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Screening of Puberty Related Differentially Expressed Genes from Ovary Tissues of Jining Grey Goat Based on Suppression Subtractive Hybridization

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Key words
Puberty, Ovary, Goat, Suppression subtractive hybridization, Differentially expressed genes

ABSTRACT

To explore the molecular mechanism of the puberty effects on the reproduction of goats, goats at different growth stages from two breeds (Jining Grey (JG) goats and Liaoning Cashmere (LC) goats) were divided into three groups with three replicates (juvenile stage (JG 30d vs. LC 30d, AO group), puberty (JG 90d vs. LC 180d, BO group) and the same age control of puberty (JG 90d vs. LC 90d, EO group). Ovary tissues were taken from goats on 30 days, 90 days and 180 days, total RNA was extracted, differentially expressed (DE) gene libraries were constructed by suppression subtractive hybridization (SSH) technology, and puberty-related differential genes were screened. A total of 184 differentially expressed genes were screened, including 57, 68 and 59 in the AO, BO and EO groups, respectively. There are 22 differentially expressed genes were directly associated to the ovary development. According to the gene function analysis in KOG database, the known genes were clustered into 10 subdivisions in A group, 8 in E group and 9 in B group under three categories, mainly in posttranslational modification, protein turnover, chaperones, energy production and conversion, and transcription process. Pathway analysis in KEGG pathway database of the known genes revealed that the five pathways that most differentially expressed genes involving: GnRH signaling pathway, oxytocin signaling pathway, melanogenesis, thyroid hormone synthesis, and insulin secretion. Online tool was used to predict the function of DEGs, the results showed that the *CYP11A1* gene might regulate the *GDF9* and other prolificacy-associated markers in ovary organs of goats, thereby affecting the development of puberty. The expression of various genes and the transduction of hormone secretion signals, improving the reproduction of goats.

INTRODUCTION

Puberty is the process by which animals maturate into an adult capable of sexual reproduction, characterized by the maturation of gametogenesis, secretion of gonadal hormones. In human, onset of puberty occurs after reactivation of the hypothalamic GnRH secretory system (De Sanctis *et al*., 2019). At puberty, the pulsatile GnRH secretion, and the subsequent episodic pituitary gonadotropin secretion, necessary for normal gonadal development and function, is triggered by the activation of the GnRH pulse generator (Dulka *et al*., 2020). Where, the GnRH pulsatile secretion is dependent on the coordinated action of the scattered GnRH neurons, controlled by stimulatory and inhibitory inputs, such as neurokinin B, kisspeptins and gamma aminobutyric acid (Plant, 2015). In sheep, the pubertal onset is marked by a decrease in estradiol-negative feedback, resulting in an increased frequency of GnRH, and subsequently LH, pulses and the increased estradiol production that in turn induces the subsequent GnRH/LH surge and first ovulation (Foster and Hileman, 2015). The immature female lamb is capable of generating requisite high-frequency GnRH/LH pulses, but this pattern is not produced because sensitivity to estradiol feedback inhibition is high (Moenter *et al*., 1991). Removal of steroid-negative feedback by ovariectomy

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(OVX) in the pre-pubertal ewe lamb results in an elevation of GnRH/LH secretion, which can readily be reduced with subcutaneous administration of E_2 (OVX+E lambs). This E_2-induced suppression reflects an inhibition of GnRH/LH pulse frequency that persists until the time when pubertal onset would normally occur (Foster and Hileman, 2015).

In summary, the mechanism of pubertal onset is complex and thought to be associated with environmental factors, neuroendocrine factors, genetic factors and their interactions (Smith, 2001). Understanding how puberty is timed is critical for developing practical strategies to synchronize lambing with market demand and for potentially decreasing the generation interval in farm animals, thereby increasing the overall production of food and fiber. The Jining Gray (JG) goat is a sexual precocious breed that reaches puberty at 60-90 days and the Liaoning Cashmere (LC) goat is a late-puberty breed that reaches puberty at 150-210 days. Our previous study showed that Jining Grey (JG) goats and Liaoning Cashmere (LC) goats provide the best materials to identify factors that regulate the maturation of the hypothalamic-pituitary-gonadal (HPG) axis (Cao *et al*., 2015). In this study, we selected the domestics JG and LC goats at different growth stages (30, 90 and 180 days) as the experimental animals. Suppression subtractive hybridization (SSH) is an effective method for isolation of specific DNA fragments that can be used to differentiate two closely related species (Diatchenko *et al*., 1996; Rebrikov *et al*., 2004). A key feature of this method is simultaneous normalization and subtraction steps that respectively equalize the abundance of DNA fragments within the target population and exclude sequences common to the two populations being compared (Gurskaya *et al*., 1996). We have therefore used the SSH method to isolate puberty-related genes in goat ovaries. These results increase our understanding of the physiological processes involved in goat reproduction. In particular, the differently expression genes in the puberty may play a role in the modulation of the ovary development.

MATERIALS AND METHODS

Ethics approval

All experimental procedures mentioned in the present study were approved by the Science Research Department (in charge of animal welfare issue) of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (IAS-CAAS) (Beijing, China). Ethical approval was provided by the animal ethics committee of IASCAAS (No. IASCAAS-AE-03).

Animals and tissue collection

All the Jining Grey (JG) goats and Liaoning Cashmere (LC) goats displayed in Table I in this study were housed in open sheepfolds and under the same nutrition condition. The ovaries tissues were collected from each goat, sacrificed after anesthesia with 3% pentobarbital sodium salt injection (20mg/kg body weight) (Merck, Darmstadt, Germany), and preserved in RNA later RNA Stabilization reagent (Qiagen, Hilden, Germany) and kept at -20°C until RNA isolation.

Table I. The detail information of experiment samples.

Goat breed	Age	Abbreviation	Number	Development stage
Jining gray goats	30-day-old	30-JG	3	Juvenile
	90-day-old	90-JG	3	Puberty
Liaoning cashmere goats	30-day-old	30-LC	3	Juvenile
	90-day-old	90-LC	3	Juvenile
	180-day-old	180-LC	3	Puberty

RNA extraction and concentration of cDNA libraries

Total RNA of ovaries was isolated using Trizol Reagent (Invitrogen, Inc. USA) according to the manufacturer's instructions and the quantity and quality were determined by electrophoresis and spectrometry. The equal quantity total RNA from three goats in each group were mixed together and used for poly A^+ RNA purification using the Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany). After that, the poly A^+ RNA was concentrated using RNA clean and concentration-5 mRNA (Zymo Research, Orange, CA, USA) to a suitable concentration.

Construction of SSH libraries

The regents for SSH were from PCR-Select cDNA Subtraction Kit (Clontech, palo alto, CA) and the procedure were carried out according to the protocols. Simply, the double strand cDNA (dscDNA) was synthesized from 2 μg poly A^+ RNA of tester and driver respectively and then were digested by *Rsa* I restriction endonuclease to obtain shorter, blunt-ends dscDNA fragments which were required for adaptor ligation and optimal for subtraction. After analysis of *Rsa* I digestion, the digested blunt-ends of tester cDNA were divided into two parts and ligated with two different cDNA adaptors (adaptor 1 ATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′ and adaptor 2R 5′-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT- 3′), respectively and adaptor will not be ligated to the driver dscDNA. After ligation, the ligation efficiency analysis was performed by PCR experiment with *GAPDH* primers (GAPDHF: 5′- AGGCTGGGGCTCACTTGAAG-3′, GAPDHR: ATGGCGTGGACAGTGGTCAT-3′) (goat GAPDH mRNA sequence-Genbank:

AJ431207) and PCR primer I (5′-CTAATACGACTCAC-TATAGGGC-3′) using the Advantage cDNA PCR kit (Clontech, palo alto, CA).

Two cycles of hybridization were followed after the ligation. In the first hybridization, 1.5 μL Rsa I-Digested Driver cDNA and 1.5μL Adaptor-Ligated Tester were hybridized in 1.0 μL 4×Hybridization buffer at 68°C for 8 hours. For the second hybridization process, 1.0 μL fresh denatured driver cDNA, 1.0 μL 4×Hybridization Buffer and 2.0 μL ddH_2O were then mixed with the two samples from the first hybridization simultaneously and incubated at 68°C overnight.

The final hybridization solution (also called the subtracted library) was employed as a template to amplify the differentially expressed sequences in the tester population by using a set of PCR primer1 and was followed by nested PCR primers (Nested PCR primer 1 5′-TCGAGCGGCCGCCCGGGCAGGT-3′, Nested PCR primer2R 5′-AGCGTGGTCGCGGCCGAGGT-3′), which does not exponentially amplify the non-adaptor (derived from driver cDNA), cDNA with the one adaptor on either end (derived from tester cDNA hybridized with driver cDNA), or cDNA with the same adaptor on both ends (derived from relatively abundant tester cDNA). For the primary PCR, 1μL sample was added to 24 μL PCR master mix prepared using the reagents supplied in the kit, and cycling conditions commenced as follows: 75°C for 5 min to extend the adaptors; 94°C for 25 sec; and 27 cycles at 94°C for 10 sec, 66°C for 30 sec, and 72°C for 1.5 min. Amplified products were diluted 10-fold in sterile water and 1 μL of diluted primary PCR products were added to 24 μL of secondary PCR master mix containing nested primers, 1 and 2R, to ensure specific amplification of double-stranded templates containing both adaptors. Secondary PCR was performed 12 cycles at 94°C for 10 sec, 68°C for 30 sec, and 72°C for 1.5 min. Primary and secondary PCR products were analyzed on a 2% agarose gel. And now the PCR mixture is enriched for differentially expressed cDNA in tester sample.

Cloning, sequencing and bioinformatics analysis

3 μL of PCR products purified using DNA clean and concentration-5 (Zymo Research, Orange, CA, USA) was ligated into pGEM-T Easy Vector (Promega, Madison, WI, USA) and transformed into the competent *E. coli* DH5α. Most white clones were picked up and cultured for PCR and sequencing. Positive mono clones were detected by PCR and sequenced by Invitrogen Corporation. The vector and adaptor sequence of sequences obtained by sequencing were remove using the UniVec database (http://www.ncbi.nlm.nih.gov/VecScreen/) to get clean sequence.

The differentially expressed ESTs were classified into known genes, known ESTs and unknown ESTs according to homologies analyzed by BLAST (www.ncbi.nlm.nih.gov/blast/blast.cgi). The differentially expressed genes (DEGs) function predicted in the Cluster of Orthologous Groups of proteins database (COG) (http://www.ncbi.nlm.nih.gov/COG/) and were classified according to the COG database. Then the DEGs were submitted to the KEGG pathway database (http://www.genome.jp/kegg/pathway.html) to analysis which pathway they participate in. The protein-protein interactions were predicted in the STRING (Search Tool for Retrieval of Interacting Genes/Proteins) net (http://string.embl.de/).

RESULTS

Construction of cDNA libraries

The results of 1.2% agarose gel electrophoresis of part of double-stranded cDNA products under different cycles are shown in Figure 1. Treated group have the brightest bands and the widest range in 23 cycles. Control group have the brightest bands in 33 cycles, but the widest range in 23 cycles. As can be seen from the figure, the optimal cycles for both treated group and control group was 23.

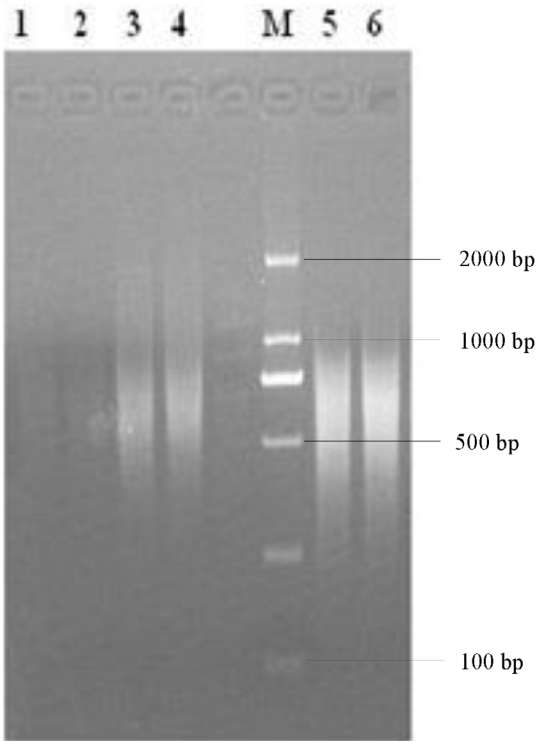

Fig. 1. Agarose electrophoresis analysis of optimal cycles of double-stranded cDNA. Electrophoresis with agarose of 1.2% concentration. M: DNA marker DL 2000 (100 bp, 250 bp, 500 bp, 750 bp, 1000 bp, 2000 bp).

Table II. The SSH groups arrangement.

Hybridization Groups	Tester	Driver
A	30-JG	30-LC
B	90-JG	180-LC
E	90-JG	90-LC

Table III. The numbers of differentially expressed ESTs in ovary from A, E and B group.

	A group	E group	B group
Known gene	62 (57.4%)	74 (69.8%)	127 (84.1%)
Known EST	45 (41.7%)	31 (29.2%)	23(15.2%)
Unknown EST	1(0.9%)	1(1.0%)	1(0.07%)
Total	108	106	151

*The number in the bracket represents the percent.

Homologies analysis of differentially expressed ESTs

108, 106 and 151 differential expressed ESTs were obtained in A group, E group and B group, respectively after the positive clones were sequenced. Total 365 EST were submitted to NR, KEGG and UNIPROT database for sequence alignment. The homology with goat, sheep, cattle, human, pig, mouse and rat were recorded and the ESTs were classified. The ESTs were classified into known gene, known EST and unknown EST, as shown in Table III, and the percentage of known gene of juvenile group is the least. In group A, the 62 ESTs of known genes represent 57 transcripts of genes, the 74 ESTs of known genes represent 59 transcripts of genes in group E, and the 127 ESTs of known genes represent 68 transcripts of genes in B group.

GO and KOG classification

Upon GO functional enrichment, 124, 68, and 55 specific GO terms in biological process, molecular function and cellular component were identified in the three groups, respectively (Fig. 2). Most DEGs in the three groups are enriched in cell, organelle, and cell part in cellular component and metabolic process and cellular process in biological process. In the cellular component classification, most DEGs are enriched in cell, cell part and organelle. In molecular function classification, most DEGs are enriched in binding, structural molecule activity and catalytic activity and metabolic process, cellular process, single-organism process are the top three subdivisions with DEGs in the biological process. The known genes in the three groups were submitted into KOG database. According to the gene function analysis in the database, the known genes were grouped into 13 divisions in A group, 15 in E group and 14 in B group (Fig. 3).

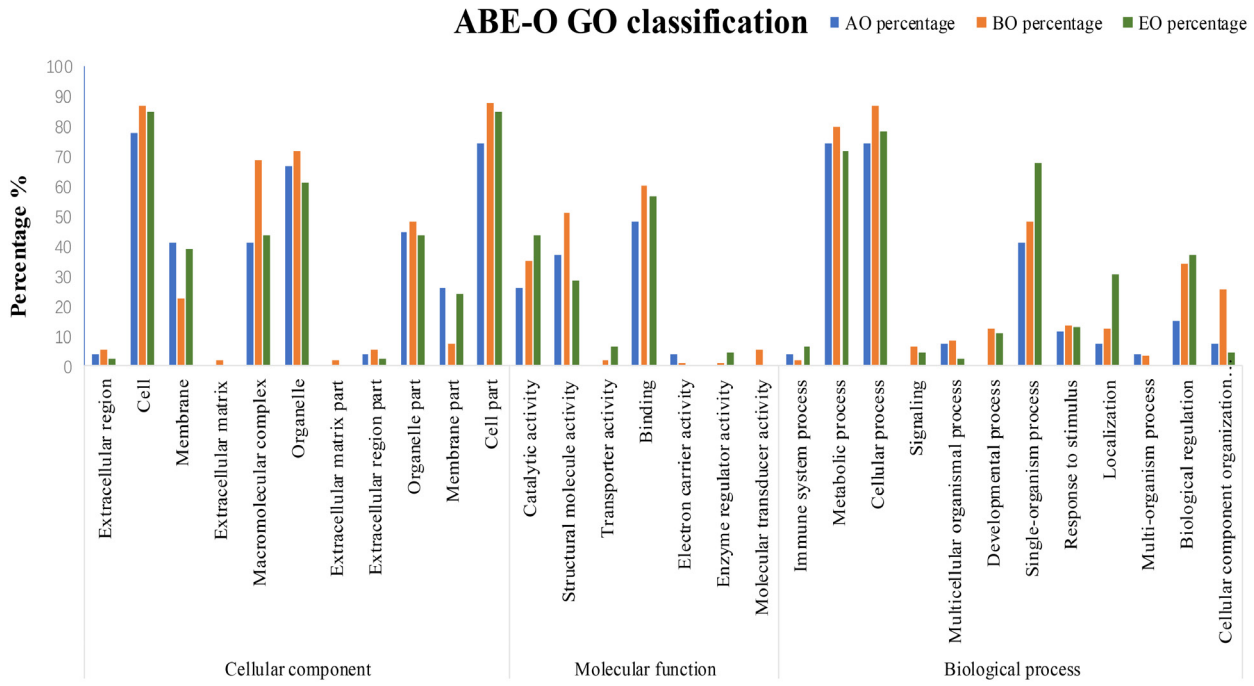

Fig. 2. GO classification of differential expression genes from the three groups of ovaries. AO, BO and EO represent the three groups in Table IV, respectively.

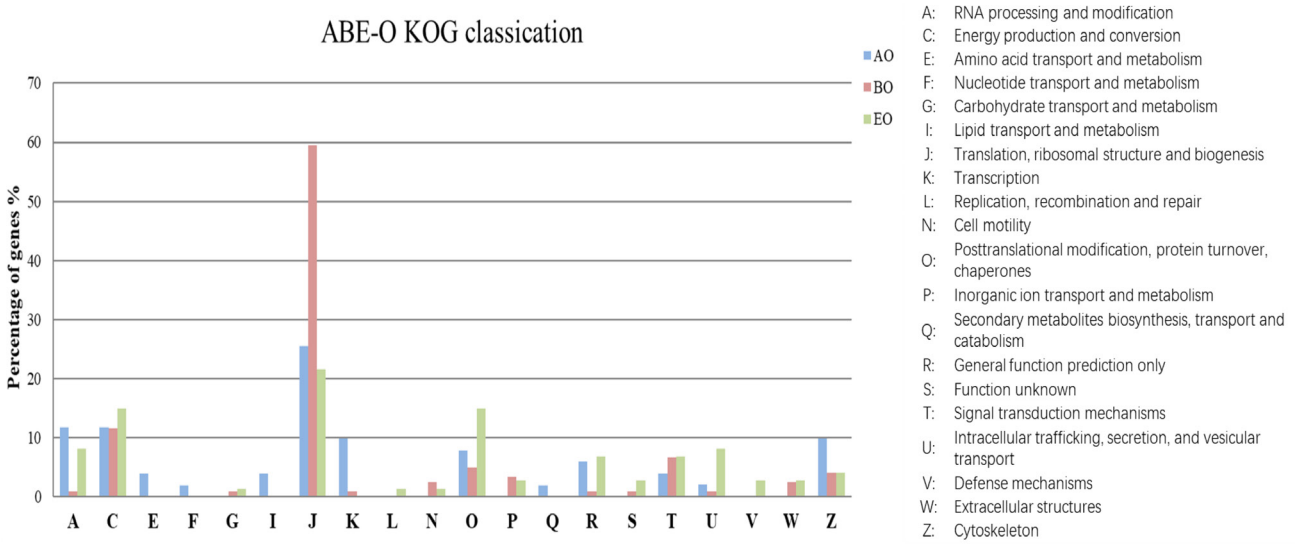

Fig. 3. KOG classification of differential expression genes from the three groups of ovaries. AO, BO and EO represent the three groups in Table II, respectively.

Table IV. The genes enriched in different KOG function in the three SSH groups.

KOG function	AO	EO	BO
Energy production and conversion	ND4, ATP6, CYTB	ATP5H, NDUFA9, ND4, COX1, ND4L, ATP6, ATP5A1	ND4, COX2, COX1, ND2, CYTB, ATP6, LDHB, ATP5B
RNA processing and modification	RPL4, DROSHA, NOP58	FUBP1, RPL4, SRSF11	RPL4
Past-transcription modification			
Amino acid transport and metabolism	IDH3B, SHMT2		
Nucleotide transport and metabolism	IMPDH2		
Lipid transport and metabolism	TECR, PLA2G7		
Transcription process	EEF1B2, IKBKAP, ASCC1, NCOR1		
Cytoskeleton	ACTG2, PNN, ARPC5L		ACTB, MYLK, ARPC2,RPS15A
Cell motility		Thymosin beta-b	Thymosin beta-b
Inorganic ion transport		FTH1	ATP1B3, FTH1
Extracellular structures		SPARC	COL3A1, LAMA4
Signal transduction	GNB2L1, FBLN1	GNB2L1, RGS2, LTBP1, PDLIM3	GNAS, GNB2L1
Posttranslational modification, protein turnover, chaperones	OSGEP, POMP, PSMD6, HSP90AA1	YWHAB, C3, YWHAQ, HSP90B1, GDI1, HSC71, HSP90AA1, PPIB	GSTM2, PPIB, RPN1, HSPA8, C3
Intracellular trafficking, secretion, and vesicular transport	TEMD10	CLTA, TM9SF3, COPA, TIMM8B, KDELR3	ARL4A

KEGG pathway analysis

The differentially expressed known genes in the three groups were submitted to KEGG Pathway database (Fig. 4 and Table V). The results revealed that most pathways that differentially expressed known genes involved in were metabolic pathways, Parkinson's disease, oxidative phosphorylation, Huntington's disease, Alzheimer's disease and GnRH signaling pathway. The

most three pathways that the DEGs in AO, EO, BO groups participated are ribosome, oxidative phosphorylation, and Parkinson's disease. Some KEGG pathways in the three groups are related with reproduction, for instance, ovarian steroidogenesis, estrogen signaling pathway, oocyte meiosis, GnRH signaling pathway, melanogenesis. In addition, the DEGs in EO and BO groups participate in progesterone-mediated oocyte maturation. Parts of DEGs in EO are related with oocyte meiosis and some DEGs in BO group are related with oxytocin signaling pathway and melanogenesis. Some pathways are related with energy metabolism, including fatty acid metabolism, glycolysis/ gluconeogenesis, PI3K-Akt signaling pathway, insulin signaling pathway, and mTOR signaling pathway.

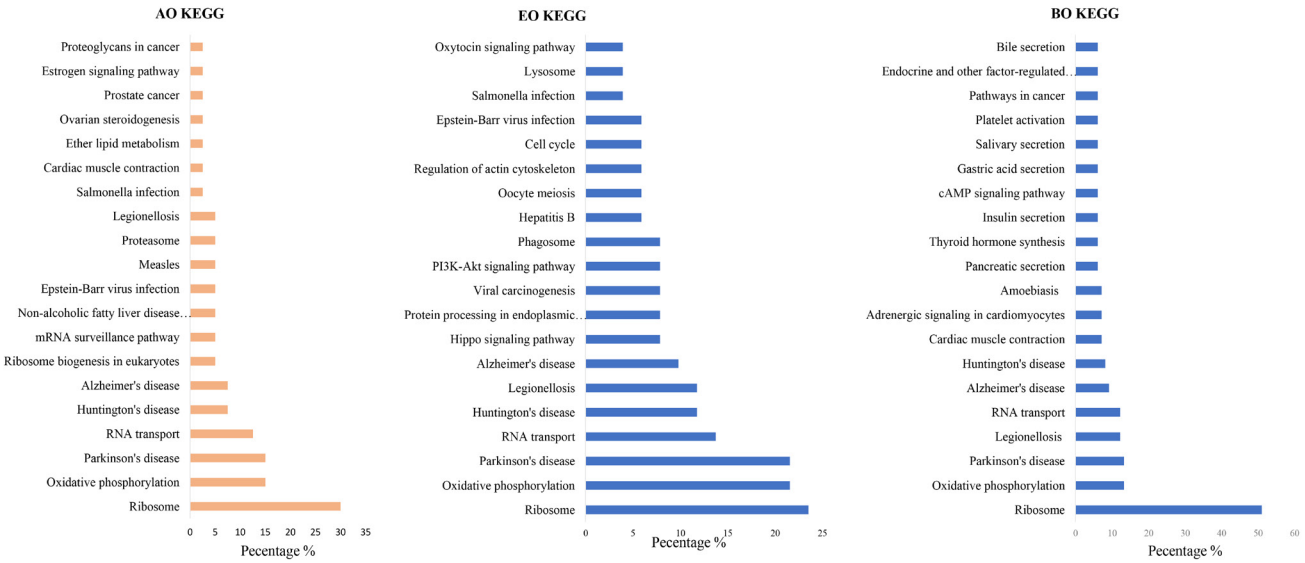

Fig. 4. KEGG pathway enrichment analysis of SSH differential genes. AO, BO and EO represent the three groups in Table II, respectively.

Table V. Parts of KEGG pathways in the three SSH groups.

AO	EO	BO
Ovarian steroidogenesis		Ovarian steroidogenesis
Estrogen signaling pathway	Estrogen signaling pathway	Estrogen signaling pathway
Steroid hormone biosynthesis	Oocyte meiosis	Oxytocin signaling pathway
	Progesterone-mediated oocyte maturation	Progesterone-mediated oocyte maturation
		GnRH signaling pathway
		Melanogenesis
		Circadian entrainment
Fatty acid metabolism	Galactose metabolism	Glycolysis / Gluconeogenesis
Biosynthesis of unsaturated fatty acids	Amino sugar and nucleotide sugar metabolism	Carbohydrate digestion and absorption
Fatty acid elongation	Glycolysis / Gluconeogenesis	N-Glycan biosynthesis
	Starch and sucrose metabolism	
	Pentose phosphate pathway	
		Insulin secretion
		Insulin signaling pathway
MAPK signaling pathway		mTOR signaling pathway
PI3K-Akt signaling pathway	PI3K-Akt signaling pathway	PI3K-Akt signaling pathway
	Glutathione metabolism	Glutathione metabolism

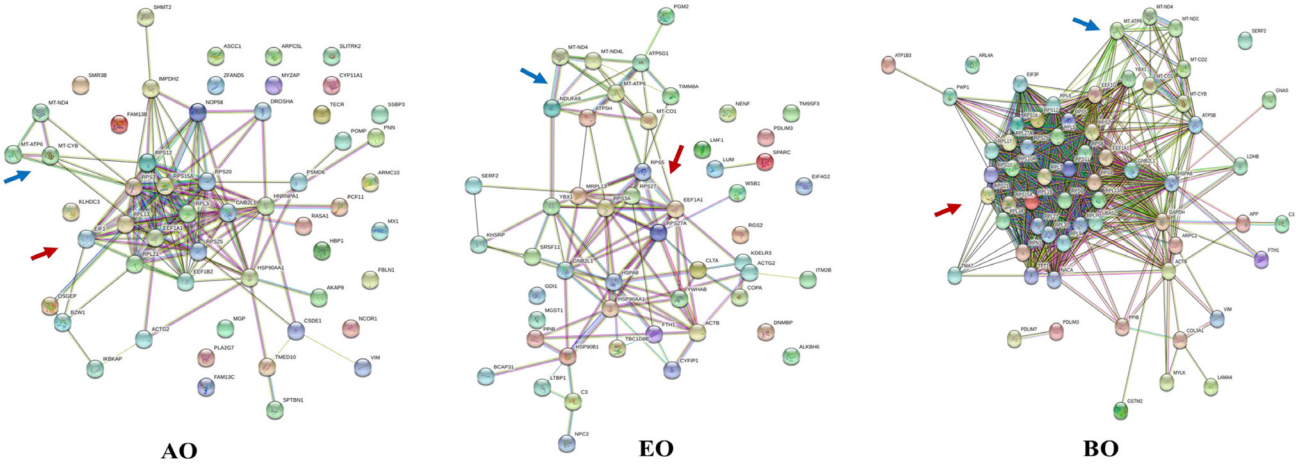

Fig. 5. The protein-protein interactions of differential expression genes from the three group of ovaries. Genes in the first red circle participate in ribosome/protein translations, genes in the second red circle participate in oxidative phosphorylation and genes in the third red circle participate in carbohydrate metabolism.

The pathways that DEGs in AO group participated are related with fat metabolism, such as fatty acid metabolism and biosynthesis of unsaturated fatty acids, while, that in EO and BO groups are related with glucose metabolism, for instance, glycolysis/gluconeogenesis, carbohydrate digestion and absorption, pentose phosphate pathway. Carbohydrate metabolism provide much energy to body and energy obtained from metabolism is usually stored temporarily within cells in the form of ATP.

Protein-protein interaction analysis

The protein interaction of DEGs in A, E and B group analyzed in STRING database were showed in Figure 5. The most obvious interaction network in the three groups is the structure of ribosome/protein translation, especially in the BO group. more proteins are need in the ovary of JG goats than that in the ovary of LC goats, even all are pubertal goats. The numbers of DEGs related with the in oxidative phosphorylation EO and BO group are more than that in AO group. The oxidative phosphorylation is the metabolic pathway in which the mitochondria in cells use their structure, enzymes, and energy released by the oxidation of nutrients to reform ATP which is the molecule that supplies energy to metabolism. This pathway is a highly efficient way of releasing energy. So, these may indicate that more genes related with energy production and storage is expressed in JG goat than that in LC goat in these stages.

Twenty-two candidate genes were related to ovary development directly or indirectly (Table VI). The STRING database was used to predict the relationship between candidate genes and *GDF9* and other prolificacy-associated markers in goat, cattle, mouse and human. The results showed that *CYP11A1* gene was interacted with *GDF9* and *BMP15* genes whatever in the four species (Fig. 6).

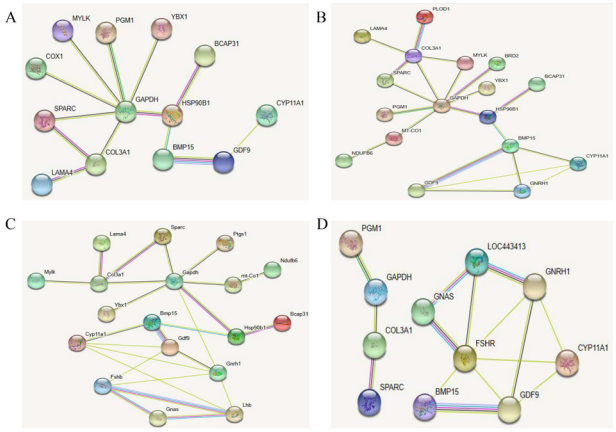

Fig. 6. The relationship prediction between *CYP11A1* and prolificacy-associated markers among four species. A: cattle; B: human; C: mouse; D: goat.

DISCUSSION

In this study, SSH technology was used to construct the differential gene libraries of different growth stages of ovaries in two breed goats. Through sequencing, the SSH library of AO group contains 108 ESTs, 52.8% of which were known genes. There were 106 ESTs in the SSH library of EO group, 55.66% of which were known genes. There were 151 ESTs in the SSH library of BO group, 45.03%

Table VI. Puberty-related important DEGs among three groups.

Gene name	Description	References
CYP11A1 (cytochrome P450 family 11 subfamily A member 1)	Cleavage of cholesterol to pregnenolone, the precursor of most steroid hormones	Terry *et al*., 2010; Wickenheisser *et al*., 2012; Meng-Chun *et al*., 2018; Moravek *et al*., 2016
GNAS	Involved as modulators or transducers in various transmembrane signaling systems	Nicolas *et al*., 2013; Hirofumi Ando *et al*., 2016
BCAP31 (B cell receptor associated protein 31)	Involved in the anterograde transport of membrane proteins from the endoplasmic reticulum to the Golgi and in caspase 8-mediated apoptosis	Preethi *et al*., 2015
HSP90B1 (heat shock protein 90 beta family member 1)	Expression of this protein is associated with a variety of pathogenic states, including tumor formation.	Li *et al*., 2016; Christophe *et al*., 2011
GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	Catalyzes an important energy-yielding step in carbohydrate metabolism	Liu *et al*., 2020
mt-co1 (mitochondrially encoded cytochrome c oxidase I)	Cooperate to transfer electrons derived from NADH and succinate to molecular oxygen	Zhen *et al*., 2015; Ghaffari *et al*., 2015
NDUFB6 (NADH: ubiquinone oxidoreductase subunit B6)	Transfers electrons from NADH to the respiratory chain	Schutt *et al*., 2019
SPARC (secreted protein acidic and cysteine rich)	Promote tumor cell invasion	Tumbarello *et al*., 2016; John *et al*., 2019
LAMA4 (laminin subunit alpha 4)	Related to cell adhesion, differentiation, migration, signaling, neurite outgrowth and metastasis	Liu *et al*., 2020
Mylk (myosin light chain kinase)	Facilitate myosin interaction with actin filaments to produce contractile activity	Wallace *et al*., 2019
COL3A1 (collagen type III alpha 1 chain)	Mutations in this gene are associated with Ehlers-Danlos syndrome types IV, and with aortic and arterial aneurysms.	Engqvist *et al*., 2019
YBX1 (Y-box binding protein 1)	Aberrant expression of the gene is associated with cancer proliferation in numerous tissues.	Campbell *et al*., 2017; Lv, 2020
PGM1 (phosphoglucomutase 1)	Mutations in this gene cause glycogen storage disease type 14.	Balakrishnan *et al*., 2019
COX1 (cytochrome c oxidase subunit I)	Cooperate to transfer electrons derived from NADH and succinate to molecular oxygen	Barnard *et al*., 2018
EIF3F (eukaryotic translation initiation factor 3 subunit F)	Specifically targets and initiates translation of a subset of mRNAs involved in cell proliferation	Rafael *et al*., 2018
SLITRK2 (SLIT and NTRK like family member 2)	Involved in synaptogenesis and promotes excitatory synapse differentiation	Chen *et al*., 2020
ZFX (zinc finger protein X-linked)	As a transcriptional regulator for self-renewal of stem cell types	Song *et al*., 2020
JMJD6 (Jumonji domain containing 6, arginine demethylase and lysine hydroxylase)	Identified as a putative phosphatidylserine receptor involved in phagocytosis of apoptotic cells	Siu *et al*., 2009
RGS2 (regulator of G protein signaling 2)	Acts as a mediator of myeloid differentiation and may play a role in leukemogenesis	Jiang *et al*., 2016; Matsuo *et al*., 2013
Ikbkap (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein)	Involved in neurogenesis.	Yang *et al*., 2019
bzw1 (basic leucine zipper and W2 domains 1)	Enhances histone H4 gene transcription but does not seem to bind DNA directly.	Xu *et al*., 2020; Liu *et al*., 2018
rpl4-b (60S ribosomal protein L4-B)	Translates the encoded message by selecting cognate aminoacyl-transfer RNA (tRNA) molecules	Kolkova *et al*., 2013

of which were known genes. At the same time, the KOG annotation of these libraries showed that the most DEGs are enriched in the translation, ribosomal structure and biogenesis, especially in the B group (59.5%). In addition, 11.77%, 14.7% and 14.87% DEGs are related with energy production and conversion. These may indicate that more genes expressed in ovaries used for energy production and conversion in juvenile and pubertal JG goats than that in LC goats. The percentages of DEGs enriched in each KOG function in the three groups are different. Compared with group B (0.83%), more genes related with RNA processing and modification expressed in the ovaries of juvenile JG goat (A group, 11.77%) and pubertal JG goat (E group, 8.11%) group, which may indicate that the biology process in ovaries of juvenile and pubertal JG goats need more transcripts participating in past-transcription modification. 3.92%, 1.96% and 4% of the DEGs in A group are related with Amino acid, nucleotide and lipid transport and metabolism respectively, but no in E and B group. 9.8% of the DEGs in group A, but only 0.82% in B group and no in E group, take part in transcription process. And the percentage of DEGs related with cytoskeleton in A (9.8%) group is higher than that in B (4.13%) and E (4.05%) group. These may indicate that more active metabolism occurred in the juvenile JG goats' ovaries.

Also, the percentages of DEGs enriched in some KOG stem are higher in B and E group but lower or no in A group. Some DEGs related with cell motility are in B and E group but not in A group, maybe because that these DEGs have some roles in the follicle motility and development in the pubertal ovaries. 2.7%, 2.7% and 3.3%, 2.47% of the DEGs in E and B group are related with inorganic ion transport and metabolism, and but extracellular structures, respectively, but no in A group, and whether the related inorganic ions and the extracellular structures correlating with ovaries function need further research. The percentages of the DEGs related with signal transduction in E (6.76%) and B (6.61%) group are higher than that in A (3.92%) group. The percentage of the DEGs related with Posttranslational modification, protein turnover, chaperones, and intracellular trafficking, secretion, and vesicular transport in E (14.87%, 8.11%) group is higher than that in A (7.84%, 1.98%) and B (4.96%, 0.83%) group, respectively. These genes may have some undiscovered roles in ovaries among the onset of puberty, or some of them are unique to JG goats for their high prolificacy.

The KEGG pathway analysis of annotation genes showed that the leptin-insulin signal pathway and PI3K-Akt signaling pathway are found in the three groups. Besides this one, insulin secretion, insulin signaling pathway and mTOR signaling pathway are found related with DEGs in B group. These DEGs related with energy may have some undiscovered roles in the onset of puberty. Also, some DEGs in B and E groups take part in the glutathione metabolism pathway. Glutathione plays a key role in the onset of puberty and oocyte growth. The distribution of glutathione in the hypothalamus was increased to the maximum at puberty and decreased to the adult level thereafter. Administration of glutathione to immature female rats brings about a preponement of puberty. The pituitary, ovaries and uterine mass was increased significantly and the pituitary FSH levels were increased in ovariectomized-steroid primed rats (Vali Pasha *et al*., 1989; Vali-Pasha, 2007). The glutathione concentration in oocytes from prepubertal gilts was significantly higher than that in cyclic oocytes (Pawlak *et al*., 2015). Oocytes with higher glutathione concentration have more developmental advantage, such as the higher rate of male pronucleus formation, larger size and proper pattern of mitochondria distribution, and higher blastocyst yield (De Matos and Furnas, 2000; Wu *et al*., 2007; Jiao *et al*., 2013; Abazari-Kia *et al*., 2014). The DEGs in BO and EO group involved in the glutathione metabolism, but no in juvenile group, may indicated that the glutathione metabolism may have some effect on the high fecundity and/or precocious puberty of Jing Grey goats. These data are consistent with the possibility that the kisspeptin/GnRH network is intact before puberty but merely inhibited by heightened sensitivity to estradiol negative feedback. In the immature female sheep, preovulatory gonadotropin surges do not occur spontaneously. Although the surge system is capable of function from a very early age, it remains dormant until puberty because of inadequate tonic (pulsatile) LH secretion. Pulses of LH, along with FSH, are ultimately responsible for the production of estradiol by the ovarian follicle.

According to the information of frequencies in the SSH cDNA libraries, the functional information and location in the protein interaction, some important candidate genes were selected as the targets for further study. More differential expressed ESTs and more known gene were in group B than that in group A and E. Many genes appeared more than once in group B and E, and are most related with translation process. For example, *EEF1A* gene appeared 9 and 6 times in the ovaries of the B and E group; guanine nucleotide-binding protein, *alpha-stimulating activity polypeptide 1 (GNAS)* gene appeared 5 times in the B group but none in A and B group. *NADH dehydrogenase subunit 4(ND4)* gene appeared 3 and 4 times in B and E group. In B group, the genes that appeared more than once are more related with ribosomal protein.

GNAS gene, appeared 5 times in the BO group, participated in GnRH signaling pathway. The *GNAS*

gene encodes the α-subunit of the stimulatory guanine nucleotide-binding protein (Gsα), which transduces signals from a G protein-coupled receptor. *GNAS* has been shown to elevate intracellular cAMP levels by stimulating adenylyl cyclase, which provokes cellular proliferation through the protein kinase A-ERK signal pathway (Landis *et al*., 1989). Several studies reported that some McCune-Albright Syndrome (MAS) patients with mutation in *GNAS* gene displayed precocious puberty (Mariot *et al*., 2011). *GNAS* gene also have important role in oocyte mature. Gs-alpha activity in the oocyte is required to maintain meiotic arrest within the ovarian follicle (Mehlmann *et al*., 2006; Mehlmann, 2013). KEGG pathway analysis revealed that *GNAS* gene takes part in the GnRH signaling pathway in our results. So, *GNAS* may be an important gene for the onset of puberty.

Secreted protein acidic and rich in cysteine (SPARC) was originally identified as a bone-specific protein. SPARC is a secreted, multi-domain protein, containing an amino-terminal acidic domain that binds hydroxyapatite and calcium ions, a follistatin-like domain containing multiple cysteine residues, and a carboxy-terminal extracellular calcium-binding (EC) domain containing two EF-hand motifs (Sasaki *et al*., 1998). Exogenous *SPARC* can promote apoptosis in ovarian cancer cells and elevated *SPARC* expression has been shown to occur in the activated stroma surrounding ovarian tumors (Brown *et al*., 1999; Paley *et al*., 2000), leading to the suggestion that it may be modulating the ovarian tumor microenvironment through regulation of matrix metalloproteinases, inflammation, and pro-migratory cytokines (Said *et al*., 2007). So, *SPARC* gene may be an important gene for the onset of puberty in goat.

CYP11A1 encoded the cholesterol side chain cleavage enzyme (P450scc), and cleaves the cholesterol side chain, converting cholesterol to pregenolone, the precursor of androgens, estrogens, and progesterone (Hu *et al*., 2002; Terry *et al*., 2010). The pentanucleotide repeat in the 5' regulatory region of *CYP11A1* has been shown to be associated with ovarian androgen excess and polycystic ovary syndrome (PCOS) (Pusalkar *et al*., 2009). Elevated steady state *CYP11A1* mRNA abundance in PCOS cells results from increased transactivation of the *CYP11A1* promoter and increased *CYP11A1* mRNA stability (Wickenheisser *et al*., 2012). Meng-Chun Shih *et al*., found that the promoter mutation of *CYP11A1* did not decrease life span and caused no defect in mouse reproduction (Meng-Chun *et al*., 2018). Most of studies showed that *CYP11A1* gene associated with ovary disease and development, which gene might quite be an important candidate gene related to puberty.

CONCLUSION

Overall, several biological processes were selected by SSH, and of which energy production and conversion may be an initiating factor for pubertal onset in the goat. Many genes related with energy production and conversion and GnRH signal pathway may have significant roles in the puberty onset, of which, *CYP11A1*, *GNAS* and *SPARC* will be our candidates for further research.

Data availability

The original data of the paper are available from the corresponding author upon request.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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