Long-read sequencing reveals the effect of follicle-stimulating hormone on the mRNA profile of chicken granulosa cells from prehierarchical follicles

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ABSTRACT Follicle selection is an important step in the laying process of chicken, which is closely related to the laying performance and fecundity of hens. Follicle selection mainly depends on the regulation of follicle-stimulating hormone (**FSH**) secreted by pituitary gland and the expression of follicle stimulation hormone receptor. To uncover the role of FSH in chicken follicle selection, in this study, we analyzed the changes in the mRNA transcriptome profiles of FSH-treated chicken granulosa cells from prehierarchical follicles by long-read sequencing Oxford Nanopore Technologies (**ONT**) approach. Among the 10,764 genes detected, 31 differentially expressed (**DE**) transcripts of 28 DE genes were significantly upregulated by FSH treatment. These DE transcripts (**DETs**) were mainly related to the steroid biosynthetic process by GO analysis and enriched in pathways of ovarian steroidogenesis and aldosterone synthesis and secretion by KEGG analysis. Among these genes, the mRNA and protein expression of TNF receptor associated factor 7 (***TRAF7***) was upregulated after FSH treatment. Further study revealed that TRAF7 stimulated the mRNA expression of steroidogenic enzymes steroidogenic acute regulatory protein (***StAR***) and cytochrome P450 family 11 subfamily A member 1 (***CYP11A1***) genes and the proliferation of granulosa cells. This is the first study to investigate differences in chicken prehierarchical follicular granulosa cells before and after FSH treatment by using ONT transcriptome sequencing, which provides a reference for a more comprehensive understanding of the molecular mechanism of follicle selection in chicken.

Key words: chicken, follicle selection, granulosa cell, FSH, TRAF7

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INTRODUCTION

Chicken is an oviparous animal. The ovary of chicken is a dynamically developing reproductive organ and plays a decisive role in follicle development and ovulation (Mfoundou et al., 2021). There are 3 types of ovarian follicles differing in diameter, including slow-growing follicles (1−5 mm in diameter), prehierarchical follicles (6−8 mm in diameter, also called small yellow follicles), and hierarchical follicles (also called preovulatory follicles) (Johnson, 2015). When a hen reaches sexual maturation, from a pool of growing follicles, one small yellow follicle in the diameter of 6 to 8 mm will be selected to become dominant and continues maturation until it ovulates (Johnson, 2012), the process of which is called follicle selection.

Follicle selection in chicken is finely regulated by hormones from hypothalamus-pituitary-ovary axis, among which follicle-stimulating hormone (**FSH**) plays an essential role by stimulating the expression of FSH receptor and regulating the activity of genes involved in this process. Upon follicle selection, in the form of nerve impulse, gonadotrophin-releasing hormone secreted by hypothalamus acts on the anterior pituitary to stimulate the synthesis and secretion of FSH (Shen et al., 2017), which subsequently promotes the proliferation and differentiation of granulosa cells and stimulates the synthesis of progesterone. With the development of follicles, the response of dominant follicle (usually the small yellow follicle with the highest *FSHR* expression) to FSH is enhanced, and the expression of *FSHR* is significantly increased, thus promoting the selection, development, and maturation of follicles (Chu et al., 2018; Chen et al., 2020).

In chicken, the proliferation and differentiation of granulosa cells are major features of follicle selection.

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For follicles being selected, in response to FSH, the expression of steroidogenic acute regulatory protein (***StAR***) and cytochrome P450 family 11 subfamily A member 1 (***CYP11A1***) genes increase in the granulosa cells, resulting in the production of steroid hormones including progesterone via cyclic adenosine monophosphate (Francoeur et al., 2021), while for granulosa cells from prehierarchical follicles, they are suppressed by factors and/or signaling cascades, such as mitogen-activated protein kinase signaling (Woods and Johnson, 2005). As an oocyte-derived factor, growth differentiation factor 9 (**GDF9**) promotes FSH-induced progesterone production in chicken granulosa cells and regulates the expression of *FSHR* and *STAR* (Li et al., 2019). In cooperation with GDF9, forkhead box L2 is involved in the intracellular FSHR transcription and granulosa cell proliferation via an autocrine regulatory mechanism (Qin et al., 2015). Recently, FSH is reported to increase the survival of granulosa cells by suppressing DNA damage through the CHK2/p53 pathway (Zhou et al., 2022).

By previous transcriptomic analysis on the expression differences between chicken small yellow follicles differing in FSHR expression, we analyzed the mechanisms underlying follicle selection in chicken and revealed the role of Wnt4 and SOWAHA in chicken follicle selection (Wang et al., 2017; Zhong et al., 2021). However, due to that chicken follicle is a complex tissue consisting of oocyte and somatic cells including theca and granulosa cells, and that granulosa cells exhibit changes both in cell number (proliferation) and function (differentiation), it is essential to identify genes specifically expressed in chicken granulosa cells that are involved in follicle selection. Recently, long-read sequencing using Oxford Nanopore Technologies (**ONT**) approach is more and more widely used to identify differentially expressed genes and transcripts (Lu et al., 2016). Therefore, in this study, by ONT approach, we analyzed the changes in the transcriptomes of chicken granulosa cells from prehierarchical follicles after treatment with FSH and identified the function of TNF receptor associated factor 7 (***TRAF7***) in granulosa cells of chicken follicles.

MATERIALS AND METHODS

Animals and Sample Collection

Hy-line brown hens 38 wk of age with regularly laying for at least 1 mo were used in this study. The hens were housed individually in laying batteries, with free access to feed and water, under a photoperiod of 16 h light and 8 h dark. These hens were sampled randomly from the farm, and euthanized in the lab by cervical dislocation immediately. Then, the ovaries including all sized follicles were carefully obtained from each chicken, prehierarchical follicles were collected to prepare granulosa cells. The Institutional Animal Care and Use Ethics Committee of Shandong Agricultural University reviewed and approved all procedures described in this study (No. SDAUA-2022-36). This study was performed in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology of China.

Cell Culture and Treatment With FSH

Primary granulosa cells were prepared and cultured according to reference (Wang et al., 2017). Briefly, prehierarchical follicles including small yellow follicles collected above were treated with 0.1% collagenase II (MP Biomedicals, Santa Ana, CA) at 37°C for 8 min to disperse follicular granulosa cells. The isolated granulosa cells were then planted in a 24-well culture plate containing 1 mL of M199 complete medium with high glucose (Gibco, Camarillo, CA) plus 10% fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel), and were subsequently treated with either 10 ng/mL FSH (experimental group) or equal amount of PBS (control group) for 24 h. FSH treatment concentration and time was designed according to reference (Wang et al., 2017).

Oxford Nanopore RNA Sequencing and Quantification of Gene Expression Level

Total RNA was extracted from 2 groups (experimental group and control group) of chicken granulosa cells from prehierarchical follicles using a MicroElute Total RNA Kit (Omega, Norcross, GA). Three biological replicates from the same sample of prehierarchical granulosa cells for each group were obtained. Quality evaluation, and library construction were performed according to the standard protocol provided by Oxford Nanopore Technologies. Long-read sequencing was then conducted for the libraries with the Oxford Nanopore PromethION flow cells (FLO-PRO002) in Benagene Company (Wuhan, China). After removal of the low-quality reads using NanoFilt (v2.6.0) and nanoQC (v0.9.2), the remaining reads were subjected to the identification and classification of full-length transcripts followed by alignment to the chicken reference genome GRCg6a with the aid of Pychopper (v2.4.0) and Pinfish (v0.1.0) packages under default settings, respectively. The abundance of genome-matched transcripts was calculated and normalized as fragments per kilobase transcript per million mapped reads (**FPKM**).

Gene Ontology Terms, KEGG Pathway Enrichment, and Alternative Splicing Analysis

The statistically significant differentially expressed (**DE**) transcripts were screened according to the criteria of adjusted P value (padj) threshold of <0.05 and $|\log_2(\text{Fold Change})|>1$ using the DEGseq software. Hierarchical clustering analysis was performed using the R language package gplots according to the RPKM values of differential transcripts in different groups, and colors represent different clustering information, such as the

similar expression pattern in the same group, including similar functions or participating in the same biological process (**BP**). All DE transcripts were selected for gene ontology (**GO**) and Kyoto encyclopedia of genes and genomes (**KEGG**) pathway analyses. GO was performed with KOBAS software (v 3.0), which provides label classification of gene function and gene product attributes (http://www.geneontology.org) and covers cellular component (**CC**), molecular function (**MF**), and BP domains. As for KEGG analysis, KOBAS3.0 software (http://www.genome.jp/kegg) was used to map the DE transcripts and the enrichment of different pathways. GO and KEGG analyses were assessed by Fisher t test, q value <0.05 was considered as significantly different. Combined with differential expression analysis of transcripts, the types of alternative splicing (**AS**) and the number of each type differentially expressed alternative splicing were identified by diffsplice function of SUPPA2 software (v 2.3).The significant differentially expressed AS were selected by $P < 0.05$.The transcriptome data have been deposited with the NCBI Sequence Read Archive (**SRA**, https://www.ncbi.nlm.nih.gov/sra/docs/) under accession number PRJNA892218.

Overexpression

The entire coding region of chicken *TRAF7* gene was amplified using the forward primer containing the *Xba*I site and reverse primer containing the *Hind*III site as follows: forward: 5′-GCTCTAGACTGCCAGAGCAAACTTCC-3′ and reverse: 5′-CCAAGCTTAGAACTAGGGCGGCTCTC-3′ (*Xba*I and *Hind*III linker sequences are underlined). The polymerase chain reaction (**PCR**) amplification procedure was composed of 94°C for 5 min as the denaturing step, followed by 35 thermal cycles of 94°C for 30 s, annealing at 60°C for 30 s and 72°C for 2 min. The final extension was at 72°C for 5 min. The polymerase PrimeSTAR (TaKaRa, Dalian, China) was used to ensure high fidelity. The PCR fragments were generated by double enzyme digestion and ligated with pcDNA3.1(+) expression vectors (Invitrogen, Carlsbad, CA) by T4 DNA ligase, which were transformed into DH5α (TransGen Biotcch, Beijing, China) competent cells. After being confirmed by bidirectional sequencing and purified using an EndoFree Plasmid Purification Kit (Qiagen, Valencia, CA), these plasmids were used for transfection. Empty pcDNA3.1(+) vector was used as control.

Cell Transfection and FSH Treatment

Chicken granulosa cells isolated from prehierarchical follicles were transfected with pcDNA3.1-TRAF7 overexpression plasmid when they were grown to 80% confluency using NanoFectin Transfection Reagent (Shanghai ExCell Biology, Shanghai, China). As for FSH treatment, the cells were cultured with serum-free M199 medium in the absence or presence of different concentration of FSH (Sigma, St. Louis, MO). Twenty-four hours after transfection and FSH treatment, the cells were lysed for RNA or protein extraction.

Real-Time Quantitative PCR

Total RNA from the cultured cells was extracted using a MicroElute Total RNAKit (Omega, Norcross, GA) and the quality of the total RNA samples was tested by gel electrophoresis and spectrophotometry. The cDNA was synthesized using a PrimescriptRT reagent Kit with gDNA Eraser (TaKaRa, Dalian, China), and the resultant cDNA was stored at −20°C for mRNA expression analysis. Real-Time quantitative PCR (**qRT-PCR**) was conducted on an MX3000p instrument (Stratagene, La Jolla, CA) using the SYBR premix ExTaq (TaKaRa, Dalian, China). Melting curves were used to confirm the specificity of each product, and the PCR efficiencies were determined by analysis of 2-fold serial dilutions of cDNA that were designed to detect all the signals in the spanning region. The efficiencies were nearly 100%, and therefore, the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) for calculating the relative gene expression levels was used, and *β-actin* gene was used as the internal control. Primer sequences used for qRT-PCR are shown in Table 1.

Cell Proliferation Assay

The proliferation of granulosa cells was detected using an Enhanced Cell Counting Kit-8 AssayKit (Beyotime, Beijing, China). Approximately 6×10^3 cells were seeded in every well of a 96-well plate. The cells were transfected with the pcDNA3.1-TRAF7 or empty pcDNA3.1 when the cells reached 60% confluence. At 0, 24, 48, and 72 h after transfection, 100 μL of medium with 10 μL of CCK8 was added to each well, and then the plates were incubated for a further1 h at 38°C. The absorbance was evaluated using an ELx808Absorbance Reader at 450 nm.

Western Blotting

Phosphatase inhibitors and protease inhibitors were added into cell lysates (cell lysis buffer for Western and IP, Beyotime, Beijing, China) at a ratio of 1:50 to lyse granulosa cells treated with different concentration of FSH hormones. Proteins were obtained by centrifugation at 12,000 RPM at 4°C for 5 min. Protein concentration was determined by the bicinchoninic acid assay (BCA Protein Array kit, TIANGEN Biotech, Beijing, China). An equal amount of protein was separated by running on 4 to 15% SDS gel (BeyoGelTM Plus Precast PAGE Gel for Tris-Gly System) electrophoresis under denaturing and nonreducing conditions and then transferred to nitrocellulose filter membrane (PVDF). At room temperature, the membrane was sealed with confining liquid (NcmBlot Rapid Transfer Buffer) for 10 min and then incubated with rabbit customized

Table 1. Primers used in this study.

Gene	Accession number (GenBank)	Primer sequence	Annealing Temperature (°C)
StAR	NM_204686	Forward: 5′TGCCTGAGCAGCAGGGATTTATCA Reverse: 5′TGGTTGATGATGGTCTTTGGCAGC	56
CYP11A1	NM_001001756	Forward: 5′ACTTCAAGGGACTGAGCTTTGGGT Reverse: 5′AGTTCTCCAGGATGTGCATGAGGA	56
ZP3	NM_204389.2	Forward: 5′GTGCTCCAGTCATCACTCAT Reverse: 5′CACACAACACCGTCACCTT	56
TRAF7	NM_001012528.2	Forward: 5′CACACTACAGCACAGTTGGC Reverse: 5′ACAAGAACGCTCGCTTCA	58
HSD3B1	NM_205118.1	Forward: 5′AAGGTGTCAATGATGGAAGC Reverse: 5′CCAAAGAGGAGCAAACCAG	56
RDH12	XM_421193.6	Forward: 5′AGAATGGACAGAGCCAGGA Reverse: 5′TGGGAGTCAATCATCTTGGT	56
DIO3	NM_001122648.2	Forward: 5′GAAATCCCAGTTAGCACTTGC Reverse: 5′CAGCCACGCTCTGTCAATA	56
ANKRD46	XM_004939977.3	Forward: 5′GCAAGAACGAGTGGTGTTG Reverse: 5′CGCCTGTTAGAGAGTGGTTT	56
PLBD1	XM_416206.6	Forward: 5′ACCGTCATCAAGGCTCTTC Reverse: 5′CAACCGTGTCTCATTCAGC	56
ARG2	NM_001199704.1	Forward: 5′CAGCATTGCGGGTTCTTA Reverse: 5′CAGAGGAAATACACAACACAGG	56
β-actin	NM_205518	Forward: 5′ TGGATGATGATATTGCTGC	58

StAR, steroidogenic acute regulatory protein; *CYP11A1*, cytochrome P450 family 11 subfamily A member 1; *ZP3*, zona pellucida sperm-binding protein 3; *TRAF7*, TNF receptor associated factor 7; *HSD3B1*, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1; *RDH12*, retinol dehydrogenase 12; *DIO3*, iodothyronine deiodinase 3; *ANKRD46*, ankyrin repeat domain 46; *PLBD1*, phospholipase B domain containing 1; *ARG2*, arginase 2.

TRAF7 primary antibody (1:1,000) in a 5% bovine serum albumin/PBS solution for 2 h at room temperature, and β-actin-HRP rabbit monoclonal antibody (1:1,000; Beyotime, Beijing, China) was used for standardization. After washing in PBST (G-Biosciences, St. Louis, MO), the membranes were incubated with horseradish peroxidase-conjugated goat antirabbit immunoglobulin G antibody (1:1,000; Beyotime, Beijing, China) in a 5% bovine serum albumin/PBS solution for 2 h at room temperature and washed with PBST (Coolaber, Beijing, China). The membrane is dipped into the luminescent solution (BeyoECLPlusA:B=1:1) and developed with a C300 developer. The obtained protein bands were analyzed by the Image J software, and the expression level of the target protein under different treatments was determined according to the gray value.

Statistical Analysis

All data are presented as the mean ± standard error of the mean. The differences between groups were determined by 1-way analysis of variance followed by Duncan's test using the SPSS software (SPSS Inc., Chicago, IL). $P < 0.05$ was considered as significantly different.

RESULTS

Differentially Expressed Transcripts Regulated by FSH

By Oxford Nanopore RNA sequencing, we compared the mRNA transcription profiles between chicken granulosa cells of prehierarchical follicles treated with FSH and control. Among the total of 10,764 genes that respond to FSH stimulation in granulosa cell were detected, 31 differentially expressed transcripts (**DETs**) of 28 differentially expressed genes (**DEGs**) were identified according to the criteria of $|\log_2(\text{Fold Change})| > 1$ and padj <0.05, which were all significantly upregulated (Figure 1A and Table 2). GO analysis showed that most of these DETs were related to the steroid biosynthetic process (Figure 1B and Table 3). KEGG analysis showed that these DETs were mainly enriched in pathways of ovarian steroidogenesis and aldosterone synthesis and secretion (Figure 1C and Table 4). Among these DETs, the expression changes of 9 genes that are likely involved in follicle selection were shown in Figure 1D.

Quantitative real-time PCR on 10 DEGs showed that their expression level was similar to the sequencing data, including *StAR, CYP11A1, ZP3* (zona pellucida sperm-binding protein 3), *TRAF7, HSD3B1* (hydroxy-delta-5-steroid dehydrogenase, 3beta- and steroid delta-isomerase1), *RDH12* (retinol dehydrogenase 12), *DIO3* (iodothyronine deiodinase 3), *ANKRD46* (ankyrin repeat domain 46), *PLBD1* (phospholipase B domain containing 1), *ARG2* (arginase 2), suggesting that the RNA sequencing result was reliable (Figure 1E).

Analysis of Alternative Splicing

The Oxford Nanopore RNA sequencing has the advantage of detecting alternative splicing due to that it can obtain long reads. In this study, 7 types of AS events were detected in chicken granulosa cells of prehierarchical follicles, including skipping exon, mutually exclusive exon, alternative 5′ splice sites, alternative 3′ splice sites,

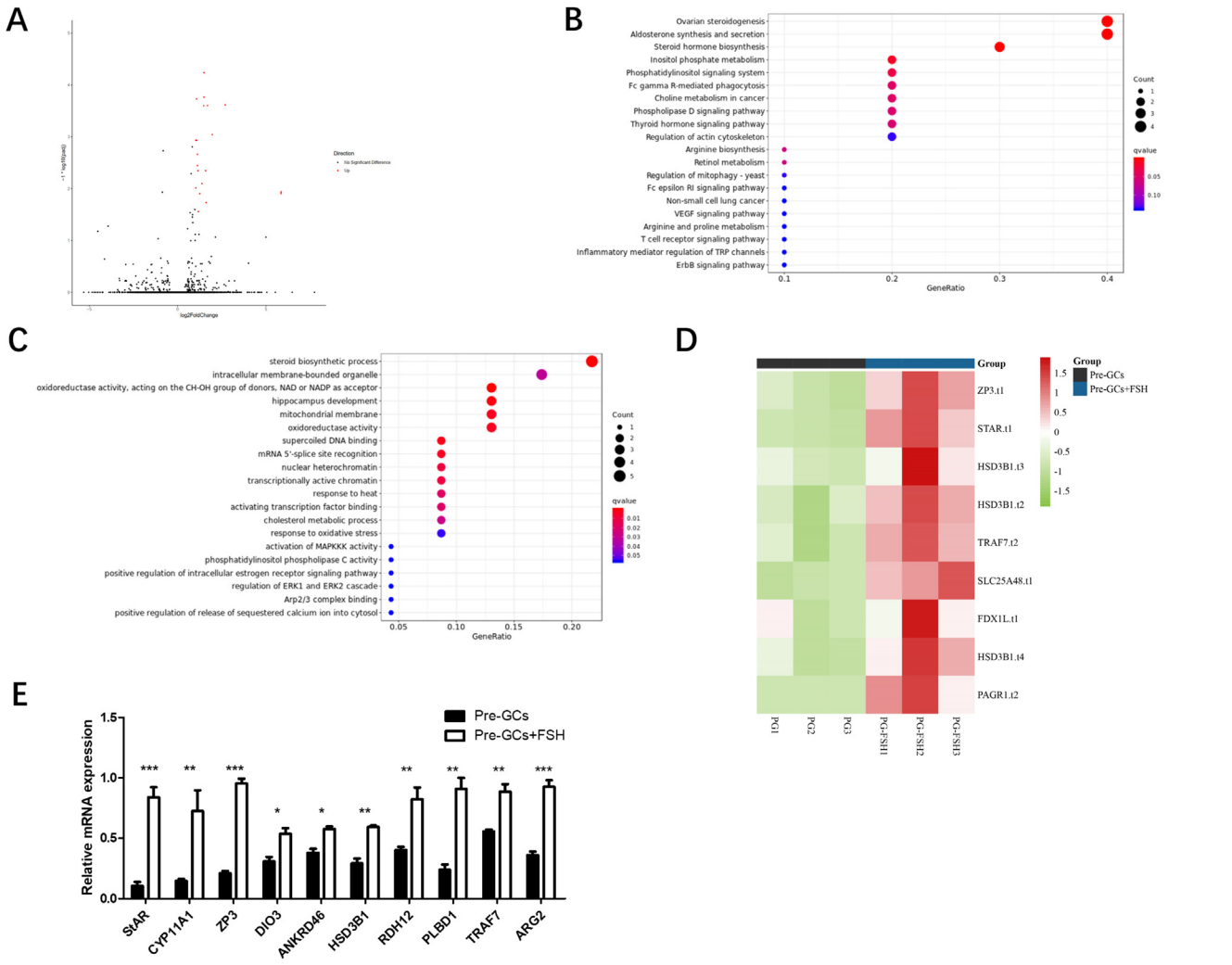

Figure 1. The differentially expressed transcripts (DETs) between FSH-treated granulosa cells of chicken prehierarchical follicles and controls. (A) Volcano map of total gene expression. The GO (B) and KEGG (C) analysis diagram of DEGs. The DETs and expression comparison of DEGs that are likely involved in follicle selection are shown via heatmap (D). (E) Validation by qRT-PCR of 10 DEGs obtained by RNA-seq.

retained intron, alternative first exons, and alternative last exons (Figure 2A), and alternative 3′ splice sites accounted for 25.57%, being the most frequent type (Figure 2B). For the alternative splicing events of DETs in the granulosa cells of prehierarchical follicles after FSH treatment, alternative 5′ splice sites accounted for 26.47% at most (Figure 2C).

Expression of TRAF7 Gene After FSH Treatment in Prehierarchical Follicular Granulosa Cells

The granulosa cells from chicken prehierarchical follicles were treated with different concentrations of FSH (0, 10, 50, 100 ng/mL), and the expression of TRAF7 mRNA and protein was detected. The results indicated that FSH could stimulate the expression of *TRAF7*, and that at the concentration of 10 ng/mL, FSH significantly increased both the mRNA (Figure 3A) and protein (Figure 3B) expression levels of *TRAF7* ($P < 0.05$). This is consistent with the Oxford Nanopore RNA sequencing result (Table 2).

Effect of TRAF7 on Follicle Selection Related Genes in Chicken Granulosa Cells of Prehierarchical Follicles

We next tested the function of *TRAF7* gene in chicken follicle selection. After overexpression of chicken *TRAF7* gene in chicken granulosa cells of prehierarchical follicles, the mRNA expression of essential steroidogenic enzymes *StAR* ($P < 0.05$) and *CYP11A1* ($P < 0.01$) was significantly increased (Figure 4), likely promoting the production of steroid hormones.

Effects of TRAF7 on the Proliferation of Chicken Granulosa Cells of Prehierarchical Follicle

To examine whether TRAF7 affect granulosa cell proliferation of prehierarchical follicles, we analyzed the cell proliferation dynamics after transfection with the overexpression vector pcDNA3.1-TRAF7. We found that, from 48h to 72h, *TRAF7* overexpression significantly

Table 2. Significantly upregulated transcripts in FSH-treated chicken granulosa cells of prehierarchical follicles.

Gene	Gene ID	Transcript	TPM control	TPM FSH	$\log_2$(Fold Change)	padj
AGR2	420596	ARG2.t2	5.31	8.86	1.0799	0.00116
DIO3	395939	DIO3.t1	2.13	4.70	1.4987	5.8E-05
FDX1L	422172	FDX1L.t1	2.28	3.65	1.0347	0.00116
FGL2	768821	FGL2.t2	1.66	3.31	1.3478	5.8E-07
FUNDC1	418558	FUNDC1.t3	0.00	0.65	5.8591	0.01155
GPC5	418795	GPC5.t2	12.17	20.75	1.1632	0.0045
HAPLN1	396475	HAPLN1.t4	0.82	1.42	1.1258	0.00217
HSD3B1	396015	HSD3B1.t2	1.56	3.79	1.6353	8.6E-08
		HSD3B1.t3	2.41	6.38	1.7163	1.5E-08
		HSD3B1.t4	0.49	1.13	1.5881	0.0045
IQGAP2	427211	IQGAP2.t10	1.06	1.74	1.128	0.00359
MIR202	777897	MIR202.t2	9.59	19.87	1.3799	0.00799
NELL2	417799	NELL2.t9	3.50	5.62	1.0664	0.00018
PAGR1	107051071	PAGR1.t2	0.00	0.77	5.8528	0.01234
PIP5K1B	427243	PIP5K1B.t9	1.03	1.82	1.1735	0.02758
PLBD1	417967	PLBD1.t6	1.28	2.96	1.5633	4.1E-11
PSIP1	431605	PSIP1.t2	4.87	11.99	1.6934	1.3E-09
		PSIP1.t4	2.76	5.62	1.3903	6.1E-07
RDH12	423274	RDH12.t1	4.09	8.29	1.3877	1.3E-09
SLC25A48	416308	SLC25A48.t1	0.36	0.90	1.6936	0.00025
STAR	395421	STAR.t1	0.23	1.71	3.2663	5.6E-09
TFPI2	420561	TFPI2.t1	7.40	12.95	1.1542	6.4E-07
TRAF7	416555	TRAF7.t2	0.18	0.93	2.6986	0.00024
UMOD	404754	UMOD.t2	1.26	2.98	1.6132	0.01853
ZP3	378906	ZP3.t1	59.49	190.21	2.0522	1.9E-35
LOC422926		LOC422926.t2	1.24	2.63	1.4841	0.00025
novel170		novel170.t1	2.12	4.05	1.2543	0.01245
novel184		novel184.t1	5.38	8.61	1.0321	0.00963
novel512		novel512.t1	0.90	2.60	1.9648	0.0009
novel527		novel527.t2	2.95	6.47	1.4958	0.00017
novel664		novel664.t1	179.23	327.48	1.2194	6.4E-06

increased the proliferation of chicken granulosa cells from prehierarchical follicles ($P < 0.01$) (Figure 5).

DISCUSSION

Follicle selection is an important step in the laying process of chicken, which is closely related to the laying performance and fecundity of chicken. As an essential component of chicken ovarian follicles, the proliferation and differentiation of granulosa cells of prehierarchical follicles plays important roles in follicle selection, and is regulated by FSH through binding to its receptor. Therefore, in this study, for the first time, using Oxford Nanopore Technologies approach, we analyzed the effect of FSH on the mRNA profiles of chicken granulosa cells of prehierarchical follicles and further analyzed the role of TRAF7 in follicle selection.

Among the 31 DETs identified, 3 *HSD3B1* transcripts (t2, t3, and t4) and 2 *PSIP1* transcripts (t2 and t4) were upregulated by FSH in the granulosa cells of prehierarchical follicles. During chicken follicle selection, the differentiation of granulosa cells is characterized by the synthesis of progesterone, which is stimulated by FSH. Progesterone is synthesized from cholesterol by the actions of StAR, CYP11A1 and HSD3B, of which HSD3B1 is critical for progesterone synthesis, by converting pregnenolone into progesterone in the assistance of cofactor NAD^+ (Zhu et al., 2019). The increased expression of *HSD3B1* after treatment with FSH in the granulosa cells of prehierarchical follicles, as was observed in this study, is consistent with the effect of

Table 3. Information about the significantly enriched GO terms of DETs.

ID	Description	*q* value	Transcript
GO:0006694	Steroid biosynthetic process	9.00072E-08	STAR.t1/HSD3B1.t3/HSD3B1.t2/FDX1L.t1/HSD3B1.t4
GO:0016616	Oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	0.000276437	HSD3B1.t3/HSD3B1.t2/HSD3B1.t4
GO:0021766	Hippocampus development	0.001534763	HSD3B1.t3/HSD3B1.t2/HSD3B1.t4
GO:0097100	Supercoiled DNA binding	0.00253122	PSIP1.t2/PSIP1.t4
GO:0031966	Mitochondrial membrane	0.004132731	HSD3B1.t3/HSD3B1.t2/HSD3B1.t4
GO:0000395	mRNA 5′-splice site recognition	0.004132731	PSIP1.t2/PSIP1.t4
GO:0016491	Oxidoreductase activity	0.004132731	HSD3B1.t3/HSD3B1.t2/HSD3B1.t4
GO:0005720	Nuclear heterochromatin	0.010487883	PSIP1.t2/PSIP1.t4
GO:0035327	Transcriptionally active chromatin	0.010564447	PSIP1.t2/PSIP1.t4
GO:0009408	Response to heat	0.018396996	PSIP1.t2/PSIP1.t4
GO:0033613	Activating transcription factor binding	0.019715735	PSIP1.t2/PSIP1.t4
GO:0008203	Cholesterol metabolic process	0.027565887	STAR.t1/FDX1L.t1
GO:0043231	Intracellular membrane-bounded organelle	0.032140917	HSD3B1.t3/HSD3B1.t2/TRAF7.t2/HSD3B1.t4

Table 4. Information about the significantly enriched KEGG pathways of DETs.

ID	Description	*q* value	Transcript
KO04913	Ovarian steroidogenesis	2.20074E-05	STAR.t1/HSD3B1.t3/HSD3B1.t2/HSD3B1.t4
KO04925	Aldosterone synthesis and secretion	2.78003E-05	STAR.t1/HSD3B1.t3/HSD3B1.t2/HSD3B1.t4
KO00140	Steroid hormone biosynthesis	0.000101711	HSD3B1.t3/HSD3B1.t2/HSD3B1.t4
KO00562	Inositol phosphate metabolism	0.01254423	SLC25A48.t1/PIP5K1B.t9
KO04070	Phosphatidylinositol signaling system	0.027849794	SLC25A48.t1/PIP5K1B.t9
KO04666	Fc gamma R-mediated phagocytosis	0.047436209	SLC25A48.t1/PIP5K1B.t9
KO05231	Choline metabolism in cancer	0.047436209	SLC25A48.t1/PIP5K1B.t9

FSH on progesterone synthesis (Wang et al., 2017). Similarly, the mRNA level of chicken *HSD3B* is higher in F1 follicles compared to small white follicles (Zhu et al., 2015). PSIP1 (PC4 and SFRS1 interacting protein 1), also called lens epithelium-derived growth factor (**LEDGF**), plays important roles in the formation of transcription complexes in active chromatin, transcriptional activation of specific genes, regulation of mRNA splicing, DNA repair, and cellular survival against stress and is a key protein contributing to several human pathologies, including acquired immunodeficiency syndrome, leukemia, cancer, ocular diseases, and Rett syndrome (Ortiz-Hernandez et al., 2020). However, its role in chicken follicle development is unknown. The upregulation of *PSIP1* by FSH in the granulosa cells of prehierarchical follicles suggests an important role in chicken follicle selection.

In this study, DETs were mainly enriched in the synthesis of steroid hormones as revealed by both GO and KEGG analysis, suggesting an essential role of FSH in stimulating progesterone production. Based on the transcriptome sequencing of Oxford Nanopore Technologies, the alternative splicing and fusion genes were accurately analyzed and new isomers were identified to achieve accurate quantification of the expression level of transcripts (Deamer et al., 2016). The present study also revealed that alternative 5′ splice sites was mostly affected splicing types by FSH in the granulosa cells of prehierarchical follicles. Other studies also revealed that

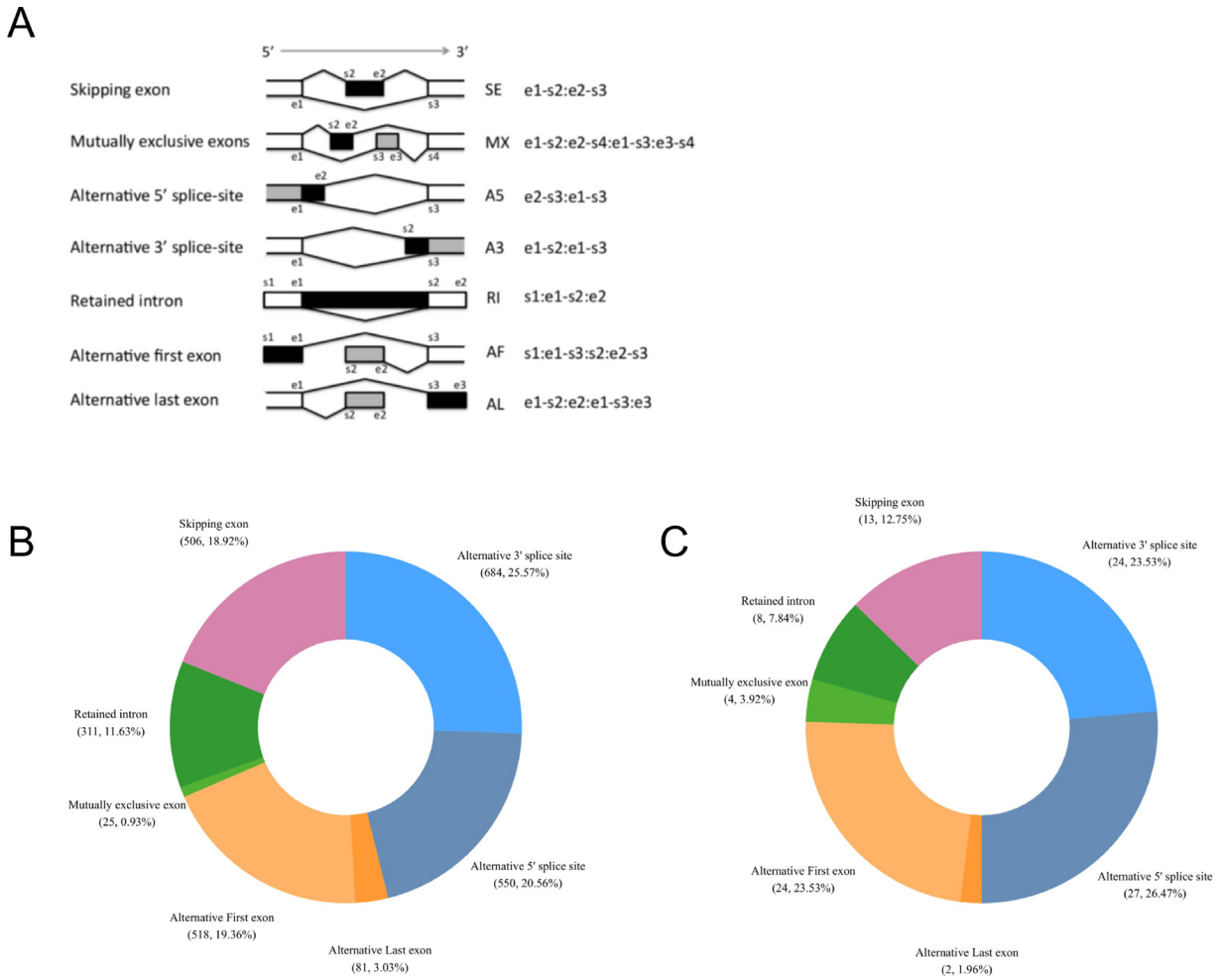

Figure 2. The results of alternative splicing analysis of transcripts in chicken granulosa cells of prehierarchical follicles. (A) Seven types of alternative splicing. (B) The number and proportion of alternative splicing events. (C) The number and proportion of alternative splicing events affected by FSH treatment.

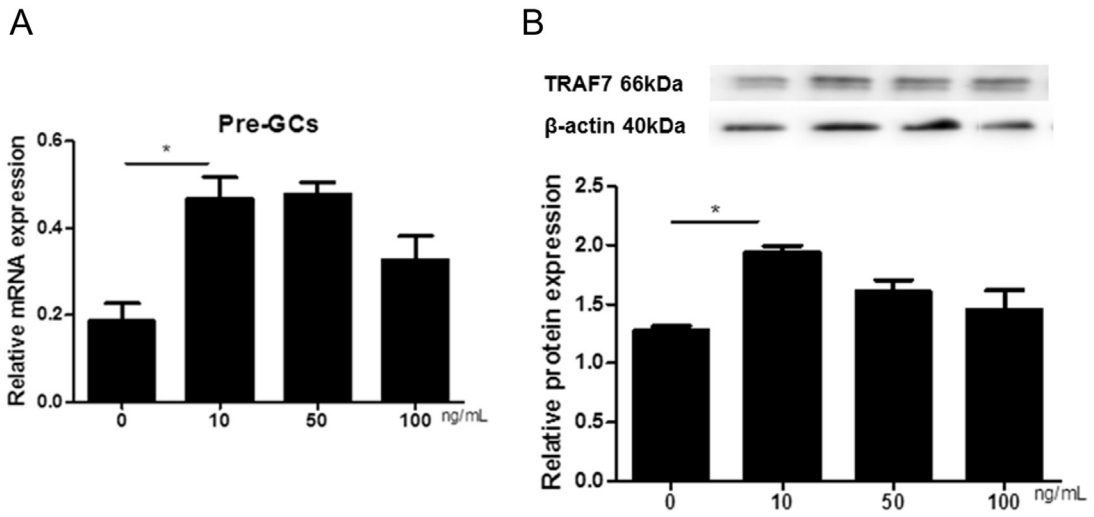

Figure 3. Effect of FSH on TRAF7 mRNA (A) and protein (B) expression level in chicken granulosa cells of prehierarchical follicles. $^{*}P < 0.05$.

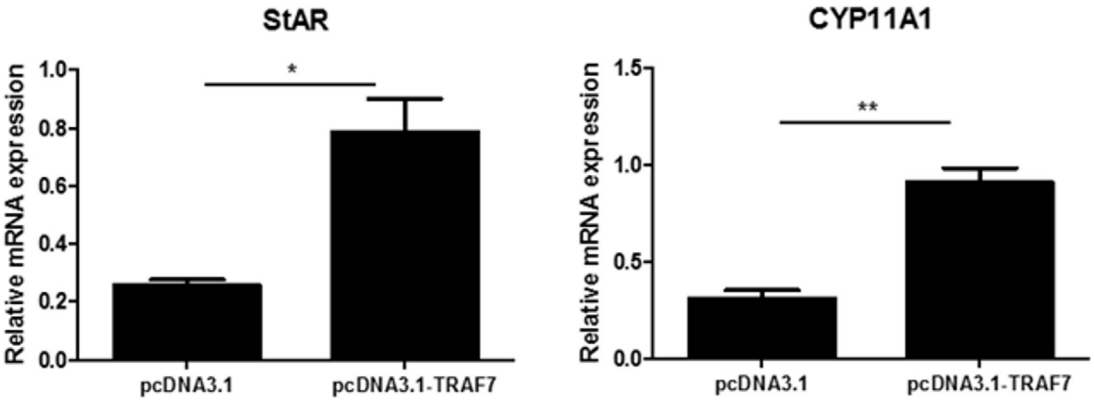

Figure 4. Effect of TRAF7 on the expression of follicular development related genes in chicken granulosa cells of prehierarchical follicles. $^{*}P < 0.05$, $^{**}P < 0.01$.

different transcripts of the same gene displayed different expression patterns in granulosa cells. WNT4-α, 1 of 2 alternatively spliced products of *WNT4*, plays an important role in follicle development and has a significant effect on the proliferation of granulosa cells in goats (Wang et al., 2022). In chicken, 2 DETs of *ANXA6*, ANXA6.t1 and ANXA6.t4, display opposite changes in the granulosa cells of prehierarchical follicles, may play different roles (Li et al., 2023). The regulatory mechanisms underlying the alternative splicing of *HSD3B1* and *PSIP1* as revealed by present study and their functions in follicle selection requires further investigations.

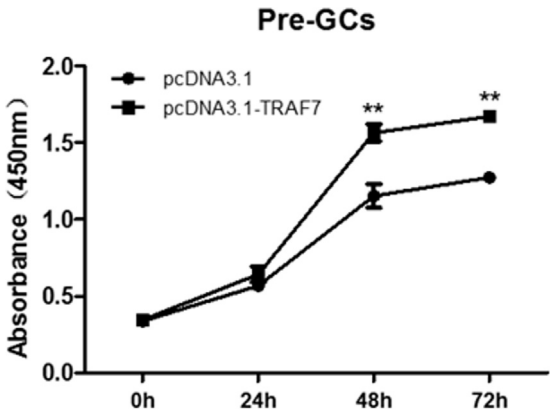

Figure 5. Effect of TRAF7 on the proliferation of chicken granulosa cells of prehierarchical follicles. $^{**}P < 0.01$.

From the transcriptome sequencing data, it was found that the mRNA expression of *TRAF7* was significantly upregulated in FSH treated granulosa cells of chicken prehierarchical follicles. TRAFs are a family of adaptor proteins that interact with cell surface receptors or other signaling molecules (Bradley and Pober, 2001) to affect cell survival, proliferation, differentiation, death and multiple biological regulatory processes (Zirlik et al., 2007; Pedros et al., 2018). TRAF7 is a 670-amino-acid member of the TRAF family, consisting of a RING finger domain at the N terminus, a noncanonical TRAF domain, a coiled-coil (**CC**) domain and a 7 carboxyl-terminalWD40 repeats at the C terminus (Bouwmeester et al., 2004; Xu et al., 2004). TRAF7 is also related to the occurrence of many diseases, such as meningioma (Clark et al., 2013), breast cancer (Kim and Shohet, 2015), and tsc2-pkd1 continuous deletion syndrome (Napetschnig and Wu, 2013). In this study, we found that the mRNA and protein expression levels of TRAF7 significantly increased with FSH treatment, overexpression of *TRAF7* produced a significant increase effect on

the expression of *StAR* and *CYP11A1* and significantly promoted the proliferation of chicken granulosa cells prehierarchical follicles, suggesting that TRAF7 could participate in the process of follicle selection by affecting the proliferation and differentiation of granulosa cells.

CONCLUSIONS

In this study, we investigated the differences in the mRNA profile of chicken granulosa cells of prehierarchical follicles after FSH treatment by using ONT transcriptome sequencing. Thirty-one differentially expressed transcripts were found to be involved in ovarian steroidogenesis, steroid hormone biosynthesis and other related pathways. The expression of *TRAF7* was upregulated by FSH, which subsequently stimulated the expression of *StAR* and *CYP11A1* and the proliferation of chicken follicle granulosa cells of prehierarchical follicles, suggesting a promoting role in chicken follicle selection.

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DISCLOSURES

The authors declare no conflict of interest.

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