Gene Expression Analysis of Pregnant Specific Stage in the Miniature Pig Ovary

Seong-Jo Yun, Won-Gun Noh, Jong-Taek Yoon and Kwan-Sik Min†

Animal Biotechnology, Graduate School of Bio. & Information Technology, Institute of Genetic Engineering, Hankyong National University, Ansung 456-749, Korea

ABSTRACT

The miniature pig is considered to be a better organ donor breed for xenotransplantation than other pig breeds because the size of the organs of the miniature pig is similar to that of humans. In this study, we aimed at identifying differentially expressed genes in the miniature pig ovary during pregnancy. For this, we used the miniature pig ovary model, annealing control primer-based reverse transcription polymerase chain reaction (PCR), quantitative real-time PCR (qRT-PCR), and northern blotting analysis. We identified 13 genes showing differential expression on the based of pregnancy status and validated 8 genes using qRT-PCR. We also sequenced the full-length cDNA of ephrin receptor A4 (EphA4), which had a significant difference in expression level, and validated it by northern blotting. These genes may provide a better understanding of the cellular and molecular mechanisms during pregnancy in miniature pig ovary.

(Key words : Differentially expressed gene (DEG), Ovary, Ephrin receptor A4)

INTRODUCTION

Somatic cell nuclear transfer (SCNT) is a useful tool for producing genetically modified pigs. In recent years, the miniature pig has been regarded as a better organ donor breed for xenotransplantation than other pig breeds because the size of its organs is similar to that of humans (Koo *et al.,* 2009). Also, miniature pigs are smaller and easier to handle than common domestic pigs, and they have been used in a variety of fields, such as medical and pharmacological research (Lai *et al.,* 2002). However, less information regarding the production of cloned and genetically modified pigs is available for miniature pigs compared to that available for common domestic pigs (Dor *et al.,* 2004). Also, abortion is observed at various stages of pregnancy after transfer of cloned embryos from somatic cells to recipients (Tsunoda and Kato, 2000).

These have been studied in pigs, but detailed molecular mechanisms in the miniature pig remain poorly understood. To better understand the genetic and physiological process of the miniature pig ovary, we used a gene expression approach to identify differences in the ovarian transcriptome in relation to pregnancy status. Hormonal interactions of the hypothalamic-pituitary-ovarian-uterine axis account for normal reproduction in female pigs (Madej *et al.,* 2005). These hormonal events are tightly regulated by steroid hormones such as estrogen and progesterone from the ovaries (Bazer *et al.,* 1998).

The purpose of this study was to use annealing control primer-based reverse transcription polymerase chain reaction (ACP RT-PCR), and quantitative real-time PCR (qRT-PCR) to determine the differential expression of genes and to sequence isolated genes in the miniature pig ovary during pregnancy.

MATERIALS AND METHODS

Animals and Tissue Sampling

Ovariectomy of miniature pigs was performed at 0 (non-pregnancy), 30, 60, and 110 days of pregnant, using one animal at each time.

RNA Extraction and First Strand cDNA Synthesis

Total RNA was extracted from ovary tissue samples using Trizol reagent (Invitrogen, Grand Island, NY, USA), according to the manufacturer's instructions. Total RNA was used for the synthesis of first-strand cDNAs by reverse transcriptase. Reverse transcription was performed for 1.5 h at 42℃ in a final reaction volume of 20 μl containing 3 μg of the purified total RNA, 4 μl of 5 × reaction buffer (Promega, Madison, WI, USA), 5 μl of dNTPs (each 2 mM), 2 μl of 10 μM dT-ACP1 [5′-CTGTGAATGCTGCGACTACGATIIIIIT(18)-3′], 0.5 μl

† Corresponding author : Phone: +82-31-670-5421, ksmin@hknu.ac.kr

of RNasin® RNase Inhibitor (40 U/μl; Promega), and 1 μl of Moloney murine leukemia virus reverse transcriptase (200 U/μl; Promega). First-strand cDNAs were diluted by the addition of 80 μl of ultra-purified water for the GeneFishingTM PCR, and stored at −20 ℃ until further use.

ACP-based Gene Fishing PCR

Differentially expressed genes were screened by an ACP-based PCR method (Kim *et al.*, 2004) using the GeneFishing differentially expressed gene (DEG) kits (Seegene, Seoul, South Korea). Briefly, second-strand cDNA-synthesis was conducted at 50℃ during one cycle of first-stage PCR in a final reaction volume of 20 μl containing 3~5 μl(about 50 ng) of diluted first-strand cDNA, 1 μl of dT-ACP2 (10 μM), 1 μl of 10 μM arbitrary ACP, and 10 μl of 2×Master Mix (Seegene). The PCR protocol for second-strand synthesis was one cycle at 94℃ for 1 min, followed by 50℃ for 3 min, and 72℃ for 1 min. After second-strand DNA synthesis was completed, the second-stage PCR amplification protocol was 40 cycles of 94℃ for 40 s, followed by 65℃ for 40 s, 72℃ for 40 s, followed by a 5 min final extension at 72℃. The amplified PCR products were separated in 2% agarosegel stained with ethidiumbromide.

Cloning and Sequencing of DEGs

Differentially expressed bands were extracted from the gel by using the GENECLEAN II Kit (Q-BIO gene, Carlsbad, CA, USA), and were directly cloned into a TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. The cloned plasmids were sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), using an M13 forward primer (5'-CGCCAGGGTTTTCCCAGTCACGA-3') and M13 reverse primer (5'-AGCGGATAACAATTTCACACAGGA-3').

Quantitative Real-Time PCR

qRT PCR was performed in triplicate in 384-well plates. A 384-well high-throughput analysis was performed by using the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, www.appliedbiosciences.com) and whit-colored 384-well plates (ABgene, Hamburg, Germany) for intensification of the fluorescent signals by a factor of 3. The system operates using a thermal cycler and a laser that is directed via fiber optics to each of the 384 sample wells. The fluorescenceemission from each sample was collected by a charge-coupled device-camera, and the quantitative data were analyzed using the Sequence Detection System software (SDS version 2.0, PE Applied Biosystems). Reaction mixtures contained 10 pmol/ul of each primer and 2x SYBR Green PCR Master Mix (PE Applied Biosystems), which includes HotStarTaqt DNA polymerase in an optimized buffer, the dNTP mix (with dUTP additive), SYBRs Green I fluorescent dye, and ROX dye as a passive reference.

Each of the 384-well qRT-PCR plates included serial dilutions (1, 0.5, and 0.25) of cDNA, which were used to generate relative standard curves for genes. All primers were amplified using the same conditions. Thermal cycling conditions were 50℃ for 2 min and 95℃ for 10 min, followed by 40 cycles of 95℃ for 30 s, and 60℃ for 30 s, and 72℃ for 30 s. To exclude the presence of unspecific products, a melting curve analysis of products was performed routinely after finishing amplification by high-resolution data collection during an incremental temperature increase from 60℃ to 95℃ with a ramp rate of 0.21℃ /s. We then converted real-time PCR cycle numbers to gene amounts (ng) on the basis of the equation. Real-time PCR analysis was performed on an Applied Biosystems Prism 7900 Sequence Detection System.

Northern Blot Analysis

Total RNAs (20 μg) from different stages of pregnancy were loaded in each lane and electrophoresed on a 1% morpholinepropanesulfonic acid-formaldehyde agarose gel. RNA was transferred overnight onto a nylon membrane in 20× saline sodium citrate (SSC). The cDNA probe was labeled with digoxigenin (DIG)-UTP using a DIG DNA labeling kit (Roche, Indianapolis, IN, USA). After transfer, the RNA was fixed to the blot by baking at 120℃ for 30 min. Prehybridization (30 min) and hybridization were carried out overnight at 68℃ using PerfectHyb® hybridization solution (Toyobo, Japan). The blot was washed in low stringency buffer [2 × SSC and 0.1% sodium dodecyl sulfate (SDS)] twice for 5min each. The blot was washed in high stringency buffer (0.1 × SSC and 0.1% SDS) 2 times for 15 min each at 68℃. After stringency washing, the blot was visualized by alkaline phosphatase reaction (Roche) and exposure to X-ray film (Kodak, Rochester, NY, USA).

Molecular Cloning of Ephrin Receptor A4

Porcine ovary cDNA was used as a template for PCR to obtain full-length cDNA for ephrin receptor A4 (EphA-4). We designed primers in conserved regions from expressed sequence tag (EST) sequences, and PCR was carried out using the Takara LA Taq Polymerase kit and 10 pmol of both forward and reverse primers, as per the manufacturer's instructions (Takara). The PCR product was cloned into a pCR® 2.1-TOPO® vector (Invitrogen) and transformed into DH5α™-T1^{R} (Invitrogen). cDNA inserts were sequenced with the ABI PRISM 3100 Genetic. Web-based basic local alignment search tool (BLAST) searches were performed against the

GenBank.

RESULTS

Identification of Differentially Expressed Genes in the Ovary at the Different Stages of Pregnancy

To identify DEGs in the ovary at different stages of pregnancy, we performed ACP RT-PCR analysis using a combination of 120 arbitrary ACP primers and 2 oligo dT primers (dT-ACP1 and dT-ACP2). From the analysis, we selected 13 PCR products showing differential expression on the basis of pregnancy status. Among them, expression of 6 genes (DEG 1, 3, 5, 8, 9, and 11) was higher in pregnant sows (days 30, 60) than that in the non-pregnant sow (day 0) (Fig. 1).

The expression of the other 6 genes (DEG 4, 6, 7, 12, 13 and 14) was higher in the non-pregnant sow (day 0) than in that in the pregnant sows (day 30, 60) (Fig. 1). Finally, the expression level for DEG 16 was higher in the pregnant sow (day 30) than in the non-pregnant (day 0) and pregnant (day 60) sows (Fig. 1).

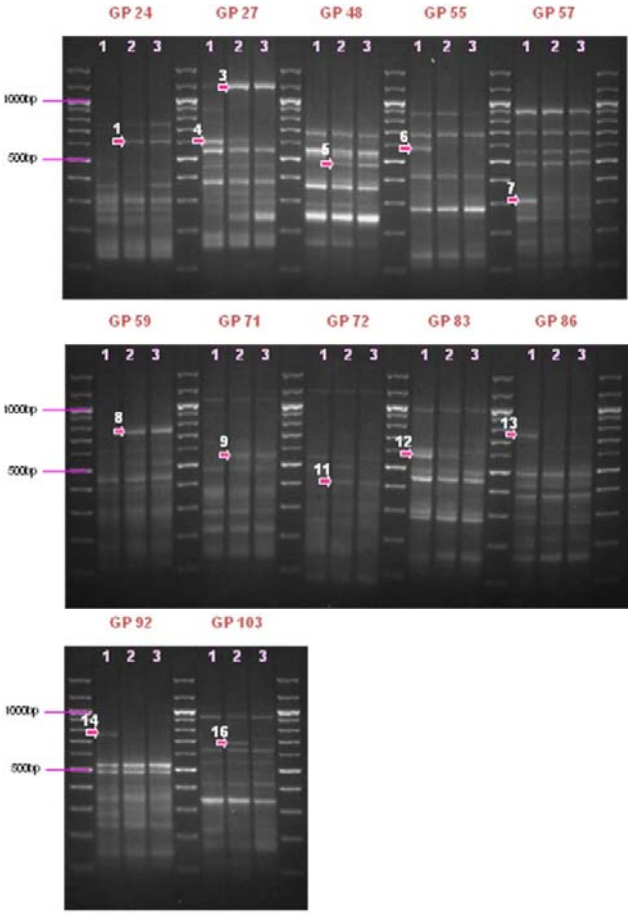

Fig. 1. Images of DEGs in the miniature pig ovary on Day 0 (1), Day 30 (2) and Day 60 (3) of pregnancy amplified by ACP RT-PCR using arbitrary ACP primers. Arrows indicate the PCR products of DEGs. The 13 genes were screened for cloning and sequencing.

Cloning, Sequence Analysis and BLSAT Search of Differentially Expressed Genes

We cloned the PCR products into the PCR 2.1 vector. All of these differentially expressed clones were then analyzed by cDNA sequencing and BLAST search of the GenBank database was conducted with the National Center for Biotechnology Information server. The DEGs were identified as DEG 1 (EPHA4), DEG 3 (transmembrane emp 24 transport; TMED3), DEG 4 (sulfotransferase family cytosolic 1A, phenol-preferring, member 1; SULT1A1), DEG 6 (lg α heavy chain constant region; Ig α C), DEG 8 (selenium binding protein), DEG 11 (neuron specific gene family member 1; nsg), DEG-12 (lg α heavy chain constant region : Ig α C), DEG 13 (keratin; KRT), and DEG 14 (Ig γ 2a constant region; IgG2a), while DEG 5, DEG 7, DEG 9, and DEG 16 were unknown genes (Table 1).

Validation of Differentially Expressed Genes by Real-Time RT-PCR

Real-time RT-PCR analysis was performed on ovary tissue to confirm the differential expression of the selected cDNA fragments and to assess validation of mRNA levels of 8-selected DEG clones. Primers were designed against 8 of the genes to examine differential expression (Table 2). The normalization was performed with the housekeeping gene, glyceraldehydes-3-phophate dehydrogenase (*GAPDH).* Among the 8 clones that were validated for ovary tissue, 2 clones (D01, D08) showed higher mRNA levels as the pregnancy progressed (days 30, 60 and 110) (Fig. 2). Clones D04, D07, D09, and D16 showed lower mRNA levels as the pregnancy progressed (Fig. 2). The mRNA level of one clone (D11) was not significantly different during the pregnancy stages (Fig. 2). Interestingly, clone D13 only showed a significantly higher mRNA level on day 60 of pregnancy (Fig. 2).

Nucleotide Sequence and Northern Blot Analysis of EphA 4

Bovine EphA 4 cDNA was cloned by step by step PCR method by using 1st strand cDNA synthesized from bovine ovary. The nucleotide sequence of the 2,961-bp insert was determined. We identified an open reading frame of a 986-amino-acid polypeptide. The nucleotide sequence determined in this study has been deposited in the GenBank databases and assigned accession number NM_001134967. Bovine EhpA 4 cDNA display high homolog to those reported for human (92 %), mouse (89%), rat (89%), and chicken (85%). The ex-

Table 1. Gene name abbreviation and NCBI accession No. by DEG sequence

DEG no.	GenBank accession no	Gene name	Gene abbreviation	Size (bp)
1	DY432233.1	EPH receptor A4 (EPHA4), mRNA	EPHA4	536
3	DN115943.1	Transmembrane emp 24 transport	TMED3 P24B	1,116
4	AY193893.1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	SULT1A1	530
5	AJ941488.1	Unknown	-	392
6	SSU12594	Immunoglobulin alpha heavy chain constant region	IgA C alpha	498
7	DV228333.1	Unknown	-	191
8	DN103014.1	Selenium binding protein	Selenbp2	680
9	DN120261.1	Unknown		466
11	AY609871.1	Contig1	Contig1	328
12	SSU12594	Immunoglobulin alpha heavy chain constant region	IgA C alpha	499
13	DN107773.1	Keratin	KRT	638
14	U03779.1	Ig gamma 2a constant region	IgG2a	685
16	CK460775.1	Unknown	-	611

Table 2. Sequence of forward and reverse primers for genes used in qRT-PCR

Gene name	Forward primer sequence	Reverse primer sequence	Size
D01	GCA GAA GGA GAG AAG CGA CA	GTC CTC TTC AAG CTG TTG GG	90 bp
D04	CAC ACG TCC TTC CAG GAG AT	CCA GTG ATG CCT TTC CTC AT	110 bp
D07	GGG AAG GTC ACG GTC AAG TA	CTT CCT GGC AGT CGT AGA GG	103 bp
D08	TTG GAG GAC CAG GAG CTA AA	ATC GAT CTG CAG CAT CAC AG	195 bp
D09	TTG AAC AAG ACT CCG TGC TG	TGT GAT CAG GAG GGT GAG TG	101 bp
D11	CTT AGC GAG ATG GGC AGG T	CAA AGC AGC ATT AGC AAA CAG A	143 bp
D13	ACC AGC GTG CTA AGT TGG AG	GTA GGT GGC GAT CTC GAT GT	193 bp
D16	TCA ACT CAT TTG GAC ACC ACA	TTC CAC ATG TCC CAA CCT TT	118 bp

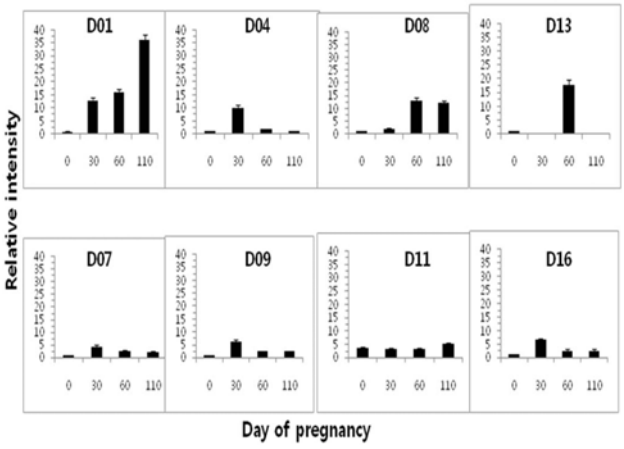

Fig. 2. Levels of mRNA for DEGs by qRT-PCR reaction. All expression levels given are relative to GAPDH (normalized).

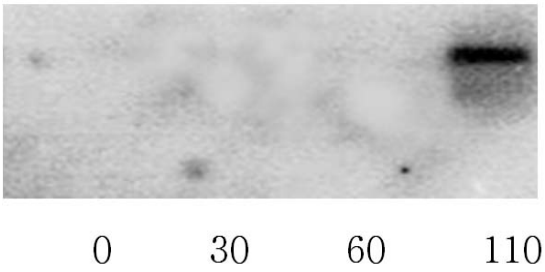

Fig. 3. Northern blot hybridization analysis of EphA4 in the miniature pig ovary on day 0, 30, 60, and 110 of pregnancy.

pression patterns of EphA4 mRNAs in different pregnancy stages were validated by northern blotting. Expression of EphA4 mRNAs was detectable only from day 110 of pregnancy (Fig. 3).

DISCUSSION

In this study, we isolated novel and unknown cDNA differentially expressed in the ovary tissue of miniature pigs at day 0 (non-pregnancy), 30, and 60 of gestation. Miniature pig ovary was taken additionally on day 110 of the pregnancy to perform real-time RT-PCR analysis. Using ACP RT-PCR, we identified 13 genes that were differentially expressed depending on pregnancy status (Fig. 1) (Table 1). The ACP RT-PCR method involves ACPs consisting of a tripartite structure with a 30-end target core sequence, a polydeoxyinosine linker, and a 50-end non-target universal sequence (Hwang *et al.*, 2003). Application of this method to the discovery of DEGs generates highly reproducible, authentic, and long PCR products because of the high annealing specificity (Kim *et al.*, 2004). Although this technique is much less complete and global than others, it is simple, easy, reliable and cost-efficient for identifying DEGs (Ka *et al.*, 2009). Accordingly, we used ACP RT-PCR to profile genes differentially expressed in the miniature pig ovary tissues. Real-time RT-PCR analysis was performed to assess validation on mRNA levels of 8 selected DEG clones (Fig. 2). The real-time RT-PCR approach is well suited for validation of differential expression since it is quantitative and rapid and requires 1000-fold less RNA than conventional assays (Mangalathu *et al.*, 2001).

We detected differences in EphA4 (DEG 01) in the ovary of the pregnant miniature pig compared with that of the non-pregnant animals. EphA4 was measured using qRT-PCR analysis. It showed significantly increased expression as pregnancy progressed (Fig. 2); however, the expression patterns of EphA4 mRNAs validated by northern blotting were detectable only from day 110 of pregnancy (Fig. 3); these were not detectable during other terms (days 0, 30, and 60), possibly because of low levels of ovary expression. Although there is no previous evidence of EphA4 and its function in miniature pig ovary during pregnancy, porcine corpora luteas produce angiogenic factors throughout the estrous cycle and early pregnancy (Reynolds *et al.*, 1992; Ricke *et al.*, 1995), indicating that corpora luteas produce an angiogenic factor(s) at all stages of luteal development. EphA4 is a receptor tyrosine kinase and a class A type receptor. It is a member of the ephrin family and binds both class A and class B ephrins (Frisen *et al.*, 1999). Eph receptors and ephrins have emerged as essential regulators of angiogenesis *in vivo*, through mediating interactions between cells that contact each other (Adams *et al.*, 1999; Gerety *et al.*, 1999; Wang *et al.*, 1998). Moreover, EphA4 plays an important role in blood vessel organization and regulates new vessel formation and guidance (Yona *et al.*, 2006). Accordingly, we suggest that EphA4 might induce angiogenesis in the miniature pig ovary as pregnancy progresses. The GenBank accession number for the complete coding region sequences of EphA4 reported in this paper is NM_001134967.

Sulfotransferase (SULT) 1A1 enzyme, DEG 04 (Table 1), is a member of the sulfotransferase family that alters biological activities of numerous carcinogenic and mutagenic compounds through sulfation. There was a decrease in expression levels of SULT1A1 in the miniature pig ovary as pregnancy progressed (Fig. 2). Since estrogen may modify the activity of SULT1A1 enzymes (Yunfei *et al.*, 2002), it is possible that SULT1-A1 is associated with estrogen. Selenium-binding proteins have been implicated in cell-growth regulation, intra-Golgi protein transport, and lipid metabolism (Serguei *et al.*, 2008). SELENBP2 was shown to bind selenium and acetaminophen, but the exact physiological role of this protein has not been well characterized (Papp *et al.*, 2007). In this study, SELENBP2, DEG 8 (Table 1), expression tended to be higher in the miniature pig ovary as pregnancy progressed (Fig. 2). Thus, we believe that the expression of SELENBP2 mRNA could be associated with binding of selenium during the pregnancy period.

Interestingly, clone DEG 13 showed a significantly higher only on day 60 of pregnancy (Fig. 2). Clone DEG 13 showed high homology with *Sus scrofa* keratin (Table 1). Keratins, a complex family of proteins composed of 20 members in humans (Ramaekers *et al.*, 1992), have a specific distribution pattern in normal epithelia, which is often retained in neoplasms derived from them (Carla *et al.*, 1995). Keratins have been previously identified to be involved in ovarian epithelial cell biology (Auersperg *et al.*, 2001). Although their roles in the ovary are still being investigated, results indicate that clone DEG 13 are expressed on day 60 in ovarian tissues and represent promising targets for a pregnancy specific gene. However, further investigation is required to determine whether clone D13 has a role in the regulation of ovarian function during mid-pregnancy. Clone DEG 11 showed high homology with *Sus scrofa* Contig1 (Table 1). Although expression of clone D11 was detected during pregnancy, its mRNA level was not significantly different during the pregnancy stages (Fig. 2). Because of the lack of information for genes expressed in the miniature pig ovary during pregnancy, attempts to explain Contig1 would only be speculative. However, Contig1 may be pivotal during pregnancy.

Finally, DEG 7, DEG 9, and DEG 16 showed no similarity with known GenBank database entries (Table 1). The expression level of these clones tended to decrease in the miniature pig ovary as pregnancy progressed (Fig. 2). Accordingly, molecular cloning for the-

se unknown genes and their functions should be further analyzed. We identified known and unknown genes that were differentially expressed during pregnancy, indicating stage-specific roles. Because this study was performed on a limited number of miniature pigs, further confirmation on a much larger population and at various gestation stages will be needed to confirm these results. These genes may provide a better understanding of the cellular and molecular mechanisms in the miniature pig ovary during pregnancy.

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