Animal Reproduction Science 115 (2009) 39–48

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Analysis of gene expression in granulosa cells of ovine antral growing follicles using suppressive subtractive hybridization

A. Qin Chen[a,b], Zheng Guang Wang[b], Zi Rong Xu[b], Song Dong Yu[b,*], Zhi Gang Yang[a]

[a] College of Fisheries and Life, Shanghai Ocean University, Shanghai 200090, PR China
[b] College of Animal Science of Zhejiang University, Key Laboratory for Molecular Animal Nutrition, Ministry of Education, Hang Zhou 310029, PR China

ARTICLE INFO

Article history:
Received 11 March 2008
Received in revised form 19 October 2008
Accepted 24 October 2008
Available online 11 November 2008

Keywords:
Gene expression
Granulosa cells
Ovary
SSH

ABSTRACT

Follicular growth, development and ovulation are highly ordered processes that involve the expression of many genes under precise temporal and spatial regulation. However, information on stage-specific gene expression during the antral follicle phase in sheep is not well understood. In the present study, suppressive subtractive hybridization (SSH) was performed to screen genes that were differentially expressed in the granulosa cells between large follicles (LF, >5 mm) and small follicles (SF, 3–5 mm), and subtractive cDNA library was constructed. Furthermore, with dot-blot analysis, a total of 90 clones randomly selected from the library were proven to be differentially expressed in the granulosa cells. Among these, 38 exhibited high homology to known genes, 14 sequences were corresponding to novel expressed sequence tags (ESTs). Four ESTs, LAPTM4A, SERPINE2, GSTA1, and INHBA, were further examined the reproducibility of the SSH data by the real-time quantitative PCR. Results confirmed an increase expression of respective mRNA in granulosa cells of large follicles compared with that of small follicles. It is concluded that we have identified several genes (known or unknown) that may effect follicular growth, dominance or ovulation in ewes.

* Corresponding author. Tel.: +86 571 86985347; fax: +86 571 86994963.
E-mail address: yzgicaq@hotmail.com (S.D. Yu).

0378-4320/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.anireprosci.2008.10.022

1. Introduction

Ovarian antral follicles emerge and grow in a wave-like pattern in sheep, with 3–4 waves per cycle and the emergence of each follicular wave is preceded by a peak in serum follicle-stimulating hormone (FSH) concentrations (Ginther et al., 1995; Bartlewski et al., 1999; Duggavathi et al., 2006). Studies have shown that growth of antral follicles beyond 2.5 mm in diameter cannot occur in the absence of gonadotrophins (McNatty et al., 1990; Picton et al., 1990; Picton and McNeilly, 1991; Evans, 2003). The pre-ovulatory phase of the estrous cycle in ewes is characterized by selection of one to several dominant follicles and atresia of subordinate follicles coincident with decreasing FSH concentrations in peripheral plasma (Evans, 2003). On the other hand, selection, dominance, and ovulation of the follicle are complex processes that require the coordinated expression of a large number of genes (Sarit et al., 2005). The expression patterns of steroidogenic enzymes, in sheep, have been studied in ovaries collected either at different of stages of the estrous cycle or from the slaughterhouse from ewes at unspecified stages of the cycle (Duggavathi et al., 2006). The components of insulin-like growth factor (IGF) and transforming growth factor-β (TGF-β) system were shown to be related with the follicular growth, selection, and atresia in cycling sheep at the mRNA level (Hastie et al., 2004; Hastie and Haresign, 2006). In addition, endothelial nitric oxide synthase and connexin 37 may play a role in follicular development and ovulation as well as in luteal tissue growth, differentiation, and regression (Gonzalez-Bilska et al., 2006; Borowczyk et al., 2006).

Although some genes involved in the processes of growth, development of ovine follicle were well characterized, no screens for genes that effect sequential development and differentiation during the final stage of antral follicular development have been reported. PCR-based suppressive subtractive hybridization (SSH) techniques are highly sensitive for identifying differences in gene expression. In recent years, SSH has been utilized to identify genes that are expressed in different development stages of ovarian follicles in rat (Espey and Richards, 2002), cattle (Fayad et al., 2004; Ndiaye et al., 2005b; Diouf et al., 2006), and chicken (Seol et al., 2006). In the present study, we employed SSH to identify changes in gene expression between large follicles (LF, >5 mm, tester) and small follicles (SF, 3–5 mm, driver) during the final stage of ovine antral follicular development. A better knowledge of gene expression patterns during follicle selection and ovulation in sheep would yield insights into the molecular pathways controlling ovulation and as a preamble to improve the fertility of sheep.

2. Materials and methods

2.1. Experimental animal model, tissue collection

Experiments were conducted in accordance with the Animal (Scientific Procedures) Act of PR China. The experiments were conducted during the breeding season in Zhejiang Province, China. Ten adult non-pregnant, cycling does of Hu breed; 2–3 years of age were used for the experiment. Hu sheeps are well recognized for early sexual maturity, aseasonal breeding, and prolificacy. The animals were fed alfalfa hay and pellets, and water was available ad libitum. All ewes were pre-synchronized to control the time of ovulation using an intravaginal pessary (EAZI-BREEDTM CIDR®; Pharmacia & Upjohn, New Zealand) containing 300 mg progesterone for 12 days. Day 0 of the estrous cycle was determined by using vasectomized rams. The animals were received a total of 300 IU pig FSH at 12 h intervals, administered twice daily with i.m. injections for 3 days at a constant dose of 50 IU/treatment on days 13–15 of the estrous cycle. Ewes were slaughtered in the late follicular phase. Visible antral follicles on the surface of the ovaries were dissected from the ovarian stroma and initially separated by size into two groups: small follicles (3–5 mm) and large follicles (>5 mm). Follicular fluid and granulosa cells with oocytes were collected separately from SF or LF as described previously (Rosales-Torres et al., 2000) with minor modifications. Follicles were washed with phosphate buffered saline (PBS) and cut open with small iris scissors, and gently scraped with a blunt probe into the cold PBS. Follicular contents were then processed to recover cell-free follicular fluid and granulosa cells. Follicular contents were centrifuged at 2000 × *g* for 3 min. The supernatant (follicular fluid) was collected. The granulosa cell pellet was washed in cold PBS and centrifuged again at 2000 × *g* for 3 min, and 1 ml Trizol was

Table 1
Primer sequences used for real-time PCR (RT-PCR).

Gene	Primer sequence	Tm	Product size (bp)
LAPTM4A	F 5′-ACTTTGTATCGAGCCCTGTG-3′ R 5′-GCAGTGAAGATGCCTGAGAA-3′	55	140
GSTA1	F 5′-ACAAACCGCTATCTCCCTG-3′ R 5′-GGTCCAGCTCTTCCACATAG-3′	60	130
INHBA	F 5′-CCAGCCAATGTCCTTGAAAC-3′ R 5′-CATACGGATTGCCTGTGAGC-3′	53	286
SERPINE2	F 5′-AGAAATAGTGAAATCTTTAGCCTC-3′ R 5′-ACAGCAAACCTTGTCTAGCA-3′	53	118
GAPDH	F 5′-CCTGCCAAGTATGATGAGAT-3′ R 5′-GTGAGTGTCGCTGTTGAAGT-3′	53	120

added to the pellet. The pelleted cells were resuspended by gentle vortexing and then flash-frozen in liquid nitrogen and stored at −80 °C. Concentrations of E2 and P4 were analyzed by EIA using 96-well ELISA plates (Corning Glass Works, Corning, NY, USA), as described previously (Purinton and Wood, 2002; Wood and Giroux, 2003).

2.2. RNA isolation and suppression-subtractive hybridization

Total RNA was isolated from granulosa cells of the ovine follicles using TRIzol (Invitrogen, Auckland, New Zealand). The RNA quality was determined by UV spectrophotometry (260 and 280 nm). A two-directional (forward and reverse) SSH was performed using MessageAmpII aRNA Amp Kit (Amnion, Inc., USA) and PCR-Select cDNA Subtraction Kit (Clontech Lab, Inc., Palo Alto, CA). After second strand cDNA synthesis, samples were digested with RsaI to generate shorter, blunt-ended, double-stranded cDNA fragments, necessary for adaptor ligation. Subtraction was performed in two directions: cDNA derived from LF served as tester and SF cDNA served as driver and vice versa. RsaI-digested tester cDNA was ligated with either Adaptor 1 or Adaptor 2R for use in the subtraction. Adaptor-ligated tester cDNA was denatured and hybridized with excess driver cDNA. Fresh denatured driver DNA was added, and a second hybridization was performed. The samples were PCR amplified; the primary PCR was 27 cycles, and the secondary was 12 cycles. The amplified cDNA fragments from the secondary PCR amplification, PCR products were cloned into the pTAK-101 kit (ToYoBo). To evaluate the efficiency of the subtraction, the relative amounts of GAPDH cDNA present in the subtracted and unsubtracted cDNA populations after SSH were examined by PCR amplification using the GAPDH forward and reverse primers (Table 1).

2.3. Differential hybridization screening and analysis of cloned SSH cDNA

In order to confirm differential expressions of the clones, cDNA dot-blots were performed. Nested primers 1 and 2R (included in the SSH kit) were used to PCR amplify 384 white colonies selected from each of the two subtracted libraries. To check the size of the insert and ensure that only one insert was present, 5 μl of each PCR was electrophoresed on 1% agarose gels. PCR products were dot-blotted onto nylon membranes in duplicate (Amersham Pharmacia Biotech, CK). The forward- and reverse-subtracted PCR products were purified, digested with RsaI to remove the adaptor sequences, and used as templates for synthesizing probes for differential screening. The RsaI digested products were purified and were labeled with ^{32}Pα-dATP. Membranes were pre-hybridized for 2.5 h at 68 °C and hybridized with probes at 68 °C overnight. The differentially hybridizing cDNA clone was performed by BLAST against GenBank data banks (NR and EST). A cDNA sequence was considered homologous to a GenBank sequence when at least 100 base pairs (bp) matched with an *E* probability value of less than e^{-10}.

2.4. Quantification of specific transcripts by real-time PCR

Expression of the clones of interest, namely LAPTM4A, SERPINE2, GSTA1, and INHBA, was evaluated by real-time quantitative RT-PCR. Primers were designed using the Primer5.0 (Table 1). Total cDNA was generated using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and random primers, and 1 μl was used in a real-time reaction (25 μl) using SYBR® Premix Ex Taq™ (TakaRa). The PCR was carried out in the iCycler iQ Real-time PCR Detection System (Bio-Rad, Belgium). GAPDH was used to normalize for variations in the amount of starting material, which has been previously for use as internal controls in real-time PCR. Dissociation curve analysis was run after each real-time experiment to ensure that there was only one product and that no primer dimers were present. To control for false positives, a reverse transcriptase-negative control was run for each template and primer pair. Real-time PCR products were verified on a 1% agarose gel.

2.5. Statistical analysis

All data are expressed as means ± S.E.M. Statistical differences were analyzed using Student's *t*-test. Differences were considered significant at $P < 0.05$ or less.

3. Results

3.1. Suppression-subtractive hybridization

The mean diameter of LF ($n = 13$) and SF ($n = 15$) is 5.2 ± 0.2 mm and 4.2 ± 0.4 mm, respectively (Chen et al., 2009). Concentrations of E2 in follicular fluid of the LF were much higher than that in the SF (139.6 ± 19.6 vs. 50.6 ± 8.7, $P < 0.05$) (Chen et al., 2009). Total RNAs from the LF and SF were synthesized into double-strand cDNAs, which were shown as a smear of 0.3–7.0 kilobases (kb). After digestion by RsaI, the smear moved to 0.1–2.0 kb (data not shown). The PCR product of GAPDH appeared to be detectable on the agarose gel after amplification for 33 cycles and 23 cycles in the subtracted and the unsubtracted cDNA pools, respectively. The data indicated that the amount of GAPDH transcript was reduced over 2^7-fold after subtraction (Fig. 1).

After ensuring that the GAPDH gene had been extensively removed in the subtracted pools, the subtracted cDNAs were cloned and transformed into DH5α competent cells. Seven hundred and sixty-eight clones were selected at random and the inserts were reamplified by PCR (Fig. 2A). The amplified cDNAs were dotted onto Hybond N1 membranes and hybridized separately with ^{32}P-labeled unsubtracted cDNAs from LF or SF, and positive signals were detected (Fig. 2B). Approximately 90 clones were analyzed by DNA sequencing and then compared against the GenBank/EMBL databases using the online Blast system. Of 87 ESTs that provided sequence information, 38 different genes corresponded to known biological function, 15 corresponded to complete or partial sequences of undefined biological function (Tables 2 and 3), and 14 were novel ESTs (Table 4). Three clones were rejected due to inadequate quality of the nucleotide sequences.

3.2. Real-time PCR

In order to confirm that genes identified by SSH are differentially expressed in LF compared to SF, RT-PCR was be conducted in parallel to verify the validity of the SSH data. Expression of four genes (GSTA1, INHBA, SERPINE2, and LAPTM4A) in sheep was determined by real-time PCR using specific

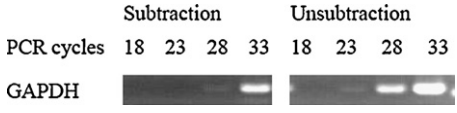

Fig. 1. Evaluation of the subtraction efficiency by amplifying the cDNA of GAPDH in both the subtracted and unsubtracted cDNA pools using PCR. The products amplified for different cycles were electrophoresed on agarose gel.

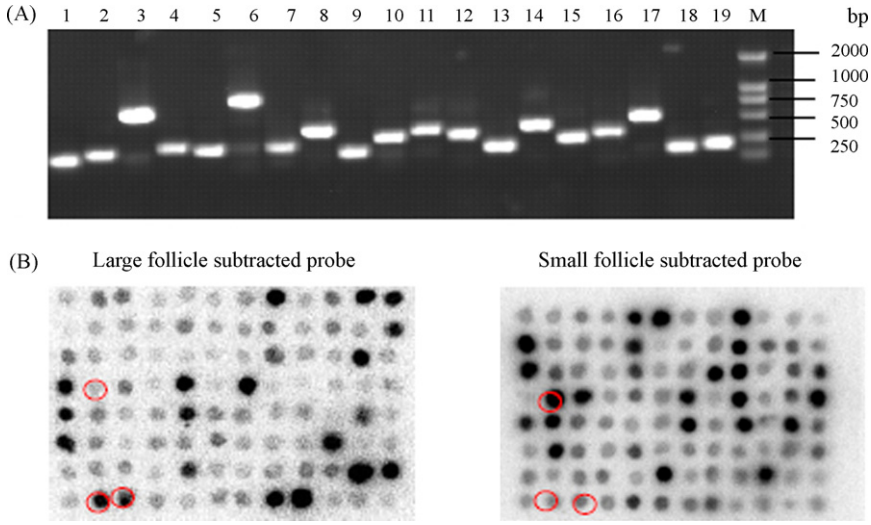

Fig. 2. Screening of the subtracted library. (A) The subtracted library was screened by PCR. 1–19 were PCR results by NP1/NP2R primers; M was DNA ladder marker. (B) Results of dot-bolt hybridization showing the forward-subtracted products dotted onto membrane 1 hybridized with the radioactive-labeled granulosa cells cDNA from LF (left panel) or SF (right panel). Dot-labeled were selected as differentially expressed.

sets of primers. All these genes exhibited some increase in expression in the large follicles; however, they presented no or faint expression in the small follicles (Fig. 3). The expression patterns obtained by RT-PCR reflected the results obtained by SSH, demonstrating a low false positive rate associated with SSH in this experiment.

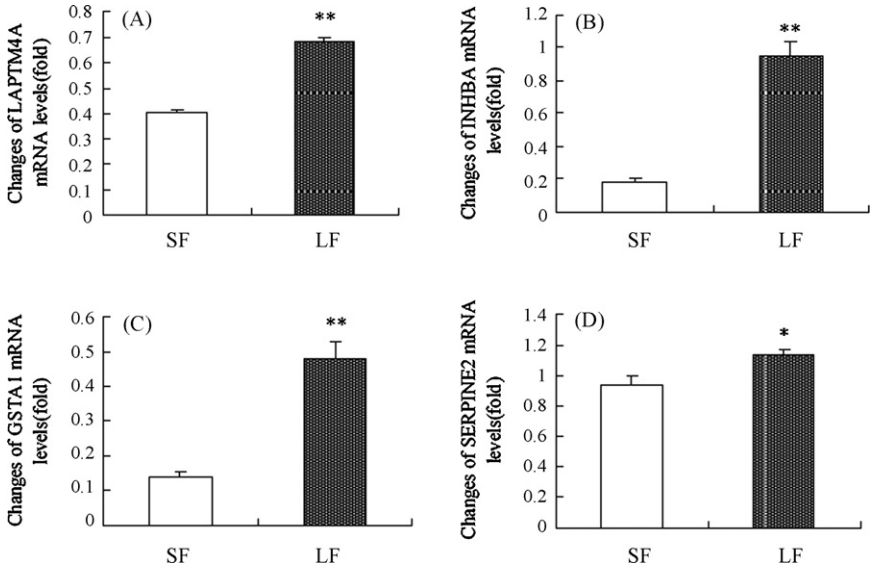

Fig. 3. Quantitative RT-PCR of some differential clones in granulosa cells. Total RNAs from the granulosa cells of LF and SF were subjected to RT-PCR as described in Section 2. RNA samples were normalized using the GAPDH gene, which did not show differential expression between samples.

Table 2
Identity of up-regulated genes differentially expressed in ovine granulosa cells of LF.

Frequency	Identity[a]	GenBank no.[b]	Blast database no.	Molecular function
2	BT osteoglycin (OGN)	EY202182, EY202193	BT030567	Growth factor activity
1	BT glutathione S-transferase subunit isoform I (GSTA1)	EY202183	U49179	Transferase activity
1	BT similar to ADP-ribosylation factor GTPase activating protein 3 (ARFGAP3)	EY202184	NM_001075974	ARF GTPase activator activity
1	BT protein tyrosine phosphatase type IVA, member 2 (PTP4A2)	EY202187	BC151453	Protein tyrosine phosphatase activity
2	BT actin, beta (ACTB)	EY202189	BT030480	Protein binding
4	BT vimentin (VIM)	EY202199	NM_173969	Protein binding
1	BT B-cell translocation gene 1, anti-proliferative (BTG1)	EY202202	BC142147	Enzyme binding
1	BT cytochrome c oxidase subunit VIIb (COX7B)	EY202203	BC103174	Cytochrome-c oxidase activity
1	OA clone TO-DOWN-B6-2 elongation factor-1 alpha (EEF1A1)	EY202208	DQ223573	Translation elongation factor activity
2	OA inhibin beta-A-subunit gene (INHBA)	EY202209	EF192431	Activin inhibitor activity
1	BT proteasome (prosome, macropain) subunit, beta type, 1 (PSMB1)	EY202210	NM_001038539	Peptidase activity
1	Predicted: CF similar to calumenin (CALU) precursor, transcript variant 8	EY202211	XM_853685	Calcium ion binding
1	BT sterol-C4-methyl oxidase-like (SC4MOL)	EY202213	NM_001098863	C-4 methylsterol oxidase activity
1	OA laminin receptor 1 (LAMR1)	EY202212	NM_001105263	ND[c]
1	BT lysosomal-associated protein transmembrane 4 alpha (LAPTM4 A)	FD480235	NM_205799	Lysosomal membrane fraction
1	BT similar to guanine nucleotide-binding protein gmma10 (GNG10)	FD480236	BC103019	Signal transducer activity
1	BT nascent polypeptide-associated complex alpha subunit (NACA)	FD480237	NM_001014916	DNA binding
1	BT serine protease inhibitor clade E member 2(SERPINE2)	FD480241	AY581202	Serine-type endopeptidase inhibitor activity
1	BT matrin 3 (MATR3)	FD480242	NM_001102486	RNA binding
3	HM zinc finger, MIZ-type containing 1 (ZMIZ1)	FD480238	NM_020338	Metal ion binding
1	MM 16 days embryo head cDNA, RIKEN full-length enriched library, clone: C130068E13 ROD1 regulator of differentiation 1	FD480245	AK048512	RNA binding
1	OA follistatin (FST)	FD480246	M63123	BMP signaling pathway
1	BT similar to ribosomal protein L30 (RPL30)	FD480248	NM_001034434	Structural constituent of ribosome
1	Predicted: BT similar to 60S ribosomal protein L5 (LOC784446)	EY202205	XM_001251892	5S rRNA binding
2	BT ribosomal protein L5 (RPL5),	FD480249	NM_001035306	5S rRNA binding
1	Predicted: CF similar to KH domain containing, RNA binding, signal transduction associated 1	EY202179	XM_544442	ND[c]
1	Predicted: BT similar to KIAA14 51 protein	EY202206	XR_028250	ND
1	Predicted: BT similar to ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	FD480240	XR_027599	ND
1	BT CF-24-HW fat cDNA library *Bos taurus* cDNA	FD480253	DV782038	ND
1	BT chromosome 14 open reading frame 147	FD480254	BC134492	ND
1	BT isolate UoG-BovSAGE- UK7L mRNA sequence	FD480255	AY999166	ND
2	Predicted: BT similar to ENSANG P00000009498	EY202180, EY202181	XM001252235	ND
1	OA partial mRNA for hypothetical protein	FD480256	AJ783860	ND
1	BT hypothetical protein MGC128614	FD480259	BC103275	ND
1	010914OSTA088085HT OSTA *Ovis aries* cDNA	FD480264	EE863018	ND
1	HS cDNA FLJ23877 fis, clone LNG13624	EY202190	AK074457	ND

[a] BT, *Bos taurus*; HS, *Homo sapines*; OA, *Ovis aries*; MM, *Mus musculus*; CF, *Canis familiaris*.
[b] GenBank accession number of differentially expressed ovine SSH cDNA clones.
[c] No biological data available based on gene ontology.

Table 3
Identify of down-regulated genes differentially expressed in ovine granulosa cells of LF.

Frequency	Identity[a]	GenBank no.[b]	Blast database no.	Molecular function
2	BT cytochrome c oxydase subunit 4 (COX4)	EY202192	NM_001001439	Cytochrome-c oxidase activity
5	BT hypoxia-inducible factor 1, alpha subunit (HIF1A)	EY202197, EY202201	NM_174339	Protein heterodimerization activity
4	Predicted: BT nucleolin, transcript variant 1 (NCL)	EY202198	XM_614626	RNA binding
1	BT glycerol-3-phosphate dehydrogenase 2 (GPD2)	FD480243	BC148085	Glycerol-3-phosphate dehydrogenase activity
2	BT F-box protein 8 (FBXO8)	FD480244	BT020954	ARF guanyl-nuleotide exchange factor activity
1	BT karyopherin alpha 2 (RAG cohort 1), importin alpha (KPNA2)	FD480247	NM_001034449	Protein transporter activity
1	Predicted: BT similar to zinc finger protein 711, transcript variant 2	EY202186	XM_604385	Transcription regulator activity
1	BT ribosomal protein S3 (RPS3)	FD480250	NM_001034047	RNA binding
1	OA clone TO-DOWN-L13-6 ribosomal protein S12	EY202185	DQ223559	ND[c]
2	OA clone TO-DOWN-D24-7 ribosomal protein S27a	FD480251, FD480252	DQ223562	ND
1	Predicted: BT hypothetical protein LOC783293	FD480257	XR_027730	ND
1	BT DEAH (Asp-Glu-Ala-His) box polypeptide 36	FD480258	NM_001080251	ATP binding
1	BT cDNA clone IMAGE: 8311708	FD480260	BC140566	ND
2	OA mitochondrion	FD480262, FD480263	AY858379	ND
1	020731OFLT074068HT OFLT *Ovis aries* cDNA	FD480265	EE768795	ND

[a] BT, *Bos taurus*; HS, *Homo sapines*; OA, *Ovis aries*; MM, *Mus musculus*; CF, *Canis familiaris*.
[b] GenBank accession number of differentially expressed ovine SSH cDNA clones.
[c] No biological data available based on gene ontology.

Table 4
The new ESTs from the present study that were submitted to GenBank.

Clone no.	GenBank no.
420	EY202207
101	FD480266
113	FD480267
164	FD480268
197	FD480269
236	FD480270
285	FD480271
294	FD480272
478	FD480273
489	FD480274
541	FD480275
553	FD480276
656	FD480277
341	FD480278

4. Discussion

Because each stage of follicular development is characterized by patio-temporal activation specific subsets of genes (many of which need to be identified), it is necessary that we have a better understanding of expression patterns of stage-specific gene. In the present study, we constructed subtracted cDNA library from ovine antral follicles to display genes that are differentially expressed in granulosa cells of large follicles compared to these of small follicles. To our knowledge, this is the first report to determine genes involved in ovine granulosa cells of ewes with high throughput technology.

Several genes, such as INHBA, FST, and laminin have been identified to be expressed in granulosa cells of ovarian follicle during the follicular development in sheep. Previous study showed strongly mRNA level for INHBA and FST in granulosa cells of healthy large antral follicles at various stages of the sheep estrous cycle (Engelhardt et al., 1993). The expression of mRNA encoding the inhibin, a subunit is positively related to follicular size (Barid and Campbell, 2001). Laminin has been shown to be one of the most abundant extracellular matrix (ECM) components within the basement membrane (Le Bellego et al., 2002). During the follicular and the pre-ovulatory phases of the estrous cycle, the increasing levels of laminin and high levels of mature $\alpha_6\beta$ integrin in granulosa cell layers of healthy antral follicles might support their final development (Le Bellego et al., 2002). It is suggested that during the follicular and the pre-ovulatory phases of the estrous cycle, the increasing levels of laminin in granulosa cells of large antral follicles might support their final development to ovulation (Berkholtz et al., 2006). The identification of these genes thus provides an important validation of the physiological model and the analytical techniques used herein.

Other genes identified by the SSH procedure are already known to be expressed in granulosa cells of ovarian follicles, but no report has yet indicated that these genes are differentially expressed during follicular development in sheep. This group includes GSTA1, SERPINE2, LAMPT4A, and ARFGAP3. GST is a multigene family of related proteins that have been divided to five classes of alpha, mu, pi, sigma, and theta. The biological action of GSTs is to provide protection against cellular oxidative stress (Ketterer, 1998). Expression of GSTA is associated with granulosa cells and theca cells, and that levels of GSTA mRNAs are modulated by FSH and LH in bovine follicles during the pre-ovulatory period (Rabahi et al., 1999). Expression of GSTA isoenzymes may be linked to cell types involved in steroid synthesis or metabolism and could be related to cellular oxidative stress induced by reactive oxygen species generated during steroid metabolism (Ketterer, 1998). Because steroidogenic activity increases as follicular development occurs, an increase in GSTA mRNA in healthy growing dominant follicles compared to small follicles would be expected (Fayad et al., 2004). In the present study, GSTA1 expression was found to be significantly higher in granulosa cells of LF compared to these in SF. Increased expression of GSTA1 by granulosa cells of LF in the present study may be partially responsible for the cellular oxidative stress induced by reactive oxygen species generated during steroidogenesis.

Previous studies have displayed SERPINE2 mRNA is regulated in a spatio-temporal pattern with highest steady state levels in granulosa cells of growing dominant follicles and high expression of SERPINE2 may contribute to follicular growth in cattle (Bédard et al., 2003; Fayad et al., 2004). Expression and protein secretion of SERPINE2 are hormonally regulated by FSH (Cao et al., 2006). Estradiol and SERPINE2 secretion were highly positively correlated, but estradiol did not alter SERPINE2 expression (Cao et al., 2006). These results indicates that SERPINE2 is involved in the regulation of atresia. The LAPTM4A shares a number of characteristics with other lysosome-associated proteins namely LAPTM4B and LAPTM5, which are thought to be involved in regulation of cell proliferation and cell survival. In human and mouse species, LAPTM4A protein is relatively abundant in the lysosomal membrane fraction (Hogue et al., 2002). Recently, the mRNA of LAPTM4B was demonstrated to be expressed in bovine granulosa cells of dominant follicles (Ndiaye et al., 2005a). To our knowledge, this is the first characterization of LAPTM4A gene expression in the ovine ovary and first evidence for this gene involving in follicle development.

In our study, 14 ESTs did not match any homologous sequences in the GenBank. In additional, the majority of the unique genes we identified appear homologized to bovine known gene and several expected follicular development related-genes uncovered in this study. There are many reasons for these. On the one hand, it is likely to no orthologs existing in the GenBank, sequence conservation among ovine genes and those in the GenBank was generally low. On the other hand, these are related

to its low level of expression, to the small difference in level of expression between large follicles and small follicles, and to additional sequencing of the library.

In summary, we have identified several genes that may contribute toward understanding the mechanisms involved with final ovarian follicular growth, selection, dominance, and ovulation in ewes. It will be necessary to further analyze their spatio-temporal expression pattern at the mRNA and protein levels in the developing ovarian follicle.

Acknowledgments

The authors thank Center of analysis & measurement of Zhejiang University for providing iCycler iQ Real-time PCR Detection System. This research was supported by grants from Zhejiang Science and Technology Committee (Grant Agreement No. 2006C12037).

References

Barid, D.T., Campbell, B.K., 2001. Follicle selection in sheep with breed differences in ovulation rate. Mol. Cell. Endocrinol. 145, 89–95.

Bartlewski, R.M., Beard, A.P., Cook, S.J., Chandolia, R.K., Honaramooz, A., Rawlings, N.C., 1999. Ovarian antral follicular dynamics and their relationships with endocrine variables throughout the oestrous cycle in breeds of sheep differing in prolificacy. J. Reprod. Fertil. 115, 111–124.

Bédard, J., Brûlé, S., Price, C.A., Silversides, D.W., Lussier, J.G., 2003. Serine protease inhibitor-E2 (SERPINE2) is differentially expressed in granulosa cells of dominant follicle in cattle. Mol. Reprod. Dev. 64, 152–165.

Berkholtz, C.B., Lai, B.E., Woodruff, T.K., Shea, L.D., 2006. Distribution of extracellular matrix proteins type I collagen, type IV collagen, fibronectin, and laminin in mouse folliculogenesis. Histochem. Cell. Biol. 126, 583–592.

Borowczyk, E., Johnson, M.L., Bilski, J.J., Borowicz, P., 2006. Gap junctional connexin 37 is expressed in sheep ovaries. Endocrine 30, 223–230.

Cao, M., Nicola, E., Porteal, V.M., Price, C.A., 2006. Regulation of serine protease inhibitor-E2 and plasminogen activator expression and secretion by follicle stimulating hormone and growth factors in non-luteinizing bovine granulosa cells in vitro. Matrix Biol. 25, 342–354.

Chen, A.Q., Yu, S.D., Wang, Z.G., Xu, Z.R., Yang, Z.G., 2009. Stage specific expression of bone morphogenetic protein type I: effects of follicle-stimulating hormone on ovine antral follicles. Anim. Reprod. Sci. 111, 391–399.

Diouf, M.N., Lefebvre, R., Silversides, D.W., Sirois, J., Lussier, J.G., 2006. Induction of alpha-caveolin-1 (alpha CAV1) expression in bovine granulosa cells in response to an ovulatory dose of human chorionic gonadotrophins. Mol. Reprod. Dev. 73, 1353–1360.

Duggavathi, R., Janardhan, K., Singh, J., Singh, B., Barrett, D.M.W., Davies, K.L., Bagu, E.T., Rawlings, N.C., 2006. Patterns of expression of steroidogenic enzymes during the first wave of the ovine estrous cycle as compared to the preovulatory follicle. Anim. Reprod. Sci. 91, 345–352.

Engelhardt, H., Smith, K.B., McNeilly, A.S., Baird, D.T., 1993. Expression of mRNA for inhibin/activin subunits and ovarian secretion of inhibin and oestradiol throughout the sheep oestrous cycle. Biol. Reprod. 49, 281–294.

Espey, L.L., Richards, J.S., 2002. Temporal and spatial patterns of ovarian gene transcription following an ovulatory dose of gonadotropin in the rat. Biol. Reprod. 67, 1662–1670.

Evans, A.C.O., 2003. Ovarian follicle growth and consequences for fertility in sheep. Anim. Reprod. Sci. 78, 289–306.

Fayad, T., Levesque, V., Sirois, J., Silversides, D.W., Lussier, J.G., 2004. Gene expression profiling of differentially expressed genes in granulosa cells of bovine dominant follicles using suppression subtractive hybridization. Biol. Reprod. 70, 523–533.

Ginther, O.J., Kot, K., Wiltbank, M.C., 1995. Associations between emergence of follicular waves and fluctuations in FSH concentrations during the estrous cycle in ewes. Theriogenology 43, 689–703.

Gonzalez-Bilska, A.T., Navanukraw, C., Johnson, M.L., Arnold, D.A., Reynolds, L.P., Remder, D.A., 2006. Expression of endothelial nitric oxide synthase in the ovine ovary throughout the estrous cycle. Reproduction 132, 579–587.

Hastie, P.M., Haresign, W., 2006. Expression of mRNAs encoding insulin-like growth factor (IGF) ligands, IGF receptors and IGF binding proteins during follicular growth and atresia in the ovine ovary throughout the oestorus cycle. Anim. Reprod. Sci. 92, 284–299.

Hastie, P.M., Onagbesan, O.M., Haresign, W., 2004. Co-expression of messenger ribonucleic acids encoding IGF-I, IGF-II, type I and IIIGF receptors and IGF-binding proteins (IGFBP-1 to -6) during follicular development in the ovary of seasonally anoestrous ewes. Anim. Reprod. Sci. 84, 93–105.

Hogue, D.L., Nash, C., Ling, V., Hobman, T.C., 2002. Lysosome-associated protein transmembrane 4 alpha (LAPTM4 alpha) requires two tandemly arranged tyrosine-based signals for sorting to lysosomes. Biochem. J. 365 (Pt3), 721–730.

Ketterer, B., 1998. Glutathione S-transferases and prevention of cellular free radical damage. Free Radic. Res. 28, 647–658.

Le Bellego, F., Pisselet, C., Huet, C., Monget, P., Monniaux, D., 2002. Laminin-alpha(6)beta(1) integrin interaction enhances survival and proliferation and modulates steroidogenesis of ovine granulosa cells. J. Endocrinol. 172, 45–59.

McNatty, K.P., Heath, D.A., Hudson, N., Clarke, I.J., 1990. Effect of long-term hypophysectomy on ovarian follicle populations and gonadotrophin-induced adenosine cyclic 3′,5′-monophosphate output by follicles from Booroola ewes with or without the F gene. J. Reprod. Fertil. 90, 515–522.

Ndiaye, K., Carriere, P., Sirois, J., Silversides, D.W., Lussier, J.G., 2005a. Molecular characterization of the lysosomal-associated protein transmembrane-4 beta (LAPTM4B) reveals a differential expression pattern in granulosa cells of bovine dominant follicles. Biol. Reprod. SI, 197 (Abstract).

Ndiaye, K., Fayad, T., Silversides, D.W., Sirois, L., Lussier, J.G., 2005b. Identification of downregulated messenger RNAs in bovine granulosa cells of dominant follicles following stimulation with human chorionic gonadotropin. Biol. Reprod. 73, 324–333.

Picton, H.M., McNeilly, A.S., 1991. Effect of basal and pulsatile LH release on FSH-stimulated follicle growth in ewes chronically treated with gonadotrophin-releasing hormone agonist. J. Enodrinol. 128, 449–456.
Picton, H.M., Tsonis, C.G., McNeilly, A.S., 1990. FSH causes a time-dependent stimulation follicle growth in ewes chronically treated with gonadotrophin- releasing hormone agonist. J. Enodrinol. 126, 297–308.
Purinton, S.C., Wood, C.E., 2002. Oestrogen augments the fetal ovine hypothalamus–pituitary–adrenal axis in response to hypotension. J. Physiol. 544, 919–929.
Rabahi, F., Brule, S., Sirois, J., Beckers, J.F., Silversides, D.W., Lussier, J.G., 1999. High expression of bovine alpha glutathione S-transferase (GSTA1, GSTA2) subunits is mainly associated with steroidogenically active cells and regulated by gonadotropins in bovine ovarian follicles. Endocrinology 140, 3507–3517.
Rosales-Torres, M.A., Avalos-Rodríguez, A., Vergara-Onofre, M., Hernández-Pérez, O., Ballesteros, L.M., García-Macedo, R., Ortíz-Navarrete, V., Rosado, A., 2000. Multiparametric study of atresia in ewe antral follicles: histology, flow cytometry, internucleosomal DNA fragmentation, and lysosomal enzyme activities in granulosa cells and follicular fluid. Mol. Reprod. Dev. 55, 270–281.
Sarit, F., Ada, D., Abraham, A., 2005. Ovarian transcriptomes as a tool for a global approach of genes modulated by gonadotropic hormones in human ovarian granulosa cells. Endocrine 26, 259–265.
Seol, H.S., Sato, K., Murakami, H., Toyomizu, M., Akiba, Y., 2006. Changes in gene expression involved in energy utilization during chicken follicle development. Anim. Reprod. Sci. 95, 283–294.
Wood, G.E., Giroux, D., 2003. Central nervous system prostaglandin endoperoxide synthase-1 and -2 responses to oestradiol and cerebral hypoperfusion in late-gestation fetal sheep. J. Physiol. 549, 572–581.