Luteinization of porcine preovulatory follicles leads to systematic changes in follicular gene expression

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Abstract

The LH surge initiates the luteinization of preovulatory follicles and causes hormonal and structural changes that ultimately lead to ovulation and the formation of corpora lutea. The objective of the study was to examine gene expression in ovarian follicles ($n=11$) collected from pigs (*Sus scrofa domestica*) approaching estrus (estrogenic preovulatory follicle; $n=6$ follicles from two sows) and in ovarian follicles collected from pigs on the second day of estrus (preovulatory follicles that were luteinized but had not ovulated; $n=5$ follicles from two sows). The follicular status within each follicle was confirmed by follicular fluid analyses of estradiol and progesterone ratios. Microarrays were made from expressed sequence tags that were isolated from cDNA libraries of porcine ovary. Gene expression was measured by hybridization of fluorescently labeled cDNA (preovulatory estrogenic or -luteinized) to the microarray. Microarray analyses detected 107 and 43 genes whose expression was decreased or increased (respectively) during the transition from preovulatory estrogenic to -luteinized ($P<0.01$). Cells within preovulatory estrogenic follicles had a gene-expression profile of proliferative and metabolically active cells that were responding to oxidative stress. Cells within preovulatory luteinized follicles had a gene-expression profile of nonproliferative and migratory cells with angiogenic properties. Approximately, 40% of the discovered genes had unknown function.

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Introduction

The growth and development of the preovulatory follicle requires a coordinated series of events that lead to cell proliferation, oocyte development, and estrogen synthesis (Evans 2003, Hunter *et al.* 2004, Berisha & Shams 2005). Gonadotropins (both luteinizing hormone (LH) and follicle-stimulating hormone (FSH)) play a central role in the processes. Growth factors may act directly to control follicular growth or may act indirectly by modifying the sensitivity of follicular cells to FSH and LH (Lucy 2000, Richards *et al.* 2002a,b, Webb *et al.* 2003, Fortune *et al.* 2004). The release of the LH surge near the onset of estrus redirects preovulatory development and causes the biochemical differentiation of the follicle (luteinization) and ovulation (Richards *et al.* 2002, Murdoch & Gottsch 2003). There is a shift from estrogen to progesterone synthesis, thinning and folding of the follicle wall, and dispersion of the granulosa cell layer (Smith *et al.* 1994, Murphy *et al.* 2001). Growth factors, proteases, and protease inhibitors coordinate

tissue remodeling and angiogenesis that are essential for ovulation and the formation of the corpus luteum (Smith *et al.* 2002, Tamanini & DeAmbrogi 2004). Perhaps, the most notable morphological change occurs within the microvasculature of the theca cell layer where there is hyperemia, edema, and extravagation of erythrocytes (Smith *et al.* 1994, Murphy *et al.* 2001).

Luteinization of follicular cells and ovulation of the follicle are complex processes that require the coordinated expression of a large number of genes (Sarit *et al.* 2005). Some genes involved in the processes are well characterized, but there are other genes whose role in the process is unknown. Gene-expression analysis using microarrays is a method of identifying genes involved in biological processes (Eisen & Brown 1999, Koopman *et al.* 2002). We developed a microarray that contained expressed sequence tags (EST) found in porcine ovary (Jiang *et al.* 2004). The objective of this study was to use the microarray to systematically evaluate genes expressed in porcine ovary and to examine their

respective roles during the luteinization of porcine preovulatory follicles.

Materials and Methods

Animals and follicle collection

The University of Missouri Animal Care and Use Committee approved the procedures that we describe. Pigs with known estrus dates were watched for signs of impending estrus (development of a red and swollen vulva) beginning on estrous cycle day 17. Two pigs that were approaching estrus, but had not expressed estrus were used for the collection of preovulatory estrogenic follicles (follicles collected before luteinization). Two additional pigs were allowed to come into estrus and on the second day of estrus were used for the collection of preovulatory luteinized follicles (follicles collected after luteinization; the LH surge in pigs occurs on the first day of estrus; Esbenshade *et al.* 1982). Ovaries were collected after the pigs were killed by electrocution. Individual intact follicles (>6 mm diameter) were dissected from the ovaries (six follicles before the LH surge ($n=3$ follicles from each of two sows) and five follicles after the LH surge ($n=3$ and 2 follicles from two sows)). The follicular fluid was aspirated from the follicle using a tuberculin syringe. The follicle wall was placed into a tube and snap frozen in liquid nitrogen. The follicular fluid was analyzed for estradiol and progesterone concentrations by RIA (Liu *et al.* 2000) to determine the extent of luteinization for individual follicles.

Preparation of cDNA microarrays

Eleven cDNA libraries were used to generate EST from porcine ovary (Jiang *et al.* 2004). The cDNA libraries were created using tissue from fetal, neonatal, and prepubertal porcine ovaries, pubertal ovaries on different days of the estrous cycle (day 0 (follicle), 5 (ovary), and 12 (follicle and corpus luteum)), and follicles isolated from weaned sows (2, 4, 6, and 8 mm diameter). The cDNA sequences from the 11 libraries were clustered into a single nonredundant UniGene set using the tclcluster program (Scheetz *et al.* 2003). The UniGene set contained all of the singleton EST as well as a single representative member for each cluster (clusters were groups of independent clones encoding the same EST; the representative member was the clone with the longest sequence read). The UniGene set contained a total of 8009 genes. The cDNA corresponding to each EST were PCR-amplified from bacterial stocks that contained the representative plasmid. The PCR contained 0.25 mM dNTP and 200 ng M13 forward and reverse primers and 0.85 U Biolase DNA polymerase (Bioline, Randolph, MA, USA). The PCR conditions were 3 min at 95 °C initial denaturation

followed by 30 cycles of 30 s at 94 °C denaturation, 30 s at 55 °C annealing, and 3 min at 72 °C extension. The PCR was purified using MultiScreen-PCR Plates (Millipore, Billerica, MA, USA) according to manufacturer's instructions. The purified PCRs were dried in a vacuum centrifugation apparatus and resuspended in 10 µl 3× SSC.

Microarray slides were printed in the same facility and under the same conditions that were described in a previous paper from our group (Whitworth *et al.* 2005). Gold Seal glass microscope slides (Fisher Scientific, Hampton, NH, USA) were coated with 0.02% poly-L-lysine (Sigma) in 0.05× PBS and aged for 15 days at room temperature under desiccant (Eisen & Brown 1999). The cDNA was printed onto the slides by using a pick and place robot. Printed slides were rehydrated and cross-linked at 120 mJ/cm² for 20 s (Spectrolinker, Spectronics Corp., Westbury, NY, USA). Slides were blocked with 0.018% succinic anhydride and 0.043 M sodium borate in 1-methyl-2-pyrrolidinone and stored in a desiccator until hybridization.

Total RNA purification, cDNA labeling, and hybridization of arrays

Eleven individual follicles were used for RNA isolation. Total cellular RNA was extracted using the TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. The integrity and purity of the extracted RNA were verified by measuring the ratio of absorbencies at 260 and 280 nm and by visual inspection of an RNA aliquot on a Tris–borate/EDTA (0.09 M Tris–borate, 0.02 M EDTA) ethidium bromide-stained agarose gel.

At least two replicates for each RNA sample were subjected to the microarray analysis (29 microarrays contributed to the dataset). A control RNA sample was generated by combining equal amounts of total RNA from 2, 4, 6, and 8 mm porcine follicles as well as corpus luteum and corpus hemorrhagicum. The test samples (preovulatory estrogenic and -luteinized) were labeled with Cy5 and the control sample was labeled with Cy3. Indirect labeling of cDNA with amino allyl dUTP was done according to the procedure of Hegde *et al.* (2000) with the following modifications. Equal concentrations of 3-amino allyl dUTP and dTTP were used in the RT of 15 µg total RNA. The purified cDNA was incubated with approximately 0.02 mg Cy5 (test sample) or Cy3 (control) for 1 h at room temperature before the purification with PCR purification columns (Qiagen). The reactions were dried in a vacuum centrifugation apparatus and resuspended in 26 µl of hybridization buffer containing 48% formamide, 4.8× SSC, 0.1% SDS and 20 µg poly A. The cDNA was denatured by incubating at 95 °C for 3 min and allowed to cool to room temperature before being transferred onto a microarray slide. Microarray slides were hybridized overnight at 42 °C and washed (4 min at 42 °C in

1 × SSC, 0.2% SDS; 4 min at room temperature in 0.1 × SSC, 0.2% SDS; 4 min at room temperature in 0.1 × SSC and rinsed with water). The microarrays were then dried by centrifugation before scanning was done with a GenePix 4000B Scanner (Axon Instruments, Inc., Union City, CA, USA).

The intensity of each spot was determined using GenePix Pro (Version 4.0) software (Axon Instruments, Inc.). Raw data were imported into GeneSpring microarray analysis software (Silicon Genetics, Redwood City, CA, USA). Genes with intensities less than 160 in the control channel (Cy3) in at least half of the arrays were filtered out ($n=2665$). Welch *t*-test and Benjamini and Hochberg multiple testing correction with 1% false discovery rate were performed to determine the genes whose expression changed after luteinization in Lowess normalized and log transformed data. Fold changes in gene expression after luteinization were also determined using GeneSpring software.

Annotation of cDNA clones

A list of cDNA clones and corresponding GenBank Accession numbers are available on the project website (<http://genome.rnet.missouri.edu/Swine>). Each clone was previously submitted to GenBank and therefore has been clustered by the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) into the porcine UniGene database. The UniGene cluster and cluster annotation was used for preliminary assignment of name and function for each cDNA clone. The cDNA clones whose expression was found to be differentially expressed were further annotated through a manual annotation procedure. The sequence of individual clones was aligned (Blast analysis) against the RefSeq_rna database within NCBI (Wheeler *et al.* 2006). The highest homology alignments were examined for alignment score, the sense of the alignment (plus/plus vs plus/minus) and whether the alignment corresponded to the 3' tail of the RefSeq mRNA. The cDNA clones that we used were 3' cDNA (Jiang *et al.* 2004). Thus, clones with a high alignment score (generally >200) that were plus/minus and whose

alignment was to the 3' tail of the RefSeq mRNA were annotated with the official gene name as found within RefSeq. The porcine RefSeq was preferentially used but, in the absence of porcine data, then other species were used (primarily human RefSeq). Clones whose alignments had low score (generally <200) or whose alignments appeared fragmented relative to the RefSeq alignment were annotated as unknown. Clones that were described as 'weakly similar to' or 'moderately similar to' a known gene was also considered unknown within the context of this work.

Real-time RT-PCR

Real-time RT-PCR was used to validate the microarray results. The test RNA and the control samples used in the microarrays were reverse transcribed using Superscript II reverse transcriptase (Invitrogen). The reactions were carried out using 5 µg total RNA, 3 µg random hexamer, and 1.25 µM oligo dT primers. Cytochrome P450 17A1 (CYP17A1), LH/choriogonadotropin receptor (LHCGR), 3β-hydroxysteroid/δ-5-δ-4 isomerase (3B-HSD), plasminogen activator inhibitor-1 (PLANH1) and ribosomal protein L19 (RPL19) were amplified in an ABI Prism 7700 machine (Applied Biosystems, Foster City, CA, USA) using the QuantiTect Sybr Green PCR Kit (Qiagen) and specific PCR primers (Table 1). The threshold cycle (C_T) for the test samples was subtracted from the C_T for the control sample to obtain the change (Δ) in C_T (ΔC_T). The amount of each mRNA (relative to the control sample) was calculated by using the equation, relative expression = amplification efficiency ΔC_T . The amplification efficiencies (derived from an analysis of C_T in a serially diluted samples; data not shown) were 2.2, 2.4, 2.2, 2.0, and 2.3 for CYP17A1, 3B-HSD, LHCGR, PLANH1, and RPL19 respectively.

Results

Hormone concentrations in follicular fluid

Follicles collected from pigs that were approaching estrus (preovulatory estrogenic) or on day 2 of estrus (preovulatory luteinized) were different for estradiol and

Table 1 GenBank number, gene name, primer location, and oligonucleotide primer sequence for the individual genes whose expression (as measured by RT-PCR) was used to validate the data generated by the analyses of microarray.

GenBank	Gene name	Primer	Primer sequence	Primer location	Amplicon (bp)
NM_214428	CYP17A1	Sense	5'-CATGCCTGAGCGTCTCT-3'	1333–1350	71
		Antisense	5'-CGAAGGGCAAGTAGCTCAATG-3'	1403–1383	
NM_214449	LHCGR	Sense	5'-GCCGAACCTTTATAGACGGAAGGA-3'	2006–2028	123
		Antisense	5'-CATGACAGTGGAACTACTGACATTGTAA-3'	2128–2102	
NM_001004049	3B-HSD	Sense	5'-CCAAGTCTCAGTTGCTGATTCTGA-3'	1527–1550	125
		Antisense	5'-TGCGTGAAGGGATACATACTTTTAA-3'	1652–1627	
NM_213910	PLANH1	Sense	5'-GGAAAGGGAATATGACCAGACTCA-3'	991–1014	110
		Antisense	5'-CATATCCGTCATTCCCAAGTTCT-3'	1100–1078	
AF435591	RPL19	Sense	5'-GTACTGCCAATGCTCGAATGC-3'	224–244	67
		Antisense	5'-AGCCGGCGCAGAATTCT-3'	290–274	

progesterone concentrations in follicular fluid (Table 2). Follicular fluid collected from follicles before estrus had nearly ten-fold greater follicular fluid estradiol concentrations when compared with follicular fluid from follicles collected on the second day of estrus ($P<0.01$). The relative concentration of steroids in follicular fluid was reversed for progesterone. Follicles collected before estrus had lower follicular fluid progesterone concentrations compared with follicles collected on day 2 of estrus ($P=0.10$).

Gene-expression analyses

The GenBank identifier for the printed clone, the UniGene cluster for the printed clone, expression relative to preovulatory estrogenic (negative denotes a decrease in expression after luteinization; positive denotes an increase in expression after luteinization), and the annotation for individual genes are presented in Tables 3 and 4. Relative to preovulatory estrogenic, microarray analyses detected 107 and 43 genes whose expression was decreased (Table 3) or increased (Table 4) respectively, in preovulatory luteinized follicles at a minimum threshold of $P<0.01$. For genes whose expression decreased (i.e. were high in preovulatory estrogenic and then low in luteinized), the predominant functional classifications were cytoskeletal proteins and regulators of cytoskeleton; nuclear proteins (chromatin components) and nucleic acid-binding proteins; metabolic enzymes, mitochondrial transporters, and proteins involved in the oxidative stress response; ligands, receptors, and receptor pathways (predominantly the cAMP response system); and cell proliferation/differentiation. For genes whose expression increased (i.e. were low in preovulatory estrogenic and high in luteinized), the predominant functional classifications were proteins that are involved in cell adhesion and migration, cell growth inhibition, or angiogenesis. There were functional classifications that were shared across both expression profiles (decreased or increased) but contained different genes (i.e. CYP17A1 and 3B-HSD) that were in the same functional class (steroidogenic

enzymes), but expression was decreased and increased (respectively) in the analyses). Functional classifications shared across both expression profiles (Tables 3 and 4) contained genes whose proteins were involved in lipid transfer/steroidogenesis, proteolysis (proteases and protease inhibitors), and metabolism.

Of the 150 EST that were differentially expressed, 58 EST were classified as having low homology to known genes using the criteria that were used for annotation (Table 5). Forty-one of the EST had a decrease in expression from preovulatory estrogenic to preovulatory luteinized (negative-fold change).

Validation and evaluation of microarray analyses

The expression of four genes (two whose expression had a negative-fold change and two whose expression had a positive-fold change) was tested by using RT-PCR for the purpose of validating the microarray analyses. In each case, the relative change in gene expression was similar in magnitude and direction when measured by RT-PCR and microarray (Fig. 1). The relative expression of RPL19 (a selected house-keeping gene (Lee *et al.* 2002) that was not differentially expressed in the microarrays) was similar for preovulatory estrogenic (1.2 ± 0.1 -fold) and preovulatory luteinized (1.0 ± 0.1 -fold).

Genes from the present study whose expression was found to decrease after luteinization (i.e. were high in preovulatory follicle and then low in luteinized follicles) were evaluated for the distribution of their respective EST, that were sequenced from the original cDNA libraries (Jiang *et al.* 2004). For contigs with at least five members (i.e. a modest sample size), the predominant cDNA libraries from which the aforementioned EST arose were 6 and 8 mm follicle (Table 6). There were three genes (*RPL13A*, *H3F3B*, and *TPM3*) whose EST did not appear to be preferentially represented in the 6 and 8 mm follicle libraries from Jiang *et al.* (2004). Other than CO946724 (CAPNS1; one in p2mm and four in p6mm), the genes that had increased expression in the preovulatory luteinized follicles had fewer than five members in the follicular libraries (p2mm, p4mm, p6mm, and p8mm) of Jiang *et al.* (2004).

Table 2 Estradiol and progesterone concentrations (lsmean $\pm$ s.e.m.) in follicular fluid for follicles whose mRNA was isolated and used for microarray analyses.

Pig number	Follicular status	Follicular diameter (mm)	N	Follicular fluid estradiol (ng/ml)	Follicular fluid progesterone (ng/ml)
1	Preovulatory estrogenic	>6	3	259 $\pm$ 30	131 $\pm$ 18
2	Preovulatory estrogenic	>6	3	234 $\pm$ 15	142 $\pm$ 40
3	Preovulatory luteinized	>6	3	21 $\pm$ 0.5	2070 $\pm$ 170
4	Preovulatory luteinized	>6	2	51 $\pm$ 2.1	499 $\pm$ 40

Discussion

We measured gene expression in porcine preovulatory follicles before (preovulatory estrogenic) and after (preovulatory luteinized) luteinization by using a microarray approach. One hundred and fifty genes whose expression was either down- or upregulated in response to luteinization were found in these analyses (Tables 3–5). The regulated genes fell into a variety of functional categories. Of interest was the fact that the functional categories were largely distinct for the different follicle classes (preovulatory estrogenic and -luteinized) owing

Table 3 GenBank accession number for the microarray-printed cDNA, UniGene number for the GenBank clone, fold change in gene expression (relative to preovulatory estrogenic), gene symbol, gene name, and *P*-value (for change in expression) for genes whose expression decreased (negative-fold change) from preovulatory estrogenic to preovulatory luteinized. Genes are shown as groups within functional categories.

GenBank	UniGene	Fold	Gene symbol	Gene name	<i>P</i> -value
Steroidogenesis, steroid metabolism, and steroid response					
CO949101	Ssc.1417	−25.6	LRP8	Low-density lipoprotein-related receptor protein 8	<0.0001
CO948336	Ssc.29043	−2.6	NR5A2	Nuclear receptor subfamily 5, group A, member 2	<0.001
CO946044	Ssc.279	−2.7	STAR	StAR protein	<0.01
CO944136	Ssc.2887	−5.1	POR	P450 (cytochrome) oxidoreductase	<0.01
CO946141	Ssc.15060	−6.5	CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	<0.0001
CO943852	Ssc.16041	−2.7	SUS2DD	Dimeric dihydrodiol dehydrogenase	<0.01
Cytoskeletal protein or regulator of cytoskeleton					
<i>Cytoskeletal component</i>					
CO943677	Ssc.13956	−1.8	TPM1	Tropomyosin 1 (α)	<0.001
CO946602	Ssc.1829	−1.8	TPM3	Tropomyosin 3	<0.001
CO947940	Ssc.941	−1.9	ACTA2	Actin α-2	<0.01
CO947522	Ssc.30968	−1.4	MACF1	Microtubule-actin crosslinking factor-1	<0.01
CO943806	Ssc.11770	−1.5	PDLIM7	PDZ and LIM domain 7 (enigma)	<0.001
<i>Cytoskeletal regulator</i>					
CO947842	Ssc.5356	−1.5	MARK1	MAP/microtubule affinity-regulating kinase 1	<0.01
CO946486	Ssc.27615	−1.8	OBSCN	Obscurin	<0.01
CO955649	Ssc.23247	−1.7	MYLK	Myosin light polypeptide kinase	<0.01
CO943228	Ssc.950	−1.6	BASP1	Brain abundant, membrane attached signal protein 1	<0.001
Nuclear protein or nucleic acid-binding protein					
<i>Nuclear proteins (chromatin components)</i>					
CO952666	Ssc.18486	−1.5	H1F0	H1 histone family member 0	<0.01
CO942264	Ssc.11548	−1.5	H3F3B	H3 histone family 3B	<0.01
CO952549	Ssc.21844	−1.3	E3-3	Nuclear protein E3-3	<0.01
CO945043	Ssc.226	−1.7	HMGB2	High-mobility group box 2	<0.01
CO945389	Ssc.19964	−1.5	HIRIP5	HIRA interacting protein 5	<0.01
<i>Nucleic acid-binding proteins</i>					
CO944038	Ssc.3911	−1.8	RY1	Putative nucleic acid-binding protein RY-1	<0.0001
CO943262	Ssc.17290	−1.8	HNRPA1	Heterogenous nuclear ribonucleoprotein A1	<0.001
CO944059	Ssc.9133	−3.1	REXO2	RNA exonuclease 2 homolog	<0.0001
CO943622	Ssc.2708	−2.2	RNAUXA	Small nuclear RNA adaptor	<0.001
Cell proliferation/differentiation					
CO949669	Ssc.30068	−4.4	SIPAI	Signal-induced proliferation-associated 1 like 1	<0.0001
CO943413	Ssc.3010	−1.5	MADD	MAP-kinase activating death domain	<0.01
CO948371	Ssc.15749	−3.1	CCND2	Cyclin D2	<0.0001
CO945596	Ssc.22394	−12.3	YAP1	Yes-associated protein 1	<0.0001
CO947263	Ssc.2663	−1.5	WBP1	WW domain binding protein 1 (binds Yes-associated protein)	<0.001
<i>Wnt signalling</i>					
CO945662	Ssc.9131	−1.5	LRRFIP2	Leucine rich repeat interacting protein 2	<0.01
CO945850	Ssc.29807	−1.4	TLE2	Transducin-like enhancer of split 2 homolog	<0.01
CO948051	Ssc.16819	−2.0	ENC1	Ectodermal-neural cortex	<0.0001
Metabolic enzymes, mitochondrial transporters, and control of oxidative stress					
<i>Metabolic enzymes</i>					
CO947714	Ssc.3039	−1.7	FADS2	Fatty acid desaturase 2	<0.01
CO955632	Ssc.21101	−3.7	ALAS1	Aminolevulinatase δ-synthase-1	<0.0001
CO946902	Ssc.16315	−6.0	CS	Citrate synthase	<0.0001
CO955084	Ssc.22000	−2.6	CBR1	Carbonyl reductase 1	<0.0001
CO947943	Ssc.24494	−2.1	HDC	Histidine decarboxylase	<0.001
<i>Mitochondrial transporters and proteins</i>					
CO946105	Ssc.5284	−2.5	SLC25A4	Solute carrier family 25 member 4	<0.0001
CO946590	Ssc.16638	−1.4	SLC25A6	Solute carrier family 25 member 6	<0.01
BF713441	Ssc.21244	−1.3	TOM7	Translocase of outer mitochondrial membrane 7	<0.01
CO944130	Ssc.1241	−2.4	HSPE1	Heat shock 10kDa protein 1 (Chaperonin 10, mitochondrial)	<0.0001
<i>Proteins in the oxidative stress response</i>					
CO952474	Ssc.12390	−1.4	SOD1	Superoxide dismutase 1 (SOD1)	<0.01
CO944924	Ssc.1490	−1.5	PRDX3	Peroxisome oxidoreductin 3 (PRDX3)	<0.01
CO949707	Ssc.29035	−2.8	GCLC	Glutamate-cysteine ligase, catalytic subunit	<0.0001
BF711914	Ssc.8799	−3.8	MGST1	Microsomal glutathione S-transferase 1	<0.0001
CO948129	Ssc.217	−1.4	ESD	Esterase D (formylglutathione hydrolase)	<0.01
Ligands, receptors, and pathways					
<i>Ligand</i>					
CO944667	Ssc.8909	−3.4	INHBB	Inhibin, β-B	<0.0001
CO946617	Ssc.4152	−6.3	AKR1C3	Prostaglandin F synthase	<0.0001

Table 3 Continued

GenBank	UniGene	Fold	Gene symbol	Gene name	P-value
CO947399	Ssc.1310	-2.1	PTGES	Prostaglandin E synthase	<0.0001
<i>G protein coupled receptors</i>					
CO949616	Ssc.16311	-4.4	LHCGR	Luteinizing/chorionic gonadotropin hormone receptor	<0.001
BF704285	Ssc.5038	-2.0	LGR4	Leucine-rich repeat-containing G protein-coupled receptor 4	<0.01
BF704220	Ssc.8706	-4.7	RGS3	Regulator of G-protein signaling 3	<0.0001
CO945501	Ssc.1056	-2.2	PRKAR2B	cAMP-dependent protein kinase, regulatory, type II, β	<0.0001
CO942872	Ssc.29749	-2.1	AKAP7	A kinase anchor protein 7	<0.0001
<i>Tyrosine kinase receptors</i>					
CO943457	Ssc.8648	-1.8	GRB14	Growth factor receptor-bound protein 14	<0.001
<i>JAK/STAT pathway</i>					
CO943444	Ssc.23054	-1.8	JAK3	Janus kinase 3	<0.001
Cell membrane component					
CO945267	Ssc.942	-2.3	GJA1	Gap junction protein, α -1	<0.001
CO950396	Ssc.31172	-4.5	SYND1	Syndecan 1	<0.0001
CO946596	Ssc.28961	-3.1	SLC4A3	Solute carrier family 4, member 3	<0.0001
CO949001	Ssc.30657	-5.4	SLC41A1	Solute carrier family 41, member 1	<0.0001
Protein synthesis					
CO942463	Ssc.17024	-1.4	RPL13A	Ribosomal protein L13a	<0.01
CO946401	Ssc.27429	-5.6	CPEB2	Cytoplasmic polyadenylation element-binding protein-2	<0.0001
CO943882	Ssc.1439	-1.4	EEF1D	Eukaryotic translation elongation factor-1 δ	<0.01
Proteases and protein inhibitors					
CO946472	Ssc.15674	-4.7	CTSL2	Cathepsin L2	<0.0001
CO947028	Ssc.2073	-2.1	UCHL1	Ubiquitin carboxyl-terminal esterase L1	<0.0001
CO943317	Ssc.19571	-1.9	ABHD4	Abhydrolase domain containing 4	<0.001
CO942251	Ssc.16342	-4.2	PN-1	Nexin 1 (protease inhibitor)	<0.0001

to the unique transition that occurs during luteinization. Genes that were downregulated during luteinization (i.e. were high in preovulatory estrogenic and low in preovulatory luteinized) fell largely into the categories of cytoskeletal proteins or regulators of the cytoskeleton; nuclear proteins or nucleic acid-binding proteins; metabolic enzymes, mitochondrial proteins, and proteins involved in oxidative stress; ligands, receptors, and receptor pathways (predominantly cAMP); and cell proliferation/differentiation proteins. Genes whose expression increased after luteinization fell largely into the categories of cell adhesion/migration, cell growth inhibition, and angiogenesis. There were 58 EST that were differentially expressed, but gene function could not be categorized because it was unknown (Table 5).

The LH surge in pigs occurs near the onset of estrus (Esbenshade *et al.* 1982). Ovaries were collected shortly before estrus (preovulatory estrogenic) and on the second day of estrus (preovulatory luteinized; follicles presumably exposed to the LH surge). Collection of preovulatory luteinized follicles on the second day of estrus has been done in the past (Fricke *et al.* 1996) and this model was used in the present study. The status of individual follicles (estrogenic or luteinized) was verified by the analysis of estradiol and progesterone concentrations in the follicular fluid. As expected, the follicular fluid collected from follicles before estrus (preovulatory estrogenic) had high estradiol concentrations. Follicular fluid collected from follicles after the onset of estrus had relatively low estradiol concentrations and high progesterone concentrations (preovulatory luteinized). The follicular concentrations of estradiol and

progesterone presented herein were similar to those reported in our previous publication on gene expression in preovulatory porcine follicles (Liu *et al.* 2000). Distinct regulation of genes involved in steroidogenesis were also observed and were predictable given the shift from estrogen synthesis to progesterone synthesis that occurred in the isolated follicles (Table 2). StAR protein, CYP17A1 (17 α -hydroxylase/17,20 lyase) and P450 oxidoreductase (POR; P450 oxidoreductase, a flavo-protein that donates electrons to P450 enzymes) were elevated in preovulatory estrogenic follicles. The expression of NR5A2 (liver receptor homolog-1), a transcription factor that activates transcription of steroidogenic genes, including aromatase (Sirianni *et al.* 2002, Mendelson *et al.* 2005) was also greater in preovulatory estrogenic follicles. 3- β -Hydroxysteroid dehydrogenase (3 β -HSD) increased in preovulatory luteinized follicles; a change in gene expression that is known to occur during luteinization (Conley *et al.* 1995, Driancourt *et al.* 1998). There was also a predictable change in INHBB, AKR1C3, and PTGES (inhibin, β -B, prostaglandin F synthase, and prostaglandin E synthase; high in preovulatory follicles; Jiang *et al.* 2004). Previous studies of porcine ovary had found greater aromatase mRNA in follicles approaching estrus (Liu *et al.* 2000), but the arrays used in this study did not contain the gonadal isoform of aromatase (Corbin *et al.* 2004). Thus, the failure to detect a change in aromatase may reflect our error in not including the appropriate aromatase variant for ovary. Finally, dimeric dihydrodiol dehydrogenase (SUS2DD or AKR1C1) was differentially expressed in preovulatory estrogenic follicles.

Table 4 GenBank accession number for the microarray-printed cDNA, UniGene number for the GenBank clone, fold change in gene expression (relative to preovulatory estrogenic), gene symbol, gene name, and *P*-value (for change in expression) for genes whose expression increased (positive-fold change) from preovulatory estrogenic to preovulatory luteinized. Genes are shown as groups within functional categories.

GenBank	UniGene	Fold	Gene symbol	Gene name	<i>P</i> -value
Lipid transfer/steroidogenesis					
CO946215	Ssc.3517	1.5	PLTP	Plasma phospholipid transfer protein	<0.01
CO946466	Ssc.14393	2.9	3B-HSD	3 β -hydroxysteroid dehydrogenase	<0.001
Cell adhesion and migration					
CO955624	Ssc.54	2.1	CD9	CD9 antigen	<0.0001
BF712968	Ssc.17325	5.0	CD24	CD24 antigen	<0.0001
CO949594	Ssc.20465	1.8	LGALS3	Galectin 3	<0.01
CO994242	Ssc.8162	3.1	PTX3	Pentraxin-3 PTX	<0.0001
CO946724	Ssc.7158	4.7	CAPNS1	Calpain I light subunit	<0.0001
CO948855	Ssc.13061	3.2	FBNP1L	Formin-binding protein 1-like	<0.0001
CO953942	Ssc.38257	2.0	EFNA5	Ephrin A5	<0.0001
CO949185	Ssc.10338	1.5	NCK1	NCK adaptor protein 1	<0.01
Cell growth inhibition					
CO948274	Ssc.236	3.7	PPP2CB	Protein phosphatase 2A- β subunit	<0.0001
CO993620	Ssc.8609	2.4	PDCD4	Programmed cell death 4	<0.01
CO942640	Ssc.3392	2.4	ZFP36L2	Zinc finger protein 36, C3H type-like 2 (tristetraprolin)	<0.0001
BF711292	Ssc.14392	3.0	MSMB	PSP94-like protein	<0.01
CO954834	Ssc.328	1.5	JAK1	Janus kinase 1	<0.01
CO942970	Ssc.800	1.8	ATP1A1	(Na ⁺ , K ⁺)-ATPase α -subunit	<0.01
Angiogenesis					
CO956362	Ssc.7297	1.6	MAOB	Monoamine oxidase B	<0.01
CO949287	Ssc.8775	1.4	LAP3	Leucine aminopeptidase 3	<0.01
CO954557	Ssc.3189	2.4	DSCR1	Down syndrome critical region gene 1	<0.01
CO993631	Ssc.9781	8.7	PLANH1	Plasminogen activator inhibitor-1	<0.0001
CO947953	Ssc.12241	8.3	ANXA2	Annexin A2	<0.01
CO955098	Ssc.24144	1.4	ACBR1B	Activin A receptor, type IB	<0.01
Metabolism					
CO951010	Ssc.1151	2.1	ELOVL5	Elongation of long chain fatty acids, family member 5	<0.001
CO944033	Ssc.14530	10.3	ACADL	Long-chain acyl-CoA dehydrogenase	<0.0001
CO943165	Ssc.4123	1.7	PSMC2	Proteasome 26S subunit ATPase 2	<0.01

Dihydrodiol dehydrogenase metabolizes progesterone to the inactive 20 α -hydroxyprogesterone (Jez *et al.* 1997) demonstrating that progesterone may be metabolized to an inactive state in the preovulatory estrogenic follicle.

The phenotype of the preovulatory estrogenic follicle, as revealed by these analyses (Table 3), is one of a tissue whose cells are actively synthesizing cytoskeletal components, regulators of the cytoskeleton, and nuclear proteins (chromatin proteins, proteins associated with chromatin, and nucleic acid-binding proteins). Cytoskeletal and nuclear proteins are structural components of the cell and the high level of gene expression may reflect the proliferative nature of the cells in the preovulatory estrogenic follicle relative to the preovulatory luteinized follicle (Fricke *et al.* 1996). The high level of cytoskeletal protein gene expression may also be a consequence of gonadotropin stimulation because gonadotropins were shown to increase cytoskeletal gene expression *in vitro* (Grieshaber *et al.* 2003). Markers of proliferative cells whose expression was high in preovulatory estrogenic follicles included SIPA1, cyclin D2 (CCND2), and components of the Wnt/ β -catenin signaling pathway. The importance of cyclin D2 expression to granulosa cell function was unequivocally demonstrated by the CCND2 knockout mouse that had a defect in granulosa cell proliferation (Sicinski

et al. 1996). The Wnt/ β -catenin signaling pathway controls additional aspects of granulosa cell proliferation. Misregulation of the Wnt/ β -catenin pathway leads to ovarian tumorigenesis (Boerboom *et al.* 2005) and knockout of pathway components leads to infertility in mice through a defect in corpus luteum development (Hsieh *et al.* 2005). Two different genes (*YAP1* and *WBPI*) whose gene products bind Yes (a Src family nonreceptor tyrosine kinase proto-oncogene; Summy & Gallick 2003) were differentially expressed (high in preovulatory estrogenic follicles).

The second most striking feature of gene expression within the preovulatory estrogenic follicle was the highly metabolic nature of the cells and the evidence for an ongoing oxidative stress response. Three molecular transporters of the mitochondrion were differentially expressed including two adenine nucleotide transporters (SLC25A4 and SLC25A6). Citrate synthase (CS), a mitochondrial enzyme of the tricarboxylic acid cycle, was differentially expressed as well. Oxidative stress in preovulatory follicles is probably created by the highly metabolic environment within follicular cells and the generation of reduced cofactors for steroidogenesis (i.e. NADPH; Miller 2005). Alleviating oxidative stress in follicular cells is important for cell survival because the accumulation of reactive oxygen

Table 5 GenBank accession number for the microarray-printed cDNA, UniGene number for the GenBank clone, fold change in gene expression (relative to preovulatory estrogenic), and *P*-value (for change in expression) for genes with low homology to known genes.

GenBank	UniGene	Fold	P-value	GenBank	UniGene	Fold	P-value
CO948355	Ssc.1417	-19.5	<0.0001	CO956225	Ssc.9560	-1.7	<0.001
CO946569	Ssc.14541	-18.0	<0.0001	CO947296	Ssc.11297	-1.7	<0.01
CO948261	Ssc.36726	-15.6	<0.0001	CO952791	Ssc.30857	-1.7	<0.01
CO946735	Ssc.31012	-8.6	<0.0001	CO946414	Ssc.27571	-1.6	<0.0001
CO942498	None	-4.9	<0.0001	BF713306	Ssc.6265	-1.6	<0.01
CO945514	Ssc.1432	-4.9	<0.0001	CO947783	Ssc.17946	-1.6	<0.01
CO946352	Ssc.1064	-4.6	<0.0001	CO949334	None	-1.5	<0.01
CO945414	Ssc.11983	-4.2	<0.0001	CO947319	Ssc.15372	-1.5	<0.01
CO946311	Ssc.26802	-4.2	<0.0001	CO943064	Ssc.20343	-1.5	<.01
CO943616	Ssc.29676	-3.8	<0.0001	CO954385	Ssc.25882	-1.4	<.01
CO945822	None	-3.7	<0.0001	CO945143	Ssc.19458	-1.3	<.01
CO943658	Ssc.29662	-3.6	<0.0001	CO954541	Ssc.2776	1.4	<0.01
BF713298	None	-3.3	<0.0001	CO993615	Ssc.14780	1.5	<0.01
CO942983	Ssc.30917	-3.2	<0.0001	CO943174	Ssc.925	1.6	<0.01
BF704331	Ssc.8747	-3.1	<0.001	CO956122	Ssc.6365	1.7	<0.0001
CO955627	None	-2.8	<0.0001	CO956886	Ssc.6155	1.8	<0.0001
CO944535	Ssc.11694	-2.5	<0.0001	CO944585	None	1.8	<0.01
BF703366	Ssc.4591	-2.5	<0.01	CO943169	Ssc.1181	1.8	<0.01
CO948656	Ssc.30039	-2.5	<0.01	BF713006	None	2.0	<0.001
CO946458	None	-2.3	<0.0001	CO955876	Ssc.2112	2.1	<0.0001
CO947502	Ssc.4193	-2.2	<0.001	BF703217	Ssc.9269	2.1	<0.0001
CO943161	Ssc.21150	-2.2	<0.001	CO956925	Ssc.12085	2.4	<0.001
CO943587	Ssc.3626	-2.1	<0.0001	CO994083	Ssc.7438	2.5	<0.001
CO947196	Ssc.31026	-2.0	<0.0001	CO994172	Ssc.30563	2.6	<0.001
CO948294	Ssc.8563	-2.0	<0.01	BF711868	Ssc.8789	2.8	<0.01
CO948119	None	-2.0	<0.01	CO946926	Ssc.27253	3.2	<0.01
BF703256	Ssc.914	-1.8	<0.01	BF712989	Ssc.6093	3.5	<0.0001
CO945564	None	-1.8	<0.0001	BF711981	Ssc.18298	4.2	<0.0001
CO944761	Ssc.2961	-1.7	<0.0001	BF704319	Ssc.8528	6.0	<0.0001

species causes apoptosis (Tsai-Turton & Luderer 2006). Relative to the preovulatory luteinized follicle, the preovulatory estrogenic follicle expressed a high level of superoxide dismutase 1 (SOD1) and peroxiredoxin 3 (PRDX3); two enzymes that combat oxidative stress (Chang *et al.* 2004). The *SOD1* gene is required for ovarian function as *SOD1* knockout mice are subfertile, but this could be related to low blood gonadotropin concentrations in the knockout model (Matzuk *et al.* 1998). Reduced glutathione is an antioxidant that relieves oxidative stress. It is synthesized within the follicle to act locally and is also transported into and stored within the oocyte (Luberda 2005). Two enzymes that lead to the synthesis of glutathione, glutamate-cysteine ligase catalytic subunit, (GCLC and esterase D, ESD), were differentially expressed (high in preovulatory estrogenic follicle). GCLC is perhaps the more noteworthy of the two because it is the rate-limiting enzyme in glutathione synthesis. We also detected high expression of microsomal glutathione S-transferase 1 (MGST1); an enzyme with the capacity to reduce oxidative stress, but is also involved in prostaglandin and leukotriene synthesis (Hayes *et al.* 2005).

The third feature of the gene-expression profile within the preovulatory estrogenic follicle (relative to preovulatory luteinized) was the expression of genes within the G-protein-coupled cAMP second messenger system.

LH (as opposed to FSH) is the primary gonadotropin driving the terminal differentiation of preovulatory follicles in the pig (Guthrie 2005). Our previous studies demonstrated that preovulatory estrogenic follicles

Figure 1 Relative gene expression (lsmean $\pm$ S.E.M.) for four genes that were differentially expressed in preovulatory luteinized and -estrogenic follicles. The gene expression was analyzed by both microarray and RT-PCR. Two genes with decreased expression after luteinization (*CYP17A1* and *LHCGR*) and two genes with increased expression after luteinization (*3B-HSD* and *PLANH1*) are shown. The relative expression for the microarray data is from the GeneSpring Lowess normalization and analysis used in this study. The relative expression for the RT-PCR data was calculated by the ΔC_T method and is expressed relative to the control (reference) sample used for the microarray study.

Table 6 GenBank accession number for the microarray-printed cDNA, UniGene number for the GenBank clone, contig number from Jiang *et al.* (2004), gene symbol, gene name, and the number of sequenced clones from the original porcine cDNA libraries (Jiang *et al.* 2004). Clones with EST as Gene symbol were expressed sequence tags with low homology to known genes. The listed genes had expression that decreased from preovulatory estrogenic to preovulatory luteinized (negative-fold change in present study).

GenBank	UniGene	Contig	Gene symbol	Gene name	p2mm	p4mm	p6mm	p8mm
BF711914	Ssc.8799	36	MGST1	Microsomal glutathione S-transferase 1	2	3	10	37
CO946617	Ssc.4152	273	AKR1C3	Prostaglandin F synthase	4	4	25	7
CO946472	Ssc.15674	651	CTSL2	Cathepsin L2	6	1	9	14
CO946569	Ssc.14541	18	EST	None	2	0	7	15
CO942463	Ssc.17024	1133	RPL13A	Ribosomal protein L13a	6	6	5	3
CO944059	Ssc.9133	372	REXO2	RNA exonuclease 2 homolog	1	3	5	7
CO947940	Ssc.941	222	ACTA2	Actin α -2	1	0	4	7
CO946590	Ssc.16638	163	SLC25A6	Solute carrier family 25 member 6	1	0	6	3
CO946141	Ssc.15060	5149	CYP17A1	Cytochrome P450 17A1	1	0	4	5
CO944924	Ssc.1490	416	PRDX3	Peroxisome oxidoreductin 3 (PRDX3)	2	1	2	5
CO949101	Ssc.1417	294	LRP8	Low-density lipoprotein-related protein 8	0	0	4	5
CO946735	Ssc.31012	5178	EST	None	0	0	4	3
CO942264	Ssc.11548	1470	H3F3B	H3 histone family 3B	4	1	0	1
CO946602	Ssc.1829	5195	TPM3	Tropomyosin 3	0	2	2	1
CO943882	Ssc.1439	760	EEF1D	Eukaryotic translation elongation factor-1 δ	1	1	1	2
CO949616	Ssc.16311	1894	LHCGR	Luteinizing hormone receptor	0	0	0	5

(6 mm) had high LH receptor (LHCGR) expression (Liu *et al.* 2000). A similar observation was made in this study (Table 3). In addition to elevated LHCGR, there was increased expression for several genes with the cAMP pathway in the preovulatory estrogenic follicle. One gene was structural (AKAP7; a scaffold protein that binds the protein kinase A (PKA) regulatory subunit; Malbon *et al.* 2004). Two of the identified genes were regulators of cAMP signaling with an inhibitory tone. For example, the protein product of RGS3 is a GTPase activator that is inhibitory toward G-protein signaling (Ishii & Kurachi 2003). The *PRKAR2B* gene encodes one of the regulatory subunits of the PKA complex. The *PRKAR2B* gene is an early response gene for gonadotropin-treated ovarian cells (Chu *et al.* 2002). The expression of *PRKAR2B* and *RGS3* in preovulatory estrogenic follicles may imply a mechanism through which the preovulatory follicle restrains the cAMP signal and prevents premature luteinization.

The LH surge initiates a physiological program that leads to ovulation and the formation of the corpus luteum. Microarray analyses detected some of the underlying genome-level changes that support the transition within the follicle. Cytoskeletal, nuclear, metabolic, and proliferative genes had high expression in preovulatory estrogenic follicles and reduced expression in preovulatory luteinized follicles (see above). The genes that had high expression in the preovulatory luteinized follicles fell into different functional categories when compared with preovulatory estrogenic follicles. The functional categories of genes that were highly expressed in preovulatory luteinized follicles were reminiscent of the cellular processes that have been classically ascribed to follicular cells after the LH surge (Smith *et al.* 1994, Murphy *et al.* 2001).

The predominant functional category for increased gene expression in preovulatory luteinized follicles was cell adhesion and migration (Table 4). The category included cell surface antigens (CD9, CD24, LGALS3, and PTX3) as well as regulatory molecules that control cell migration (CAPNS1, FNBP1L, EFNA5, and NCK1). Of the cell surface molecules, CD24 and pentraxin 3 (PTX3) are particularly relevant to ovarian biology. CD24 is characteristic of migratory cells; and is a known marker for tumorigenesis in a variety of tissues (including ovary; Kristiansen *et al.* 2004). PTX3 is a secreted protein that binds extracellular matrix and is released in response to growth differentiation factor-9 (GDF-9). PTX3 expression is specifically increased after the LH surge (Varani *et al.* 2002). The PTX3 knockout mouse is subfertile because of defective ovulation and defective formation of the cumulus-oocyte complex (Varani *et al.* 2002).

Cells within the porcine follicle initiate migration and the remodeling of the follicle wall before ovulation (Murphy *et al.* 2001). The migratory nature of cells within the preovulatory luteinized follicle was clearly suggested by the increase in expression for calpain (CAPNS1; Franco & Huttenlocher 2005), ephrin A5 (EFNA5; Surawska *et al.* 2004), forming binding protein (FNBP1L; Watanabe & Higashida 2004), and NCK1 (Miyamoto *et al.* 2004); genes whose proteins have well-characterized roles in cell migration. The *ATP1A1* gene (Na⁺/K⁺ transporter) is included in this category as well because of the recent work implicating the function of this gene in controlling cell movement (Rajasekaran *et al.* 2005).

The second largest class of differentially expressed genes in the luteinized preovulatory follicle was for cell growth inhibition (antiproliferation). The luteinized follicular tissue was presumably a mixture of granulosa

(believed to be nondividing), theca (dividing), and endothelial (actively dividing) cells. Given the diversity of cell types and mitotic states, it was somewhat surprising that cell growth inhibitory molecules were detected in these studies. Nonetheless, several genes were identified and their existence suggests an active process through which some cells within the preovulatory follicle become nonproliferative. Perhaps, the most notable members of this class were protein phosphatase 2A β -subunit (PPP2CB), a Ser/Thr phosphatase that inhibits cell growth by inactivating the MAPK signal (Theodosiou & Ashworth 2002), and PDCD4, a tumor suppressing gene that acts by suppresses carbonic anhydrase (Goke *et al.* 2004). The remaining members of the class have known tumor suppressor activities as well but do so through different mechanisms. Tristetraprolin (ZFP36L2) destabilizes growth factor mRNA and increases mRNA turnover (Stoecklin *et al.* 2003) and microseminal protein β , MSMB or PSP94; one of the most highly expressed genes in porcine corpus luteum from our previous work; Jiang *et al.* 2004) is an inhibitor of vascular endothelial growth factor (VEGF) signaling (Lamy *et al.* 2005). Janus kinase 1 can have proliferative or antiproliferative properties depending on the nature the activating ligand (Verma *et al.* 2003).

The final functional class that was differentially expressed in preovulatory luteinized follicles was comprised of genes whose proteins play a role in angiogenesis. The process through which angiogenesis is initiated within the luteinized follicles has been described (Murphy *et al.* 2001) and is of interest because of the rapid development of a vascular bed in the growing corpus luteum (Driancourt *et al.* 1998.). Two of the enzymes in this group (MAOB and LAP3) are vasodilatory; monoamine oxidase metabolizes vasoactive amines to inactive forms and is found in high amounts within the preovulatory follicle and corpus luteum (Yoshimoto *et al.* 1986) and LAP3 (also known as cysteine amino peptidase or oxitocinase) metabolizes oxytocin, vasopressin, and angiotensin III (Tsujimoto & Hattori 2005) and is a marker for malignant gynecological tumors (Ino *et al.* 2004). The importance of VEGF in the angiogenic cascade that occurs in preovulatory luteinized follicles is well documented (Tamanini & De Ambrogio 2004) and DSCR1, a differentially expressed gene shown here, is an early response gene for VEGF (Iizuka *et al.* 2004). PLANH1, the primary inhibitor of plasminogen activator that has been implicated in tumor growth and cellular remodeling (Stefansson *et al.* 2003) and annexin a2 (ANXA2), a plasminogen–plasminogen activator co-receptor with angiogenic properties (Kim & Hajjar 2002), were also found to be differentially expressed. The upregulation of both PLANH1 and ANXA2 is curious given their contrasting functions within the plasminogen–plasminogen activator system that has functional roles in both ovulation and

angiogenesis (Murdoch & Gottsch 2003). Their coexistence may simply reflect the delicate enzymatic balance that exists during cellular remodeling. The activin A receptor is included in this class because of the role of the transforming growth factor- β superfamily in angiogenesis (Lebrin *et al.* 2005).

Analyses of gene expression by microarray typically yield a long list of genes with diverse functions that can present a confusing picture of cellular physiology. We have attempted to simplify the process by applying stringent statistical criteria to our analyses (diminishing the number of spurious results) and also by carefully evaluating each printed clone against a curated database (RefSeq of the NCBI). This process has hopefully created a robust list of genes whose expression changes during luteinization of the porcine follicle. Many of the discovered genes have recognized roles in the process, whereas others could be viewed as novel. We grouped genes into functional categories. These grouping could be criticized because genes can assume many biological roles. The categories that we developed were based on a literature search for each gene in this study. Two additional points should be raised. The first is that many of the genes that we identified here were also found to be highly expressed in preovulatory follicle libraries from our original publication (Jiang *et al.* 2004; Table 6). The two studies used entirely different approaches and samples and achieved a degree of commonality in their results. An obvious limitation was that we sequenced a limited number clones in the original study and therefore could not estimate the true frequency of EST for all clusters in the original libraries. The second point is that a related study in cattle yielded an expression profile that overlapped with ours. Ndiaye *et al.* (2005) examined gene expression within the bovine dominant follicle before and after human chorionic gonadotropin (hCG) treatment. They discovered 23 known genes that were decreased by hCG in their differential display analyses. Five of the genes that they described (*CYP19A1*, *LRP8*, *GJA1*, *LHCGR*, and *JAK3*) were identical to ours and two additional genes (*INHBA* and *SERPINE2*) were very closely related. Thus, approximately one-third of the known genes discovered by Ndiaye *et al.* (2005) were identical or highly similar to ours. The results of a related study in mice (McRae *et al.* 2005) in which serial analyses of gene expression (SAGE) was used to study changes in granulosa gene expression were less consistent with ours. The mouse study employed pregnant mare serum gonadotropin followed by a luteinizing dose of hCG. Twelve of the reported genes (*STAR*, *TPM1*, *ACTA2*, *FADS2*, *INHBB*, *LHCGR*, *GJA1*, *SYND1*, *CTSL2*, *3B-HSD*, *ANXA2*, and *LRP8*) can be found in our lists, but only three of the genes (*FADS2*, *INHBB*, and *GJA1*) undergo the same directional change within the two studies. The significance of this observation

depends on the technical (SAGE vs microarray) and biological (pig vs mouse; whole follicle vs granulosa cells) basis for the inconsistency. In all likelihood, none of the studies presents a complete list of genes that are differentially expressed during luteinization. The microarray technology that we used and the approaches used by others (differential display and SAGE) have their limitations. As for microarray, the cDNA are printed on glass and many genes are screened out because of low-intensity signals (about one-third in this study). The genes described herein had the qualities of relatively high signal intensity, low between animal and follicle variance, and high differential expression. Technology that employs greater precision and sensitivity will need to be developed to improve the gene-expression profile and increase our knowledge of luteinization.

Fifty-eight of the discovered genes were classified as EST or unique sequences (Table 5). Some of the genes were highly differentially expressed. For example, clones CO946569 and CO946735 had a fold change from preovulatory estrogenic to -luteinized of -18.0 and -8.6 respectively. These clones belong to contigs whose members were highly represented in the 6 and 8 mm follicle libraries from our original publication (Table 6; Jiang *et al.* 2004). The clones were clustered in the Porcine UniGene cluster of GenBank as Ssc.14541 (CYP19A3, aromatase) and Ssc.31012 (transcribed locus). The annotation associated with the first clone (aromatase) is a good example of the problems encountered with gene annotation in public databases. This clone and a large number of clones within Ssc.14541 have relatively low homology to porcine aromatase. We have attempted to avoid gene annotation problems in this study by individually examining each clone for homology to genes in the RefSeq database. The list of genes with unknown function that we present (Table 5) may contain genes whose functions fit into the categories described in this paper. Their protein products need to be identified and characterized.

In summary, genes with differential expression before and after luteinization were discovered by using a microarray approach. Some of the differentially expressed genes had functions that were already known and their regulation was consistent with the published literature. Most of the discovered genes, however, have never been previously associated with luteinization. Cells within preovulatory estrogenic follicles had a gene-expression profile that was distinctly different from cells within preovulatory luteinized follicles. Some of the differentially expressed genes in this study may be novel candidate genes for proteins that function in cell proliferation or cell growth inhibition, oxidative stress, cell migration, and early stages of angiogenesis.

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