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MiR-223 inhibits proliferation and steroid hormone synthesis of ovarian granulosa cell via the AKT signaling pathway by targeting CRIM1 in chicken

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ABSTRACT Within the poultry industry, hens' reproductive performance is of great economic significance. The development and growth of follicles is a key aspect of hen egg production, and ovarian follicle growth and development are closely associated with granulosa cells (**GCs**) proliferation and the synthesis of steroid hormones. It has been confirmed by numerous studies that microRNAs (**miRNAs**) play important roles in the steroid hormone synthesis and proliferation of GCs. In this study, we examined the main miRNAs influencing hens' ability to reproduce, identified the miR-223 that is mainly expressed in atretic follicles based on sequencing, and investigated its role in GCs. Then, we used miR-223 mimic and inhibitor to knockdown or overexpress miR-223 expression. The result showed that miR-223 significantly inhibits both the steroid hormone synthesis and the proliferation of GCs. Subsequently, the results of the dual luciferase reporter experiment and bioinformatics prediction demonstrated that cysteine rich transmembrane BMP regulator 1 (**CRIM1**) was a downstream target gene of miR-223, and overexpression of miR-223 prevented CRIM1 expression. The function of CRIM1 was further investigated, and we observed a significant reduction in the synthesis of steroid hormones and the proliferation of GCs after transfection with CRIM1 siRNA. The opposite function of miR-223 was observed for CRIM1 in our study. Additionally, we demonstrated the involvement of the miR-223/CRIM1 axis in GCs through modulation of the AKT signaling pathway. Our data demonstrate the pivotal role of the miR-223 in the proliferation and steroid hormone synthesis of chicken GCs, which helps to explain how non-coding RNA (ncRNA) affects chicken reproductive function.

Key words: miR-223, CRIM1, proliferation, steroid hormone synthesis, AKT signaling pathway

2024 Poultry Science 103:103910
https://doi.org/10.1016/j.psj.2024.103910

INTRODUCTION

The production efficiency of animal husbandry mainly depends on the animals' reproductive capacities, which are largely determined by the development of the animals' ovaries and follicles. Usually, animals with well-developed follicles have higher ovulation rates. Egg production is an important evaluation of poultry performance and is strongly related to development of follicles. The development and selection of poultry follicles determines the number of mature follicles. However, in most cases of follicle development, 99% of unselected follicles undergo follicular atresia (Matsuda, et al., 2012). Numerous studies have demonstrated that the ovary's steroid hormone synthesis and GC proliferation can be used as biomarkers of follicular atresia.

GC is the primary source of Estrogen and Progesterone (**P4**), and they can regulate follicle development by secreting cytokines, and further affect the reproductive performance of animals (Pangas and Matzuk, 2004). Steroid hormones, including estradiol (**E2)** and P4, play important regulatory role in the ovary through specific receptors, including the normal development of follicles and the maturation of oocytes (Drummond, 2006). In bovine GCs, high secretion of E2 before ovulation promotes ovulation and after ovulation, the secretion level of P4 increases, promoting the transformation of GC and ruptured follicles into the luteal phase (Zhang et al., 2019). The synthesis of steroid hormones involves various enzymes and proteins. Cholesterol is first converted into pregnenolone by the Cholesterol side chain cleavage enzyme, and pregnenolone is then converted into estradiol in the ovary by cytochrome P450 aromatase.

Received March 27, 2024.
Accepted May 23, 2024.

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Additionally, P4 can enter the mitochondrial inner membrane, mediated by Steroidogenic acute regulatory protein (**STAR**), and be converted into progesterone through the catalysis of 3β-hydroxysteroid Dehydrogenase (**3βHSD**) (Hu, et al., 2023). Besides hormone regulation, the maturation of follicle development is accompanied by changes in follicle morphology, with follicles gradually developing into primary, secondary, luminal, and preovulatory follicles. At the same time, GC transition from a monolayer to a multilayer cubic structure (Fortune, 1994). These findings collectively highlight the crucial role of steroid hormone secretion and proliferation in GC in chicken follicle development. miRNA is a ncRNA with a length of 20-24nt, which binds to the 3′UTR region of the target gene to inhibit the translation of target gene proteins and promote the degradation of target gene mRNA. Many studies have found that miRNA affects the function of GC by targeting its target gene, such as miR-30a-5p inhibits GC death via targeting Beclin1 (He, et al., 2022), miR-27b-3p inhibits E2 secretion of goose GC by targeting cytochrome P450 family 1 subfamily B member 1 (**CYP1B1**) (Hu, et al., 2023) and miR-130b-3p promotes GC proliferation by targeting SMAD family member 4 (**SMAD4**) (Bao, et al., 2021). Therefore, miRNAs are very important for follicle growth and development, but the role of a large number of miRNAs in follicle development remains unclear.

In our previous sequencing data found that miR-223 was highest expression in atretic follicles than normal follicles (He, et al., 2022). Therefore, the results of this study provide new insights into the mechanism by which miR-223 regulates the biological function of GCs, further evidence for the effect of miRNAs on follicular development in hens, and also provided a regulatory molecular marker for improving the reproductive ability of poultry.

MATERIALS AND METHODS

Ethics Statement and Animals

Thirty 200-day-old Tianfu laying hens were selected as the experimental animals for this investigation. Thirty 200-day-old laying hens broilers were euthanized with cervical vertebrae dislocation. The Animal Welfare Committee at Sichuan Agricultural University granted ethical approval for all animal experimentation. (Approval Number: 2023202025).

Granulosa Cells Isolation and Culture

Initially, the follicles (largest yellow preovulatory F3-F1 follicles) were removed from the ovaries and the blood membrane on the surface of the follicles was removed. The follicles were then placed in PBS (Hyclone, Logan, UT) to shake off the GC after a small knife incision was made on one side. 0.1% Type II collagenase (BioFroxx, Einhausen, Germany) was then utilized for 10 minutes at 37°C to digest all of the GCs. These tissue pieces were then passed through a 70 μm screen filter. The cells were subsequently harvested by centrifugation at 1500 × g and then introduced into the fresh medium. Subsequently, the cells were cultured at 37°C and 5% CO_2 in a culture plate with 10% Fetal Bovine Serum (**FBS**) (Gibco, Grand Island, NY) and 1% double antibody mediums (Solarbio, Beijing, China).

Plasmids Construction and Transfection

An hour before transfection, double antibody-free medium (F12-DMEM containing 10% FBS) was substituted for the full culture medium (F12-DMEM including 10% FBS, 1% penicillin, and 1% streptomycin). Transfection was carried out when the cell fusion reached 50% to 60%. Subsequently, following the manufacturer's instructions, the miR-223 mimic, mimic NC, miR-223 inhibitor, inhibitor NC, si-CRIM1, and siRNA NC were transfected using Opti-MEM (Gibco) and Lipofectamine 3,000 (Invitrogen, Carlsbad, CA). The miR-223 mimic, mimic NC, miR-223 inhibitor, inhibitor NC, si-CRIM1, and siRNA NC were mixed with Optim-MEM for 5 min. Reconstitute lipofectamine 3000 and incubate the mixture for 20 minutes at room temperature. Subsequently, they were individually introduced into the cell culture plates. The sequences of these constructs are listed in Table 1. Three duplicates of each treatment were set up, and the medium substituted after eight hours of transfection.

RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR

To extract GC's RNA, Trizol Reagent (Invitrogen) was utilized initially 24 hours after transfection. Subsequently, Takara PrimeScript RT reagent kit (Takara, Dalian, China) and one-step miRNA synthesis kit (Takara, Beijing, China) were utilized to synthesize cDNA and miRNA, respectively. Following that, qRT-PCR was carried out using the Takara SYBR Green Master Mix. Each sample included 3 duplicates, and the cDNA internal control gene, β-actin, was determined to be the same as the miRNA internal control gene, U6. The relative expression of the qRT-PCR data was analyzed using $2^{-\Delta\Delta Ct}$. The primer sequences are listed in Table 2.

Table 1. The oligonucleotide sequences used in the present study.

Name	Primer sequences (5'→3')
miR-223 mimic	UGUCAGUUUGUCAAAUACCCC
Mimic NC	UUGUACUACACAAAAGUACUG
miR-223 inhibitor	GGGGUAUUUGACAAACUGACA
Inhibitor NC	CAGUACUUUUGUGUAGUACAA
si-CRIM1-703	GCCUGGCUAUUUGAAUAUATT
si-CRIM1-908	GAGUGUCUCUCUGGUUUAUTT
si-CRIM1-1790	GGGUGUGUUAUCUGCAAAUTT
si-CRIM1-NC	UUCUCCGAACGUGUCACGUTT

Table 2. The primer pairs used in the present study.

Gene	Primer Sequences (5′ to 3′)	Product size, bp	Accession number
β-actin	F: GTCCACCGCAAATGCTTCTAA R: TGCGCATTTATGGGTTTTGTT	78	NM_205518.1
CYP19A1	F: ATGAGATACCAGCCAGTTG R: TGCGTCCAATGTTGAGAA	112	NM_001001761.3
CYP11A1	F: CTCTTCAGCCTCCTTGTG R: CAGTCGGATGCTCTTCAG	225	NM_001001756.1
STAR	F: TGCCATCTCCTACCAACA R: CATCTCCATCTCGCTGAAG	190	NM_204686.2
PCNA	F: AACACTCAGAGCAGAAGAC R: GCACAGGAGATGACAACA	225	NM_204170.2
CDK2	F: CCAGAACCTCCTCATCAAC R: CAGATGTCCACAGCAGTC	171	NM_001199857.1
CCND1	F: CTCCTATCAATGCCTCACA R: TCTGCTTCGTCCTCTACA	165	NM_205381.1
CCND2	F: AACTTGCTCTACGACGACC R: TTCACAGACCTCCAACATC	150	NM_204213.1
U6	F:GGGCCATGCTAATCTTCTCTGTA R:CAGGTCCAGTTTTTTTTTTTTTT	\	\

Protein Extraction and Western Bolting

Total protein was recovered from GC using the Total Protein Tissue or Cell Total Protein Extraction kit (Solarbio, Beijing, China) and the protein concentrations was measured using a Bicinchoninic acid (**BCA**) protein assay kit (BestBio, Shanghai, China) after 48-hour transfection period. Following the process of separating the proteins by SDS-polyacrylamide gel electrophoresis (**SDS-PAGE**), the proteins were subsequently transferred to PVDF membranes (Millipore, Massachusetts, USA). Experiments were then carried out on the PVDF membrane. The PVDF membrane was then used for experiments. After treating the primary antibody at 4°C for 12 h over night, the secondary antibody was incubated at 4°C for 1 h. The corresponding antibody concentrations are listed in the Table 3. Finally, adding the ECL luminescence solution (Beyotime), the protein bands were found and captured on camera using the Image Lab program (National Health Institute, Bethesda, MD). In the experiment, tubulin acts as a loading control.

Target Gene Prediction

Target genes of miR-223 were predicted from DIANA (http://diana.imis.athena-innovation.gr/), miRDB (http://mirdb.org/) and TargetScan (http://www.targetscan.org/) databases. A Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) was created with genes obtained from 3 databases to find crossovers for further study.

Table 3. Antibody dilution concentration.

Antibody	Company	Accession number	Dilution ratio
anti-CYP19A1	ABclonal (Wuhan, China)	A12684	1:800
anti-STAR	ABclonal (Wuhan, China)	A16432	1:1000
anti-β-Tubulin	Zenbio (Chengdu, China)	200608	1:5000
anti-AKT	Bioss	Bs-0115R	1:1000
anti-p-AKT	Bioss	bs-o876r	1:1000

Enzyme-Linked-Immunosorbent Serologic Assay

Following a 48 h transfection period, the medium was collected, and using the ELISA kit (MEIMIAN, Yancheng, Jiangsu) in accordance with the manufacturer's instructions, the concentrations of E2 and P4 were determined.

Flow Cytometry Cell Cycle Analysis

Collagenase type II (BioFroxx, Einhausen, Germany) was employed to digest the cells for 2 min at 37°C after the supernatant was removed 24 h after transfection. After stopping the reaction by the same volume of medium, the supernatant was aspirated into a 1.5 mL centrifuge tube and centrifuged for 10 minutes at 2,000 rpm. Ultimately, 70% ethanol was used to fix the precipitate following a PBS wash. The flow cytometry study was conducted using the BD AccuriC6 flow cytometer (BD Biosciences).

5-Ethynyl-2′-deoxyuridine Assay

Following transfecting cells in 96-well plates, EdU analyses were carried out using Cell-Light TM EdU Apollo567 In Vitro Kits (RiboBio, Guangzhou, China) in accordance with the manufacturer's method. Brief summary: each well received 50 mM EdU reagent, and it was incubated for 2 h at 37°C with 5% CO_2, and Hoechst 33342 was used as a counterstain for 30 minutes on the cell nuclei. Three at random spots were observed using an IX53 biological microscope (Olympus, Japan), and the number of stained cells was determined by Image-Pro Plus software (Media Cybernetics).

Cell Counting Kit-8

Chicken GC were seeded and transfected with an inhibitor or mimic in 96-well plates. Following transfection, 10 μL of CCK-8 solution (Meilunbio, Shanghai, China) were given to each well every 12 h. Each group repeats the same pattern 8 times during the period of 4 time points. At 450 nm, absorbance was determined with a Thermo Scientific TM Varioskan LUX (San Jose, CA).

Dual-Luciferase Reporter Assay

Dual luciferase report research was performed on chicken fibroblast cell line (DF-1 cells). We used the 3′UTR sequence of CRIM1 that we obtained from NCBI to construct reporter genes for the 2 types of wild-type and mutant Dual luciferase. Following culture on a 48-well plate, DF-1 cells were co-transfected with miR-223 mimic or mimic NC and CRIM1-WT or CRIM1-MT. Luciferase luminometric activity was detected 48 hours after the culture, in accordance with the requirements provided by the dual luciferase kit (Promega, Madison, WI).

Statistical Analysis

All data in this experiment were analyzed by SPSS 17.0 (SPSS, Inc., Chicago, IL). Each experiment had at least 3 biological replicates, and data are presented as least squares ± standard error of the mean (**SEM**). Unpaired *t*-test was used for comparison between 2 groups, and one-way ANOVA was used for comparison between multiple groups for statistical significance between groups. Significant differences were considered at $P < 0.05$ (* means $P < 0.05$; ** means $P < 0.01$).

RESULTS

The Expression Pattern of miR-223 in Chicken

In order to investigate the expression pattern of miR-223, the expression pattern of miR-223 in different chicken tissues was confirmed via qRT-PCR. The result showed that the miR-223 was highly expressed in ovary tissue (Figure 1A). Furthermore, by analyzing our previous sequencing data (He, et al., 2022), we found that the expression level of miR-223 was highest in atretic follicle. The results of qRT-PCR also showed that the expression level of miR-223 in broody follicles (**BF**) and was significantly higher than that in laying follicles (**LF**) (Figure 1B).

Effects of miR-223 on Steroid Hormone Synthesis of Chicken Follicular GCs

Firstly, miR-223 expression in chicken GC was successfully reduction and increase after treatment with miR-223 inhibitors and miR-223 mimic (Figures 2A and 2B). Furthermore, the mRNA levels of Cytochrome P450 family 19 subfamily A member 1 (CYP19A1) and steroidogenic acute regulatory protein (STAR) were significant reduction in the miR-223 mimic transfected group (Figure 2C), but knockdown of miR-223 enhanced the expression of 3 steroid hormone-related genes (Figure 2D). WB also showed similar results. A noticeable reduction of steroid hormone-related protein after overexpression of miR-223 (Figures 2E and 2F), and the content of steroid hormone-related proteins was increase after knockdown of miR-223 (Figures 2G and 2H). Moreover, ELISA experiment showed that the production and release of P4 and E2 were significant reduction after overexpression of miR-223 (Figure 2I), while the

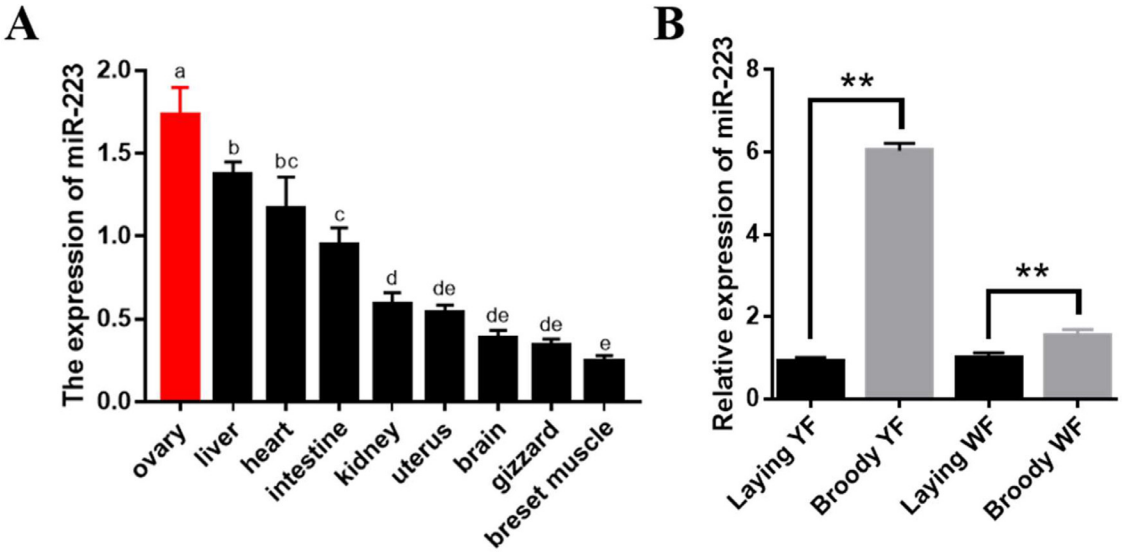

Figure 1. The expression pattern of miR-223 in chicken. (A) Tissue-specific expression of miR-223 in chicken. Bars not sharing the same letter labels are different ($p < 0.05$). (B) Expression of miR-223 in different chicken follicles. Abbreviations: YF, yellow follicle; WF, white follicle. Data were showed as Mean ± SEM, $^*P < 0.05$, $^{**}P < 0.01$.

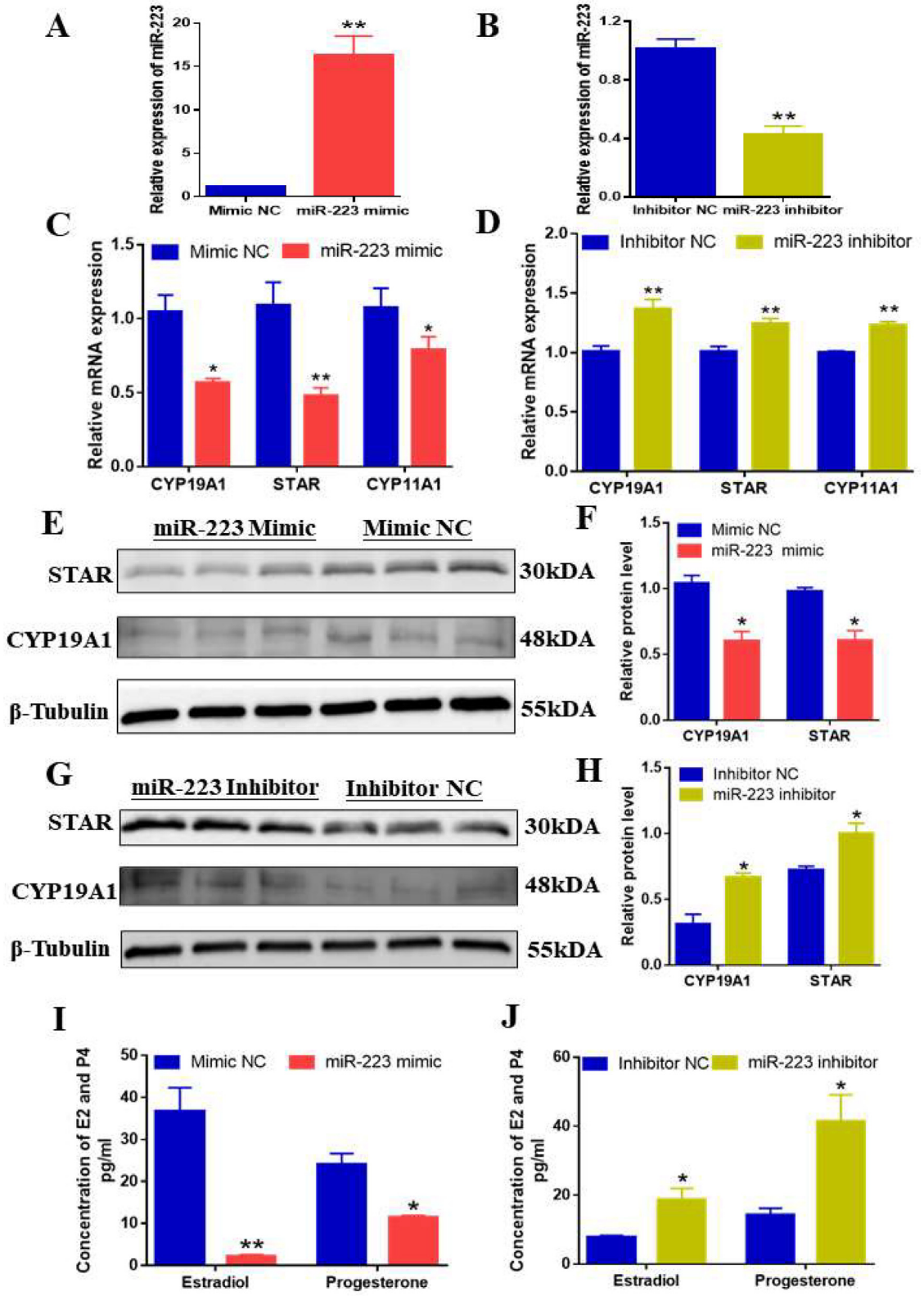

Figure 2. miR-223 inhibits steroid hormone synthesis of GCs. (A) The expression of miR-223 after overexpression of miR-223. (B) The expression of miR-223 after knockdown of miR-223. (C and D) Relative RNA expression of steroid hormone-related genes in GC after transfection with miR-223 mimic and miR-223 inhibitor. (E and F) The protein content of CYP19A1 and STAR in GC after overexpression of miR-223. (G and H) Protein content of CYP19A1 and STAR in GC after knockdown of miR-223. (I) E2 and P4 expression levels in GC after overexpression of miR-223. (J) The expression levels of E2 and P4 in GC after knockdown the miR-223. Data were showed as Mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

levels of E2 and P4 were increased after miR-223 knockdown (Figure 2J). All of these results point to the critical function of miR-223 in chicken GC hormone production.

Effects of miR-223 on Proliferation of Chicken Follicular GCs

In addition to its role in steroid hormone synthesis, we also investigated the effects of miR-223 on the proliferation of chicken GCs. qRT-PCR analysis revealed that miR-223 knockdown led to an increase in the RNA expression of Cyclin-dependent kinase 2 (**CDK2**), Cyclin D1 (**CCND1**), Cyclin D2 (**CCND2**) and Proliferating cell nuclear antigen (PCNA) (Figure 3A). However, the expression of proliferation-related genes was increase upon the transfection of miR-223 mimic (Figure 3B). In addition, the results of CCK-8 showed that the number of proliferating cells was significant reduction at 24h and 48h after the addition of exogenous miR-223 (Figure 3C), while the proliferating GC was significant increase at 36h and 48h after the knockdown of miR-223 (Figure 3D). EdU results also showed similar results, exogenous miR-223 resulted in a reduction in the number of proliferating GC (Figures 3E and 3F), while knockdown resulted in an increase in the number of proliferating GC (Figures 3G and 3H). According to flow cytometry analysis, overexpression of miR-223 inhibited GC transition from G0/G1 phase to S and G2/M phase (Figure 3I), conversely, miR-223 knockdown had the opposite result (Figure 3J). In summary, miR-223 plays an inhibitory role in the proliferation of chicken GCs.

CRIM1 is a Direct Target of miR-223 in Follicular GCs

In order to further explore the potential mechanism of miR-223 regulation of follicle development, we used TargetScan, miRDB, and DIANA to forecast the target genes of miR-233. Subsequently, there were 28 genes displayed in the intersection of Venn analysis (Figure 4A). Among the 28 genes, according to the previous sequencing data, we selected five genes (CRIM1, RPSK6KB1, EIF1, FBXW7, and RORB) whose expression trend between BF and LF was opposite to that of miR-223 for further verification. Upon the results of qRT-PCR analysis showed that knockdown of miR-223 significant increase the level of CRIM1 mRNA, while overexpression of miR-223 did the opposite (Figures 4B and 4C). To confirm this result, we constructed CRIM1-WT and MT vectors for dual luciferase reporting assay (Figure 4D). The luciferase analysis showed that exogenous miR-223 significant reduction luciferase activity in DF-1 cells transfected with CRIM1-WT, but had no effect on luciferase activity of CRIM1-MT (Figure 4E). These results suggest that miR-223 targeting CRIM1 plays a regulatory role in GCs.

Effects of CRIM1 on Steroid Hormone Synthesis of Chicken Follicular GCs

To investigate the role of the CRIM1 gene in chicken GCs, 3 siRNAs (si-703, si-908, and si-1090) were transfected into chicken GC in order to modulate the expression of CRIM1 (Figure 5A). According to qRT-PCR research, si-703 (CRIM1 siRNA) was the most effective. Following knockdown of the CRIM1, a significant decrease in the RNA expression of steroid hormone-related genes was verified by qRT-PCR experiment (Figure 5B). In addition, after CRIM1 knockdown, ELISA experiment showed significantly reduction synthesis and release of P4 and E2 (Figure 5C). This was also proved by WB results, which showed significant decrease in steroid hormone-related protein expression after transfection of si-CRIM1 (Figure 5D and 5E). These findings suggest the critical function of CRIM1 in steroid hormones synthesis in chicken GCs.

Effects of CRIM1 on Proliferation of Chicken Follicular GCs

We then investigated how CRIM1 influenced the proliferation of chicken GCs. Upon the successful knockdown of CRIM1 in GCs, a noticeable reduction in the RNA expression of cell proliferation-related genes was observed. (Figure 6A). CCK-8 also showed that the proliferation of GCs were reduction at 24h and 48h after CRIM1 knockdown (Figure 6B). Subsequently, flow cytometry analysis showed that CRIM1 knockdown hindered GC transition from G0/G1 phase to S and G2/M (Figure 6C). This was also demonstrated by EdU experiment, when CRIM1 was knockdown, led to a reduction in the number of proliferating GCs (Figures 6D and 6E). Together, these findings highlight CRIM1 key role in the proliferation of GC in chickens.

miR-223 May Mediates the AKT Signaling Pathway by Targeting CRIM1

In order to evaluate that miR-223 preformed regulatory role through mediating the AKT signaling pathway, we verified this by overexpressing miR-223 and knocking down miR-223 and CRIM1. Compared with the control group, the p-AKT/AKT ratio was significant reduction after overexpressing miR-223 (Figures 7A and 7B). On the contrary, the p-AKT/AKT ratio was significant increase after transfection miR-223 inhibitor (Figure 7C and 7D). In addition, the p-AKT/AKT ratio was significant increase after transfection si-CRIM1 (Figures 7E and 7F). These results suggest that miR-223 may exert regulatory effects on the development of GCs through modulation of the AKT signaling pathway.

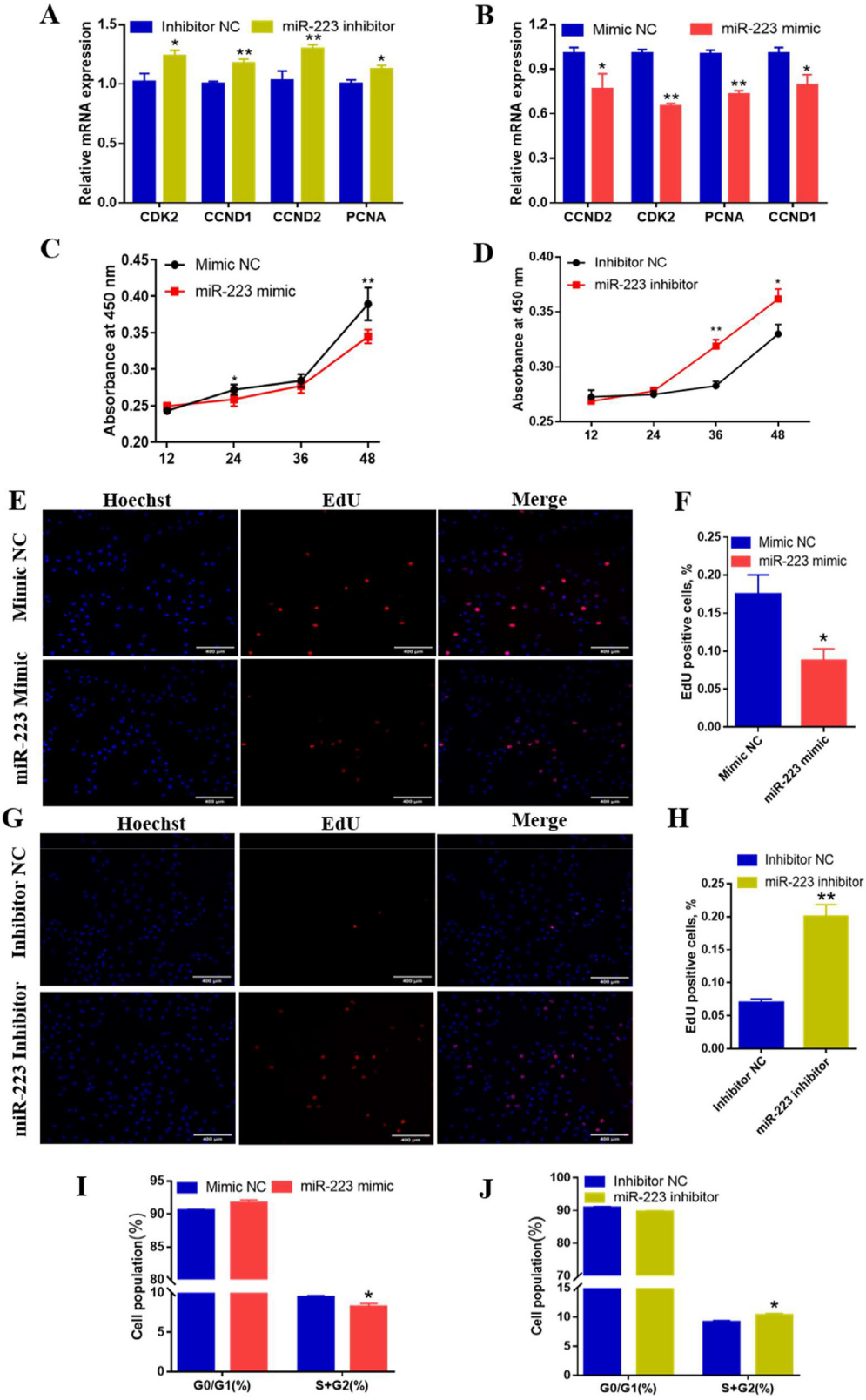

Figure 3. miR-223 inhibits GC proliferation. (A) Relative RNA expression of proliferation-related genes in GC with knockdown of miR-223. (B) Relative RNA expression of proliferation-related genes in GC after overexpression of miR-223. (C) The results of CCK-8 showed the proliferation of GC at 12h, 24h, 36h and 48h after overexpression of miR-223. (D) The results of CCK-8 showed the proliferation of GC at 12h, 24h, 36h and 48h after miR-223 knockdown. (E and F) EdU staining showed the number of proliferating GC after overexpression of miR-223. (G and H) EdU analysis showed the amount of GC proliferation after miR-223 knockdown. EdU (red) fluorescence indicates proliferation. Hoechst (blue) fluorescence indicates nuclei (I) GC flow cytometry cell cycle analysis and statistical results of miR-223 overexpression. (J) GC flow cytometry cell cycle analysis and statistical results following miR-223 knockdown. Data were showed as Mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

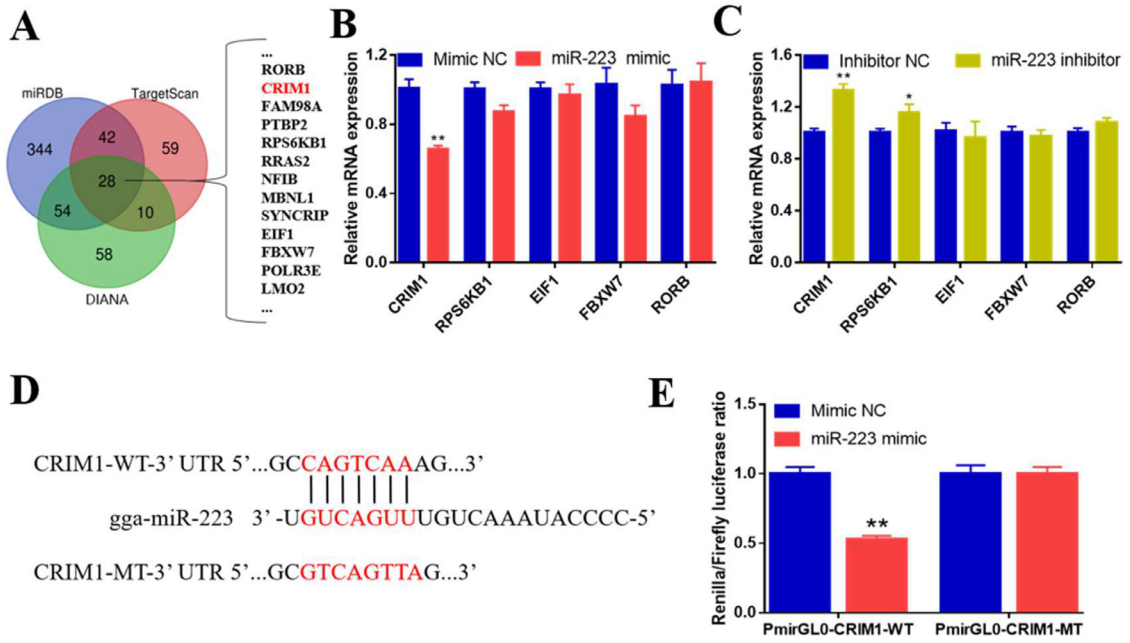

Figure 4. miR-223 target genes prediction. (A) The target genes of miR-223 were predicted using miRDB, Targetcan and DIANA, and the results of the 3 sites were integrated. (B) Relative mRNA expression levels of 5 candidate target genes after overexpression of miR-223. (C) Relative mRNA expression of five candidate target genes after miR-223 knockdown. (D) The CRIM1 3′ UTR binding sites analyzed. The seed sequences are highlighted in red. (E) Co-transfection of wild-type or mutant-type CRIM1 3′ UTR with a miR-223 mimic or mimic-NC was used to perform luciferase analyzes in GCs. Data were showed as Mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

DISCUSSION

Animal reproductive efficiency can be strongly linked to the economic impacts of livestock production. In recent years, miRNAs have been widely reported to be associated with the development of follicles, but there are few studies on poultry. Current studies have shown that miRNAs can regulate the development of follicles by regulating key factors in the process of follicular development. For example, miR-214-3p can promote cell proliferation, and estradiol hormone synthesis in porcine GC (Shi, et al., 2020), and miR-31 and miR-143 promote steroid hormone synthesis and inhibit apoptosis in bovine GC (Zhang, et al., 2019). Studies on miRNA in

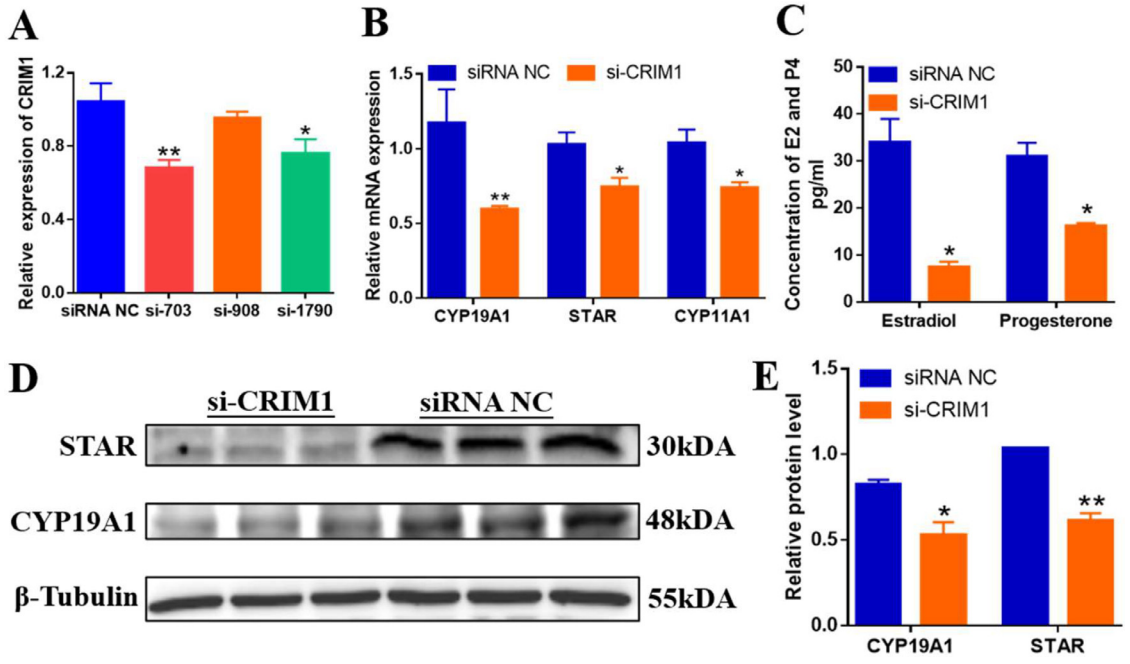

Figure 5. CRIM1 knockdown inhibits steroid hormone synthesis of GCs. (A) The knockdown efficiency of CRIM1 in chicken GC by transfected with 3 siRNAs. (B) Relative RNA expression of steroid hormone-related genes in GC after transfection with si-CRIM1. (C) ELISA experiment revealed the levels of E2 and P4 in GC after CRIM1 knockdown. (D and E) The protein content of CYP19A1 and STAR in GC after CRIM1 knockdown. Data were showed as Mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

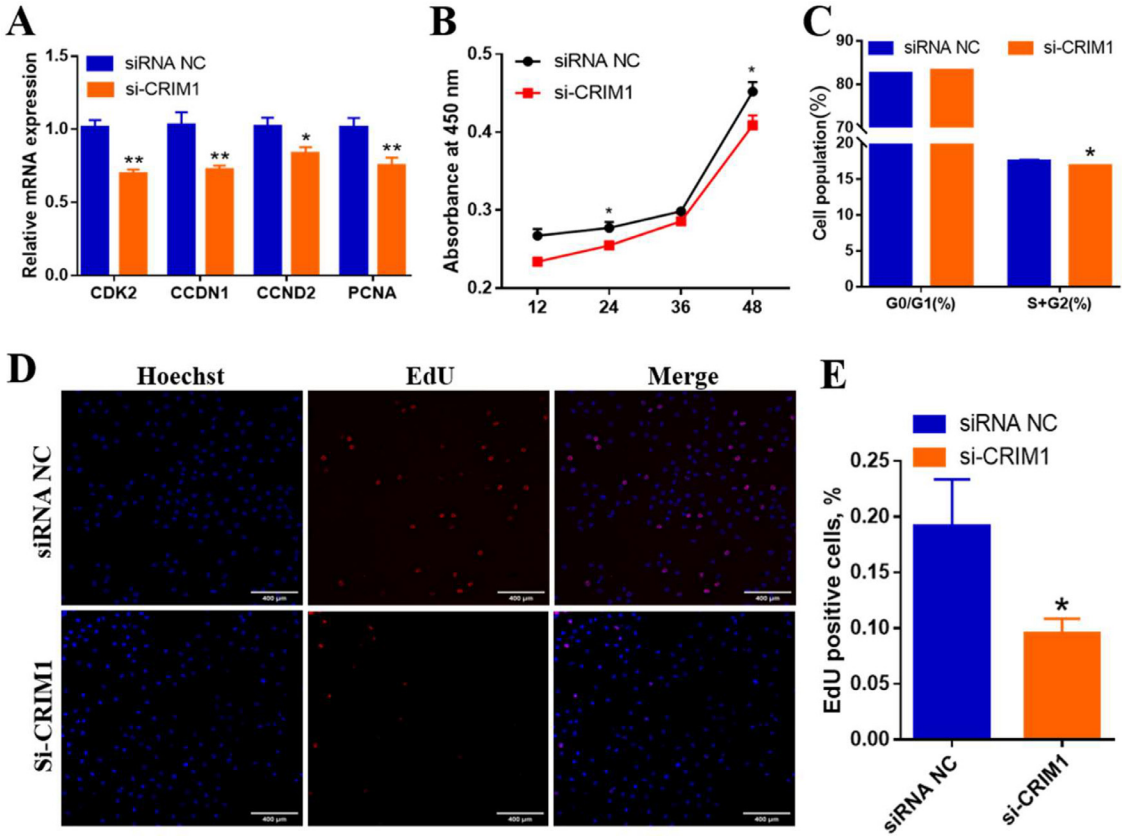

Figure 6. CRIM1 knockdown inhibits the proliferation of chicken GCs. (A) mRNA expression of proliferation-related genes after CRIM1 knockdown. (B) The results of CCK-8 showed the proliferation of GC at 12, 24, 36, and 48h after CRIM1 knockdown. (C) GC flow cytometry cell cycle analysis and statistical results following CRIM1 knockdown. (D and E) EdU analysis showed the proliferation of GC after transfection with si-CRIM1. EdU (red) fluorescence indicates proliferation. Hoechst (blue) fluorescence indicates nuclei. Data were showed as Mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

poultry have also been reported, such as miR-34c-5p promotes GC apoptosis by targeting BCL2 apoptosis regulator (**BCL2**) (Hou, et al., 2022), and miR-3188 promoted proliferation of GC (Zhou, et al., 2021). Furthermore, miR-23b-3 inhibits proliferation and steroid hormone synthesis of chicken GC by targeting growth differentiation factor 9 (**GDF9**) (Wei, et al., 2022). The above results show that miRNA plays an important biological role in follicular development. However, many miRNAs control the development of GC is still a mystery, therefore, it is necessary to explore and verify the regulatory mechanism of miRNAs on chicken follicles. Our previous sequencing data analysis revealed that miR-223 had the highest expression level in atretic follicles (He, et Al., 2022). Following qRT-PCR analysis, we discovered that miR-223 was strongly expressed in ovaries relative to other tissues and organs. Therefore, we suspect it may have an impact on ovarian development.

The synthesis of steroid hormones and the proliferation of GC are crucial for the development of chicken follicles. GC is a primary site for steroid hormone synthesis within the ovary. The CYP11A1, and STAR genes are commonly utilized to assess the ovary's capacity for steroid hormone production. Cholesterol serves as the raw material for this synthesis (Yu, et al., 2021). Specifically, CYP11A1 is a crucial enzyme responsible for the conversion of cholesterol into pregnenolone, which is then transported into the mitochondria by the STAR protein for P4 synthesis. Similarly, CYP19A1 is a key enzyme in the formation of estradiol (Drummond, 2006). In this study, we found that miR-223 can reduce the expression of steroid hormone-related genes and proteins in GCs, as well as the synthesis of E2 and P4. Although direct evidence linking miR-223 to steroid synthesis is lacking, some studies have reported that miR-223 over-expression suppresses the expression of cytochrome b5. This protein is known to facilitate steroid hormone synthesis by modulating the activity of key enzymes such as CYP17A and 3βHSD (Storbeck et al., 2013; Takahashi et al., 2014), aligning with our experimental results.

Cell proliferation is primarily regulated by genes such as CCND1, CCND2, CDK2, and PCNA. Cyclins, widely distributed in eukaryotic cells, include CCND1 and CCND2, which belong to the D-type cell cycle family (Chang, et al., 2018). The cyclin-dependent kinase (CDK) family plays a pivotal role in regulating cell cycle transitions, activated by various cyclins at specific times to drive cell cycle progression. CDKs are essential for cell proliferation in nearly all animals (Malumbres and Barbacid, 2009). Our findings indicate that miR-223 over-expression down-regulates the expression of proliferation-related genes and leading to reduction cell proliferation activity. Previous studies have demonstrated miR-223′s inhibitory effect on cell proliferation, such as its suppression of HCC cell proliferation (Zhang and

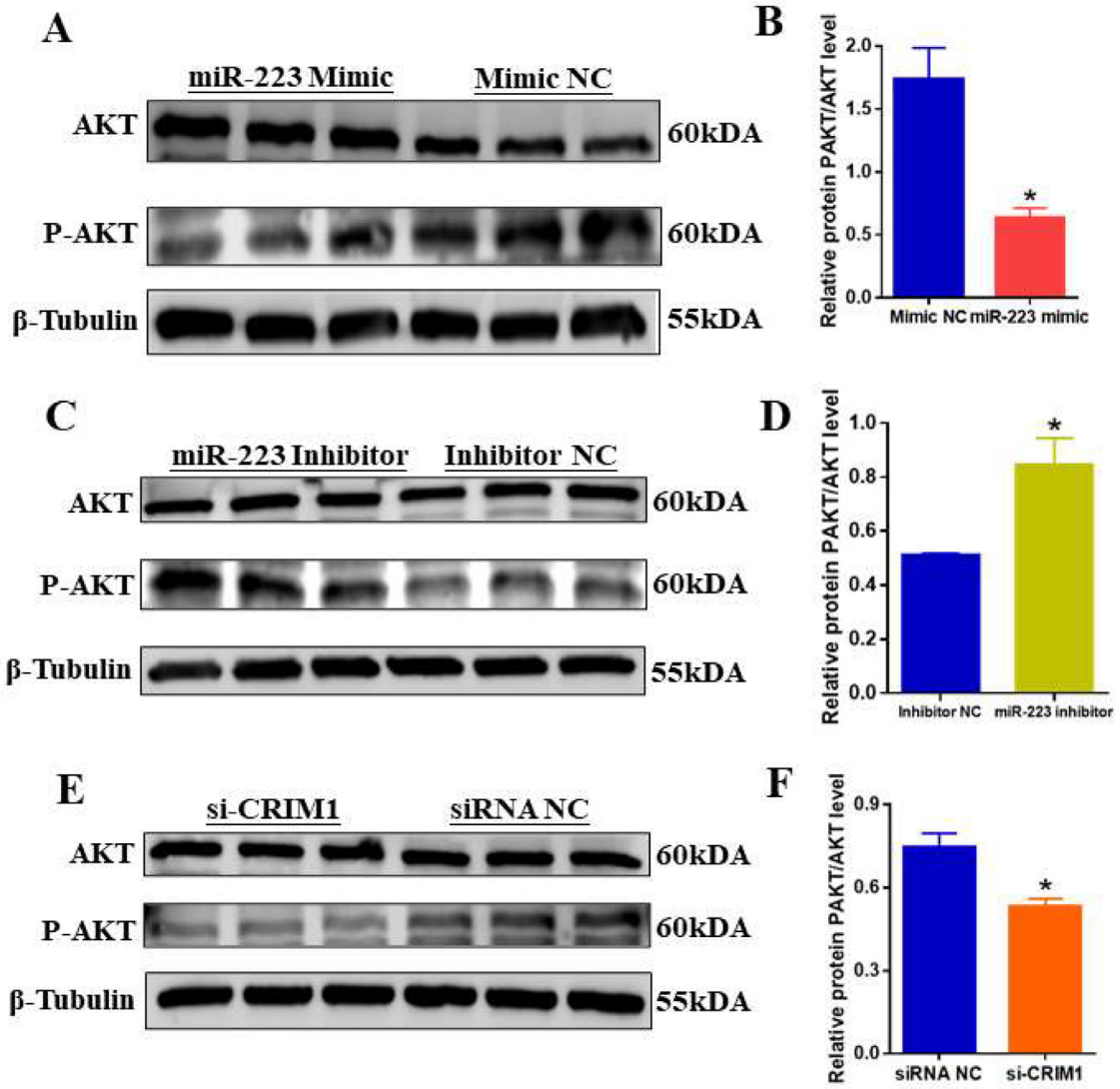

Figure 7. miR-223 may mediates the AKT signaling pathway by targeting CRIM1. (A and B) The protein ratio of p-AKT/AKT after overexpression of miR-223. (C and D) The protein ratio of p-AKT/AKT after miR-223 knockdown. (E and F) The protein ratio of p-AKT/AKT after interference with si-CRIM1. Data were showed as Mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$.

Zhang, 2018), nasopharyngeal carcinoma cell proliferation (Lu, et al., 2013), and eosinophil progenitor proliferation (Yang, et al., 2015), aligning with our results.

CRIM1 is a transmembrane protein rich in multiple cysteines, which is involved in cell adhesion, polarity, proliferation, and other functions (Pennisi, et al., 2007; Tam, et al., 2018). CRIM1 has been shown to regulate the key growth factor TGF-β and promote the proliferation of epicardium-derived cells (EPDCs) (Iyer, et al., 2016). CRIM1 can also influence cell adhesion, polarity and proliferation in the lens by regulating integrin-signaling (Tam, et al., 2018). Although studies have shown that CRIM1 is closely related to the development of GC (Brązert, et al., 2020), its specific function in GC has not been explored. Therefore, we found that the proliferation of GC and the synthesis of steroid hormones were down-regulated after CRIM1 was knocked down, shedding light on its potential role in in follicular development.

Previous studies found that CRIM1 functioned as an early response gene to regulate the growth of blood vessels via the AKT signaling pathway (Nakashima and Takahashi, 2014). The AKT signaling pathway is an intracellular signaling pathway that promoted cell proliferation, metabolism, and growth (Hsueh et al., 2015). Numerous studies showed that miRNA and its target genes could regulate GC via the AKT signaling pathway. For example, miR-103 disrupts PI3K/AKT pathway activation by targeting IRS1, resulting in a decrease in GCs (Mu, et al., 2021). miR-29 targets PTX3 to promote GC proliferation by activating PI3K/AKT/mTOR signaling pathway (Wang, et al., 2020). In our study, we found that the p-AKT/AKT ratio was decrease after over-expression miR-223 and knockdown CRIM1. Conversely, the p-AKT/AKT ratio increased after knockdown miR-223. This indicated that the miR-223/CRIM1 axis may functioned in GC via mediating the AKT signaling pathway.

In summary, this study explored the effects of miR-223 on the development of GCs, which provided a basis for the subsequent research on the function of miRNA and enriched the function of miRNA on

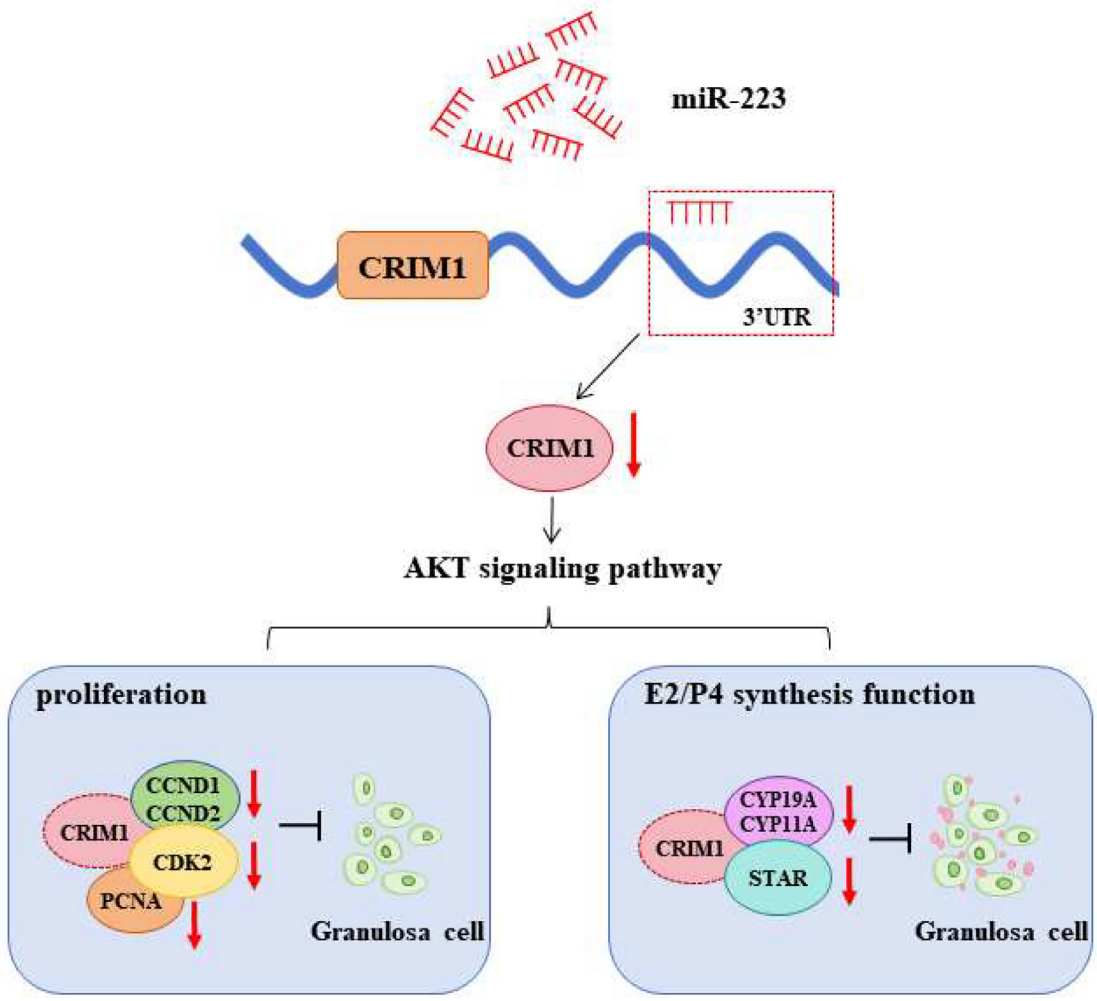

Figure 8. Model diagram of miR-223 target CRIM1 to regulate follicular proliferation and steroid hormone synthesis via mediating the AKT signaling pathway.

chicken GCs. Furthermore, further study will be necessary to identify more miRNAs related to follicular development, which can act as a foundation for additional molecular genetic studies aimed at enhancing hen reproductive efficiency.

CONCLUSIONS

In conclusion, as shown in Figure 8, we found that miR-223 affects GC proliferation and steroid hormone synthesis by targeting the CRIM1 via mediate the AKT signaling pathway.

ACKNOWLEDGMENTS

Financial support for this study was provided by the National Key Research and Development Program of China (2021YFD1300600), the Sichuan Science and Technology Program (2023NSFSC1940, 2021YFYZ0007, 2021YFYZ0031, 2022YFYZ0005 and 2023NSFSC1145) and the China Agriculture Research System of MOF and MARA (CARS-40).

DISCLOSURES

The authors declare no conflicts of interest.

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