Master's Thesis

Fibroblast Growth Factor 10 Markedly Improves *In Vitro* Maturation and Embryo Development of Porcine Cumulus-Oocyte Complexes

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GRADUATE SCHOOL

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Fibroblast Growth Factor 10 Markedly Improves Improves *In Vitro* Maturation and Embryo Development of Porcine Cumulus-Oocyte Complexes

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ABSTRACT

Growth factors synthesized by ovarian somatic cells affect cumulus cell expansion and oocyte maturation *in vitro*. Fibroblast growth factor 10 (FGF10) regulates the maturation of mammalian COCs. In this study, we investigated the effects of 0, 5, 10, 50, and 100 ng/mL FGF10 (5F, 10F, 50F, and 100F, respectively) on cumulus cell expansion, oocyte maturation, and embryo development *in vitro*. The percentage of fully expanded cumulus cells at metaphaseII (MII) was significantly higher in the 10F-treated group than in the control. Compared to the controls, the mRNA expression level of the cumulus cell expansion-related gene hyaluronan synthase 2 (*SHAS2*) in oocytes at germinal vesicle breakdown (GVBD) increased significantly in the 10F-, and 50F-treated group, while the mRNA expression level of the protease cathepsin B (*CTSB*) in oocytes at MII decreased in the 10F-treated group. The percentage of oocytes with normal spindle formation was higher in the 10F-, and 50F-treated group than in the other groups at GVBD; it was also higher in 5F-, and 10F-treated groups than in control, 5F-, and 50F-treated groups at MII. Compared to the other treatment groups, the mRNA expression levels of *GDF9* and *BMP15* in oocytes at GVBD, as well as those of *BMP15* and *CCNB1* in oocytes at MII, increased in the 10F-treated group. The cleavage rate, blastocyst formation rate, and total cell number were significantly higher in the 5F-, 10F-, and 50F-treated groups than in the other groups. These results demonstrate that FGF10 markedly improves cumulus cell expansion, oocyte maturation, and embryo development.

Key words: *fibroblast growth factor 10*, *porcine*, *parthenogenesis*, *embryo*

1. INTRODUCTION

In vitro matured oocytes are used for parthenogenetic activation (PA), *in vitro* fertilization (IVF), and somatic cell nuclear transfer (SCNT). To improve oocyte quality, the most efficient methods have been extensively tested and used *in vitro*. Previous studies were performed to increase understanding of various characteristics of oocyte maturation, and the importance of cyclic adenosine monophosphate (cAMP) (Govindan JA et al., 2009) and Akt (Tomek W et al., 2005) signaling; co-culture systems (Yoon JD et al., 2015); cumulus cells; follicular size; protein synthesis (Sun QY et al., 2001); and the addition of gonadotropins and/or growth factors to media (Prochazka R et al., 2011) has been established. Of these, growth factors have received the most attention in many experiments.

Growth factors synthesized by ovarian somatic cells affect cumulus cell expansion and oocyte maturation *in vitro* (Song HJ et al., 2011). For example, epidermal growth factor (EGF), follicle-stimulating hormone (FSH), and estradiol play important roles in the nuclear and cytoplasmic maturation of sheep oocytes *in vitro* (Guler A et al., 2000). EGF also stimulates the maturation and developmental competence of embryos in the cat (Thongkittidilok C et al., 2015), sheep (Ni H et al., 2015), pig (Uhm SJ et al., 2010), and mouse (Downs SM, 1989). Furthermore, the addition of leukemia inhibitory factor in oocyte maturation medium enhances both nuclear and cytoplasmic maturation of bovine (MO X et al., 2014), porcine (Dang-Nguyen TQ et al., 2014), and sheep (Ptak G et al., 2006) oocytes, while keratinocyte growth factor directly stimulates granulosa cell proliferation and indirectly inhibits granulosa cell differentiation (Parrott JA et al., 1998).

Fibroblast growth factor (FGF) is a growth factor that regulates the maturation of mammalian cumulus–oocyte complexes (COCs) by improving the expression of cumulus cell expansion-related genes in pigs (Schams D et al., 2009), rodents (Valve E et al., 1997), and cattle (Zhang K et al., 2010). There are four FGF receptors (FGFR1, FGFR2, FGFR3, and FGFR4) and 23 FGF ligands in cumulus cells and oocytes (Pomini Pinto RF et al., 2014) that produce different actions. For example, FGF7 stimulates oocyte growth in co-cultures of COCs and granulosa cells (Cho JH et al., 2008). FGF8

suppresses FSH-induced estrogen production by granulosa cells (Miyoshi T et al., 2012). FGF10 influences the expression of cathepsin B (*CTSB*) and sprouty homolog 2 (*SPRY2*) in cumulus cells and bone morphogenetic protein 15 (*BMP15*) in bovine oocytes (Zhang K et al., 2010). Furthermore, the addition of FGF10 to oocyte maturation medium improves oocyte maturation *in vitro*, decreases the percentage of apoptotic oocytes, and increases the relative expression of developmentally important genes (Pomini Pinto RF et al., 2014). The combination of BMP15 and FGF10 stimulate the expansion of *in vitro* matured bovine COCs by driving glucose metabolism toward hyaluronic acid production and controlling the expression of genes involved in the ovulatory cascade (Caixeta ES et al., 2013).

In this study, we investigated the effects of FGF10 treatment during *in vitro* maturation (IVM). Cumulus cell expansion was graded and gene expression in cumulus cells was investigated at germinal vesicle breakdown (GVBD) and metaphase II (MII). In oocytes, spindle and chromosome formation and maternal gene expression were examined. During the *in vitro* development of porcine parthenogenetic embryos induced by FGF10-supplemented IVM medium, the cleavage rate, blastocyst formation rate, and total cell number were determined. We report that FGF10 treatment during porcine oocyte maturation improves cumulus cell expansion, oocyte maturation, and embryo development *in vitro*.

2. MATERIALS & METHODS

2.1. Oocyte collection and *in vitro* maturation

Pre-pubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory in saline supplemented with 75 mg/mL penicillin G and 50 mg/mL streptomycin sulfate within 2 h at 32–35.0℃. COCs were aspirated from follicles with a diameter of 2–8 mm using an 18-gauge needle and a disposable 10 mL syringe. COCs were washed three times in tissue culture medium (TCM)-199 HEPES containing 0.1% (w/v) bovine serum albumin (BSA). Groups of 50 COCs were matured in 500 μL of TCM-199 (Gibco, Grand Island, NY, USA) containing Earle's salts, 0.57 mM cysteine, 10 ng/mL EGF, 0.5 μg/mL FSH, 0.5 μg/mL luteinizing hormone, and 10% (v/v) porcine follicular fluid under mineral oil for 44 h at 38.8℃ in a humidified atmosphere of 5% (v/v) CO_2 and 95% (v/v) air. TCM-199 was supplemented with 0, 5, 10, 50, and 100 ng/mL FGF10 (Con, 5F, 10F, 50F, and 100F, respectively).

2.2. Parthenogenetic activation and *in vitro* culture

Matured COCs were denuded of cumulus cells by mechanical pipetting with 0.1 mg/mL hyaluronidase. The denuded oocytes were activated with 5 μM Ca-ionomycin for 5 min. After culturing in porcine zygote medium (PZM, defined medium for porcine embryos)-5 containing 7.5 μg/mL cytochalasin B (Sigma, St. Louis, MO, USA) for 3 h, embryos were washed three times in PZM-5 containing 0.4% (w/v) BSA and cultured in the same medium for 6 days at 38.8℃ in a humidified atmosphere of 5% (v/v) CO_2 and 95% (v/v) air. On day 2, embryos were examined under a microscope. Cleaved embryos were then transferred to a new drop of medium and cultured until day 6.

2.3. Immunofluorescence

To investigate the morphology of meiotic spindles and the pattern of chromosomes in porcine COCs at GVBD or MII during IVM for 22 or 44 h, the cumulus cells of these COCs were removed. Oocytes were then fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4℃ and incubated with 0.5% (v/v) Triton X-100 at 38.8℃ for 30 min. After blocking with 1% (w/v) BSA in PBS for 1 h, oocytes were incubated with a fluorescein isothiocyanate-conjugated anti-α-tubulin antibody (diluted 1:200 in PBS containing 0.1% (w/v) BSA, Sigma, Cat. No. F2168) overnight at 4°C. Nuclei were stained with Hoechst 33342 (1 μg/mL) for 30 min, and oocytes were washed three times with PBS containing 0.1% (w/v) BSA. Oocytes were mounted onto glass slides and examined under an inverted Olympus IX-71 microscope (Tokyo, Japan). At least 30 oocytes were examined per treatment group.

2.4. Real-time quantitative RT-PCR

To quantify the mRNA expression levels of target genes in cumulus cells and oocytes, COCs were denuded and then cumulus cells and oocytes at GVBD and MII were separated. The cumulus cells were collected by centrifugation at 2,000 rpm for 2 min. mRNA was isolated from the cumulus cells and oocytes of 20 COCs using the Dynabeads mRNA Direct Kit (DynalAsa, Oslo, Norway). First-strand cDNA synthesis was achieved by reverse transcription of mRNA using an oligo(dT)12-18 primer and SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed in a DNA Engine OPTICON 2 system (MJ Research, Waltham, MA, USA) in a final reaction volume of 20 μL that contained SYBR Green and a double-stranded DNA-binding fluorophore (qPCR Kit; FINNZYMES, Espoo, Finland). The primers used for real-time PCR are listed in Table 1. The real-time PCR conditions were as follows: 10 min at 94℃, followed by 39

cycles of 30 s at 94℃, 30 s at 60℃, and 55 s at 72℃, and a final extension of 5 min at 72℃. Relative gene expression was analyzed by the $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001) after normalization to the relative *GAPDH* mRNA level.

Table 1. Primers used for real-time PCR

Gene	GenBank accession no.	Primer sequence	Annealing temperature (°C)	Product size (bp)
GAPDH	AF017079	F: GGGCATGAACCATGAGAAGT R: AAGCAGGGATGATGTTCTGG	60	230
GDF9	AY626786	F: GAGCTCAGGACACTGTAAGC R: CTTCTCGTGGATGATGTTCTG	60	272
BMP15	NM_001005155	F: CCCTCGGGTACTACACTATG R: GGCTGGGCAATCATATCCT	60	192
CCNB1	L48205	F: TTGACTGGCTAGTGCAGGTT R: CTGGAGGGTACATTTCTTCA	60	177
SHAS2	NM_213053.1	F: ACTGTCCAGTTAGTAGGTCTCA R: ACATGTACAACACCGAGTAGAG	54	100
CTSB	NM_001097458.1	F: CTCTAGGAACGAGAAGGAGAT R: CCAGACTTATACTGCAGGAAG	54	99

Abbreviations: bp, base pair; F, forward; R, reverse.

2.5. Statistical analysis

The general linear model (GLM) procedure within the SAS software package (SAS Institute Inc., Cary, NC, USA) was used to analyze data from all experiments. Significant differences were determined by the Tukey multiple-range test. A paired Student's *t*-test was used to compare relative mRNA expression levels between treatment groups. *P*-values of <0.05 were considered significant.

3. RESULTS

3.1. Effect of FGF10 on cumulus cell expansion

We investigated the effect of FGF10 treatment on cumulus cell expansion in porcine COCs at GVBD or MII. COCs were treated with 0, 5, 10, 50, and 100 ng/mL FGF10 (Con, 5F, 10F, 50F, and 100F, respectively). Cumulus cell expansion in the various treatment groups was confirmed by microscopy and classified into one of three grades at GVBD or MII. Cumulus cell expansion was assessed by using identical stereomicroscope settings at a 100× magnification. The color images were then altered to black-and-white images to avoid the visual distinction following brightness difference.

The criteria for grading were according to Zhang *et al.* (2010). The degree of cumulus cell expansion at GVBD was graded as follows: grade I, hardly expanded cumulus cells; grade II, slightly expanded cumulus cells; and grade III, greatly expanded cumulus cells. The cumulus cell expansion at GVBD, the percentage of grade II cumulus cells was higher than grade I and III cumulus cells. However, there was no significant difference in all treatment groups ($p < 0.05$; Figure 1A).

The degree of cumulus cell expansion at MII was graded as follows: grade IV, clumped expanded cumulus cells; grade V, partially expanded cumulus cells; and grade VI, fully expanded cumulus cells. The percentage of grade IV expanded cumulus cells at MII was lower in the 10F-treated group (23.9% ± 3.3%) than in the other groups (Con, 32.8% ± 2.5%; 5F, 24.7% ± 4.3%; 50F, 27.5% ± 3.5%; and 100F, 29.4% ± 2.0%). There was no difference in the percentage of grade V COCs in all treatment groups (Con, 37.2% ± 9.3%; 5F, 30.6% ± 8.5%; 10F, 32.1% ± 8.1%; 50F, 38.2% ± 9.7%; and 100F, 38.0% ± 9.5%). The percentage of grade VI COCs was significantly higher in the 10F-treated group (22.4% ± 3.2%) than in the other groups (Con, 15.7% ± 1.9%; 5F, 23.5% ± 5.4%; 50F, 21.1% ± 3.5%; 100F, 16.1% ± 2.9%; $p < 0.05$; Figure 1B).

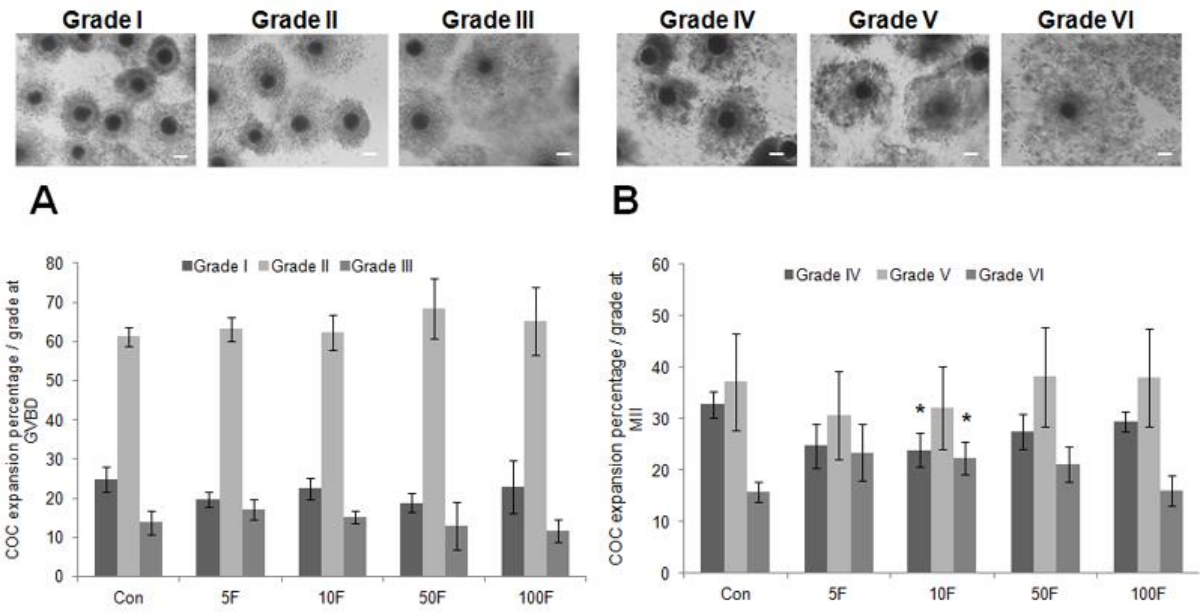

Figure 1. Effect of FGF10 on the expansion rate of porcine COCs. COCs were matured in the presence of increasing concentrations of FGF10 *in vitro*. The degree of cumulus cell expansion was classified into one of three grades at GVBD **(A)** and MII **(B)**. Data were derived from four independent replicates for each treatment ($p < 0.05$). Bar = 100 μm.

We investigated the mRNA expression of the cumulus cell expansion-related genes hyaluronan synthase 2 (*SHAS2*) and *CTSB* in cumulus cells at GVBD or MII. *SHAS2* transcript level was increased in 10F-, and 50F-treated groups compared to control, 5F-, and 100F-treated groups at GVBD (Figure 2A), and decreased significantly in the 10F-treated group at MII (Figure 2B). Although *CTSB* expression was similar in all treatment groups at GVBD (Figure 2A), it was the lowest in the 10F-treated group at MII (Figure 2B, $^{a\text{-}b}p < 0.05$).

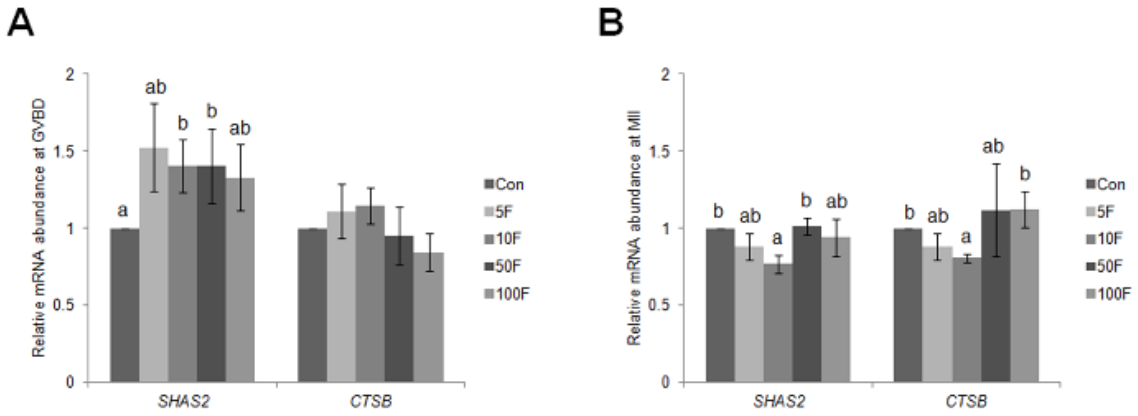

Figure 2. Relative mRNA expression levels of cumulus cell expansion-related genes (S*HAS2* and *CTSB*) in porcine oocytes at GVBD **(A)** and MII **(B)** following IVM in the presence of increasing concentrations of FGF10. Data were derived from three independent replicates for each treatment ($^{a-b}$ $p < 0.05$)

3.2. Effect of FGF10 on chromosome alignment and spindle organization

To examine the effects of FGF10 on spindle formation and chromosome assembly, α-tubulin and nuclei were stained in porcine oocytes at GVBD or MII. Based on their morphology, oocytes were classified as normal or abnormal at GVBD or MII. The criteria for classifying the morphology of spindles and chromosomes were according to Lenie *et al.* (2008). The abnormal oocytes were classified by the α-tubulin and nuclei morphology at both GVBD and MII stage of porcine oocytes (Figure 3A). After IVM, there was no difference in the survival rate in all treatment groups (Con, 88% ± 8%; 5F, 88% ± 8%; 10F, 93% ± 5%; 50F, 93% ± 3%; and 100F, 85% ± 3%; Table 2).

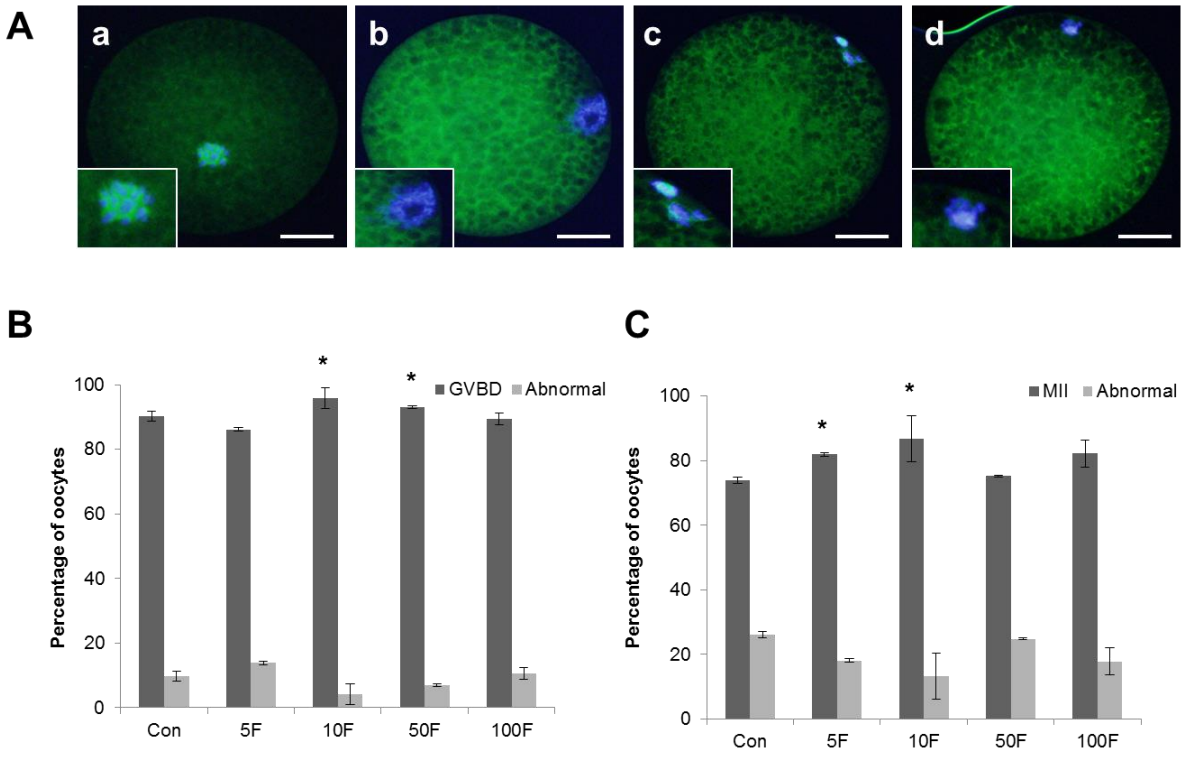

Figure 3. Morphological classification of microtubule and chromosome patterns of porcine oocytes at GVBD **(A)** and MII **(B)** during IVM in the presence of increasing concentrations of FGF10. Oocytes were classified as normal or abnormal at GVBD (normal, a; abnormal, b) and MII (normal, c; abnormal, d). Epifluorescent images of chromosomes stained blue with Hoechst 33342 and microtubules stained green with Alexa Fluor 488 were merged. Data were derived from three independent replicates for each treatment ($p < 0.1$). Bar = 40 μm.

However, the polar body (PB) emission rate was significantly higher in 5F-, 10F-, and 100F treated groups (5F, 81.9% ± 0.6%; 10F, 88.0% ± 5.9%; and 100F, 82.2% ± 4.2%) than in control and 50F-treated groups (Con, 74.0% ± 1.0%; 50F, 75.3% ± 0.3%; Table 2; $^{a\text{-}b}p < 0.05$). At GVBD, the percentage of oocytes displaying normal spindle formation and nuclear status was higher in 10F- and 50F-treated groups (10F, 95.8% ± 3.1%; 50F, 93.0% ± 0.4%) than in the other groups (Con, 90.2% ± 1.5%; 5F, 86.1% ± 0.6%; and 100F, 89.4% ± 1.8%; Figure 3B; $p < 0.1$). At MII, the percentage of oocytes displaying normal spindle formation and nuclear status was the highest in the 10F-treated group, although these results were not statistically significant, and significantly higher in 5F- and 100F-treated groups than control and 50F-treated groups (Con, 71.7% ± 2.3%; 5F, 80.2% ± 1.7%; 10F, 81.8% ± 6.4%; 50F, 74.8% ± 0.5%; and 100F, 79.6% ± 3.5%; Figure 3C; $p < 0.1$).

Table 2. Effect of FGF10 treatment on porcine oocyte nuclear maturation *in vitro* (r = 6)

Group	No. of GV	No. (%)* of	
		survived	PB emission
Con	300	264 (88.0 ± 8.0)	195 (74.0 ± 1.0)[a]
5F	300	264 (88.0 ± 8.0)	216 (81.9 ± 0.6)[b]
10F	300	279 (93.0 ± 5.0)	243 (88.0 ± 5.9)[b]
50F	300	279 (93.0 ± 5.0)	210 (75.3 ± 0.3)[a]
100F	300	255 (85.0 ± 3.0)	210 (82.2 ± 4.2)[b]

Abbreviations: GV, germinal vesicle; PB, polar body, $*p < 0.05^{a\text{-}b}$

3.3. Effect of FGF10 on the maternal gene expression in porcine oocytes

To examine the effect of FGF10 on porcine oocyte maturation, we investigated the mRNA expression levels of maternal genes in porcine oocytes at GVBD or MII. At GVBD, the mRNA expression of growth differentiation factor 9 (*GDF9*) and *BMP15* significantly increased in 10F-treated groups compared to the other groups. *CCNB1* expression was the highest in the control and higher in the 10F-treated group than in the 5F-, 50F-, and 100F-treated groups (Figure 4A, $^{a-d}p < 0.05$). The mRNA expression of *BMP15* and *CCNB1* in oocytes at MII increased in the 10F-treated group. At the MII stage, *GDF9* expression increased in 5F- and 10F-treated groups compared to the other groups (Figure 4B, $^{a-e}p < 0.05$).

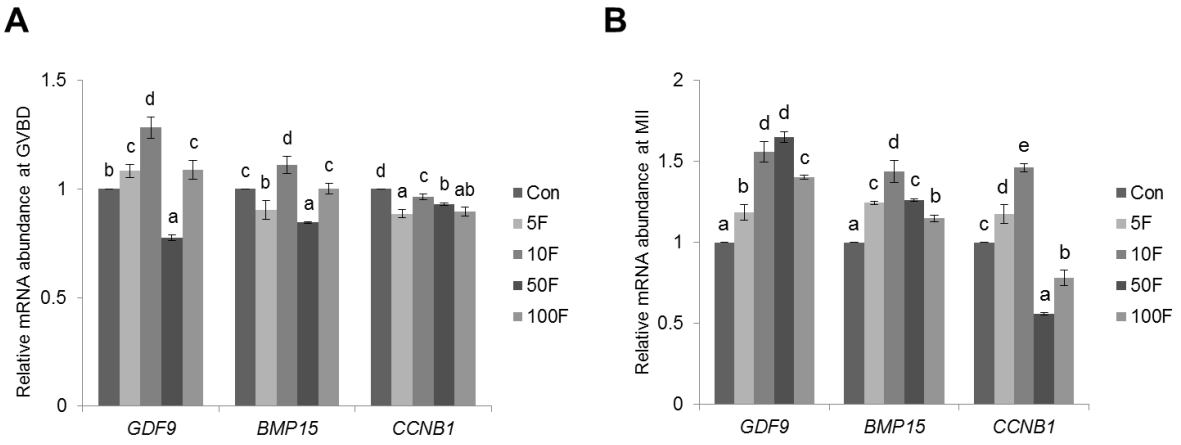

Figure 4. Relative mRNA expression levels of maternal genes (*GDF9*, *BMP15*, and *CCNB1*) in oocytes at GVBD (A) and MII (B) following IVM in the presence of increasing concentrations of FGF10. GAPDH was used as an internal standard, and significant differences from the control are indicated ($^{a-e}p < 0.05$). Data were derived from three independent replicates for each treatment.

3.4. Effect of FGF10 on porcine embryo developmental capacity *in vitro*

Matured oocytes from each treatment group were subjected to PA. The cleavage rate, blastocyst formation rate, and total cell number were determined. On day 2, the percentage of 2–4-cell stage embryos was significantly higher in the 10F-treated group (67.9% ± 6.8%) than in the other groups (Con, 53.7% ± 5.9%; 5F, 59.3% ± 3.7%; 50F, 64.5% ± 7.5%; and 100F, 63.8% ± 5.3%; Table 3; $^{a-b}p < 0.05$). The percentage of blastocyst formation was also higher in the 10F-treated group (72.5% ± 5.8%) than in the other groups (Con, 45.6% ± 4.2%; 5F, 58.8% ± 6.4%; 50F, 55.8% ± 5.3%; and 100F, 55.4% ± 5.4%; Table 3; $^{a-c}p < 0.05$). To confirm the effect of FGF10 treatment on embryo development during IVM, blastocysts were stained with Hoechst on day 6. The total cell number in blastocysts was significantly higher in the 10F-treated group (83.7 ± 9.7) than in the other groups (Con, 69.0 ± 6.0; 5F, 68.0 ± 7.0; 50F, 62.7 ± 4.8; and 100F, 73.1 ± 5.9; Table 3; $^{a-b}p < 0.05$).

Table 3. The effect of FGF10 treatment on porcine embryo development during IVM (r = 6)

Group	No. of MII oocytes	No. (%)* of embryos developed to		Total Cell No.*
		Day 2	Day 6	
		≥ 2–4 cell	≥ blastocyst / 2C	
Con	234	125 (53.7 ± 5.9)^{a}	58 (45.64 ± 4.2)^{a}	69.0 ± 6.0^{ab}
5F	226	134 (59.3 ± 3.7)^{ab}	80 (58.84 ± 6.4)^{b}	68.0 ± 7.0^{ab}
10F	228	156 (67.9 ± 6.8)^{b}	106 (72.54 ± 5.8)^{c}	83.7 ± 9.7^{b}
50F	223	144 (64.5 ± 7.5)^{ab}	87 (55.83 ± 5.3)^{b}	62.7 ± 4.8^{a}
100F	224	147 (63.8 ± 5.3)^{ab}	79 (55.41 ± 5.4)^{ab}	73.1 ± 5.9^{ab}

Abbreviations: MII, metaphase II, *$p < 0.05^{a-c}$

4. DISCUSSION

Oocyte maturation is one of the most important events for successful embryo production *in vitro* (Zhu G et al., 2008), and several investigators have attempted to improve oocyte IVM (Carabatsos MJ et al., 1998 and Lee SE et al., 2014). This study investigated the effects of FGF10 on porcine oocyte IVM and development. The addition of FGF10 to the IVM medium increased the cumulus cell expansion and relative gene expression (S*HAS2* and *CTSB*). It also affected oocyte meiotic progression by improving the nuclear status, chromatin assembly, and the expression of the maternal genes *GDF9, BMP15*, and *CCNB1*. FGF10 treatment during IVM influences both embryo developmental capacity and blastocyst quality. This study demonstrated that FGF10 treatment increased the percentage of normally matured porcine oocytes and that these oocytes had an improved developmental capacity.

Cumulus cells that mature and expand normally are important for the IVM of porcine COCs. Appropriate cumulus cell differentiation appears to be required for normal oocyte development (Eppig JJ, 2001). At the time of ovulation, cumulus cells are fully expanded and encompass an oocyte that has progressed to MII (Downs SM, 1989). The rate of cumulus cell expansion was significantly higher in the 10F-treated group than in the other groups at MII (Figure 1, $^{a\text{-}b}p < 0.05$), but there was no significant difference at GVBD. We confirmed the expression of the cumulus cell expansion-related genes *SHAS2* and *CTSB* at GVBD and MII in cumulus cells. *SHAS2* significantly increased in the FGF10-treated group at GVBD (5F, 10F, 50F, and 100F), although it was lower in the 10F-treated group than in the other groups at MII. There was no significant difference in The transcript level of *CTSB* FGF10-treated and control groups at GVBD. However, *CTSB* expression was significantly lower in the 10F-treated group than in the other groups at MII (Figure 2, $^{a\text{-}b}p < 0.05$). HAS2 induces the formation of large hyaluronic acid coats that surround cells and a protective cementing gel that is deposited into intracellular spaces, whose degradation is a causative factor for tissue damage (Xing G, 2014). CTSB plays a major regulatory role in multiple aspects of mammalian cell death (Angela J et

al., 2010) by inducing apoptosis in oocytes, at least in part, through the stimulation of caspase 3 (Balboula AZ et al., 2013). In addition, the *CTSB* mRNA expression level is higher in cumulus cells harvested from poor developmental competence groups (Bettegowda A et al., 2008). Collectively, these results demonstrated that FGF10 treatment affects the IVM of porcine cumulus cells by increasing the expansion of cumulus cells and enhancing expression of S*HAS2* and *CTSB* at both GVBD and MII.

Chromosome condensation, which is the first visible process to occur during maturation, is essential for the correct packaging of chromatin fibers into chromosomes and their proper segregation during meiotic maturation (Jelinkova L et al., 2006). The centrosome serves as the poles for mitotic and meiotic spindles and as the microtubule-organizing center during interphase (Hewitson L et al., 1997). Proper microtubule nucleation and spindle organization regulate cell cycle progression during meiotic maturation, fertilization, and early embryo cleavage (Yang SW et al., 2012). Time-dependent nucleus and spindle alignment coordinates oocyte maturation and embryonic development (Rienzi L et al., 2012). For example, spontaneous arrest before MII in the absence of PB extrusion associates with abnormalities in the morphology of cortical microfilaments in approximately 50–60% of immature oocytes at metaphase in both pigs and cattle (Somfai T et al., 2011). During nuclear maturation, the addition of 10F to IVM medium significantly affected the rate of PB emission in MII-stage oocytes (Table 1). In our study, GVBD- or MII-stage oocytes were stained to investigate the nuclear status and spindle alignment. At both GVBD and MII, the percentages of oocytes with normal and abnormal nuclear formation were affected in the 10F-treated group (Figure 3, $^{a\text{-}c}p < 0.05$). Taking these results together, treatment with 10F during the IVM of porcine COCs improves the expansion of cumulus cells, the expression of cumulus cell expansion-related genes (S*HAS2* and *CTSB*), and the alignment of chromosome and spindles at GVBD or MII. These results demonstrate that FGF10 treatment affects the expansion of cumulus cells and the IVM of porcine oocytes.

Several genes are linked to oocyte competence. At GVBD and MII, the relative expression of the maternal genes *GDF9*, *BMP15*, and *CCNB1* was affected in oocytes treated with FGF10 during IVM. GDF9 is a paracrine factor that is secreted by oocytes to regulate several key granulosa cell

enzymes involved in cumulus cell expansion and maintenance of an optimal oocyte microenvironment (Elvin JA et al., 1999). *BMP15* is involved in the regulation of oocyte developmental competence (Wang QL et al., 2013). Cyclin B, a key factor that controls the resumption of meiosis, is an integral part of the active maturation-promoting factor molecule (Siraed MA et al., 1998). In this study, we examined the expression of oocyte competence-related genes at GVBD and MII in porcine oocytes treated with FGF10. At GVBD, the relative mRNA expression levels of *GDF9* and *BMP15* were significantly higher in the 10F-treated group than in the other groups. The *CCNB1* mRNA expression level was lower in all FGF10-treated groups than in the control (Figure 4A, $^{a-d}p < 0.05$). In MII-stage oocytes, the mRNA expression levels of *BMP15* and *CCNB1* were significantly higher in the 10F-treated group, and the *GDF9* mRNA expression level was significantly higher in the 50F-treated group than in the other groups (Figure 4B, $^{a-e}p < 0.05$). Collectively, these observations indicate that FGF10 treatment contributes to cytoplasmic maturation during porcine oocyte maturation *in vitro* by increasing maternal gene expression.

FGFs are a potential paracrine signaling molecule, and FGF10 mRNA and protein expression were detected in oocytes of preantral follicles and theca cells (Burantini J et al., 2007). Ovarian angiogenesis plays an important role in the sequence of events leading to ovulation, and angiogenic factors, such as vascular endothelial growth factor and basic fibroblast growth factor, are present in follicular fluid (Malamitsi-Puchner A et al., 2001). In human ovarian follicles, FGF7 was detected in follicular fluid with a possible physiological role in folliculogenesis (Osuga Y et al., 2001). The basic medium that is normally used to culture porcine oocytes *in vitro* already contains a lot of growth factor, nutrients and hormones. Although basic medium is used to culture porcine oocytes and to improve their developmental capacity, many experiments were performed using EGF, LIF, and even FGFs (Song HJ et al., 2011, Dang-Nguyen TQ et al., 2014 and Pomini Pinto RF et al., 2014). However, there is no study on the effects of FGF10 on porcine oocyte maturation and embryo development. For this reason, we performed this study and confirmed synergistic effects of FGF10 compared to only follicular fluid or FGF10. These results agree with other studies, which show that the addition of FGF10 to maturation medium improves embryo quality by increasing the expression

of genes important for development and survival (Pomini Pinto RF et al., 2014). During the IVM of porcine oocytes, FGF10 can improve the efficiency of oocyte maturation and embryo developmental capacity. The number of cells in blastocysts is an important indicator of embryo quality, and FGF10 treatment increases the total cell number in bovine embryos (Zhang K et al., 2010). The cleavage rate was highest in the 10F-treated group, and the blastocyst formation rate increased significantly in 5F-, 10F-, and 50F-treated groups (Figure 5A and B, $^{a-c}p < 0.05$). The total cell number was significantly higher in the 10F-treated group than in the other groups (Figure 5C, $^{a-b}p < 0.05$). These results show that FGF10 treatment during IVM improves the developmental capacity of porcine embryos, and 10 ng/mL was the most effective concentration.

In conclusion, our data demonstrate that treatment with 5–50F during IVM significantly accelerates the maturation of porcine oocytes by improving the cumulus cell expansion rate, nuclear maturation rate, cytoplasmic maturation, and target gene expression in cumulus cells and oocytes. In terms of embryo development, treatment with 10F affected the cleavage and blastocyst formation rates and the total number of cells. Based on these results, we conclude that FGF10-treated oocytes will improve the quality of *in vitro*-produced embryos and the subsequent production of transgenic animals.

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ABSTRACT IN KOREAN

포유동물의 난자는 체외에서 성숙시켜 단위발생, 체외수정 체세포 핵이식 등의 다양한 실험에 사용될 수 있으며, 성숙된 난자의 품질은 배아의 발달 효율을 결정짓는 중요한 요소로 작용한다. 성장인자는 난소의 체세포로부터 합성되어 체외에서 난구세포의 확장과 난자의 성숙에 영향을 미치는 것으로 알려져 있으며, 본 실험에서 사용한 섬유아세포성장인자는 포유류의 난자-난구 복합체의 성숙을 조절하는 인자로서 알려져 있다. 본 연구에서 우리는 0, 5, 10, 50 그리고 100 ng/ml (Con, 5F, 10F, 50F and 100F)의 섬유아세포 성장인자의 처리가 체외에서 난구세포의 확장, 난자의 성숙 및 배아 발달에 미치는 영향을 조사하였다. MII 시기에 완전히 확장된 난구세포의 비율은 10F 처리군에서 대조군에 비해 유의적으로 높게 나타나는 것을 확인하였다. 난구세포의 확장과 관련되어 있는 하이알루로난 생성효소 2 (hyaluronan synthase 2, *HAS2*) 유전자의 발현정도는 GVBD 시기에 10F 와 50F 처리군에서 대조군에 비해 유의적으로 높게 나타났으며, MII 시기에는 단백질 가수분해효소 카텝신 B (protease cathepsin B, *CTSB*)의 발현이 10F 처리군에서 확연하게 감소하였다. GVBD 시기에 정상적인 핵형을 보이는 난자는 10F 와 50F 처리군이 5F, 10F, 100F 와 대조군에 비해 훨씬 높았으며, MII 시기에는 50F 처리군이 가장 높게 나타났다. 10F 처리군에서 GVBD 시기에는 *GDF9* 과 *BMP15* 의 발현이, MII 시기에서는 *BMP15* 와 *CCNB1* 의 발현이 증가되었다. 난할과 배반포 형성률 및 총 세포수는 5F, 10F 및 50F 처리군에서 유의적으로 높게 나타났다. 이러한 결과는 FGF10 이 난구세포의 확장과 난자의 성숙, 이어지는 배아의 발달을 확연하게 증가시킨다는 것을 나타내고 있다. 이에 따라, 본 실험에서 사용된 물질인 FGF10 은 추후 형질전환 복제동물 생산, 배아 줄기세포 연구 및 세포 치료제 개발을 위한 난자실험에 적용 할 수 있을 것으로 기대된다.

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