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Gonadotropins Improve Porcine Oocyte Maturation and Embryo Development through Regulation of Maternal Gene Expression

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ABSTRACT

The present study assessed the effect of FSH and LH on oocyte meiotic, cytoplasmic maturation and on the expression level and polyadenylation status of several maternal genes. Cumulus-oocyte complexes were cultured in the presence of FSH, LH, or the combination of FSH and LH. Significant cumulus expansion and nuclear maturation was observed upon exposure to FSH alone and to the combination of FSH and LH. The combination of FSH and LH during entire IVM increased the mRNA level of four maternal genes, *C-mos*, *Cyclin B*1, *Gdf*9 and *Bmp*15, at 28 h. Supplemented with FSH or LH significantly enhanced the polyadenylation of *Gdf*9 and *Bmp*15; and altered the expression level of *Gdf*9 and *Bmp*15. Following parthenogenesis, the exposure of oocytes to combination of FSH and LH during IVM significantly increased cleavage rate, blastocyst formation rate and total cell number, and decreased apoptosis. In addition, FSH and LH down-regulated the autophagy gene *Atg*6 and upregulated the apoptosis gene *Bcl-xL* at the mRNA level in blastocysts. These data suggest that the FSH and LH enhance meiotic and cytoplasmic maturation, possibly through the regulation of maternal gene expression and polyadenylation. Overall, we show here that FSH and LH inhibit apoptosis and autophagy and improve parthenogenetic embryo competence and development.

(Key words : apoptosis, autophagy, embryo development, maternal gene, oocyte maturation)

INTRODUCTION

In mammals, meiotic resumption and subsequent ovulation are controlled by two gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Richards, 1980). Previous studies reported that gonadotropins influence the resumption of meiotic maturation and cumulus expansion in porcine cumulus-oocyte complexes (COCs) *in vitro*. Further addition of gonadotropins to the culture medium increased developmental competence, suggesting that gonadotropins could improve cytoplasmic maturation (Sun *et al*., 2003). Exposure to FSH alone also affected the nuclear and cytoplasmic maturation of sow oocytes. Cleavage and blastocyst development rates showed that FSH was most effective in the first 20 hr of maturation culture (Schoevers *et al*., 2003). Although most *in vitro* maturation (IVM) protocols currently utilize FSH, LH, or a combination of both, the effect of gonadotropins on IVM and subsequent early embryo development is still controversial and little is known about their molecular mechanism.

During the growth period, the oocyte synthesizes and accumulates transcripts and proteins that are vital to growth, maturation and embryo development (Lonergan *et al*., 2003, Lonergan *et al*., 2003). Translation of these transcripts is generally associated with changes in polyadenylation (Bettegowda *et al*., 2007). Several oocyte genes, such as growth differentiation factor 9 (*Gdf9*) and bone morphogenetic protein 15 (*Bmp15*), play crucial roles in oocyte maturation; these two members of the Transforming growth factor (TGF) superfamily regulate granulosa cell functions including proliferation, differentiation and cumulus expansion (Otsuka *et al*., 2011, Sugiura *et al*., 2010). *C-mos* is a proto-oncogene first identified as a regulator of oocyte maturation in pigs (Newman *et al*., 1996). Mos is a serine/threonine kinase that activates the cascade through direct phosphorylation of the MAPK activator MAPK kinase (Prasad *et al*., 2008). Mos acts almost exclusively in the second meiotic metaphase arrest and is an essential component of the cytostatic factor in mouse oocytes (Hashimoto *et al*., 1994). Another essential regulator of meiosis resumption is formed by

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Cyclin B1, which gene product complexes with p34 (cdc2) to form the maturation-promoting factor (MPF) (Sartor *et al*., 1992). It has been reported that two key cell cycle regulator mediated the activity MPF through MAPK pathway by their cytoplasmic polyadenylation in porcine oocyte (Zhang *et al*., 2009).

Apoptosis and autophagy are common features of mammalian development. They are tightly regulated processes that play a fundamental role in cell growth, development and homeostasis. Recent reports have shown that apoptotic genes are expressed during the preimplantation stage of embryo development in human, and bovine embryos (Cui *et al*., 2004, Fear *et al*., 2011, Metcalfe *et al*., 2004). *Bcl-2* gene products act as essential regulators of the apoptotic signal and may either suppress (*Bcl-2*, *Bcl-xL*) or promote (*Bak*, *Bad*) the induction of apoptosis (Hetz, 2010). *Caspases* are cysteine proteases involved in programmed cell death that have recently been found to have vital functions in mouse preimplantation development (Busso *et al*., 2010). Autophagy is initiated by class III phosphoinositide 3-kinase and theautophagy gene *Atg6*. In addition, other systems are involved, including the ubiquitin-like protein *Atg8* that is known as microtubule-associated protein 1 light chain 3 (*Lc3*) in mammalian cells (Schmid *et al*., 2007). A recent study also suggests that apoptosis and autophagy are involved in early embryo development (Boumela *et al*., 2011).

Hence, to identify the molecular mechanism (s) triggered by FSH and LH, the effects of FSH and LH on the expression and polyadenylation of maternal genes and on the developmental competence of matured oocytes following parthenogenesis were assessed. Our results show that FSH and LH affect porcine oocyte maturation, regulate maternal gene expression and improve embryo developmental competence by inhibiting autophagy and apoptosis.

MATERIALS AND METHODS

All chemicals were obtained from Sigma-Aldrich Co. unless otherwise indicated. Each experiment was repeated at least three times.

1. Collection of Porcine Oocytes, I*n Vitro M*aturation (IVM) and Embryo Culture

COCs aspirated from 3 to 6 mm follicles were matured in IVM medium based with tissue culture medium (TCM) 199 under paraffin oil at 38.5℃ in a humidified atmosphere of 5% CO_2 inair. FSH (F2293, Sigma,) (5 μg/ml) and LH (L5269, Sigma) (5 μg/ml) were used in experiment. All experimental groups are showed in Table 1. Following maturation, oocytes were activated for parthenogenesis with 5 μM Ca^{2+} ionophore and cultured in North Carolina State University (NCSU) 37 medium with 0.4% (w/v) Bovine Serum Albumin (BSA) at 38.5℃ in an atmosphere of 5% CO_2. During culture, the percentages of 2cell, 4cell and blastocysts were measured.

2. Western Blot Analysis

Pig oocytes were denuded in 0.1% Hyaluronidase and wash three times in PVA/PBS and stored in −80℃ until use. Before western blot, oocytes were thawed at room temperature, and lysised by 25 μl Laemmli Sample Buffer. Total protein content was subsequently separated by electrophoresis through a Mini-PROTEAN TGXTM Precast Gel for 2 hr at 100 V. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% BSA in Tris Buffered Saline (TBS) for 1 hr, the membrane was incubated with primary phospho-p44/42 MAPK antibody (1 : 500) for 1 hr at room temperature. After washing three times in TBS with 1.0% Tween-20 (TBST), the membrane was incubated for 1 hr with horseradish peroxidase (HRP)-linked anti-rabbit IgG diluted 1 : 2000 in blocking solution at room temperature. Finally, after three 10 min washes in TBST, bands were visualized using Enhanced Chemiluminescece Luminol Reagent.

3. Real-Time RT-PCR with SYBR Green

To detect gene expression, mRNA was extracted from porcine oocytes or blastocyst using Dynabeads mRNA Direct Kit based the instructions. Primer sequences of maternal genes,

Table 1. List of treatment groups supplemented with FHS and/or LH

Treatmet groups	Period of oocyte maturation			
	0～22 h		23～44 h	
	FSH	LH	FSH	LH
NG (0～44)*	-	-	-	-
FSH (0～22)	+	-	-	-
FSH+LH (0～22)	+	+	-	-
FSH+LH (0～44)	+	+	+	+

* No gonadotropins.

apoptotic and autophagic-related genes are shown in (Table 2). Target genes expression were checked with PCR. Gene expression was normalized to porcine *Gapdh* mRNA and quantified using the 2-ddCt method.

4. Poly (A) Tail (PAT) Length Assay

The PCR-based poly (A) tail (PAT) assay was conducted according to the method of Salles and Strickland (Salles *et al*., 1995) to determine maternal transcript poly (A) tail length. Poly A+ RNA was isolated from oocytes at GV, GVBD, MI or MII stage which sampled at different time points (0, 18, 28, and 44 hr, respectively) of IVM using Dynabeads mRNA Direct Kit. First-strand cDNA was synthesized by the reverse transcription of mRNA with Oligo (dT)-Anchor as the primer (5'-GCGAGCTCCGCGGCCGCG- T_{12}-3') (Salles *et al*., 1995). The specific upstream primer sequences were shown in (Table 3). The PCR reactions were performed in 20 µl volumes containing 1 × PCR buffer, 1 µl of each primer, 75 mM of each dNTP, 2.0 mM $MgCl_2$, 0.5 UTaq DNA polymerase, and 4 µ lofc DNA (equalto1oocyte). The amplification protocol was initiated for 5 min at 93℃, followed by 33 cycles of 30 sec at 93℃, 1 min at 60℃ and 0 sec at 72℃, and was completed by a final extension of 5 min at 72℃. The PCR products were electrophoresed on 2.0% agarose gel stained with 0.5 mg/ml ethidium bromide (EB) and visualized by exposure to ultraviolet light.

5. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End Labeling (TUNEL) Assay

The parthenote blastocystes were fixed, washed in PBS/ PVA (0.1% PVP in PBS) and permeabilized in 0.3% Triton X-100 for 1 hr at room temperature (RT). Terminal of DNA

Table 2. List of primers used for real-time RT-PCR

Genes	GenBank acc. No.	Primer sequence (5'~3')	Length (bp)	Annealing temp(℃)
Gapdh	AF017079	F: GGGCATGAACCATGAGAAGT R: AAGCAGGGATGATGTTCTGG	230	60
C-mos	NM_001113219	F: TGGGAAGAAACTGGAGGACA R: TTCGGGTCAGCCCAGTTCA	121	60
Cyclin B1	L48205	F: TTGACTGGCTAGTGCAGGTTC R: CTGGAGGGTACATTTCTTCATA	177	60
Gdf9	AY626786	F: GAGCTCAGGACACTGTAAGCT R: CTTCTCGTGGATGATGTTCTG	272	60
Bmp15	NM_001005155	F:CCCTCGGGTACTACACTATG R: GGCTGGGCAATCATATCCT	192	60
Casp3	NM_214131	F: GAGGCAGACTTCTTGTATGC R: CATGGACACAATACATGGAA	237	60
Bak	AJ001204	F: CTAGAACCTAGCAGCACCAT R: CGATCTTGGTGAAGTACTC	151	60
Bcl-xL	AF216205	F: GGAGCTGGTGGTTGACTTTC R: CTAGGTGGTCATTCAGGTAAGG	249	60
Lc3	NM_001190290	F:CCGAACCTTCGAACAGAGAG R:AGGCTTGGTTAGCATTGAGC	206	60
Atg6	NM_001044530	F:AGGAGCTGCCGTTGTACTGT R:CACTGCCTCCTGTGTCTTCA	189	60

Table 3. List of primers used for PAT assay

Genes	GenBank acc. No.	Primer Sequence (5’-3’)
C-mos	NM_001113219	GCTGAACTGGGCTGACCCGAAAC
Cyclin B1	L48205-BLAST	TCTTGATAATGGTGAATGGACACCA
Gdf9	AY626786	CTGCGTACCTGCCAAGTACAGCC
Bmp15	NM_001005155	CCCTCGGGTACTACACTATG

were labeled with fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase enzyme in the dark for 1 hr at 37℃. Nuclei were stained with 40 μg/mL Hoechst 33342, After washing in PBS/PVP, the embryos mounted with slight coverslip compression and observed by confocal microscopy. Total cell number and apoptosis cell were measured. Apoptosis index (Apoptosis cell number/total cell number) were compared among each group.

6. Statistical Analysis

The general linear models (GLM) procedure in SAS program was used to analyze the data from all experiments. Western blotting images were digitized by Gel analyzer 4.0 (Media Cybernetics, USA). Significant differences were determined using Tukey’s multiple range test and $P<0.05$ was considered significant.

RESULTS

1. Effect of Gonadotropins on Cumulus Expansion and Porcine Oocyte *In Vitro* Maturation

The effect of FSH or FSH + LH on nuclear progression and cumulus expansion in porcine oocytes after 22 hr of IVM is shown in Fig. 1. Pig oocytes exposed to FSH or FSH+LH showed an increase in cumulus expansion at 22 hr (Fig. 1A), while control COCs clumped together and cumulus cells appeared black and shrunken (Fig. 1A). Gonadotrophin treatment enhanced meiotic resumotion of oocytes at 22 hr (Fig. 1B). Both FSH (43.75% ± 4.30) and FSH + LH (32.00% ± 7.30) decreased the percentage of germinal vesicle (GV)-stage oocytes compared with control group (61.54% ± 4.70%, $P<0.05$). Most of oocytes in the FSH + LH group reached into germinal vesicle breakdown (GVBD) stage and significantly higher than that in control group (64.00% ± 7.60% vs. 26.92% ± 2.60, $P<0.05$).

The percentage of oocytes arrested at the metaphase II stage was shown in Fig. 1C, metaphase II (MII) oocytes in the FSH (0～22), FSH + LH (0～22) and FSH + LH (0～44) groups after 44 hr were 68.97% ± 6.00, 89.66% ± 2.40 and 92.00% ± 5.70, respectively, significant higher than control group (47.37% ± 3.30). However, oocytes arrested at GV or GVBD stage in control group were higher than that in gonadotropins treatment groups.

2. Effect of Gonadotropins on MAP Kinase Phosphorylation and p44/42 Level

To study the effect of FSH and LH on the MAPK pathway during porcine oocyte maturation, the protein level of phosphorylated extracellular signal-regulated kinase (p-ERK)1/2 was measured by Western blotting. Fig. 2 shows ERK1/2 activation in porcine oocytes. Differences in ERK1/2 activation were observed between the three oocyte maturation protocols. Moreover, FSH alone or in combination with LH promoted the phosphorylation of ERK1/2 at 18h. ERK1/2 activation was remarkably increased from 18 hr to 44 hr in the each experimental groups. ERK1/2 were significantly activated at 28h in FSH + LH (0～22) and (0～44) group than NG or FSH group. Although phosphorylated ERK1/2 were higher in FSH (0～22) than that in FSH (0～44) at 28h, there were no significantly different at 44h between the two group, but higher than NG and FSH (0～22) group.

3. Effect of Gonadotropins on Parthenote Developmental Competence and Number of Cells in Blastocysts

To further assess oocyte developmental competence, all MII stage oocytes were parthenogenetically activated. During parthenogenesis, cleavage rates differed between the groups (58.82% ± 4.20, 72.22% ± 5.30, 70.00 ± 3.20 and 35.29% ± 2.00 for FSH (0～22), FSH + LH (0～22), FSH + LH (0～44) and negative control groups, respectively). Blastocyst rates were 13, 41, 38 and 6%, respectively. Similar percentages of four-cell stage were observed, except for the FSH + LH (0～22 and 0～44) group, which showed the highest percentage (Fig. 3A). The FSH + LH (0～22 and 0～44) group showed

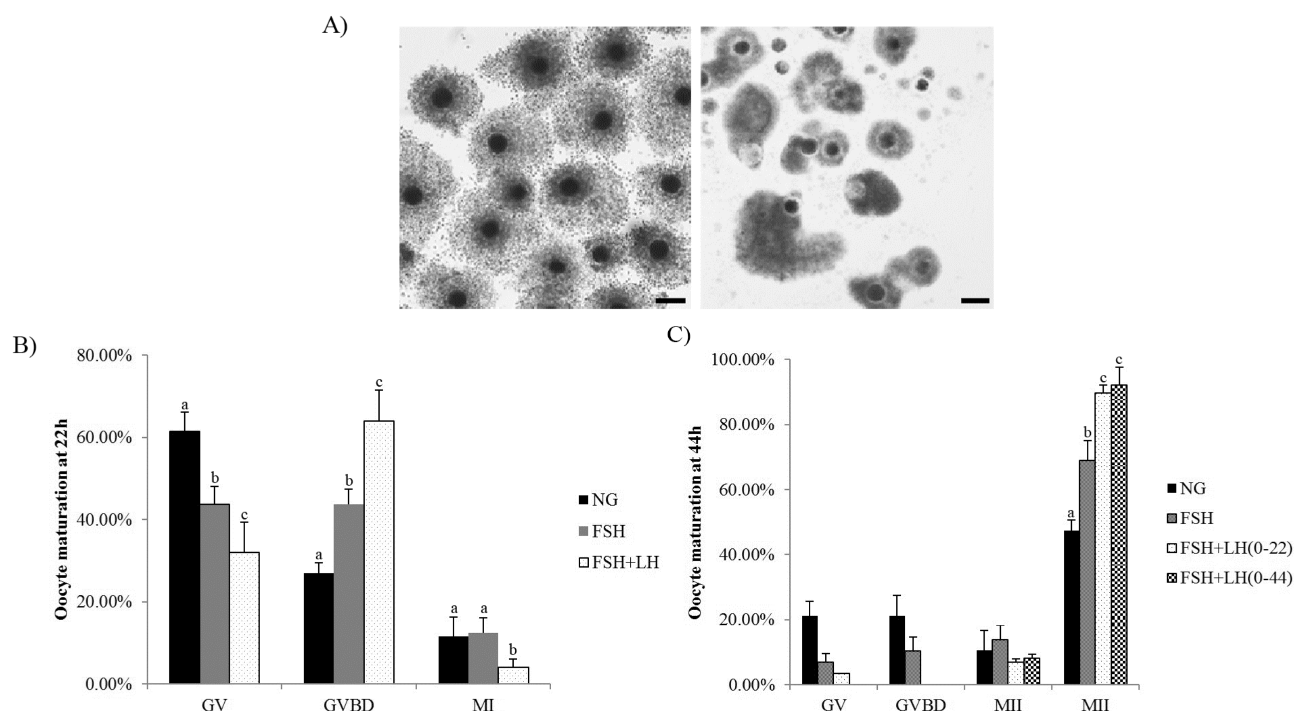

Fig. 1. (A) Cumulus expansion *in vitro*. Only good-quality COCs were selected. COCs were further cultured in IVM medium with or without FSH and LH and observed under a microscope at 22 h. (B) COC nuclear maturation *in vitro*. The oocytes were exposed to different gonadotropin protocols (FSH, FSH+LH or no gonadotropins) and sampled at 22 h. The nuclear maturation status of denuded oocytes was determined by Hoechst 33342 staining. (C) COC nuclear maturation *in vitro* at 44 h. COCs were cultured in medium supplemented with FSH and LH. Data are expressed as the percentage ± SEM of three independent repetitions of the experiments. Different letters over bars in the same nuclear stage indicate significant differences (*P*<0.01). Scal bar = 200μM

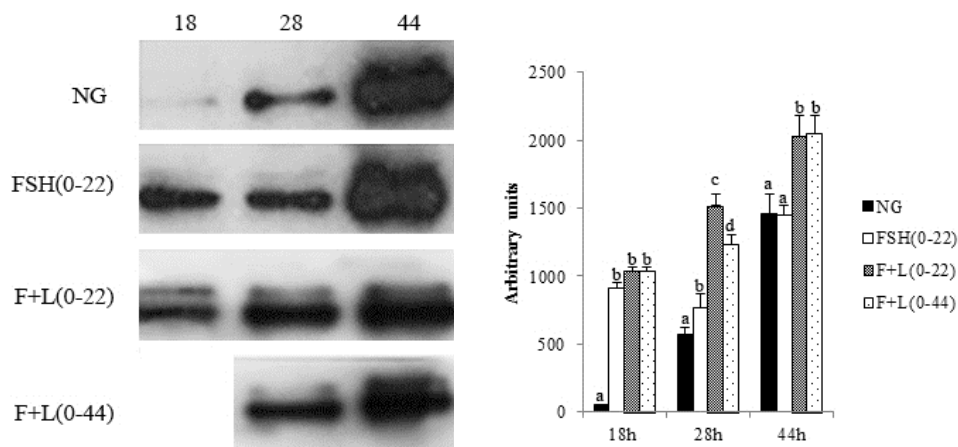

Fig. 2. Mitogen-activated protein kinase (MAPK) phosphorylation in pig oocytes during *in vitro* maturation. The oocytes were sampled at four time points (0, 18, 28 and 44 h). Fifty oocytes were used per lane. The culture times and treatments are indicated at the top of the figure. Note that each Western blot image shown is of the same membrane sequentially immunoblotted for the proteins indicated. NG: non-treatment group; FSH (0~22): oocytes matured in IVM medium with FSH from 0~22h; F + L (0~22): oocytes matured in IVM medium with FSH + LH for 22h; F + L (0~44): oocytes matured in IVM medium in the present of FSH and LH for 44h.

higher development of early embryos than other two groups.

To further investigate the effect of FSH and LH on oocyte developmental competence, the number of cells per blastocyst and the apoptosis index (number of apoptotic cells/total number of cells) were measured. FSH + LH (0～22) significantly increased total cell number than NG and FSH group, but no significantly different compared with blastocyst in FSH + LH (0～44) group. In the contrast, apoptosis index of NG showed the relatively higher rates (Fig. 3B & C) than all of other groups.

4. Effect of Gonadotropins on Apoptotic and Autophagic mRNA Expression in Blastocysts

The effect of FSH and LH on the mRNA expression of apoptotic genes and autophagy genes was assessed in porcine parthenotes developing *in vitro* (Fig. 4). All three protocols increased the mRNA level of the anti-apoptotic gene *Bcl-xL* ($P<0.05$) and decreased the mRNA level of the pro-apoptotic gene *Casp3* compared to the NG group, especially the FSH (0～22) and FSH + LH (0～44) protocols ($P<0.05$). No difference in *Bak* was observed among the groups. *Atg6* and *Lc3* mRNA (autophagosome markers) was detected in all the groups (Fig. 4B). *Atg6* mRNA levels were lowest in the FSH + LH (0～44) group and all three protocols decreased *Atg6* mRNA levels compared to the NG group ($P<0.05$). No differences in *Lc3* were observed.

5. Effect of Gonadotropins on Maternal mRNA Expression during Oocyte Maturation

The effect of FSH and LH on maternal gene expression patttern during IVM was further investigated. *Gdf9* expression pattern was similar in all the groups, and with a specific higher expression at 28 hr. The expression pattern of *Bmp15* was similar to that of *Gdf9* in all groups. *Cyclin B1* expression was relatively low during the early stages of IVM and significantly increased up to 28 hr in all the groups. In the presence of LH, *Cyclin B1* expression was maintained from 28 hr to 44 hr. *C-mos* expression was relatively low during the early stages

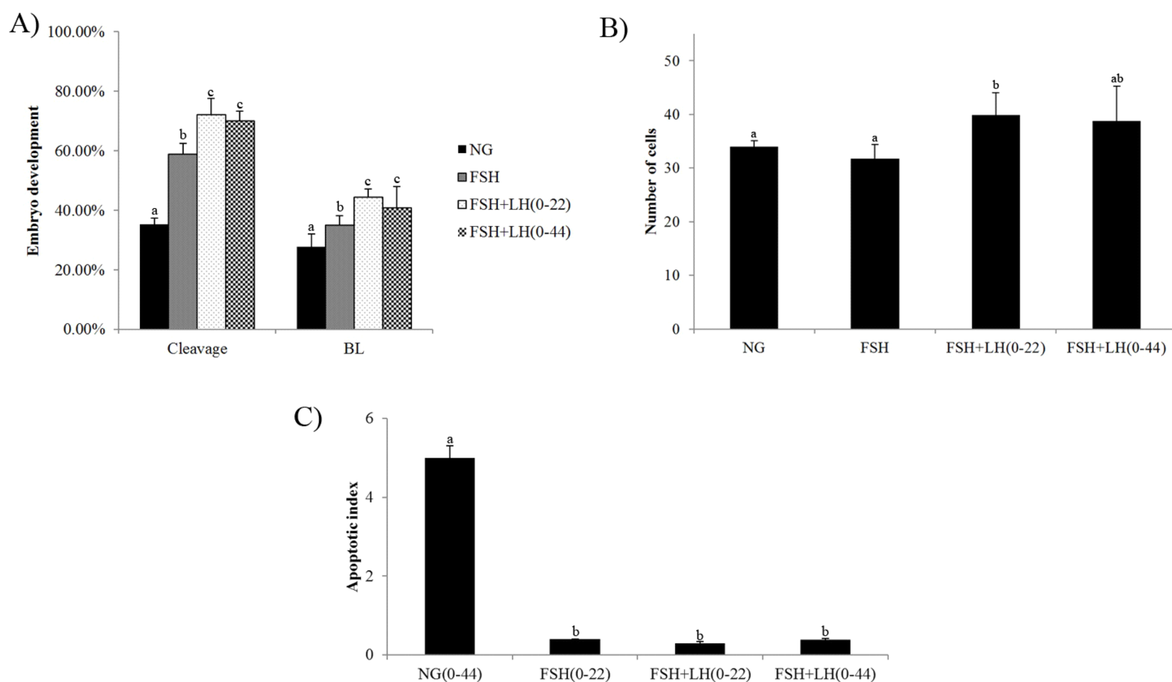

Fig. 3. Developmental rates, total cell numbers and apoptotic rates in blastocysts. (A) *In vitro* development of MII oocytes to the blastocyst stage following parthenogenetic activation. The data show cleavage, and BL (blastocyst) stages, respectively. (B) Comparison of the total cell number per blastocyst in four experimental groups. (C) Comparison of the apoptotic rate (number of apoptotic cells/ total number of cells) of blastocysts. Different letters over bars in the same nuclear stage indicate significant differences ($P<0.01$). Values are the means ± SEM of four separate experiments. BL: blastocyst; NG: non-treatment group; FSH (0~22): oocytes matured in IVM medium with FSH from 0~22h; F + L (0~22): oocytes matured in IVM medium with FSH + LH for 22h; F + L (0 ~ 44): oocytes matured in IVM medium in the present of FSH and LH for 44h.

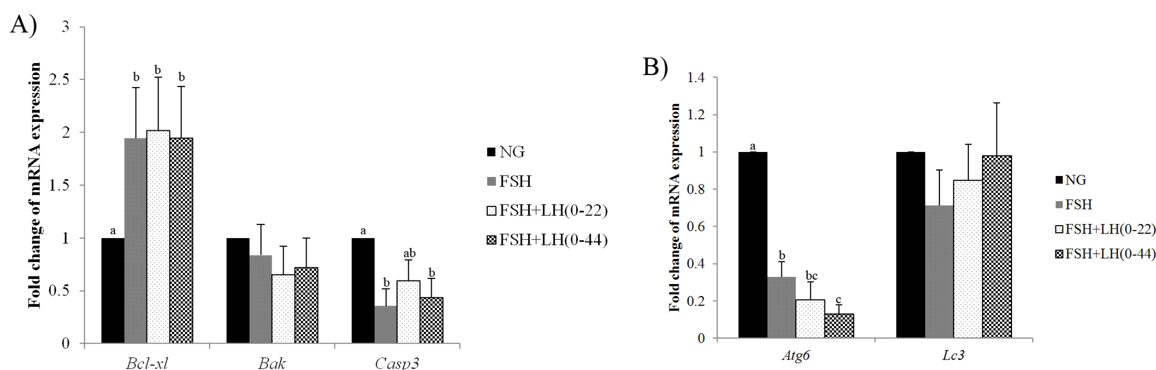

Fig. 4. Relative abundance of apoptosis genes (*Bcl-xL*, Bak and *Casp3*) by real-time RT-PCR in blastocysts (A). Relative abundance of autophagy genes (*Atg6* and *Lc3*) in blastocysts (B). Gapdh was used as the internal reference. Different letters over bars in the same nuclear stage indicate significant differences (*P*<0.01). Values are the means ± SEM of four separate experiments.

of IVM and significantly increased up to 44 hr in the FSH (0 ~22) groups (Fig. 5).

6. Effect of Gonadotropins on Maternal Gene Polyadenylation during Oocyte Maturation

The poly (A) tail length of important maternal genes (*Gdf9* and *Bmp15*), *C-mos* and a MPF component (*Cyclin B1*) was assessed in response to FSH and LH during IVM. Gonadotropins can affect maternal mRNA polyadenylation (Fig. 6). Polyadenylation status was different in the presence of gona-

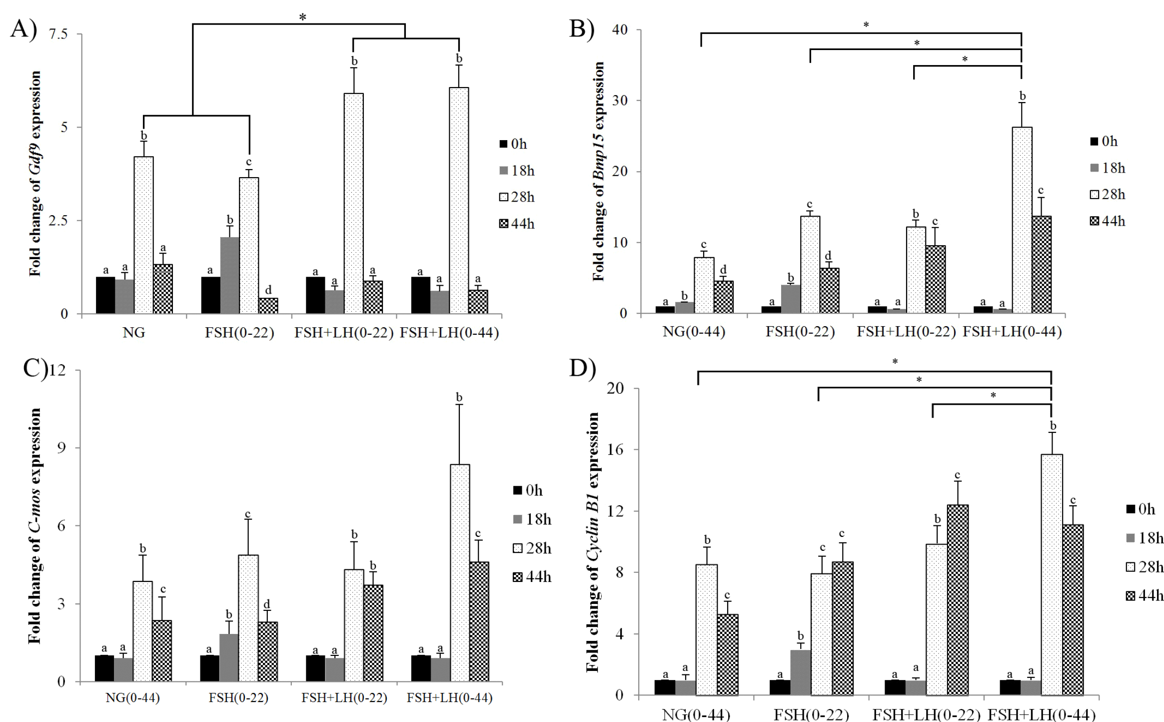

Fig. 5. Expression patterns of *C-mos*, *Cyclin-B1*, *Bmp15* and *Gdf9* mRNA during pig oocyte *in vitro* maturation (IVM) by real-time PCR. *Gdf9* mRNA was detected at four time points (0, 18, 28 and 44 h) in all groups (A). *Bmp15* (B), *C-mos* (C) and *Cyclin B1* (D) mRNAs were detected at the same time point. mRNA expression was normalized to Gapdh. Data are expressed as the percentage ± SEM of three independent repetitions of the experiments. Different letters over bars in the same nuclear stage indicate significant differences (*P*<0.05). * represent significant difference (*P*<0.05) over different groups at the same time point. NG: non-treatment group; FSH (0~22): oocytes matured in IVM medium with FSH from 0~22h; F + L (0~22): oocytes matured in IVM medium with FSH + LH for 22h; F + L (0~44): oocytes matured in IVM medium in the present of FSH and LH for 44h.

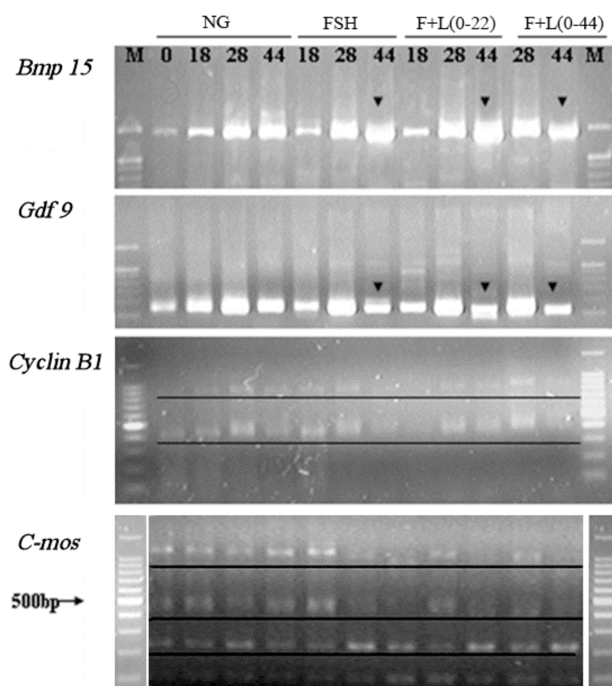

Fig. 6. Dynamic changes in poly(A) tail length in four maternal genes during pig oocyte *in vitro* maturation (IVM) by the PCR-based poly (A) tail (PAT) assay. Protocols studied: GV stage (0 h), 18 h, 28 h and 44 h of IVM with gonadotropins in culture medium. *Gdf9* and *Bmp15* underwent deadenylation, especially at 44 h (i.e., the smears for *Gdf9* and *Bmp15* were shortened at 44 hr). By contrast, *C-mos* and *Cyclin B1* showed polyadenylation beginning at 18 h.

dotropins compared to the negative control group, regardless of whether polyadenylation or deadenylation occurred during IVM. PAT assays revealed more intensive polyadenylation signals for four genes. *C-mos* and *Cyclin-B1* underwent similar polyadenylation pattern during oocyte maturation with all the protocols, except with the LH protocol. In particular, *Cyclin B1* was intensively polyadenylated at 28 hr in the presence of gonadotropins, while the polyadenylation of *C-mos* started at 18 hr. *Gdf9* and *Bmp15* deadenylation was observed during oocyte maturation in the presence of gonadotropins, especially at 44 hr (MII stage). Herein, FSH played an important role in this process during the first half of oocyte maturation.

DISCUSSION

In mammalian ovarian follicles, FSH/LH signaling is essential for follicle growth (Richards, 1994) and steroidogenesis (Seger *et al*., 2001). Recent data indicated that LH binds to its receptors on granulosa cells and stimulates expression of epidermal growth factor (EGF)-like peptides (Park *et al*., 2004). These peptides (amphiregulin, epiregulin) are produced as membrane-bound pro-peptides that require a proteolytic step to be released into the follicular environment (Ashkenazi *et al*., 2005). They then act directly on mural granulosa as well as cumulus cells and stimulate resumption of meiosis and expansion of the cumulus. The EGF-like peptides are also produced in cumulus cell of *in vitro* cultured cumulus-oocyte complexes upon stimulation by FSH and trigger resumption of meiosis. However, it had been reported that FSH regulate meiotic resumption through increase cyclic denosine monophosphate (cAMP), a principle molecular through protein kinase A (PKA) pathway, in sequence, inhibit meiotic resumption. But in our results, FSH increased meiotic resumption compared with control group. The effect of FSH on oocyte maturation was biphasic, initially inhibitory and then stimulatory. FSH induces a transient increase in cAMP levels and regulates gap junction communication between oocytes and cumulus cells to control PKA and GPR3 activities, thereby creating an inhibitory phase. Prior to FSH-induced meiotic maturation, cAMP, PKA type I and GPR3 returned to basal levels, and phosphodiesterase 3A activity and MAPK phosphorylation increased markedly(Li *et al*., 2012). However, FSH induced meiotic resumption in coordination with LH via PKA and protein kinase C (PKC) to modulate GVBD. It has been demonstrated that LH increased intracellular Ca^{2+} concentration. The increase in intracellular Ca^{2+} probably results predominately from the ability of the LH receptor to activate phospholipase C (PLC), and further results in the cleavage of phosphatidylinositol 4,5-bisphophate into inositol triphosphate (IP_3) and diacylglycerol (DAG), which act as a necessary co-activator for many PKC isoforms. Activation of PKC were required by meiotic resumption of pig oocytes. PKA and PKC are two important protein kinases in regulation meiotic resumption via MAPK pathway, phosphorylated MAPK is required by GVBD. In our experiment profile of p-MAPK in the early stage of meiotic resumptionin dicated the mechanism of FSH and LH on GVBD.

Oocytes maturation include nuclear and cytoplasmic maturation. Nuclear maturation is very easy to be identified, parameters of IVF and embryos development always be used to evaluate cytoplasm maturation, but changes in molecular level still need been explored. Our results showed that FSH and LH significantly increased the embryo development presented by blastocyst rate, total cell number and apoptosis. Recent impor-

tant studies have demonstrated that both BMP15 and GDF9 increased oocytes developmental competence independently (Hussein *et al*., 2006), despite the fact that they elicit different intracellular responses. This suggests that both the SMAD 1/5/8 (activated by BMP15) and SMAD 2/3 pathway (activated by GDF 9) in cumulus cells (CCs) are someway involved in regulating development competence of the oocytes. As both GDF9 and BMP15 required for development in bovine(Juengel *et al*., 2009) and for fertility in sheep(Hanrahan *et al*., 2004), gonadotropins probably activate both TGF-β superfamily signaling pathways in CCs, in contrast to mice, where murine oocytes predominately activate SMAD 2/3. So better quality of oocytes after gonadotropins treatment might caused by high GDF9 and BMP15 synthesis.

Synthesis and degeneration consisted the balance system for protein content in oocytes maturation. MOS and CYCLIN B1 are important molecular effect on MPAK and MPF pathway synthesized from mRNA stored in cytoplasm of oocytes(Liang *et al*., 2007). Oocyte maturation mainly controlled by the two kinases via phosphorylation of some downstream proteins (e.g. lamin and so on) to induce GVBD. In addition, GDF9 and BMP15 are important maternal genes that regulate cumulus cell proliferation, expansion and oocytes development ability (Hussein *et al*., 2006). We investigated the dynamics of the four maternal genes in the entire duration of maturation among different protocols. Similar pattern between *C-mos* and *Cyclin B1* were observed. Expression of *C-mos* and *Cyclin B1* increased at 28h and at 28h, no obvious different expression among control and other groups. But expressions of *Gdf9* and *Bmp15* at 28h after gonadotropins treatment were higher than that in control group. Our data in here suggest that maternal genes are involved in maturation process.

Translation efficiency is mainly controlled by the polyadenylation status and integrity of mRNA (Burgess *et al*., 2010), thus, to investigate the mechanism involved on gene expression and regulation, polyA length of mRNA of the four maternal genes were assayed. Our results showed that those of *Gdf9* and *Bmp15* were shortened during *in vitro* maturation, particularly at 44 hr in the presence of FSH alone or the combination of FSH and LH, indicating that *Gdf9* and *Bmp15* have underwent deadenylation. This result is consistent with previous polyadenylation studies in pig and bovine oocytes, which showed a shorter poly (A) tail at the MII stage than at the GV stage (Zhang *et al*., 2009) and may be attributable to the very high levels of *Gdf9* and *Bmp15* mRNA that already exist in GV oocytes and may be less affected by deadenylation. The addition of FSH and LH to the culture medium changed the timing of maternal gene expression, suggesting an important role for cellular deadenylation in the regulation of mRNA expression.

In conclusion, the addition of FSH and LH to IVM medium enhances meiotic and cytoplasmic maturation, by regulating the maternal genes expression and polyadenylation. Moreover, FSH and LH improve pre-implantation developmental competence and further reduce apoptosis and autophagy.

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