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—Original—

Supplementation of Maturation Medium with Folic Acid Affects DNA Methylation of Porcine Oocytes and Histone Acetylation of Early Developmental Stage Embryos

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Abstract: The present study examined the effect of supplementation of the maturation medium with folic acid on the developmental competence of porcine oocytes as well as on the global DNA methylation of oocytes and histone acetylation in early developmental stage embryos. Supplementation of the maturation medium with 100 μM folic acid improved the development ratio of the oocytes to the blastocyst stage compared with those of oocytes that were cultured with either 0 or 10 μM folic acid. The global DNA methylation levels of the oocytes that matured with 100 μM folic acid were higher than those of oocytes that were cultured without folic acid, while the global DNA methylation levels of the oocytes decreased during maturation. Addition of either folic acid or 1.2 mM *N*-acetyl-cysteine (NAC) to the maturation medium significantly reduced the levels of reactive oxygen species in the oocytes. Supplementation of maturation medium with folic acid significantly enhanced the acetylation level of H4K8 and decreased the expression of histone deacetylase 1 (HADC1), whereas the addition of NAC did not affect the acetylation levels of H4K8 or HADC1. In addition, supplementation of maturation medium with 1.2 mM NAC did not improve the development rate to the blastocyst stage. In conclusion, folic acid added to the maturation medium affects not only the DNA methylation state of oocytes, but also the histone acetylation of early developmental stage embryos, as well as improving the developmental competence of the oocytes.

Key words: Folic acid, DNA methylation, Porcine oocyte, Histone acetylation

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Introduction

One of the major events in early embryonic development is zygotic genome activation (ZGA). This event occurs at the 4–8 cell stage in porcine embryos [1]. ZGA is associated with global histone modification, including the acetylation of histones and the demethylation of DNA [2]. The acetylation levels of histones in early developmental stage embryos are reported to be related to the developmental competency of somatic nuclear transfer porcine embryos [3]. Tricostatin A treatment of 8- to 16-cell-stage bovine embryos that are produced *in vitro* increases the level of histone acetylation and improves embryo development [4]. However, it is unclear what factors, including oocyte culture conditions, affect the levels of histone acetylation of embryos at the time of ZGA.

Oocyte quality is closely related to developmental competence, and the acquisition of oocyte competence accompanies spatiotemporal changes in histone acetylation and DNA methylation [5–7]. The methylation level of DNA increases with oocyte growth [5, 8, 9], and bovine oocytes that are derived from large antral follicles have higher DNA methylation levels than those that are derived from small follicles [8]. It has been suggested that DNA methylation continues up to approximately metaphase stage 2 in mice [10]. Folic acid is a member of the vitamin B family and serves as a methyl donor [11]. The presence of a methyl donor such as folic acid in culture profoundly affects the DNA methylation state. Oocytes that develop under methyl-donor deficient conditions have low developmental competency and a slight but significant decrease in the methylation state of their imprinted genes [12]. The concentration of folate in plasma has been found to positively affect the fertilization out-

come of oocytes [13].

Folic acid also acts as an antioxidant [14]. Kim et al. [15] reported that oocytes that are cultured in a medium that contains folic acid show increased GSH content and improved developmental ability. On the basis of these studies, it is plausible that the folic acid in maturation media affects the oocyte redox state as well as DNA methylation. However, it is not clear whether folic acid in the maturation medium affects DNA methylation of oocytes and the acetylation levels of histones in 4- to 8-cell-stage embryos or whether the effect of folic acid on histone acetylation is attributable to its antioxidant properties or methyl donor properties. *N*-acetyl-cysteine (NAC) is a potent antioxidant that has been proven to reduce oxidative stress in oocytes and improve embryo development [16]. In the present study, we confirmed the previously reported beneficial effect of adding folic acid to the maturation medium on the developmental competence of porcine oocytes. In addition we examined the effect of the supplementation of maturation medium with folic acid on the level of DNA methylation and reactive oxygen species (ROS) of the oocytes, as well as on the levels of histone H4 acetylation and the expression of histone deacetylase 1 (HADC1) in 4- to 8-cell-stage embryos. We also examined the effect of NAC on the levels of ROS in oocytes, the levels of histone H4 acetylation, and the expression of HADC1 in 4- to 8-cell-stage embryos.

Materials & Methods

Chemicals and media

All the chemicals were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise indicated. The medium used for *in vitro* maturation (IVM) was North Carolina State University 37 solution [17], containing 0.6 mM cysteine and follicular fluid (10% v/v). The media that were used for parthenogenetic activation and *in vitro* culture were based on PZM4 [18].

Oocyte collection

Ovaries were obtained from prepubertal gilts at a local slaughterhouse, and were transported to the laboratory at 37 °C within 1.5 h in phosphate-buffered saline (PBS) that contained 10 IU/ml penicillin G potassium and 0.1 g/ml streptomycin sulfate. The oocytes were aspirated from the follicles (diameter, 3–6 mM) using an 18-gauge needle that was connected to a 10-ml syringe. Only cumulus cell-oocyte complexes (COCs) with compact CCs were selected and cultured in maturation medium (10 oocytes/100 µl droplet).

In vitro maturation, activation, and in vitro culture

For the first 20 h of the maturation period, the oocytes were cultured in maturation medium that contained 1 mM dibutyryl cAMP (dbcAMP: Sigma Chemical Co., St Louis, USA), 10 IU/ml equine chorionic gonadotropin (eCG, ASKA Pharma Co. Ltd, Tokyo, Japan), and 10 IU/ml human chorionic gonadotropin (hCG, Fuji Pharma Co. Ltd, Tokyo, Japan). They were then transferred to maturation medium that lacked both dbcAMP and the hormones, and were cultured for 24 h. *In vitro* maturation was performed at 38.5 °C under an atmosphere of 5% CO_2 and 95% air. After maturation, the oocytes were separated from the enclosing cumulus cells by gentle pipetting in the maturation medium containing 0.2% hyaluronidase (Sigma Chemical Co.). To determine oocyte developmental competence, the oocytes were subjected to parthenogenetic activation on the basis of the premise that a high rate of polyspermic fertilization might prevent assessment of their intrinsic oocyte developmental abilities. The oocytes were activated in culture medium that contained ionomycin (10 µg/ml), and were then incubated for 6 h in culture medium that contained cytochalasin B (10 µg/ml) and cycloheximide (10 µg/ml). After incubation, the oocytes were washed and transferred into a culture drop (10 oocytes/50 µl) and incubated for 7 days to obtain blastocysts. *In vitro* culture was performed at 38.5 °C under an atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

Immunostaining analysis for the detection of 5-methylcytosine

Immunostaining of 5-methyl-cytosine was performed as described below. The oocytes were permeabilized in 0.1% Triton X for 30 min prior to fixation in 2% paraformaldehyde/PBS. Then, they were treated for 10 min with 4N HCl containing 0.1% Triton X, followed by neutralization in 100 mM Tris-HCl (pH 8.5). The oocytes were incubated in blocking solution and incubated overnight with the primary antibody (anti-5-methyl cytosine mouse monoclonal antibody, 1:500; Epigenetek), followed by 1 h of incubation with the secondary antibody (goat anti-mouse IgG, 1:1000; Alexa Fluor 555, Cell Signaling). The fluorescence intensity of the spindles was measured using the Image-J software, and the fluorescence intensity per spindle was calculated.

ROS measurement

ROS levels in the oocytes were measured using ROS Detection Reagents (Invitrogen) according to the manufacturer's instructions. For these measurements, dihydrocalcein AM, which freely permeates cell membranes and is oxidized to emit green fluorescence, was used.

The fluorescence of the oocytes was observed under a digital fluorescence microscope and the intensity of the fluorescence was measured using the Image-J software.

Immunostaining analysis for detection of acetylated histone H4K8 and HADC1

Three days after activation, the embryos were fixed overnight at 4 °C in PBS-polyvinyl alcohol (PVA) containing 4% paraformaldehyde (Funakoshi, Tokyo, Japan). After several washes in PBS-PVA, the embryos were permeabilized for 30 min at room temperature with PBS-PVA containing 0.25% Triton X-100, and were then incubated in blocking solution (PBS containing 5% BSA, 1% Tween 20, and 5% goat serum; Funakoshi, Tokyo, Japan) for 1 h at room temperature. This step was followed by treatment with primary antibodies (rabbit polyclonal antibodies against acetylated [Ac] H4K8, 1:500; Millipore, Milford, MA, USA) and HADC1 (1:500; Millipore) by adding each of them individually to the blocking solution and incubation overnight at 4 °C. After the embryos had been incubated with each primary antibody, they were vigorously washed in 5% BSA in PBS-PVA and incubated in a blocking solution that contained the secondary antibody (FITC-conjugated goat anti-rabbit IgG, 1:1000; Millipore) for 1 h at room temperature. After washing, the embryos were mounted with an antifade reagent containing DAPI (Pro-long gold antifade reagent with DAPI; Invitrogen, OR, USA) on glass slides and observed under a digital fluorescence microscope (BZ-8000; Keyence, Tokyo, Japan); fluorescence images of the oocytes were captured. A negative control was made following the above-mentioned procedure without a primary antibody. The fluorescence intensities of embryos were measured using the Image-J software (NIH, Bethesda, MD, USA) and divided by the total number of nuclei to obtain the fluorescence intensity per nucleus.

Experimental design

Experiment 1: The effect of supplementation of the maturation medium with folic acid on the nuclear maturation of the embryos was examined. Twenty oocytes were cultured in a medium that contained 0, 10, or 100 μM folic acid, and nuclear maturation was examined. This experiment was repeated 4 times.

Experiment 2: The effect of supplementation of the maturation medium with folic acid on the development rate to the blastocyst stage and on the total cell numbers of the blastocysts was examined. Twenty-five oocytes were activated and cultured for 7 days. This experiment was repeated 5 times.

Experiment 3: We examined the effect of supplementation of the maturation medium with folic acid on global DNA methylation levels in oocytes. Oocytes were cultured in IVM medium containing 0 or 100 μM folic acid. At the end of IVM, approximately 20 oocytes were randomly selected from each experimental group and subjected to immunostaining. This experiment was repeated 2 times.

Experiment 4: We examined the effect of supplementation of the maturation medium with folic acid and NAC on the amount of ROS in oocytes. Oocytes were matured in medium containing 100 μM folic acid, 1.2 mM NAC, or no supplementation. At the end of IVM, the ROS in the oocytes were examined. Twenty oocytes were used for the comparison. This experiments was repeated 2 times.

Experiment 5: We examined the effect of supplementation of the maturation medium with folic acid and NAC on the expression levels of Ac-H4K8 and HADC1 in early developmental stage embryos. Three days after activation, 4- to 8-cell-stage embryos were randomly selected from each experimental group and were subjected to immunostaining. Approximately 20 embryos were used for each replicate. This experiments was repeated 2 times.

Experiment 6: We examined the effect of the supplementation of maturation medium with NAC on the nuclear maturation rate and the development rate to the blastocyst stage. Approximately 50 oocytes were cultured. Twenty-five randomly selected oocytes were used for the assessment of nuclear maturation, and the remaining 25 oocytes were activated and cultured for 7 days. This experiment was repeated 5 times.

Statistical analysis

The data were compared using Tukey's *post hoc* test and one-way ANOVA. The maturation and development rates were arc-sine transformed prior to analysis. *P* values of less than 0.05 were considered to be statistically significant.

Results

In experiment 1, almost 80–90% of the oocytes reached metaphase 2 stage both in the presence and absence of folic acid (Table 1). In experiment 2, we compared the developmental competence of oocytes between the presence and absence of folic acid in the maturation medium. After activation, 12.7% of the oocytes that were cultured in medium containing 100 μM folic acid developed to the blastocyst stage, a figure which was significantly greater than the percentage of oocytes that were cultured with 0 or 10 μM folic acid ($P < 0.05$). In contrast, the total cell numbers of the blastocysts were similar among the groups (Table 2). Experiment 3 examined whether cultur-

Table 1. Effect of supplementation of maturation medium with folic acid on the rate of nuclear maturation

Folic acid (μM)	No. of replicates	No. of oocytes cultured	M2 oocytes (%) Average ± S.E.
0	4	80	83.8 ± 5.4
10	4	80	88.8 ± 2.2
100	4	80	86.3 ± 6.5

ing oocytes with folic acid affects global DNA methylation of *in vitro* matured oocytes. As seen in Fig. 1, global DNA methylation decreased significantly during oocyte maturation but was maintained at a significantly higher level in the oocytes that were cultured with folic acid than in those that were cultured in medium without folic acid. In experiment 4, we examined whether NAC or folic acid affects the amount of ROS in oocytes. The ROS levels in the oocytes are shown in terms of green fluorescence intensity in Fig. 2A. When the oocytes matured in medium containing folic acid or NAC, the amount of ROS in the oocytes was significantly lower than that in the oocytes that matured in medium without these supplements. The results of experiment 5 are shown in Figs. 3 and 4. Ac-H4K8 and HADC1 expression were observed in the nucleus. Supplementation of maturation medium with folic acid significantly increased the acetylation level of H4K8 in the embryos but decreased the acetylation level of HDAC1, whereas NAC did not affect the acetylation levels of H4K8 or HADC1 (Fig. 3 and 4). In experiment 6, we examined the effect of supplementation of the maturation medium with NAC on the developmental competence of the oocytes. Neither the nuclear maturation rate nor the development rate to the blastocyst stage was increased by NAC supplementation (Table 3).

Discussion

In the present study, supplementation of maturation medium with folic acid increased the global DNA methylation of oocytes and the development rate to the blastocyst stage. Supplementation with folic acid or NAC decreased the amount of ROS in matured oocytes, whereas only folic acid increased the acetylation levels of H4K8 and decreased the level of HADC1 in 4- to 8-cell stage embryos.

Kim *et al.* [15] reported that supplementation of maturation medium with folic acid increases the level of GSH in oocytes as well as the development rate to the blastocyst stage. They suggested that folic acid acts as an antioxidant, thereby improving oocyte quality. Consis-

Fig. 1. Level of global DNA methylation in oocytes. Oocytes immediately after collection (GV) and after *in vitro* maturation with or without 100 μM folic acid (FA) were immunostained against 5-methyl cytosine. The experiment was repeated 2 times, and no significant difference was found between the replicates. The average fluorescent intensity per spindle of oocytes that had matured without FA (control) was defined as 1.0. a–c, Fig. 1A and B: Representative figures of bright fluorescence and fluorescence field images of immunostained specimens. Fig. 1C: The relative level of global DNA methylation (number of oocytes: GV=40, control=35, and FA=34). The bar represents the average ± S.E. value. The average fluorescence intensity per spindle of oocytes that had matured without FA (control) was 378 defined as 1.0. a–c, Different letters indicate significant differences ($P<0.05$).

Fig. 2. Level of reactive oxygen species (ROS) in oocytes. Oocytes were matured with 100 μM folic acid, 1.2 mM NAC, or without these supplements. The amount of ROS in the oocytes was measured in 2 replicates, between which no significant difference was observed. Fig. 2A and B: Representative figures of bright fluorescence and fluorescence field images of stained specimens. Fig. 2C: The relative level of ROS in the oocytes (number of oocytes: control=30, NAC=35, and FA=38). The bar represents the average ± S.E. value. The average of the fluorescence intensities of the oocytes that were cultured without supplements was defined as 1.0. a–b, Different letters indicate significant differences ($P<0.05$).

Fig. 3. Level of acetylation of H4K8 in embryos originating from oocytes that were cultured with 1.2 mM *N*-acetyl-cysteine (NAC), 100 μM folic acid (FA), or without these supplements. Four- to eight-cell- stage embryos were subjected to immunostaining against Ac-H4K8 in 2 replicates. No significant difference was observed between the replicates. Fig. 3A and B: Representative figures of bright fluorescence and fluorescence image of the embryos. Fig. 3C: The relative acetylation levels of H4K8 in the embryos (number of embryos: control=49, NAC=48, and FA=48). The bar represents the average ± S.E. value. The average fluorescence intensity per nucleus of the embryos that originated from oocytes that were cultured without supplements (Control) was defined as 1.0. a–b, Different letters indicate a significant differences ($P<0.05$).

Fig. 4. Level of HADC1 in embryos that originated from oocytes that were cultured with 1.2 mM *N*-acetyl-cysteine (NAC), 100 μM folic acid (FA), or without these supplements. Four- to eight-cell-stage embryos were subjected to immunostaining against HADC1 in 2 replicates. No significant difference was observed between the replicates. Fig. 4A and B: Representative figures of bright fluorescence and fluorescence images of the embryos. Fig. 4C: The relative expression levels of HADC1 in the embryos (number of embryos: control=38, NAC=35, and FA=38). The bar represents the average ± S.E. value. The average data for the fluorescence intensity per nucleus of the embryos that originated from oocytes that were cultured without supplements (Control) was defined as 1.0. a–b, Different letters indicate a significant differences ($P<0.05$).

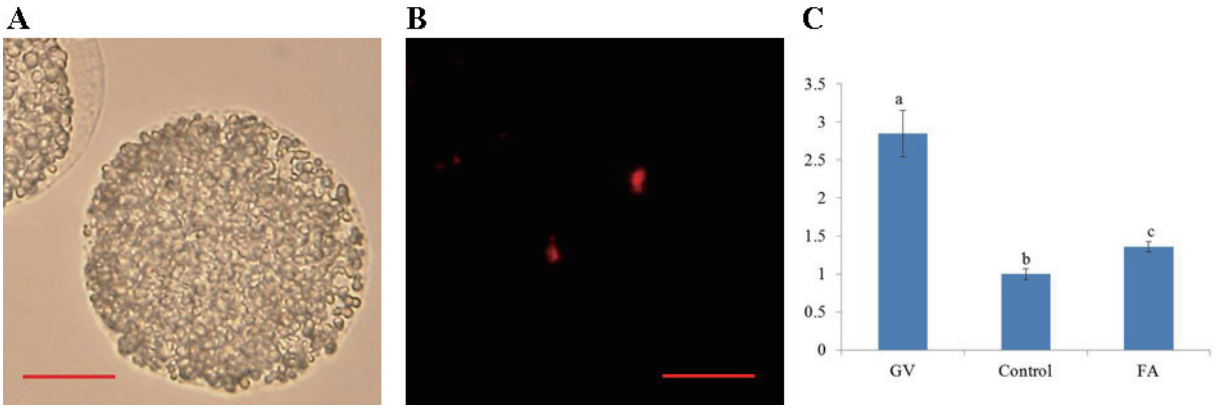

Fig. 1.

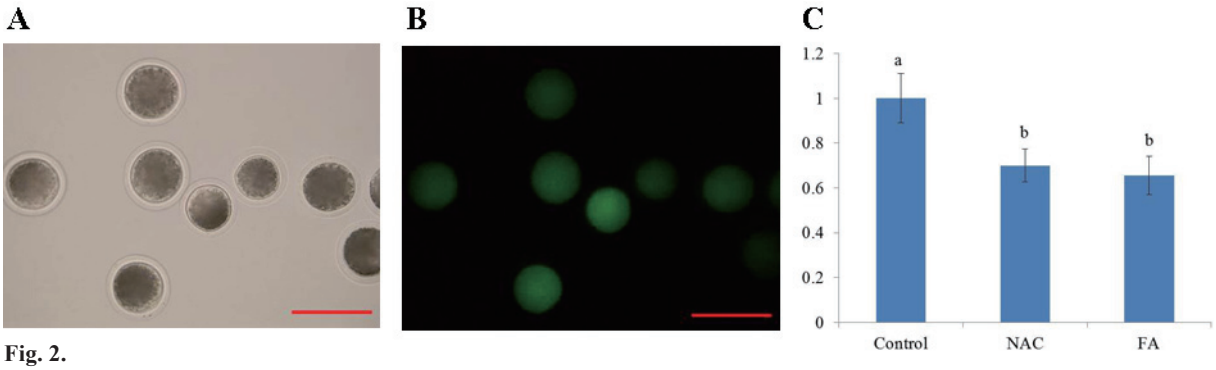

Fig. 2.

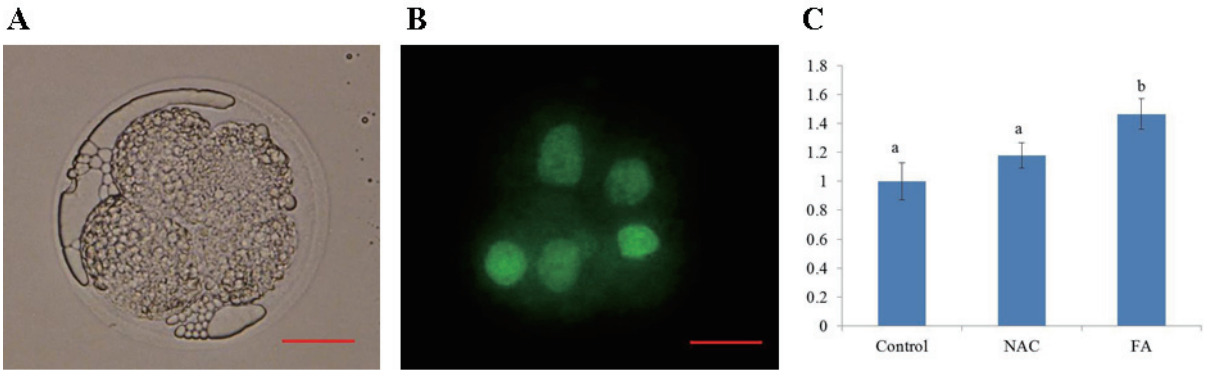

Fig. 3.

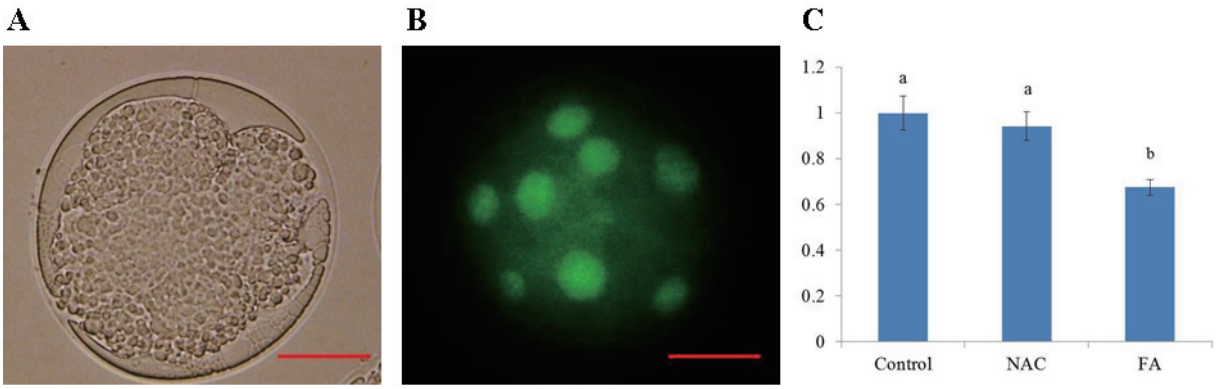

Fig. 4.

Table 2. Effect of supplementation of maturation medium with folic acid on the developmental rate to the blastocyst stage

Folic acid (μM)	No. of replicates	No. of oocytes activated	Blastocysts (%) Average ± SE	Total cell no. of blastocysts Average ± SE
0	5	122	5.6 ± 0.4[a]	40.0 ± 6.1
10	5	139	4.9 ± 0.4[a]	40.6 ± 5.1
100	5	136	12.7 ± 2.2[b]	40.5 ± 2.4

a–b, Different superscripts differ significantly (P<0.05).

Table 3. Effect of supplementation of maturation medium with NAC on the rate of nuclear maturation and the development rate to the blastocyst stage

NAC (mM)	No. of replicates	No. of oocytes	M2 oocytes (%) Average ± SE	Blastocysts (%) Average ± SE	Total cell no. of blastocysts Average ± SE
0	5	250	70.4 ± 2.0	6.4 ± 1.6	31.4 ± 1.7
1.2	5	250	73.6 ± 3.7	4.0 ± 1.3	36.3 ± 5.2

tent with these data, our results show that folic acid decreased the amount of ROS in oocytes. Folic acid has also been reported to act as a donor for DNA methylation. Ikeda *et al.* [19] reported that supplementation of the culture medium with homocysteine, which is another methyl donor, increases the global DNA methylation of bovine embryos. In addition, in mouse embryos that were cultured in a methyl donor-deficient medium, low methylation levels of DNA imprinting were observed [12]. To the best of our knowledge, this is the first study to report that supplementation of maturation medium with folic acid affects the level of global DNA methylation in mammalian oocytes. Methylation levels have been shown to increase along with oocyte growth in cows and mice [8, 9]. Once oocytes resume meiotic maturation, their methylation levels decrease during oocyte maturation, and demethylation continues once they undergo aging [20]. In addition, the level of DNA methylation further decreases as oocytes age [21]. In the present study, while the level of global DNA methylation was low at the end of the maturation period compared with the level that was seen immediately after collection, DNA methylation was maintained at higher levels in oocytes that were cultured with folic acid than in those cultured without folic acid. Under the present culture conditions, germinal vesicle breakdown occurred at 21 h of the maturation period and nuclear maturation occurred after following 23 h. It is not known whether porcine oocytes that are cultured with folic acid increase their levels of *de novo* DNA methylation during the first 21 h of maturation or whether the oocytes maintain their the DNA methylation level after following 23 h of the maturation period. Furthermore, considering the fact that DNA methylation is well regulated, depending on the tissue type, development time, DNA sequence, and genomic region, the significance of high global DNA methylation has yet to be elucidated.

One of the focus areas of our study was the mechanism by which culture condition of oocyte maturation affects histone acetylation in early developmental stage embryos. In the present study, we examined the levels of acetylation of H4K8 in 4- to 8-cell-stage embryos when ZGA occurs. We also demonstrated that embryos derived from oocytes that were cultured with folic acid had higher acetylation levels of H4K8 than those that were derived from oocytes cultured without folic acid. Shiratsuki et al. [4] reported that treatment of 8- to 16-cell-stage bovine embryos with tricostatin A at the time of genomic activation increased acetylation levels of histones and the development rate to the blastocyst stage, as well as the inner cell mass/ trophoblastic cell ratio of the blastocysts. On the basis of these observations, we believe that the high level of acetylation observed in the present study may have contributed to the high development rate. Because folic acid also exhibits an antioxidative effect, we examined the possibility that the antioxidant properties of folic acid increase the acetylation of histones as well as the development rate to the blastocyst stage. When oocytes were cultured in medium containing NAC, the amount of ROS in the oocytes decreased and the development rate to the blastocyst stage increased [16]. However, in the present study, NAC reduced the amount of ROS, but neither the acetylation levels of H4K8 nor the development rate to the blastocyst stage were affected. The reason why NAC did not affect the development rate is unclear, but Whitaker et al. [16] reported that addition of 1.5 mM NAC into the maturation medium improved

male pronucleus formation and the blastulation ratio. We examined the effect of 1.2 mM NAC on the developmental competence of the oocytes following parthenogenetic activation. We think that the low level of NAC or differential conditions might have been responsible for the lack of effect observed in the present study. Based on the present data, we conclude that the high acetylation levels of H4K8 were not derived from the antioxidative effect of folic acid. It has been reported that the downregulation of HADC1 results in high H4K5 acetylation in mouse embryos [22], while low levels of HADC1 result in high levels of H4K14 acetylation in cows. In line with these results, the present study demonstrated that supplementation of the maturation medium with folic acid decreases the levels of HADC1 in 4- to 8-cell-stage embryos, whereas NAC does not affect the level of HADC1. These data indicate that low HADC1 expression is the cause of high H4K8 acetylation and support our hypothesis that the presence of folic acid in porcine maturation medium influences the histone acetylation of early developmental stage embryos. However, the mechanism connecting low HADC1 expression in embryos with high global DNA methylation of oocytes remains to be elucidated.

In conclusion, supplementation of culture media with folic acid improves oocyte quality. In addition, folic acid in the maturation medium is responsible for global DNA hypermethylation in oocytes and a high level of histone acetylation in early developmental stage embryos.

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