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Research Article

Synergistic inhibition of *csal1* and *csal3* in granulosa cell proliferation and steroidogenesis of hen ovarian prehierarchical development†

Hongyan Zhu[1,2], Ning Qin[1,3], Xiaoxing Xu[4], Xue Sun[1,3], Xiaoxia Chen[1], Jinghua Zhao[1], Rifu Xu[1,3,*] and Birendra Mishra[4,*]

[1]Department of Animal Genetics, Breeding and Reproduction, College of Animal Science and Technology, Jilin Agricultural University, Changchun, China [2]Department of Animal Genetics, Breeding and Reproduction, College of Animal Husbandry and Veterinary, Jinzhou Medical University, Jinzhou, China [3]Modern Agricultural Technology International Cooperative Joint Laboratory of the Ministry of Education, Changchun, P. R. China and [4]Department of Human Nutrition, Food and Animal Sciences, University of Hawaii at Manoa, Honolulu, Hawaii, USA

***Correspondence:** Rifu Xu, College of Animal Science and Technology, Jilin Agricultural University, Xincheng Avenue 2888, Changchun, Jilin Province, P. R. China. Tel: +8613944104559; Fax: +86-0431-84532813; E-mail: poultryxu@jlau.edu.cn. Birendra Mishra, Department of Human Nutrition, Food, and Animal Sciences, University of Hawaii at Manoa, 1955 East-West Road, Rm 216, Honolulu, Hawaii, USA. Tel: +1-808-956-7021; Fax: +1-808-956-7021; E-mail: bmishra@hawaii.edu

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Abstract

SALL1 and SALL3 are transcription factors that play an essential role in regulating developmental processes and organogenesis in many species. However, the functional role of SALL1 and SALL3 in chicken prehierarchical follicle development is unknown. This study aimed to explore the potential role and mechanism of *csal1* and *csal3* in granulosa cell proliferation, differentiation, and follicle selection within the prehierarchical follicles of hen ovary. Our data demonstrated that the *csal1* and *csal3* transcriptions were highly expressed in granulosa cells of prehierarchical follicles, and their proteins were mainly localized in the cytoplasm of granulosa cells and oocytes as well as in the ovarian stroma and epithelium. It initially revealed that both *csal1* and *csal3* may be involved in chicken prehierarchical follicle development via a translocation mechanism. Furthermore, our results showed an abundance of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* mRNA in granulosa cells, and the proliferation levels of granulosa cells from the prehierarchical follicles were significantly increased by siRNA-mediated knockdown of *csal1* or/and *csal3*. Conversely, the overexpression of *csal1* or/and *csal3* in the granulosa cells led to a remarkably decreased of them. Moreover, *csal1* and *csal3* together exert a much stronger effect on the regulation than any of *csal1* or *csal3*. These results indicated that *csal1* and *csal3* play synergistic inhibitory roles on granulosa cell proliferation, differentiation, and steroidogenesis during prehierarchical follicle development in vitro. The current data provide a basis of molecular mechanisms of *csal1* and *csal3* in controlling the prehierarchical follicle development and growth of hen ovary in vivo.

Summary Sentence

The transcription factors *csal1* and *csal3* might play a synergistic inhibitory role on granulosa cell proliferation, differentiation, and steroidogenesis during PF development.

Key words: *csal1*, *csal3*, synergistic inhibition, proliferation, differentiation, follicle selection

Introduction

Ovarian follicle development in the hen (*Gallus gallus*) is a highly complex process involving the actions of numerous endocrine, paracrine, and autocrine factors in a spatial and temporal manner and is characterized by dramatic changes during the follicle selection (cyclic recruitment) [1, 2]. A strictly controlled follicular hierarchy is maintained in the hen. Only a small percentage of follicles into the preovulatory hierarchy are selected from prehierarchical follicles (PFs) (6–8 mm in diameter), reach maturity, and undergo ovulation. During this process, proliferation and differentiation of granulosa cells (GCs) play crucial roles in PF development, selection, and growth [3, 4]. CyclinD1 (*CCND1*) plays a central role in the regulation of proliferation by regulating cell cycle progression in humans [5, 6]. *CCND1* is the target gene of beta-catenin protein (β-catenin), and nuclear accumulation of mutated β-catenin in hepatocellular carcinoma is associated with increased cell proliferation [7]. As a key downstream effector of the canonical Wnt signaling pathway, β-catenin also contributes to cell proliferation and tumorigenesis [8]. It is suggested that *CCND1* and β-*catenin* (*Bcat*) are the promising biomarkers for cell proliferation of GCs within ovarian PFs. Several other factors, such as steroidogenic acute regulatory protein (*StAR*) and cytochrome p450, family11, subfamily A, polypeptide 1(*CYP11A1*) have been proved to promote the growth and cell differentiation of GCs in chicken hierarchal follicles and steroidogenesis [4, 9, 10]. Prior to follicle selection (9–12 mm in diameter), the granulosa layer remains in an undifferentiated state as shown by the undetectable to very low mRNA levels of *CYP11A1* and *StAR*; however, immediately after selection, the *CYP11A1* and *StAR* mRNA levels dramatically increase in the granulosa cell layer during the developmental stages [4, 11, 12]. Follicle-stimulating hormone (FSH) induces GC proliferation, differentiation, and follicle selection by stimulating FSH receptors (*FSHRs*), *StAR* and *CYP11A1* expression, and steroidogenesis [10, 13, 14]. The *FSHR* mRNA is highest in the granulosa layer from the 6–8 mm follicles for selection [4, 10, 15]. Therefore, the increased expression levels of *FSHR* transcription in the GCs of PFs (6–8 mm in diameter) have generally been accepted as an indicator to initiate follicle selection [3, 4, 10]. Furthermore, accumulating evidence indicates that the spalt gene family may be involved in ovarian follicle development and growth [16–20].

Previous studies have documented that four members of spalt, spalt1 (sal1), sal2, sal3, and sal4, are required for the specification of head and tail regions in Drosophila embryos [21]. The spalt members are characterized by multiple C2H2-type zinc-finger motifs (ZFs) serving as trans-regulators of gene expression during growth and development [22, 23]. In chicken, three homologues of the spalt family have been described so far, *csal1* (also named SALL1), *csal3* (SALL3), and *csal4* (SALL4) [24, 25, 27]. Expression of *csal1* is predominantly detected in the developing heart, mesoderm, ectoderm, and neural tube of the early embryo [24, 25], whereas *csal3* is found in the neural tissue, limb buds, mesonephros, and cloaca [26, 27]. The SALL proteins have been described as transcriptional repressors because the N-terminal region of the protein contains a highly conserved 12-amino acid motif, which is sufficient for the recruitment of nucleosome remodeling and deacetylase corepressor complex (NuRD) [28], of which SALL1 is capable of binding to β-catenin and activates synergistically a reporter construct responding to the Wnt signaling pathway through recruitment of remodeling factors to heterochromatin [29]. Moreover, SALL2 binds and represses *CCND1* promoters and is recognized as a novel mechanism by which SALL2 exerts a negative regulatory role in cell proliferation associated with the regulation of cell cycle progression [19]. SALL2 is deregulated and is proposed as a tumor suppressor in human ovarian cancer [17, 18, 30]. This information has revealed that the SALL family is related to ovarian development. However, there is no direct evidence to prove that the component of SALL family plays an essential role in ovarian follicle development in chickens.

To delineate the functions and regulatory mechanisms of *csal1* and *csal3* in regulating the ovarian follicular development in chicken, we examined the expression profile of *csal1* and *csal3* in PFs. Furthermore, the effects of *csal1* and *csal3* on GCs proliferation and expression of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* genes that contribute to cell proliferation, differentiation, and steroidogenesis were investigated.

Materials and methods

Chicken and sample collection

The Hy-Line Brown layers were utilized from the College of Animal Science and Technology of Jilin Agricultural University. All hens were reared in laying batteries under standard husbandry practices and were exposed to a 16L: 8D photoperiod as previously described [4]. Laying hens at 21 weeks of age (n = 20) were selected from the population and euthanized for ovarian follicle sampling. According to Gilbert et al. [31] and Knight et al. [33], follicles within the ovary were grouped into PF (from less than 1 to 8 mm in diameter) and were classified as small white follicles (less than 1 mm), large white follicles (2–5 mm) and small yellow follicles (6–8 mm), and preovulatory follicles that are arranged in a hierarchical order to clearly demarcate the stage of the development of each follicle from large yellow follicles (9–12 mm) to large yellow preovulatory follicles (13–40 mm, named F5, F4, F3, F2, and F1, respectively) [32]. After slaughter, the ovary from each hen was immediately removed and placed into ice-cold 0.9% NaCl solution. GCs from the PFs in various sizes (1–3.9, 4–4.9, 5–5.9, 6–6.9, and 7–8 mm in diameters) and preovulatory follicles were taken from the hen ovaries and then dispersed for culture as previously described [34, 35, 36]. A representative portion of each ovary was taken and snapped frozen immediately in liquid nitrogen and stored at −80 °C until analysis. All animal experimentations were carried out in accordance with the guidelines (Permission No. GR(J)18-010) approved by the Institutional Animal Care and Use Committee of Jilin Agricultural University (Changchun, China).

Immunofluorescence assay

Immunofluorescence staining was used to investigate the expression of *csal1* and *csal3* proteins in ovarian PFs. Briefly, follicles were collected and were fixed in 4% paraformaldehyde (pH 7.4),

embedded in paraffin after dehydration and then cut into 4 mm tissue sections. The sections were mounted on silanized glass slides, deparaffinated and rehydrated as described [9], pretreated to unmask antigen using 10 mM citrate buffer (pH 6.0) for 10 min at 98 °C, and blocked with 5% goat serum. Sections were then incubated at 4 °C overnight with anti-*csal1* antibody (1:100, Abcam, Cambridge, MA, USA) or anti-*csal3* antibody (1:50, Novus, Littleton, CO, USA) as shown in Supplemental Table S1 followed by goat anti-rabbit fluorescence antibody coupled to Cy3 (1:100, Boster Biological Technology, Wuhan, China) for a 60-min incubation. Nuclei counterstain was with DAPI (4′, 6-diamidino-2-phenylindole) (Sigma, St Louis, MO, USA) for 5 min. After being washed thrice with PBS, slides were coverslipped using a mounting solution. All fluorescence images were captured using the same exposure time by fluorescence microscopy system (Zeiss), and the pictures were merged using Axio Vision Rel. 4.8 software (Carl Zeiss Ltd., Oberkochen, Germany) as previously reported [37].

Cell culture

The GCs isolated from the PFs (6–8 mm diameters) were cultured in Medium199 (M199; Gibco, Waltham, MA, USA) supplemented with 10% (v/v) fetal calf serum (Gibco) at 37 °C with 5% CO_2 in humidified chambers. The cultured GCs used in this experiment have been purified and quantified in our laboratory [4, 10]. The specificity of the GCs has been identified by the H & E staining procedure and fluorescence staining analysis [9, 12].

Quantitative real-time PCR analysis (qPCR)

Total RNA was isolated from the various-sized PFs and *csal1*, *csal3*-transfected cells at 1 × 10^6 cells/well using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. Total RNA (100 ng) was reverse transcribed using a reverse transcription kit (TaKaRa, Dalian, China). Expressions of *csal1*, *csal3*, *CCND1*, *Bcat*, *StAR*, *CYP11A1*, *FSHR,* and *18S rRNA* were analyzed using qPCR. RT-qPCR primers were also from TaKaRa (Table 1). SYBR Green RT-qPCR assay for the target genes was performed in optical 96-well plates using the SYBR Premix EX Taq^{II} (TaKaRa, DRR081A) and 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA), according to the manufacturer's instructions. Results were detected quantitatively in real-time by relative quantitation of two standard curves. Using the $2^{-\Delta\Delta Ct}$ method, the mRNA expression results were normalized against 18S rRNA as an internal control. All experiments were performed three times, each time in triplicate.

Western blotting

Western blotting for *csal1* and *csal3* was performed. GCs were washed, harvested, lysed with a lysis buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% SDS, 1% Triton X-100, 10 μg/ml of leupeptin, 1 mM of aprotinin, and 1 mM of PMSF) on ice for 30 min, and centrifuged at 14 000 g at 4 °C for 15 min. Proteins were resolved by SDS-PAGE, transferred onto PVDF membranes, blocked at room temperature for 1 h in 5% BSA, and then incubated overnight at 4 °C on a rotator with the primary antibodies: *csal1* (1:500, Abcam, Cambridge, MA, USA), *csal3* (1:500, Novus, Littleton, CO, USA), and β-actin (1:200, Boster Biological Technology, Wuhan, China), which was used as a loading control. Membranes were washed four times with TBST and incubated for 1 h with appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000, S Boster Biological Technology, Wuhan, China) (Supplemental Table S1). Immunoreactivity was visualized with an enhanced chemiluminescence detection reagent (ECL,ThermoScientific, Rockford, USA). All experiments were performed three times, each time in triplicate.

Construction of expressing vector

The chicken *csal1* and *csal3* cDNA sequences (NM_204707.1 and NM_204647.1) were amplified by specific primers (Table 2) for *csal1* and *csal3* genes from a chicken cDNA library by PCR, and cloned into a pUC57-Simple plasmid (Sangon Biotech Co., Ltd., Shanghai, China). After the recombined plasmids were identified by restriction enzyme (*EcoR* I and *Pme* I, TaKaRa, Dalian, China) digestion assay (Supplemental Figure 1), sequencing of the targeted fragments was performed. Then, the *csal1* and *csal3* cDNA sequences were subcloned separately into pYr-adshuttle-4 expressive vector containing an N-terminal hemagglutinin epitope (HA) tag (Biobuffer Biotech Service Co. Ltd., Wuhan, China) to generate the pYr-adshuttle-4-*csal1*/*csal3* expression construct as previously described [4].

Cell transfection

The transfection for the *csal1* and *csal3* gene expression using the recombinant plasmid vector pYr-adshuttle-4-*csal1*/*csal3* was performed as previously reported [4, 10]. Briefly, the GCs were randomly grouped and transfected with pYr-adshuttle-4-*csal1*/*csal3* and pYr-adshuttle-4 blank vector using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA). The cultures (10^5 cells/well in a 24-well plate) were conducted in a basal medium containing 1 μl/ml polybrene (hexadimethrine bromide, Sigma) and incubated at 37 °C with 5% CO_2 according to the manufacturer's instructions [4]. After 24 h of continuous culture, the GCs were collected, and the transfection efficiency was confirmed by qPCR and western blotting.

RNAi

Specific siRNA sequences targeting the chicken *csal1* and *csal3* gene were designed using InvivoGen siRNA Wizard v3.1 (http://www.invivogen.com/sirnawizard/). All designed siRNA sequences were blasted against the chicken genome database to eliminate the cross-silence phenomenon with nontarget genes. The most effective *csal1*- and *csal3*-specific siRNAs were further screened by RT-qPCR and Western blotting. Cells transfected with scramble siRNA that does not target any gene were used as the negative control (Table 3). As mentioned above, GCs were plated in 24-well plates, and the siRNAs were transfected into the cultured cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Granulosa cell proliferation assay

BrdU (5′-bromo-2′-deoxyuridine) Cell Proliferation Assay Kit (Invitrogen, Carlsbad, CA, USA) was performed according to the manufacturer's instructions to detect the variation of cell proliferation after cell transfection for *csal1* or *csal3* expression briefly after cell culture in a CO_2-incubator at 37 °C overnight. Subsequently, the supernatant in each well was removed by pipetting and washed twice with PBS. The cells were fixed by adding 100 μl of 4% formaldehyde in PBS to each well for 15 min at room temperature. Then 0.5% Triton X-100 in PBS was added to each well for 20 min at room temperature for permeabilization, and the DNA was denatured with the denaturing agent for 30 min at 37 °C. Subsequently, the diluted anti-BrdU-peroxidase was added and kept at 37 °C for 1 h. The

Table 1. The sequences of genes for RT-qPCR.

Gene	Primer sequences (5′-3′)	Accession no.	Size (bp)
csal1	5′-CCCAGGAAGCAAGGTGTA-3′ 5′-CGTTGGCATGTCCGTATT-3′	NM_204707.1	167
csal3	5′-CTCCTCATCGG CTGTTGG-3′ 5′-GTGGTGGGCTGTCTGGTT-3′	NM_204647.1	197
18S rRNA	5′-GAAACGGCTACCACATCC-3′ 5′-CACCAGACTTGCCCTCCA-3′	AF173612.1	169
FSHR	5′-TCCTGTGCTAACCCTTTCCTCTA-3′ 5′-AACCAGTGAATAAATAGTCCCATC-3′	NM_205079.1	207
StAR	5′-AGCAGATGGGCGACTGGAAC-3′ 5′-GGGAGCACCGAACACTCACAA-3′	AF220436.1	148
CCND1	5′-AAACGATTCCTCTGACCGCA-3′ 5′-GGTCATTGCAGCCAGATTCC-3′	NM_205381.1	125
CYP11A1	5′-TCCGCTTTGCCTTGGAGTCTGTG-3′ 5′-ATGAGGGTGACGGCGTCGATGAA-3′	NM_001001756.1	112
Bcat	5′-CGGAGGACAAACCACAAGACTAC-3′ 5′-TATCCGCCAGAGTGGAAAGAAC-3′	NM_205081.1	184

Table 2. The sequences of *csal1* and *csal3* for construction of expressing vector.

Gene	Recombinant vector	Primer pairs (the forward and reverse, 5′-3′)	Restriction sites
csal1	pUC57-simple-*csal1*	Forward:5′CCGGAATTCGCCACCATGTACCCATACGATGTTCCAGATTACGCTTCG-3′	*EcoRI*
		Reverse:5′AGCTTTGTTTAAACGGCGCGCCGGCTAATTTGTTACGATTTCTTTGTT-3′	*Pme I*
csal3	pUC57-simple-*csal3*	Forward:5′CCGGAATTCGCCACCATGTACCCATACGATGTTCCAGATTACGCTTCT-3′	*EcoRI*
		Reverse:5′AGCTTTGTTTAAACGGCGCGCCGGTTAATTTATGCCAATCTCTTTATTAT-3′	*Pme I*

Note: The underlined nucleotides indicate the location corresponding to each of the restriction sites. The Kozak sequence is indicated with a dotted line.

Table 3. siRNA sequences for the target sites in the *csal1* and *csal3* gene.

Gene	Sequences
csal1	5′-GCUUCCAGCAUUAACAAUATT-3′
csal3	5′-GCGAGUACACCCAAUAUAUTT-3′
Scrambled	5′-UUCUCCGAACGUGUCACGUTT-3′

reaction was completed by adding a DAPI solution for 5 min. Subsequently, the redundant supernatant was washed four times with PBS. Fluorescence images were captured using a fluorescence microscopy system (Zeiss), and the pictures were merged using Axio Vision Rel. 4.8 software (Carl Zeiss Ltd., Oberkochen, Germany). The number of BrdU+ cells was expressed as a percentage and calculated relative to the total number of cells counted in the microscope fields observed in the negative control. Twenty fields were analyzed, and each condition was averaged. Each experiment was performed in triplicate and repeated three times. The method was according to that of our groups [12].

Statistical analysis

The statistical analyses were performed using the SPSS 17.0 software package (IBM, Armonk, NY, USA). All experiments were repeated at least three times using different batches of sampled hens. To quantify the mRNA expression using the qPCR analysis, four amplified products per hen from independent reactions were utilized. All groups of quantitative variables were checked for normal distribution by the Kolmogorov-Smirnov test. Since the conditions of normality of distribution and homogeneity of variance were satisfied, these data were analyzed with a one-way ANOVA followed by a Dunnett Multiple Comparison test. In the experiment with less than three groups, a Student *t*-test was performed for comparisons between the treatment and control groups after confirmation of normal distributions for nonparametric analysis. Statistical significance was declared at $P < 0.01$ or $P < 0.05$.

Results

Immunolocalization of *csal1* and *csal3* in the chicken ovarian follicles

In order to examine the biological roles of *csal1* and *csal3* factors in chicken follicle development, we initially localized *csal1* and *csal3* proteins in various-sized prehierarchichal ovarian follicles by immunofluorescence. As shown in Figure 1, the *csal1* protein was found to be predominantly expressed in the GCs and oocytes of the PFs as well as in the ovarian stroma and epithelium, and the protein was mostly localized in the cytoplasm rather than in the nucleus. As shown in Figure 2, the expression and distributions of *csal3* protein were very similar to the *csal1*. This result indicated that both *csal1* and *csal3* might be involved in the hen ovarian PF development in a translocation mechanism.

Expression of *csal1* and *csal3* genes in GCs of the PF

The RT-qPCR analysis showed that the *csal1* and *csal3* mRNAs were highly expressed in the GCs of all PFs sampled from 1–3.9 up to 7–8 mm in diameters. The highest expression of *csal1* mRNA was observed in GCs of the ovarian follicles with 6–6.9 mm

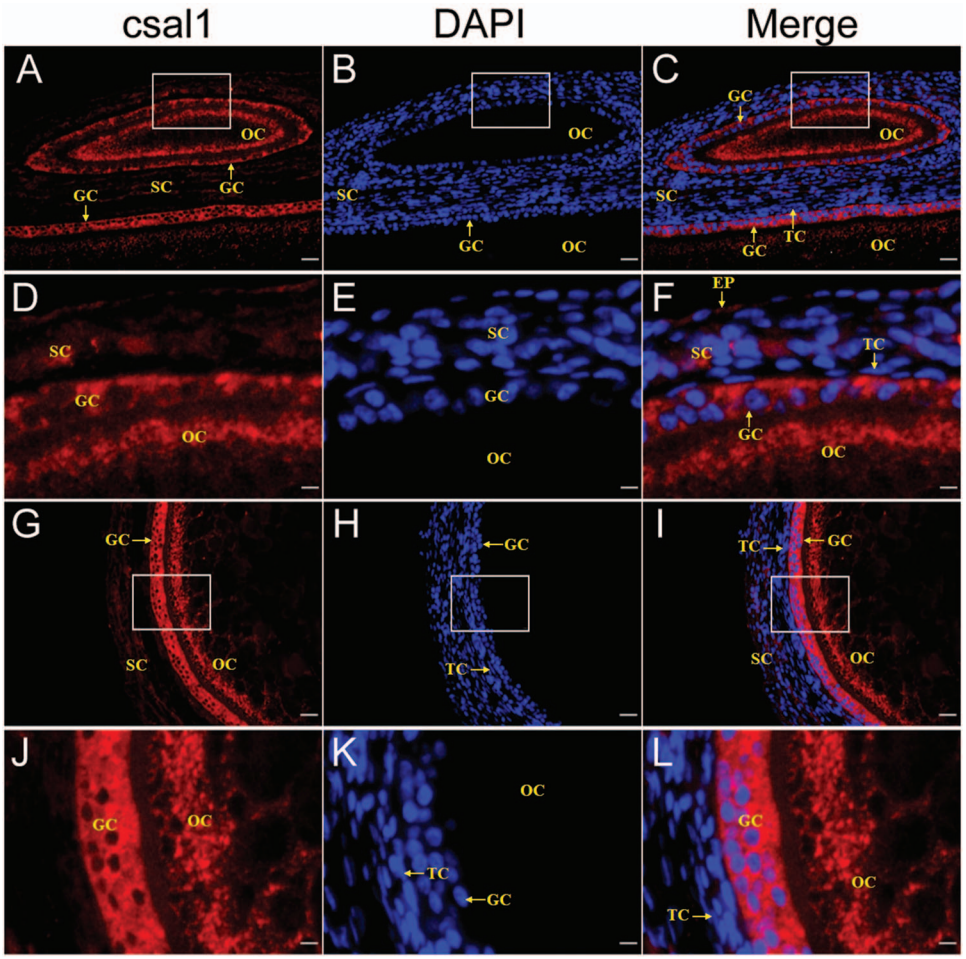

Figure 1. Immunofluorescence localization of *csal1* in the prehierarchichal follicles of the chicken ovary. Paraformaldehyde-fixed tissue sections were probed with anti-chicken *csal1*. The positive *csal1* signal was detected as red staining. Intense immunofluorescence staining was detected in the GCs, oocytes, and in the ovarian stroma and epithelium in the various-sized prehierarchichal follicles (A, D, G, and J). Blue staining represents artificial coloring of the nuclei with DAPI staining (B, E, H, and K). Scale bars, 20 μm (A, B, C, G, H, and I); 5 μm (D, E, F, J, K, and L). (A)–(F) were from the PF of 1–3.9 mm in diameter and (G)–(L) were from the PF of 6–6.9 mm in diameter. Oocyte (OC), GC, theca cell (TC), somatic cells (SCs), and epithelium (EP) are indicated in the images.

(1.865 ± 0.174) and the lowest in the ovarian follicles with 4–4.9 mm (0.516 ± 0.193). The highest expression of *csal3* mRNA was determined in GCs of the ovarian follicles with 7–8 mm (2.470 ± 0.318) and the lowest in the ovarian follicles with 1–3.9 mm (1.098 ± 0.232) (Figure 3). Although the mRNA expression patterns of *csal1*gene appeared to be different from the *csal3*, the gradually increasing abundance of *csal1* and *csal3* mRNA in the follicles from 1–3.9 up to 7–8 mm in diameters was observed.

csal1 plays an inhibitory role in granulosa cell proliferation and the expression of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* genes

To determine the roles of *csal1* gene in ovarian follicular development, siRNA-mediated knockdown of *csal1* gene was performed by transfection of *csal1*-specific siRNA into the GCs (Figure 4A and B). Our findings showed that the expression levels of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* mRNA abundance were significantly increased ($P < 0.01$) after the GCs were transfected with *csal1*-specific siRNA (Figure 4C). Moreover, as the expression of *csal1* gene was knocked down, the expression of *csal3* mRNA was also noticeably downregulated (Figure 4D). These results suggested that *csal1* may mainly serve as a suppressor in the GC proliferation as well as in GC differentiation and steroidogenesis within chicken ovaries by synergistic action with the transcription factor *csal3*.

Furthermore, to delineate the suppressive effect of *csal1* gene on the GC proliferation, a BrdU cell proliferation assay was utilized to determine the cell proliferation rate. BrdU is a nucleoside analog of thymidine, which incorporates into newly synthesized DNA in proliferating cells during the synthesis of DNA [38]. As shown in Figure 4E and F, a significant enhancement of GCs proliferation was observed by knocking down of the *csal1* gene ($P < 0.01$) compared to the negative control.

Conformity of the suppressive roles of *csal1* on granulosa cell proliferation, differentiation, and steroidogenesis

To further confirm the exact role of *csal1* gene in the follicle development as evaluated by *csal1* knockdown, overexpression of *csal1* gene was carried out by transfecting the reconstructed vector pYr-adshuttle-4-*csal1* into the GCs. The expression of mRNA and protein were determined by RT-qPCR and western blot analysis, respectively. As shown in Figure 5A and B, the expression of *csal1*

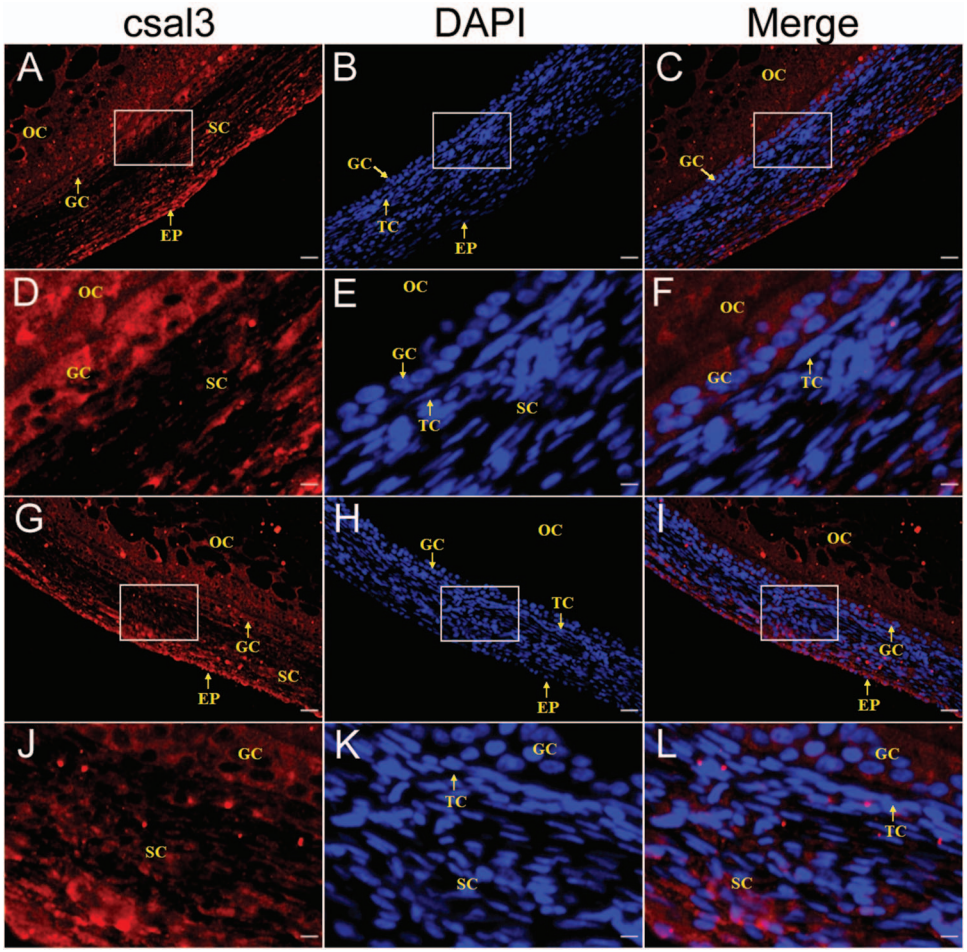

Figure 2. Immunofluorescence localization of *csal3* in the prehierarchichal follicles of the chicken ovary. Paraformaldehyde-fixed tissue sections were probed with anti-chicken *csal3*. The positive *csal3* signal was detected as red staining. Intense immunofluorescence staining was detected in the GCs, oocytes, and in the ovarian stroma and epithelium in the various-sized prehierarchichal follicles (A, D, G, and J). Blue staining represents artificial coloring of the nuclei with DAPI staining (B, E, H, and K). Scale bars, 20 μm (A, B, C, G, H, and I); 5 μm (D, E, F, J, K, and L). (A)–(F) were from the PF of 1–3.9 mm in diameter, (G)–(L) were from the PF of 5–5.9 mm in diameter. Oocyte (OC), GC, theca cell (TC), somatic cells (SC), and epithelium (EP) are indicated.

mRNA and protein was remarkably elevated in the cells at 24 h of post-transfection with an expression vector ($P < 0.01$). Under the stimulation of the overexpressed *csal1*, expression of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* mRNA was significantly decreased ($P < 0.01$) (Figure 5C). Moreover, the cell proliferation rate of the GCs is dramatically decreased in the overexpressed *csal1* group compared to the negative control ($P < 0.01$, Figure 5E and F). These results consolidated that *csal1* plays an inhibitory role in GC proliferation, differentiation, and steroidogenesis during the PF growth and development in vitro. Unexpectedly, the expression level of *csal3* mRNA was significantly upregulated simultaneously as *csal1* was overexpressed (Figure 5D). It was inferred that transcription factor *csal1* acts synergistically with a *csal3* molecule in the follicle development.

The similar effect of *csal3* on granulosa cell proliferation and the expression of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* genes to *csal1* in the GCs

To explore the functions of *csal3* gene in ovarian follicular development, siRNA-mediated knockdown of *csal3* gene was conducted by transfection of *csal3*-specific siRNA into the GCs from the PFs as shown in Figure 6A and B. Knockdown of *csal3* in the GCs was performed to investigate how *csal*3 affects the expression of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR*. Interestingly, the expression levels of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* mRNA were remarkably enhanced ($P < 0.01$, Figure 6C). As the expression of *csal3* was knocked down, the expression of *csal1* mRNA was also significantly decreased (Figure 6D). Moreover, knockdown of *csal3* significantly increased the GC proliferation ($P < 0.01$, Figure 6E and F). The current results are coincidently consistent with the data of knockdown *csal1* in the GCs. Furthermore, silencing of *csal3* in the GCs also significantly downregulated the expression of *csal1*.

Conformity of the suppressive roles of *csal3* on granulosa cell proliferation, differentiation, and steroidogenesis to those of *csal1*

To further testify the suppressive role of transcription factor *csal3* on granulosa cell proliferation, differentiation, and steroidogenesis, transfection of the recombined expression vector pYr-adshuttle-4-*csal3* into the GCs was performed (Figure 7A and B). The expression levels of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* mRNA were

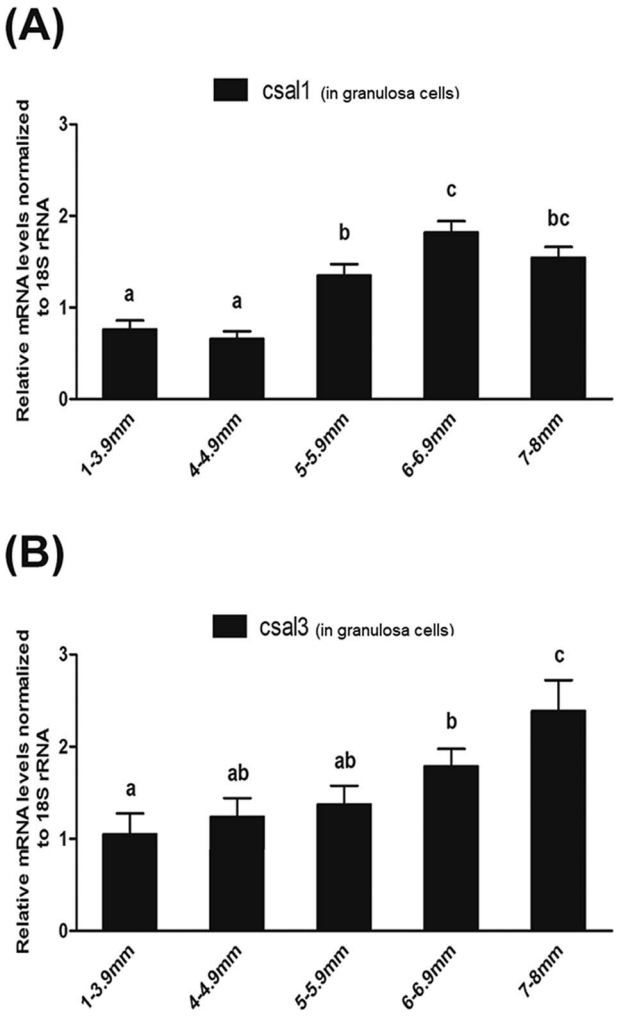

Figure 3. Expression of *csal1* and *csal3* genes in GCs of the PF. The expression of *csal1* (A) and *csal3* (B) was analyzed using RT-qPCR, and values were normalized to the *18S rRNA*. The bar graphs show the mean ± SD. Data labeled with different letters are significantly different from each other ($P < 0.05$).

remarkably decreased under the overexpression of *csal3* (Figure 7C). This result is coincidently consistent with the data of overexpressed *csal1* in the GCs. Furthermore, when overexpression of *csal3* was conducted in the GCs, the expression level of *csal1* was also significantly upregulated (Figure 7D). Moreover, the cell proliferation rate was remarkably reduced ($P < 0.01$) in the *csal3* overexpressed group compared to the negative control (Figure 7E and F), which is concordant with that of *csal1* overexpression. Taken together, these results provided strong evidence that *csal3* plays an inhibitory role in GC proliferation in vitro through a synergistic action with the *csal1*.

Synergistic effect of *csal1* and *casl3* on granulosa cell proliferation, differentiation, and steroidogenesis

To further substantiate the synergistic effect of *csal1* and casl3 on granulosa cell proliferation, differentiation, and steroidogenesis, knockdown or overexpression of *csal1* and *csal3* in the cultured GCs was conducted. The cells transfected with *csal1*- and *csal3*-specific siRNAs had significantly increased expression of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* mRNA, and GC proliferation (Figure 8A–F). Conversely, after the stimulation by transfecting the reconstructed expression vectors of pYr-adshuttle-4-*csal1* and pYr-adshuttle-4-*csal3* into the GCs, the expression of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* mRNA, and the proliferation level of GCs were more remarkably downregulated than the groups transfected only with the pYr-adshuttle-4-*csal1* or pYr-adshuttle-4-*csal3* vector (Figure 8G–L). Collectively, these results further corroborated that *csal1* and *csal3* play a synergistic inhibitory effects on GC proliferation, differentiation, and steroidogenesis in PF growth and development.

Discussion

In recent years, an increasing number of studies have investigated the factors that control follicular development and selection of undifferentiated PFs into the differentiated preovulatory follicle hierarchy [4, 9, 10, 13]. The biological roles of members of the SALL family in ovarian development have attracted much attention [17, 18, 30]. Previous studies have well-documented that members of the SALL family involve in many developmental processes, such as embryonic development, organogenesis, and tissue specification [16, 20, 39–41]. Moreover, the transcription factors SALL1 and SALL3 are involved in the human ovarian cancer suppression [17, 18, 42]. However, the exact roles of *csal1* and *csal3* in chicken ovarian follicular development remained unclear. In this study, we initially investigated the expression profiles of chicken *csal1* and *csal3* mRNA in the ovarian PFs (1–3.9 to 7–8 mm in diameters) by using qPCR analysis. Although mRNA expression patterns of *csal1*gene appeared to be different from the *csal3*, the gradually increasing high expression level of *csal1* and *csal3* mRNA in the follicles from 1–3.9 up to 7–8 mm in diameters indicated that *csal1* and *csal3* exert an indispensable role in the regulation of chicken PF growth and development. Furthermore, *csal1* and *csal3* proteins were predominantly localized in the cytoplasm of oocytes and GCs, ovarian stroma, and epithelium (Supplemental Figures 2 and 3), suggesting that *csal1* and *csal3* may be involved in both the oocyte growth and development and the granulosa cell proliferation through a translocation mechanism in PFs of the hen ovary as previously reported [25].

To provide direct evidence for substantiating *csal1* and *csal3* proteins in the regulation of the PF development, we utilized an in vitro system to investigate the effect of *csal1* and *csal3* on cell proliferation, differentiation, follicle selection, and steroidogenesis within GCs from the PF. It is well known that ovarian GCs are essential for follicular growth, development, and follicular atresia [43]. The controlled ovarian cell proliferation is critical for the normal function of the ovary. However, uncontrolled GC proliferation and decreased cell death and differentiation can lead to hyperplasia of the granulosa layer and the formation of granulosa cell tumors [44]. However, the occurrence and alteration of GC proliferation and differentiation are subjected to the control of many intrinsic and extrinsic factors, such as positive/negative feedbacks of hormone secretion and intrafollicular growth factor production [13, 45]. As aforementioned, *csal1* and *csal3* molecules were presumed to be involved in ovarian PF growth and development by regulating GC proliferation. To date, there is no direct evidence to confirm that *csal1* and *csal3* are involved in GC proliferation and ovarian development, but it was reported that human SALL1 synergistically activates canonical Wnt signaling and the N-terminal truncated SALL1 downregulates the synergistic transcriptional enhancement for Wnt signal by native SALL1 [29]. WNT/β-catenin signaling is involved in cell proliferation and differentiation [46, 47], ovarian development

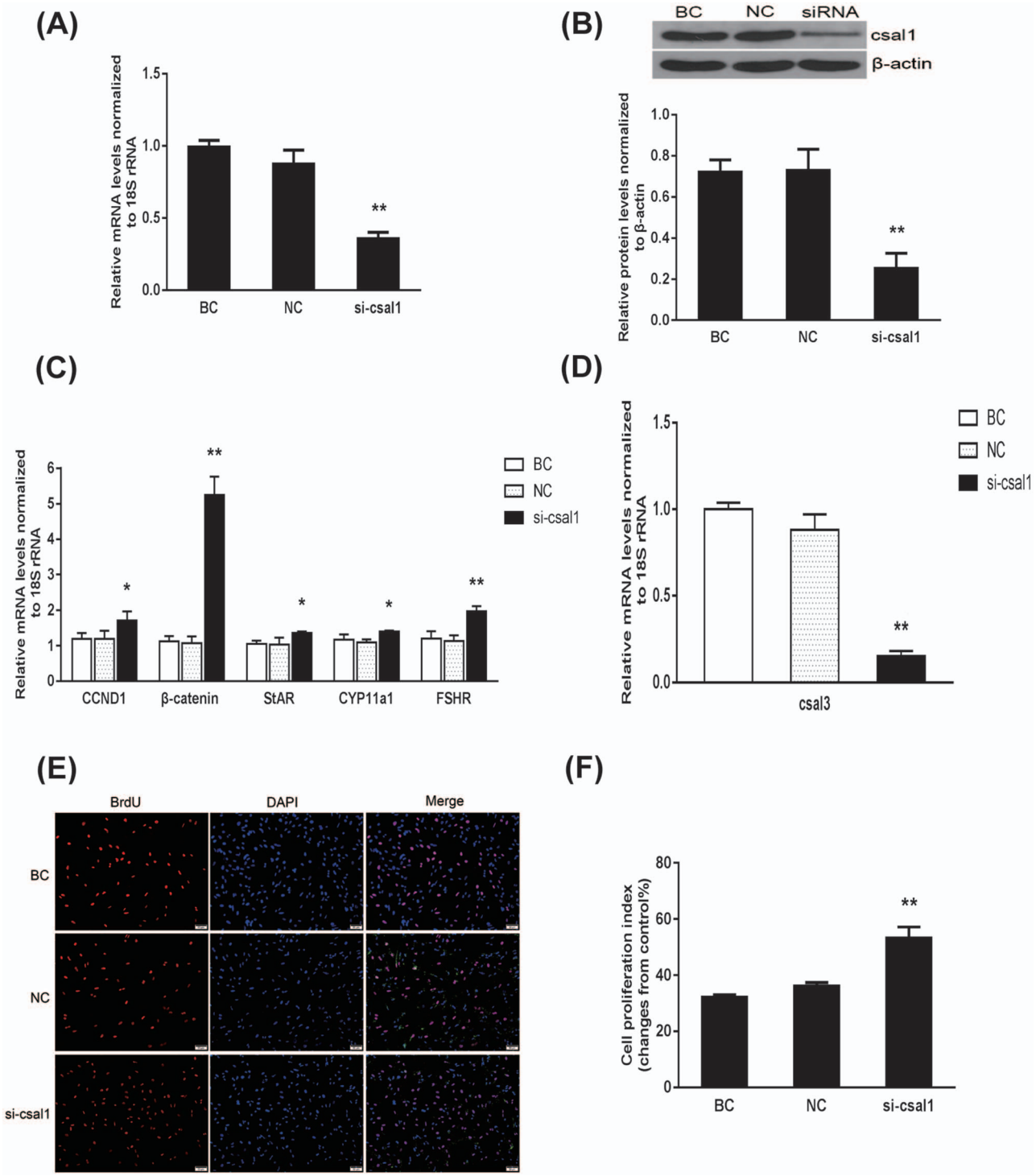

Figure 4. Effects of silencing *csal1* on the expression of *CCND1, Bcat*, *StAR, CYP11A1,* and *FSHR* mRNA and granulosa cell proliferation. GCs from the PFs (6–8 mm in diameter) were transfected with specific siRNA targeting *csal1* gene, scrambled siRNA (NC, negative control), and no siRNA (BC, blank control). (A) The expression of *csal1* gene before and after the GCs transfected with specific siRNA was analyzed using RT-qPCR. (B) Expression levels of *csal1* protein in the GCs before and after the specific siRNA interference (RNAi) were detected by western blotting. The β-actin was used as the loading control. (C) The influence of silencing *csal1* on *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* mRNA abundances in the GCs was determined. (D) The influence of silencing *csal1* on *csal3* mRNA abundances in the GCs was examined. (E) Chicken GCs were transfected with *csal1*-specific siRNA, scrambled siRNA (NC, negative control), and absence of any siRNA (BC, blank control). The effects of *csal1* knockdown on the GC proliferation were detected by BrdU assay. All cell nuclei show blue fluorescence indicative of DAPI staining; the BrdU-labeled cells showed red fluorescence indicating their newly synthesized DNA (×200). (F) Quantification of granulosa cell proliferation rate after cells transfected with the *csal1* specific siRNA. Values are presented as mean ± SD. Asterisk indicates that the values are significantly different at $^{**}P < 0.01$, 1, $^{*}P < 0.05$.

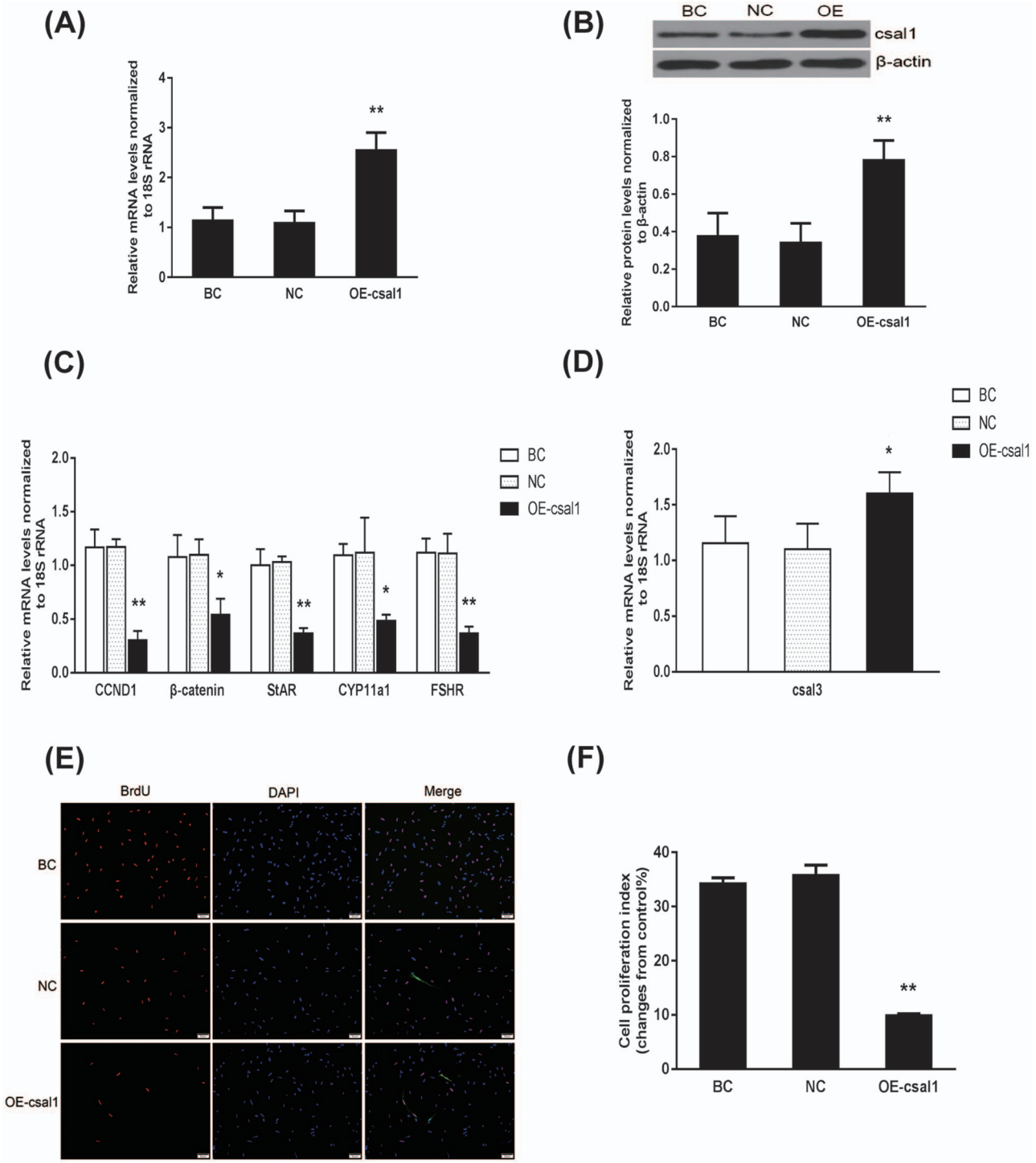

Figure 5. Repression of *csal1* on granulosa cell proliferation and the expression of *CCND1*, *Bcat*, *StAR*, *CYP11A1,* and *FSHR* genes in GCs. The GCs were transfected with reconstructed pYr-adshuttle-4-*csal1* plasmids (OE, overexpression group), pYr-adshuttle empty vector (NC, negative control), and no plasmid (BC, blank control). (A) The expression of *csal1* gene before and after the GCs transfected with pYr-adshuttle-4-*csal1* expression vector was examined by RT-qPCR. The values on the bar graphs are the mean ± SD. (B) Expression levels of *csal1* protein in the GCs before and after the transfection with pYr-adshuttle-4-*csal1* vector were detected by western blotting. The β-*actin* was used as the loading control. (C) The influence of *csal1* overexpression on *CCND1, Bcat*, *StAR*, *CYP11A1,* and *FSHR* mRNA abundances in the GCs was examined. (D) The influence of *csal1* overexpression on *csal3* mRNA abundances in the GCs was examined. (E) The effects of *csal1* overexpression on the GC proliferation were detected by BrdU assay. All cell nuclei show blue fluorescence indicative of DAPI staining; the BrdU-labeled cells showed red fluorescence indicating their newly synthesized DNA (×200). (F) Quantification of granulosa cell proliferation rate after cells transfected with the pYr-adshuttle-4-*csal1* plasmid. Asterisk indicates that the values are significantly different at $^{**}P < 0.01$, $^{*}P < 0.05$.

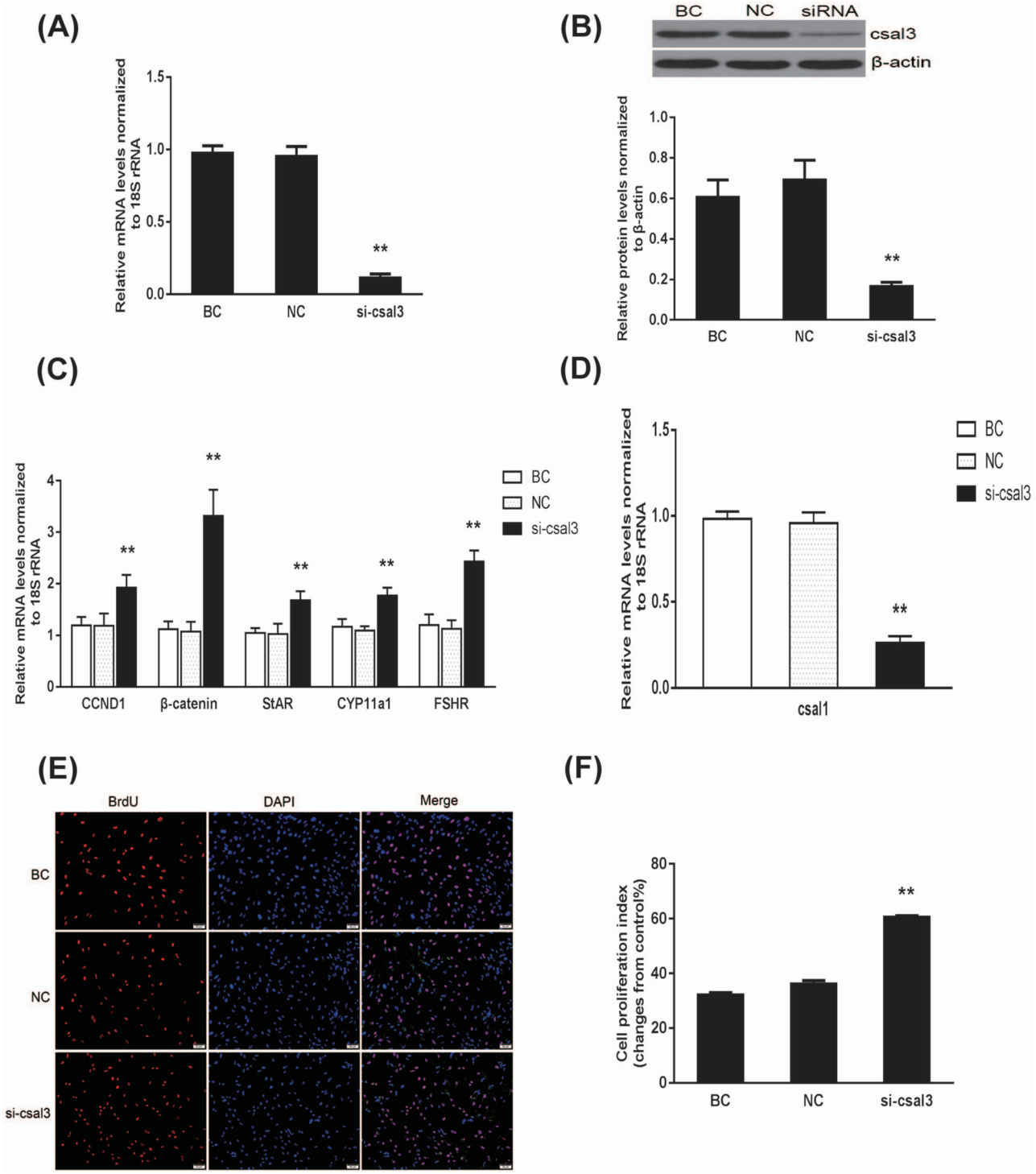

Figure 6. Effects of silencing *csal3* on granulosa cell proliferation and the expression of *CCND1, Bcat*, *StAR, CYP11A1,* and *FSHR* mRNA. The GCs were transfected with specific siRNA targeting *csal3* gene, scrambled siRNA (NC, negative control), and no siRNA (BC, blank control). (A) The expression of *csal3* gene before and after the GCs transfected with specific siRNA was analyzed using RT-qPCR. The values on the bar graphs are the mean ± SD. (B) Expression levels of *csal3* protein in the GCs before and after the specific siRNA interference (RNAi) were detected by western blotting. The β-actin was used as the loading control. (C) The influence of silencing *csal3* on *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* mRNA abundances in the GCs was examined. (D) The influence of silencing *csal3* on *csal1* mRNA abundances in the GCs was examined. (E) The effects of *csal3* knockdown on the GC proliferation were detected by BrdU assay. All cell nuclei show blue fluorescence indicative of DAPI staining; the BrdU-labeled cells showed red fluorescence indicating their newly synthesized DNA (×200). (F) Quantification of granulosa cell proliferation rate after cells transfected with the *csal3*-specific siRNA. Asterisk indicates that the values are significantly different at $^{**}P < 0.01$, $^{*}P < 0.05$.

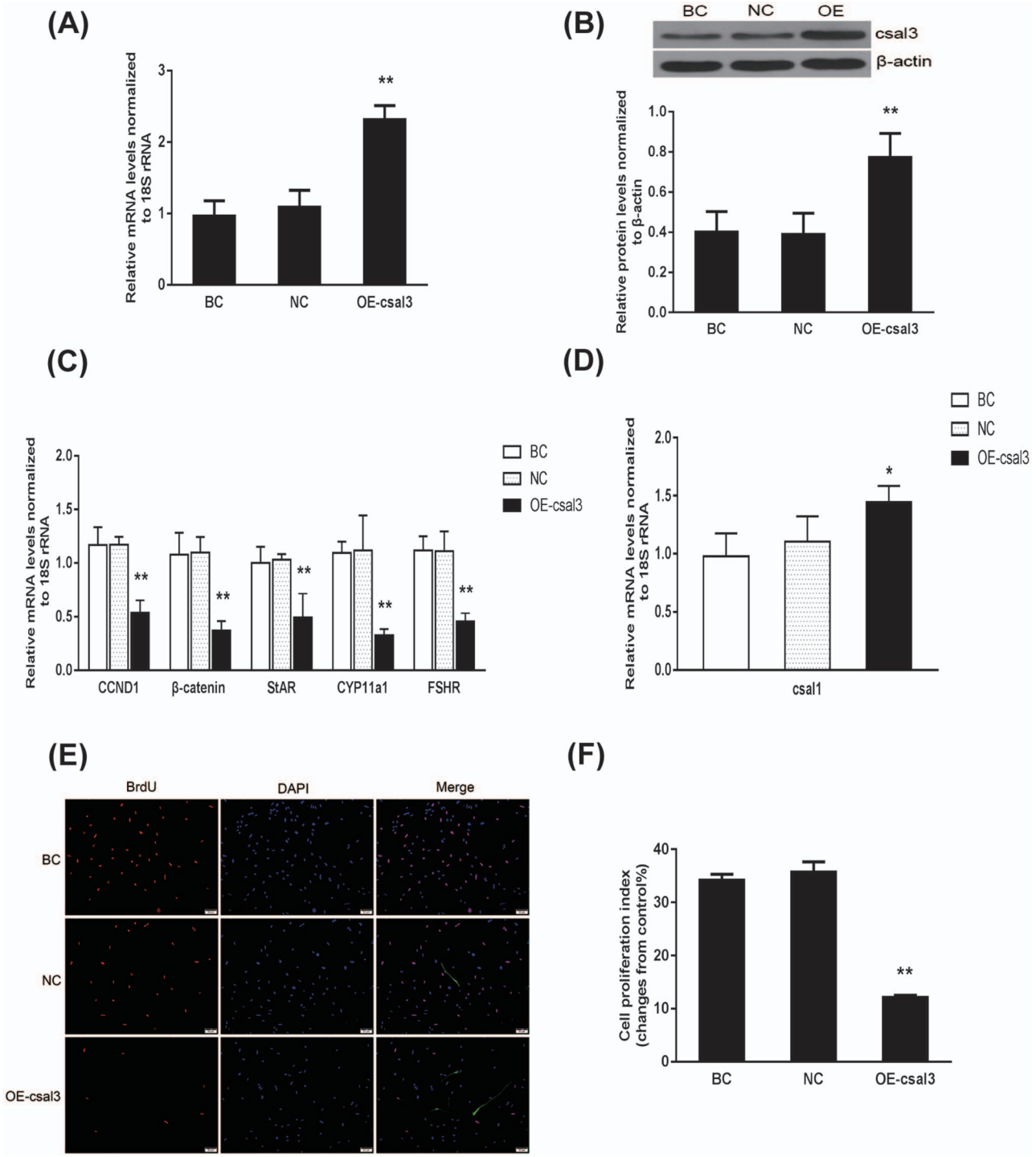

Figure 7. The inhibitory effect of *csal3* on granulosa cell proliferation and the expression of *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR*. The GCs were transfected with reconstructed pYr-adshuttle-4-*csal3* plasmids (OE, overexpression group), pYr-adshuttle-4 empty vector (NC, negative control), and no plasmid (BC, blank control). (A) The expression of *csal3* gene before and after the GCs transfected with pYr-adshuttle-4-*csal3* expression vector was examined by RT-qPCR. The values on the bar graphs are the mean ± SD. (B) Expression levels of *csal3* protein in the GCs before and after the transfection with pYr-adshuttle-4-*csal3* vector were detected by western blotting. The β-actin was used as the loading control. (C) The influence of *csal3* overexpression on *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* mRNA abundances in the GCs was examined. (D) The influence of *csal3* overexpression on *csal1* mRNA abundances in the GCs was examined. (E) All cell nuclei show blue fluorescence indicative of DAPI staining; the BrdU-labeled cells showed red fluorescence indicating their newly synthesized DNA (original magnification ×200). (F) Quantification of granulosa cell proliferation rate after cells transfected with the pYr-adshuttle-4-*csal3* plasmid. Asterisk indicates that the values are significantly different at $^{**}P < 0.01$, $^{*}P < 0.05$.

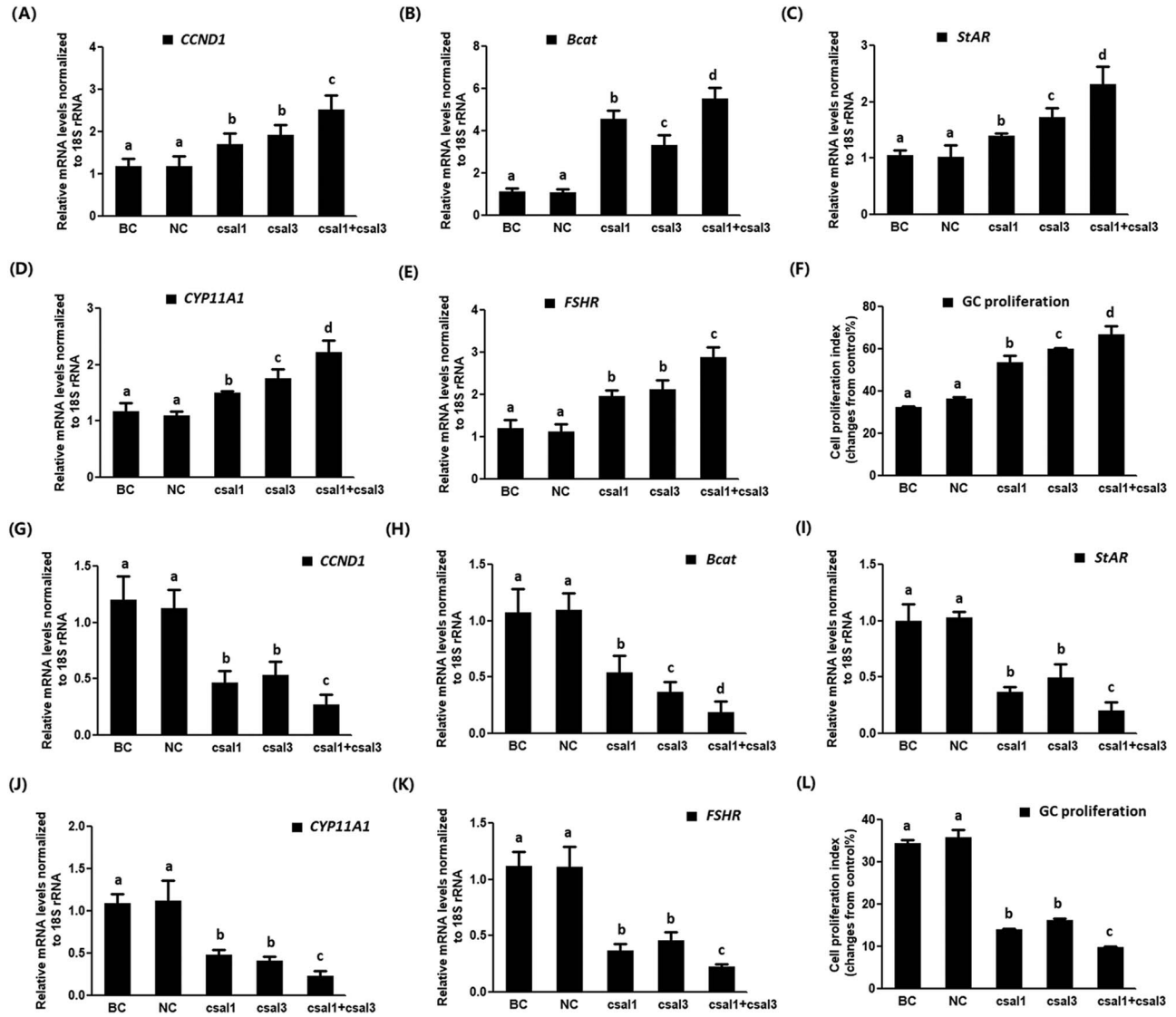

Figure 8. Synergistic inhibition of *csal1* and *csal3* in granulosa cell proliferation and the expression of *CCND1*, *Bcat*, *StAR*, *CYP11A1,* and *FSHR*. The GCs were transfected with specific siRNA targeting *csal1* or/and *csal3* genes, scrambled siRNA (NC, negative control), and no siRNA (BC, blank control) (A–F). The GCs were transfected with reconstructed pYr-adshuttle-4-*csal1* or/and pYr-adshuttle-4-*csal3* plasmids, pYr-adshuttle-4 empty vector (NC, negative control), and no plasmid (BC, blank control) (G–L). Values are presented as mean ± SD. Data labeled with different letters are significantly different from each other ($P < 0.01$).

[48], and ovarian granulosa cell tumor development [49]. SALL1 expression in human and murine breast cancer cells inhibited cancer cell growth and proliferation, whereas knockdown of SALL1 in breast cancer cells promoted cancer cell growth and proliferation [42]. Moreover, SALL2 can suppress tumorigenesis through cell cycle inhibition and induction of apoptosis [50], and the latest study proved that SALL2 is a negative regulator of cell proliferation [19]. In the current study, we found that overexpression of *csal1*/*csal3* gene in the GCs contributed to a significant decrease in cell proliferation ($P < 0.01$). Conversely, knockdown of *csal1*/*csal3* by RNAi resulted in a remarkable increase in cell proliferation ($P < 0.01$). These data attested that *csal1* and *csal3* genes exert an inhibitory effect on the development of the PFs. To our knowledge, this is the first direct evidence showing the biological roles of the *csal1* and *csal3* factors in the GC proliferation during growth and development of the PF in the chicken ovary. However, the molecular mechanism that underlies the negative regulation of *csal1* and *csal3* genes in the GC proliferation is unknown.

It has been reported that GC proliferation is highly regulated by reproductive hormones and growth factors in an endocrine, paracrine, and autocrine manner [9, 45, 51]. Therefore, endocrinal hormones, local ovarian-follicular growth factors, and signaling pathways involved in the regulation of normal ovarian granulosa cell functions may also affect the ovarian folliculogenesis, growth, and development. *CCND1* is one of the important cell cycle regulators associated with cell cycle arrest [5, 6, 52]. The decreased expression levels of *CCND1* have been demonstrated to inhibit cell proliferation, whereas the enhanced levels of *CCND1* promote cell proliferation and tumorigenesis; therefore, *CCND1* was accepted as a key regulator of cell cycle and proliferation [6]. Inhibition on *CCND1* is bound to induce cell cycle arrest and further contribute to the inhibited proliferation of the human granulosa-like tumor

cells [53]. Owing to the promotion of *CCND1* and β-catenin to cell proliferation [6, 8, 49, 53], they are known to be a critical enhancer or activator for follicular growth and development in the ovary. Furthermore, it has been reported that SALL1 and SALL3 were able to directly interplay with the *CCND1* and β-catenin proteins, respectively [41]. In the present study, the abundance of *CCND1* and *Bcat* mRNA was significantly decreased in the cultured GCs with overexpression of *csal1*/*csal3* and significantly increased under the knockdown of *csal1*/*csal3*. It initially revealed that upregulated *csal1* and *csal3* resulted in the inhibition of *CCND1* and *Bcat* mRNA expression in the cultured GCs and with a synchronous decrease of GC proliferation level in vitro. It indicated that transcription factor *csal1* and *csal3* might primarily play an inhibitory effect on the ovarian GC proliferation by downregulating *CCND1* and *Bcat* expression. Furthermore, this hypothesis is also strongly supported by the decreased expression levels of *csal1* and *csal3* in the GCs once the PFs entering the hierarchy stage (Supplemental Figure 4), in which the downregulated *csal1* and *csal3* expressions facilitate GC to proliferate further and to relieve the inhibition of *CCND1* and *Bcat* expression simultaneously.

It is well known that *FSHR* plays a decisive role in the avian follicular selection of GCs by enhancing FSH-responsiveness [3,54,55] and a relatively higher mRNA expression level of *FSHR* was evaluated in the GC layer from the 6–8 mm follicles for selection [15]. *FSHR* knockdown induces porcine GC apoptosis and follicular atresia [56]. An increased expression level of *FSHR* transcription in the GCs of PFs (6–8 mm in diameter) has been generally accepted as an indicator to initiate follicle selection [3, 4, 10]. Furthermore, at or immediately after follicle selection, the transition of GCs from an undifferentiated to a differentiated state was initiated, and increased expression of CYP11A and *StAR* plus progesterone production was accompanied [45, 57]. Therefore, the elevated expression of *FSHR* mRNA is also one of the earliest markers for differentiating GCs [45].

Granulosa cell proliferation and differentiation have been reported as a crucial cellular process during ovarian follicle growth and development after follicle selection [45]. *StAR* serves as a key molecule in steroidogenesis and a marker of granulosa cell differentiation [58]. *StAR* immunoreactivity is detected in GCs of large preovulatory follicles, but not in small and medium immature follicles [58, 59]. The steroidogenesis (predominantly progesterone production) was mediated by increased expression of mRNA encoding *StAR* and CYP11A [60]. *CYP11A1* gene encodes the P450scc enzyme that is the first and rate-limiting enzyme in the steroidogenic pathway, converting cholesterol to pregnenolone [61]. In the current study, overexpression of *csal1*/*csal3* in GCs significantly decreased the expression of *FSHR*, CYP*11A1*, and *StAR* mRNA, whereas knocking down *csal1*/*csal3* enhanced their expression. These data suggested that *csal1* and *csal3* genes might exert a suppressing role in follicle selection, GC differentiation, and steroidogenesis in the ovarian PFs.

Interestingly, we also found that when *csal1* was overexpressed, the abundance of *csal3* mRNA was synchronously elevated. On the contrary, when *csal1* was knocked down, the abundance of *csal3* mRNA was simultaneously downregulated. It presented the synergistic transcriptional enhancement or reduction for *csal1*and *csal3* genes between each other in GCs under the overexpression or RNA interference (RNAi) state. Moreover, either *csal1*or *csal3* overexpression has significantly decreased the abundance of *CCND1*, *Bcat*, *StAR*, *CYP11A1,* and *FSHR* mRNA in the follicular GCs and the proliferation levels of GCs from the PFs. Moreover, simultaneously silencing *csal1*and *csal3* leads to a much higher increase of the abundance of *CCND1*, *Bcat*, *StAR*, *CYP11A1,* and *FSHR* mRNA expression and the GC proliferation level than that in knockdown of *csal1* or *csal3* exclusively. In contrast, overexpression of both *csal1*and *csal3* contributes to a more significant decrease of the *CCND1*, *Bcat*, *StAR*, *CYP11A1,* and *FSHR* mRNA expression and the GC proliferation levels than that in overexpressing *csal1* or *csal3* singly. These data demonstrated the synergistic functional role between *csal1* and *csal3* genes on the granulosa cell proliferation, differentiation, and steroidogenesis during PF development in hen ovary. Further studies are necessary to elucidate the precise regulatory mechanisms, and whether *csal1*and *csal3* have similar functions in the ovary of older hens.

Conclusions

In sum, our study for the first time demonstrates the suppressive role of the transcription factors *csal1* and *csal3* in GC proliferation and steroidogenesis by downregulating their downstream genes: *CCND1*, *Bcat*, *StAR*, *CYP11A1*, and *FSHR* and moreover, the molecular mechanisms underlying the synergistic inhibitory effects induced by the *csal1* and *csal3* on GC proliferation, differentiation, and steroidogenesis during PF development in the hen ovary. Our ongoing study will delineate the precise regulation of *csal1* and *csal3* genes in PF development.

Conflict of interest

The authors have declared that no conflict of interest exists.

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