Journal of Animal Science, 2023, **101**, 1–12
https://doi.org/10.1093/jas/skad295
Advance access publication 1 September 2023
Cell and Molecular Biology

Integrative analyses of the mRNA expression profile reveal the involvement of *STC1* in chicken folliculogenesis

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Abstract

Efficient ovarian follicle development, maturation, and ovulation are critical for egg production performance. Previous research has underscored the importance of messenger RNAs (**mRNAs**) in regulating development and folliculogenesis in chicken ovarians. However, the molecular mechanism is not fully understood, especially in the late period of the laying cycle. In the present study, ovarian tissues from 80-week-old Hy-Line Brown layers (three with high and three with low rates of egg laying) were collected for transcriptome sequencing. A total of 306 differentially expressed genes (**DEGs**) were identified in this study, at a false discovery rate (**FDR**)-corrected *P*-value < 0.05 and a log2|fold change| (**log2|FC|**) ≥1.5. Among these DEGs, stanniocalcin 1 (***STC1*)** was mainly related to cellular processes, single-organism processes, biological regulation, metabolic processes, developmental processes, and reproductive processes. Then, we further investigated the regulation of *STC1* during chicken follicle development and found that *STC1* inhibited the proliferation and stimulated the apoptosis of follicular granulosa cells (**GCs**), and decreased the expression of progesterone (**P4**) and estradiol (**E2**). Collectively, these results suggest that *STC1* plays an important role in chicken follicle development by decreasing GC proliferation and steroidogenesis and stimulating GC apoptosis. This study contributes to the understanding of the reproductive biology of laying hens in the late period of the laying cycle and further lays a foundation for the improvement of egg production in poultry breeding.

Lay Summary

The egg production performance of chickens is an essential economic trait that differs significantly between high- and low-egg-laying breeds. In addition to external factors such as feeding, light, and environment, the periodic recruitment of pre-hierarchical follicles and the normal development of hierarchical follicles affect this difference. Thus, we used high-throughput sequencing technology to perform transcriptome analysis of ovarian tissues from 80-wk-old Hy-Line Brown layers with high- and low-egg-laying rates (**HH** and **HL**), and an association with the laying performance gene stanniocalcin 1 (***STC1*)** was found. The proliferation and apoptosis of granulosa cells (**GC**s), as the basic functional cells of ovarian follicles, are highly correlated with the normal development and regression of follicles. Therefore, this study used ovarian follicular GCs cultured in vitro to study the effects of the *STC1* gene on the proliferation, apoptosis, and secretion function of GCs and to explore its mechanism of action, laying a foundation for the study of the regulation of the *STC1* gene on follicular development.

Key words: chicken, egg production, ovary, transcriptome sequencing, *STC1*

Abbreviations: ACV, activins; DEG, differentially expressed genes; E2, estradiol; ELISA, enzyme-linked immunosorbent assay; FC, fold-change; FDR, false discovery rate; FPKM, fragments per kilobase million; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; FST, follistatin; GCs, follicular granulosa cells; GO, gene ontology; HH, Hy-Line Brown layers with high-egg-laying rates; HL, Hy-Line Brown layers with low-egg-laying rates; IGF1, insulin-like growth factor 1; INH, inhibin; *INHBA*, inhibin beta A; KEGG, kyoto encyclopedia of genes and genomes; LH, luteinizing hormone; log2|FC|, log2|fold change|; mRNA, messenger RNA; P4, progesterone; PBS, phosphate buffered saline; PG, prostaglandin; qRT-PCR, quantitative real-time PCR; RNA-seq, RNA sequencing; STC, stanniocalcin; *STC1*, stanniocalcin 1; STC2, stanniocalcin 2; *ZP3*, zona pellucida glycoprotein 3

Introduction

Eggs are considered one of the most affordable sources of animal protein (Bain et al., 2016; Lesnierowski et al., 2018). As human demand for eggs increases, the number of layers are increasing rapidly. In poultry breeds, prolonging the egg-laying cycle is an important measure to improve egg production performance. The longer the laying cycle is, the greater the production and the higher the profit we obtain. However, in production practice, we found that some laying hens have a high-egg-laying rate, and some laying hens have a low-egg-laying rate in the late laying period (80 to 100 weeks). In addition to environmental and metabolic factors, egg production performance may be related to growth, development,

Received June 30, 2023 Accepted August 30, 2023.

ovulation, atresia, and regression in the chicken ovary follicle (Etches et al., 1983). Therefore, thoroughly understanding the process of follicular development and its mechanisms could aid in significantly improving egg production.

The exploration of the molecular mechanisms of ovarian follicle development has been a hot topic of research on poultry reproductive traits, and genetic differences are the determining factors affecting poultry reproduction. For decades, breeders have used traditional quantitative genetics and other technical tools to continuously select excellent reproductive traits with great success. However, reproductive traits are controlled by multiple genes with low heritability, and relying on traditional means of selection, the generation interval is long, the process is tedious, and new genetic progress occurs slowly. With the development of modern molecular biology, especially the application of molecular marker-assisted selection, high-throughput sequencing, and other molecular biology technologies, poultry breeders have been provided with new ideas and approaches.

Granulosa cells (**GCs**) proliferation and differentiation are an important part of follicular development, and the process of follicular development maturation must be accompanied by the proliferation and differentiation of GCs, which are mutually dependent. GCs proliferation, differentiation, and apoptosis determine follicular growth, selection, and atresia (Ghanem and Johnson, 2018). However, GCs synthesize the steroid hormones progesterone (**P4**) and estradiol (**E2**) under the stimulation of gonadotropins follicle-stimulating hormone (**FSH**) and luteinizing hormone (**LH**), respectively, to regulate follicular maturation and promote ovulation (Pierce and Parsons, 1981). During follicular growth and development, the coordination between GCs and oocytes is crucial for follicular development and oocyte maturation, and information exchange is complex. GCs provide 85% of nutrient metabolic requirements for oocytes mainly through gap junctions, regulate oocyte transcriptional activity, and induce post-translational modification of oocyte proteins (Jin et al., 2006). GCs secrete and express the growth factors insulin-like growth factor 1 (**IGF1**) and the local hormones follicle-stimulating hormone receptor (**FSHR**) in a paracrine manner to regulate follicular development and maturation, and activins (**ACV**), inhibin (**INH**), and follistatin (**FST**) expressed by GCs can regulate follicular differentiation and maturation with the help of FSH (Onagbesan et al., 2009; Liu et al., 2019b). In conclusion, GCs have a very important regulatory role in the developmental process of the follicle, and this role is closely related to the normal proliferation and differentiation of GCs and their secretory function.

Stanniocalcin (**STC**) is a homodimeric glycoprotein hormone that was first discovered in the corpuscles of Stannius of the teleost kidney endocrine gland (Yeung et al., 2012). It was found that STC in teleost tissue inhibited and promoted calcium uptake and phosphate reabsorption, respectively; therefore, STC was shown to act as an endocrine regulator, regulating the balance of calcium and phosphate (Lu et al., 1994; Schein et al., 2012). STC also play an important regulatory role in developmental and pathophysiological processes such as pregnancy, lactation, and organogenesis (Varghese et al., 1998; An et al., 2020). At present, two related *STC* genes, stanniocalcin 1 (***STC1***) and stanniocalcin 2 (***STC2***) have been identified in mammals (Joshi, 2020). Interestingly, the highest level of *STC1* expression in mammals occurs in ovarian tissue, but no information is available on chicken species (Chang et al., 1996; Basini et al., 2010). Previous reports have shown that *STC1* has different expression patterns during folliculogenesis, and *STC1* has been proven to inhibit steroidogenesis (Baioni et al., 2009). Luo et al. (2004) showed that *STC1* inhibited progesterone production in mouse ovarian GCs but had no effect on estrogen production, while studies in pigs showed that it could inhibit estrogen production (Baioni et al., 2009). However, even though *STC1* plays an essential role in many cell processes, its function in chicken ovary folliculogenesis remains unclear. Therefore, the present study was undertaken to investigate the expression and function of *STC1* in chicken ovaries.

In the present study, we adopted RNA sequencing (RNA-seq) technology to evaluate the predominant genes involved in the development of growing follicles and found that *STC1* was differentially expressed in the two groups [Hy-Line Brown layers with high- and low-egg-laying rates (**HH** and **HL**)]. Then, the expression of *STC1* in vitro was detected, and functional research was performed using a chicken follicular GCs model. In addition, *STC1* was found to have a potential role in inhibiting follicular development in chickens.

Materials and Methods

Ethics statement

In this study, all of the animal experiments were approved by the Institutional Animal Care and Use Committee (**IACUC**) of Henan Agricultural University, Zhengzhou, China, and performed in strict accordance with the guidelines of the Animal Use Committee of the Chinese Ministry of Agriculture, Beijing, China (approval number: S20190196).

Animal tissue sampling, RNA extraction, and sequencing

A total of 30 Hy-Line Brown layers from the Henan Innovative Engineering Research Center of Poultry Germplasm Resource were reared under the same environmental conditions with ad libitum access to water and food before sample collection. Based on similar reproductive traits, the population was divided into high-egg-laying rate (HH1, HH2, and HH3) and low-egg-laying rate (HL1, HL2, and HL3) groups, and ovarian tissues collected at 80 wks of age, and analyzed the egg production performance differences in the high- and low-egg-laying rates by SPSS 24.0 (IBM, Among, New York, USA). Fresh tissue samples were washed in Phosphate Buffered Saline (PBS) (Cat 70011044, Gibco, Fisher Scientific, Waltham, MA, USA) and immediately stored at −80 °C after quick freezing with liquid nitrogen. Total RNA was extracted using TRIzol reagent (Cat R401-01, Vazyme, Nanjing, China) following the manufacturer's instructions, and RNA integrity was assessed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Qualified total RNA was further purified by an RNA Clean XP Kit (Cat A63987, Beckman Coulter, Inc., Kraemer Boulevard Brea, CA, USA) and RNase-Free DNase Set (Cat 79254, QIAGEN, GmBH, Germany). The cDNA libraries were constructed by Shanghai Biotechnology Corp (Shanghai, China) and sequenced on an Illumina HiSeq 2500 ((Illumina, San Diego, CA, USA). The raw reads were filtered with Seqtk (https://github.com/lh3/seqtk), and the number of all clean reads for each gene in each sample was counted with HTSeq (version: 2.0.4) (Kim et al., 2015). The fragments per kilobase of exon model per million mapped reads (**FPKM**;

Mortazavi et al., 2008) were calculated to estimate the expression level of genes in each sample using the Stringtie (version: 1.3.0) (Pertea et al., 2015, 2016). The raw transcriptome datasets for the Hy-Line Brown layers ovarian have been deposited in the NCBI Sequence Read Archive (**SRA**, http://www.ncbi.nlm.nih.gov/Traces/sra) with accession number SRR6456830.

Differential expression and functional enrichment analyses

edgeR (Robinson et al., 2010) was used to perform differential gene analysis between samples and multiple hypothesis test correction. The threshold of the *P*-value was determined by controlling the false discovery rate (**FDR**; Deol et al., 2000). In addition, we calculated the differential expression multiple based on the FPKM value, that is, fold change (**FC**). In this study, the genes with *P*-value < 0.05 and log2|FC| ≥ 1.5 were identified as differentially expressed genes (**DEGs**). Gene ontology (**GO**) and Kyoto Encyclopedia of Genes and Genomes (**KEGG**) analyses were performed by clusterProfile, and $P < 0.05$ was considered as the threshold.

Plasmid construction and siRNA

The *STC1* coding sequence was amplified from chicken follicle cDNA by PCR using gene-specific clone primers (Supplementary Table S1). The PCR product was cloned into the pcDNA3.1-EGFP vector (Cat V79020, Invitrogen, USA) within NheI and EcoRI sites. The successful *STC1* overexpression vector was confirmed by agarose gel electrophoresis and DNA sequencing. siRNA specifically against chicken *STC1* was obtained from RiboBio (Guangdong, China), and a nonspecific duplex was used as the control. The siRNAs used in this study are listed in Supplementary Table S2.

GC isolation culture and transfection

GCs were isolated and cultured from chicken follicles. First, the granulosa layers were dissected away from the pre-hierarchical follicles (6 to 8 mm) of 35-wk-old chicken, and then minced and homogenized in a centrifuge tube. To release single cells, the granulosa layers were digested with 0.25% trypsin (Cat 25200056, Gibco) for 10 min at 37 °C. After neutralization with complete medium, single cells were collected by centrifugation at 1800 × g. Subsequently, cell suspensions were seeded in cell culture medium containing DMEM/F12 (Cat SH30023.01B, HyClone, Logan, Utah, USA) supplemented with 2.5% fetal bovine serum (Cat 1099141C, Gibco) and 1% penicillin/streptomycin (Cat 15140122, Gibco). GCs were cultured at 37 °C in a 5% CO_2 humidified atmosphere. Lipofectamine 2000 (Cat 11668-027, Invitrogen) was used for transient transfection. For specific procedures, refer to the reagent specification.

Cell proliferation assays

GCs were treated with *STC1* overexpression vector and siRNA, and cell activity was detected at 12 h, 24 h, 36 h, and 48 h. First, 10 μL of Cell Counting Kit-8 solution (CCK-8) (Cat CK04, Dojindo, Kumamoto, Japan) was added into the blank, control, and treatment wells of the 96-well plate and incubated in the incubator for 2 h. Then, the absorbance at 450 nm was measured by Enzyme Labeler (Cat Synergy2, BioTek, Palo Alto, USA), and the GCs viability was calculated: (treatment wells–control wells)/(control wells–blank wells) × 100%.

A Cell-Light TM EdU DNA Cell Proliferation (EdU) (Cat C10310, RiboBio, Guangzhou, China) assay was performed on GCs treated with *STC1* overexpression vector and siRNA. In the experiment, EdU medium was first added for incubation for 2 h and then fixed with paraformaldehyde for 30 min. Subsequently, glycine and Triton X-100 were added for incubation on the decolorization shaking bed. Finally, 1×Apollo staining reaction solution and DAPI were added for observation of cell proliferation under a fluorescence inverted microscope (Cat Eclipse Ts2, Nikon, Tokyo, Japan).

Cell cycle analysis

For analysis of the cell cycle of GCs, cells were collected and fixed in 75% ethanol overnight. After ethanol fixation, the cells were stained with propidium iodide using PI/RNase staining buffer (Cat 550825, BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's manual. Subsequently, flow cytometry (Cat Accuri C6, BD Biosciences Pharmingen) was used for detection, generally counting 2~3 × 10^4 cells, and the cell cycle was analyzed by Flow-Jo7.6 software (Stanford University, Stanford, CA, USA).

Cell apoptosis assay

Forty-eight hours after transfection, chicken GCs were harvested, and a cell-counting machine (Cat 6749, Corning, NY, USA) was used for the detection of apoptotic cells based on the principle of fluorescence-activated cell sorting (FACS). According to the indicated manufacturer's protocol (Cat A211-01, Vazyme), the apoptosis rate was detected by flow cytometry (Cat Accuri C6, BD Biosciences Pharmingen) with the fluorescein isothiocyanate (FITC) and propidium iodide (PI) signals. The FlowJo v7.6 software (Stanford University, Stanford, CA, USA) was used to analyze the results.

Hormone analysis

Chicken GCs were cultured in a six-well plate with 2 mL of medium. After a 48 h of overexpression vector or siRNA treatment, the culture medium was assayed immediately or stored at −80 °C until assayed. The progesterone concentration in the conditioned medium was diluted to one-twentieth with saline and then measured using an enzyme-linked immunosorbent assay kit (**ELISA**) (Cat FY4369-B, Feiya Technology, Jiangsu, China) according to the manufacturer's instructions. PG and E2 values were determined by extrapolating from a standard curve. Each sample was measured in triplicate.

Quantitative real-time PCR

Quantitative real-time PCR (**qRT–PCR**) was performed using a TB Green Premix Ex Taq II (Tli RNaseH Plus) Kit (Cat RR820B, TaKaRa, Dalian, China). The qRT–PCR gene-specific primers were designed using the NCBI online software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and purchased from BiosSunya Co., Ltd (Zhengzhou, China), and all the qRT–PCR primers for these genes are described in Supplementary Table S3. The qRT–PCR amplification procedure was as follows: 95 °C for 3 min, 35 cycles of 95 °C for 12 s, 60 °C for 30 s, 72 °C for 30 s, and an extension for 10 min at 72 °C ($n = 3$). Quantification of the expression of all chosen mRNAs was conducted using the comparative CT ($2^{-\Delta\Delta CT}$) method with β-actin as an internal reference RNA (β-actin acts as an internal reference gene, and it is stably expressed in melanocytes; Livak and Schmittgen, 2001).

Statistical analysis

Statistical analysis was carried out by using Prism 8 software (GraphPad Software, Inc., USA). All results are presented as the means ± SEMs. The significance of the comparison between the two groups was assessed by one-sample *t*-test using SPSS 24.0 (IBM, Among, New York, USA). $P < 0.05$ was considered a significant difference.

Results

Statistics of egg production rate

The average egg production rates at 80 weeks (mean ± SEM) were 85.11% ± 0.0212 and 15.60% ± 0.071 for HH and HL chickens, respectively. The egg production rate of HH chickens was significantly higher than that of HL chickens ($P < 0.01$, Figure 1), indicating that the two groups were suitable for identifying DEGs associated with reproductive traits.

Identification and functional annotation of differentially expressed genes

In this study, we included six chicken ovarian cDNA libraries created by the Illumina HiSeq 2500 platform. After quality control, 147,492,401, 117,774,725, and 80,057,178 clean reads were generated for the three HHs, and 142,872,483 78,079,711, and 90,807,270 clean reads were generated for the three HLs. Approximately, 91% of the reads in each library were uniquely mapped to the GGA5 assembly of the chicken reference genome (Table 1).

A total of 24,881 genes were identified in both groups, including 306 differentially expressed genes (*P*-value < 0.05 and log2|FC| ≥ 1.5) that were coexpressed in the high- and low-egg-laying rate groups, with 112 mRNAs being upregulated and 194 mRNAs being downregulated in HL compared to HH (Figure 2a and b).

To explore the biological roles of the DEGs, we performed GO and KEGG enrichment analyses. A total of 175 differentially expressed genes were annotated to the biological processes, cellular components, and molecular functions of GO. Among them, in biological processes, differentially expressed genes were mainly involved in regulating cellular processes, bioregulation, metabolic processes, developmental processes, and reproduction processes; in cellular components, differentially expressed genes were associated with cells, organelles, cell membranes, and extracellular compartments; and in molecular functions, differentially expressed genes were associated with nucleic acid binding transcription factor activity, transporter protein activity, and molecular sensor activity (Figure 2c). Notably, *STC1* is enriched in cell development, cell differentiation, tissue development, hormone activity, cell migration, and other biological processes (Supplementary Table S4).

In addition, the results of KEGG analysis revealed that DEGs were enriched mainly in metabolic pathways, tight junctions, and mRNA surveillance pathways. As shown in Figure 2d, the largest category was metabolic pathways, which had 12 annotated genes. Notably, specific enrichment of genes was observed for pathways involved in reproduction regulation (Supplementary Table S5), such as the FoxO signaling pathway, TGF–beta signaling pathway, MAPK-signaling pathway, PPAR-signaling pathway, and steroid biosynthesis. In addition, several genes are involved in multiple pathways, such as PCK1 (FoxO signaling pathway, glycolysis/gluconeogenesis, adipocytokine-signaling pathway, insulin signaling pathway), and FZD6 (mTOR-signaling pathway, Wnt-signaling pathway), which indicates that these genes can be taken as functional candidate genes associated with the difference in egg production.

Verification of gene expression profiles

To verify the accuracy and reproducibility of the RNA-seq data, we selected 8 well-known candidate DEGs associated with egg reproduction, including *INHBA*, *PLAU*, *STC1*, *FZD6*, *VIP*, *OASL*, *PCK1*, and *STAR*, to conduct qRT–PCR. All eight mRNAs had similar expression patterns in comparison to the RNA-seq data (Figure 3), indicating that the RNA-seq data are reliable.

Infection efficiency

To study the function of *STC1* in the chicken ovary, we constructed an *STC1* overexpression vector and synthesized STC1-specific siRNA. The pcDNA3.1-EGFP-STC1 plasmid and STC1-specific siRNA were successfully transfected into GCs. EGFP and CY5 were detected by fluorescence microscopy in the transfected group (Figure 4a and b). qRT–PCR indicated a significant increase in *STC1* abundance in the pcDNA3.1-EGFP-STC1 group ($P < 0.01$, Figure 4c). Among the three synthesized *STC1*-specific siRNAs, si-STC1-2 most significantly silenced *STC1* expression in the chicken GCs ($P < 0.01$, Figure 4d), so we subsequently used this siRNA to conduct the following research.

STC1 inhibits cell proliferation in chicken GCs

Because the normal proliferation and differentiation of GCs are crucial for follicular development, we tested the effects of *STC1* on GC activity and proliferation. CCK-8 and the EdU assays demonstrated that *STC1* significantly repressed GC viability, while its inhibition GC proliferation ($P < 0.01$,

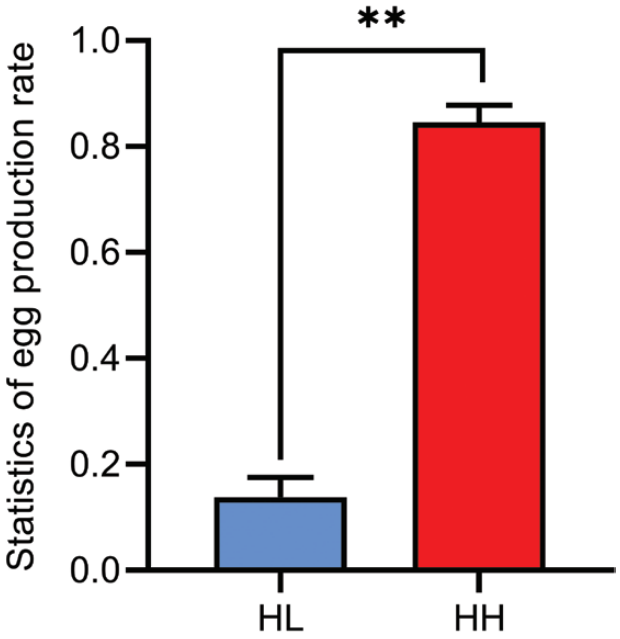

Figure 1. Comparison of the egg production rate of 80-wk-old HH and HL chickens. Data are presented as the mean ± SE ($n = 15$), and the error bar shows the standard deviation. $^{**}P < 0.01$.

Figure 5a–d). We also detected the expression of marker genes for the proliferation and differentiation of GCs, and the results showed that the overexpression of *STC1* in GCs can repress the expression of *FSHR*, *GDF9*, and *PCNA* ($P < 0.01$, Figure 5e), and that *STC1* knockdown induced the expression of *FSHR*, *GDF9*, and *PCNA* ($P < 0.01$, Figure 5f).

The overexpression of *STC1* significantly decreased the numbers of S-phase and G2/M-phase cells and induced cell cycle arrest ($P < 0.01$, Figure 5g). Conversely, *STC1* knockdown significantly increased S-phase cells relative to control group cells ($P < 0.01$, Figure 5h). In addition, *STC1* overexpression reduced the expression of *CDK1*, *CDK2*, *CCND1*, *CCND2* genes involved in the cell cycle pathway ($P < 0.01$, Figure 5i), and the siRNA of *STC1* promoted the expression of *CDK1* and *CCND1* ($P < 0.01$, Figure 5j). Together, these data indicate that *STC1* expression has a negative regulatory role in cell proliferation and decreases the cell cycle distribution of proliferating GCs.

Table 1. Characteristics of the reads from six chicken ovarian transcriptomes

Sample ID[1]	Raw reads	Clean reads	Clean ratio[2] (%)	All reads	Mapped reads	Mapped	Mapping ratio[3] (%)
HH-1	154,392,790	147,492,401	95.53	143,663,524	131,113,327	129,937,309	91.26
HH-2	123,416,200	117,774,725	95.43	114,535,538	104,276,259	103,432,922	91.04
HH-3	83,774,408	80,057,178	95.56	78,055,678	71,071,119	70,446,136	91.05
HL-1	149,512,210	142,872,483	95.56	139,337,904	127,556,400	126,481,832	91.54
HL-2	81,256,850	78,079,711	96.09	76,637,518	69,923,560	69,366,288	91.24
HL-3	94,542,336	90,807,270	96.05	88,805,590	82,822,947	82,080,840	93.26

[1]HH, 3 ovarian samples from high rates of egg production chickens; HL, 3 ovarian samples from low rates of egg production chickens.
[2]Clean ratio = clean reads/raw reads
[3]Mapping ratio = mapped reads/all reads.

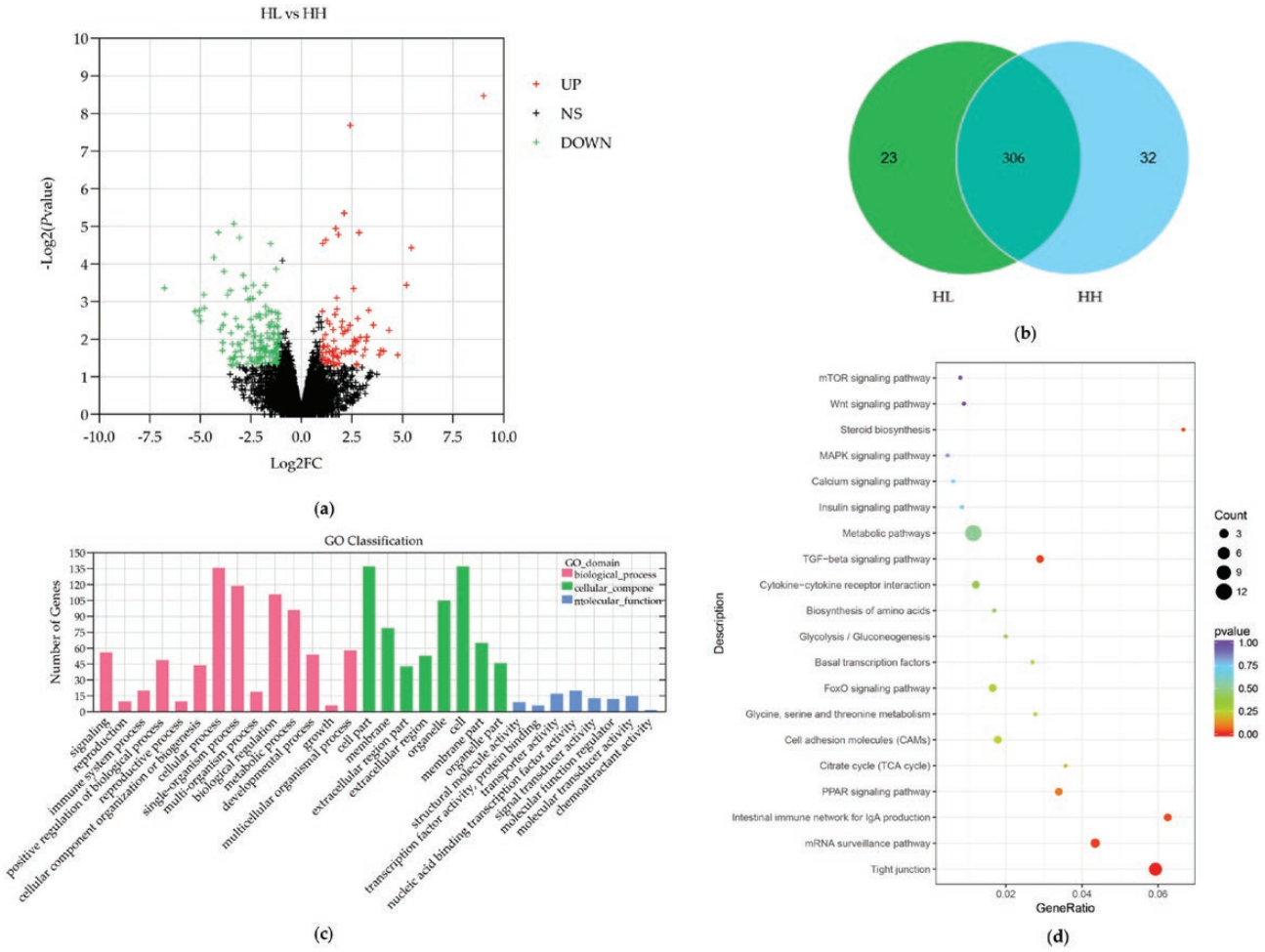

Figure 2. Differentially expressed genes between HH and HL chickens. (a) Volcano plot of differentially expressed genes between HL and HH chicken ovaries. (b) Venn diagram of coexpressed differentially expressed genes in HL and HH chicken ovaries. (c) GO enrichment of differentially expressed genes between HH and HL chicken ovaries. (d) KEGG pathway of differentially expressed genes between HH and HL chicken ovaries.

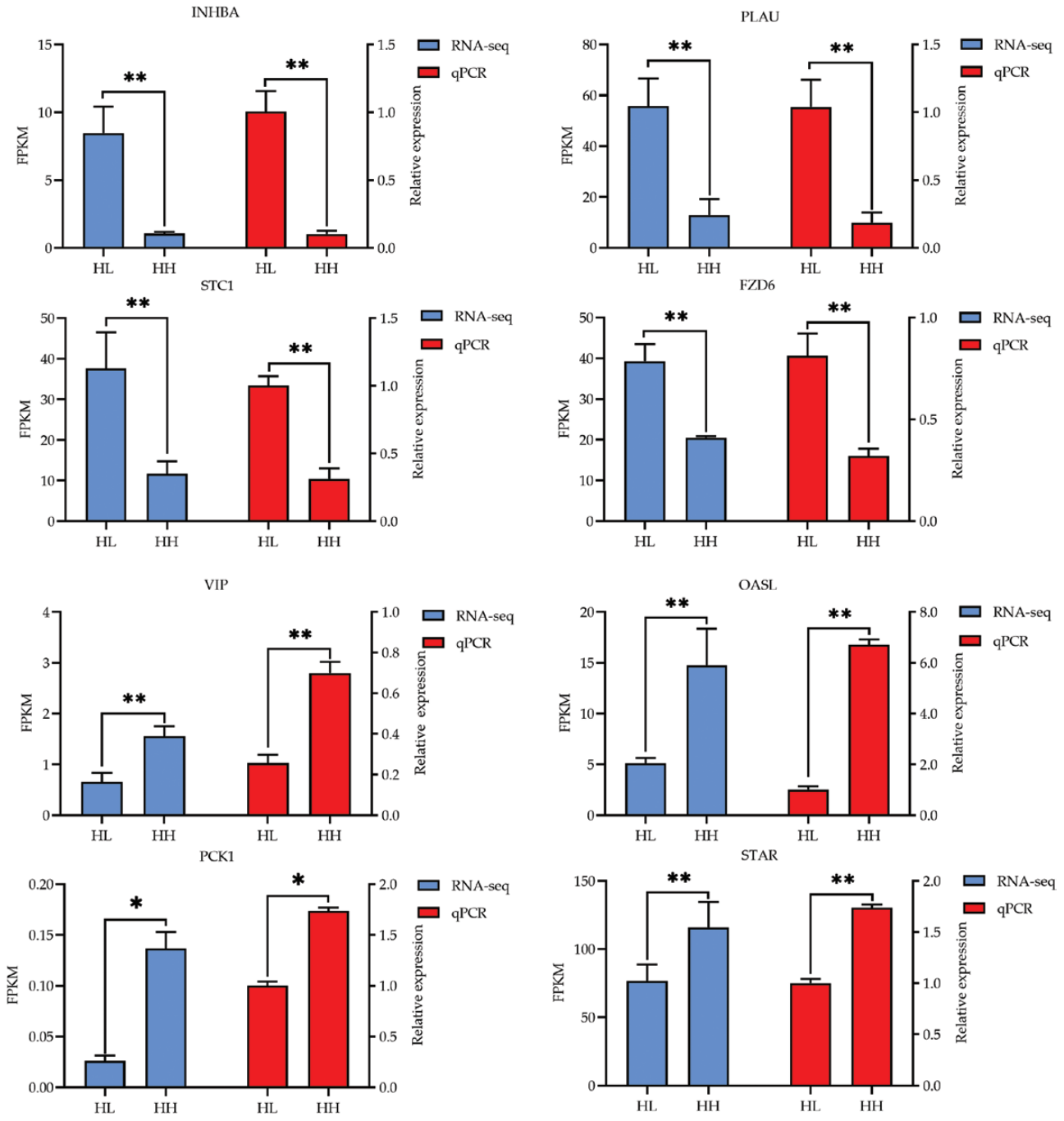

Figure 3. Quantitative validation of transcriptome sequencing results.

STC1 promotes cell apoptosis in chicken GCs

STC1 overexpression caused a significant upregulation in the apoptosis rate and proapoptotic gene *caspase 3*, *caspase 9* mRNA levels in chicken GCs and a significant downregulation of antiapoptotic gene *Bcl2* mRNA expression ($P < 0.05$, Figure 6a and b). Furthermore, the siRNA of *STC1* revealed that the GC apoptosis rate and proapoptotic gene *caspase 3* and *caspase 9* mRNA levels were significantly inhibited and antiapoptotic gene *Bcl2* mRNA level is significantly increased ($P < 0.01$, Figure 6c and d). These data revealed that *STC1* promotes apoptosis in the chicken GCs.

STC1 inhibits steroid hormone production in chicken GCs

The proliferation and apoptosis of GCs play an important role in follicular development. Another major function of GCs is to regulate the production and secretion of steroid hormones. Next, we tested the function of *STC1* in the synthesis of steroids. Overexpression of *STC1* repressed follicular development marker gene expression and the expression of genes related to the secretion of steroid hormones, such as *FSHR*, *STAR*, and *CYP11A1* ($P < 0.01$, Figure 7a). ELISA results showed that overexpression of *STC1* significantly reduced progesterone and estrogen production ($P < 0.01$, Figure 7b). However, the siRNA

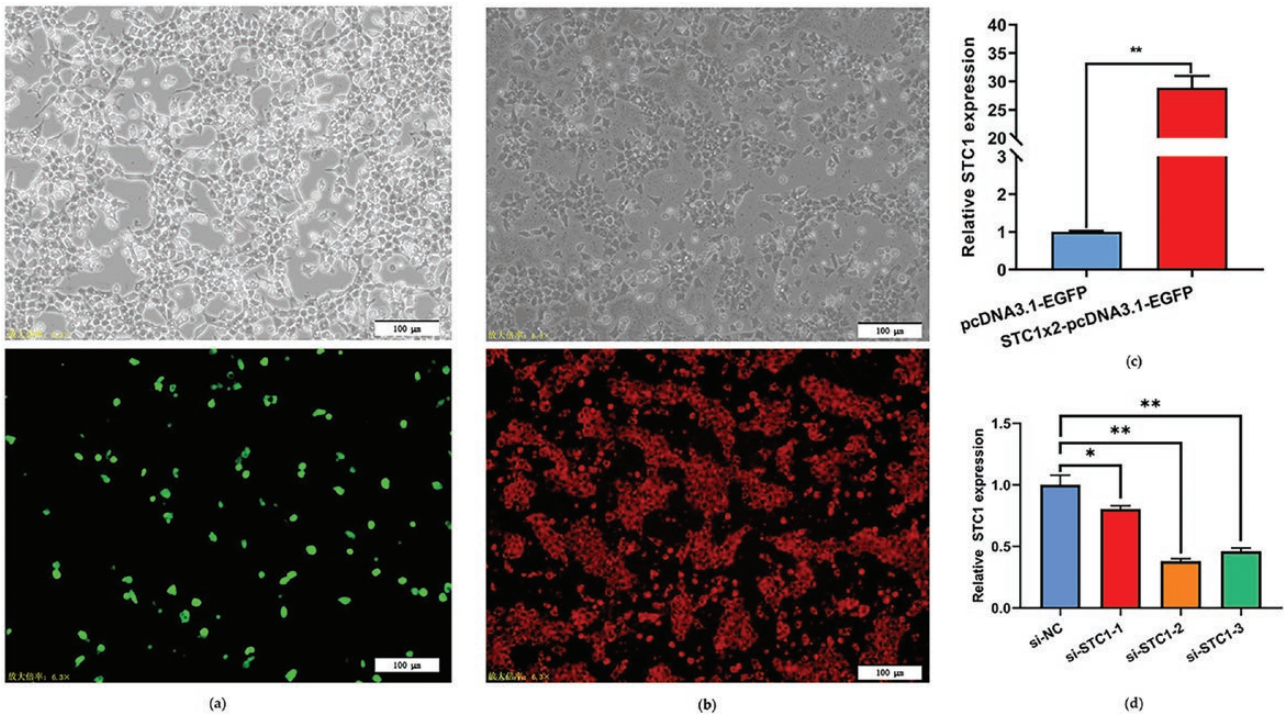

Figure 4. Detection of the transfection efficiency of *STC1* in GCs. (a) GCs expressing green fluorescent protein (GFP) could be observed after *STC1* overexpression. (b) GCs expressing red fluorescence (Cy5) could be observed after si-STC1 transfection. (c) *STC1* mRNA expression levels in GCs after *STC1* overexpression ($n = 3$). (d) *STC1* mRNA expression levels in GCs after si-STC1 transfection ($n = 3$).

of *STC1* promoted marker gene expression and the production and secretion of steroid hormones ($P < 0.01$, Figure 7c and d). To investigate the role of *STC1* in the insulin-like growth factor pathway, we assessed the expression of *PAPPA*, *IGFBP5*, and *IGF2* after overexpression and interference of *STC1* in chicken GCs. The results showed that overexpression of *STC1* could significantly downregulate the expression of *PAPPA* and *IGF2* and significantly upregulate the expression of *IGFBP5* ($P < 0.05$, Figure 7e). The siRNA of *STC1* significantly upregulated *PAPPA* and downregulated *IGFBP5* ($P < 0.01$), while *IGF2* showed no significant change (Figure 7f).

Discussion

The egg-laying rate of hens mainly depends on the normal development of follicles and ovulation in the ovary. The egg-laying process is mainly regulated by the hypothalamic–pituitary–ovarian axis, involving a series of sex steroid hormones and cytokines (Bronneberg et al., 2009; Brady et al., 2019). The development of follicles is orchestrated by numerous gene interactions and requires tight regulation of gene expression at both the transcriptional and post-transcriptional levels (Etches et al., 1983; Onagbesan et al., 2009). In recent years, an increasing number of studies have proven that high-throughput transcriptomic sequencing technology is efficient and widely used to generate whole transcriptome sequences and identify differentially expressed genes (Kang et al., 2009; Ozsolak and Milos, 2011; Terenina et al., 2017). Gaining insight into the gene expression patterns during ovarian follicle development by high-throughput sequencing could improve egg-laying performance.

The present study identified a total of 24,881 mRNAs in chicken ovaries using the Illumina sequencing method, including 306 differentially expressed mRNAs, some of which may participate in ovarian follicular development, maturation, and ovulation. GO enrichment analysis revealed nine differentially expressed genes associated with reproduction during biological processes, including two known genes, inhibin beta A *(INHBA)* and zona pellucida glycoprotein 3 (*ZP3*). KEGG analysis showed that the differentially expressed genes were significantly enriched in reproduction regulation-related pathways, including the FoxO signaling pathway, TGF-β signaling pathway, MAPK signaling pathway, PPAR signaling pathway, and steroid biosynthesis. *INHBA* is a subunit of inhibin that belongs to the transforming growth factor beta (TGF-beta) superfamily, which affects follicular development by regulating the production of inhibin (Burger et al., 1988; Bao et al., 2021). INH is a macromolecular glycoprotein hormone secreted by testicular support cells in males and ovarian GCs in females, and studies have shown that INH indirectly regulates follicular growth and development by inhibiting the synthesis and secretion of FSH (Jimenez-Krassel et al., 2003). Our transcriptome analysis showed that the expression of the *INHBA* gene was significantly increased in the ovaries of low-egg-laying Hy-Line Brown layers compared to high-egg-laying Hy-Line Brown layers. Therefore, it is hypothesized that the upregulation of *INHBA* in the ovaries of low-yielding layers may suppress the effect of FSH on reproductive activity. The *ZP3* gene is a member of the zona pellucida glycoprotein family, and mutations in the *ZP3* gene cause abnormal oocyte and follicle development in humans, resulting in female infertility (Li et al., 2022; Zhang et al., 2022). It has been shown that chicken ZP3 is synthesized in follicular GCs, and its encoded protein serves as a sperm receptor (Waclawek et al., 1998; Nishio et al., 2018). There are relatively few studies on the specific mechanism of action of the *ZP3* gene in poultry.

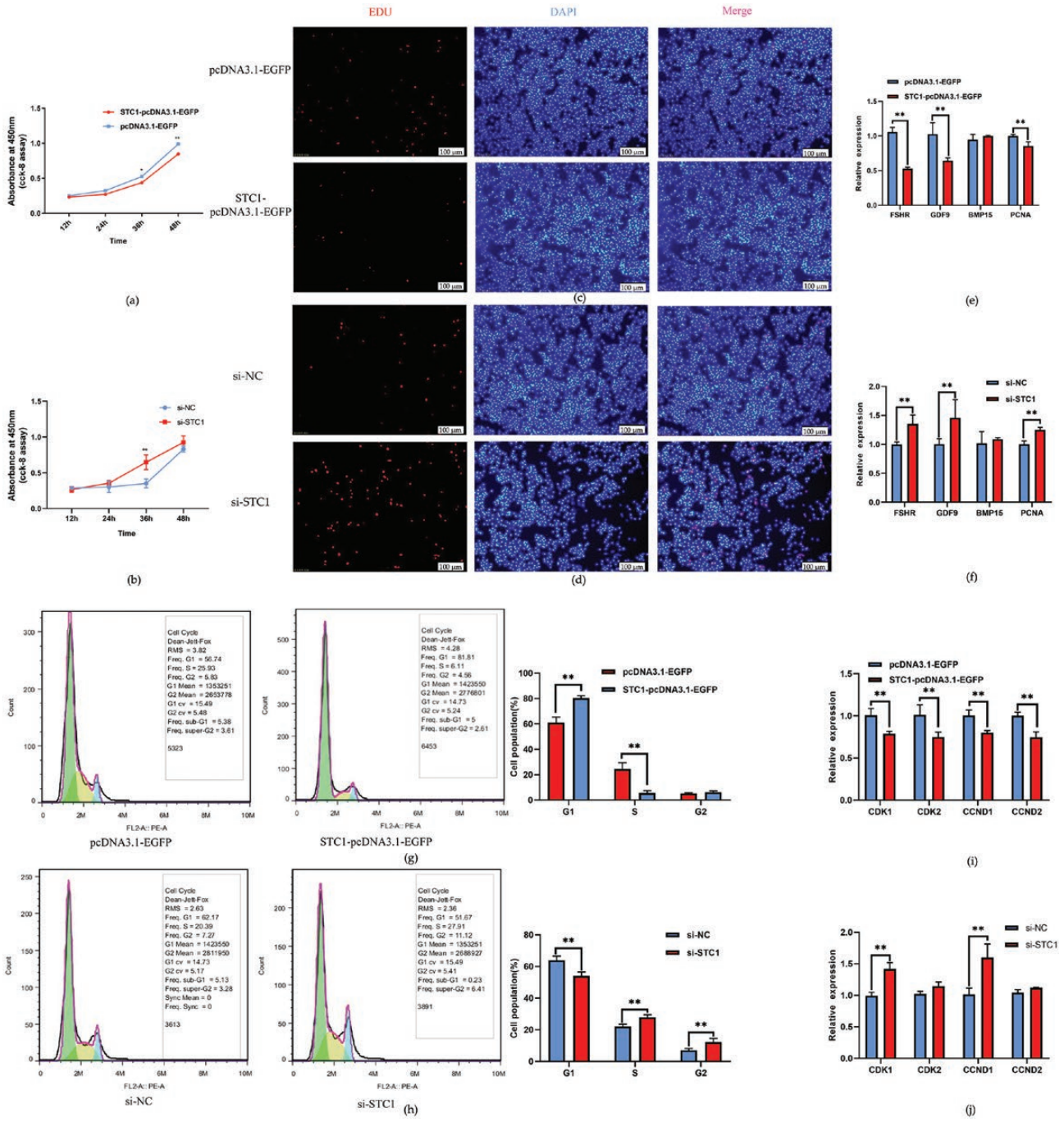

Figure 5. The inhibitory effect of *STC1* on granulosa cell proliferation. (a) Results of the CCK-8 assay after *STC1* overexpression ($n = 3$). (b) CCK-8 assay results after si-STC1 transfection ($n = 3$). (c) EdU assay results after *STC1* overexpression. (d) EdU results after si-STC1 transfection. (e) The expression of cell proliferation-related genes after *STC1* overexpression ($n = 3$). (f) The expression of cell proliferation-related genes after si-STC1 transfection ($n = 3$). (g) Results of flow cytometry after *STC1* overexpression ($n = 3$). (h) Results of the flow cytometry cycle analysis after si-STC1 transfection ($n = 3$). (i) The expression of cell cycle-related genes after *STC1* overexpression ($n = 3$). (j) The expression of cell cycle-related genes after si-STC1 transfection ($n = 3$).

We found that *STC1* was significantly enriched in cell development, cell differentiation, tissue development, hormone activity, cell migration, and other biological processes. STC1 is a glycoprotein hormone involved in mineral homeostasis that was originally found in fish in 1839 (Wagner and Dimattia, 2006; Bishop et al., 2021), whereas mammalian *STC1* shows a diverse tissue expression pattern, with the ovary exhibiting the highest level (Varghese et al., 2002; Yoshiko and Aubin, 2004). In recent years, studies have indicated that *STC1* plays a key role in cell proliferation and apoptosis (Dai et al., 2016; Pan et al., 2017). However, the exact role and molecular regulatory mechanisms of *STC1* in the follicular development of the hen ovary remain unclear. In the current study, we found that *STC1* inhibited GC proliferation and decreased the mRNA expression levels of *FSHR*, *GDF9*, *PCNA*, *CDK1*, *CDK2*, *CCND1*, and *CCND2*, demonstrating that *STC1* may suppress follicle selection, growth, and maturation by hindering GC proliferation and differentiation

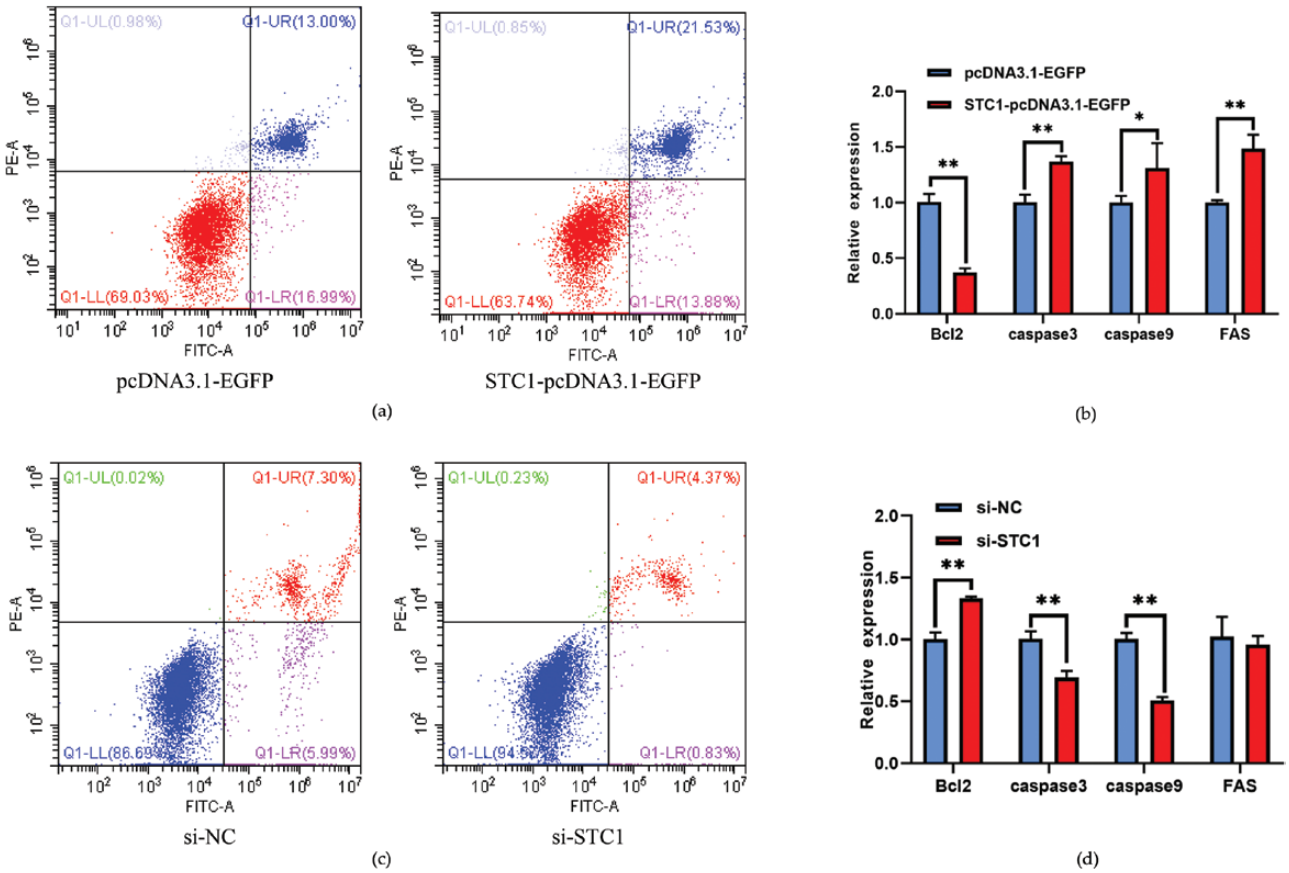

Figure 6. The promoting effect of *STC1* on granulosa cell apoptosis. (a) Results of flow cytometry apoptosis after si-STC1 transfection. (b) The expression of cell apoptosis-related genes after *STC1* overexpression ($n = 3$). (c) Results of flow cytometry apoptosis after si-STC1 transfection. (d) The expression of cell apoptosis-related genes after si-STC1 transfection ($n = 3$).

during the follicular development of the hen ovary. These data attested that the *STC1* gene exerts an inhibitory effect on the development of the follicles.

Poultry reproduction is strictly regulated by sex steroid hormones (Johnson et al., 2002). Sex steroid hormones, such as progesterone and estradiol, are involved in the regulation of ovulation, gonadal differentiation, and sexual and nesting behaviors in birds through interactions with their intracellular receptors (Ghanem and Johnson, 2019; Liu et al., 2019b). In some GC research, *STC1* has been proven to dampen gonadotropin stimulation of GC differentiation by paracrine regulation and inhibit progesterone secretion (Luo et al., 2004; Baioni et al., 2011). It is tempting to speculate that *STC1* may have a pivotal role in the development of follicles. In the current study, overexpression of *STC1* in chicken GCs inhibited the expression of *FSHR*, *STAR*, and *CYP11A1*. Conversely, the siRNA of *STC1* in chicken GCs increased the expression of *FSHR*, *STAR*, and *CYP11A1*. As expected, ELISA detection also showed decreased progesterone and estradiol accumulation in the *STC1* overexpressing group, and opposite results were obtained with siRNA of *STC1*. These results suggest that *STC1* acts as a negative regulator of follicular growth and development in the hen ovary. Several studies (Argente et al., 2017) have shown that when *STC1* acts on follicles, the IGF signaling pathway is inhibited. Recently, the stanniocalcin (STC1 and STC2) was identified as a novel inhibitor of pregnancy-associated plasma protein A (**PAPPA**) (Jepsen et al., 2015, 2016; Kløverpris et al., 2015), which is a key regulator of IGF bioactivity, by releasing IGFs from their corresponding IGF-binding proteins (**IGFBPs**; Spitschak and Hoeflich, 2018; Liu et al., 2019a). Thus, *STC1* may regulate folliculogenesis via *PAPPA* in the chicken ovary. Similarly, our results showed that *STC1* inhibited the expression of *PAPPA* and promoted the expression of *IGFBPs*, suggesting that there may be some interactions between *STC1* and *PAPPA* that regulate follicular development. Moreover, further studies focusing on the function of the chicken STC1-PAPPA-IGF axis in the ovary are needed.

Conclusion

In conclusion, our results demonstrate a novel role and mechanism of *STC1* in regulating GC proliferation, apoptosis, and steroid secretion (P4 and E2) in chicken GCs. We first verified that *STC1* inhibited the expression of *STAR* and *CYP11A1*, and suppressed the expression of *PAPPA* through the IGF pathway in chickens. These findings show that *STC1* plays a critical role in follicle development and maturation by maintaining steroid hormone homeostasis.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

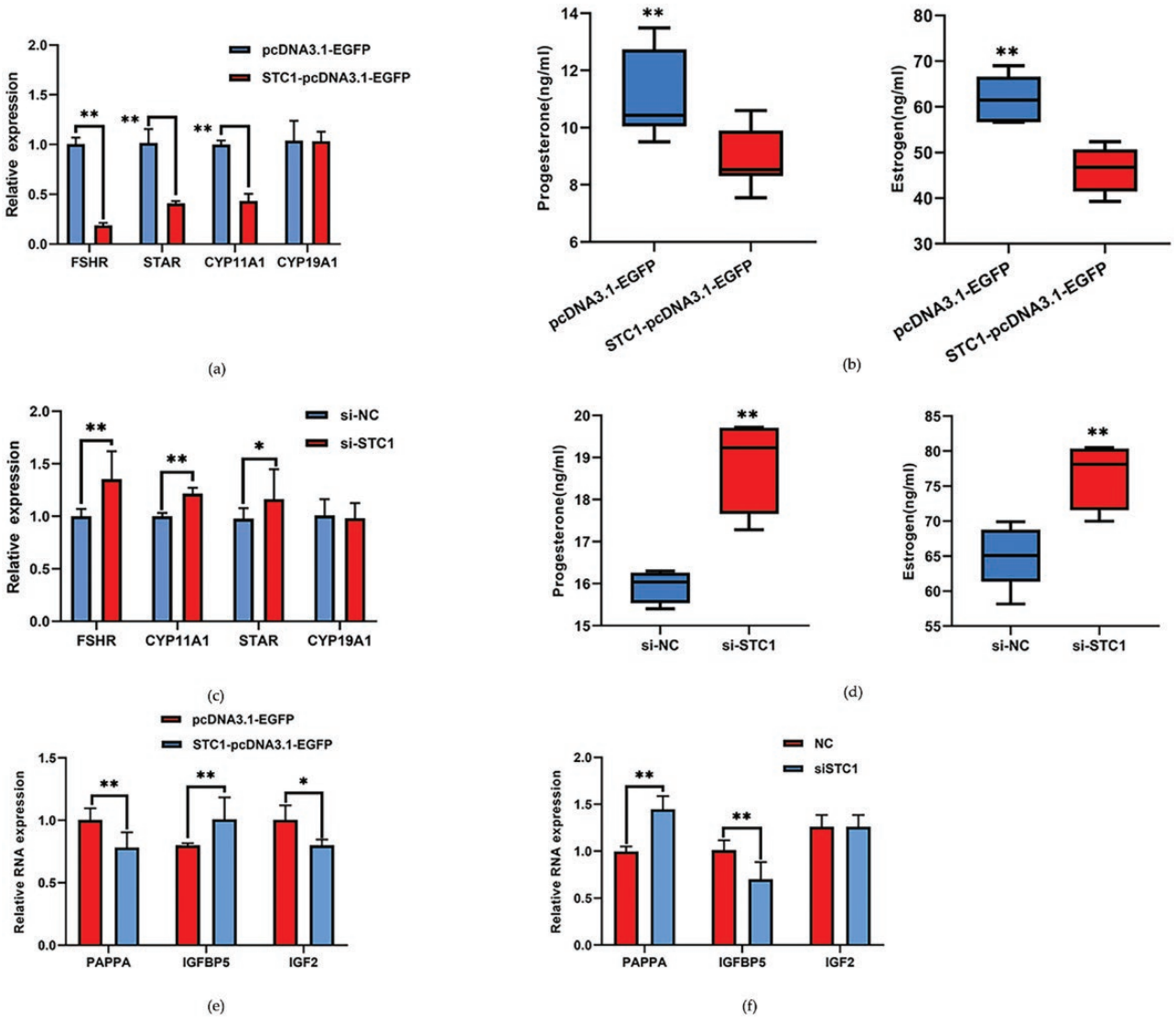

Figure 7. The inhibitory effect of *STC1* on granulosa cell steroid hormone production. (a) The expression of steroid hormone secretion-related genes after *STC1* overexpression ($n = 3$). (b) The secretion of steroid hormones after *STC1* overexpression ($n = 3$). (c) The expression of steroid hormone secretion-related genes after si-STC1 transfection ($n = 3$). (d) The secretion of steroid hormones after si-STC1 transfection ($n = 3$). (e) The expression of insulin-like growth factor pathway-related genes after *STC1*overexpression ($n = 3$). (f) The expression of the insulin-like growth factor pathway-related genes after si-STC1 transfection ($n = 3$).

Author Contributions

Junwei Sun, Pengwei Zhang, Dongxue Wang: methodology and writing-original draft. Shuaipeng Zhu, Xiangfei Ma, Zhenwei Du: investigation and software. Jiechang Zhang: formal analysis. Ruirui Jiang: validation and data curation. Shuangyuan Yang and Yadong Tian: methodology and software. Wenting Li: investigation and data curation. Xiangtao Kang and Guirong Sun: resources, supervision, and project administration. Hetian Huang and Fengbin Yan: writing-review and editing. Donghua Li: conceptualization, writing-review, and editing.

Acknowledgments

This study was supported by the Key Research Project of the Shennong Laboratory (SN01-2022-05) and the China Agriculture Research System (CARS-40-K04), National Natural Science Foundation of China (32072710 and 32102540).

Conflict of Interest Statement

All authors disclosed no relevant relationships.

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