

# Parthenogenetic activation and somatic cell nuclear transfer of porcine oocytes activated by an electric pulse and AZD5438 treatment

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## Summary

We examined the *in vitro* developmental competence of parthenogenetic activation (PA) oocytes activated by an electric pulse (EP) and treated with various concentrations of AZD5438 for 4 h. Treatment with 10  $\mu$ M AZD5438 for 4 h significantly improved the blastocyst formation rate of PA oocytes in comparison with 0, 20, or 50  $\mu$ M AZD5438 treatment (46.4% vs. 34.5%, 32.3%, and 24.0%, respectively;  $P < 0.05$ ). The blastocyst formation rate was higher in the group treated with AZD5438 for 4 h than in the groups treated with AZD5438 for 2 or 6 h (42.8% vs. 38.6% and 37.2%, respectively;  $P > 0.05$ ). Furthermore, 66.67% of blastocysts derived from these AZD5438-treated PA oocytes had a diploid karyotype. The blastocyst formation rate of PA and somatic cell nuclear transfer (SCNT) embryos was similar between oocytes activated by an EP and treated with 2 mM 6-dimethylaminopurine for 4 h and those activated by an EP and treated with 10  $\mu$ M AZD5438 for 4 h (11.11% vs. 13.40%,  $P > 0.05$ ). In addition, the level of maturation-promoting factor (MPF) was significantly decreased in oocytes activated by an EP and treated with 10  $\mu$ M AZD5438 for 4 h. Finally, the mRNA expression levels of apoptosis-related genes (*Bax* and *Bcl-2*) and pluripotency-related genes (*Oct4*, *Nanog*, and *Sox2*) were checked by RT-PCR; however, there were no differences between the AZD5438-treated and non-treated control groups. Our results demonstrate that porcine oocyte activation via an EP in combination with AZD5438 treatment can lead to a high blastocyst formation rate in PA and SCNT experiments.

Keywords: AZD5438, Cyclin-dependent kinase inhibitor, MPF, PA, SCNT

## Introduction

Parthenogenesis is a powerful method to analyse epigenetic changes in maternal genes during embryonic development (Surani & Barton, 1983; McGrath & Solter, 1984; Bos-Mikich *et al.*, 2016). Moreover,

improved parthenogenetic activation (PA) assists somatic cell nuclear transfer (SCNT) (Whitworth *et al.*, 2009). However, the efficiency of activation remains low. Efforts are continuing to identify better ways (Lee *et al.*, 2004; Diao *et al.*, 2013; Lee *et al.*, 2015; Arias *et al.*, 2016). Metaphase II (MII) arrest is partly regulated by high maturation-promoting factor (MPF) activity (Hashimoto & Kishimoto, 1988). Early events in oocyte activation occur as MPF activity decreases.

Protein inhibitors that accelerate oocyte activation and improve the blastocyst rate have been widely used (Nussbaum & Prather, 1995; Mayes *et al.*, 1995). A widely used method in bovine oocytes activation is combining an electric pulse (EP) with cycloheximide, a protein synthesis inhibitor, which prevents synthesis of MPF (Presicce & Yang, 1994). Cytochalasin B (CB),

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a microfilament polymerization inhibitor, has been investigated as means of artificial activation (Cha *et al.*, 1997). 6-Dimethylaminopurine (6-DMAP), a protein kinase inhibitor, inhibits MPF activity by blocking protein phosphorylation, which causes chromosomal abnormalities (De La Fuente & King, 1998; Van De Velde *et al.*, 1999). In addition, cyclin-dependent kinase (CDK) inhibitors such as butyrolactone I selectively inhibit the catalytic subunit of MPF and achieve a high rate of good-quality blastocysts (Dinnyés *et al.*, 2000).

AZD5438 (4-(1-isopropyl-2-methylimidazol-5-yl)-2-(4-methylsulphonylanilino)pyrimidine), a broad range CDK inhibitor, can inhibit the phosphorylation of CDK substrates and blocks cell cycling *in vitro* and significantly decreases the proliferation of human tumour cell lines (Dinnyés *et al.*, 2000; Byth *et al.*, 2009; Raghavan *et al.*, 2012). In addition, AZD5438 has undergone clinical evaluation and been investigated in healthy volunteer studies (Camidge *et al.*, 2007a,b). However, activation of mammalian oocytes using AZD5438 has not been evaluated.

The goal of this study was to investigate the development of porcine oocytes parthenogenetically activated by an EP combined with AZD5438 treatment. Optimal concentration and duration of AZD5438 treatment were determined. Then, MPF assay, karyotype analysis and RT-PCR analysis of several apoptosis- and pluripotency-related genes in embryos were conducted. We also compared the developmental competence of SCNT embryos between the AZD5438-treated and 6-DMAP-treated groups.

## Materials and Methods

### Animals

This research was approved by the Ethics Committee of Yanbian University.

### Chemicals

All chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise noted. AZD5438 was purchased from Selleck Chemicals (Houston, TX, USA).

### Oocyte retrieval and *in vitro* maturation

Ovaries were obtained from a local abattoir, transported to the laboratory within 2 h. Cumulus-oocyte complexes (COCs) were aspirated from follicles with a diameter of 2–7 mm using an 18-gauge needle. The aspirated oocytes showing a uniformly granulated cytoplasm and surrounded by at least three uniform layers of compact cumulus cells were selected.

The selected good-quality COCs were washed three times in Tyrode's lactate HEPES-buffered solution containing 0.1% polyvinyl alcohol (PVA), cultured in NCSU-37 medium supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 0.1 IU/ml human chorionic gonadotropin (hCG) and 0.1 IU/ml pregnant mare serum gonadotropin (PMSG) for 20–22 h, and cultured for another 22 h in medium that lacked PMSG and hCG at 38.5°C in 5% CO<sub>2</sub> in air.

### PA and *in vitro* culture (IVC)

All matured oocytes with an extruded first polar body were exposed to a direct current pulse (1.5 kV/cm, 60 μs) in 0.28 mol/l mannitol containing 0.1 mM MgSO<sub>4</sub>, 0.05 mM CaCl<sub>2</sub>, and 0.1% PVA. After PA, oocytes were washed with NCSU-37 medium supplemented with 4 mg/ml bovine serum albumin and incubated in culture medium containing AZD5438 or CB at 38.5°C in 5% CO<sub>2</sub> in air. Previous studies have proved that treatment with AZ5438 alone, following parthenogenetic stimulation, can prevent the release of the second polar body after activation of the oocytes, which results in diploid development. Cleavage and blastocyst formation were evaluated on days 2 and 7, respectively.

### *In vitro* MPF activity assay

The level of MPF was measured in PA oocytes treated with AZD5438 or CB at optimal concentration and duration. Oocytes (40 per treatment group) were washed several times in phosphate-buffered saline (PBS). Oocytes were repeatedly blown with a fine needle whose diameter was smaller than that of the oocytes until they ruptured. The samples were centrifuged at 150 g for 15 min at 4°C. The supernatants were collected to determine the MPF level by the Porcine MPF ELISA Kit (Kexing, Shanghai, China) following the manufacturer's protocol. In brief, 10 μl supernatant was added to each well, followed by 40 μl of dilution buffer (i.e., the sample was diluted five-fold). Plates were incubated at 37°C in the dark for 30 min. Each well was washed thoroughly with buffer and supplemented with 50 μl horseradish peroxidase conjugate reagent, except for the blank. Samples were incubated at 37°C for 30 min and then washed. After adding TMB substrate solution to each well, the plate was incubated at 37°C in the dark for 15 min. The reaction was terminated by adding 50 μl sulfuric acid. The colour changes were measured using the spectrophotometer at a wavelength of 450 nm within 15 min of the reaction being terminated, as previously reported (Li *et al.*, 2014). The concentration of MPF in the samples was determined using a standard curve.

**Table 1** Primer sequences used for gene expression analysis

Symbol	Gene	Primer sequence (5'–3')	Product size (bp)
<i>Bax</i>	Homo sapiens BCL2 antagonist/killer 1 (BAK1), transcript variant X1	Forward: CGGGACACGGAGGAGGTTT Reverse: CGAGTCGTATCGTCGGTTG	189
<i>Bcl-2</i>	BCL2 like 1 (BCL2L1)	Forward: GAAACCCCTAGTGCCATCAA Reverse: GGGACGTCAGGTCCTGAAT	196
<i>Nanog</i>	Nanog homeobox (NANOG)	Forward: TTCCTTCTCCATGGATCTG Reverse: ATCTGCTGGAGGCTGAGGTA	214
<i>Sox2</i>	SRY-box 2 (SOX2)	Forward: CGCAGACCTACATGAACG Reverse: TCGGACTTGACCACTGAG	103
<i>Oct4</i>	POU class 5 homeobox 1 (POU5F1)	Forward: AAGCAGTGACTATTCCGAAC Reverse: CAGGGTGGTGAAGTGAGG	136
RPL19	Ribosomal protein L19 (RPL19)	Forward: GCTTGCCTCCAGTGTCTC Reverse: GGCGTTGGCGATTCAT	79

### Karyotype analysis of embryos derived from PA oocytes

Blastocysts derived from the AZD5438-treated groups were subjected to karyotype analysis. Briefly, blastocysts were pre-treated with 0.2 µg/ml demecolcine for 4 h to block cell division and then incubated in a hypotonic solution of 0.075 mol/l KCl for 20 min at 37°C. Swollen blastocysts were placed on a clean glass slide immersed in stationary liquid (methanol:acetic acid = 3:1). After drying, slides were stained with 10% Giemsa (Invitrogen, Carlsbad, CA, USA) for 10 min. The stained chromosomes were imaged using oil immersion optics (Nikon) to determine the chromosome number.

### Nuclear transfer

Nuclear transfer was performed as described previously (Yin *et al.*, 2002). Briefly, mature oocytes with the first polar body were cultured in medium supplemented with 0.4 mg/ml demecolcine and 0.05 M sucrose for 1 h. Sucrose was used to enlarge the perivitelline space of the oocytes. Treated oocytes with a protruding membrane were transferred to medium supplemented with 5 mg/ml CB and 0.4 mg/ml demecolcine, and the protrusion was removed with a beveled pipette. A single donor cell was injected into the perivitelline space of each oocyte, and then electrically fused using two direct current pulses of 150 V/mm for 50 ms in 0.28 mol/l mannitol supplemented with 0.1 mM MgSO<sub>4</sub> and 0.01% PVA. Fused oocytes were cultured in NCSU-37 medium for 1 h before electro-activation and then cultured in medium supplemented with 10 µM AZD5438 or 5 mg/ml CB for 4 h. The reconstructed oocytes were activated by two direct current pulses of 100 V/mm for 20 ms in 0.28 mol/l mannitol supplemented with 0.1 mmol/l MgSO<sub>4</sub> and 0.05 mmol/l CaCl<sub>2</sub>. Activated oocytes were cultured in the medium for

6 days in an atmosphere of 5% CO<sub>2</sub> and 95% air at 39°C. Finally, the nuclei of blastocysts stained with 50 µg/ml bisbenzimidazole were imaged by fluorescence microscopy.

### Gene expression in PA oocytes

The mRNA expression levels of two apoptosis-related genes (*Bax* and *Bcl-2*) and three pluripotency-related genes (*Oct4*, *Nanog*, and *Sox2*) were measured by RT-PCR, together with ribosomal protein L19 (RPL19) as an endogenous reference gene. All samples were stored at –80°C until use. Total RNA was extracted from 34 PA oocytes treated with AZD5438 or CB using the Dynabeads<sup>®</sup> mRNA DIRECT<sup>™</sup> Kit (Life Technologies AS, Oslo 010, Norway) following the manufacturer's instructions. Purified RNA was immediately transcribed into cDNA using the SuperScript<sup>®</sup> III First-Strand Synthesis System (Invitrogen, Carlsbad CA, USA), according to the manufacturer's directions. The primers were designed based on methods reported in the literature (Liang *et al.*, 2015; Wang *et al.*, 2015; Zhao *et al.*, 2016) and expected product sizes are shown in Table 1. PCR was performed in a thermal cycler (T100<sup>™</sup> Thermal Cycle; Bio-Rad Laboratories, Inc., CA, USA). PCR products were electrophoresed in 1.5% (w/v) agarose gels. The expression levels were determined by measuring the intensity of each band.

### Experimental design

In a preliminary experiment, the extrusion of the second polar body was inhibited in more than 90% of matured oocytes subjected to PA and treated with AZD5438 for 30 min; therefore, oocytes were treated with AZD5438 and CB. Each experiment was performed more than three times.

In Experiment 1, the optimal concentration and duration of AZD5438 treatment after PA were determined. The developmental rates were compared among the

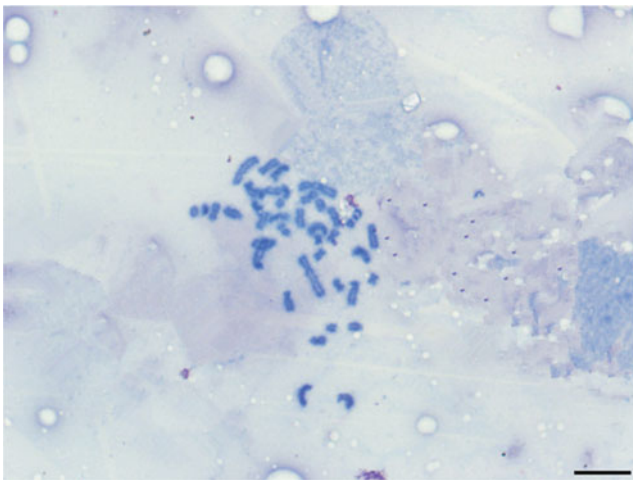
**Table 2** *In vitro* development of porcine embryos derived from parthenogenetic activation oocytes treated with different concentrations of AZD5438 for 4 h after exposure to electric pulse

Concentration of AZD5438 ( $\mu\text{M}$ )	No. of embryos cultured	No. of embryos that reached the 2–4-cell stage (%)	No. of embryos that reached the blastocyst stage (%)	No. of cells per blastocyst (mean $\pm$ SEM)
0	168	145 (85.5%)	58 (34.5%) <sup>b</sup>	39.2 $\pm$ 2.1
10	166	146 (88.0%)	77 (46.4%) <sup>d</sup>	42.0 $\pm$ 2.9
20	167	142 (85.0%)	54 (32.3%) <sup>b</sup>	41.8 $\pm$ 4.2
50	167	136 (81.4%)	40 (24.0%) <sup>c</sup>	39.2 $\pm$ 2.9

<sup>a,b,c</sup>Values with different superscripts in the same column are significantly different ( $P < 0.05$ ).

**Table 3** *In vitro* development of porcine embryos derived from parthenogenetic activation oocytes treated with 10  $\mu\text{M}$  AZD5438 for different durations after exposure to electric pulse

Duration of AZD5438 treatment (h)	No. of embryos cultured	No. of embryos that reached the 2–4-cell stage (%)	No. of embryos that reached the blastocyst stage (%)
2	171	142 (83.0%)	35 (38.6%)
4	173	146 (84.4%)	39 (42.8%)
6	172	138 (80.2%)	32 (37.2%)



**Figure 1** Karyotype analysis of a porcine blastocyst at day 7 derived from an AZD5438-treated parthenogenetic activation oocyte. A normal diploid karyotype of 38 chromosomes in metaphase is shown. Original magnification:  $\times 1000$ .

different groups. Karyotype analysis was performed of blastocysts derived from these PA oocytes.

In Experiment 2, PA and SCNT were performed using oocytes treated with AZD5438 or 6-DMAP, a commonly used protein inhibitor, and the *in vitro* development of the embryos was compared.

In Experiment 3, the mRNA expression levels of apoptosis-related genes (*Bax* and *Bcl-2*) and pluripotency-related genes (*Oct4*, *Nanog*, and *Sox2*) were checked by RT-PCR in PA oocytes treated with or without AZD5438. MPF activity was assessed in porcine PA oocytes treated with AZD5438 for 4 h or non-treated oocytes.

### Statistical analysis

Each experiment was performed at least three times. Differences between groups were determined using the SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Data expressed as proportions (i.e. percentages) were analysed using chi-squared test, nuclei numbers were analysed by T-Test (Independent Samples). All data were reported as the mean  $\pm$  standard error of the mean (SEM).  $P$ -values  $\leq 0.05$  were considered statistically significant.

### Results

#### Effect of treatment with AZD5438 at different concentrations and for different durations on the *in vitro* development of porcine PA oocytes

The percentage of blastocyst was significantly higher in the group treated with 10  $\mu\text{M}$  AZD5438 than in the groups treated with 20  $\mu\text{M}$ , 50  $\mu\text{M}$  or 5  $\mu\text{g/ml}$  CB (46.4% vs. 32.3%, 24.0%, and 34.5%, respectively;  $P < 0.05$ ; Table 2). There were no significant differences in the number of cells per blastocyst among these four groups. By contrast, the blastocyst formation rate was significantly lower ( $P < 0.05$ ) in the group treated with 50  $\mu\text{M}$  AZD5438 than in the control group. The blastocyst formation rate was higher in the group treated with 10  $\mu\text{M}$  AZD5438 for 4 h than in the groups treated with 10  $\mu\text{M}$  for 2 or 6 h. However, this was not significant (42.8%, 38.6%, and 37.2%, respectively; Table 3). In karyotype analysis, 66.67% (12/18) of blastocysts (Fig. 1) were classified as diploid in the group treated with 10  $\mu\text{M}$  AZD5438 for 4 h (Fig. 2).

**Table 4** In vitro development of porcine parthenogenetic activation embryos activated with AZD5438 and 6-DMAP

Activation treatment	No. of embryos cultured	No. of embryos that reached the 2–4-cell stage (%)	No. of embryos that reached the blastocyst stage (%)	No. of cells in blastocyst mean $\pm$ SEM
EP+6-DMAP	137	128 (93.4%)	47 (34.3%)	42.4 $\pm$ 13.1
EP+AZD5438	125	114 (91.2%)	53 (42.4%)	42.8 $\pm$ 11.5

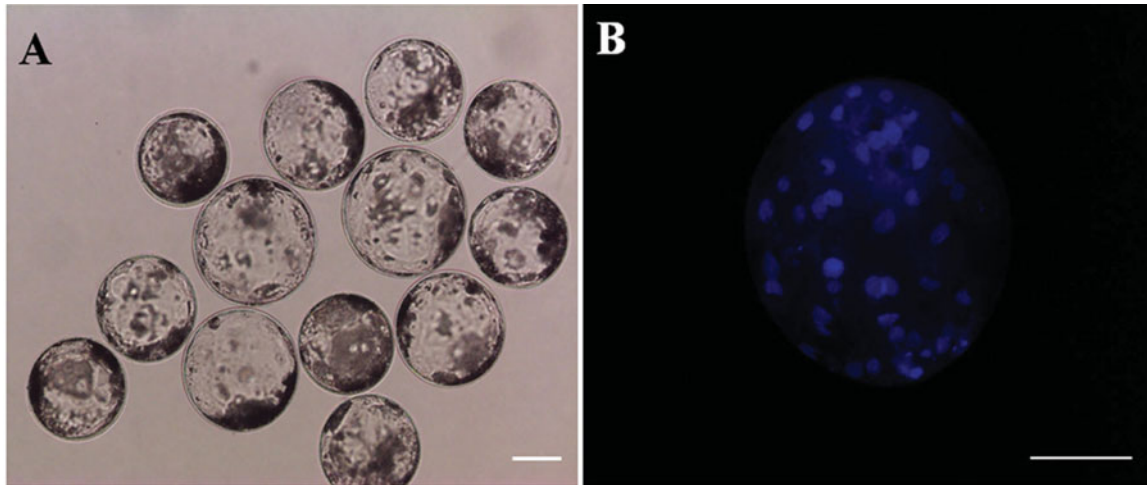
6-DMAP, 6-Dimethylaminopurine; EP, electric pulse.

**Table 5** In vitro development of reconstructed porcine somatic cell nuclear transfer embryos derived from oocytes activated by EP and treated with AZD5438, 6-DMAP or CB

Activation treatment	No. of embryos cultured	No. of embryos that reached the 2–4-cell stage (%)	No. of embryos that reached the blastocyst stage (%)	No. of cells per blastocyst (mean $\pm$ SEM)
EP + CB	95	66 (66.67%)	4 (4.21%) <sup>b</sup>	30.30 $\pm$ 7.45
EP + 6-DMAP	99	68 (68.69%)	11 (11.11%) <sup>a</sup>	42.33 $\pm$ 7.55
EP + AZD5438	97	77 (79.38%)	13 (13.40%) <sup>a</sup>	47.20 $\pm$ 6.95

CB, Cytochalasin; 6-DMAP, 6-Dimethylaminopurine. EP, electric pulse;

<sup>a,b</sup> Values with different superscripts in the same column are significantly different ( $P < 0.05$ ).



**Figure 2** (A) Blastocysts derived from AZD5438-treated parthenogenetic activation oocytes. (B) Blastocysts stained with Hoechst 33342. Scale bars = 100  $\mu$ m.

### In vitro development of PA and SCNT embryos derived from oocytes activated with an EP and treated AZD5438 or 6-DMAP

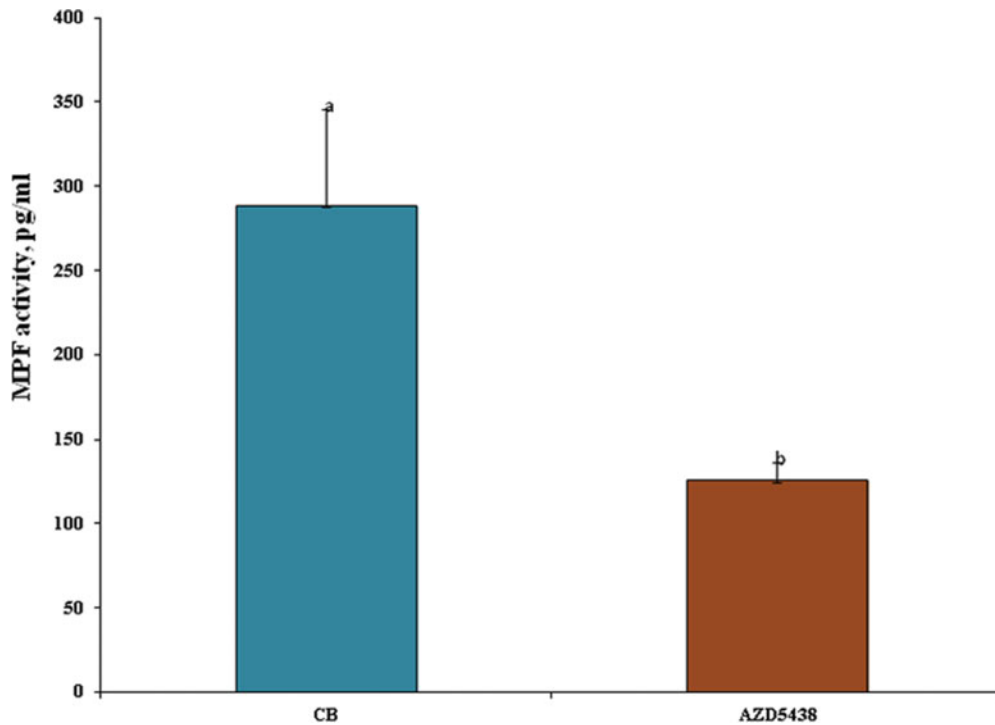
After PA, there is no significant difference between the percentage (42.4% and 34.3%,  $P > 0.05$ ; Table 4) of embryos that developed to the blastocyst stage in the AZD5438-treated and 6-DMAP-treated groups (Table 4). The cleavage rate (93.4% and 91.2%, respectively) and total number of cells per blastocyst ( $42.4 \pm 13.1$  and  $42.8 \pm 11.5$ , respectively) were similar in the AZD5438-treated and 6-DMAP-treated groups (Table 4).

After SCNT, the percentage of embryos that developed to the blastocyst stage was significantly higher in the AZD5438-treated and 6-DMAP-treated

groups than in the CB-treated group (13.4% and 11.1% vs. 4.2%,  $P < 0.05$ ; Table 5). The cleavage rate (79.4%, 68.7%, and 66.7%, respectively) and total number of cells per blastocyst ( $47.2 \pm 7.0$ ,  $42.3 \pm 7.6$ , and  $30.3 \pm 7.5$ , respectively) were similar in each groups (Table 5).

### Levels of MPF and gene expression in porcine oocytes activated by an EP and AZD5438 treatment

The level of MPF was investigated in oocytes activated by an EP and treated with AZD5438 or CB. The level of MPF was significantly lower in the AZD5438-treated group than in the CB-treated group (125.9 pg/ml vs. 288.9 pg/ml,  $P < 0.05$ ; Fig. 3).



**Figure 3** MPF concentrations in oocytes activated by an electric pulse and treated with AZD5438 or cytochalasin B for 4 h. Bars with different superscripts are significantly different.

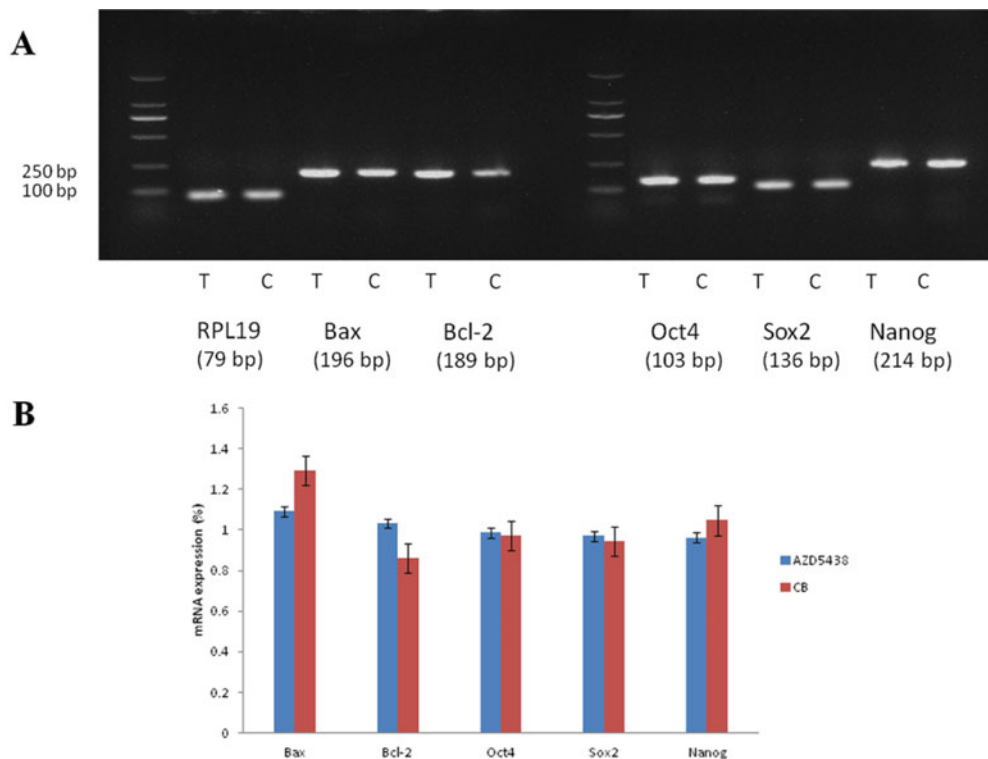
The mRNA expression level of genes related to apoptosis (*Bax* & *Bcl-2*) and pluripotency (*Oct4*, *Nanog*, and *Sox2*) were measured, together with RPL19 as an endogenous reference gene. The mRNA expression levels of these genes did not differ between the AZD5438- and CB-treated groups (Fig. 4).

## Discussion

Present results clearly show that porcine oocytes can be efficiently activated by PA followed by AZD5438 treatment. This finding is in agreement with the previous findings that PA in combination with 6-DMAP (Liu *et al.*, 1998; Saikhun *et al.*, 2004), cycloheximide (Presicce & Yang, 1994), and butyrolactone I (Dinnyés *et al.*, 2000) treatment induces activation and the subsequent development of bovine oocytes more effectively than any single treatment.

This study aimed to improve the developmental competence of porcine embryos in PA and SCNT experiments using oocytes activated by an EP and treated with AZD5438 at different concentrations and for different durations. Treatment with 10  $\mu$ M AZD5438 for 4 h significantly improved the developmental capacity of embryos following PA. Furthermore, the blastocyst formation rates in PA and SCNT experiments were similar between the group

treated with 2 mM 6-DMAP for 4 h and the group treated with 10  $\mu$ M AZD5438 for 4 h. 6-DMAP is a protein kinase inhibitor that has been widely exploited in nuclear transfer experiments and significantly enhances activation, accelerates pronuclear formation and parthenogenetic development, and inactivates cytoskeletal factor and MPF (Masui & Markert, 1971; Masui, 1991). However, 6-DMAP non-specifically affects several pathways in oocytes, induces a high incidence of chromosomal abnormality, and impairs further embryonic development (De La Fuente & King, 1998; Van De Velde *et al.*, 1999). In total, 66.67% of blastocysts derived from AZD5438-treated PA oocytes had a diploid karyotype. 6-DMAP treatment inhibits extrusion of the second polar body due to inhibition of phosphorylation necessary for the spindle apparatus (De La Fuente & King, 1998; Van De Velde *et al.*, 1999). The mechanism by which it inhibits extrusion of the second polar body is not known, although it may be related to inhibition of CDK substrate phosphorylation and blockade of cell cycling at the G2-M, S, and G1 phases (Dinnyés *et al.*, 2000). The level of MPF was significantly decreased in PA oocytes treated with AZD5438. This result is consistent with previous research, and this supports the theory that a reduced level of MPF allows porcine oocytes to progress into interphase (Masui & Markert, 1971; Masui, 1991; Lee *et al.*, 2016). Moreover, previous studies have proved that treatment with CB, following



**Figure 4** Detection of porcine *Bax*, *Bcl-2*, *Oct4*, *Sox2*, *Nanog*, and *RPL19* transcripts in parthenogenetic activation oocytes treated with AZD5438 and cytochalasin B by RT-PCR analysis (A) Expression of mRNA is shown. (B) Band intensities are shown. T = AZD5438 treatment, C = CB treatment.

parthenogenetic stimulation, enhanced developmental competence in cattle (Fukui *et al.*, 1992; Presicce & Yang, 1994) and pig (Cha *et al.*, 1997). CB can prevent the release of the second polar body after activation of the oocytes, which results in diploid development (Fukui *et al.*, 1992). Therefore, CB-treated groups were used in these comparisons as a control.

To evaluate the effect of AZD5438 on the expression of genes related to apoptosis and pluripotency, RT-PCR was conducted. A previous study demonstrated that MPF plays important roles in oocyte cytoplasmic maturation by regulating gene expression (Zhang *et al.*, 2011).

*Bax* and *Bcl-2* play vital roles in apoptosis (Yang & Rajamahendran, 2002). *Oct4*, *Nanog*, and *Sox2* are considered to be important transcription factors in the regulation of early embryonic development (Sommer *et al.*, 2009). The mRNA expression levels of these genes did not differ between the AZD5438- and CB-treated groups.

In conclusion, our results indicate that treatment with 10  $\mu$ M AZD5438 for 4 h can improve the developmental competence of porcine embryos in PA and SCNT experiments. This situation may be because AZD5438 treatment decreases MPF activity, reduces the incidence of abnormal karyotypes, and decreases the extrusion rate of the second polar body. AZD5438

improves the efficiency of porcine oocyte activation, and further studies are required to clarify its effects on early embryonic development.

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## Conflict of interest

None of the authors has any conflict of interest to declare.

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