The Effect of Epigenetic Changes on the Extrusion of the First Polar Body in Pig Oocytes During *In Vitro* Maturation

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Abstract

The present study was designed to investigate the comprehensive function of maternal factors of primordial germ cell 7 (*PGC7*) and *POU5F1*—POU class 5 homeobox 1 (*OCT4*), as well as the epigenetic modification roles on the mitosis for the extrusion of first polar body (PB1) in pig matured oocytes. First, the common distribution of histone modifications, including H3K4me2, H3K27me3, H3K9me2, and H4K12ac and DNA methylation, were detected at the high level in the nucleus. However, only one part of the chromosome was higher methylated or acetylated when the mitosis happened to extrude the PB1. When the mitosis was inhibited by the cytochalasin B (CB) treatment, the expression of *PGC7*, *OCT4*, DNA methyltransferase1 (*DNMT1*), DNA methyltransferase3b (*DNMT3b*), tet methylcytosine dioxygenase 1 (*TET1*), tet methylcytosine dioxygenase 2 (*TET2*), and tet methylcytosine dioxygenase 3 (*TET3*) could be inhibited ($p < 0.01$), and no concentrated expression of the *PGC7* and *OCT4* was observed on the chromosome, but the levels of H3K9me2 and H4K12ac were higher. In addition, when the trichostatin A was performed on the *in vitro* maturation, the extrusion of the PB1 was inhibited too. And the histone methylation (H3K9me2 and H3K27me3) could be detected all the time with relative higher level and no demethylation could be observed. However, the expression of *PGC7* and *OCT4* was lower in the chromosome. It might indicate that the maternal factor of *PGC7* and histone modification that included H4K12ac and H3K9me2 could regulate the extrusion of the PB1 and play an important role in the maturation of pig oocytes.

Keywords: histone acetylation, DNA methylation, *PGC7*, meiosis, porcine

Introduction

MATURATION OF OOCYTES is crucial for the initiate and sustains embryonic development. When the oocytes going on for maturation, they acquire the ability to resume and complete meiosis to extrude the first polar body (PB1) and have the competence to successfully undergo the fertilization process (First et al., 1988; Pomerantz et al., 2012). Commonly the extrusion of the PB1 depends on proteasomes degradation of cyclin B1, securin and the subsequent respective *CDK1* inactivation, as well as the chromosome segregation (Pomerantz et al., 2012). However, many studies have indicated that DNA methylation and histone modifications also play functional roles in oocytes maturation (Liu et al., 2011; Xu et al., 2017).

The changes of DNA methylation during oocytes maturation are essential for resetting genomic potential and

correcting developmental genes (Hargancalvopina et al., 2016), while the histone modifications are important for meiotic resumption and chromosome organization and segregation (Gu et al., 2010). However, few reports are available for the relationship between the extrusion of PB1 and the epigenetic changes in pig oocytes.

In addition, maternal factors are critical for oocytes maturation as well as the early embryo development before implantation. Two maternal factors *OCT4* (*POU5F1*) and primordial germ cell 7 (*PGC7*) are vital for the maturation oocytes and subsequently developmental ability of pig embryos (Bortvin et al., 2003; Saitou et al., 2002; Sato et al., 2002). The transcription level of *OCT4* varies significantly among individual oocytes (Monti and Redi, 2010; Pfeiffer et al., 2010) and *PGC7* plays an essential role in the development of oocytes (Bortvin et al., 2003; Saitou et al., 2002; Sato et al., 2002) for protecting

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TABLE 1. PRIMERS FOR QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION

Gene name	Gene ID	Forward primer sequence	Reverse primer sequence	Product length (bp)
<i>18S</i>	100861538	CCCACGGAATCGAGAAAGAG	TTGACGGAAGGGCACCA	122
<i>PGC7</i>	100511328	AGGATGACGGTGATGACAGT	TCATCTTCTAGACTTGGGGC	333
<i>OCT4</i>	00127461	TACAGGGTGGTGAAGTGAGGG	CAAGCAGTGACTATTTCGCAACG	166
<i>TET1</i>	100625859	TGTCGGCTTGGCAAGAAAGA	AGACCACTGTGCTGCCATTA	115
<i>TET2</i>	100521465	GTGAGATCACTACCCATCGCATA	TACTGGCACTATCAGCATCACAGG	123
<i>TET3</i>	100520484	TCTTCCGTCGTTTCAGCTACTACAG	GTGGAGGTCTGGCTTCTTCTCAA	127
<i>DNMT1</i>	606746	AGGTGAGGACATGCAGCTTT	AACTTGTTGTCTCCGTTGG	213
<i>DNMT3b</i>	100302109	GGTGACGGAGACGGTGAA	GCAGGGACGCTGGACTTT	105

DNA methylation (Nakamura et al., 2012) to affect transcriptional repression and chromatin condensation (Liu et al., 2012).

Although oocyte maturation is an important developmental process and is prerequisite for the subsequent fertilization and embryo development, the direct molecular mechanisms for the extrusion of PB1 are still unclear. Completion of the first meiosis results in formation of the PB1, and in almost all mammals, oocytes are arrested at metaphase II (MII). When oocytes were treated with cytochalasin B (CB; Tarkowski et al., 1977; Wakayama et al., 1998; Winger et al., 2015; Zijno et al., 1996), the spindles could not migrate to cortex region that resulted in inhibition of the extrusion of the PB1 (Longo and Chen, 1985).

On the contrary, histone deacetylation plays a crucial role in inhibiting oocyte maturation and meiosis (Akiyama et al., 2006; Endo et al., 2005; Ivanovska and Orr-Weaver, 2006; Yoshida et al., 2007), and the *in vitro* maturation (IVM) of oocytes could be delayed by the trichostatin A (TSA) treatment in mouse (De et al., 2004; Göttlicher et al., 2001; Krämer et al., 2003) and in pig (Zhang et al., 2018). TSA-induced hyperacetylation can disrupt chromatin and affect interactions between kinetochores and spindle microtubules (Kuroda et al., 2004; Sugiura et al., 2006). In addition, TSA could affect chromosomal segregation in different cell types (Akiyama et al., 2006; Endo et al., 2005; Ivanovska and Orr-Weaver, 2006). Therefore, TSA and CB were selected to

treat the oocytes *in vitro* for the regulation of the mitosis in the present study.

To evaluate the possible effect of epigenetic changes on the maturation of pig oocytes, some criteria, including the PB1 extrusion, epigenetic, and genetic maturation in cytoplasm (Mesbah et al., 2016), will be investigated. Hence, we consider the possible relationship between the extrusion of PB1 and the epigenetic changes in pig oocytes during IVM.

Materials and Methods

Chemical reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) unless otherwise stated.

Primer design

After querying the target gene sequence on NCBI, primer design was performed by Premier 6.0 software, and then the specificity was evaluated using Oligo 6.0 software. Then the designed primers were synthesized from the Shanghai Jierui Bioengineering Co., Ltd. Before being used, the primers were diluted with water to a working concentration of 10 $\mu\text{mol/L}$.

Samples collection and IVM

Ovaries were collected from prepubertal gilts at a local abattoir and were transported to the laboratory in 0.9% (wt/vol) NaCl at 35°C within 5 hours. In brief, cumulus-oocyte complexes (COCs) were aspirated from 2 to 6 mm follicles using an 18 gauge needle connected to a 20 mL disposable syringe. COCs with several compact layers cumulus cells (CC) were selected and washed three times

TABLE 2. THE PRIMARY ANTIBODIES USED FOR IMMUNOFLUORESCENCE

Primary antibodies	REF no.	Company
Rabbit anti- <i>PGC7</i>	ab19878	Abcom
Rabbit anti- <i>OCT4</i>	ab18976	Abcom
Rabbit anti-histone H4(acetyl K16)	ab109463	Abcom
Rabbit anti-histone H4(acetyl K12)	ab177793	Abcom
Rabbit anti-Di-Methyl-HistoneH3 (Lys9)	4658#	Cell Signaling Technology
Rabbit anti-Di-Methyl-Histone H3 (Lys4)	#9725	Cell Signaling Technology
Rabbit anti-Tri-Methyl-Histone H3 (Lys27)	#5499	Cell Signaling Technology
5-hmC (mouse anti-5-Hydroxymethylcytosine)	#51660	Cell Signaling Technology
5-mC (mouse anti-5-methylcytosine)	ab10805	Abcom

TABLE 3. THE INFLUENCE OF TRICHOSTATIN A TREATMENT ON THE OOCYTES MATURATION

TSA treatment concentration	Total oocytes (N)	Viability rate (%)	PB1 extrusion rate (%)
0 nM	162 (3)	90.81 $\pm$ 1.92	72.00 $\pm$ 1.73 ^a
5 nM	144 (3)	85.34 $\pm$ 4.50	50 ^b
50 nM	149 (3)	88.66 $\pm$ 3.84	23.89 $\pm$ 3.89 ^c
250 nM	121 (3)	91.92 $\pm$ 1.72	23.41 $\pm$ 1.33 ^c
500 nM	128 (3)	85.16 $\pm$ 0.47	27.43 $\pm$ 2.63 ^c
5 μM	145 (3)	83.29 $\pm$ 1.90	14.86 $\pm$ 0.92 ^d

PB1, first polar body; TSA, trichostatin A.

Different superscript in the same column indicates the significant difference ($p < 0.05$).

TABLE 4. THE INFLUENCE OF CYTOCHALASIN B TREATMENT ON THE OOCYTES MATURATION

<i>CB treatment concentration</i> ($\mu\text{g/mL}$)	<i>Total oocytes</i> (N)	<i>Viability rate</i> (%)	<i>PB1 extrusion rate</i> (%)
0	127 (3)	86.68 ± 1.86	61.90 ± 1.90^a
2.5	115 (3)	91.17 ± 3.42	8.96 ± 4.37^b
5	120 (3)	90.9 ± 2.1	13.73 ± 1.38^b
10	165 (3)	87.28 ± 0.94	8.43 ± 2.60^b

Different superscript in the same column indicates the significant difference.

in HEPES-buffered Tissue Culture Medium 199 (TCM-199) plus 0.8 mM L-glutamine and 2% (v:v) cattle serum (CS). The COCs were cultured in groups of 50–60 in four-well dishes containing IVM medium (TCM-199 supplemented with 10% CS, 10% (v:v) porcine follicular fluid, 0.8 mM L-glutamine, 75 mg/mL penicillin, 50 mg/mL streptomycin, 15 IU/mL pregnant mare serum gonadotropin (PMSG), and 15 IU/mL human chorionic gonadotropin (hCG)) at 38.5°C in a 5% CO₂/20% O₂ atmosphere at maximum humidity.

Oocytes matured in normal IVM medium as above were named as the control group. And the oocytes cultured in the IVM medium adding TSA with the doses of

FIG. 1. The distribution of H3K4me2 (A1), H3K9me2 (A2), and H3K27me3 (A3) in common pig oocytes.

FIG. 2. The distribution of H3K4me2 (**B1**, **C1**), H3K9me2 (**B2**, **C2**), and H3K27me3 (**B3**, **C3**) in TSA- (**B**) and CB-treated (**C**) pig oocytes. CB, cytochalasin B; TSA, trichostatin A.

0 nM/mL, 5 nM/mL, 50 nM/mL, 250 nM/mL, 500 nM/mL, and 5 µmol/mL CB at the concentration of 0, 2.5, 5, and 10 µg/mL were named as TSA treatment group and CB treatment group, respectively. After 42 hours IVM, the CCs were stripped from COCs by hyaluronidase to observe the quality of oocytes, which had intact membrane and the homogeneous cytoplasm.

Assessment of gene expression level in oocytes during IVM

Oocytes from GV to MII were collected every 12 hours (0, 12, 24, 36, and 45 hours). First, RNA was extracted from a pool of 60 oocytes using the Dynabeads® mRNA DIRECT™ Kit (Thermo Fisher, Carlsbad, CA) following the manufacturer's protocol. The total extracted RNA was loaded into the cDNA reaction and the first-strand cDNA was synthesized using the HiScript® RT SuperMix for qPCR kit (Vazyme Biotech Co., Nanjing, China) following the instruction. Quantification of each mRNA level was performed with a real-time polymerase chain reaction (RT-PCR) using AceQ® qPCR with SYBR® Green Master Mix (Vazyme Biotech Co., Nanjing, China) in a reaction volume of 20 µL, while 18S was used as internal controls. Primers for relevant genes were designed by the Primer 5 software and primer sequences are shown in Table 1.

Immunofluorescence

Oocytes from GV to MII were collected every 12 hours (0, 12, 24, 36, and 45 hours). In brief, oocytes were fixed with 4% paraformaldehyde at room temperature (RT) for 30 minutes. After being thoroughly washed three times, they were then permeabilized with 0.5% Triton X-100 for 1h followed by the incubation in blocking buffer (1% albumin from bovine serum [BSA] in phosphate-buffered solution [PBS]) at RT for 1 hour. Afterward, samples were incubated with primary antibodies shown in Table 2, respectively.

Subsequently, permeabilized oocytes were denatured with 4 N HCl for 10 minutes and were neutralized with 100 mM Tris-HCl for 10 minutes before blocking. After being washed three times in PBS, samples were further incubated with coat anti-rabbit or anti-mouse secondary antibody (1:100 dilution in 1% BSA) at RT for 1 hour. Finally, samples were washed and were stained with 10 µg/mL of Hoechst 33342 (Molecular Probes, Eugene, OR) for 10 minutes. All samples were then mounted on slides with glycerol and were observed by using a scanning microscope (Zeiss LSM 700 META).

Statistics

All analyses were performed by using SPSS 20.0 (SPSS, Inc., Chicago, IL) with one-way ANOVA. Differences among treatments were examined with the LSD Duncan test. Data

FIG. 3. The acetylation of H4K12 (A1, B1, C1) and H4K16 (A2, B2, C2) acetylationin in oocytes during IVM. (A) The control oocytes, (B) TSA-treated oocytes, and (C) CB-treated oocytes. IVM, *in vitro* maturation.

are expressed as mean $\pm$ standard error of the mean. For all analyses, significance was set at a p -value < 0.05 .

Results

The concentration selection for TSA and CB treatment on pig oocytes

TSA and CB will not adversely affect the survival rate of oocytes during maturation, but will affect the extrude

efficiency of polar bodies. As shown in Tables 3 and 4, although the viability of oocytes, which had intact membrane, compact, and homogeneous cytoplasm, was not affected by TSA or CB, the rate of oocytes with the PB1 extrusion was inhibited significantly when TSA or CB was performed on the IVM of pig oocytes.

The higher the TSA concentration is, the lower the polar body excretion rate and the greater the damage to the oocyte observed. In the lower the concentration, although it can

FIG. 4. The DNA methylation of 5mc and 5hmc in pig oocytes. (A) and (B) The control oocytes, (C) and (D) TSA-treated oocytes, (E) and (F) CB-treated oocytes.

reduce the maturation rate, the effect is not strongly different compared to the other groups. Different concentrations of polar bodies have a strong inhibitory effect on the discharge of polar bodies. Considering that the higher concentration could cause the greater damage to the oocytes, the concentrations of 50 nM TSA and 2.5 µg/mL CB were selected in the present study for further studies.

Histone acetylation and methylation of pig oocytes

As shown in Figure 1, the histone modifications were first examined in the common pig oocytes. The signal of H3K4me2, H3K9me2, and H3K27me3 was relatively strong

in the nucleus of GV oocytes before IVM. However, H3K4me2 was increased while the other two of H3K9me2 and H3K27me3 decreased at 12 hours in nucleus of pig oocytes. It is interesting to note that H3K4me2 is more concentrated on the chromosomes in nucleus of oocytes at 24 hours of IVM. With the progress of IVM at 36 and 45 hours, the signal of H3K4me2 is much stronger in PB1 than that in the nucleus when the mitosis happened. However, from 12 to 45 hours of IVM, the signal of both H3K9me2 and H3K27me3 was relatively weak in the pig oocytes. In detail, the common oocytes reached at the metaphase I (MI) middle-splitting period in 24 hours, and the first meiosis will happen in 36 hours.

FIG. 5. The distribution of PGC7 in pig oocytes during IVM. (A) The control oocytes, (B) TSA-treated oocytes, and (C) CB-treated oocytes.

When TSA was performed on the IVM of pig oocytes, the oocytes remained at the stage of MI presplitting according to the shape of the chromosome at 24 hours. Chromosomes of the oocytes in the TSA- and CB-treated groups (shown in Fig. 2B, C, respectively) did not separate and no PB1 could be observed. Further epigenetic observation showed that the methylation level of H3K4me2, H3K9me2, and H3K27me2 was kept at the higher level. Commonly in the control group, the acetylation of H4K16 will deacetylate as shown in Figure 3, however, no deacetylation of histone H4K12 and H4K16 was observed either in TSA- or CB-treated group, when chromosome condensation during the maturation of oocytes as shown in Figure 3.

DNA methylation of pig oocytes

As shown in Figure 4 of pig oocytes, 5mC existed in the whole IVM progress, but the stronger signal was detected in

one chromosome set at 36 hours. However, nearly no signal of 5hmC was detectable in pig oocytes during IVM. As described above, no separation happened in the chromosomes set when TSA or CB treatment was performed on IVM, the DNA methylation of 5mC was relatively higher in all of these chromosomes and no signal of 5hmC was observed. Therefore, the DNA methylation was kept at a higher level when the mitosis was blocked by TSA or CB treated.

PGC7 and OCT4 distribution in pig oocytes

As shown in Figures 5 and 6, PGC7 and OCT4 were evenly distributed in pig common oocytes during the early 36 hours of IVM. Once the mitosis happened from 36 hours, both PGC7 and OCT4 were more detectable in one chromosome set. However, when TSA or CB was added in IVM, only the evenly distribution of PGC7 and OCT4 was observed in oocytes during IVM whole progress. It is very

FIG. 6. The distribution of OCT4 in pig oocytes during IVM. (A) The control oocytes, (B) TSA-treated oocytes, and (C) CB-treated oocytes.

interesting that both in TSA and CB treatment groups, the DNA methylation and histone methylation were relatively higher, but noncentralized *PGC7* in one chromosome set could be observed. In conclusion, histone modification included H4K12ac and H3K9me2; maternal factors *PGC7* and *OCT4* may be related to pig oocytes maturation for the extrusion of PB1. But the relationship between *PGC7* and DNA methylation as well as H4K12ac and H3K9me2 should be explored.

Gene expression in oocytes

The expression of these genes was validated in the common pig oocytes by quantitative RT-PCR as shown in Figures 7 and 8. Results showed that the expression level of *PGC7*, *OCT4*, DNA methyltransferase1 (*DNMT1*), DNA methyltransferase3b (*DNMT3b*), tet methylcytosine dioxygenase 1 (*TET1*), tet methylcytosine dioxygenase 2 (*TET2*),

and tet methylcytosine dioxygenase 3 (*TET3*) was increased during IVM. However, when CB was performed on the IVM, the expression of all of genes *PGC7*, *OCT4*, *DNMT1*, *DNMT3b*, *TET1*, *TET2*, and *TET3* was decreased significantly at 24 hours of IVM, but no difference could be observed at 45 hours when CB was removed.

In addition, when TSA treatment was performed on the IVM, expression level of *PGC7*, *OCT4*, *DNMT1*, *DNMT3b*, and *TET1* was significantly lower at 36 hours of IVM ($p < 0.05$), but the expression levels of *OCT4*, *DNMT1*, and *TET1* were significantly increased at 45 hours of IVM ($p < 0.05$).

Discussion

In mammals, epigenetic modifications are crucial for the oocyte development, not only the regulation of chromatin structure but also the gene expression is related to the epigenetic modifications (Jenuwein and Allis, 2001; Reik et al.,

FIG. 7. The effect of TSA treatment on the gene expression in pig oocytes during IVF. Significant differences were indicated by the different letters between the treatments. Results are shown as means $\pm$ SEMs, statistically significant differences are indicated by asterisks (* $p < 0.05$ and ** $p < 0.01$). SEM, standard error of the mean.

FIG. 8. The effect of CB treatment on the expression of genes in pig oocytes during IVM. Results are shown as means $\pm$ SEMs, statistically significant differences are indicated by asterisks (* $p < 0.05$ and ** $p < 0.01$).

2001; Robertson and Wolffe, 2000). The diverse biological functions of oocytes such as cell cycle progression, DNA replication and repair, transcriptional activity, and chromosome stability are associated with histone modifications (Bloushtain-Qimron et al., 2009; Gu et al., 2010; Kageyama et al., 2007; Kouzarides, 2007; Strahl and Allis, 2000; Wang and Tilly, 2010).

Status of different lysine residues on histone H3 and H4 were examined, including acetylation of H4K12 and methylation of H3K4, H3K9 which were reported to play a vital role in oocyte maturation (Endo et al., 2005; Kageyama et al., 2007; Kim et al., 2003; Seki et al., 2005). Therefore, it was confirmed that oocyte maturation needs the accurate histone modification (Hou et al., 2008; Kageyama et al., 2007; Park et al., 2010; Qiao et al., 2010; Racedo et al., 2009; Sarmento et al., 2004).

The completion of meiotic divisions is a result of the coordination of meiotic spindle assembly and function with meiotic cell cycle progression (Albertini, 1992). The epigenetic changes during pig oocyte maturation are precisely controlled by the cell cycle (Endo et al., 2005), abnormal of which might disrupt the maturation of pig oocytes (Wang et al., 2016).

Previous study has shown that histone acetylation is a meiosis stage-dependent and lysine residue-specific process during oocyte maturation, and acetylation patterns are not identical among oocytes from different species (Gu et al., 2010). At the mitosis stage, gene expression could be propagated by the histone acetylation, the level of H3K4me2 was increased, but the DNA methylation (5-mC) and H3K9me2 were decreased (Zhao et al., 2017), while the misexpression could be caused by the aberrant H3K4me3 and H3K27me3 (Xu et al., 2017). If the discharge of the PB1 is suppressed, the level of H3K4me2 will increase, while the level of 5mC and H3K9me2 will decrease (Zhao et al., 2017).

In addition, H3K27me3 and H3K9me2 are reported as two independent repressive chromatin modifications, especially H3K27me3 has been reported as a euchromatin-specific mark (Roudier et al., 2011). Although methylation levels of H3K4me2/me3 remained high and constant throughout the cell cycle, the H3K27me3 was restricted to distal regions on metaphase and anaphase (Oliver et al., 2013). Our results showed that with the resumption of meiosis, the deacetylation of H4K12ac takes place in the condensed chromo-

some, while a similar acetylation pattern of H4 takes place during maturation in bovine oocytes (Maalouf et al., 2008).

Although histone deacetylation may be required for the orderly progression through germinal vesicle (GV) to germinal vesicle breakdown (GVBD) as it has been reported in IVM pig oocytes (Gu et al., 2010), the present study further confirmed that the accurate modification of histones, especially the H4K12ac and H3K9me2, is necessary for pig oocyte maturation.

In addition, the present study indicated that the extrusion of the first PB1 in pig oocytes might be affected not only by the modification of histones but also by DNA methylation, which is a dynamic process during oocytes' growth and development (Bo et al., 2017). Once the CB or TSA treatment was performed on the oocytes, the mitosis did not happen, but the concentrated 5mC was observed on one of the nuclei. It has been noted that the DNA methylation can be protected by PGC7 from TET family enzyme-dependent oxidation (Bian and Yu, 2014), that PGC7 binds to H3K9me2 to block the activity of the Tet3 methylcytosine oxidase in the maternal genome of the fertilized embryos.

Although the genomic regions occupied by nucleosomes containing H3K9me2 can be targeted by PGC7 (Kang et al., 2013), the relationship of PGC7 and DNA methylation, H3K9me2, and H4K12ac was first reported to be correlated with the extrusion of PB1 extrusion in pig oocytes during IVM. We supposed that the mitosis of pig maturation to extrude the PB1 might be regulated by the DNA methylation.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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