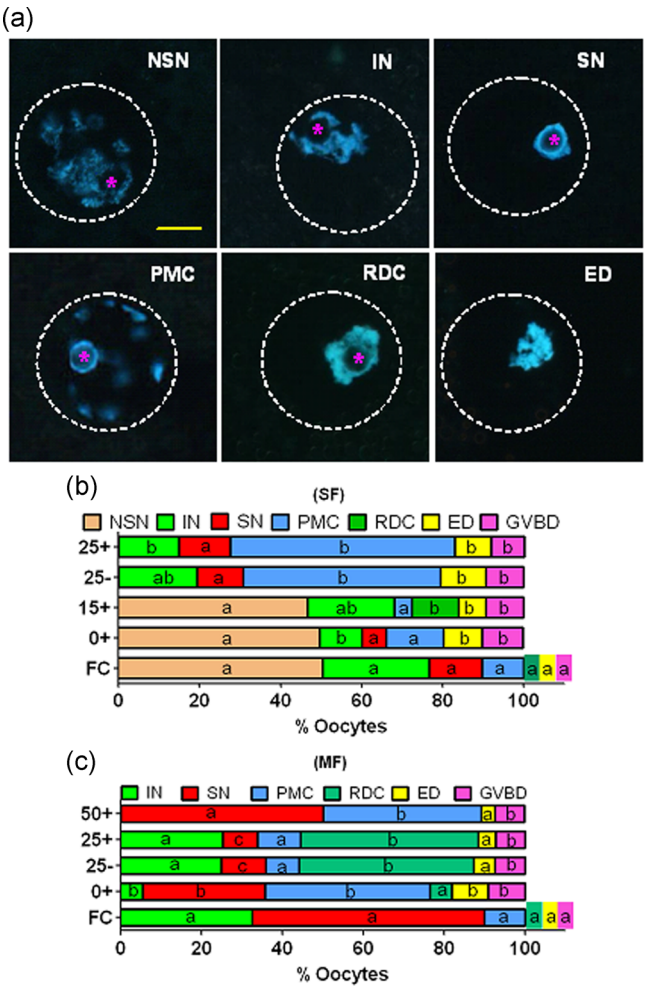

FIGURE 3 Effects of MAM with roscovitine and/or db-cAMP on chromatin configurations of SF or MF oocytes. Oocytes were blocked for 24 hr in the MAM medium containing roscovitine and/or db-cAMP before chromatin configuration examination. Panel (a) shows images of oocytes observed under a fluorescence microscope following Hoechst 33342 staining. Original magnification ×400. The bar is 10 μm and applies to all images. "*" indicates the location of the nucleolus. The chromatin configurations observed include the non-surrounded nucleolus (NSN), intermediate (IN), surrounded nucleolus (SN), prematurely condensed (PMC), re-decondensed (RDC), and early diakinesis (ED) patterns. Graphs (b) and (c) show percentages of oocytes with different chromatin configurations before (freshly collected [FC]) or after MAM with 15, 25, or 50 μM roscovitine with (+) or without (−) 1 mM db-cAMP of SF and MF oocytes, respectively. Each treatment was repeated five to six times with each replicate containing about 20 oocytes. (a and b) Values without a common letter in bars differ significantly ($p < .05$) within configurations. db-cAMP, dibutyryl cyclic adenosine monophosphate; MAM, meiotic arrest maintenance; MF, medium follicle; SF, small follicle

4.4 | Effects of chromatin configurations on global gene transcription in pig oocytes

To get as many SF oocytes with different configurations as possible, NSN and IN oocytes from SFs were observed immediately after collection while SN and PMC oocytes were observed

following MAM with 25 μM roscovitine alone (Figure 3b). While IN and SN oocytes from MFs were examined freshly after collection, RDC and PMC oocytes were examined following MAM with 25-μM roscovitine or 1-mM db-cAMP, respectively (Figure 3c). While all the NSN oocytes from SFs showed intensive transcription, and about 13–15% of the IN oocytes from either SFs or MFs showed a mild transcription, none of the oocytes with an SN or PMC configuration from either SFs or MFs showed any discernible transcription (Figure 4). About 55% of the RDC oocytes from MFs displayed intensive transcription after MAM with 25 μM roscovitine. As MAM of oocytes from MFs with 25 μM roscovitine produced the maximal numbers of RDC configurations and the highest rates of blastocyst development among different treatments, the data confirmed that MAM using roscovitine increased oocyte competence by enhancing RNA transcription.

4.5 | Optimal MAM protocols enhanced expression of oocyte competence-related genes

To further verify that MAM using roscovitine promotes RNA transcription, we compared expression of competence-related genes, maternal antigen that embryos require (*MATER*) and nuclear factor, erythroid 2 like 2 (*NFE2I2*), between transcribing oocytes showing a high percentage of RDC and/or NSN configurations and non-transcribing oocytes showing a high percentage of PMC pattern. Transcribing oocytes included SF oocytes obtained following MAM with 15 μM roscovitine + 1 mM db-cAMP and MF oocytes obtained after MAM with 25-μM roscovitine plus db-cAMP, and the non-transcribing oocytes included SF oocytes recovered after MAM using 25-μM roscovitine plus db-cAMP and MF oocytes following MAM using db-cAMP alone. The data showed that mRNA levels of both

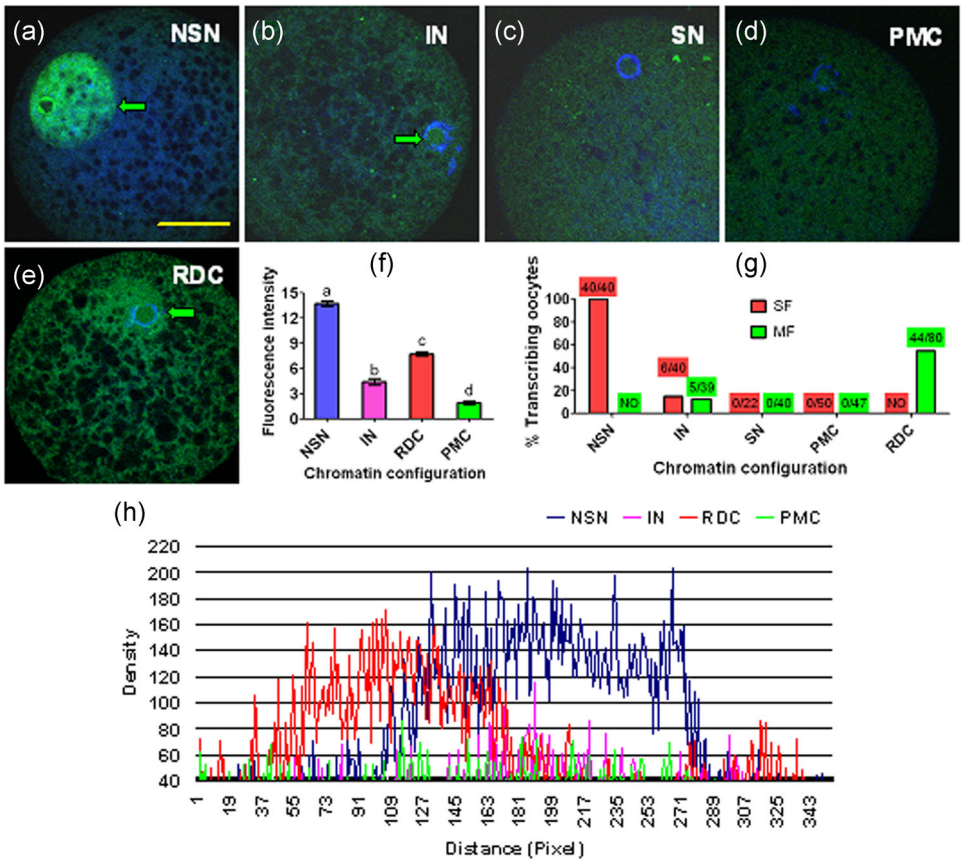

FIGURE 4 Effects of MAM with roscovitine on oocyte global gene transcription. For SF oocytes, NSN and IN were observed immediately after collection whereas SN and PMC were observed following MAM with roscovitine (25 μM). For MF oocytes, while IN and SN were examined freshly after collection, RDC and PMC were examined following MAM with roscovitine and db-cAMP, respectively. Photographs (a–e) are laser confocal (merged) images showing global RNA transcription in porcine oocytes with NSN, IN, SN, PMC, and RDC chromatin configurations, respectively. DNA and RNA were pseudocolored blue and green, respectively. RNA transcripts (arrows) were observed in NSN, IN, and RDC but not in SN and PMC oocytes. Original magnification ×630. Bar is 20 μm and applied to all images. Graph (f) shows gene transcription (fluorescence) intensity in NSN, IN, RDC, and PMC oocytes. Each treatment was repeated five times with each replicate including 1 oocyte. (a–d) Values with a different letter above bars differ significantly ($p < .05$). Graph (g) shows percentages of transcribing SF or MF oocytes with different chromatin configurations. The numbers above bars indicate numbers of transcribing/observed oocytes. "NO" indicates "Not observed." Panel (h) shows curves for gene transcription (fluorescence) intensity in NSN, IN, RDC, and PMC oocytes. db-cAMP, dibutyryl cyclic adenosine monophosphate; IN, intermediate; MAM, meiotic arrest maintenance; MF, medium follicle; NSN, non-surrounded nucleolus; PMC, prematurely condensed; RDC, re-decondensed; SF, small folli

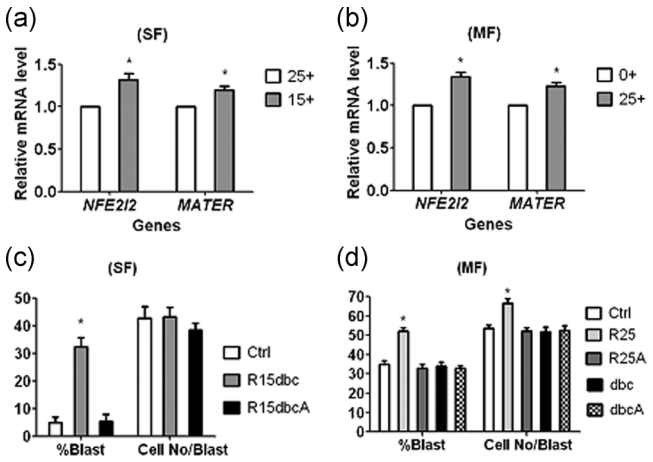

FIGURE 5 Expression of competence-related genes and the effects of α-amanitin treatment during MAM on the competence of SF and MF oocytes. In Graph (a), oocytes were blocked for 24 hr with 25 μM (25+) or 15 μM roscovitine (15+) plus 1 mM db-cAMP, and in Graph (b), oocytes were blocked with 1 mM db-cAMP alone (0+) or with 25 μM roscovitine (25+) before PCR quantification for mRNA levels. Each treatment was repeated three times with each replicate containing 14 oocytes. In Graphs (c) and (d), oocytes were blocked for 24 hr with 15 (R15) or 25 μM roscovitine (R25) and/or 1 mM db-cAMP (dbc) with or without 50 nM α-amanitin (a) before maturation culture. Control (Ctrl) oocytes were cultured continuously for 48 hr in the maturation medium. Mature oocytes were activated for embryo development. Each treatment was repeated five times with each replicate including about 40 oocytes. Percentages of MII oocytes, 2-cell embryos, 4-cell embryos, and blastocysts were calculated from oocytes put in maturation culture, MII oocytes, 2-cell embryos, and 4-cell embryos, respectively. * Indicates a significant difference ($p < .05$) from the rest of the values within blastocyst stage or cell number per blastocyst. db-cAMP, dibutyryl cyclic adenosine monophosphate; MF, medium follicle; MII, metaphase II; mRNA, messenger RNA: PCR, polymerase chain reaction; SF, small follicle

MATER and *NFE2l2* were lower ($p < .05$) in non-transcribing than in transcribing oocytes (Figure 5a,b), further confirming that our optimal MAM protocols improved oocyte competence by promoting transcription of beneficial genes, as high expression levels of both *MATER* (Monti et al., 2013) and *NFE2l2* (Jiao et al., 2016; Yoon et al., 2015) are associated with high oocyte developmental potential.

4.6 | Treatment with α-amanitin during MAM to inhibit gene transcription completely eliminated the good effect of roscovitine on oocyte developmental potential

To further verify that MAM with roscovitine increased oocyte developmental potential by enhancing gene transcription, α-amanitin treatment was used to inhibit transcription during MAM. SF or MF oocytes were blocked for 24 hr using roscovitine and/or db-cAMP with or without 200, 100, or 50 nM α-amanitin before maturation culture. Although Chen et al. (2017) had successfully inhibited transcription in mouse oocytes using 200 nm α-amanitin, we found that both 200 and 100 nm α-amanitin killed pig oocytes. We, therefore, used 50 nM to inhibit transcription of pig oocytes. In SF oocytes, while MAM with 15-μM roscovitine and 1-mM db-cAMP (R15dbc) significantly increased blastocyst rates compared with those in controls matured without MAM, the presence of amanitin (R15dbcA) completely reversed the effect by R15dbc (Figure 5c). In MF oocytes, although MAM with 25 μM roscovitine (R25) significantly increased blastula rates and cell counts per blastula, the presence of α-amanitin (R25A) completely eliminated the beneficial effect by R25 (Figure 5d). Treatment with α-amanitin during MAM with db-cAMP (dbcA) affected neither blastula rates nor cell counts per blastula, compared with MAM with db-cAMP alone (dbc). Furthermore, percentages of MII oocytes (54–58% for SF oocytes and 88–90% for MF oocytes) and 2-cell (82–85% for SF oocytes and 87–92% for MF oocytes) and 4-cell embryos (85–89% for SF oocytes and 85–88% for MF oocytes) did not differ between treatments involving or not involving α-amanitin. Thus, the data verified that MAM using roscovitine increased oocyte developmental potential by enhancing transcription and that the effect of α-amanitin treatment resulted from its efficient block on the RNA polymerase II during MAM, not from its toxicity on oocytes when used at 50 nM.

4.7 | Expression of CDK5 and CDK2 in SF and MF oocytes

The above observations demonstrated that MAM using roscovitine increased oocyte developmental potential in a dose- and follicle size-dependent manner. As the dose- and oocyte size-dependency of roscovitine action might be related to CDK contents or activities, we compared CDK2/5 contents between SF and MF oocytes. The results showed that both CDK2 and CDK5 contents were higher ($p < .05$) in MF than in SF oocytes (Figure 6). Results perfectly explained why the optimal concentration of roscovitine to improve competence by MAM was higher ($p < .05$) for MF than for SF oocytes. The results also confirmed that 15 and 25 μM were the optimal concentration of roscovitine to inhibit CDK5 while not affecting CDK2 in SF and MF oocytes, respectively, suggesting that more roscovitine was required to inhibit CDK2 than CDK5.

5 | DISCUSSION

The present results showed that 25 μM roscovitine efficiently blocked GVBD in both SF and MF oocytes. Roscovitine of 15 and 25 μM was the optimal concentration to improve the competence of SF and MF oocytes, respectively, as they inhibited CDK5 (promoted gene transcription) while not affecting CDK2 (not inducing PMC) in these oocytes. Furthermore, both the contents of CDK2 and CDK5 were higher ($p < .05$) in MF than in SF oocytes. As Adhikari et al. (2012) reported, CDK1 was the sole CDK essential and sufficient to support resumption of meiosis in mouse oocytes. In somatic cells,

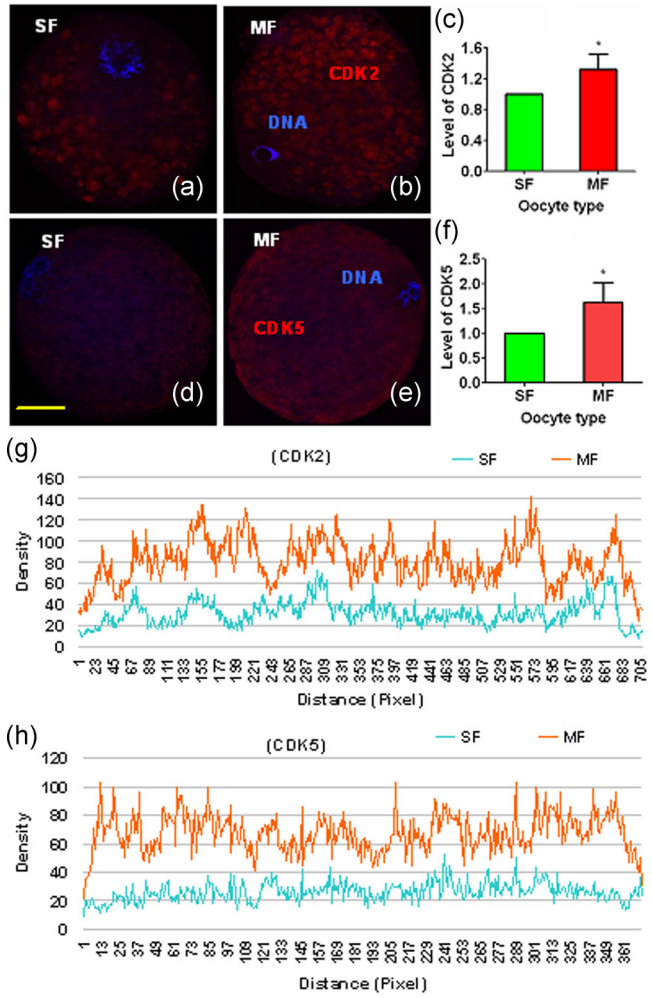

FIGURE 6 Expression of CDK2 and CDK5 in SF and MF oocytes. Micrographs A/D and B/E are merged images of SF and MF oocytes, respectively, observed under a laser scanning confocal microscope following staining with anti-CDK2 (a and b) or anti-CDK5 (d and e) antibodies (colored red) and Hoechst 33342 (colored blue). Some oocytes were processed with primary antibodies omitted to serve as negative controls. Original magnification ×400. Bar is 33 μm and applies to all images. Graphs (c) and (f) show relative levels (fluorescence intensity quantification) of CDK2 and CDK5, respectively, in SF and MF oocytes. Each treatment was repeated three times with each replicate including about 15 oocytes. *Significant difference ($p < .05$) from values in SF oocytes. Panels (g) and (h) show fluorescence intensity curves for CDK2 and CDK5, respectively, in SF (green line) or MF oocytes (red line). MF, medium follicle; SF, small follicle

while CDK2 promotes phosphorylation of histone H1 and decondensation of chromatin (Alexandrow & Hamlin, 2005), CDK5 inhibits RNA transcription via activating histone deacetylase (Li et al., 2004). Roscovitine suppresses several CDKs, such as CDK1, CDK2, and CDK5, and the half-maximal inhibitory concentration for roscovitine to inhibit CDK1, CDK2, and CDK5 was 0.65, 0.7, and 0.2 mM, respectively (Meijer et al., 1997). Chen et al. (2017) observed in mouse oocytes that the roscovitine concentration required to inhibit CDK1 was significantly higher than that for inhibition of CDK2, but they did

Oocytes	CDK1	CDK2	CDK5
SF	25 μM	25 μM	15 μM
MF	25 μM	50 μM	25 μM

FIGURE 7 A summary of optimal roscovitine concentrations required to efficiently inhibit GVBD and improve the developmental competence of SF and MF oocytes by inhibiting different CDKs. CDK, cyclin-dependent kinase; GVBD, germinal vesicle breakdown; MF, medium follicle; SF, small follicle

not examine the roscovitine concentration needed for CDK5 inhibition. Taken together, our results suggested that in the pig (a) both SF and MF oocytes need a similar level of roscovitine to inhibit CDK1 and require a higher level of roscovitine to suppress CDK2 than CDK5; (b) MF oocytes require higher levels of roscovitine to inhibit CDK2 and CDK5 than SF oocytes did; and (c) while SF oocytes need similar levels of roscovitine to suppress CDK1 and CDK2, MF oocytes require a higher level of roscovitine to inhibit CDK2 than CDK1 (Figure 7). Thus, the competence of oocytes at different developmental stages from different species may be improved by MAM with tailored roscovitine protocols.

As it is known that the competence of an oocyte is closely correlated with its GV chromatin configuration and gene transcription (Tan et al., 2009), we observed chromatin configurations and RNA transcription in SF and MF oocytes following MAM with different concentrations of roscovitine and/or 1 mM db-cAMP, to explain the different impacts that different MAM protocols had on the competence of differently sized oocytes. The results demonstrated that MAM with optimal roscovitine concentrations of 15 and 25 μM, which improved oocyte competence, promoted a significant transition from SN to RDC configuration in SF and MF oocytes, respectively. However, MAM with higher concentrations of roscovitine that did not improve oocyte competence could not support such an SN to RDC transition but evoked a PMC configuration instead. Our further observations indicated that while about 55% of the RDC oocytes displayed intensive transcription, none of the oocytes showing an SN or PMC configuration displayed any transcription. Zhang et al. (2017) also observed an SN to RDC transition with the promoted transcription of beneficial genes following MAM with roscovitine in porcine MF oocytes. Pan et al. (2018) reported that while some porcine SN oocytes from MFs re-decondensed into RDC before GVBD when conditions are favorable, PMC occurred under unfavorable conditions. Furthermore, our further observations indicated that the optimal MAM protocols enhanced the expression of oocyte competence-related genes. Taken together, our data indicated that MAM with optimal concentrations of roscovitine increased the competence of differently sized pig oocytes by facilitating an SN to RDC transition and thence gene transcription while preventing PMC.

In this study, db-cAMP was used to prevent GVBD when a low roscovitine concentration was insufficient to maintain meiotic arrest. Our data showed that when used alone, 1 mM db-cAMP could successfully inhibit GVBD in both SF and MF oocytes but it did not improve oocyte competence in either SF or MF oocytes as it could

not induce the RDC configuration (promote transcription) and/or evoked PMC. It is known that db-cAMP can activate protein kinase A (PKA). It was reported that PKA worked upstream of CDKs and it could inhibit the activities of CDK1 (Solc, Schultz, & Motlik, 2010) and CDK2 (D'Angiolella et al., 2001; Onishi & Hruska, 1997) while activating CDK5 (Chen et al., 2010; Jeon, Kim, Chung, & Kim, 2015). When used in combination with roscovitine in this study, db-cAMP did not affect the roscovitine action on oocyte competence or chromatin configuration. We all know that roscovitine suppresses CDK1 and CDK2 directly by occupying their ATP-binding pockets. Thus, roscovitine competitively inhibits ATP-binding to CDK1 (Meijer et al., 1997) and its purine portion can bind to the adenine-binding pocket of CDK2 (De Azevedo et al., 1997). The db-cAMP, however, inhibits CDK1 indirectly by activating PKA, which inactivates CDK1 also indirectly by way of the cell division cycle 25 phosphatase and the Wee1 kinase (Han & Conti, 2006). We thus postulated that when used together, roscovitine would quickly inactivate CDKs before db-cAMP could come into any play.

This study showed that when used alone, db-cAMP prevented chromatin condensation from NSN to SN in SF oocytes, but facilitated chromatin condensation into SN and PMC in MF oocytes. While our results from SF oocytes are in agreement, the results from MF oocytes are in conflict with those reported by Sun et al. (2016) who observed that treatment of pig cumulus-oocyte complexes with db-cAMP reduced the intra-oocyte cAMP level by increasing that in cumulus cells, leading to prevented chromatin condensation from NSN to SN in both SF and MF oocytes. Our explanation for why db-cAMP treatment facilitated chromatin condensation in our MF oocytes was that the gap junctions linking oocytes with their cumulus cells might be interrupted during MAM, so that the db-cAMP entering cumulus cells could not decrease the intra-oocyte cAMP level, and thus, those db-cAMP entering the oocyte would facilitate chromatin condensation without difficulty. In fact, a major drop in gap junctions has been observed in bovine oocytes recovered from medium antral follicles following in vitro culture for 6 hr (Lodde, Franciosi, Tessaro, Modina, & Luciano, 2013).

In conclusion, the successful use of SF oocytes is an urgent demand for animal embryo production and human IVF as it can reduce hormone-caused side effects (Nastri et al., 2010; Sánchez et al., 2017). This study tested whether the competence of porcine oocytes including SF oocytes could be improved by fine-tuning roscovitine concentrations during MAM to differentially regulate CDK5 and CDK2 activities. The results demonstrated that the competence of pig oocytes from SFs and MFs could be improved by MAM using 15 and 25 μM roscovitine, respectively, which promote gene transcription by inhibiting CDK5 while letting CDK2 off to prevent PMC. The results suggested that the competence of oocytes at different developmental stages from different species might be improved by MAM with tailored roscovitine protocols. Thus, the data have provided not only a novel insight into the mechanisms by which MAM improves oocyte competence but also essential data for the formulation of efficient protocols for IVM of oocytes including SF oocytes from different species.

ACKNOWLEDGMENTS

This study was supported by grants from the National Key R&D Program of China (2017YFC1001602 and 2017YFC1001601, Nos. 2017YFD0501904), the China National Natural Science Foundation (Nos. 31772599 and 31702114), the Natural Science Foundation of Shandong Province (No. ZR2017BC025), and the Funds of Shandong Double Tops Program (No. SYL2017YSTD12).

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

S. Z., Y-J. J., L-Z. P., S. G., M-J. S., G-L. W., and M-J. L. conducted the experiments; S. Z., Y-J. J., and J-H. T. analyzed the data. J-H. T. designed the experiments and wrote the manuscript. All authors reviewed the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Zhu S, Jia Y-J, Pan L-Z, et al. Meiotic block with roscovitine improves competence of porcine oocytes by fine-tuning activities of different cyclin-dependent kinases. *J Cell Physiol*. 2020;1–11.
https://doi.org/10.1002/jcp.29655