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Identification of Stage-specific Genes Related to Porcine Folliculogenesis

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ABSTRACT

Although assisted reproductive technology is very useful to develop novel and therapeutic biomaterials for reproduction, research on molecular mechanism of folliculogenesis in pig is not clear. Therefore, the alteration of gene expression during follicular development in pigs was examined in this study. The expression of folliculogenesis-related genes was quantified in preantral (250～300 μm) and antral (>300 μm in diameter) follicles, and overall gene expression was evaluated by a genome-wide microarray. The microarray results showed that 219 genes were differentially expressed, and of those, 10 and 22 known genes showed higher and less expression at the preantral stage than at antral stages, respectively. Among them, the expression of *NR0B1*, *PPARG*, *GATA4*, and *ANXA2* genes related to folliculogenesis was validated by quantitative real-time PCR analysis. The expression of *PPARG* and *GATA4* genes were increased at antral stages, but a significantly stage-specific increase ($p<0.05$) was only detected in annexin A2 (*ANXA2*) in antral-stage follicles. The expression of *NR0B1* genes was increased at preantral stage and these patterns of gene expression were comparable to the results obtained by microarray analysis. We propose that the systematical regulation of genes supporting specific follicle stage should be employed for improved *in-vitro* folliculognesis.

(Key words : Antral follicles, Gene expression, Ovary, Preantral follicles)

INTRODUCTION

Assisted reproductive technology for pigs is very useful to produce large amounts of biomaterials. *In vitro* culture of ovarian preantral follicles can be utilized to generate large number of viable oocytes, which would contribute to overcome the limitation of a conventional follicle aspiration technique. To develop an *in vitro* porcine folliculogenesis technology, various interactions between follicular somatic cells and growing oocytes have been identified (Wu *et al*., 2002; Mao *et al*., 2004; Hashimoto *et al*., 2007) and the identification of genes expressed during follicular growth is also required to understand the biological regulation of porcine folliculogenesis (Eppig, 2001; Mao *et al*., 2002; Drummond, 2006; Hashimoto *et al*., 2007; Bonnet, 2008). However, there have been no reports related with gene profiles identified during porcine folliculogenesis. Accordingly, in this study, the microarray was used to elucidate the overall difference in gene expression between the two stages of growing follicles; preantral and antral follicles. Subsequently, several novel genes related to folliculogenesis were investigated in preantral and antral follicles.

MATERIALS AND METHODS

Collection of Ovarian Follicles

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Ovaries were collected from 4 to 5-month-old cross-bred Duroc × Landrace × Yorkshire prepubertal gilts at a local slaughterhouse and transported to the laboratory in Dulbecco's phosphate-buffered saline (DPBS) (Gibco Invitrogen, Carlsbad, CA) supplemented with antibiotics. The ovarian cortices were cut into small pieces (2~5 mm^2), and the pieces were placed in a 60-mm petri dish containing 5 ml Dulbecco's modified Eagle's medium (DMEM) (Gibco Invitrogen) supplemented with 285 collagenase digestion units/ml of collagenase type IV (Sigma-Aldrich Corp., St. Louis, MO). Ovarian follicles were collected after a 1-hour treatment and classified into preantral and antral stages according to diameters.

Generation of Affymetrix Chip Data and Analysis of Microarray

For the microarray, 702 follicles were obtained from three replicates, and 107 preantral and 127 antral follicles were allotted to each replicate. Total RNA was extracted using the PicoPure RNA Isolation kit (Arcturus, Mountain View, CA), and extracted RNA was subjected to two rounds of linear amplification using the RiboAmp™ RNA amplication Kit (Arcturus, Mountain View, CA) (Feldman *et al*., 2002; Pukas *et al*., 2002; Wang *et al*., 2003). Gene expression profiling of each stage was performed using a Genechip™ (AFFYMETRIX, Santa Clara, CA) porcine microarray chip (Bolstad *et al*., 2003; Gautier *et al*., 2004). A pseudo-method (Oh *et al*., 2007) was conducted to overcome robustness of outliers or skewness of data, and then linear models for microarray data (Smyth, 2004) applied to compare individual gene expression levels between preantral and antral follicle data. A *P*-value of 0.05 was considered significant. The data of microarray analysis was annotated with the DAVID tool at the NIAID server (http://david.abcc.ncifcrf.gov/).

Analysis of the Relative mRNA Levels using Real-Time PCR

Isolated follicles were transferred into RNA later™ (Ambion, Austin, TX), and total follicular mRNA was extracted using an RNeasy™ Mini Kit (Qiagen, Valencia, CA). The cDNA was synthesized from total mRNA using the SuperScriptTM III First-Strand Synthesis System for RT-PCR (Gibco Invitrogen), and the expression of five genes related to folliculogenesis in other species (*NR0B1, ANXA2, PPARG,* and *GATA4*) was quantified by real-time PCR (ABI PRISM 7700; Applied Biosystems, Foster, CA) using the DyNAmo HS SYBR Green qPCR Kit (FINNZYME, Espoo, Finland). The real-time PCR was performed for 40 cycles at 95℃ for 30 seconds, 60℃ for 30 seconds, and 72℃ for 30 seconds. All experiments were replicated three times and normalized to the GAPDH gene. The primer sequences are shown in Table 1.

Statistical Analysis

All experiments replicated three times and a generalized linear model (PROC-GLM) in a Statistical Analysis System (SAS; Cary, NC) program was used for evaluating model effects of each treatment. When analysis of variance (ANOVA) in the SAS package detected a statistical significance, each value of the treatments was subsequently compared using the least square method. Significant differences among treatments were determined where the p value was less than 0.05.

RESULTS AND DISCUSSION

After treating porcine ovaries with collagenase, dissociated ovarian follicles were classified with diverse diameters and morphology. Whereas preantral follicles, 250~300 μm in diameter, were spherical in shape with multilayers of granulosa cells (Fig. 1A), follicles with > 300 μm in diameter were regarded as antral stage starting antrum formation (Fig. 1B). Subsequently, preantral or antral stage follicles were allocated to the following microarray analysis.

Among total 20,201 genes, 120 and 99 genes were differentially up-regulated at the preantral and antral stages, respectively. This result indicates that follicles at

Table 1. Oligonucleotide primer sequence for the relative quantification of gene mRNA by real-time PCR analysis

Gene	GeneBank_ID	Sense	Anti-sense
ANXA2	AY706383	aaaggacatcatttccgaca	tccacttgggaacatcagtt
PPARG	DQ437885	aggtttgctgaatgtgaagc	gctcatgtccgtctctgtct
GATA4	AY115491	aaagaggggattcaaaccag	tgagaaggtctgggacagag
NR0B1	NM_214387	tcagcagatccttagcgaac	actttgcacagagcatttcc
GAPDH	U48832	gtccactggtgtcttcacga	gtcatgagtccctccacgat

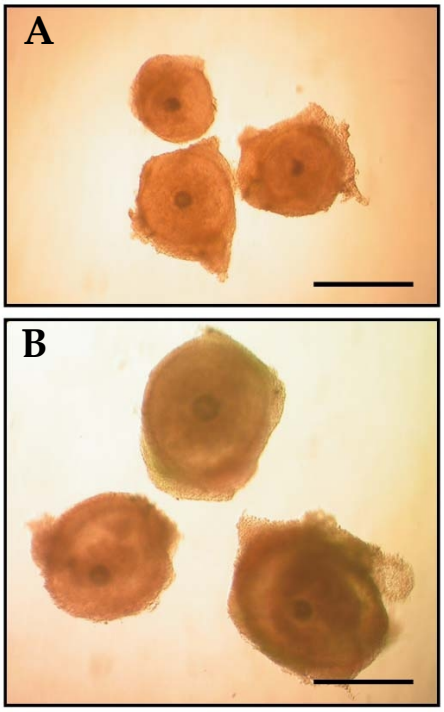

Fig. 1. Morphology of the porcine ovarian follicles used for gene expression quantification and microarray analysis. The preantral follicles were retrieved by collagenase treatment. (A) Preantral follicles (250~300 μm in diameter) were spherical in shape with multilayers of granulosa cells. (B) Antral follicles (>300 μm in diameter) had started antrum formation. Scale bar=300 μm.

these two stages have a similar gene expression profile, except for 219 genes of total 20,201 genes. When considering the genes either annotated or with known function, 10 genes (*AMCFII, RPLP1, RPL34, LOC733606, SG-2304, CDKN1B, CDH1, NR0B1, EF1ALPHA,* and *DNMT1*) and 22 genes (*SPP1, HSP70, PLAT, ATPB1, UF, C1S, GATA4, CTSH, ST3GAL4, IREB2, MMP2, LOC39-6849, HMGB1, LOC100157633, RPN2, ANXA2, B2M, RH, PPARG, VDAC1, CRNNB1,* and *MPCP-PB*) were up-regulated in preantral and antral follicles, respectively (Table 2).

Subsequently, four genes (*NR0B1, ANXA2, PPARG* and *GATA4*), which play an important role in *in-vivo* folliculogenesis (Salmon *et al*., 2005; Yang *et al*., 2008; Hara *et al*., 2011), were further analyzed for identifying genes specifically up-regulated in the preantral or antral stage follicles. All folliculogenesis-related genes evaluated were expressed at both stages of follicles, but a significant (p=0.0342) difference was only detected in *ANXA2* expression (Fig. 2). A tendency for transcriptional up-regulation in preantral follicles was observed in the *NR0B1*, whereas *PPARG* and *GATA4* expression tended to be increased at the antral stage.

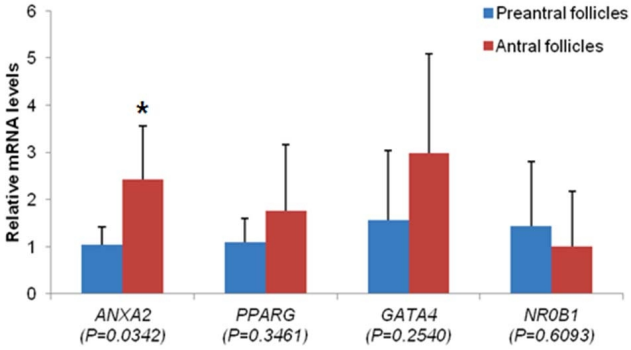

Fig. 2. Quantification of gene expression in porcine preantral and antral follicles by real-time PCR. Nuclear receptor subfamily (*NR-0B1*) gene was up-regulated in preantral compared to antral follicles. Increased expression of annexin A2 (*ANXA2*) gene was detected in antral compared to preantral follicles (* p<0.05). Expression of peroxisome proliferation-activated receptor gamma 2 (*PPARG*), and transcription factor gata-4 (*GATA*) genes appeared to be higher in antral compared to preantral follicles.

Transcriptional up-regulation of *NR0B1* in preantral follicles is synchronized with the previous reports that *NR0B1* regulates a steroidogenesis in ovarian granulosa and luteal cells (Yang *et al*., 2009) and is essential for maturation and ovulation of preantral follicles (Duggavathi *et al*., 2008). Moreover, in the antral follicles, transcriptionally up-regulated *PPARG*, being a member of the nuclear receptor superfamily (Grindflek *et al*., 1998) expressed in granulosa cells (Banerjee and Komar, 2006) and regulating survival of granulosa cells (Lovekamp-Swan and Chaffin, 2005), emphasize the importance of proliferation, activities and functions of granulosa cells in the formation of follicles with typical antral stage structure, with transcriptional up-regulation of *GATA4* used as a granulosa cell-specific marker at the antral follicle stage in various sizes (Gillio-Meina *et al*., 2003; Bocca *et al*., 2008). Finally, data on *ANXA2* expression demonstrate the regulatory role of epidermal growth factor (EGF) in porcine folliculogenesis. *ANXA2* was firstly discovered as a target for tyrosine phosphorylation by the EGF receptor (Emans *et al*., 1993; Danielsen *et al*., 2003) and EGF is also an intrafollicular regulator related to follicle atresia and suppresses apoptosis in follicular cells (Chun *et al*., 2001). These results suggest that expression of *PPARG, GATA4*, and *ANXA2* genes is useful for an indicator of antral follicles and for growth and maturation of follicular cells.

In conclusion, significant increase of *ANXA2* expression was detected in the development of preantral follicles into antral follicles, indicating that *ANXA2* might play a very important role in the antral follicle formation. Simultaneously, increase of *PPARG* and *GATA4*, and decrease of *NR0B1* expression during antral stage follicle development indicate that better folliculogenesis

Table 2. List of differentially expressed genes in preantral or antral follicles using the DAVID Gene ID conversion tool

More expression in preantral than in antral follicles			
Gene ID	Gene name	Symbol	Gene annotation
Ssc.719	Alveolar macrophage-derived chemotactic factor-II	*AMCFII*	Protein binding, Binding
Ssc.791	60s acidic ribosomal protein p2	*RPLP1*	Intracellular organelle, Intracellular part, Macromolecule biosynthetic process, Organelle, Gene expression, Cellular biosynthetic process, Translation
Ssc.803	60s ribosomal protein L34	*RPL34*	Intracellular organelle, Intracellular part, Macromolecule biosynthetic process, Organelle, Gene expression, Cellular biosynthetic process, Translation
Ssc.4122	Thymosin β-4	*LOC733606*	Intracellular organelle, Intracellular part, Organelle
Ssc.6139	Tryptophanyl-tRNA synthase	*SG2304*	Macromolecule biosynthetic process, Gene expression, Cellular biosynthetic process, Translation
Ssc.6966	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	*CDKN1B*	Intracellular organelle, Intracellular part, Organelle
Ssc.14500	Nuclear receptor subfamily 0, group B, member 1	*NR0B1*	Protein binding/localization, Macromolecule localization, Cellular process, Intracellular part, Cellular macromolecule/Primary metabolic process, Nucleic acid binding, Intracellular organelle, Regulation of biological process, Intracellular membrane-bounded organelle
Ssc.24344	DNA methyltransferase 1	*DNMT1*	Intracellular organelle, Intracellular part, Organelle, Gene expression
More expression in antral than in preantral follicles			
Gene ID	Gene name	Symbol	Gene annotation
Ssc.101	Secreted phosphoprotein 1	*SPP1*	Signal
Ssc.114	Heat shock protein 70	*HSP70*	Cellular macromolecule/protein metabolic process, Hydrolase activity, Primary/cellular metabolic process
Ssc.196	T-plasminogen activator	*PLAT*	Cellular macromolecule/protein metabolic process, Primary/cellular metabolic process, Peptidase activity, Protease, Signal, Proteolysis
Ssc.246	ATPase, Na^+/K^+ transporting, β1 polypeptide	*ATP1B1*	Hydrolase activity
Ssc.575	Uteroferrin	*UF*	Hydrolase activity, Signal
Ssc.1177	Complement component c1s	*C1S*	Cellular macromolecule/protein metabolic process, Hydrolase activity, Primary/cellular metabolic process, Peptidase activity, Protease, Positive regulation of biological process, Signal, Proteolysis
Ssc.3566	Transcription factor ε-4	*GATA4*	Zinc finger, NHR/GATA-type, Primary/cellular metabolic process, Positive regulation of biological process
Ssc.3593	Cathepsin H	*CTSH*	Cellular macromolecule/protein metabolic process, Hydrolase activity, Primary/cellular metabolic process, Peptidase activity, Protease, Signal, Proteolysis
Ssc.4387	ST3β-galactoside α2,3-sialyltransferase	*ST3GAL4*	Cellular macromolecule/protein metabolic process, Organelle membrane, Biopolymer glycosylation, Protein amino acid glycosylation, Primary/cellular metabolic process, Glycoprotein biosynthetic/metabolic process
Ssc.5713	Gelatinase a	*MMP2*	Cellular macromolecule/protein metabolic process, Hydrolase activity, Primary/cellular metabolic process, Peptidase activity, Protease, Proteolysis
Ssc.7539	High-mobility group box 1	*HMGB1*	Primary/cellular metabolic process
Ssc.10822	Eukaryotic elongation factor 1 γ-like protein	*LOC1001-57633*	Cellular macromolecule/protein metabolic process, Primary/cellular metabolic process
Ssc.11153	Ribophorin II	*RPN2*	Cellular macromolecule/protein metabolic process, Organelle membrane, Biopolymer glycosylation, Protein amino acid glycosylation, Primary/cellular metabolic process, Glycoprotein biosynthetic/metabolic process, Signal
Ssc.12241	Annexin A2	*ANXA2*	Extracellular region, Cell/Intracellular part, Binding, Intracellular organelle, Cytoplasm, Membrane bound organelle, Calcium ion binding
Ssc.12809	β2-microglobulin	*B2M*	Signal
Ssc.14472	Rh protein	*RH*	Cell, Integral/Intrinsic to membrane, Transporter activity, Transmembrane, Establishment of localization
Ssc.14475	Peroxisome proliferator-activated receptor γ 2	*PPARG*	Zinc finger, NHR/GATA-type, Primary/cellular metabolic process, Positive regulation of biological process
Ssc.16732	Voltage-dependent anion channel 1	*VDAC1*	Organelle membrane

may require systematical regulation of genes supporting development into specific follicle stage. Furthermore, identification of follicle stage-specific gene networking will help us to develop an efficient *in vitro* porcine folliculogenesis protocol by manipulating niche stimulating these gene systems.

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