



Full-Length Article

Nanopore long-read RNA sequencing reveals key genes regulating pre-hierarchical follicle development in the chicken ovary

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ABSTRACT

During the egg laying period of a hen, one pre-hierarchical follicle, approximately 6–8 mm in size, is selected into the preovulatory hierarchy for ovulatory stage development. These follicles develop from white follicles that are 2–5 mm in size. Although numerous studies have investigated follicle selection, the molecular mechanisms underlying pre-hierarchical follicle development and recruitment remain unclear. In this study, we explored the genes regulating chicken ovarian pre-hierarchical follicular development by sequencing RNA from ≤ 2 mm follicles in ovarian stroma (OS), 3–4 mm small white follicles (SWFs), and 6–8 mm small yellow follicles (SYFs) using Oxford Nanopore long-read transcriptome sequencing technology. Herein, we obtained 2,164 differentially expressed transcripts (DETs) from the three phases. The steroidogenesis and angiogenesis signaling pathways were enriched in SWFs compared with in OS. In contrast, the endocytosis, p53, FoxO, and oocyte meiotic signaling pathways were significantly enriched during SYF development compared with during SWF development. The focal adhesion, apelin, and TGF- β signaling pathways were significantly enriched in all three phases. The time-course analysis of DETs and cluster Kyoto Encyclopedia of Genes and Genomes enrichment revealed dynamic changes in gene expression during pre-hierarchical follicle development. Key examples include the genes associated with follicle initial recruitment and development (*AMH*, *GDF9*, *INHBB*, *BMPRI1B*, *STAR*, and *FST*), angiogenesis-related genes (*ANGPT2*, *VEGFA*, *VEGFC*, *VEGFD*, and *VEGFRs*), extracellular matrix remodeling-related genes (*MMP2* and *MMP10*), and Wnt signaling pathway genes (*WNT4*, *WNT9A*, *CTNBN1*, *RSPO3*, and *WIF1*). Moreover, *RSPO3* upregulated the expression of *FSHR* and *StAR* in cultured chicken Pre-hierarchical follicle granulosa cells and activated the Wnt signaling pathway. The results obtained in this study demonstrate the transcriptional dynamics during small folliculogenesis; the identified signaling pathways and DETs can be used as candidate targets for investigating pre-hierarchical follicle development in chickens.

Introduction

The sexually mature chicken ovary maintains numerous primordial follicles smaller than 1 mm diameter, which can keep with quiescent state in breeding period until activated and to growth and differentiation (Johnson and Woods, 2009; Kim et al., 2013). Part of primary follicles (1–2 mm) could develop to white follicles of diameter approximately 2–6 mm, which contain small amounts of a protein-rich white yolk and grow slowly (Waddington and Walker, 1988). The part of white follicles will accumulate lipid-rich yellow yolk, form a follicular pool composed of several small yellow follicles 6–8 mm in size (Perry and Gilbert, 1979). In peak laying periods, chicken ovarian contain dozens of

follicles with diameters between 2 and 8 mm (Gilbert et al., 1983). Hens lay an egg every day in a laying sequence (Etches, 1990). After oviposition, a single follicle was selected into the preovulatory hierarchy occurs from a cohort of prehierarchical follicles that diameter about 6–8 mm (Ghanem and Johnson, 2019). Follicles selected is related to the acquisition of granulosa cells responsiveness to follicle-stimulating hormone (FSH). The ovarian response to standard gonadotrophin stimulation depends on follicle-stimulating hormone receptor (*FSHR*), sex hormone-binding globulin (*SHBG*) and Cytochrome P450 19A1 (*CYP19*) gene synergism (Woods and Johnson, 2005).

For avian follicles, once a follicle enters the sequence of the pre-ovulatory follicle, it is high probability to develop to ovulation. This is

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difference from follicular development in mammals. What is the fate of a follicle? During the transformation of primordial follicles to primary follicles, there is a balance regulation of the activation and dormancy of primordial follicles. When avian follicles develop to the primary follicle stage, their diameter is about 1–2 mm, with a single layer of granular cells and flat membrane cells (Johnson and Woods, 2007). At this stage, some follicles may undergo non-rupture atresia, but some lucky follicles can develop into the next stage. Gradually develop and increase in size, granulosa cells and membrane cells proliferate, and egg yolk increases. However, its mechanism has not yet been clarified and needs to be studied.

Nanopore sequencing technology has made great progress compared with second-generation sequencing technologies such as illumine in terms of read length. This makes it possible to directly obtain the original transcript information without the need for short sequence splicing after RNA sequencing. Long-read RNA-seq holds immense potential for the discovery of novel transcripts, identify different splicing case. In this study, full-length transcriptome sequencing was performed on ovarian stroma (containing follicles smaller than 2 mm), small white follicles and small yellow follicles to explore the genes that change during the development of prehierarchical follicles. Discover the crucial candidate genes for the formation of the 6–8 mm small yolk follicle pool, and preliminarily study the development mechanism of prehierarchical follicles. Provide a certain theoretical basis and research materials for improving the laying performance of laying hens.

Materials and methods

Collection of ovarian follicle samples and RNA extraction

Hy-line brown hens were fed at a hen farm. Three regularly laying hens (approximately 28 weeks of age) were selected randomly. The three hens represented three biological groups. All three hens were sacrificed; their ovaries were collected and immediately stored in liquid nitrogen. The following three follicular development stages were assessed: ≤ 2 mm follicles (adhered the ovarian stroma, named OS), 3–4 mm follicles (named SWF) and 6–8 mm follicles (named SYF) (Fig. 1). RNA was extracted using an RNeasy Pure Tissue Kit (TIANGEN, Beijing, China), following the manufacturer's instructions. RNA quality and quantity were estimated using a BioPhotometer plus (Eppendorf, Hamburg, Germany) and a c300 Imager (Azure Biosystems, CA, America). All animal experiments were performed according to the recommendations in the Guidelines for Experimental Animals of the Ministry of Science and Technology of China. The Animal Care and Use Committee on the Ethics of Animal Experiments of Shandong Agricultural

University approved all animal experiments performed in this study.

cDNA library construction and sequencing using Oxford Nanopore Technologies (ONT)

The construction of cDNA libraries was performed according to the protocol provided by Oxford Nanopore Technologies Ltd. (Oxford, UK). Briefly, total RNA was extracted from follicles using TRIzol reagent (Invitrogen, CA, USA). RNA quality and quantity were estimated using a NanoDrop spectrophotometer and a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Oligo (dT) was used as a primer to reverse transcribe the objective mRNA. Full-length cDNA was amplified using low-cycle polymerase chain reaction (PCR) amplification; sequencing adapters (containing motor protein sequences) were added. After constructing the cDNA library, a certain concentration and volume of it was added to the flow cell; the flow cell was then transferred to the Oxford Nanopore PromethION sequencer for real-time single-molecule sequencing.

Analysis of the ONT sequencing data

The original sequencing data contained low-quality sequences and adapters. To obtain clean reads, sequences containing adapters less than 50 bp in length and quality less than 7 were filtered. The long-read transcripts were identified using Pypochopper (version 2.4.0). Subsequent bioinformatics analysis was based on the filtered data. Pinfish (version 0.1.0) was used to construct the non-redundant transcripts of full-length sequences. The spliced_bam2gff program was used for clustering, de-redundancy, and correction to obtain consistent sequences, which were mapped to the *Gallus* reference genome (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/002/315/GCF_000002315.6_GRCg6a/) using minimap2 (version 2.17-r941) (Li, 2018).

Identification of novel transcripts

Full-length transcriptomes can accurately identify transcript structure. Using GffCompare (version 0.11.2), we compared the transcripts obtained after full-length sequencing with the known transcripts of the *Gallus* genome to find new genes and transcripts and supplement genome annotation (Pertea and Pertea, 2020). If there were mapped reads supporting regions outside the original gene boundaries, the untranslated regions of the gene were extended up and down to correct the boundaries.

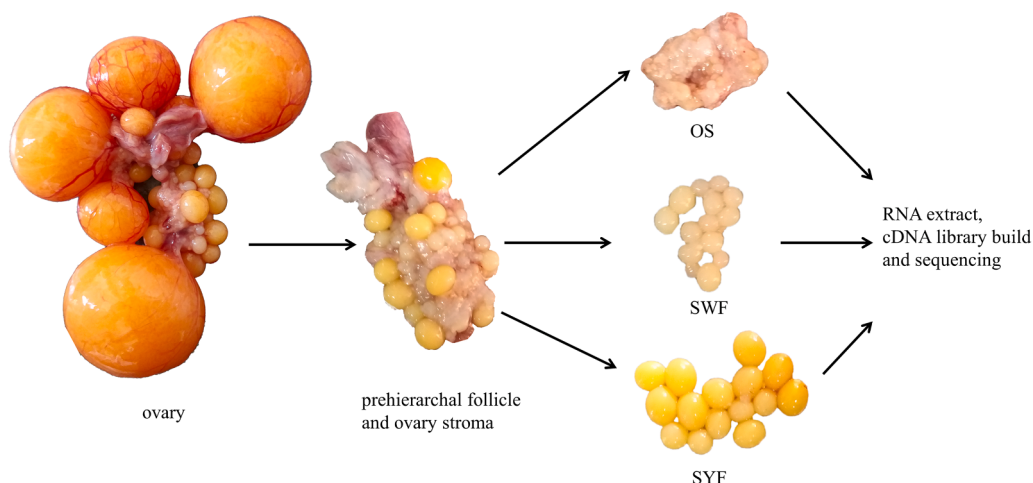


Fig. 1. The samples of hen ovary and follicles.

Alternative splicing (AS) analyses

AS events for consensus sequences that mapped to the reference genome were identified using SUPPA (version 2.3) (Trincado et al., 2018). Based on the analysis results, the transcripts were statistically analyzed for the following seven AS event types: skipping exons (SEs), mutually exclusive exons (MXs), alternative 5' (A5) splice sites, alternative 3' (A3) splice sites, retained introns (RIs), alternative first exons (AFs), and alternative last exons (ALs).

Identification of differentially expressed transcripts (DETs)

We calculated Transcripts Per Kilobase Million (TPM) to assess transcript expression levels by dividing the gene reads count by gene length (kb) and multiplying the obtained value by 1,000,000. The screening threshold was set at $P < 0.05$ and $|\log_2\text{FoldChange}| > 1$; DETs between the groups were analyzed using DESeq2 (version 1.22.2).

Functional enrichment analysis of the genes corresponding to DETs

To elucidate biological functions and signaling pathways associated with the identified DETs, we conducted functional enrichment analysis on their corresponding differentially expressed genes (DEGs). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed using the KOBAS system (version 2.0), with statistical significance set at $P < 0.05$.

Temporal pattern analysis of the genes corresponding to DETs

In the three stages, genes expression pattern shows dynamic change. We performed time-course analysis of the DEGs using BMKCloud with Mfuzz. The time-course clusters were subjected to an enrichment analysis using KOBAS (version 2.0); KEGG bubble charts were drawn.

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from chicken ovarian follicles using an RNAprep Pure Tissue Kit (TIANGEN, China). Synthesis of cDNA was performed using a PrimeScript RT reagent kit with 1.0 µg of the RNA pretreated with gDNA Eraser (Accurate Biology, Changsha, China) according to the manufacturer's protocol. The mRNA expression levels of selected DEGs were analyzed using RT-qPCR with a SYBR Premix Ex Taq™ II kit (Accurate Biology, Changsha, China). The primer sequences are listed in Table S1. The following PCR cycling conditions were used on the Light Cycler 96 Real-Time PCR system (Roche, Basel, Switzerland): initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s and annealing and extension at 60°C for 20 s. The melting curves were drawn; the quantitative analysis of the data was performed using the $2^{-\Delta\Delta CT}$ relative quantification method (Livak and Schmittgen, 2001). Quantification was performed by standardizing the reaction results with those of GAPDH.

Plasmid construction and cell treatments

The protein-encoding region of the R-spondin3 (*RSPO3*) gene was synthesized by site-directed PCR using ApexHF HS DNA Polymerase (Accurate Biology, Changsha, China). PCR products were cloned into the *Bgl*II and *Kpn*I sites of the pEGFP-C1 vector to construct an overexpression vector *RSPO3* (+). *RSPO3*-siRNA (small interfering RNA) and NC (negative control)-siRNA were synthesized by RiboBio (Guangzhou, China). Theca cells and GCs of chicken Pre-hierarchical follicles were collected from Hy-line brown chicken, as described previously by Li et al. (2023). The cells were inoculated into 24-well plates and cultured until they reached 70–90% confluence. Lipofectamine 3000 (Invitrogen, USA) was used for transfection; 500 ng of the DNA constructs were

transiently transfected. After 6 h, the transfection medium was replaced with a new complete medium for an additional 48 h. Subsequently, RNA was extracted from the transected cells and subjected to reverse transcription for RT-qPCR.

TOPFlash assay

To assess the *RSPO3*-induced activation of the Wnt pathway, a TOPFlash assay was performed. The procedures were as follows: GCs were seeded in 24-well plates and co-transfected with the TOPFlash reporter plasmid and 150 mM LiCl (positive control) or empty pEGFP-C1 vector (negative control) or 500 ng of *RSPO3*(+) overexpression plasmid using Lipofectamine™ 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA). After 36 h of post-transfection culture, the reporter activity was quantitatively assessed according to the instructions of the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA).

Statistical analyses

Differences in mRNA expression between the three tissue stages were evaluated using one-way analysis of variance followed by Duncan's multiple range test in SPSS statistics (version 17.0). For one experiment, each treatment was repeated three times; at least three independent experiments were performed. All data are presented as the mean $\pm$ standard error of the mean. $P < 0.05$ indicated a significant difference, and expressed as *. $P < 0.01$ was expressed as **.

Results

Data of nanopore full-length cDNA sequencing

The mRNA obtained from pre-hierarchical follicles and OS were sequenced. Each biological sample was collected from three individuals. The three OS samples were referred to as OS1, OS2, and OS3; the SWF samples were referred to as SWF1, SWF2, and SWF3; and the SYF samples were referred to as SYF1, SYF2, and SYF3. The original FASTQ data were filtered for low-quality reads and joints to obtain valid data for subsequent analysis. The raw data were discarded after obtaining the effective data for each sample (Table 1). All samples had a read number $> 2,000,000$; an N50 $> 1,600$ bp; and a mean read length $> 1,200$ bp. The longest mRNA of the sequencing sample was the SWF3 mRNA, with a length of 120,106 bp.

Using Pinfish (version 0.1.0) and minmap2 (version 2.17), we obtained 53,635 consistent sequences. The mean and maximum lengths of the consistent sequences were 1,341.9 and 8,721 bp, respectively; the N50 was 1,939 bp. Alignments of consistent sequences to the reference genome were classified into three types: unmapped, reads mapped to the sense strand of the genome (denoted "+"), and reads mapped to the antisense strand of the genome (denoted "-"). In the sense strand, 26,741 reads were mapped, whereas in the antisense strand, 26,775 reads were mapped (Table 2).

Table 1

Summary of effective data from OS, SYF and SYF of three biological samples.

Sample Name	ReadNum	BaseNum	N50	MeanLength	MaxLength
OS1	2,406,386	2,928,745,856	1,760	1,217.10	38,911
OS2	2,911,678	3,743,337,828	1,738	1,285.60	50,113
OS3	2,886,267	3,710,994,328	1,779	1,285.70	40,086
SWF1	3,021,180	3,832,909,840	1,739	1,268.70	27,252
SWF2	2,932,055	3,795,608,269	1,691	1,294.50	15,612
SWF3	3,244,115	4,524,221,229	1,871	1,394.60	120,106
SYF1	2,918,297	3,620,108,032	1,738	1,240.50	33,682
SYF2	2,830,006	3,549,548,980	1,664	1,254.30	19,643
SYF3	3,014,181	3,980,013,865	1,824	1,320.40	32,870

Table 2
Comparison between the consistent sequence and the reference genome.

Species name	Total reads	Total mapped	'+'	'.'
Gallus gallus	53,635	53,635 (100.0%)	26,741 (49.86%)	26,775 (49.92%)

Nanopore long-read RNA sequencing technology yields longer reads than second-generation RNA sequencing technologies. In this study, reads with lengths between 400 bp and 600 bp were most abundant, tallying 7,009 counts. (Fig. 2A). The TPM density distribution map reflects the overall expression pattern of the transcripts of each sample. The graph presented in Fig. 2B shows a non-standard normal distribution, with the area of each region as 1.0, thereby representing the sum of probabilities as 1.0. From these transcripts, we identified 2,164 known DETs, which mapped to 1,588 DEGs.

Novel transcripts

Five novel transcript types were identified from the sequencing results (Fig. 3A). The novel transcript type “fully contained with a reference intron” was most common (8,922 cases) and is denoted as “i”. The second novel transcript type, “multi-exon with at least one junction match,” was detected in 6,776 cases. “Exonic overlap on the antisense strand” was the third type (2,831 cases) and is denoted as “x”. The least common case of novel transcript type, “other same strand overlap with reference exons” was detected in 652 cases and is denoted as “o” in Fig. 3A. A total of 1,759 cases of unknown and intergenic sequences, denoted as “u” in Fig. 3A, were identified.

AS events

Based on SUPPA analysis results, the predicted number of AS events is shown in Fig. 3B. AFs were detected in 1,878 cases. SEs were detected in 1,417 cases. A5 and A3 were detected in 1,020 and 839 cases, respectively. RIs, ALs, and MXs were detected in 381, 332, and 78 cases, respectively. The proportions of AS events in OS vs. SWFs (Fig. 3C) were similar to the proportions of AS events in SWFs vs. SYFs (Fig. 3D).

Functional enrichment analysis of DEGs

The standardized data were analyzed using the DESeq2 package. Volcano plots were generated to visualize upregulated and downregulated DEGs in the comparative analyses of OS vs. SWFs and SWFs vs. SYFs. In SWFs, 406 and 130 DEGs were downregulated and upregulated, respectively, compared with in OS. *MMP10*, *CYP19A1*, and *FST* expression levels were significantly higher in SWFs than in OS. Conversely, *APOA1* and *RSPO3* expression levels were significantly

lower in SWFs than in OS. The KEGG pathway enrichment analysis of DEGs revealed several signaling pathways pertinent to follicle development and related physiological processes. Among them, the steroid hormone biosynthesis and MAPK signaling pathways were enriched in the OS vs. SWF group. Based on GO analysis, the extracellular region was enriched in the OS vs. SWF group (Fig. 4A). A comparative analysis of SWFs and SYFs revealed 113 upregulated and 308 downregulated DEGs. The SYF group exhibited markedly elevated expression of genes linked to extracellular matrix (ECM) organization, including *MMP9*, as well as the collagen genes *COL4A1* and *COL3A1*, which are critical for matrix remodeling and basement membrane integrity. Furthermore, the expression levels of key cell cycle and transcription regulators, such as *CCNB2*, *HPF1*, *AURKA*, and *MED8*, were also significantly lower in SYFs than in SWFs. The expression levels of genes linked to yolk deposition, i. e., *VLDLR* and *SNX1*, were also notably reduced in the SYF group. For DEGs of SWFs vs. SYFs, KEGG enrichment analysis showed the following pathways: endocytosis, steroid hormone biosynthesis, p53, and FoxO signaling pathways. The GO terms of extracellular matrix and cell adhesion were significantly enriched in the SWF vs. SYF group (Fig. 4B).

Time-course KEGG enrichment analysis of DEGs

All DEGs identified in this study were classified into four clusters using Mfuzz (Fig. 5A). These clusters exhibited unique expression patterns across the three developmental stages. Genes in cluster 1 exhibited the highest expression levels in SWFs. Enrichment analysis revealed that this gene cluster was significantly enriched in the spliceosome and endocytosis pathways (Fig. 5B); key DEGs included *SNX2* and *ALDH7A1*. Genes in cluster 2 exhibited lowest expression at the SYF stage. These genes are associated with RNA transport, ribosome biogenesis in eukaryotes, and the autophagy signaling pathway (Fig. 5C). The expression levels of DEGs in cluster 3 increased gradually across the three developmental stages, with significantly enriched Wnt signaling, focal adhesion, ECM–receptor interaction, and TGF- β signaling pathways (Fig. 5D). DEGs in cluster 4 exhibited the highest expression in OS, with enriched MAPK and phagosome signaling pathways (Fig. 5E).

Clustering of DEGs in follicular development during the three developmental stages

To further delineate stage-specific expression patterns, Venn diagrams were employed to visualize distinct DEGs across the three developmental stages. As shown in Fig. 6A; 671, 132, and 176 DEGs were uniquely found in the OS vs. SYF, SWF vs. SYF, and OS vs. SWF comparison groups, respectively. Notably, 17 DEGs were commonly identified in these three pairwise comparisons between the two follicular development stages, including *INHBB*, *MMP9*, *GDF9*, and *RLN3*, which have been extensively documented for their roles in pre-hierarchical follicle development. These 17 genes were enriched in the

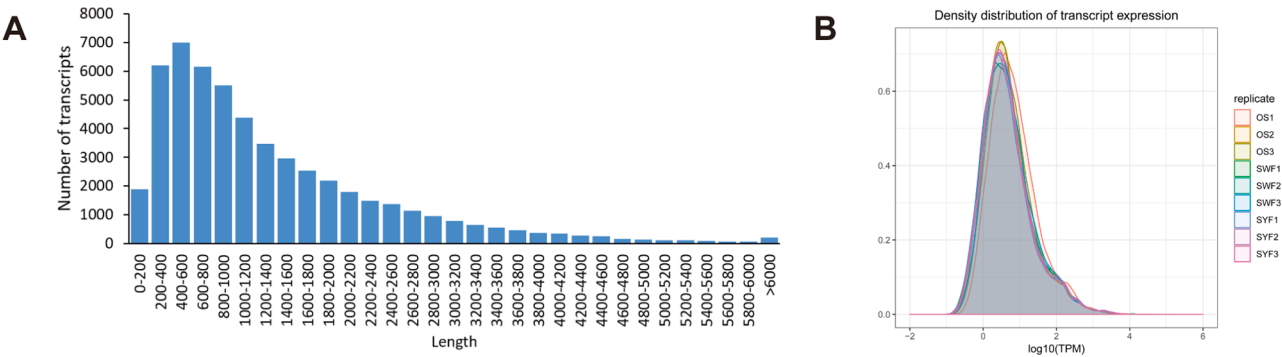


Fig. 2. General sequencing information. (A) Numbers of various transcripts length of sequencing with 9 samples. (B) Density distribution of transcripts expression between 9 samples.

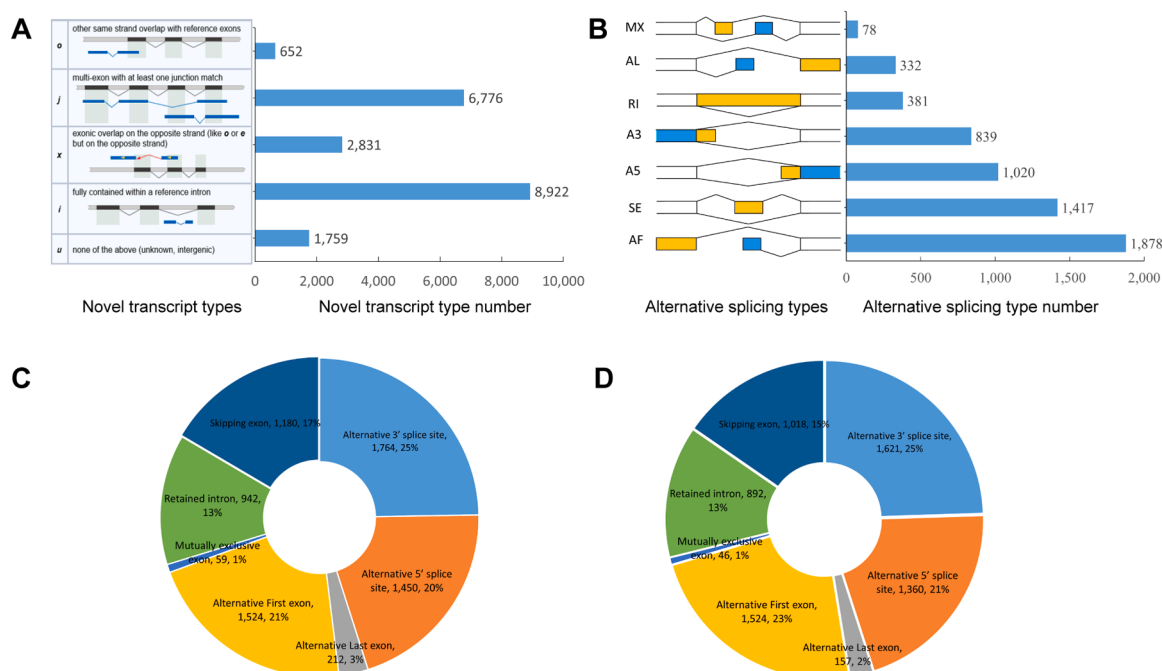


Fig. 3. Identification of new transcripts and AS events. (A) Numbers of various new transcripts types and their corresponding proportions based on all full-length transcripts. (B) Numbers of various AS events and their corresponding proportions based on all full-length transcripts. (C) The number and proportion of alternative splicing events compared to OS and SWF. (D) The number and proportion of alternative splicing events compared to SWF and SYF.

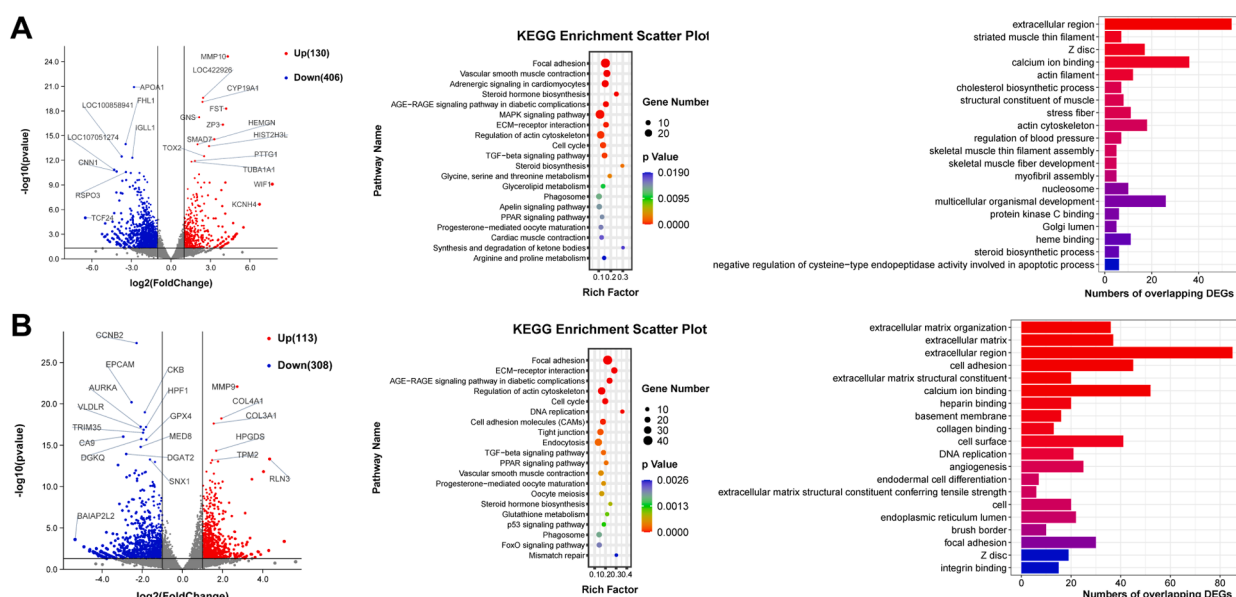


Fig. 4. Scatterplot of annotated differently expressed genes and enriched signaling pathways between OS, SWF and SYF. (A) The DEGs analysis between ovary stroma (OS) and small white follicles (SWF). (B) The DEGs analysis between small white follicles (SWF) and small yellow follicles (SYF). From left to right are the volcano plot, KEGG and GO chart.

cytokine–cytokine receptor interaction, neuroactive ligand–receptor interaction, and TGF- β signaling pathways (Fig. 6B). In total, 671 unique DEGs were identified in the OS vs. SYF group (Fig. 6C). These genes were differentially expressed only in the OS vs. SYF group and included *COL4A6*, *THBS2*, *CYP17A1*, and *ANGPT4*. They were enriched in the ECM–receptor interaction, O-glycan biosynthesis, and steroid hormone biosynthesis signaling pathways. In total, 132 unique DEGs were identified in the SWF vs. SYF group (Fig. 6D). These genes included *HES4*, *RAD51*, *CA9*, and *MYL2*, which were enriched in the Fanconi anemia, nitrogen metabolism, and β -alanine metabolism signaling pathways. In

total, 175 DEGs, including *APOA1*, *FABP4*, *VEGFD*, *HSPB1*, and *TGFB2*, were found in the OS vs. SWF group (Fig. 6E). These genes were enriched in the vascular smooth muscle contraction, peroxisome proliferator-activated receptor (PPAR), Gly–Ser–Thr metabolism, motor protein, and focal adhesion signaling pathways. These findings highlight that although these genes are active at all stages, their differential regulation likely underlies differences in follicle development.

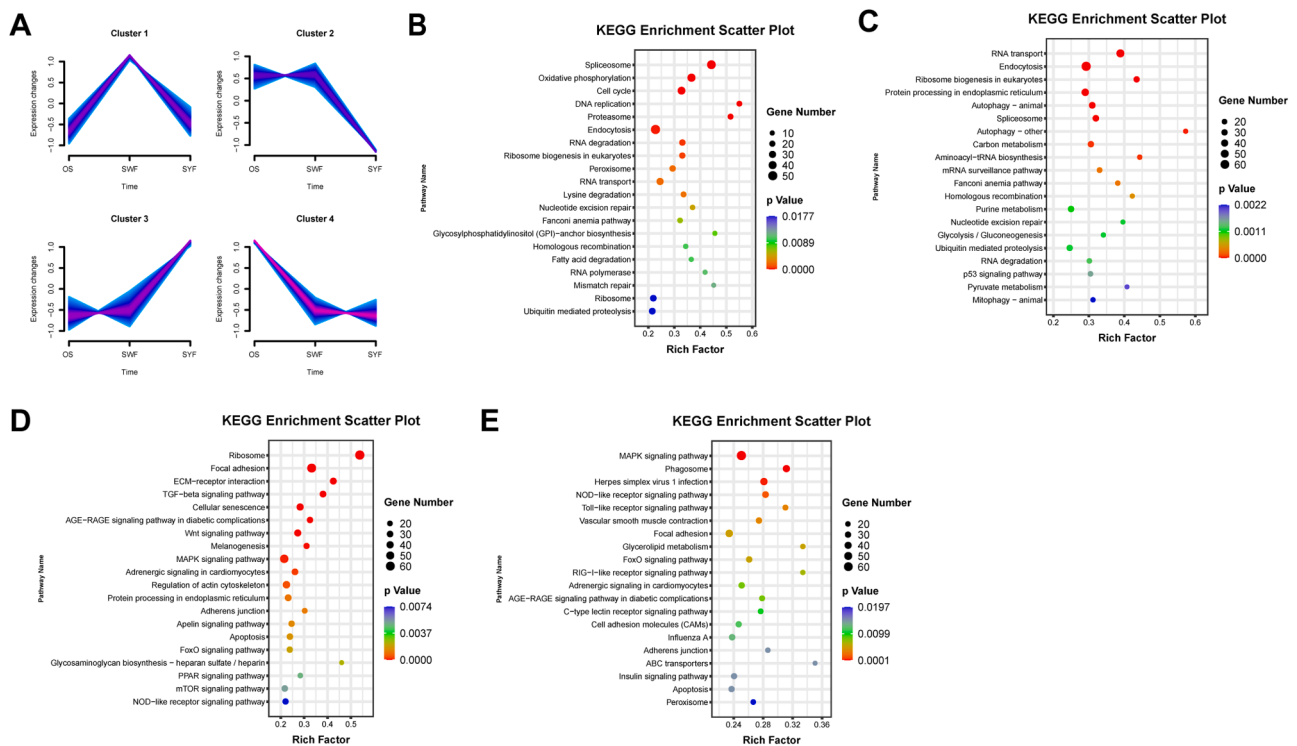


Fig. 5. The time-course of transcripts in the sequencing and the time-course cluster KEGG analysis based on the cluster. (A) Four cluster were enriched. The values of expression change indicate the expression level normalizing the TPM values of transcripts. (B) The KEGG analysis of cluster 1. (C) The KEGG analysis of cluster 2. (D) The KEGG analysis of cluster 3. (E) The KEGG analysis of cluster 4.

Validation of candidate genes using RT-qPCR

Based on the development characteristics of avian follicles and KEGG/GO analysis results, 19 critical transcripts that were involved in pre-hierarchical follicle development were selected. *AMH*, *GDF9*, *FST*, *BMPR1B*, and *INHBB* were selected for their association with follicle recruitment. During the pre-hierarchical developmental stage, the steroid hormone biosynthesis pathway was remarkably enriched. *StAR* emerged as a critically associated gene in this process. In this study, the expression of *StAR* increased markedly with follicular development. The contribution of dynamic stromal remodeling to follicle development was also investigated. The matrix metalloproteinase (MMP) genes *MMP2* and *MMP10* were detected. The angiogenesis pathway-related transcripts include *VEGFA*, *VEGFC*, *VEGFD*, *FLT1*, *KDR*, and *FLT4*. For the Wnt signaling pathway, we selected the *WNT4*, *WNT9A*, *WIF1*, *FZD1*, *RSPO3*, and *CTNNB1* genes for RT-qPCR-based validation of their expression levels. RNA sequencing features of the above-mentioned transcripts are shown in Table 3. The primer sequences are listed in Table S1. Among them, *AMH*, *BMPR1B*, *FST*, *INHBB*, *VEGFA*, *VEGFC*, *FLT1*, *KDR*, *MMP10*, *StAR*, *WNT4*, *WIF1*, and *WNT9A* mRNA levels were upregulated in SWFs compared with in OS. *GDF9*, *VEGFD*, *FLT4*, *MMP2*, *RSPO3*, and *CTNNB1* expression levels were downregulated in SWFs (Fig. 7). When follicles developed to the SYF stage, the *FST*, *INHBB*, *VEGFA*, *VEGFD*, *FLT1*, *FLT4*, *MMP2*, *MMP10*, *StAR*, *RSPO3*, *CTNNB1*, and *WNT9A* genes exhibited high expression, whereas the expression of the corresponding transcripts was downregulated in SYFs compared with in SWFs (Fig. 8). Hence, the RT-qPCR results were consistent with RNA sequencing results.

RSPO3 regulates genes implicated in steroid hormone synthesis

The candidate gene *RSPO3* was highly expressed in SWFs, with significantly higher expression in GCs than in theca cells (Fig. 9A). Its function in follicle development was preliminarily investigated using its

overexpression and siRNA constructs. Transfection with the *RSPO3* overexpression vector significantly increased *RSPO3* mRNA levels in chicken pre-hierarchical follicle cells (Fig. 9B). In contrast, at 48 h after *RSPO3*-siRNA transfection, *RSPO3* expression decreased compared with that in the NC-siRNA group (Fig. 9C). In chicken pre-hierarchical follicle GCs, *RSPO3* significantly upregulated *FSHR* expression but had no significant effect on *CYP11A1* or *StAR* expression (Fig. 9D). RNA interference-mediated knockdown of *RSPO3* significantly downregulated *FSHR* and *StAR* expression, whereas *CYP11A1* levels remained unchanged (Fig. 9E). All three genes play crucial roles in steroid hormone synthesis, which indicates that *RSPO3* promotes steroid hormone expression in chicken pre-hierarchical follicles.

RSPO3 regulates the Wnt signaling pathway

After *RSPO3* gene overexpression, a significant increase in the downstream Wnt signaling pathway gene *CTNNB1* expression was observed (Fig. 10A). Additionally, a TOPFlash assay showed that reporter activity was significantly higher in the *RSPO3* overexpression group than in the pEGFP-C1 control group, consistent with the effect of LiCl positive control (Fig. 10B). Together, these results demonstrate that *RSPO3* activates the Wnt signaling pathway in chicken pre-hierarchical follicles.

Discussion

During pre-hierarchical follicle development, the number of follicles at each stage and the fate of development will affect the pool of SYFs (6–8 mm in diameter). Some SYFs in the pool will be selected to undergo further development to ovulatory follicles. Active primordial follicles are the basis for continuous laying production. However, if pre-hierarchical follicle atresia probability is greatly increased, reproduction performance will decline (Knight and Glistler, 2006). The three fates of primordial follicles can be summarized as follows: maintaining an

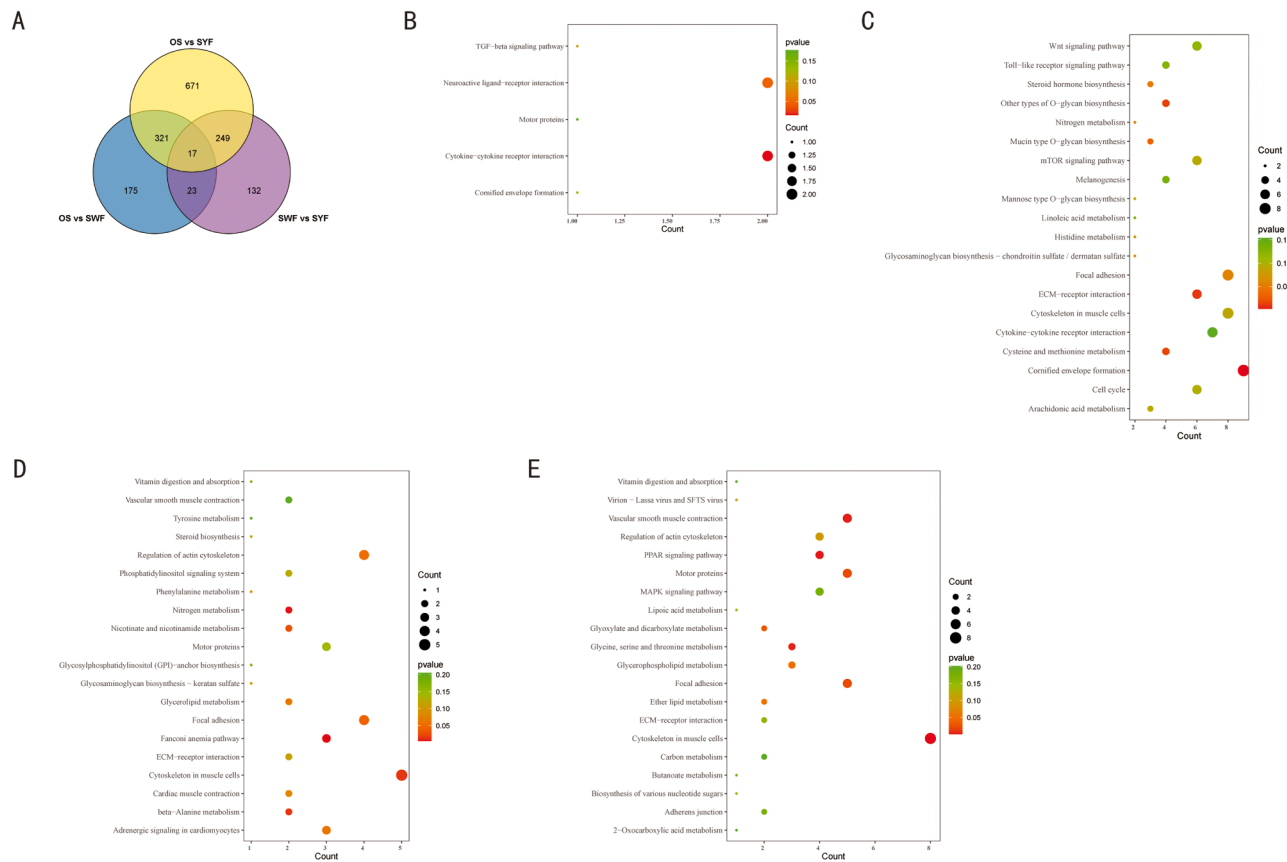


Fig. 6. Venn diagram analysis of the DEGs from the three comparison groups. (A) Venn diagram comparing the distribution of DEGs between OS, SWF and SYF. (B) The KEGG analysis of intersections of all groups (17 genes). (C) The KEGG analysis of genes in the yellow region (671 genes). (D) The KEGG analysis of genes in the violet region (132 genes). (E) The KEGG analysis of genes in the blue region (175 genes).

Table 3
The candidate different expression genes.

Gene name	Initial recruitment				Prehierarchal Follicular Development			
	OS	SWF	P-Value	log ₂ FoldChange	SWF	SYF	P-Value	log ₂ FoldChange
AMH.t1	18.378	47.439	6.54E-05	1.342	47.439	16.662	2.74E-05	-1.205
GDF9.t1	15.181	3.279	0.000665	-2.253	3.279	0.284	0.050087	-3.120
BMPR1B.t2	0.425	3.373	0.027698	3.089	3.373	1.635	0.416748	-0.773
FST.t3	2.862	52.083	4.10E-15	4.103	52.083	62.5461	0.085937	0.569
INHBB.t2	0.00	2.8223	0.014403	4.374	2.822	9.476	0.005851	2.050
STAR.t1	12.813	58.531	2.75E-10	2.137	58.531	115.946	1.56E-09	1.275
MMP2.t1	25.409	23.411	0.550522	-0.227	23.411	46.653	2.69E-06	1.316
MMP10.t2	11.216	221.926	1.37E-23	4.329	221.927	534.413	0.006091	1.536
VEGFA.t2	1.717	3.767	1.017386	0.439	3.767	5.246	0.7703722	0.399
VEGFC.t1	0.353	1.746	0.169322	2.252	1.746	0.689169	0.402465	-1.059
VEGFD.t1	7.292	1.125	0.000856	-2.772	1.125	1.563	0.490906	0.783
FLT1.t1	0.381	1.326	0.125078	1.798	1.326	1.998	0.567974	0.902
KDR.t1	1.143	1.651	0.647559	0.375	1.652	2.175	0.266402	0.669
FLT4.t1	1.302	0.601	0.263003	-1.211	0.601	1.565	0.080516	1.699
RSPO3.t5	17.781	1.608	1.32E-07	-3.592	1.608	2.239	0.44546	0.748
WNT4.t1	12.051	14.994	0.524671	0.377	14.994	4.829	0.023603	-1.378
WNT9A.t2	0.792	7.761	0.003143	3.312	7.761	14.049	0.025097	1.155
WIF1.t3	0.000	19.339	1.41E-08	7.317	19.349	14.819	0.867918	-0.079
CTNNB1.t1	10.277	7.993	0.302654	-0.429	7.993	11.699	0.016743	0.851

inactive state and maintaining the length of female reproductive life, directly dying from a dormant state, and initial recruitment to form a primary follicle. Follicular atresia probability remains high at all stages during follicle development, except for the preovulatory follicle stage. The follicle bank is greatly lost owing to atresia; only a few hundred primordial follicles remain that eventually develop to ovulation in domestic chickens. Whether in mammals or birds, a physiological process of primordial follicle activation in follicular development exists (Kim,

2012; Zhao et al., 2017). The primordial follicle is activated and grows into the primary follicle (initial recruitment), which is related to the formation of inner membrane cells and the transition that occurs via the development of a single inner layer of GCs and incorporation of a multicellular theca layer (Johnson, 2015). Continuous recruitment activities keep the number of follicles before ovulation in dynamic balance. Although various growth factors have been implicated in initiating or inhibiting follicle recruitment in mammals, they have apparently not

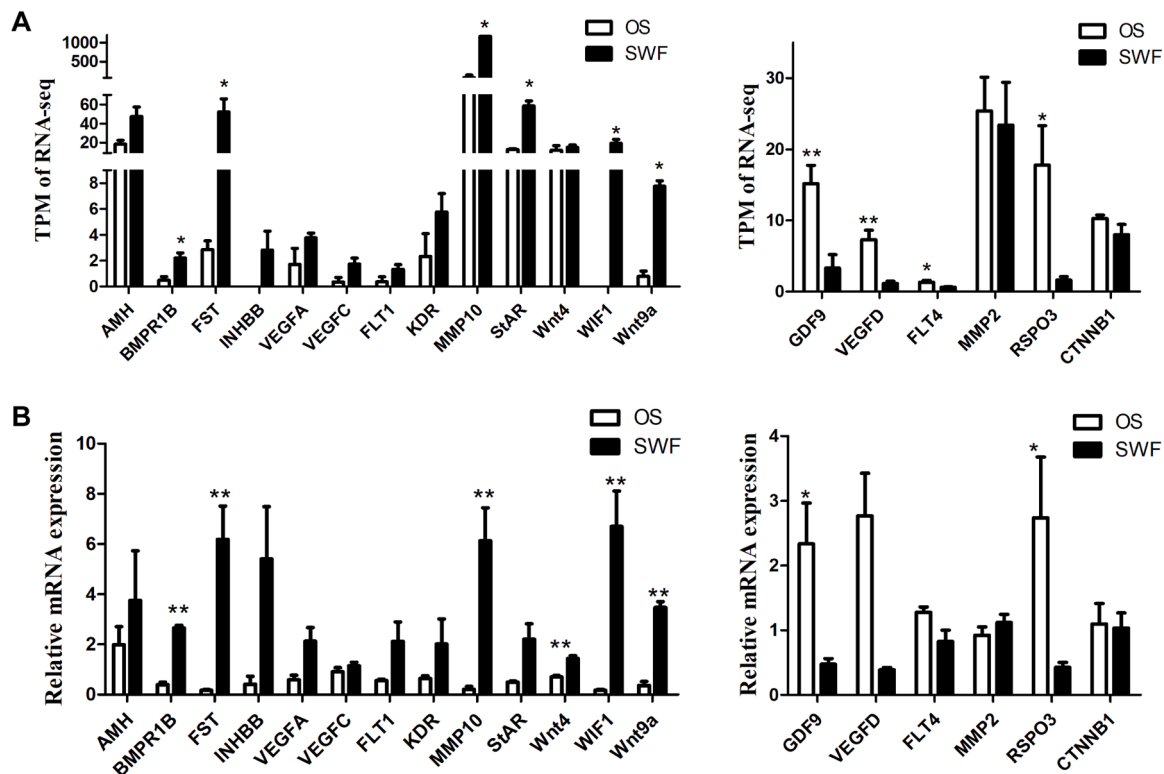


Fig. 7. RT-qPCR validation of differentially expressed genes identified by RNA-Seq. (A) The TPM of genes between ovary stroma (OS) and small white follicles (SWF), left is the upregulated genes and the right is the downregulated genes. (B) The relative mRNA expression of genes between ovary stroma (OS) and small white follicles (SWF).

been investigated in the avian ovary. Anti-Müllerian hormone (AMH) maintains follicles in a quiescent state and prevents premature primordial follicle pool failure in mammalian studies (Münsterberg and Lovell-badge, 1991; Prapa et al., 2015). The granular layer is the main source of AMH in small follicles of hens, indicating that AMH exists in the early stages of follicular development and is expressed in very small follicles (approximately 150 μ m) (Chen et al., 2020). According to studies on mammalian follicles, *FSH*; *TGF- β* ; activin/inhibin; and other genes may contribute in developing primary follicles. Rupture atresia may occur in pre-hierarchical follicles (2–8 mm in diameter). In this study, AMH expression correlated positively with the degree of follicular development and was highest in SWFs. This result may be related to its secretion by GCs, whose number increased significantly in SYFs. *BMPR1B* mRNA abundance correlated positively with plasma estradiol levels, suggesting that its estrogen-mediated regulation may be associated with normal folliculogenesis (Glister et al., 2010). In the present study, we found that *BMPR1B* expression increased with follicular development, with *BMPR1B.t4* and *BMPR1B.t11* being highly expressed in SWFs and SYFs, respectively. Follistatin, secreted by GCs, binds to activin with high affinity to neutralize its bioactivity and regulate early follicular development (Kaiser and Chin, 1993; Nakamura et al., 1990; Vos et al., 2014). In the present study, *FSTL4.t3* was highly expressed in OS, whereas *FST* was highly expressed in both SWFs and SYFs. *GDF9* is derived from oocytes; therefore, its expression was highest in OS. These genes play regulatory roles in the development of primordial and early follicles. Furthermore, the ECM–receptor interaction, steroid hormone biosynthesis, and phagosome signaling pathways promote the development of growing follicles and are related to the high-yield performance of laying hens (Shi et al., 2024; Zhou et al., 2020), which was also confirmed in our study.

Enrichment of several signaling pathways and vital genes was observed during the formation of the SYF pool. In the ovary, extensive tissue remodeling is required during follicular development and

follicular wall-breaking at the time of ovulation. Extracellular proteases, such as serine proteases and MMPs, are believed to be crucial in these processes (Liu et al., 1998), as they require extensive matrix remodeling. According to existing studies, the MMP family contributes to follicular development and ovulation (Belotti et al., 2003; Mazaud et al., 2005). In the present study, *MMP10* expression increased rapidly with follicular development. The expression of genes related to angiogenesis, steroid hormone synthesis, and blood vessel development increases significantly during follicular development (Ma et al., 2020). Pig antral follicles produce substantial amounts of vascular endothelial growth factor (VEGF) during their growing phase in response to gonadotropic stimulation (Mattioli et al., 2001). GCs are the main source of VEGF production. The activation of porcine follicles is associated with VEGF production and vascular endothelium migration. VEGF can promote the transformation of primary follicles into secondary follicles and the development of preantral follicles (Anasti et al., 1998; Yang and Fortune, 2007), indicating that angiogenesis plays a critical role during all stages of follicle development. During avian follicle development, blood vessels can be observed with the naked eye at the pre-hierarchical follicular stage. Insufficient blood vessel supply in the membrane cell layer has been reported to be a major cause of follicular atresia (Acosta, 2007; Kim et al., 2016). In the present study, the receptors and ligands of the VEGF family that are related to vascular development exhibited differential patterns during early follicular development. *VEGFA/C*, *FLT1*, and *KDR* expression increased with the development of follicles. *ANGPT2.t1* expression was limited to SWFs, whereas *ANGPT2.t4* expression was upregulated with follicular development. Regulation of the expression patterns of these genes should be explored in future studies. A comparative transcriptome analysis of large white follicles, SYFs, and large yellow follicles revealed the following important signaling pathways: neuroactive ligand–receptor interaction, cell adhesion molecule, apelin, PPAR, and cAMP signaling pathways (Chen et al., 2021). In the present study, we observed the enrichment of the

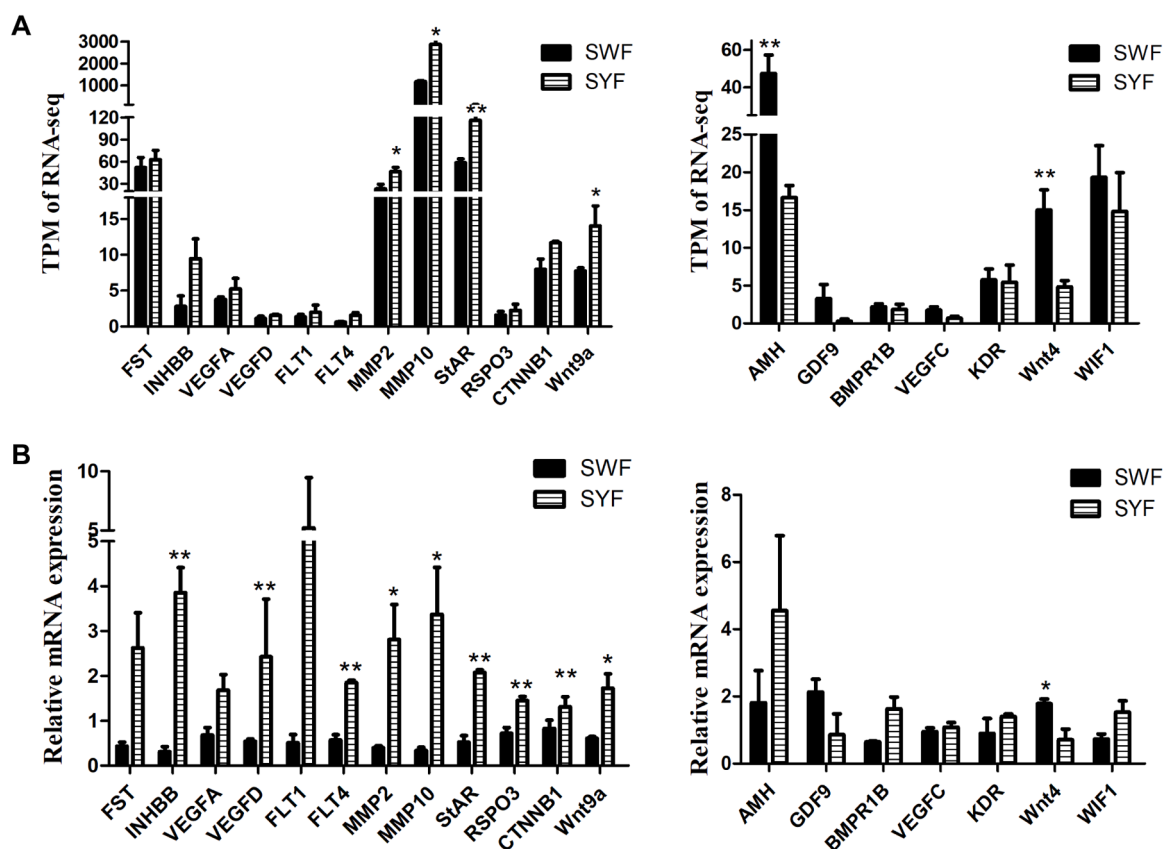


Fig. 8. RT-qPCR validation of differentially expressed genes identified by RNA-Seq. (A) The TPM of genes between small white follicles (SWF) and small yellow follicles (SYF), left is the upregulated genes and the right is the downregulated genes. (B) The relative mRNA expression of genes between small white follicles (SWF) and small yellow follicles (SYF).

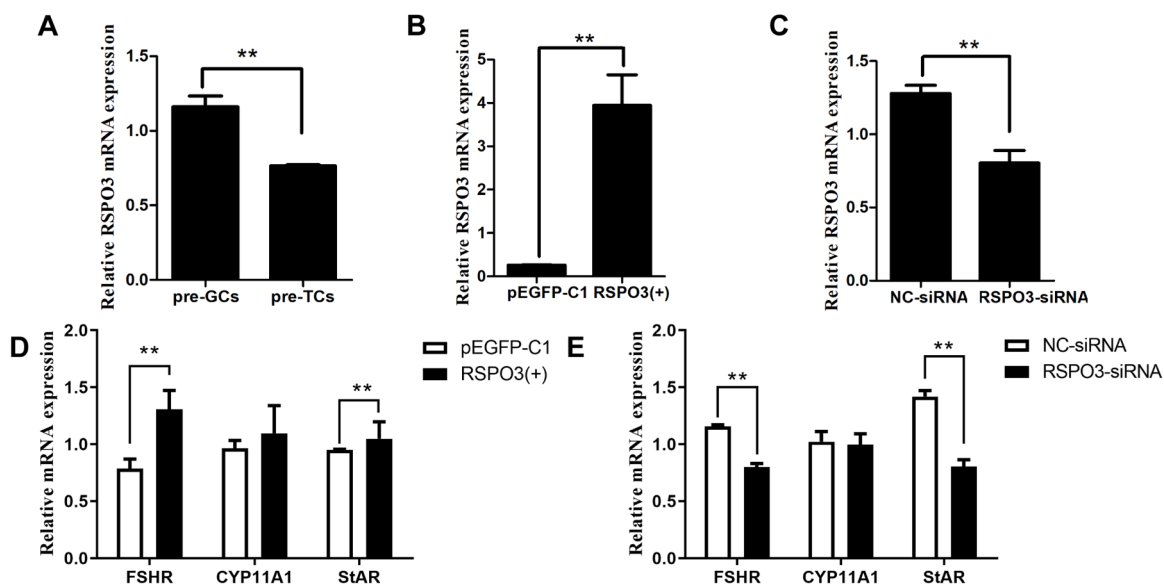


Fig. 9. *RSPO3* regulated steroid hormone synthesis genes in chicken prehierarchical follicle cells. (A) Relative expression abundance of the *RSPO3* gene in granulosa cells and theca cells of prehierarchical follicles. (B) The expression of *RSPO3* in granulosa cells which were transfected with *pEGFP-C1* or *pEGFP-C1-RSPO3*. (C) The expression of *RSPO3* in granulosa cells which were transfected with *NC-siRNA* or *RSPO3-siRNA*. (D) Granulosa cells were transfected with *pEGFP-C1* or *pEGFP-C1-RSPO3* which shows as *RSPO3* (+), and the expression of *FSHR*, *CYP11A1* *StAR* were detected by RT-qPCR. (E) Granulosa cells were transfected with *NC-siRNA* or *RSPO3-siRNA*, and the expression of *FSHR*, *CYP11A1* *StAR* were detected by RT-qPCR. **indicates $P < 0.01$.

apelin signaling pathway, which impacts steroid secretion and cell proliferation in cattle and pigs (Gupta et al., 2023; Rak et al., 2017). The Gly-Ser-Thr metabolism governs amino acid synthesis and degradation

and is intrinsically linked to energy production, nucleotide synthesis, and oxidative stress responses in cells. It coordinates processes that support GC proliferation and hormone synthesis, thereby regulating

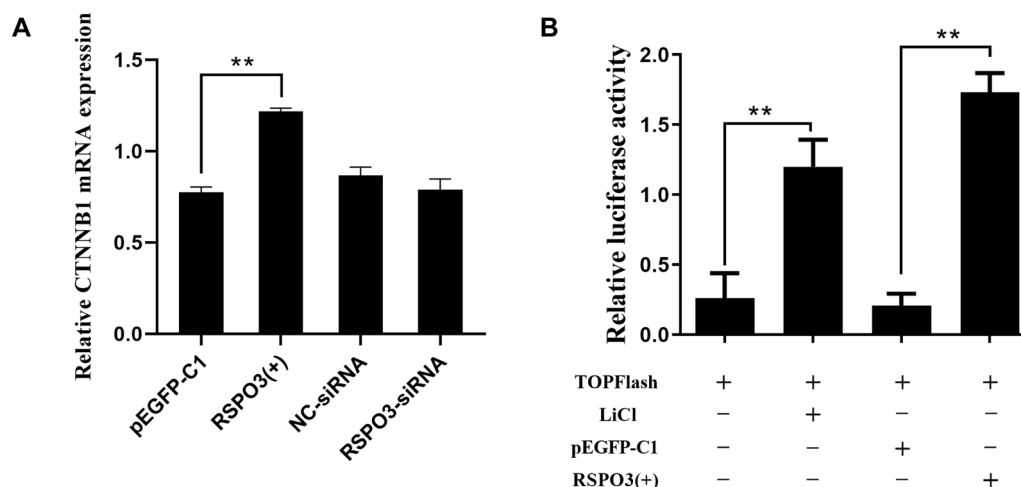


Fig. 10. RT-qPCR and TOPFlash assay detection *RSPO3* regulate the Wnt signaling pathway. (A) GCs were treatment with pEGFP-C1/*RSPO3*(+), or NC-siRNA/*RSPO3*-siRNA, and *CTNNB1* gene was detected by RT-qPCR. **indicates $P < 0.01$ (B) The TOPFlash activity following *RSPO3* expression was assessed by a dual luciferase reporter assay.

follicular development (Du et al., 2025). The Fanconi anemia pathway was enriched among the downregulated gene sets in the SYF group during temporal analysis. This pathway impacts reproductive reserve by regulating genomic stability (Xu et al., 2023). Its downregulation in SYFs may exert regulatory effects related to genomic stability.

Wnt signaling plays a pivotal role in mammalian gonad development, sex differentiation, and follicle formation and maturation (Arend et al., 2013; Gifford, 2015; Jääskeläinen et al., 2010). As a new member of the Wnt pathway discovered in recent years, there have been some studies on the regulation of Wnt by genes in the *RSPO* family (Kamata et al., 2004). In humans and other mammals, the *RSPO* family plays a key role in ovarian follicular development by regulating cell proliferation and apoptosis, with this function mediated largely through effectors such as β -catenin (Chassot et al., 2008; De et al., 2014). *RSPO2* promotes the transformation of primary follicles into secondary follicles in rats and inhibits the proliferation and differentiation of GCs after its knockout (Cheng et al., 2013; Liu et al., 2019). *RSPO2/RSPO3* expression gradually increases with follicle development and promotes GCs development in pig ovarian follicles (Huang et al., 2021; Zhou et al., 2021). In the present study, it was verified that several Wnt signaling pathway genes were expressed differently at different stages of pre-hierarchical follicles. Based on these results, it can be inferred that the transcripts of the Wnt signaling pathway exhibit varying expression trends during the development of pre-hierarchical follicles in chickens. In the present study, *RSOP3* was found to be involved in chicken ovarian pre-hierarchical follicle development by regulating *FSHR*. *RSPO3* activated the Wnt/ β -catenin pathway, as evidenced by the induction of *CTNNB1* and TOPFlash reporter. These results suggest that the *RSPO3* gene may act as a Wnt signaling pathway activator to regulate follicle development. However, the internal molecular mechanisms should be explored in future studies.

Conclusions

The above-mentioned genes can be used as candidate genes for studying pre-hierarchical follicular development. The results obtained in this study reveal that steroidogenesis and angiogenesis are prominent during the growth and development of SWFs. The endocytosis, Wnt, p53, and Fanconi anemia pathways are crucial signaling pathways in SYFs, whereas the TGF- β signaling pathway is involved in the entire process of pre-hierarchical follicle development. Additionally, our results demonstrate that the apelin signaling, Gly-Ser-Thr metabolism, and Fanconi anemia pathways, as well as the *RSPO3* gene, play crucial regulatory roles in pre-hierarchical follicular development in chickens.

This study establishes a foundation for further investigation of the regulatory mechanisms of candidate genes involved in pre-hierarchical follicular development, thereby enhancing our understanding of the hen reproductive system development.

Data availability

Transcriptome datasets for this study are available in the NCBI database under the accession number PRJNA1420151. They are also available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Xiaoyun Cao: Writing – original draft, Data curation. **Wenting Xu:** Software. **Hongrui Zhang:** Software. **Jiale Zhang:** Validation. **Yunliang Jiang:** Resources. **Xianyao Li:** Resources. **Li Kang:** Writing – review & editing, Project administration.

Disclosures

The authors have no conflicts of interest to declare.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2026.106657.

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