Research Article

Synergistic inhibition of csa11 and csa13 in granulosa cell proliferation and steroidogenesis of hen ovarian prehierarchical development†

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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 csa11 and csa13 in granulosa cell proliferation, differentiation, and follicle selection within the prehierarchical follicles of hen ovary. Our data demonstrated that the csa11 and csa13 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 csa11 and csa13 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 csa11 or/and csa13. Conversely, the overexpression of csa11 or/and csa13 in the granulosa cells led to a remarkably decreased of them. Moreover, csa11 and csa13 together exert a much stronger effect on the regulation than any of csa11 or csa13. These results indicated that csa11 and csa13 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 csa11 and csa13 in controlling the prehierarchical follicle development and growth of hen ovary in vivo.
Summary Sentence
The transcription factors csa1 and csa3 might play a synergistic inhibitory role on granulosa cell proliferation, differentiation, and steroidogenesis during PF development.

Key words: csa1, csa3, 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 prevoluntary 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 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, family 11, subfamily A, polypeptide 1 (CYP11A1) have been proved to promote the growth and cell differentiation of GCs in chicken hierarchical 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 (FSHRe), 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 (sall1), 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, csa1 (also named SALL1), csa3 (SALL3), and csa4 (SALL4) [24, 25, 27]. Expression of csa1 is predominantly detected in the developing heart, mesoderm, ectoderm, and neural tube of the early embryo [24, 25], whereas csa3 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 csa1 and csa3 in regulating the ovarian follicular development in chicken, we examined the expression profile of csa1 and csa3 in PFs. Furthermore, the effects of csa1 and csa3 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 diameter) 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 experiments 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 csa1 and csa3 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, deparaffinized 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% CO2 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 csa1, csa3, 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\(^\text{TM}\) (TaKaRa, DRR081A) and 7300 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA), according to the manufacturer’s instructions. Results were determined quantitatively in real-time by relative quantitation of two standard curves. Using the 2\(^{-\Delta\Delta C_{T}}\) 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 csa1 and csa3 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: csa1 (1:500, Abcam, Cambridge, MA, USA), csa3 (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).

Immuno-reactivity 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 csa1 and csa3 cDNA sequences (NM_204707.1 and NM_204647.1) were amplified by specific primers (Table 2) for csa1 and csa3 genes from a chicken cDNA library by PCR, and cloned into a pUC57-Simple plasmid (Sangon Biotech Co., Ltd., Shanghai, China). After the recombinated plasmids were identified by restriction enzyme (EcoRI and PmeI, TaKaRa, Dalian, China) digestion assay (Supplemental Figure 1), sequencing of the targeted fragments was performed. Then, the csa1 and csa3 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 csa1 and csa3 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% CO2 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 csa1 and csa3 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 csa1- and csa3-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 cultivated 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 csa1 or csa3 expression briefly after cell culture in a CO2-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
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 prehierarchival 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 diameter.
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, and H); 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 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

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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 csal3 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
The 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).

Synergistic effect of *csal1* and *csal3* on granulosa cell proliferation, differentiation, and steroidogenesis

To further substantiate the synergistic effect of *csal1* and *csal3* 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 *GCs was conducted. The cells transfected with *csal1*- and *csal3*-specific siRNAs had significantly increased expression of *GC* proliferation in vitro through a synergistic action with the results provided strong evidence that To further substantiate the synergistic effect of *csal1* and *csal3*, the expression level of *GC* proliferation 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, *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).

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 interact 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 csall/csal3 and significantly increased under the knockdown of csal1/csal3. It initially revealed that upregulated csall 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 csall 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 csall 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 [13]. 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 CYP11A1 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 CYP11A1 [60]. CYP11A1 gene encodes the P450sc enzyme that is the first and rate-limiting enzyme in the steroidogenic pathway, converting cholesterol to pregnenolone [61]. In the current study, overexpression of csall/csal3 in GCs significantly decreased the expression of FSHR, CYP11A1, and StAR mRNA, whereas knocking down csall1/csal3 enhanced their expression. These data suggested that csall1 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 csall1 was overexpressed, the abundance of csal3 mRNA was synchronously elevated. On the contrary, when csall1 was knocked down, the abundance of csal3 mRNA was simultaneously downregulated. It presented the synergetic transcriptional enhancement or reduction for csall1 and csal3 genes between each other in GCs under the overexpression or RNA interference (RNAi) state. Moreover, either csall1 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 csall1and 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 csall1 or csal3 exclusively. In contrast, overexpression of both csall1and 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 csall1 or csal3 singly. These data demonstrated the synergistic functional role between csall1 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 csall1and 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 csall1 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 csall1 and csal3 on GC proliferation, differentiation, and steroidogenesis during PF development in the hen ovary. Our ongoing study will delineate the precise regulation of csall1 and csal3 genes in PF development.

Conflict of interest

The authors have declared that no conflict of interest exists.

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