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Comparison of Gene Expression between Cumulus Oocyte Complexes and Naked Oocytes by Suppression Subtractive Hybridization in Swine

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ABSTRACT : In the antral follicle phase, several layers of cumulus cells surround the oocyte and play an important support and regulation role in oocyte development and maturation via intercellular communications and interactions between oocytes and cumulus cells. However, information on stage specific gene expression in swine during the phase is not well understood. To investigate the function of cumulus cells during *in vitro* maturation of porcine oocytes and gene expression, suppression subtractive hybridization (SSH) was performed to screen genes that were differentially expressed between cumulus-oocyte complexes (COCs) and naked oocytes (NOs). Utilizing mRNAs from *in vitro* maturation oocytes, a SSH cDNA library from COCs as the tester and NOs as the driver was constructed. The SSH cDNA library was then screened using dot blot analysis. Results showed that a total of 70 clones randomly selected from the library were differentially expressed. Among these, 41 exhibited high homology to known genes and 11 were novel expressed sequences tags (ESTs). Four differentially expressed genes, including *bfgf*, *sprouty 2*, *egr* and *btc*, were further studied by real time quantitative PCR; results confirmed an increased expression of respective mRNA in COCs compared with NOs, which suggests that these factors may play an important role in oocyte development and maturation. (**Key Words :** Suppression Subtractive Hybridization (SSH), Gene Expression, Cumulus Cells, Porcine)

INTRODUCTION

When oocytes are obtained for *in vitro* maturation and fertilization (IVM-IVF), not all of them have the ability to develop into an embryo. The incapability to sustain further development may be associated with incomplete maturation of oocytes during folliculogenesis (Hyttel et al., 1997; Sirard et al., 2001). Although great efforts have been made to recreate *in vitro* the follicular environment that allows complete maturation of the porcine oocyte, current procedures only produce a blastocyst development rate of about 35% (Schoevers et al., 2007). The mechanisms responsible for the coordinated maturation of the follicle and the enclosed oocyte are not yet understood. However, it is clear that the cells surrounding oocyte affect the ability of oocyte to support the first stages of development (Buccione et al., 1990; Matzuk et al., 2002). Selection of cumulus-oocyte complexes (COCs) based on morphologic characteristics of the cumulus cell layers has been effective in enhancing the blastocyst rates for *in vitro* production procedures (Vanderhyden et al., 1992).

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Bi-directional communication between oocytes and cumulus cells is essential for the development and function of ovarian follicles and promotes the production of mature oocytes competent. The cumulus cells are a subgroup of granulosa cells that surround oocyte in an antral follicle and, because of their close proximity to the oocyte, play an important role in regulating oocyte maturation (Downs et al., 1995: Vozzi et al., 2001), since they regulate the meiotic progression (Ward et al., 2002) and protect oocytes against oxidative stress through enhancement of the ooplasmic gluthatione (GSH) content (Downs, 2001; Eppig et al., 2002). In addition, there is an evidence that indicates that gap junctional communication (GJC) is one of the routes by which the cumulus cells transmit the signals to oocyte (Carabatsos et al., 2000). Low molecular substances such as ions, nucleotides, and amino acids can also traverse from cumulus cells to oocytes and vice versa via GJC (Brower et al., 1982; Wassanman et al., 1994). Although cumulus cells provide essential nutrients and stimuli for oocyte growth and development, oocytes are not merely passive recipients of such support, but rather active regulators of follicular development. Recent studies demonstrate that growth and differentiation factor-9 (GDF-9), a well-characterized oocyte derived growth factor, mediates the oocyte effect on

cumulus expansion and the normal development of COCs (Bodin et al., 2007; Pei et al., 2007). In addition, GDF-9, as well as bone morphorgenic protein-15 (BMP-15) from oocytes, mediates other regulatory effects of the oocyte on cumulus cells (Bodin et al., 2007).

As oocyte is in dialogue with the surrounding cumulus cells via paracrine and GJC signalling, we hypothesized that differences in intra-follicular processes which are responsible for oocyte development are reflected in the gene expression pattern between cumulus cells and oocytes. However, the complex mechanisms associating with cell-to-cell communications are still not completely understood, in swine only very recently have been made attempts to assess and define the genetic factors. In the present study, we constructed a porcine COCs and naked oocytes (NOs) cDNA library and contrasted their mRNA expression profiles to isolate genes that are expressed in the COCs. It will enhance our understanding of the underlying molecular mechanism in which the roles played by cumulus cells to enclosed oocytes, especially that of cell-to-cell communication and coordinate responses.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA), unless specified otherwise.

Preparation of oocytes

Ovaries from prepubertal gilts were collected at a local slaughterhouse and transported to the laboratory within 1 h in a warm 0.85% sterile saline solution. The cumulus-oocyte complexes (COCs) were aspirated from antral follicles 2-5 mm in diameter using an 18-gauge needle and then pooled in 10ml test tubes placed in a water bath (37 °C) for settling. After being washed three times with Tyrode's lactate-Hepes, oocytes with uniform ooplasm and compact cumulus cells were used. Some COCs were digested in 0.1% hyaluronidase at 39°C for 5 min, then the cumulus cells were stripped off by gentle pipetting to obtain naked oocytes (NOs). After COCs were washed with the maturation medium, TCM-199 containing 10 IU/ml human Chorionic Gonadotropin (hCG), 10 IU/ml equine Chorionic Gonadotropin (eCG), 1 mM L-glutamine, 10% (v/v) fetal calf serum, and 10% (v/v) pig follicular fluid, a total of 20-40 COCs were transferred to 200 ml of the maturation medium under mineral oil in a 35 mm Petri dish and cultured in a CO_2 incubator (5% CO_2 in air at 39°C for 42-48 h.

Primers

The primers used in this study were summarized in Table 1. All primers were provided from the PCR-Selected cDNA Subtraction kit (Clontech, Palo Alto, CA). In Suppression subtractive hybridization method, PCR primer 1, Nested PCR primer1 and 2R were used for amplifying inserts as well as for sequencing. The PCR primer 1 was used in the primary RCR amplifications. The Nested PCR primer1 and 2R were used in the secondary PCR amplifications. To evaluate the efficiency of the ligation reaction, GAPDH primers were used.

mRNA extraction

mRNAs were extracted from 20 COCs and 35 NOs, respectively, using the Dynabeads mRNA DIRECT kit (Dynal, Great Neck, NY) according to the manufacturer's instructions. All oocytes were lysed and mixed with Dynabeads oligo (dT25). Then, repetitive washing and elution of Poly (A)+RNA was followed. Poly (A)+RNA was isolated with the PolyATtract mRNA Isolation System III (Promega, NewYork, USA). The quality of sample total RNA was vital to the successful construction of the SSH cDNA libraries. Thus, the quality of RNA was monitored by non-denaturing gel electrophoresis and quantified by the absorbance at OD260/OD280.

Double stranded cDNA synthesis

First-strand cDNA synthesis, cDNA amplification by LD PCR and *Rsa I* Digestion were performed using the Super SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Reverse transcription reaction was carried out at 42°C for 1.5 h. Purification of Digested cDNA was performed using QIAquick PCR Purification Kit (Qiagen, Valencia, CA).

Table 1. Primer sequences used for PCR analysis

Target	Primer sequence
PCR primer 1	Forward: 5'-CTAATACGACTCACTATAGGGC-3'
	Reverse: 5'- CTAATACGACTCACTATAGGGC-3'
GAPDH	Forward: 5'-ACCACAGTCCATGCCATCAC-3'
	Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'
Nested PCR primer 1	5'-TCGAGCGGCCGCCCGGGCAGGT-3'
Nested PCR primer 2R	5'-AGCGTGGTCGCGGCCGAGGT-3'

Suppression subtractive hybridization

Suppression subtractive hybridization was performed using the PCR-Select cDNA Subtraction Kit (Clontech) as described previously (Fayad et al., 2004). Tester and driver cDNA was produced from mRNA for COCs and NOs. Briefly, synthesized cDNA was digested with the restriction enzyme *RsaI* and the tester cDNA populations were divided into two tubes and ligated to either adaptor 1 or adaptor 2R. The subtractive hybridization was performed by adding 1.5 μl driver cDNA to each tube, one containing 1.5 μl of adaptor 1 and the other containing 1.5 μl adaptor 2R-ligated tester cDNA in 1 μl 4X hybridization buffer. Primary PCR amplifications were conducted for each tester using subtracted products following the second hybridization or the diluted unsubtracted cDNA. Cycling conditions were commenced as follows: 75°C for 5 min to extend the adaptors, 94°C for 25 s, and 27 cycles at 94°C for 10 s, 66°C for 30 s, and 72°C for 1.5 min. Secondary PCR was performed 12 cycles at 94°C for 10 s, 68°C for 30 s, and 72°C for 1.5 min. To check the efficiency of SSH, a PCR was performed with primers of house keeping gene GAPDH and the 1:10 diluted cDNA of subtracted or unsubtracted as template according to the manufacture's recommendations. The cycle number is 18-43.

Screening by dot blot analysis

Bacterial colony PCR was performed on the GeneAmp PCR System-9700 (Dynal) with 1 μl of bacteria culture in a total volume of 25 μl (400 nM each of NP1 and NP2R primers, 0.2 mM of each dNTP, 0.5 μl *Taq* polymerase Mix, 1PCR buffer). Then, the forward and reverse subtracted cDNA were digested with *RsaI* to remove the SSH adapters. The adapter-free cDNA was labeled by dig high prime labeling and detection starter kit (Clontech) by a random priming method. An aliquot (1 μl) of each positive PCR product (10 ng) was dropped on to a nylon membrane (Clontech) in duplicate, and fixed by irradiation under a UV transilluminator (Vilber Lourmat, Marne-La-Valle´e, France) for 8 min. Detailed operation for hybridization was carried out according to the manufacturer's protocol.

Sequencing analysis

Following dot blot hybriddization, the differentially expressed sequences were sequenced using special sets of primers by Sagon Corporation (Sangon). Homology searches against GenBank databases were done using the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) at the National Center for Biotechnology Information (NCBI).

Real-time PCR

Expression of the clones of interest, namely bfgf, sprouty 2, egr and btc was evaluated by real-time quantitative RT-PCR. RT-PCR was performed using a 7500 real-time PCR System (Applied Biosystems, Foster City, CA) using a two-step Quantitect SYBR Green RT-PCR Kit (Qiagen) according to the manufacturers instructions. After reverse transcription, an initial PCR activation step was carried out at 95°C for 15 min. The subsequent PCR conditions were as follows: 40 cycles of 15 s at 95°C, 30 s at 55°C and 35 s at 72°C. After amplification, the melting curve of these PCR products was a cycle of 15 s at 95°C, 1 min at 60°C and 15 s at 95°C. All assays were carried out in triplicate. The relative mRNA levels in COCs and NOs were normalized to those of GAPDH as previously described (Ajay et al., 2007).

Data analysis

All abstracts harboring the gene symbols were downloaded in text format from Pubmed on NCBI. Data mining was performed using the programs of Word Stat 4.0 (ProvalisResearch, Montreal, QC, Canada), After all the downloaded texts were transformed to the special format by Word Stat 4.0 software, a statistical analysis was performed to get the output of the frequency percentum of each keyword targeting each bait gene by Sim Stat 4.0 (ProvalisResearch, Montreal, QC, Canada) software. All values are expressed as means±SD. Two-tailed Student's t-test was used to determine statistical differences, and $p< 0.05$ or less was considered significant.

RESULTS

Quality and efficiency of SSH

As shown in Figure 1, the band intensity of 28S rRNA was approximately twice that of 18S rRNA, indicating that total RNA was of high quality (Figure 1A). House keeping gene GAPDH reached saturation after 23-43 cycles of amplification in the unsubtracted control, while the bands were just invisible from the subtracted templates under the same condition, showing that transcripts in the same abundance in driver and tester had been efficiently suppressed after SSH (Figure 1B).

Sequence analysis

For further analysis of the subtracted genes, 300 clones were randomly selected and bacterial colony PCR were performed (Figure 2A). The amplified cDNA was dotted onto nylon membranes and hybridized separately with ^{32}P-labeled forward or reverse subtracted cDNA. Of 300 clones, 70 gave different signal intensities when probed with the forward and reverse subtracted cDNA probes (Figure 2B). All 70 clones were sequenced and subjected to BLAST analysis against the GenBank databases. Of the 70 differentially expressed sequences, 41 corresponded to 23 known genes (Tables 2 and 3), 11 were novel ESTs.

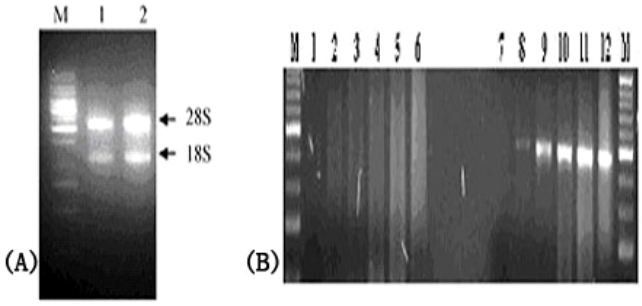

Figure 1. Quality and efficiency of suppression subtractive hybridization. (A) Lanes 1 indicated subtracted samples. Lanes 2 indicated unsubtracted sample. Lane M: 1 kb size DNA Ladder marker. (B) Lanes 1-6 indicated subtracted samples. Lanes 7-12 indicated unsubtracted sample. Lanes M: 1 kb size DNA Ladder marker; Lanes 1 and 7: 18 cycles; Lanes 2 and 8: 23 cycles; Lanes 3 and 9: 28 cycles; Lanes 4 and 10: 33 cycles; Lanes 5 and 11: 38 cycles; Lanes 6 and 12: 43 cycles.

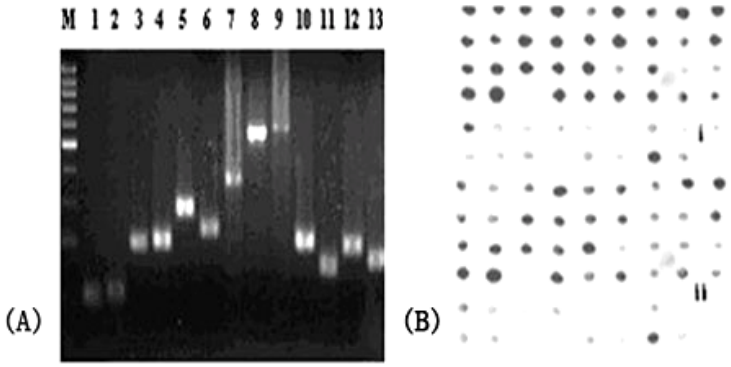

Figure 2. Screening of the subtracted library. (A) The subtracted library was screened by PCR. M was 1 kb DNA Ladder Marker; 1-13 were PCR results by NP1/NP2R primers. (B) The subtracted library was screened by dot blot. (I) The PCR fragments of COCs were hybridized by forward-subtracted cDNA probes; (II) the PCR fragments of NOs were hybridized by forward-subtracted cDNA probes.

Real-time RT-PCR confirmation

To confirm the results of SSH, four different clones that gave strong positive signals on dot-blot analysis were selected for real-time RT-PCR using specific sets of primers. Figure 3 showed results of RT-PCR. It was found that the mRNA levels of bfgf, sprouty 2, egr and btc in COCs were significantly higher than in NOs, respectively.

DISCUSSION

Suppression subtractive hybridization (SSH) has been widely used in the screening of differentially expressed

Table 2. Up-regulated genes differentially expressed in the cumulus-oocyte complexes

Size/bp	GenBank no.	Blast database no.	Gene symbol (name)	Identities (%)
832	YN576201	CB713942	Secreted frizzled-related sequence protein 2(sfrp2)	99
537	YN576202	XP0010764	Programmed cell death 1 (pdcd1)	93
476	YN576205	NM-367547	Lectin,galactose binding soluble 3 (lgbs3)	85
679	YN576206	XM480754	Cassette, sub-family B,member 1A (csfB1A)	89
573	YN576207	XP001869	Proliferating cell nuclear antigen (pcna)	91
405	YN576209	BX095685	Basic fibroblast growth factor (bfgf)	87
467	YN576210	BC080978	Regulator of chromosome condensation 1(rcc1)	81
546	YN576215	BC048698	cGMP dependent protein kinase II; cGKII (prkg2)	96
342	YN576217	AY767767	Betacellulin (btc)	92
437	YN576219	NM-789645	Sprouty 2	93
732	YN576221	NC-000643	A disintegrin and metalloproteinase 8 (adam8)	99
505	YN576223	NM-780957	Epiregulin (egr)	99
324	YN576225	CK458211	Bone morphogenetic protein 4 (bmp4)	91

Table 3. Down-regulated genes differentially expressed in the cumulus-oocyte complexes

Size/bp	GenBank no.	Blast database no.	Gene symbol (name)	Identities (%)
560	GY476595	AJ709867	Aquaporin 3 (aqp3)	99
583	GY476723	BC007252	Zona pellucida glycoprotein 2 (zp2)	99
254	YN576226	BC006454	Survivin (svn)	99
678	YN576227	BC025476	Ribosomal protein S3 (rps3)	95
357	YN576228	BC045763	Cyclin D2 (ccnd2)	83
545	YN576229	XP06898	Ig -binding protein 2 (Ighmbp2)	100
470	YN576231	NM-076567	Activated leukocyte cell adhesion molecule (aloam)	81
325	YN576242	NM-232854	Zinc finger and BTB domain containing 16 (zbtb 16)	93
446	YN576247	AR-282745	F-box protein 8 (fbp8)	93
321	YN576251	EF536546	Casein kinase 2, alpha 1 polypeptide (csnk2a1)	100

genes in developmental studies (Diatchenko et al., 1996). The method could be used to identify differentially expressed oviductal and embryonic genes or expressed sequence tags (ESTs) in various species (Robert et al., 2000; Zeng et al., 2003; Chen et al., 2009). Our study represents the first use of the SSH method to contrast the gene expression between cumulus oocyte complexes and naked oocytes in swine. Applying this method, we constructed a porcine subtracted cDNA library by the driver cDNA. Twenty three differentially expressed genes were confirmed by dot blot analysis and sequenced. It was believed that the bi-directional communication between oocytes and cumulus cells was essential for the development and function of ovarian follicles and promotes the production of mature oocytes competent to undergo fertilization. Therefore, in swine the detection of the differentially expressed genes during this event would assist to study molecular mechanisms of oocyte early development and maturation.

In this report, some known genes expressed in cumulus cells or cumulus oocyte complexes of ovarian follicle were isolated from the subtracted COCs cDNAs, including *bmp4*,

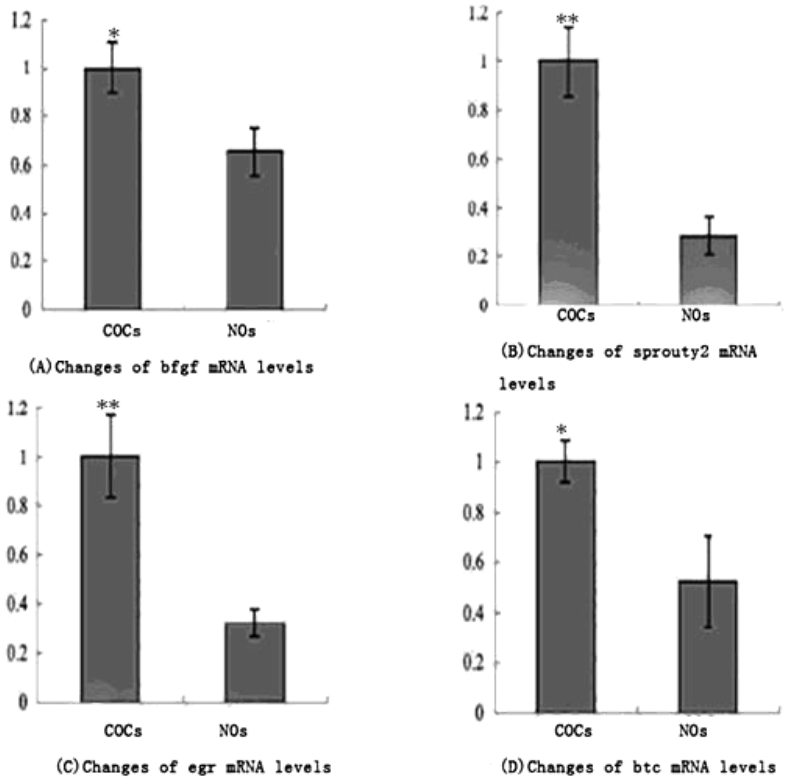

Figure 3. Comparison of four differentially expressed genes between cumulus oocyte complexes (COCs) and naked oocytes (NOs) using Quantitative real-time RT-PCR analysis. Relative mRNA expression level of (A) bfgf, Basic fibroblast growth factor; (B), sprouty 2; (C) egr, Epiregulin; and (D) btc, Betacellulin was presented by dividing the intensity of gene with GAPDH. Values are means± SD of three independent experiments. * $p<0.05$ and ** $p<0.01$ in comparison with NOs.

aqp3, *ccnd2* and *svn* gene. Previous study demonstrated the expression of BMP-4 in the various stages of porcine follicular development (Brankin et al., 2005). mRNA level of BMP-4 decreased significantly in oocytes during primordial-primary-secondary follicle transition in neonatal porcine ovaries, suggesting that BMP-4 may act as a survival factor for the follicle compartment cells (Shimizu et al., 2004). In addition, AQP-3 is a water/solute channel that can transport water and neutral solutes, involved in regulatory volume changes and osmoreception, in the mouse model, Aqp3 expression was highlighted to play a role in oocyte quality, with reduced Aqp3 expression associated with lower fertilization rates from stimulated oocytes (Meng et al., 2008). Ccnd2 is an important cell cycle regulator and plays an essential role in cumulus cell proliferation as a null mutation in ccnd2 in mice impairs cumulus cell proliferation and leads to small follicles unable to ovulate (Sicinski et al., 1996), it was reported that in humans c*cnd2* mRNA level increased significantly in COCs at 8-12 h and then decreased at 24h during culture (Aafke et al., 2008). It was reported that survivin (SVN) is an essential antiapopotic gene and is expressed in pre-implantation mouse embryos in all stages of development by inhibiting an apoptotic pathway involving caspases (Kawamura et al., 2003). In bovine, survivin mRNA was highly expressed in the GV oocyte, significantly decreased after fertilization and then gradually increased starting at the 8-cell stage (Kilsoo et al., 2008). The identification of these genes thus provides an important validation of the physiological model and the analytical techniques used herein.

Interestingly, in our study, two EGF-like family genes (*egr* and *btc*) and two fibroblast family genes (*sprouty2* and *bfgf*) were isolated from the subtracted COCs cDNAs. These genes account for about 17% (4/23) of our total differentially expressed genes by SSH, suggesting that the encoding proteins of these genes may exert important actions during folliculogenesis. Previous studies indicated that the epiregulin, as well as the betacellulin, were induced by LH in granulose cells and then, via a paracrine mechanism, activated EGF receptor (EGFR) present in cultured murine COCs (Pakarainen et al., 2005) and in follicle-enclosed rat oocytes (Ashkenazi et al., 2005). In addition, sprouty 2, as a modulator of signal transduction of fibroblast growth factor, found in chick embryo (Hacohen et al., 1998) played an important role in the development of cumulus cells (Khan et al., 2005). The identification of these genes thus provides an important validation of some physiological signing pathways, since EGF/FGF-like growth factors, as intermediates for LH-mediated induction of oocyte maturation by the activation of MAPK pathways downstream, have been shown to induce oocyte maturation and cumulus cell expansion (Conti et al., 2006).

Unfortunately, in our study, no indication was found for under-expression of BMP-15 or GDF-9, since cumulus expansion is necessary for a proper oocyte development (Zhang et al., 1995) and is induced by the oocyte factors BMP-15 and GDF-9 (Galloway et al., 2000; Elvin et al., 2000; Dragovic et al., 2007), there might be an oocyte factor other than BMP-15 or GDF-9 that induces cumulus expansion in pig. Our analyses revealed that specific genes associated with neuronal cell activity (prkg2) and immune cell functions (pdcd1, Ighmbp2 and adm8) are differentially expressed in COCs by NOs. Their specific functions in COC expansion and oocyte maturation remain to be defined.

The potential roles of the present reported genes and other novel genes in porcine reproductive physiology remain elusive. With the fragments of these genes in hand, our next step is to characterize these genes and study their roles on oocyte and follicular development.

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