

Expression profiles of key candidate genes involved in steroidogenesis during follicular atresia in the pig ovary

Zengxiang Pan · Jinbi Zhang · Fei Lin ·
Xueshan Ma · Xuguang Wang · Honglin Liu

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Abstract More than 99 % of follicles in mammalian ovaries undergo a degenerative process known as atresia, and thus only a limited number of ovarian follicles actually ovulate after full growth and development. The endocrinological regulatory mechanisms involved in follicular development have been studied extensively, but the precise and systematic molecular mechanisms of steroidogenesis enzymes involved in atresia are unclear. In the present study, we examined whether and how the steroidogenesis enzymes are involved in porcine ovary follicular atresia. Expression of steroidogenic acute regulatory protein, CYP11, CYP17, 3 β -hydroxysteroid dehydrogenase (3 β -HSD), CYP19, as well as related pituitary and ovarian hormone receptors were quantified in ovaries. During porcine follicular atresia, expressions of P450 cholesterol side chain cleavage enzyme, progesterone and androgen receptors increased significantly during the late atretic stage, while the expression of aromatase and follicle-

stimulating hormone receptors decreased significantly in the early stage. These data suggested that the regulation of aromatase by follicle-stimulating hormone might induce follicular atresia, and that progesterone and androgen production further promoted follicular atresia. Additionally, a correlation analysis indicated a large and complex interactive network among these genes and the endocrinological microenvironment of the follicles. Significant correlations were observed between expression of steroidogenic enzymes and their receptors, and also between progesterone and 17 β -estradiol (E2) levels in follicular fluid. Taken together, these results suggest that CYP19 plays a role during early atresia by regulating the production of E2, whereas CYP11 and 3 β -HSD increase atresia progression by increasing progesterone levels.

Keywords Pigs · Ovary · Follicles · Follicular atresia · Steroidogenesis enzymes

Zengxiang Pan and Jinbi Zhang contributed equally to this paper.

Z. Pan · J. Zhang · F. Lin · X. Ma · X. Wang · H. Liu (✉)
College of Animal Science and Technology, Nanjing
Agricultural University, Nanjing 210095, China
e-mail: liuhonglin@njau.edu.cn

Z. Pan
e-mail: owwa@njau.edu.cn

J. Zhang
e-mail: kibyzhang@gmail.com

F. Lin
e-mail: 2008205002@njau.edu.cn

X. Ma
e-mail: xueshan@163.com

X. Wang
e-mail: xuguang@yahoo.com

Introduction

Like all mammals, the porcine primordial follicle pool is produced in the fetal ovary around 35 days after birth [1]. After puberty, a number of primordial follicles start to grow during each estrous cycle in adult females [2]. At the beginning, primary follicles form and develop into secondary follicles that subsequently form tertiary follicles by mitosis of granulosa and thecal cells [3]. Tertiary follicles grow at an exponential rate due to a large increase in the proliferation of granulosa cells and an increase in the size of the antrum. Then, superior follicles are selected to continue on their path to the pre-ovulatory stage, and finally oocytes are ovulated [4]. During the growth and development, most of porcine follicles undergo atresia at

different stages of development. Although approximately 420,000 primordial follicles are available at puberty [5], more than 99 % of follicles fail to ovulate.

Follicular development is a complex biological process regulated by the interaction of various factors from granulosa cells, theca cells, and oocytes [6]. Gonadal steroid hormones, such as testosterone (T), progesterone (P4), and 17 β -estradiol (E2), play an important role in follicular development and atresia. The enzymes involved in steroid hormone synthesis and their relationship with follicular development has been studied extensively in a variety of animals, but the relationship between steroidogenic enzymes and follicular atresia is poorly understood.

Ovarian steroidogenesis occurs primarily in the theca and granulosa cells surrounding the oocyte [7]. The theca cells reside in the outermost layer of the follicle where P4 and androgens are synthesized from cholesterol. Androgens are primarily aromatized to E2 after diffusing across the basement membrane into the granulosa cell layer. Granulosa cells undergo rapid mitosis under estrogen stimulation, resulting in the growth of granulosa cell layers and enlargement of the follicular antrum. In some sense, this process determines the fate of recruited follicles: continued development or atresia. Additionally, it is generally believed that luteinizing hormone (LH) stimulates androgen production, whereas follicle-stimulating hormone (FSH) stimulates aromatase expression, and thereby E2 synthesis, in the granulosa cells [7]. The effectiveness of LH and FSH depends primarily on the number of FSH and LH membrane receptors, which begin to be expressed in the granulosa and theca cells at the secondary follicle stage.

Steroidogenic enzymes are those involved in the biosynthesis of the gonadal steroid hormones, such as P4, T, and E2 [8]. These related pathways are summarized in Fig. 1. The rate-limiting step for all steroid production is the translocation of cholesterol from the relatively sterol-rich outer mitochondrial membrane to the relatively cholesterol-poor inner mitochondrial membrane. This step is mediated primarily by the steroidogenic acute regulatory protein (StAR, encoded by the StAR gene). Steroidogenic stimuli rapidly induce StAR expression, which catalyzes the intermembrane transfer of cholesterol to P450 cholesterol side-chain cleavage enzyme (P450_{scc}, encoded by the CYP11A1 gene), thereby initiating steroidogenesis [9]. The first enzymatic step in steroid hormone production is the conversion of cholesterol to pregnenolone by cytochrome P450_{scc}, which catalyzes three sequential oxidation reactions followed by cleavage of six carbon side chains [10]. As precursor substrate, pregnenolone is subsequently metabolized to P4 by 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerases (3 β -HSDs, encoded by 3 β -HSD genes). All other steroid hormones are derived from these basic steroids [11]. The cytochrome P450 17 α -hydroxylase/

17,20-lyase enzyme (P450_{c17}, encoded by the CYP17 gene) catalyzes two mixed-function oxidase reactions, 17 α -hydroxylation and C17-20 bond cleavage. In these reactions, pregnenolone or P4 are used as substrates for androgen synthesis [12]. Cytochrome P450 aromatase (P450_{arom}, encoded by the CYP19 gene) uses androstenedione or T as a substrate to yield estrogens, such as estrone or E2 [8, 13]. All of these genes are key candidates for involvement in follicular atresia in pig ovaries.

We conducted this study to improve our understanding of the regulatory steroidogenic enzyme pattern involved during follicular atresia in pig ovaries and to provide data for steroid biochemistry and physiology studies, as well as for animal production and reproductive performance investigations. We separated follicles into three categories: healthy, early atresia, and progressive atresia using a uniform standard associated with multiple factors. The mRNA expression patterns of the steroidogenic enzymes (StAR, CYP11, CYP17, 3 β -HSD, CYP19) and the ovarian gonadotropin receptors (FSHR, LHR) were quantified by real-time reverse transcription polymerase chain reaction (RT-PCR). Finally, we used correlation analysis to identify the possible complex interactive network among these genes and the endocrinological microenvironment of the follicles.

Materials and methods

Follicles preparation and the study design overview

Ovaries were obtained from mature sows (weight, >120 kg; $n = 26$) at a local slaughterhouse and were washed in phosphate-buffered saline (PBS; pH 7.3). Tissue samples were transported to the laboratory within 1 h in an

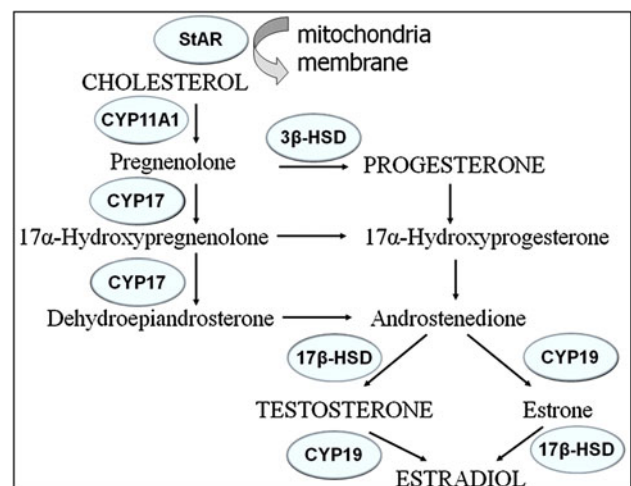


Fig. 1 Biosynthesis of steroid hormones in gonads. *Note:* Individual enzymes are *circled*. Final steroid hormone products are in *capital letters*

insulated container maintained at 37 °C. Antral follicles, approximately 3–5 mm in diameter, were dissected from the ovaries using fine forceps and a surgical dissecting microscope (SZ51; Olympus, Tokyo, Japan). Totally, 239 antral follicles were obtained in this study. An overview of the experiment is presented in Fig. 2.

Fluid collection and follicular components recovery

Each follicle was opened using fine watchmaker forceps, Follicular fluid (including antral granulosa cells) and the follicle wall layers (including thecal and granulosa walls) from each follicle were separated by centrifugation ($6,000\times g$, 5s) using a home-made microsieve inside an eppendorf tube, and then resuspended immediately in follicular fluid. A 2- μL aliquot of follicular fluid from each follicle was diluted in 40 μL PBS in another tube for granulosa cells (GC) density analysis. The remaining follicular fluid was centrifuged again ($6,000\times g$, 20s); then, 2–5 μL of supernatant was removed quickly, diluted 100 times, and kept at $-80\text{ }^{\circ}\text{C}$ for later hormone assay. The granulosa wall layers and cells remaining in the precipitate were stored at $-80\text{ }^{\circ}\text{C}$ for later RNA extraction.

Follicle classification

We used three different criteria to classify each follicle into three groups (healthy, early atresia, progressive atresia), and based on a uniform determination among the following three criteria (the morphological features of follicle, the GC density and the P4/E2 ratio), we identified healthy, early atretic, and progressively atretic follicles respectively (Table 1).

The number of the antral granulosa cells in follicular fluid was counted using a hemocytometer, and the density was calculated based on the counting results. Densities of $<250/\mu\text{L}$ were classified as healthy, 250–1,000/ μL were classified as early atretic, and $>1000/\mu\text{L}$ were classified as progressively atretic, based on preliminary findings and experience.

E2 and P4 levels in the follicular fluid from each follicle were retrospectively measured using chemiluminescent kits (Shenzhen Labkit Bioscience Co., Ltd., Shenzhen, China) to confirm the follicle classification. Follicles with a P4/E2 ratio of <5 were classified as healthy, 5–20 as early atretic, and >20 as progressively atretic, according to previous findings [14, 15] and our experience. Follicles without fluid were collected separately and stored in liquid nitrogen for subsequent RNA extraction.

Finally, after above uniform determination among the three criteria, we picked out 30 follicles from the total 239 antral follicles, representing healthy ($n = 10$), early atretic ($n = 10$), and progressively atretic follicles ($n = 10$) respectively.

RNA isolation and quantitative RT-PCR

After the uniform determination among above three criteria, we picked out 30 follicles from the total 239 antral follicles, representing three pooled biological sample for healthy ($n = 10$), early atretic ($n = 10$), and progressively atretic follicles ($n = 10$) respectively. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). The RNA was used for agarose gel (containing 1.5 % formaldehyde) electrophoresis to examine RNA quality. First-strand cDNA was synthesized using the M-MLV Reverse Transcriptase kit (Promega, Madison, WI, USA),

Fig. 2 Flowchart of the study design

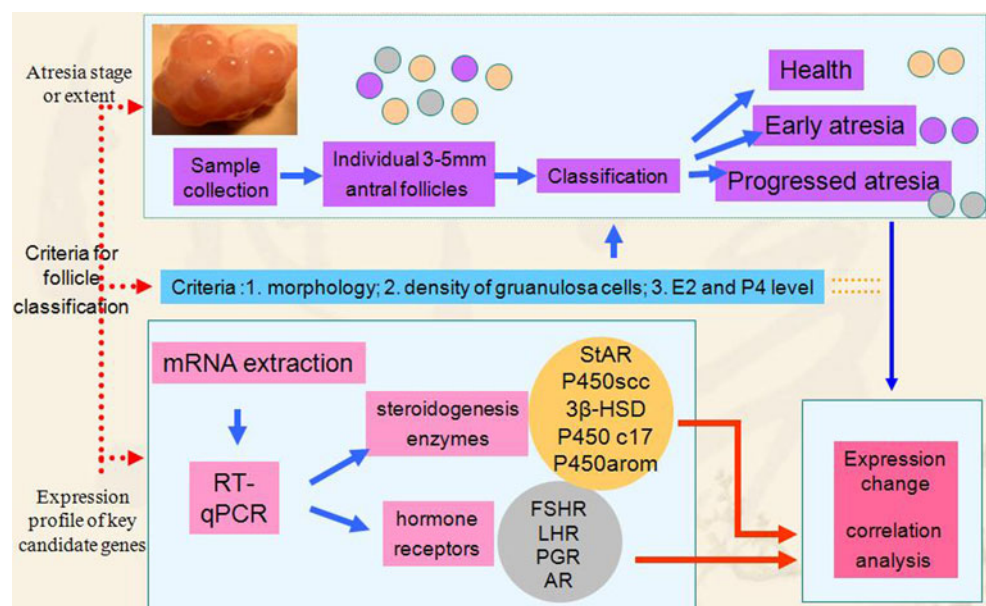


Table 1 The morphological criteria for follicle classification

	Transparency	Color	Coc	GC layers
Health (H)	Clear	Rosy	Visible	Close
Early Atresia (EA)	Hemorrhagic	Dark Orange	Hard to identify	Crenation or partly fell into the antrum
Progressively Atretic (PA)	Milky	Grey	Fell into the antrum	Severely fell off and formed deposits

Table 2 Primer pairs used for the quantitative RT-PCR

Gene	Accession Number	Primer sequences (5' → 3')	Annealing temperature	Amplicon length (bp)
StAR	NM_213755	ACTTTGTGAGTGTCCGGCTGTA CGCTTTCGCAGGTGATT	58.6	274
CYP11	DN837451.1	AGACACTGAGACTCCACCCCA GACGGCCACTTGTACCAATGT	65.0	120
CYP 17	NM_214428	CAAGACGAACGCAGAAAG AGAGGCAGAAGTCTCCACA	58.0	121
CYP 19	NM_214431	GCTGCTCATTGGCTTAC TCCACCTATCCAGACCC	60.8	187
3 β HSD	NM_001004049	ACGACACACCTCCCAAAAG CCTCACACCCACATCCACG	59.5	485
FSHR	NM_214386	AGTCCCTCGGTTCTTAT CATCTTTCAGGGTGAT	57	219
LHR	NM_214449	CGCAAGGAGACCAAATAAT ATGAGGAAACGAGGCACTG	55.8	273
AR	NM_214314.1	ATTGAGCCAGGCGTAGTGT CCAGGTCAGGAGCAAAGT	55.7	268
PGR	S49016.1	CTCACAGCGTTTCTACCA TCACCATCCCTGCCAATA	54.5	179
GAPDH	AF017079	GATGGTGAAGGTCGGAGTG CGAAGTTGTCATGGATGACC	58.0	500

StAR Sus scrofa steroidogenic acute regulatory protein, *CYP11 Sus scrofa* cDNA clone T23F7 similar to cytochrome P450, *CYP 17 Sus scrofa* cytochrome P450 17A1, *CYP 19 Sus scrofa* cytochrome P450 19A3, *3 β HSD Sus scrofa* hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1, *FSHR Sus scrofa* follicle stimulating hormone receptor, *LHR Sus scrofa* luteinizing hormone/choriogonadotropin receptor, *AR Sus scrofa* androgen receptor, *PGR* progesterone receptor, *GAPDH Sus scrofa* glyceraldehyde 3-phosphate dehydrogenase

according to the manufacturer's protocol. Steroidogenic enzymes, FSHR, LHR, PGR and AR were detected by real-time PCR using the SYBR Premix Ex Taq (Takara Bio, Ohtsu, Japan), following the manufacturer's protocol. Primers were designed based on the porcine mRNA sequences from the GenBank database for all these genes, using the primer Premier 5 software (PREMIER Biosoft Int., Palo Alto, CA). All primers were synthesized by Invitrogen (Shanghai, China). Porcine Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. The expression level of each target gene was analyzed according to previously described methods [16, 17]. The PCR amplification products were analyzed by melting curve analysis and 2 % agarose gel electrophoresis, and the results were analyzed using the LightCycler

software (ver. 3.5; Roche Diagnostics, Mannheim, Germany). Standard curve methods were used to calculate the relative gene expression ratio of a target gene. For each gene, controls for each primer set containing no cDNA were included on each plate, and the reaction was repeated three times for every sample on each plate. The amplification profiles of each gene are shown in Table 2.

Statistical analysis

Data were described as mean \pm SEM and statistically analyzed using SPSS 17.0 for windows statistical package (SPSS Inc., Chicago, IL). The significance level is set as 0.05 for *P* value. The relative mRNA expression levels among the different stages of atresia were analyzed by

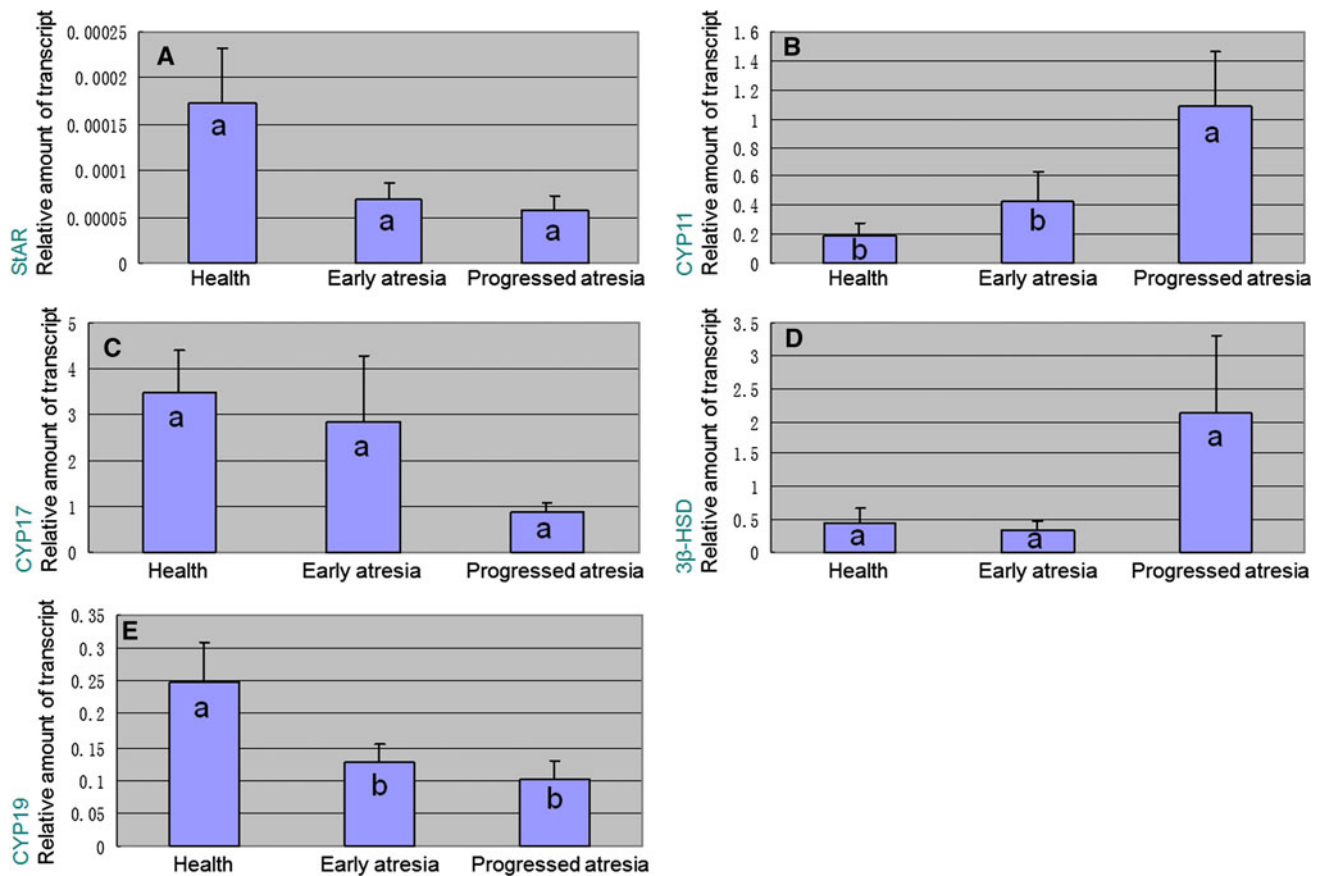


Fig. 3 Changes in steroidogenic enzyme expression in atresia. **a, b, c, d** and **e** represent the mRNA expression profiles of StAR, CYP11, CYP17, 3β-HSD, and CYP 19, respectively. The *lowercase letter* in

the histogram denoted statistically significant differences between different follicle physiological stages with the significant level $P < 0.05$. The *bar* above the histogram indicated the SEM

ANOVA process, followed by the Tukey–Kramer test as a multiple comparison test. The relationships among the mRNA expression of target genes, and the association between changes in gene expression and the corresponding follicular physiological indices, were examined by calculating the pearson correlation coefficient.

Results

Steroidogenic enzyme expression during porcine follicular atresia

Expression profiles of the key steroidogenesis enzymes in this study are shown in Fig. 3a–e for StAR, CYP11, CYP 17, 3β-HSD, and CYP 19, respectively.

StAR and CYP17 gene mRNA expression tended to be higher in healthy follicles and tended to decrease when follicles were undergoing early and progressive atresia, but the differences were not statistically significant. The mRNA level of CYP11 gene was significantly lower ($P < 0.05$) in healthy follicles than that in progressively

atretic follicles, but there was no significant differences between healthy and early atretic follicles.

3β-HSD gene expression initially decreased and then tended to increase during atresia, but the difference was not statistically significant ($P > 0.05$). CYP19 gene mRNA expression was significantly higher in healthy follicles and decreased significantly when follicles underwent early and progressive atresia ($P < 0.05$).

Gonadal hormone receptor expression during porcine follicular atresia

Expression profiles of key gonadal hormone receptors in this study are shown in Fig. 4a, b, representing the progesterone receptor (PGR) and androgen receptor (AR), respectively. PGR gene mRNA expression was low in healthy follicles and increased significantly in progressively atretic follicles, but not in early atretic follicles. AR gene expression increased significantly ($P < 0.05$) in progressively atretic follicles, but from healthy and early atretic stage, AR mRNA level showed a tendency toward stabilization or changed slightly.

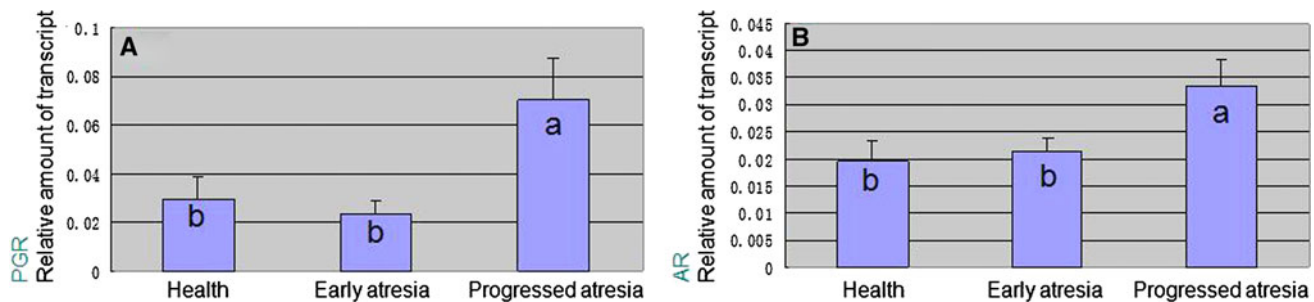


Fig. 4 Change in gonadal hormone receptor gene expression during atresia. **a** and **b** represent the mRNA expression profiles of PGR and AR respectively. The *lowercase letter* in the histogram denoted

statistically significant differences between different follicle physiological stages with the significant level $P < 0.05$. The *bar* above the histogram indicated the SEM

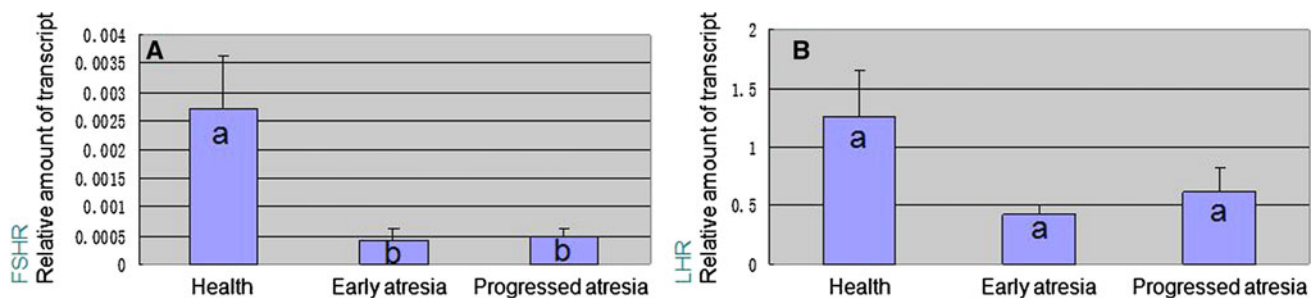


Fig. 5 Changes in pituitary hormone receptor gene expression during follicular atresia. **a** and **b** represent the mRNA expression profiles of FSHR and LHR respectively. The *lowercase letter* in the histogram

denoted statistically significant differences between different follicle physiological stages with the significant level $P < 0.05$. The *bar* above the histogram indicated the SEM

Pituitary hormone receptor expression during porcine follicular atresia

FSHR and LHR expression profiles are shown in Fig. 5a, b. FSHR expression decreased significantly during both stages of atresia ($P < 0.05$), whereas LHR gene expression did not.

Correlation analysis among physiological indices and related gene mRNA expression

Correlation analysis between physiological indices and gene expression

A correlation analysis was conducted on 30 follicle samples. The density of follicular granulosa cells, P4 and E2 levels in follicular fluid, and the expression of related genes, including steroidogenic enzymes, steroid hormone receptors, and pituitary hormone receptors, was investigated (Table 3). The correlation analysis showed that follicular diameter was not significantly associated with any of these variables. Follicular granulosa cell density was not significantly correlated with P4 or E2 content, but was positively correlated with the P4/E2 ratio ($r = 0.438$; $P = 0.047$). Additionally, granulosa cell density was significantly negatively correlated ($P = 0.036$) with CYP19 gene expression and significantly

positively correlated ($P = 0.001$) with CYP11 gene mRNA expression. P4 levels in follicular fluid were positively correlated with gene expression of CYP11 ($P = 0.009$), 3β -HSD ($P = 0.002$), and PGR ($P = 0.031$) and negatively correlated with FSHR gene expression ($P = 0.035$). Follicular E2 level was significantly positively correlated with StAR ($P = 0.011$), CYP19 ($P = 0.013$), and LHR ($P < 0.001$) expression.

Correlation analysis among mRNA expression of related genes

The expression of the steroidogenic enzymes, steroid hormone receptor, and pituitary hormone receptor genes in 30 samples was studied. The correlation analysis (Table 4) showed that StAR mRNA expression was significantly and positively correlated with LHR gene expression ($P = 0.001$); CYP11 gene expression was significantly and positively correlated with 3β -HSD ($P = 0.035$), PGR ($P = 0.003$), and the AR gene ($P = 0.045$); 3β -HSD gene expression was significantly and positively correlated with PGR ($P < 0.001$); CYP19 gene expression was significantly and positively correlated with FSHR ($P = 0.038$) and LHR gene ($P = 0.017$) expression; and AR gene expression was significantly and positively correlated with PGR gene expression ($P = 0.001$).

Table 3 Correlation between physiological indices and gene expression ($n = 30$)

		StAR	CYP11	3 β HSD	CYP17	CYP19	FSHR	LHR	AR	PGR
P4	<i>r</i>	-0.228	0.467**	0.539**	-0.268	-0.186	-0.386*	-0.208	0.349	0.394*
	<i>P</i> value	0.226	0.009	0.002	0.152	0.325	0.035	0.270	0.059	0.031
E2	<i>r</i>	0.456*	-0.198	-0.148	0.051	0.446*	0.247	0.684**	-0.176	-0.164
	<i>P</i> value	0.011	0.293	0.434	0.788	0.013	0.188	0.000	0.352	0.385
Ratio P4/E2	<i>r</i>	-0.084	0.410*	0.155	-0.232	-0.168	-0.274	-0.222	0.224	0.247
	<i>P</i> value	0.658	0.025	0.414	0.218	0.375	0.143	0.239	0.235	0.187

The *r* represented a coefficient of pearson correlation

** Showed a significant correlation at the 0.01 level (two-tailed); * Indicated a significant correlation at the 0.05 level (two-tailed)

Table 4 Correlation among mRNA expression levels of related genes ($n = 30$)

		StAR	CYP11	3 β HSD	CYP17	CYP19	FSHR	LHR	AR	PGR
StAR	<i>r</i>	1								
	<i>P</i> value									
CYP11	<i>r</i>	-0.162	1							
	<i>P</i> value	0.392								
3 β HSD	<i>r</i>	-0.202	0.386*	1						
	<i>P</i> value	0.285	0.035							
CYP17	<i>r</i>	0.227	-0.234	-0.188	1					
	<i>P</i> value	0.228	0.214	0.319						
CYP19	<i>r</i>	0.231	-0.172	-0.126	0.020	1				
	<i>P</i> value	0.220	0.363	0.508	0.917					
FSHR	<i>r</i>	-0.183	-0.084	-0.029	0.035	0.381*	1			
	<i>P</i> value	0.334	0.658	0.881	0.854	0.038				
LHR	<i>r</i>	0.575**	-0.154	-0.112	-0.091	0.434*	0.130	1		
	<i>P</i> value	0.001	0.417	0.557	0.633	0.017	0.495			
AR	<i>r</i>	-0.090	0.369	0.343	0.013	0.007	-0.352	-0.096	1	
	<i>P</i> value	0.635	0.045	0.063	0.944	0.971	0.056	0.613		
PGR	<i>r</i>	-0.031	0.525**	0.706**	-0.230	-0.185	-0.093	-0.185	0.575**	1
	<i>P</i> value	0.873	0.003	0.000	0.222	0.328	0.627	0.328	0.001	

The *r* represented a coefficient of pearson correlation

** Showed a significant correlation at the 0.01 level (two-tailed); * Indicated a significant correlation at the 0.05 level (two-tailed)

Discussion

If gene expression levels in healthy follicles are considered as a value of 1, the changes observed in early and progressively atretic follicles were showed as relative rates (Fig. 6). The results showed a positive correlation between StAR expression and the E2 level in follicular fluid, suggesting that the decline in StAR expression might inhibit E2 secretion. The increase in P4 suggested that it was not a predominant factor for inhibiting steroid production; both CYP11 and 3 β -HSD expression levels were positively correlated with P4 level and PGR expression. Thus, changes in two key enzymes during P4 synthesis may lead directly to the increase in P4 levels. These changes occurred primarily during the period between the early and

late atretic stages, suggesting that they might play a role in promoting atresia. The mRNA level of CYP17 decreased, but not significantly, whereas AR gene expression increased significantly. The expression level of CYP19 was positively correlated with FSHR and LHR expression, as well as E2 levels. Furthermore, the changes in these four factors occurred mainly during the period between the healthy and early atretic stages. Together, these results suggest that pituitary hormones regulate aromatization enzyme expression and subsequent follicular atresia.

StAR gene function in follicular atresia

The main function of StAR in the ovary is to transport cholesterol from the outer to the inner membrane of

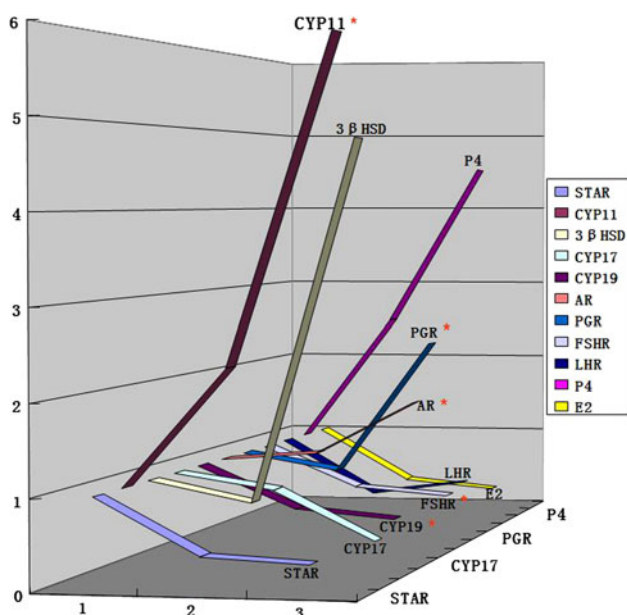


Fig. 6 Relative rates of expression in the three stages of porcine follicles. 1, 2, and 3 in the *horizontal coordinate* represent the healthy, early atretic, and progressively atretic stages, respectively

mitochondria or cytoplasm where P450scc is located. This process is the rate-limiting step of steroid hormone synthesis [18, 19]. StAR gene mRNA expression declined significantly when follicles were undergoing early atresia, whereas the change between the early atretic stage and that of progressive atresia was not significant, suggesting that changes in steroidogenesis occur before or within the early stages of follicular atresia. Furthermore, although a positive correlation was observed between StAR gene mRNA expression and E2 level in follicular fluid, the increasing P4 level suggested that the decrease in StAR was not a major limitation on E2 production. Thus, this rapid regulatory process is more likely to regulate follicular atresia activity through other pathways than through the steroidogenic pathway.

CYP11, 3 β -HSD and PGR gene function in follicular atresia

CYP11 expression levels changed the most during this experiment. CYP11 expression increased although not significantly as follicles entered early atresia, however, further significant increase was observed in progressively atretic follicles. At the same time, 3 β -HSD gene expression tended to increase but without significant difference during the progressively atretic stage. It is clear that the P450 cholesterol scc enzyme and 3 β -HSD are the two main P4 synthesis enzymes [20]. The former uses cholesterol to synthesize 17-acetylene testosterone, whereas the latter is involved in further metabolism of P4 [21, 22]. Hormone

measurements showed that the change pattern of hormone level was the same as CYP11 and 3 β -HSD expression. CYP11 and 3 β -HSD were also positively correlated with P4 levels. Additionally, PGR expression increased significantly in progressively atretic follicles. These results suggest that when follicles undergo atresia, CYP11 and 3 β -HSD are regulated to increase P4 synthesis directly, leading to the progression of atresia.

CYP17 and AR gene function in follicular atresia

Changes in CYP17 gene expression from healthy to early atretic follicles were insignificant, and they showed a further declined tendency when follicles underwent progressive atresia although the statistic result indicated no significant differences at $P = 0.05$. The main trend in CYP17 expression was consistent with the findings of Wesley and Braw-Tal in sheep and cattle, respectively [23, 24]. The CYP17 gene encodes the cytochrome P450 17 α -hydroxylase/17,20-lyase enzyme, which use pregnenolone or P4 as a substrate for androgen synthesis [25]. Follicular androgens act as two-way regulators during atresia. They directly accelerate atresia but also help follicles maintain normal development by acting as a substrate for E2 synthesis [26]. Thus, a decrease in CYP17 during atresia may reduce E2 production. It seems more likely that the AR increases after early atresia, which improves androgen efficiency even after a decrease in CYP17 expression.

CYP19 gene function in follicular atresia

The CYP19 gene encodes aromatase, a key enzyme in E2 production; thus, its expression in granulosa cells directly determines E2 secretion. Consistent with the study by Huet and Wesley [27], CYP19 expression declined during atresia. However, the decline mainly occurred at the early atretic stage, and there was no significant difference between early atretic and progressively atretic stage. This changing trend was the same as that for E2 in previous studies [27], which suggests that E2 production determines the developmental fate of follicles prior to atresia or at the very early stage of atresia. The finding that expression did not change significantly during the later stage of atresia indicates that E2 may not play a major role maintaining or promoting atresia. It is also possible that E2 secretion was reduced before follicles reached the progressive stage atresia.

FSHR and LHR gene function in follicular atresia

Both FSHR and LHR were expressed during the early stage of follicular atresia, whereas their expression significantly decreased (FSHR) or tended to decrease (LHR) in atretic follicles in comparison to the healthy ones. The LHR

responds to LH by stimulating transcriptional activity of P450c17 to catalyze P4 into androstenedione [24, 28]. The FSHR regulates P450arom in granulosa cells to synthesize E2 from androstenedione [29]. Dominant follicles not only have an increased blood supply compared with other follicles, but also show a stronger response to hormones, such as FSH and LH. Additionally, the steroid hormone concentrations in the follicular fluid of dominant follicles is higher, and StAR and P450arom expression is also higher than in subordinate follicles, which ensures adequate cholesterol supply and E2 production [30]. In our study, the significant positive correlation between LHR expression and follicular fluid E2 levels suggested that the decrease in LHR decreased steroidogenic enzyme expression. The significant positive correlation between FSHR and CYP19 gene expression suggests that FSH plays an important role in aromatase expression. Furthermore, the negative correlation between FSHR expression and P4 levels suggest that decreased expression of FSHR limits synthesis of steroid hormones at the P4 or androgen stage, resulting in early and progressive atresia.

In conclusion, we systematically described the steroidogenic enzyme mRNA expression profile during porcine follicular atresia, and analyzed their potential relationships with the occurrence and progress of follicular atresia. We illustrated the associations among follicle physiological status, steroidogenic enzymes, and their corresponding receptors during porcine follicular atresia, and demonstrated a comprehensive relationship between changes in these related factors and follicular atresia. The regulatory network and mechanisms involved in ovarian follicular atresia are a complex issue that needs to be more fully explored. With a better understanding of follicular atresia-specific factors and their interactive mechanisms, researchers will gain insights not only into follicular atresia but also into female fertility. These insights are likely to have wide applications in livestock husbandry production, assisted reproductive technologies, and human health. These data will also be useful for clarifying diseases mechanism associated with ovulatory disorders.

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References

- Miyano T (2004) Mammalian oocyte growth outside the ovary. Proceedings of the first workshop of the Asian reproductive biotechnology. Asian Reproductive Biotechnology 1, pp 4–7
- Hirshfield AN (1991) Development of follicles in the mammalian ovary. Int Rev Cytol 124:43–101
- Grant SA, Hunter M, Foxcroft G (1989) Morphological and biochemical characteristics during ovarian follicular development in the pig. Reproduction 86(1):171–183
- Manabe N, Inoue N, Yasufumi G, Matsuda F, Maeda A, Sugimoto M (2004) Follicle selection In: Porcine ovaries: regulation of granulosa cell apoptosis during atresia. Proceedings of the first workshop of the Asian reproductive biotechnology. Asian Reproductive Biotechnology 1, pp 17–21
- Schwarz T, Kopyra M, Nowicki J (2008) Physiological mechanisms of ovarian follicular growth in pigs-A review. Acta Vet Hung 56(3):369–378
- Guthrie HDCB, Welch GR, Zakaria AD, Johnson LA (1995) Atresia in follicle grown after ovulation in the pig: measurement of increased apoptosis in granulosa cells and reduced follicular fluid estradiol-17 β . Biol Reprod 52:920–927
- LaVoie HA, King SR (2009) Transcriptional regulation of steroidogenic genes: StARD1, CYP11A1 and HSD3B. Exp Biol Med 234(8):880–907
- Payne AH, Hales DB (2004) Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 25(6):947–970
- Christenson LK, Stouffer RL, Strauss JF (2001) Quantitative analysis of the hormone-induced hyperacetylation of histone H3 associated with the steroidogenic acute regulatory protein gene promoter. J Biol Chem 276(29):27392–27399
- Guo IC, Shih MC, Lan HC, Hsu NC, Hu MC, Chung B (2007) Transcriptional regulation of human CYP11A1 in gonads and adrenals. J Biomed Sci 14(4):509–515
- Kazeto Y, Ijiri S, Matsubara H, Adachi S, Yamauchi K (2003) Molecular cloning and characterization of 3 [beta]-hydroxysteroid dehydrogenase/[Delta] 5-[Delta] 4 isomerase cDNAs from Japanese eel ovary. J Steroid Biochem Mol Biol 85(1):49–56
- Storbeck KH, Swart AC, Swart P (2009) CYP17 causes hypocortisolism in the South African Angora goat. Mol Cell Endocrinol 300(1–2):121–125
- Murray AA, Swales AKE, Smith RE, Molinek MD, Hillier SG, Spears N (2008) Follicular growth and oocyte competence in the in vitro cultured mouse follicle: effects of gonadotrophins and steroids. Mol Hum Reprod 14(2):75–83
- Maeda A, Inoue N, Matsuda-Minehata F, Goto Y, Cheng Y, Manabe N (2007) The role of interleukin-6 in the regulation of granulosa cell apoptosis during follicular atresia in pig ovaries. J Reprod Dev 53(3):481–490
- Sugimoto M, Manabe N, Kimura Y, Myoumoto A, Imai Y, Ohno H, Miyamoto H (1998) Ultrastructural changes in granulosa cells in porcine antral follicles undergoing atresia indicate apoptotic cell death. J Reprod Dev 44(1):7–14
- Lu FZ, Jiang ZY, Wang XX, Luo YH, Li XF, Liu HL (2010) Role of the insulin-like growth factor system in epiphyseal cartilage on the development of Langshan and Arbor Acres chickens, Gallus domesticus. Poult Sci 89(5):956–965
- Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25(4):402–408
- Miller WL (2007) Steroidogenic acute regulatory protein (StAR), a novel mitochondrial cholesterol transporter. Mol Cell Biol Lipids 1771(6):663–676
- Gyles SL, Burns CJ, Whitehouse BJ, Sugden D, Marsh PJ, Persaud SJ, Jones PM (2001) ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory (StAR) gene. J Biol Chem 276(37):34888–34895
- Sanne JL, Krueger KE (1995) Expression of cytochrome P450 side-chain cleavage enzyme and 3 β -hydroxysteroid dehydrogenase in the rat central nervous system: a study by polymerase chain reaction and in situ hybridization. J Neurochem 65(2):528–536

21. Eimerl S, Orly J (2002) Regulation of steroidogenic genes by insulin-like growth factor-1 and follicle-stimulating hormone: differential responses of cytochrome P450 side-chain cleavage, steroidogenic acute regulatory protein, and 3 β -hydroxysteroid dehydrogenase/isomerase in rat granulosa cells. *Biol Reprod* 67(3):900–910
22. Zhang YY, Yang L (2009) Interactions between human cytochrome P450 enzymes and steroids: physiological and pharmacological implications. *Expert Opin Drug Metab Toxicol* 5(6):621–629
23. Braw-Tal R, Roth Z (2005) Gene expression for LH receptor, 17 {alpha}-hydroxylase and StAR in the theca interna of preantral and early antral follicles in the bovine ovary. *Reproduction* 129(4):453–461
24. Garrett WM, Guthrie HD (1996) Expression of androgen receptors and steroidogenic enzymes in relation to follicular growth and atresia following ovulation in pigs. *Biol Reprod* 55(5):949–955
25. Liu Y, Yao ZX, Papadopoulos V (2005) Cytochrome P450 17 alpha hydroxylase/17, 20 lyase (CYP17) function in cholesterol biosynthesis: identification of squalene monooxygenase (epoxidase) activity associated with CYP17 in Leydig cells. *Mol Endocrinol* 19(7):1918–1931
26. Walters K, Allan C, Handelsman D (2008) Androgen actions and the ovary. *Biol Reprod* 78(3):380–389
27. Huet C, Monget P, Pisselet C, Monniaux D (1997) Changes in extracellular matrix components and steroidogenic enzymes during growth and atresia of antral ovarian follicles in the sheep. *Biol Reprod* 56(4):1025–1034
28. Cardenas H, Jimenez E, Pope W (2008) Dihydrotestosterone influenced numbers of healthy follicles and follicular amounts of LH receptor mRNA during the follicular phase of the estrous cycle in gilts. *Reproduction* 135(3):343–350
29. Hsueh AJW, Billig H, Tsafirri A (1994) Ovarian follicle atresia: a hormonally controlled apoptotic process. *Endocr Rev* 15(6):707–724
30. Belin F, Goudet G, Duchamp G, Gérard N (2000) Intrafollicular concentrations of steroids and steroidogenic enzymes in relation to follicular development in the mare. *Biol Reprod* 62(5):1335–1343