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Effect of maternal gene expression on porcine oocytes in vitro maturation

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돼지 미성숙 난자 모계 유전자 발현이 체외성숙에 미치는 영향

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Abstract Understanding of the maternal transcriptome increased to elucidate the underlying molecular mechanism of normal oocyte maturation, which depends on a precise sequence of changes in maternal genes expression. Previous reports that the translational potential of a maternal mRNA is generally determined by the length of the poly(A) tail, and deadenylation is usually the first sign of mRNA degradation. However, in vitro cultured system has the underlying molecular mechanisms remain unclear. We determined whether the role of molecular basis, four important maternal genes, C-mos, cyclin-B1 (regulatory subunit of MPF), BMP15 and GDF9, were selected for detection of their precise mRNA expression patterns by real-time PCR and for determination of their polyadenylation status by poly(A) tail PCR during oocyte maturation. In the present study. the abnormal expression of maternal mRNAs prior to zygotic genome activation, which results in suppression of the corresponding protein level, may be responsible for, at least in part, a profound defect in further embryonic development. Reasonable expression of maternal gene is crucial for proper oocyte maturation and further embryonic development.

요 약 난자 세포의 정상적인 성숙과정을 이해하려면 모계유래 유전자 발현 증가의 분자 생물학적 기전을 밝혀내야 한다. 이것은 모계 유전자의 염기서열의 변화와 밀접한 관계가 있다. 전 연구결과에 의하면 돼지 난자 체외 성숙 과정에서의 모계 유전자 mRNA 발현은 통상적으로 poly(A) 꼬리 길이와 아데닐산 중합반응에 의하여 검증된다. 하지만 포유동물 체외성숙 과정에서는 아직까지 밝혀진 것이 없다. 따라서 본 연구목적은 성숙단계 난모세포에서의 분자생물학적 기전을 해명하고자, 4개의 중요한 모계유전자발현을 real-time PCR기법으로 확인하여 poly(A) 꼬리 길이와 아데닐산중합반응의 변화를 확인하였다. 본 연구에서 접합체 유전자 활성화 단계에서 모계 유전자의 비정상적인 발현과 이것에 상응하는 단백질 수준의 억제는 일부 혹은 대부분 유전자 손실에 의하여 초래된 것임을 알 수 있었다. 따라서 이상적인 모계 유전자 발현은 난자 세포의 성숙 및 더 나가서 초기 배아 발달에 중요한 역할을 하는 것임을 확인 하였다.

Key Words : Porcine oocytes, Maternal gene, Polyadenylation, In vitro maturation

1. Introduction

During meiotic maturation, mammalian oocytes accumulate a larger than necessary pool of maternally derived transcripts for oocyte maturation and early embryogenesis. An increased understanding of the maternal transcriptome is needed to elucidate the underlying molecular mechanism of normal oocyte maturation, which depends on a precise sequence of changes in maternal genes expression. The translational potential of a maternal mRNA is generally determined by the length of the poly(A) tail, and deadenylation is usually

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the first sign of mRNA degradation [1]. The in vivo overexpression of poly(A) polymerase (PAP), which further intensified the level of cellular polyadenylation, resulted in embryonic lethality in Drosophila [2].

To date, few maternal oocyte genes in human or mouse oocytes have been characterized. Among these maternal transcripts, C-mos, cyclin-B1, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15, also known as GDF9B) are well-studied genes considered to be markers of female germ cells. C-mos is a proto-oncogene identified as a regulator of oocyte maturation in human [3] mouse [4] and pig [5] . Mos is an active component of the cytostatic factor (CSF) responsible for the arrest of meiosis at MII stage [6]. As a mitogen activated protein (MAP) kinase kinase kinase, Mos activates extracellular signal-regulated protein kinase (ERK) 1 and 2. A functional study suggested that Mos also mediates the activity of maturation/M phase promoting factor (MPF), another essential regulator of meiosis resumption formed by cyclin B1 and Cdc2 kinase, through the MAPK pathway and stabilization of cyclin B1 [6]. It has been reported that the dynamic change in levels of cyclin B1 is mainly controlled by cytoplasmic polyadenylation during mouse [7] and bovine [8] oocyte maturation. However, no such study has yet been conducted in pig oocytes. GDF9 and BMP15 belong to the transforming growth factor-b (TGF-b) superfamily, which contains many members with important roles in regulating fertility [9]. GDF9 and BMP15 were currently identified as oocyte-secreted factors involved in folliculogenesis and oocyte maturation, as well as in cooperative regulation of granulose cells [10].

In the present study, cordycepin (3′-dA), a potent polyadenylation inhibitor or cyclopiazonic acid (CPA), a specific inhibitor of Ca2+-ATPase were used to detect the overall effects of polyadenylation or Ca2+-ATPase inhibition on in vitro porcine oocyte meiotic maturation. To further reveal the underlying molecular basis, four important maternal genes, C-mos, cyclin-B1 (regulatory subunit of MPF), BMP15 and GDF9, were selected for detection of their precise mRNA expression patterns by real-time PCR and for determination of their polyadenylation status by poly(A) tail PCR during oocyte maturation.

2. Materials and Methods

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl solution supplemented with 75mg/ml penicillin G and 50mg/ml strepotomycin sulfate at 35°C. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles . Approximately 60 COCs were matured in 500 ml tissue culture medium (TCM)-199 containing 0.1% PVA (w/v), 3.05mM D-glucose, 0.91mM sodium pyruvate, 0.57mM cysteine, 10 ng/ml epidermal growth factor (EGF, Sigma, St. Louis, MO, USA), 10 IU/ml PMSG, 10 IU/ml hCG, 75 mg/ml penicillin G, and 50 mg/ml strepotomycin sulfate, under mineral oil at 38.5°C for 44 hr in a humidified atmosphere of 5% CO2 in air.In the drug treatment groups,3′-dG(2mg/ml) or 3′-dA (5, 2, and 1 mg/ml) were added in the culture medium. In another experiment, 10 uM CPA was added to the culture medium.

2.1 Real-time PCR with SYBR green

mRNAsfrom porcine oocytes derived by in vitro maturation were isolated with Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway), according to the manufacturer's instruction. The firststrand cDNA synthesis was achieved by reverse transcription of mRNA using the Oligo(dT)12-18 primer and SuperScript TM II Reverse Transcriptase (Invitrogen Co., Grand Island, NY). Real time PCR using a DNA Engine OPTICOJ 2 (MJ Research, Waltham, MA, USA)) instrument was performed in a final reaction volume of 20 ml with SYBR Green, The relative quantification of gene expression was analyzed using the 2-ddCt method by normalization to internal porcine b-actin mRNA expression.

2.2 PAT Assay: analysis of poly(A) tail lengths by PCR

For determination of maternal transcript poly(A) tail length, the PCR-based poly(A) tail (PAT) assay was carried out according to the method of Salles and Strickland with some minor modifications. Poly Aþ RNAs from pools of 25 denuded pig oocytes sampled at different time points (0, 18, 28, 44 hr) during IVM were isolated by Dynabeads mRNA Direct Kit (Dynal Asa, Oslo, Norway). The first-strand cDNA was synthesized by

reverse transcription of isolated mRNAs with Oligo(dT)-Anchor as the primer (5'-GCGAGCTCCGCGGCC -GCGT12-30.

Subsequent PCR was performed using Oligo(dT) -Anchor and gene-specific upstream primers for the test maternal transcripts. The PCR reactions were performed in 20 ml reactions containing 1X PCR buffer, 1ml of each primer, 75mMof each dNTP, 2.0mM MgCl2, 0.5UTaqDNA polymerase (Promega, Madison, WI) and 4ml of cDNA (equal to 1 oocyte). The amplification protocol was initiated with 5 min at 93°C, followed by 33 cycles of 30 sec at 93°C, 1 min at 60°C, and 50 sec at 72°C, and was completed by a final extension of 5 min at 72°C. PCR products were electrophoresed on 2.0% Agarose gel stained with 0.5 mg/ml ethidium bromide.

2.3 Data analysis

At least three replicates were performed for each treatment. Statistical analyses were conducted using an analysis of variance (ANOVA) and differences between treatment groups were evaluated with Duncan's multiple comparison test. Data were expressed as mean ± SEM and $p<0.05$ was considered to be statistically significant.

3. Results and Discussion

3.1 Maternal gene expression in porcine oocytes

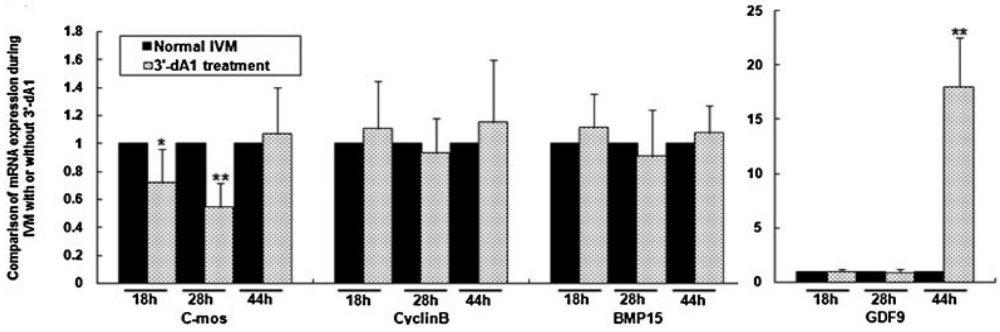
Expression patterns of C-mos, cyclin-B1 (regulatory subunit of MPF), BMP15 and GDF9 were analyzed during pig oocyte maturation by the sensitive real-time PCR method. These four genes represented three different expression patterns. C-mos and BMP15 were maintained at relatively high levels throughout oocyte maturation, with a specific higher expression at 28 hr. Cyclin-B1 expression was relatively low during early stages of IVM, and then significantly increased up to 44 hr. Transcription of GDF9 remained constant at remarkably high levels during 0–28 hr of oocyte maturation, but was dramatically decreased at 44 hr.

3.2 mRNA levels of Cyclin B1 isoforms and Cdc2 in porcine oocytes

The mRNA expression patterns of both cyclin B1 isoforms and cyclin B1-L alone were similar, showing an initial, relatively consistent, low level during the early stages of IVM followed by a significant increase at 30 and 44 hr. Expression of Cdc2 was stable during 0–30 hr of maturation, and then sharply decreased at 44 hr.

3.3 Effect of cordycepin (3′-dA) on maternal gene expression

Cordycepin (3′-dA), a potent polyadenylation inhibitor, was used to detect the overall effects of polyadenylation inhibition on in vitro porcine oocyte meiotic maturation. In 1 mg/ml 3′-dA-treated oocytes, C-mos and cyclin-B1 mRNA levels were unchanged during oocyte maturation. As demonstrated by comparative analysis, the expression of cyclin-B1 and BMP15by pig oocytes was unchanged, while C-mos and GDF9 expression patterns during maturation were different between the untreated group and the 3-dA1 treated group (Fig. 1). In 3′-dA1-treated

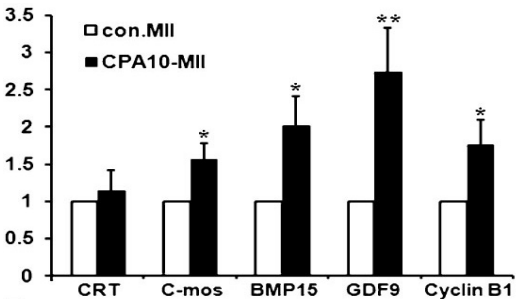


[Fig. 1] Comparison of C-mos, cyclin-B1, BMP15 and GDF9 levels during pig oocyte IVM with or without 3′-dA (1 mg/ml) treatment. Gene expression in IVM oocytes was set as onefold. Statistically significant differences are indicated by *P<0.05 and **P<0.01.

oocytes, C-mos mRNA level was significantly suppressed during 0–28 hr, and GDF9 mRNA expression was sustained at high levels at 44 hr, but was sharply down-regulated in untreated IVM at 44 hr (Fig. 1).

3.4 Effect of cyclopiazonic acid (CPA) on maternal gene expression

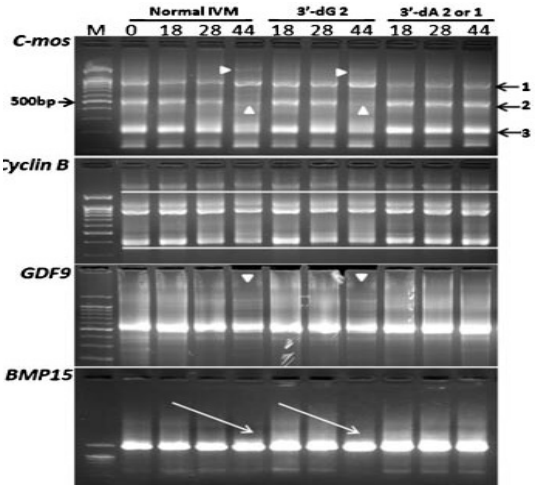
Cyclopiazonic acid (CPA) is a specific inhibitor of Ca2+-ATPase, and thereby facilitates the release of Ca2+ from intracellular stores. CPA consequently elevates intracellular calcium levels through mobilization of intracellular deposits and through the influx of extracellular Ca2+ (store-operated Ca2+ entry; Demaurex et al., 1992). To investigate the mechanism responsible for the inhibitory effect of CPA on oocyte maturation at the molecular level, the mRNA expression patterns and polyadenylation status of important maternal genes were investigated by PCR analysis. In mature oocytes treated with CPA, the maternal genes C-mos, BMP15, GDF9, and cyclin B1 showed significantly increased expression levels compared with the control groups. The expression level of calreticulin showed no change (Fig. 2).



[Fig. 2] The effect of cyclopiazonic acid (CPA) on the expression of maternal genes.
Pig MII-stage oocytes maturated with or without 10 mM of CPA were analyzed by real-time RT-PCR. The expression level of mRNAs in the control oocytes was taken as one-fold, and all expression levels were normalized to internal b-actin levels. Statistically significant differences are indicated by *P<0.05 and **P<0.01.

3.5 Polyadenylation status of maternal mRNAs in porcine oocytes

This experiment was designed to assess the poly(A) tail length of important maternal genes (C-mos, GDF9, BMP15) and mRNAs encoding proteins for MAPK (p42 and p44) and MPF (cyclin-B1 and Cdc2), and to detect the effect of a polyadenylation inhibitor on their polyadenylation status during pig in vitro oocyte maturation. Addition of 3′-dG in culture medium did not influence the oocyte maturation rate (Table 1) or the maternal mRNAs polyadenylation process. In polyadenylation inhibitor-treated (2 or 1 mg/ml) oocytes, all the genes displayed a polyadenylation pattern similar to that observed in germinal vesical (GV) oocytes (0 hr), regardless of whether polyadenylation or deadenylation occurred during IVM. C-mos and cyclin-B1 were shown to undergo polyadenylation during oocyte maturation. Particularly, C-mos was intensively polyadenylated at 44 hr after IVM, while the polyadenylation of cyclin-B1 started at 28 hr (Fig. 3).



[Fig. 3] Dynamic changes in poly(A) tail length of selected maternal transcripts during pig oocyte in vitro maturation (IVM) by the PCR-based poly(A) tail (PAT) assay.

[Table 1] Effect of polyadenylation inhibition on porcine oocyte maturation in vitro

Treatment (44 hr)	N (COCs)	GV%	GVBD%	MI%	MII%
Normal IVM	100	0^{a}	7^{a}	17^{a}	76^{a}
3′-dG 2 μg/ml	76	1.31^{a}	6.57^{a}	14.47^{a}	77.63^{a}
3′-dA 5 μg/ml	60	100^{d}	0^{d}	0^{d}	0^{d}
3′-dA 2 μg/ml	129	29.45^{b}	18.6^{b}	26.35^{b}	25.58^{b}
3′-dA 1 μg/ml	71	16.9^{c}	14.08^{b}	32.39^{c}	36.62^{c}

4. Conclusion

Previous reports have focused on maternal gene expression and polyadenylation in porcine oocyte maturing in vitro. Little information is available about maternal gene function and polyadenylation/ deadenylation status during porcine oocyte meiotic maturation. Studies showed that abnormal expression of maternal mRNAs prior to zygotic genome activation, which results in suppression of the corresponding protein level, may be responsible for, at least in part, a profound defect in further embryonic development. Reasonable expression of maternal gene is crucial for proper oocyte maturation and further embryonic development.

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<Research Interests>
Animal Reproductive Physiology