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Transcriptome for small yellow follicles reveals bone morphogenetic protein 15 involved in chicken follicle selection

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Chenxi Zhang[1,2#], Junlai Shi[1,2#], Shu Wang[1,2], Kaiyuan Tian[1,2], Xintao Jia[1,2], Guirong Sun[1,2], Yadong Tian[1,2], Yedan Gai[3], Kejun Wang[1,2*], Xiangtao Kang[1,2*] and Wenting Li[1,2*]

[1] College of Animal Science and Technology, Henan Agricultural University, Zhengzhou 450001, China
[2] The Shennong Laboratory, Zhengzhou 450046, China
[3] College of Animal Science and Veterinary Medicine, Shenyang Agricultural University, Shenyang 110866, China
[#] Authors contributed equally: Chenxi Zhang, Junlai Shi
[*] Corresponding authors, E-mail: wangkejun.me@163.com; xtkang2001@263.net; liwenting_5959@hotmail.com

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Abstract

Sustaining egg production in late-laying chickens can extend their egg-laying cycle. Follicle selection is a pivotal process that determines the egg production rate, acting as the ultimate regulatory checkpoint to ensure only the most viable follicles progress to ovulation. This study aimed to identify and elucidate the role of a potential gene in follicle selection. Follicles measuring 6–8 mm were harvested from high egg-yielding (H) and low egg-yielding (L) Taihang chickens at 43 weeks of age for RNA sequencing to identify DEGs. The role of *BMP15* in follicle selection was investigated by adding recombinant BMP15 protein to GCs. As a result, 99 DEGs were identified, and *BMP15* was selected as the candidate gene for its role in follicle selection. The addition of recombinant BMP15 activated the SMAD1 signaling pathway, upregulated the expression of bone morphogenetic protein receptor type 1B (*BMPR1B*) and follicle-stimulating hormone receptor (*FSHR*), and increased cyclic adenosine monophosphate (cAMP) levels. However, BMP15 significantly repressed the expression of steroidogenic acute regulatory protein (*STAR*) and cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*) and slightly decreased the progesterone (P4) levels. Furthermore, the co-administration of follicle-stimulating hormone (FSH) with recombinant BMP15 resulted in a significant decrease in *BMPR1B* expression and a marked increase in *FSHR, STAR,* and *CYP11A1* expression, which led to a comparable change in P4 levels. These findings demonstrated that *BMP15* is involved in follicle selection by enhancing *FSHR* expression and the cAMP level. *BMP15* interacts with FSH to regulate follicle selection from the pre-hierarchical to hierarchical follicles.

Introduction

The ovary is a vital component of the chicken's reproductive system, with egg-laying rates depending on follicle development and ovulation. Post-maturity, the ovary harbors a spectrum of follicle sizes, categorized as follows: primordial follicles (< 0.08 mm), primary follicles (0.08–1 mm), undifferentiated pre-hierarchical follicles (1–8 mm), and preovulatory/hierarchical follicles (> 9 mm)[1]. Despite the abundance of follicles, most undergo atresia, with only a select few maturing into preovulatory status. Follicle selection is critical as it determines the fate of follicles in the ovary—either advancing in the follicular hierarchy or undergoing atresia. The small yellow follicle (SYF) stage marks a critical juncture in follicle selection, with granulosa cells (GCs), the oocyte-adjacent somatic cells, playing a nurturing role in follicle maturation[2]. GC differentiation is a hallmark of follicle selection, as they synthesize and secrete progesterone and express high levels of *FSHR*[3], which plays an indispensable role in follicle selection.

Follicle selection in chickens is a complex process coordinated by multiple factors, including the neuroendocrine system, hypothalamic-pituitary-gonadal axis, and the oocytes themselves. The hypothalamus secretes GnRH, prompting the pituitary to release FSH, which then targets *FSHR* on ovarian GCs, igniting downstream pathways and enhancing gene transcription vital for steroidogenesis, including cytochrome P450 family 11 subfamily A member 1 (*CYP11A1*), steroidogenic acute regulatory protein (*STAR*). The expression of these genes plays an important role in follicle selection and GC differentiation by regulating steroid hormone synthesis in the ovary, including progesterone (P4), and estradiol (E2)[4]. FSH and its receptor (*FSHR*) are closely related to chicken follicle development and egg-laying rate[5]. The forkhead box L2 (*FOXL2*) gene also plays an important regulatory role in follicle selection and hierarchical follicles development[6], and is significantly associated with egg-laying traits[7]. Anti-Mullerian hormone (*AMH*) can inhibit the downstream signaling pathway of *FSHR* in GCs, regulating GC differentiation and follicle selection[8]. Additionally, the oocyte itself plays a crucial role in regulating follicle selection. Oocytes release oocyte-specific factors that act as signals to ovarian GCs. For example, oocytes secrete growth differentiation factor 9 (*GDF9*) outside of the cells, which then binds to receptors on the surface of GCs, activates the SMAD signaling pathway, and regulates expression of downstream genes as a ligand-activated transcription factor complex, thus involving the follicle selection process[9]. However, the key genes underlying the mechanism of follicle selection in hen ovarian follicles have not been fully elucidated.

BMP15, also known as *GDF9B*, is conserved across various species. In sheep, *BMP15* is carried by the X chromosome and is also known as *FecX*, which mutations are associated with follicle development abnormalities[10]. And the variation affects litter size[11]. Similarly, *BMP15* activates the SMAD1/5/8 pathway to promote GC proliferation in mice[12].

In recent years, transcriptome analysis has revealed and identified many genes relevant to follicular development and selection. Several differentially expressed genes (DEGs), and were identified in different stage follicles of Jilin Black chicken Lohmann Brown layer, including *STMN4, FABP3, ROBO2, RSPO4, SLC6A15,* and *SLITRK3*[13]. In the small yellow follicles and F6 follicles of Hy-Line Brown chickens, several DEGs related to follicle selection have been identified, including *VLDLR1, WIF1, NGFR, AMH, BMP15, GDF6*, and *MMP13*[1].

To ensure the continued ovulation of follicles in the late stage of egg production and identify potential genes related to follicle selection in the late-laying period, our study, focusing on 6–8 mm follicles from late-laying period Taihang chickens, leverages RNA-seq to identify potential genes associated with follicle selection. The findings on BMP15's role in follicle selection may provide novel strategies to enhance follicular recruitment in aging hens, thereby improving egg yield during the late laying period. Follicle selection is a pivotal process that determines the egg production rate, and BMP15-mediated regulation of GC differentiation offers a molecular target for optimizing this process.

Materials and methods

Animals and sample collection

We collected egg production records from 704 Taihang chickens, aged 20 to 43 weeks. All birds were reared in laying battery cages under standardized environmental conditions, including identical diet, lighting, temperature, humidity, and management practices. The number of eggs laid was recorded from 20 weeks of age to ensure consistency in data collection. Groups were divided into high egg-yielding (H) and low egg-yielding (L) based on egg production. During the late egg-producing period (43 weeks of age), three Taihang hens were randomly selected and obtained with H and L. Chickens were euthanized, and yellow follicles 6 to 8 mm in diameter were categorized as SYFs, immediately snap-frozen in liquid nitrogen then stored at −80 °C for RNA extraction.

RNA extraction, library construction, and sequencing

Total RNA was extracted from follicles using TRIzol (TaKaRa, Dalian, China) for each sample. RNA integrity was assessed using 1% agarose gel electrophoresis, where clear 28S and 18S rRNA bands were observed without smearing. Additionally, RNA quality was further evaluated using a NanoDrop-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, Calif., USA). RNA samples with an integrity number greater than 8.0 and an optical density ratio of 260/280 nm between 1.8 and 2.0 were selected for further experiments. During library construction, mRNA was enriched from total RNA by poly-A oligo-attached magnetic beads. Double-stranded complementary DNAs were synthesized with random hexamer primers and purified with AMPure XP beads. Then the ends of the double strand were repaired, and an A-tailed link connector was added to the 3' end, followed by PCR amplification to construct a cDNA library. The insert size was determined using Agilent 2100, and the concentration of the library was quantified by quantitative PCR. The mRNA libraries were sequenced using an Illumina HiSeq 2500 at Allwegene Technologies. The quality of RNA-Seq reads was evaluated using Fast QC.

RNA-Seq data analysis

To obtain clean reads, we removed low-quality reads from raw reads and filter them. And follow up analyses were based on clean reads. Concurrently, the Q20, Q30, and GC content of the clean data were calculated. The clean reads were mapped to the chicken reference genome (GRCg6a) using the sequence alignment program HISAT 2.1.0[14].

Differential expression

We used HTSeq (ver. 0.6.1) software to count the reads mapped to each gene. The featurecounts software was utilized to calculate the read counts of mRNAs in each sample[15]. The TPM (Transcript per Kilobase per Million mapped reads) value was used to evaluate the expression level of mRNAs in each library. The edge R package was used to identify differentially expressed genes, applying a significance threshold of FDR < 0.05 and | log2 FoldChange | > 1 differential expression, to identify DEGs.

GO and KEGG analyses

To investigate the DEGs biological function, we conducted Gene Ontology (GO: www.geneontology.org) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG: www.kegg.jp) pathway analyses. These annotations were used to explore the biological functions and pathways associated with the DEGs. A hypergeometric test was employed to ascertain the statistical significance of the enrichment of GO terms and KEGG pathways. A p-value < 0.05 was considered to indicate significant enrichment.

Quantitative real-time PCR

To confirm the accuracy and repeatability of the RNA-Seq results, transcription levels of six selected genes in follicles were assessed by using quantitative real-time reverse transcriptase PCR (qRT-PCR). Total RNA was reverse transcribed into cDNA using a PrimeScript™ RT Reagent Kit (TaKaRa, Dalian, China). Quantitative Real-Time PCR (qRT-PCR) was performed in triplicate using the SYBR Premix Ex Taq II kit (TaKaRa, Dalian, China) on a LightCycler 96 instrument (Roche, Indianapolis, IN, USA). Primers were designed to span exon-exon junctions, ensuring specificity for a single amplicon, as detailed in Table 1. The mRNA expression levels were normalized using the $2^{-\Delta\Delta Cr}$ method with β-actin as the internal control. All experiments were carried out in triplicate.

GCs isolation and culture

GCs were isolated from 6-8 mm follicles of Taihang chickens with an average egg-production rate at 43 weeks of age, following a previously published method[16]. The cells were cultured in Dulbecco's modified

Table 1. Primer list.

Target	Primer sequence (5'-3')	Product length (bp)
BMP15	F: ATGCTGGAGCTGTACCAACG R: CACGTACCAGCGACCTGC	135
BMPR1B	F: CTAGGATTAGAGGGCTCGGAC R: GGCCTTATGGTGAATGTTTCCTT	167
FSHR	F: CTGAGTTACACCGTGAGGTCT R: TGGTGAGGACAAATCTCAGTTC	242
CYP11A1	F: GGCGTGCTCCTCAAGACAG R: ACAAAGTCCTGGCTCACCTG	125
STAR	F: AGAACTGAGCTCCACTGCAC R: GCCTGGAGCTGAGCAGG	214
SMOC1	F: TCCAAACCACTCTGTGCCTC R: TTTGGCCAGCATCTTTGCAC	124
ZP4	F: GTGCTGACTGCTTGGGATACT R: CAGCTGGTGTAGGAAACGGA	122
ZP2	F: ATTGGGTGCTCAGACAGCTC R: CAAGTCAGACATGCGCGTT	150
MATN3	F: AAGACTTGCTCAAGAGCCACA R: GTGAGTAGAACGCTGGCTCA	194
GDF-9	F: AGGGACCCGATTACAGGAGAC R: CTCAACCCACTTGCGCTTTC	252
β-actin	F: CACGGTATTGTCACCAACTG R: ACAGCCTGGATGGCTACATA	200

Eagle's medium with high glucose (H-DMEM) (HyClone, USA), supplemented with 2.5% fetal bovine serum (FBS) (BI, Israel), and 1% penicillin-streptomycin (Solarbio, China).

The experimental design included six groups, each with three replicates, and each treatment was conducted in triplicate cultures. GCs were seeded in 6-well dishes and incubated for 12 h. Subsequently, the medium was refreshed with H-DMEM containing 0.1% bovine serum albumin (BSA) and 1% penicillin-streptomycin solution. Different treatments were then applied to the cells: 50 ng/ml Recombinant BMP15 Protein (R&D Systems, Minneapolis, MN), 50 ng/ml recombinant BMP15 protein combined with 100 ng/ml recombinant human Noggin (NOGGIN) (R&D Systems, Minneapolis, MN), or 50 ng/ml recombinant BMP15 protein combined with 25 ng/ml FSH (R&D Systems, Minneapolis, MN). After 24 h, the medium was replaced with fresh H-DMEM containing 0.1% BSA and 1% penicillin-streptomycin solution, and 50 ng/ml recombinant BMP15 protein was reapplied to the group treated with NOGGIN.

GCs were harvested for RNA extraction and subsequent qRT-PCR analysis. P4 levels in the culture medium were quantified using chicken progesterone (P4) enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng, Nanjing, China). The cAMP activity was measured using cAMP-Glo™ Assay (Promega, USA).

Statistical analysis

The data are presented as the mean ± SEM. Data were analyzed using SPSS 24.0 software (IBM, Chicago, IL, USA). A t-test was used to determine significant differences between two groups (* $p < 0.05$; ** $p < 0.01$), and one-way ANOVA followed by a post hoc test-Least Significant Difference (LSD), was used to determine significant differences among three or more groups (different letters indicate significant differences, $p < 0.05$). Graphics were drawn using GraphPad Prism 8.0 software (San Diego, CA, USA).

Results

Summary of egg production of high- and low-yielding Taihang chickens

An analysis of total egg-laying records was performed on 704 Taihang hens, aged 20 to 43 weeks. During the period from 20 to 38 weeks, the median egg production was 72 eggs, with the first and third quartiles being 55 and 81 eggs, respectively (Fig. 1a). From 39 to 43 weeks, the median was 36 eggs, with the first and third quartiles being 29 and 41 eggs, respectively (Fig. 1b). Individuals with a total of egg production from 20-38 weeks above 72 eggs, and those with egg production from 39–43 weeks below 29 eggs and above 41 eggs were classified as the low egg-yielding group (L) and high egg-yielding group (H), respectively. Finally, there were 13 hens in the L group and 74 in the H group. A significance analysis was conducted to validate the feasibility of egg production from 39–43 weeks between the H and L groups. The egg production showed the most significant differences ($p < 0.01$) (Table 2).

Sequencing results and quality control

We established libraries for both high and low egg-yielding groups using follicles of 6-8 mm from Taihang chickens at 43 weeks of age. A total of 62,748,991 and 92,837,832 raw reads were obtained from the H group and L group, respectively. After removing low-quality reads, we obtained 60,990,303 (H) and 905,616,94 (L) clean reads, which were subsequently used for analysis (Supplementary Table S1).

Differential expression analysis

A total of 99 DEGs were identified, characterized by significant changes in expression levels with |log2FoldChange| > 1 and FDR < 0.05. This included five upregulated and 94 downregulated genes (Supplementary Table S2). Among these genes, eight DEGs (*Wnt6, BMP15, ZP4, MNR2, ZP2, WNT4, FOXL2,* and *GDF-9*) were implicated in follicle development and selection (Table 3). Notably, the expression level of *BMP15* mRNA was significantly downregulated with a log2 (fold change) of -8.10, suggesting a potentially key role in the selection process of chicken follicles. However, the precise function of BMP15 in this context remains to be fully elucidated. To confirm the RNA-seq findings, six genes were selected and quantified by qRT-PCR, which showed similar expression patterns as observed in the sequencing data (Fig. 2).

Functional analysis of differentially expressed genes

To identify the potential biological functions of DEGs in H and L groups, we conducted enrichment analysis using GO and KEGG pathway databases. DEGs were enriched in terms of related to reproductive process, cell proliferation, binding, transcription regulator activity, molecular transducer activity, transporter activity, and catalytic activity (Fig. 3, Supplementary Table S3).

The KEGG pathway analysis revealed several enriched terms, including Wnt signaling pathway (gga04310), mTOR signaling pathway (gga04150), cell cycle (gga04110), TGF-beta signaling pathway (gga04350), oocyte meiosis (gga04114), FoxO signaling pathway (gga04068), cytokine-cytokine receptor interaction (gga04060), regulation of actin cytoskeleton (gga04810), and the calcium signaling pathway (gga04020) (Fig. 4, Supplementary Table S4).

Function of BMP15 in chicken follicle selection

To explore the function of BMP15 in follicle selection, we added recombinant BMP15 protein to GCs. The expression levels of BMPR1B, a specific receptor for BMP15, increased by 22-fold ($p < 0.001$), confirming the successful addition of the protein (Fig. 5a). Additionally, the upregulation of *SMAD1*, a component of the SMAD1/5/8 signaling pathway, indicated activation of the pathway (Fig. 5b). The expression levels of genes that are involved in follicle development, such as *FSHR, CYP11A1* and *STAR*, also increased. Furthermore, the results indicated an increase in cAMP activity and progesterone (P4) concentration, both

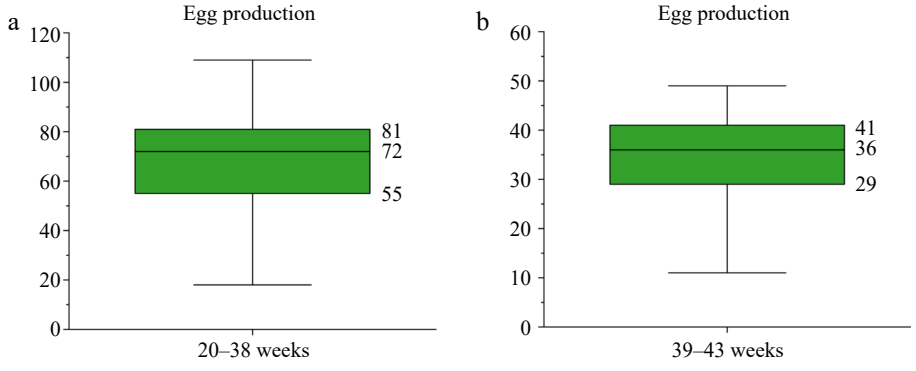

Fig. 1 Egg production of 704 Taihang chickens. (a) Egg production from 20 to 38 weeks. (b) Egg production from 39 to 43 weeks.

Table 2. Average egg production.

	High egg-yielding (H)	Low egg-yielding (L)
20–38 weeks	82.92 ± 8.76	83.69 ± 10.42
39–43 weeks	43.03 ± 2.00**	12.00 ± 3.71

The data are expressed as the mean ± SEM (* $p < 0.05$; ** $p < 0.01$)

Table 3. The list of DEGs in follicle development and selection.

Gene name	Description	log2FC	FDR	Type
Wnt6	Wnt family member 6	−6.0031	8.56E-03	Down
MNR2	Homeodomain protein	−11.8637	1.97E-02	Down
BMP15	Bone morphogenetic protein 15	−8.1033	5.56E-03	Down
ZP2	Zona pellucida glycoprotein 2	−8.7247	7.86E-03	Down
ZP4	Zona pellucida glycoprotein 4	−8.745	7.86E-03	Down
WNT4	Wnt family member 4	−5.5719	3.46E-03	Down
FOXL2	Forkhead box L2	−1.8021	2.01E-02	Down
GDF-9	Growth differentiation factor 9	−6.44	4.14E-02	Down

of which are known to promote GC differentiation during follicle selection. There was a significant increase in cAMP activity and *FSHR* expression (Fig. 5c, d). These changes in gene expression and indices indicated that BMP15 was involved in GC differentiation and affected follicle selection. However, *BMP15*'s influence on steroid hormone synthesis appeared to be inhibitory, as evidenced by the significant reduction in *STAR* and *CYP11A1* expression (Fig. 5e, f). P4 concentration showed a decreasing trend, the change was not statistically significant (Fig. 5g).

Subsequently, NOGGIN was added to the culture after 24 h of BMP15 treatment. The addition of NOGGIN resulted in a significant decrease in the expression of *BMPR1B* (Fig. 5a), indicating an effective disruption of the BMP15-BMPR1B binding. The expression of *SMAD1* also decreased (Fig. 5b), suggesting that the SMAD1/5/8 signaling pathway was inhibited. Following NOGGIN addition, *FSHR* expression significantly decreased (Fig. 5d), and there was non-significant trend towards reduced cAMP activity and P4 concentration (Fig. 5c, g). The expression of *STAR* and *CYP11A1* had a similar trend. These results showed that inhibiting BMP15 leads to a reduction in signaling pathway activity and key gene expression.

To further confirm the role of *BMP15*, we performed a recovery experiment. Following the disruption of BMP15-BMPR1B binding with NOGGIN, we added recombinant BMP15 protein. Notably, the expression level of *BMPR1B* was significantly increased compared to treatment with NOGGIN alone (Fig. 5a), indicating the effectiveness of the recovery experiment. Moreover, there was a notable increase in the levels of *SMAD1, FSHR, STAR, CYP11A1*, cAMP, and P4 upon BMP15 restoration (Fig. 5b–g). These results strongly suggest that BMP15 fosters GC differentiation, thereby regulating follicle selection.

Given FSH's pivotal role in follicle development, especially in selection, we explored its impact on BMP15-mediated processes. We treated GCs with both recombinant BMP15 and FSH, using BMP15-only treatment as a control. Interestingly, the expression of *BMPR1B* was reduced by half (Fig. 6a), indicating that FSH suppresses *BMP15* and *BMPR1B* expression. While *SMAD1* showed an upward trend, the change was not statistically significant (Fig. 6b). However, FSH significantly enhanced the expression of follicle development genes, such as *FSHR, STAR*, and *CYP11A1* (Fig. 6d–f). Moreover, the activity of cAMP and the concentration of P4 were significantly improved (Fig. 6c, g). These findings indicated that FSH, despite inhibiting *BMP15* expression, can still facilitate GC differentiation.

Discussion

In recent years, the egg production rate of commercial laying hens during the peak period has been close to the physiological limit. To enhance the utilization rate of these hens, it is essential to extend their egg-laying cycles and boost production rates in the later stages. Follicle selection is an important process that affects the egg production performance and fecundity of hens. It is directly related to the egg production of chickens, 6–8 mm follicles were the key period for determining follicle selection. To

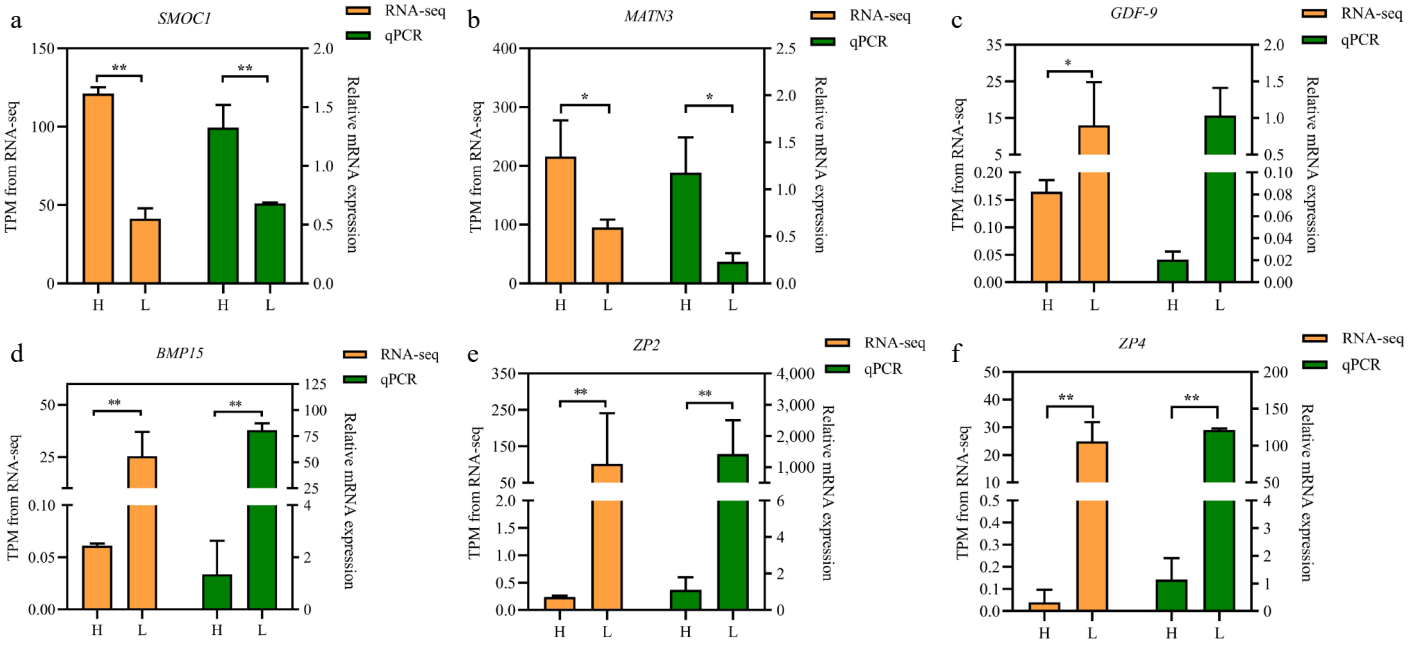

Fig. 2 Comparison of the gene expression of RNA-seq with qPCR of 6 randomly selected genes. (a) *SMOC1*, (b) *MATN3*, (c) *GDF-9*, (d) *BMP15*, (e) *ZP2*, and (f) *ZP4*. Note: The left and right axes represent the gene expression using TPM units by RNA-seq and the expression levels verified by qPCR, respectively. The yellow column indicates TPM value; the green column indicates qPCR. All experiments are performed in triplicate, and the data are expressed as the mean ± SEM (* $p < 0.05$; ** $p < 0.01$).

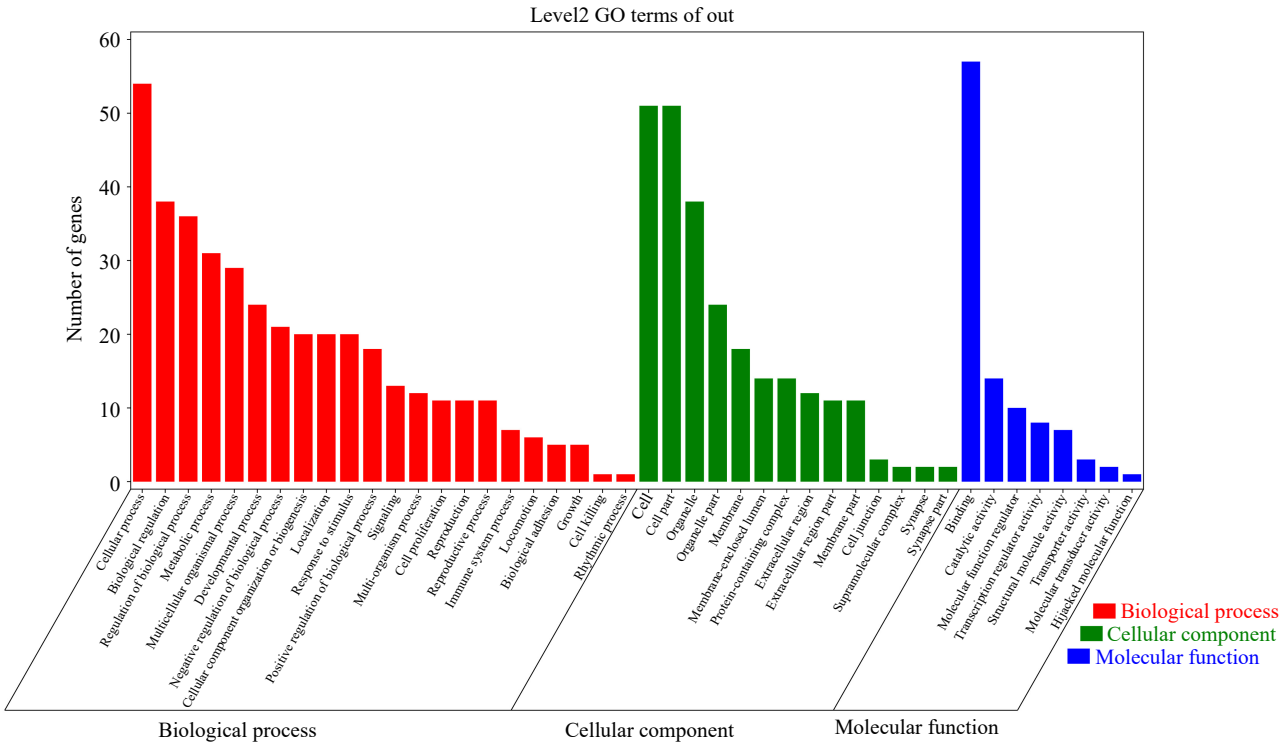

Fig. 3 GO terms enrichment analysis of DEGs.

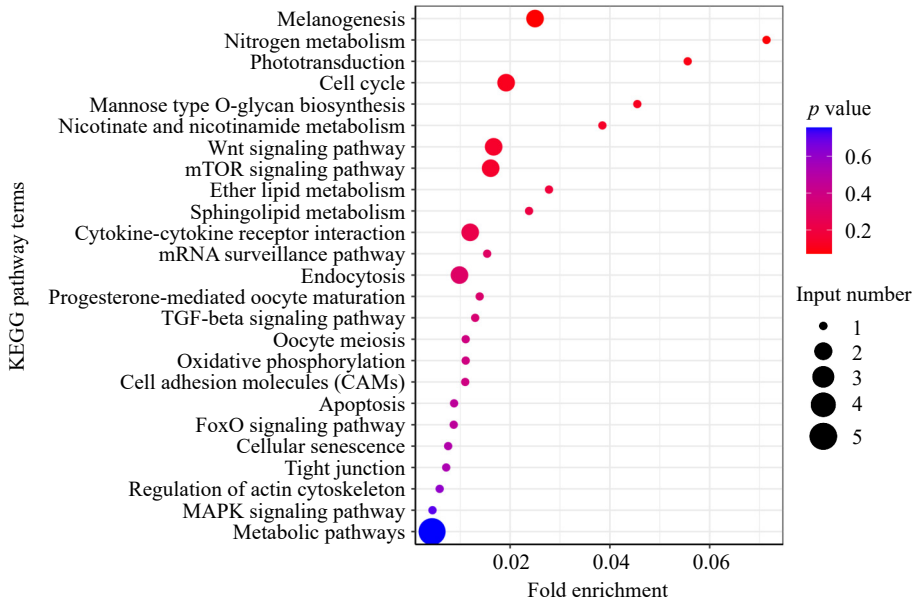

Fig. 4 KEGG pathway enrichment analysis of DEGs.

ensure continued ovulation of follicles in the late stage of egg production and to identify genes associated with follicle selection during this period, we collected 6-8 mm follicles of Taihang chicken with high- and low-yield during the late laying period for RNA-seq. The majority of DEGs were downregulated genes, suggesting that downregulated genes may play a significant role in follicle selection. Furthermore, *Wnt6, MNR2, BMP15, ZP2, ZP4, WNT4, FOXL2,* and *GDF-9* have been reported to be involved in follicle development and selection[17–21]. Further enrichment and analysis of these DEGs revealed several pathways likely critical to the egg-laying performance of chickens. Among these, the CAMs have been identified as one of the most important pathways associated with high and low egg production[1]. The Wnt signaling pathway plays a crucial role in follicle selection[6]. Additionally, the calcium signaling pathway and cytokine-cytokine receptor interaction are involved in follicle development and follicle atresia[22]. The mTOR signaling pathway and FoxO signaling pathway regulate the cell cycle, proliferation, and apoptosis of chicken follicular GCs[23]. The TGF-β signaling pathway is involved in initiating follicle selection during the follicle selection period[24].

BMP15, a specific factor released by oocytes, belongs to the TGF-β family, and is specifically expressed in oocytes[25]. In mammals, *BMP15* plays a crucial role in regulating GC differentiation, as well as participating in the regulation of the FSH signaling pathway[26,27], and follicle development[28]. Like most BMP ligands, BMP15 binds to its

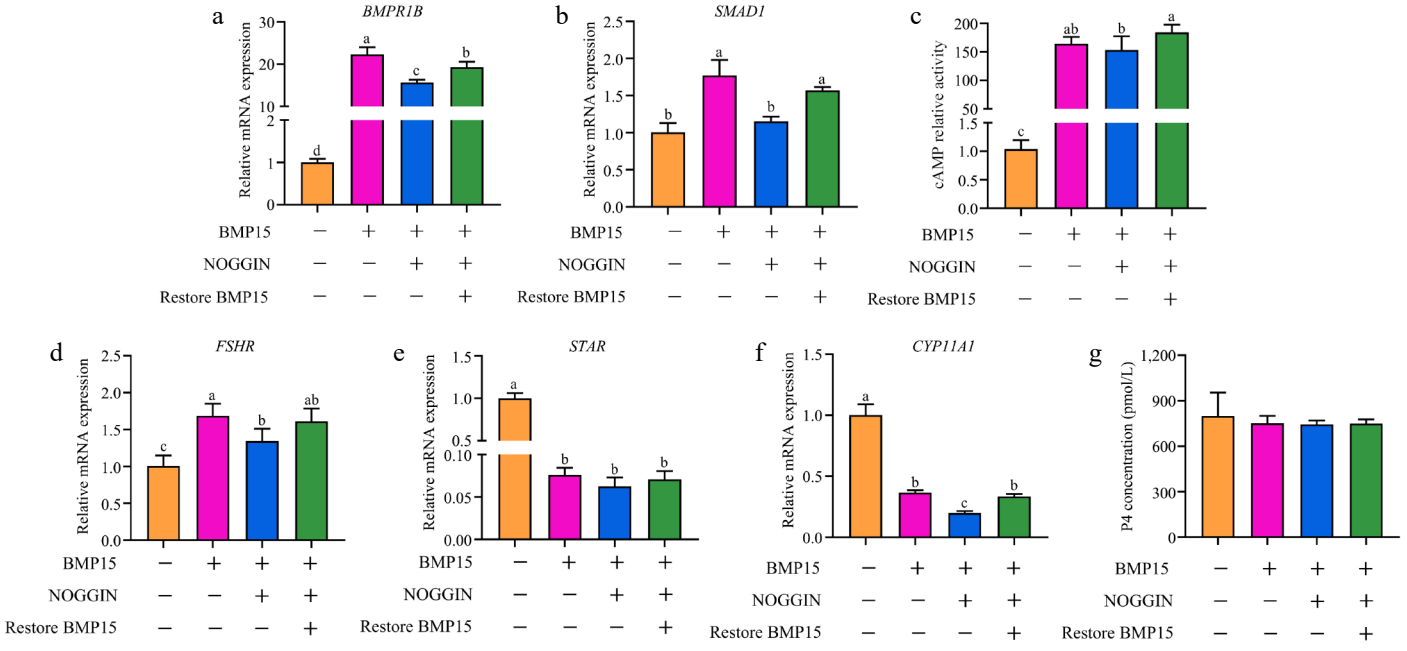

Fig. 5 Effects of recombinant BMP15 on gene expression in GCs. (a) *BMPR1B*, (b) *SMAD1*, (d) *FSHR*, (e) *STAR*, and (f) *CYP11A1*. (c) cAMP relative activity, and (g) P4 concentration. Note: All experiments were performed in triplicate, and the data are expressed as the mean SEM (different letters indicate significant difference, $p < 0.05$).

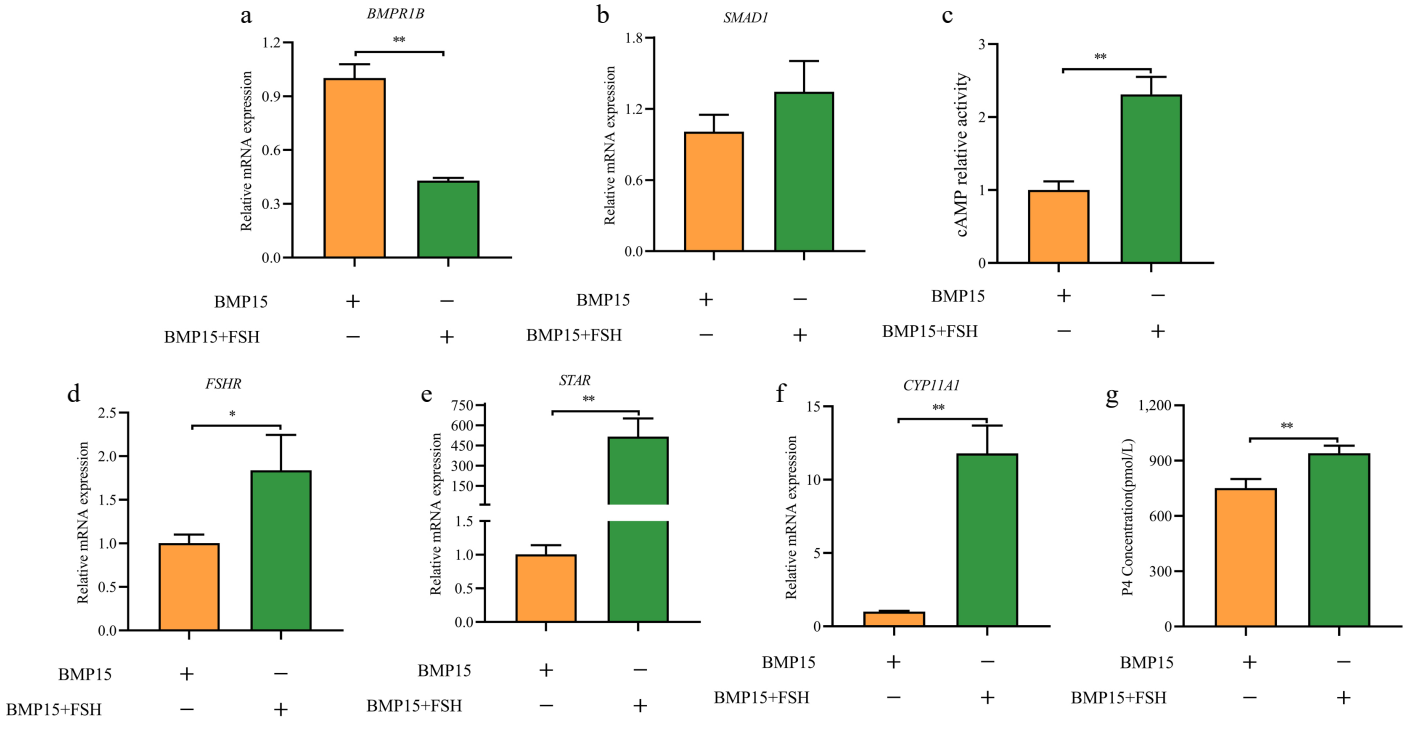

Fig. 6 FSH and BMP15 affected the expression of related genes. (a) *BMPR1B*, (b) *SMAD1*, (d) *FSHR*, (e) *STAR*, and (f) *CYP11A1*. (c) cAMP relative activity, and (g) P4 concentration. Note: All experiments were performed in triplicate, and the data are expressed as the mean SEM (* $p < 0.05$; ** $p < 0.01$).

specific receptors, BMPR1B and BMPR2, leading to phosphorylation and activation of downstream signaling pathways[29]. Multiple lines of evidence indicate that *BMP15* plays a role in regulating follicle development in various species. For instance, in sheep and cattle, *BMP15* mutations are associated with altered ovulation rates and follicular development abnormalities[10,30]. In mice, *BMP15* and *GDF9* synergistically regulate folliculogenesis and ovarian function[31]. Similarly, research in chickens has indicated that *BMP15* influences ovarian reserve and egg production[32].

As a signal factor released by oocytes, BMP15 binds to the specific receptor BMPR1B and activates the SMAD1/5/8 signaling pathway[33]. In a previous study, the addition of recombinant BMP15 protein to mouse GCs activated the SMAD1/5/8 signaling pathway[34]. *BMP15* plays an irreplaceable role in the regulation of follicular growth and development and granulosa cell growth and differentiation[35]. We observed that the expression levels of *BMPR1B* and *SMAD1* were significantly increased when recombinant BMP15 protein was added to GCs, leading to the activation of the SMAD1 signaling pathway. The

biological function of recombinant BMP15 protein has been previously demonstrated[36], while NOGGIN is known to be an antagonist of bone morphogenetic proteins (BMPs). It works by blocking epitopes on BMPs required for binding to both type I and type II receptors, thereby inhibiting BMP bioactivities[37]. To confirm the role of BMP15 in follicle selection, we disrupted the binding between BMP15 and BMPR1B by adding NOGGIN, which resulted in a significant reduction in *BMPR1B* expression levels and inhibited the SMAD1 signaling pathway. We then restored the addition of recombinant BMP15 protein, resulting in an increase in *BMPR1B* expression levels and re-activation of the SMAD1 signaling pathway. These results suggest that *BMP15* plays a critical role in follicle selection by activating the SMAD1 signaling pathway via *BMPR1B* in GCs.

GC differentiation commences as follicles are selected to become hierarchical, with their capacity to synthesize and secrete P4 escalating alongside the gradual increase in *FSHR* and cAMP levels. The increased levels of *FSHR* and cAMP in GCs are considered indicative of follicle selection[4]. A marked increase in *FSHR* expression in GCs of small yellow follicles suggests that follicle selection may be imminent. In our study, we found that *BMP15* can significantly increase the levels of *FSHR* and cAMP, thereby enhancing the sensitivity of GCs to FSH. It is possible that GCs are primed to await the FSH signal to exposure to BMP15. Our findings are corroborated by studies in other species, where *BMP15* upregulated *FSHR* expression in GCs[38]. *BMP15* is also known to promote *FSHR* levels through the SMAD signaling pathway, influencing follicle growth. Specifically, BMP15 binds to its receptor BMPR1B, initiating a signaling cascade that leads to the phosphorylation of SMAD1/5/8. Once phosphorylated, SMAD1 forms a complex with SMAD4 and is translocated into the nucleus, where it regulates the transcription of target genes, including *FSHR*. Previous studies in mammals have demonstrated that BMP15 modulates *FSHR* expression through SMAD-dependent transcriptional activation[12]. However, *BMP15* may suppress the expression level of *FSHR* in mice, indicating that the role of *BMP15* could vary among species. Additionally, the expression levels of *STAR* and *CYP11A1* were significantly decreased after adding recombinant BMP15 protein, with the P4 concentration remaining unchanged. Previous studies have shown that *BMP15* can reduce the levels of *STAR, E2*, and P4[39]. Two possible mechanisms may explain this effect. First, *BMP15* binds BMPR1B and through the SMAD1/5/8 pathway may directly inhibit the transcription of steroidogenic genes. Previous studies have shown that TGF-β superfamily members, including BMPs, can negatively regulate steroidogenesis by interfering with SF-1 (Steroidogenic Factor-1), a critical activator of *STAR* and *CYP11A1*[40]. Alternatively, *BMP15* may repress *STAR* and *CYP11A1* through an indirect feedback mechanism. *BMP15* enhances *FSHR* expression and increases cAMP levels, which are essential for follicle selection. However, excessive cAMP activation may also trigger a negative regulatory loop, preventing premature or excessive progesterone synthesis in pre-hierarchical follicles. This is consistent with findings in mammals, where *BMP15* has been reported to fine-tune FSH responsiveness while keeping steroidogenesis in check[41]. When BMP15 was added alone, it had no influence on the synthesis of steroid hormones[27]. This may be the GCs of pre-hierarchical follicles are not yet differentiated and lack the capability to synthesize and secrete P4[4].

FSH, a glycoprotein secreted by the anterior pituitary gland, affects GCs and regulates follicular development[42]. Studies have shown that FSH promotes GC growth, inhibits their apoptosis[43], and enhances the synthesis and secretion of P4[44]. It has been observed that the responsiveness to FSH was suppressed in pre-hierarchical follicles, however, as pre-hierarchical follicles are selected into hierarchical follicles, some signals are activated, and responsiveness to FSH is initiated by increasing cAMP production in GCs[3]. To investigate the effects of FSH on *BMP15* in GCs, we added both factors to the cells and compared the results to those obtained with BMP15 alone. Our analysis revealed a significant decrease in *BMPR1B* expression, suggesting that FSH may inhibit *BMPR1B* and *BMP15* expression levels, indicating that FSH antagonizes *BMP15* signaling while promoting steroidogenesis. This observation suggests that FSH functions as a stage-specific regulator of follicular development, facilitating the transition from BMP15-driven early follicle growth to FSH-dependent late-stage differentiation. Specifically, FSH may repress BMP15 expression through the cAMP-PKA pathway, which is activated during follicle maturation[45]. Increased cAMP levels stimulate steroidogenic enzymes (STAR, CYP11A1) while downregulating oocyte-derived BMP15, allowing granulosa cells to fully respond to FSH stimulation[46]. The interaction between *BMP15* and FSH may serve as a developmental checkpoint, ensuring that pre-hierarchical follicles remain BMP15-dependent until selected for further growth, at which point FSH signaling takes precedence.

In our results of RNA-seq results, *BMP15* expression was significantly higher in the high egg-yielding group compared to the low egg-yielding group. The high level of FSH in the bodies of high egg-yielding chickens may suppress *BMP15* expression. While the *SMAD1* level exhibited a slight upward trend, the effect was not statistically significant. Additionally, we also observed marked changes in the expression levels of *FSHR, CYP11A1, STAR, P4*, and cAMP. Notably, the expression levels of *FSHR, STAR*, and *CYP11A1* were significantly increased, indicating that GCs began responding to FSH signaling and synthesizing P4. Furthermore, there was an upward trend in the concentration of P4 and cAMP activity. Based on these results, we suppose that *BMP15* promotes follicle selection, enabling pre-hierarchical follicles to be chosen to enter the hierarchical follicles. Subsequently, GCs begin to respond to FSH, leading to GC differentiation and secretion of P4. As follicles progress to the hierarchical stage, the expression of *BMP15* decreases. Consequently, the *BMP15* and FSH pathways appear to act in concert during the development of the follicle from the pre-hierarchical to the hierarchical stage (Fig. 7).

These insights have practical applications in poultry breeding and reproductive management. Given *BMP15*'s role in enhancing FSH sensitivity and controlling steroidogenesis, it could serve as a genetic marker for selective breeding programs to improve egg production sustainability. Moreover, interventions aimed at modulating *BMP15* expression may help maintain follicle recruitment and extend the productive laying cycle in hens.

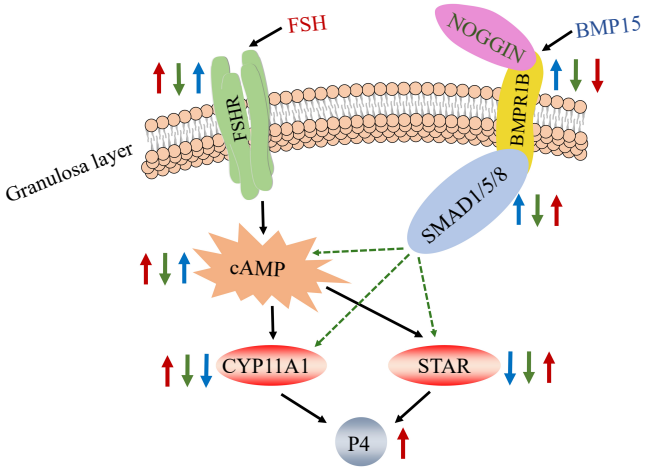

Fig. 7 Regulatory network model of BMP15 and FSH in chicken GCs. Note: The blue arrow shows that other genes expression levels after adding recombinant BMP15 protein; the green arrow shows that other genes expression levels after adding NOGGIN on the base of recombinant BMP15 protein added; the red arrow shows that other genes expression levels after adding FSH on the base of recombinant BMP15 protein added.

Conclusions

Transcriptome analysis of 6–8 mm follicles from Taihang chickens with high- and low-yield revealed the role of *BMP15* in chicken follicle selection. *BMP15* was found to increase the levels of *BMPR1B*, *FSHR*, and cAMP, activating the *SMAD1* signaling pathway, but decrease the expression of *STAR* and *CYP11A1*, and its expression was inhibited by FSH. Moreover, it was observed that from the pre-hierarchical to hierarchical follicles *BMP15* interacts with FSH signaling to regulate follicle selection. This study demonstrated that *BMP15* promotes follicle selection by enhancing *FSHR* expression and increasing cAMP levels. These findings on BMP15's role in follicle selection may provide novel strategies to enhance follicular recruitment in aging hens, thereby improving egg yield during the late laying period.

Ethical statements

All the animal experiments were approved by the Animal Care and Use Committee of Henan Agricultural University, identification number: 11-0085, approval date: 2019/3/3. The research followed the 'Replacement, Reduction, and Refinement' principles to minimize harm to animals. This article provides details on the housing conditions, care, and pain management for the animals, ensuring that the impact on the animals is minimized during the experiment.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Zhang C, Kang X, Li W; data collection: Wang S, Tian K, Sun G, Wang K; analysis and interpretation of results: Zhang C, Tian Y, Gai Y; draft manuscript preparation: Zhang C, Shi J, Wang K, Li W. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data in this study are included in the article content and the supplementary files. Transcriptome data were uploaded to the NCBI database sequence read archive, Accession no. PRJNA1080244 (www.ncbi.nlm.nih.gov/bioproject/PRJNA1080244).

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Conflict of interest

The authors declare that they have no conflict of interest.

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