miR-27b-3p inhibits estrogen secretion of goose granulosa cells by targeting *CYP1B1* through the AMPK signaling pathway

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ABSTRACT Although miR-27b-3p has been evidenced to regulate the proliferation, apoptosis, and differentiation of a variety of mammalian cell types, its actions and mechanisms on ovarian cell steroidogenesis remains largely unknown in both mammalian and avian species. In this study, we aimed to determine the expression profiles of miR-27b-3p in granulosa cell layers during goose ovarian follicle development and to reveal its actions on estrogen ($\mathbf{E_2}$) secretion of goose granulosa cells as well as the underlying regulatory mechanisms. It was observed that miR-27b-3p was ubiquitously expressed throughout follicle development but exhibited much higher levels in hierarchical- than in prehierarchical follicles. In cultured granulosa cells from the fourth through second largest preovulatory (F4-F2) follicles of goose, up- and downregulation of miR-27b-3p by using its mimic and inhibitor significantly decreased and increased E_2 secretion, respectively. Meanwhile, the mRNA levels of *STAR* and *CYP19A1* were significantly reduced while those of *CYP11A1* and *3βHSD* were elevated in the mimic-transfected granulosa cells. By comparison, downregulation of miR-27b-3p enhanced the mRNA levels of *STAR* but had no significant effects on those of *CYP19A1*, *CYP11A1*, and *3βHSD*. Results from bioinformatic prediction and luciferase reporter assay demonstrated that *CYP1B1* was a downstream target of miR-27b-3p. Although the siRNA-mediated downregulation of *CYP1B1* did not significantly change E_2 secretion by goose granulosa cells, it reduced the mRNA levels of *STAR* and *CYP19A1* as well as those of *LKB1* and *AMPKα*, which are involved in the AMPK signaling pathway. Taken together, these data suggest that miR-27b-3p plays an inhibitory role in E_2 secretion by goose F4-F2 granulosa cells, at least in part, by targeting *CYP1B1* through the AMPK signaling pathway.

Key words: miRNA-27b-3p, estrogen secretion, *CYP1B1*, AMPK signaling, goose

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INTRODUCTION

Steroid hormones play essential roles in regulation of ovarian activities such as normal follicular development and oocyte maturation (Drummond, 2006). Compared to chickens and ducks having an average annual egg production number of >250, domestic geese (*Anser cygnoides*) show a poorer egg production performance, generally producing an annual number of 30 to 50 eggs per year. One of the main causes is due to the strong incubation behavior, which leads to the degeneration of ovaries and oviducts and greatly hinders goose egg production performance (Shi et al., 2008). It has been reported that the contents of steroid hormones such as estrogen ($\mathbf{E_2}$) and progesterone ($\mathbf{P_4}$) significantly change in ovarian follicles of geese during the egg-laying and incubation periods, highlighting their critical roles in maintaining the follicular hierarchy and normal ovarian functions (Yu et al., 2016). In chicken ovarian follicles, the biosynthesis of steroid hormones mainly occurs in the granulosa and theca cell layers, and significant changes in their morphology and steroidogenic capacity are observed during follicle maturation (Johnson, 2014). Specifically, theca cell layers are the major site of steroidogenesis in nonhierarchical follicles, while both granulosa and theca cell layers are steroidogenic in hierarchical follicles (Johnson, 2015). A 3-cell model has been proposed for steroidogenesis in chicken ovarian

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hierarchical follicles. P_4 synthesized by granulosa cell layers are transported to theca interna where they are converted to testosterone (**T**), which is subsequently aromatized to E_2 in theca externa (Porter et al., 1989; Nitta et al., 1991). Noticeably, granulosa cell layers of hierarchical follicles can also synthesize and secrete E_2 to regulate oocyte maturation and follicle development (Hu and Zadworny, 2017). Although the key genes responsible for ovarian cell steroidogenesis have been revealed in domestic birds (Johnson and Bridgham, 2001; Onagbesan et al., 2009), the microRNAs (**miRNA**s)-mediated post-transcription regulatory mechanisms of granulosa cell steroidogenesis are poorly understood, especially in goose.

The miRNAs are a class of endogenously initiated noncoding RNAs having a length of ~22 nucleotides (nt), and they can post-transcriptionally control expression of target genes by binding to their 3′-untranslation region (**UTR**) sequences, leading to either the cleavage or translational repression of target mRNAs (Saliminejad et al., 2019). There is accumulating evidence that miRNAs can regulate the synthesis and secretion of steroid hormones. For instance, miR-132 is involved in the cyclic adenosine 3′,5′-monophosphate (**cAMP**) signaling pathway and promotes E_2 synthesis via the translational repression of the nuclear receptor subfamily 4 group A member 2 (*NURR1*) in mouse granulosa cells (Wu et al., 2015). miR-326 suppresses both E_2 production and expression of cytochrome P450 aromatase (*CYP19A1*) by binding to the cAMP response element binding protein 1 (*CREB1*) and CCAAT/enhancer-binding proteins β (*C/EBP-β*) in buffalo granulosa cells (Chaurasiya et al., 2020). miR-143 affects the biosynthesis of both P_4 and E_2 by targeting follicle-stimulating hormone (**FSH**) receptor (***FSHR***) in bovine granulosa cells (Zhang et al., 2019). In chicken granulosa cells, miR-30a-5p not only stimulates the synthesis of P4 and E2 but also inhibits cell autophagy and apoptosis (He et al., 2022), while miR-23b-3p inhibits cell proliferation and steroid hormone synthesis by downregulating the expression of growth differentiation factor 9 (***GDF9***) (Wei et al., 2022). In chicken theca cells, miR-26a-5p promotes cell proliferation by targeting the trinucleotide repeat containing 6A (*TNRC6A*) gene (Kang et al., 2017). Besides, in a recent study, we identified a number of miRNAs including miR-27b-3p that are differentially expressed in granulosa cell layers from goose ovarian follicles before and after selection (Li et al., 2019). Moreover, miR-27b-3p is abundantly expressed in cattle follicular cells and oocytes, which secrete high levels of steroid hormones (Pasquariello et al., 2020). miR-27b-3p has also been demonstrated to play important roles in regulating cell proliferation, apoptosis, differentiation, angiogenesis, and adipogenesis (Lin et al., 2009; Wang et al., 2009; Veliceasa et al., 2015; Ling et al., 2018). However, the effects and mechanisms of miR-27b-3p on avian granulosa cell steroidogenesis still remain largely unknown.

In the present study, we firstly determined the expression profiles of miR-27b-3p in granulosa cell layers isolated from goose ovarian follicles at different stages of development. Then, we investigated the effects of up- and downregulating miR-27b-3p on the secretion of E_2 and expression of several steroidogenic-related genes in cultured granulosa cells from goose F4-F2 follicles. Finally, we explored the underlying regulatory mechanisms of miR-27b-3p by predicting its potential target genes and verifying the roles of target gene in regulating goose granulosa cell steroidogenesis.

MATERIALS AND METHODS

Ethics Statement

All experimental procedures involving the manipulation of geese in this study were conducted in concordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China). This study has been reviewed and approved by the Sichuan Agricultural University Animal Ethical and Welfare Committee (Approval No.: 20190035).

Experimental Animals and Sample Collection

Females from the maternal line of Tianfu meat goose during the egg-laying periods were used in all experiments. All geese had free access to water and food and were kept under natural conditions of temperature and light at the Waterfowl Breeding Experimental Farm of Sichuan Agricultural University (Ya'an Campus, Sichuan, China). A total of 18 female geese hatched from the same batch and showing normal ovarian follicular hierarchies after slaughter were used for tissue collection and cell culture. Among them, ovarian follicles of 3 geese were categorized into the prehierarchical (2−4-, 4−6-, 6−8-, and 8−10 mm in diameter) and hierarchical (F5-F1; F5 < F4 < F3 < F2 < F1 in diameter) follicles according to their diameter and developmental stages. Then, granulosa cell layers were isolated from these follicular categories according to our previously described methods (Hu et al., 2020) to investigate the developmental expression patterns of miR-27b-3p. Briefly, after being washed with 0.9% NaCl solution, hierarchical follicles were cut with a scalpel blade along the line of the stigma, and intrafollicular contents including granulosa cell layers were immediately inverted into a dish containing culture medium. With respect to the prehierarchical follicles, each follicle was slit and inverted using fine forceps, granulosa cell layers were separated by gently shaking or peeling off from the inverted follicular tissue in culture medium. Besides, granulosa cell layers of the F4-F2 follicles were isolated from the remaining goose ovaries for in vitro culture. All collected follicular tissue samples were rapidly frozen in liquid nitrogen and finally stored at −80°C until RNA extraction.

Granulosa Cell Culture and Transfection

The harvested F4-F2 granulosa cells were cultured in vitro as previously described (Hu et al., 2020). Briefly, after being cut into small pieces and digested with 0.1% collagenase (Sigma, Aldrich, MO), granulosa cell suspensions were added with the Dulbecco's Modified Eagle's Medium/Nutrient Mixture (DMEM/F12, Hyclone, Carlsbad, CA) medium containing 10% fetal bovine serum (FBS; Sigma, St Louis, MO) and 1% antibiotics (1% penicillin and 1% streptomycin mixture; Gibco, NY) to terminate the digestion, filtered through a 200-mesh sieve, and centrifuged at 1,200 g/min for 10 min. Then, the supernatant was discarded and the cell pellets were suspended with above medium. The number of granulosa cells was counted using a hemocytometer, and the cells were finally seeded into 12-well culture plates at a density of about 6×10^5 cells/well and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When reaching a confluency of ~80%, the cells were transfected with the miR-27b-3p mimic (30, 90, or 150 nM), inhibitor (50, 100, or 150 nM), and the corresponding negative controls (GenePharma Co., Ltd, Shanghai, China), respectively, using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Besides, to reveal the regulatory mechanism of cytochrome P450 1b1 (*CYP1B1*), which was predicted as a key target gene of miR-27b-3p, in goose granulosa cells, the siRNAs (namely si-221, si-322, and si-542) targeting the *CYP1B1* gene as well as the siRNA-NC at the optimal concentrations of 80 nM, which were synthesized and purified with HPLC by GenePharma Co., Ltd (Shanghai, China), were transfected into goose granulosa cells using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA). The oligonucleotide sequences for the mimic, mimic NC, inhibitor, and inhibitor NC of miR-27b-3p as well as the *CYP1B1* siRNAs were listed in Table 1. After a 24-h transfection, the cells were harvested for detecting the expression levels of target genes, and the culture media were collected for measuring E_2 levels. Each experiment was independently performed at least 3 times and each treatment was repeated in triplicate.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from each sample using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. The purity and concentration of RNA were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA), and its integrity was assessed by visualization of the 28S/18S rRNA ratio after electrophoresis on 1.5% agarose gels. Approximately 1 μg RNA from each sample was reverse transcribed using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instruction.

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

Name	Sequence (5′→3′)
miR-27b-3p mimic	UUCACAGUGGCUAAGUUCUGC
mimic NC	UUCUCCGAACGUGUCACGUTT
miR-27b-3p inhibitor	GCAGAACUUAGCCACUGUGAA
inhibitor NC	CAGUACUUUUGUGUAGUACAA
si-*CYP1B1*-221	CCACGACCAACACCUUCAUTT
si-*CYP1B1*-322	CCAGAGGACUUUGACCCAATT
si-*CYP1B1*-542	CCAUUAAACCGAAGCCAUUTT
si-*CYP1B1*-NC	UUCUUCGAACGUGUCACGUTT

Quantitative Real-Time PCR

The quantitative real-time PCR (**qRT-PCR**) primer pairs for both protein-coding genes and miR-27b-3p were listed in Table 2. For the protein-coding genes, the *GAPDH* and *β-ACTIN* genes were used as the internal controls. The qRT-PCR was performed on the CFX96 Real-Time PCR Detection System (Bio-Rad) using the SYBR Premix Ex Taq II (Takara Biotechnology Co., Ltd., Dalian, China). For the miR-27b-3p, the qRT-PCR was performed using the Hairpin-it Real-Time PCR Kit (GenePharma, Shanghai, China) according to the manufacturer's instructions, and the *U6* was used as the internal control. All reactions were conducted under the following conditions: predenaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing/extension at the optimal temperature of each primer pair for 30 s. The no-template controls and negative controls without reverse transcriptase were also included in all qRT-PCR runs. Target specificity for each primer set was validated by melting curve analysis, and the identity of all amplicons was verified by sequencing. All samples were amplified in triplicate, and the relative expression levels of target genes and miRNAs were normalized to the respective internal controls using the comparative Cq method (ΔΔCq) (Schmittgen and Livak, 2008).

Target Gene Prediction and Luciferase Reporter Assay

The target genes of miR-27b-3p were predicted using a combination of miRanda, miRTarBase, and TargetScan databases, and the binding region of miR-27b-3p in the 3′-UTR of goose *CYP1B1* gene (GeneBank Accession No.: XM_013170764.1) was predicted on RNAHybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/). To construct the luciferase reporter plasmid, the 3′-UTR of *CYP1B1* containing the wild-type miR-27b-3p binding region (*CYP1B1*-WT) were cloned into the pmirGLO luciferase reporter vector (Promega, Madison, WI), and the primer pairs used for plasmid construction were shown in Table 2. The 3′-UTR of *CYP1B1* containing the mutant miR-27b-3p binding region (*CYP1B1*-MUT) was constructed by GenePharma Co., Ltd (Shanghai, China). The Chinese hamster ovary cell line (**CHO**) was purchased

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

Gene symbol	GenBank accession no.	Primer sequence (5′→3′)	Amplicon (bp)
Quantitative real-time PCR for protein-coding genes			
CYP1B1	XM_013170764.1	F: CCAACACCTTCATAATGGGCTAC R: CGAGAAAATCATCACGCTGCTA	179
STAR	KF958133.1	F: AGAATCTTGACCTCTTTGACGCTG R: GAGACGGTGGTGGATAACGGA	87
CYP11A1	KY463321.1	F: AGGGAGAAGTTGGGTGTCTACGA R: CGTAGGGCTTGTTGCGGTAGT	89
3βHSD	KC310447.1	F: GACCTGGGGTTTGGAATTGAG R: TAGGAGAAGGTGAATGGGGTGT	170
CYP19A1	KY091839.1	F: CTGGTCCTGGTCTCGTGCGTAT R: GATGTGTCAAGCATGATCCGTCTC	139
AMPKα	XM_013181736.1	F: CACCGAAGAGGAAGTTCTAAGC R: TTCAGGATGAGGGCGAGAC	181
CAMKK2	XM_013173329.1	F: CGCTTTATCTACCCGTCCCT R: GAGCCCTTCCCGATCTCAT	167
LKB1	XM_013200088.1	F: ATCTGATTTAGGCGTAGCA R: ATAGACCCGTTGTAATGTTGT	182
STRADA	XM_013196794.1	F: GCTCTTCAACCACCCTAAC R: ATTCACTCATCCCATCCATA	140
TAK1	XM_013180128.1	F: TATTCCAAGCCTAAACGA R: TGGTCCCGAGGTAGTGAT	210
GAPDH[*]	MG674174.1	F: TTTCCCCACAGCCTTAGCA R: GCCATCACAGCCACACAGA	86
β-ACTIN[*]	M26111.1	F: CAACGAGCGGTTCAGGTGT R: TGGAGTTGAAGGTGGTCTCG	93
Quantitative real-time PCR for *miR-27b-3p*			
miR-27b-3p		F: ATACGGACATTCACAGTGGCTAAG R: TATGGTTGTTCACGACTCCTTCAC	
U6[#]		F: ATTGGAACGATACAGAGAAGATT R: GGAACGCTTCACGAATTTG	
Construction of the luciferase reporter vector for *CYP1B1*			
CYP1B1		F: cgagctcACACAGTAGTGCTGTAAC R: ctctagaCAAGGCACTAATTGACCA	508

[*]and [#] indicate the housekeeping genes for protein-coding gene and miRNA, respectively.F, forward primer; R, reverse primer.

from Kunming Institute of Zoology (Kunming, China). When the CHO cells reached a confluence of ∼70%, 100 ng of the *CYP1B1*-WT or *CYP1B1*-MUT vector was co-transfected with 90 nM of the miR-27b-3p mimic or its negative control using Lipofectamine 3000 Reagent, respectively. After a 24-h transfection, the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), and the activity of Firefly luciferase was normalized to that of Renilla luciferase. Each experiment was at least repeated in triplicate.

Estrogen Measurement

After a 24-h transfection, the culture media were collected to detect the E_2 concentrations using a goose E_2 ELISA Kit (Neobioscience, Beijing, China) according to the manufacturer's protocols. Each sample was analyzed in triplicate, and the sensitivity of E_2 in this assay was 1.0 pg/mL.

Statistical Analysis

All data were expressed as the Mean ± S.D. (Standard Deviation). Statistical analysis was performed by Student's *t*-test or Duncan's multiple range test using SPSS 22.0 software. The *P*-values below 0.05 and 0.01 were considered statistically significantly and extremely significantly different.

RESULTS

Expression Profiling of miR-27b-3p in Granulosa Cell Layers During Goose Ovarian Follicle Development

As shown in Figure 1, we detected the expression levels of miR-27b-3p in granulosa cell layers during goose ovarian follicle development, which suggested that miR-27b-3p was ubiquitously expressed in granulosa cell layers isolated from all examined categorized ovarian

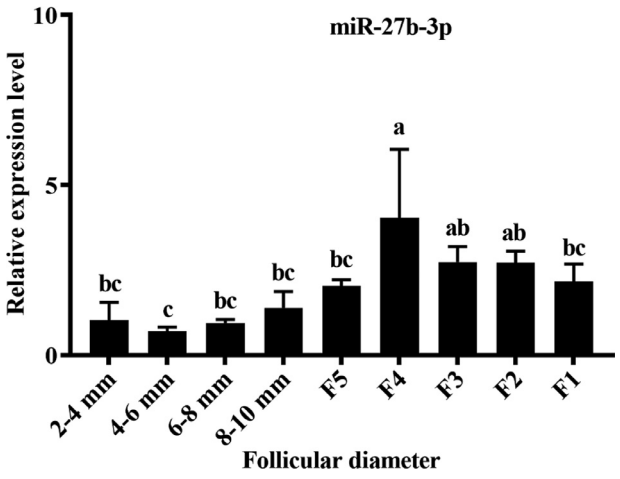

Figure 1. Expression profiles of miR-27b-3p in granulosa cell layers of goose ovarian different sized follicles. Different lowercase letters indicate significant differences in the miR-27b-3p expression levels among granulosa cell layers of different size follicles ($P < 0.05$).

follicles. Its expression reached the highest levels in granulosa cell layers of the F4 follicle, which was significantly higher than all prehierarchical follicular categories and the F1 follicle ($P < 0.05$). During the prehierarchical stage, levels of miR-27b-3p remained almost unchanged ($P > 0.05$) but rose gradually as the follicular diameter increased. Expression of miR-27b-3p was generally higher in hierarchical- than in prehierarchical follicles but declined during the accretion phase from the F4 through F1 follicles.

Effects of Up- and Downregulation of miR-27b-3p on Estrogen Secretion and Involved Gene Expression in Goose Granulosa Cells

Since miR-27b-3p was expressed at much higher levels in the F4-F2 follicles, we further explored its role in regulation of granulosa cell steroidogenesis in this follicular category. As shown in Figure 2A, the endogenous expression levels of miR-27b-3p in the in vitro cultured F4-F2 granulosa cells were significantly upregulated after transfection of its mimic at any of 3 examined concentrations ($P < 0.05$). Meanwhile, its levels were significantly downregulated after transfection of its inhibitor at the concentrations of 100 and 150 nmol/L ($P < 0.01$; Figure 2B). Thus, we used 90 nmol/L of the miR-27b-3p mimic and 150 nmol/L of the inhibitor in our subsequent experiments. It was observed that upregulation of miR-27b-3p by transfection of 90 nmol/L mimic significantly decreased the secretion of E_2 by the F4-F2 granulosa cells ($P < 0.05$; Figure 2C), while downregulation of its expression by transfection of 150 nmol/L inhibitor significantly increased E_2 secretion ($P < 0.05$; Figure 2D). With respect to changes in expression of key genes involved in E_2 synthesis, compared to the mimic NC group, the mRNA levels of steroidogenic acute regulatory (*STAR*) and *CYP19A1* were significantly reduced ($P < 0.05$) while those of P450 cholesterol side-chain cleavage enzyme (*CYP11A1*) and 3β-hydroxysteroid dehydrogenase (*3βHSD*) were significantly elevated ($P < 0.05$) in granulosa cells transfected with the miR-27b-3p mimic (Figure 3). By comparison, transfection of the miR-27b-3p inhibitor significantly enhanced ($P < 0.05$) expression of *STAR* but did not significantly change ($P > 0.05$) that of *CYP11A1, 3βHSD*, and *CYP19A1* in granulosa cells when compared to the inhibitor NC group (Figure 3).

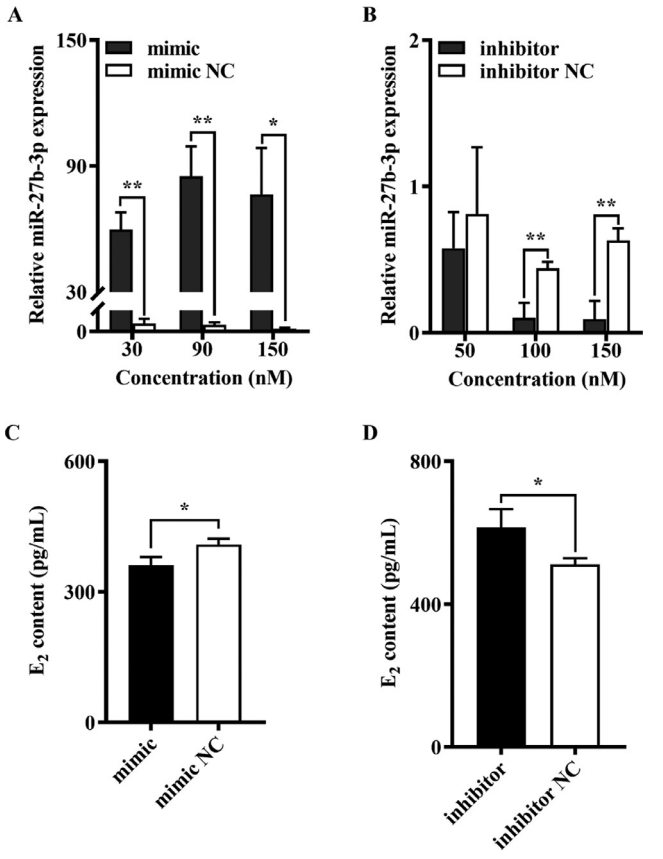

Figure 2. miR-27b-3p inhibits E_2 secretion by goose F4-F2 granulosa cells. (A) Effects of different concentrations of the miR-27b-3p mimic on the miR-27b-3p expression in goose granulosa cells. (B) Effects of different concentrations of the miR-27b-3p inhibitor on the miR-27b-3p expression in goose granulosa cells. (C) Effects of upregulation of miR-27b-3p by using its mimic on E_2 secretion by goose granulosa cells. (D) Effects of downregulation of miR-27b-3p by using its inhibitor on E_2 secretion by goose granulosa cells. $^*P < 0.05$; $^{**}P < 0.01$.

CYP1B1 is Identified as A Target Gene of miR-27b-3p

Considering that miR-27b-3p significantly affected E_2 secretion by goose F4-F2 granulosa cells, it was of importance to identify the potential target genes that may assist in exerting its suppressive effects. A combination of miRanda, miRTarBase, and TargetScan databases (Lewis et al., 2005; Betel et al., 2008; Chou et al., 2018) were used to predict the target genes of miR-27b-3p. Among the predicted candidate targets of miR-27b-3p, we found that the *CYP1B1* gene has been previously reported to be related to E_2 synthesis (Faiq et al., 2014). Meanwhile, the 3′-UTR of *CYP1B1* mRNA was predicted to contain one putative binding site for miR-27b-3p on the RNAHybrid website (Figure 4A, top panel). To further verify whether *CYP1B1* is a target gene of miR-27b-3p, the *CYP1B1* mRNA 3′-UTR containing

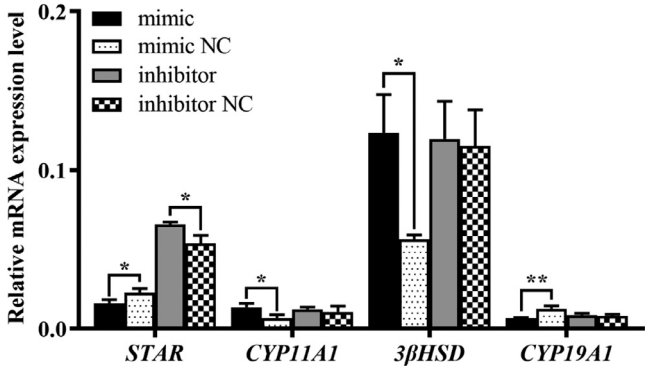

Figure 3. Effects of up- and downregulation of miR-27b-3p on the mRNA expression of several key genes involved in steroid hormone synthesis. $^*P < 0.05$, $^{**}P < 0.01$.

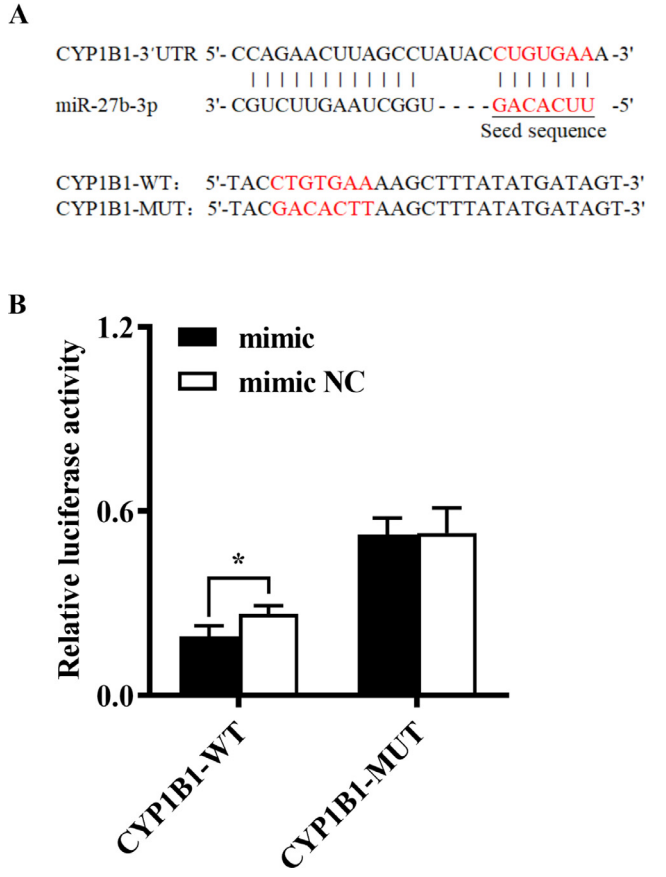

Figure 4. *CYP1B1* is verified as a target gene of miR-27b-3p. (A) Sequence alignment of the wild-type (WT) and mutant (MUT) binding sites of miR-27b-3p in the 3′-UTR of goose *CYP1B1* gene. Both the WT- and MUT binding sites of miR-27b-3p were marked in red. (B) Effects of transfection with the miR-27b-3p mimic on the luciferase activity of *CYP1B1*-WT and *CYP1B1*-MUT when compared to the mimic NC group. $^{*}P < 0.05$.

either the wild-type (**WT**) or mutant (**MUT**) binding site of miR-27b-3p was cloned into the luciferase reporter vector, respectively (Figure 4A, bottom panel). The results showed that the luciferase activity significantly decreased ($P < 0.05$) in the CHO cells co-transfected with the *CYP1B1*-WT vector and miR-27b-3p mimic than in those co-transfected with the *CYP1B1*-WT vector and mimic NC. However, there was no significant difference ($P > 0.05$) in the luciferase activity between the cells co-transfected with the *CYP1B1*-MUT vector and miR-27b-3p mimic and those co-transfected with the *CYP1B1*-MUT vector and mimic NC (Figure 4B).

Effects of siRNA-Mediated Downregulation of CYP1B1 on Estrogen Secretion and Involved Gene Expression in Goose Granulosa Cells

To determine the role of *CYP1B1* in regulation of E_2 secretion, 3 siRNAs specifically targeting different coding regions of *CYP1B1* (si-221, si-322, and si-542) were designed to curtail its endogenous expression in granulosa cells of goose F4-F2 follicles, and their interference efficiency was assessed by examining the *CYP1B1* mRNA expression levels. As shown in Figure 5A, compared to the si-NC group, these three siRNAs knocked down the mRNA expression of *CYP1B1* by 30.6%, 40.2%, and 52.7%, respectively, and we used the si-542 that showed the highest interference efficiency in the following experiments. Compared to si-NC, treatment with si-542 did not significantly change E_2 secretion in the F4-F2 granulosa cells (Figure 5B). However, the mRNA levels of several key steroidogenic genes were altered by this treatment (Figure 5C). Specifically, compared to si-NC, the mRNA levels of *STAR* and *CYP19A1* were reduced ($P < 0.05$), those of *3βHSD* were elevated ($P < 0.05$), while those of *CYP11A1* were not altered ($P > 0.05$) in granulosa cells transfected with si-542.

Since the predicted candidate targets of miR-27b-3p were significantly enriched in the AMP-activated protein kinase (**AMPK**) signaling pathway, which has been previously reported to play a critical role in regulating chicken granulosa cell steroidogenesis (Tosca et al., 2006b; Hu et al., 2017), we further investigated the effects of siRNA-mediated downregulation of *CYP1B1* on the mRNA levels of genes involved in this pathway (Figure 5D). It was observed that compared to si-NC, the mRNA expression levels of *AMPKα* and serine/threonine kinase 11 (*LKB1*) were significantly reduced ($P < 0.05$), those of Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (*CAMKK2*) and transforming growth factor β-activated kinase 1 (*TAK1*) were significantly elevated ($P < 0.05$), while those of STE20-related kinase adaptor α (*STRADA*) were not significantly changed ($P > 0.05$) in goose granulosa cells transfected with si-542.

DISCUSSION

MiR-27b-3p is one of the abundantly expressed miRNAs in granulosa cell layers of both subordinate and dominant follicles during the bovine estrous cycle, implying its important roles in regulating granulosa cell functions (Salilew-Wondim et al., 2014; Gebremedhn et al., 2015). In the present study, we found that miR-27b-3p was expressed at significantly higher levels in granulosa cell layers of goose hierarchical follicles than in those of prehierarchical follicles, which indicated that miR-27b-3p could be involved in regulation of goose ovarian follicle development. It has been previously reported that the steroidogenic capacity of granulosa cells is higher in hierarchical- than in prehierarchical follicles, and granulosa cells of follicles prior to selection are arrested in an undifferentiated state and are commonly considered to be steroidogenically incompetent (Johnson, 2015; Deng et al., 2018). Thus, we further investigated the actions of miR-27b-3p in control of steroid hormone production in goose granulosa cells of the F4-F2 follicles. The results showed that the mimic-mediated upregulation of miR-27b-3p reduced while the inhibitor-mediated downregulation of miR-27b-3p enhanced granulosa cell E_2 secretion, demonstrating

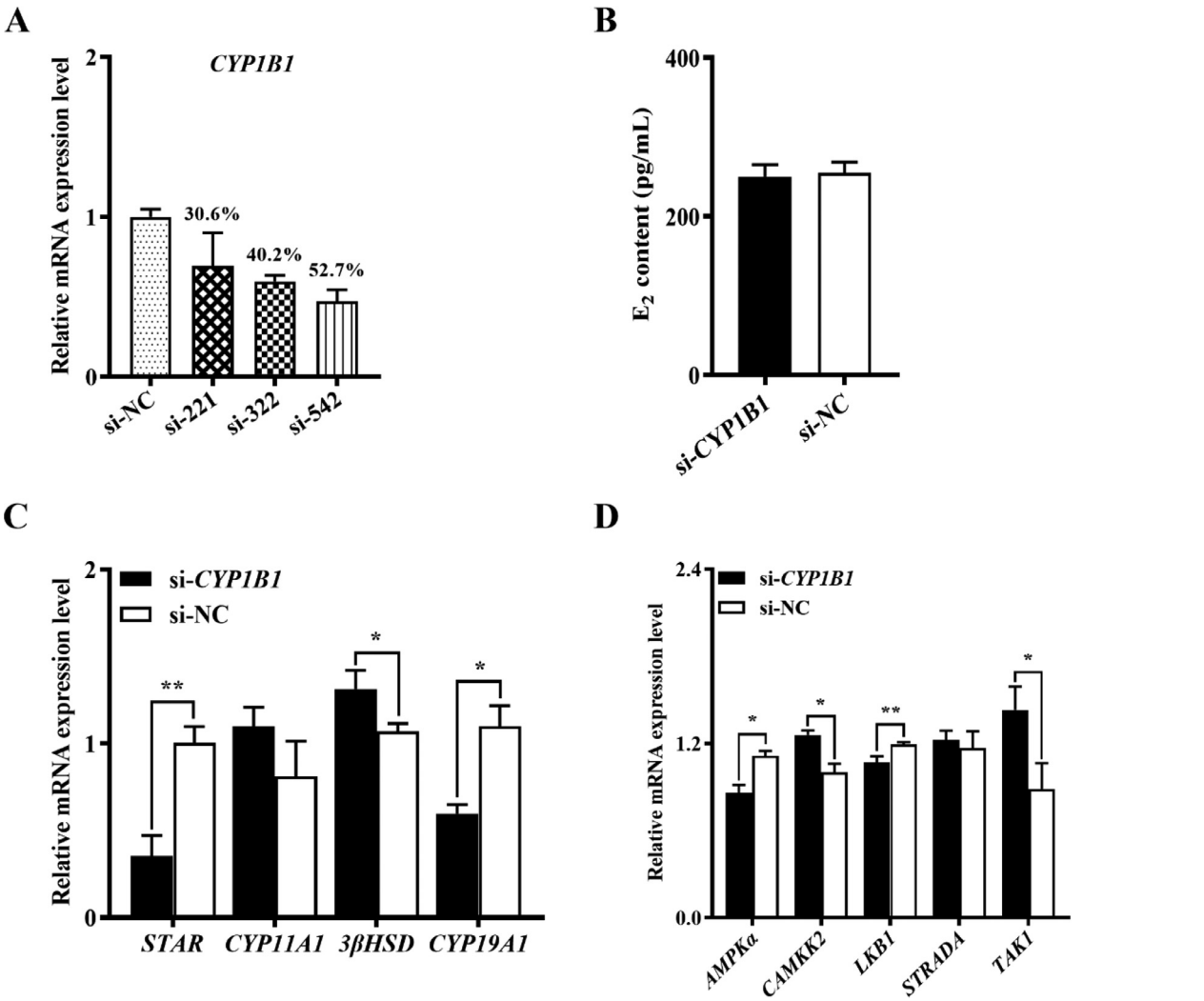

Figure 5. Effects of interference of *CYP1B1* on E_2 secretion by goose F4-F2 granulosa cells. (A) Effects of transfection with three siRNAs specifically targeting *CYP1B1* (namely si-221, si-322, and si-542) on the mRNA expression of *CYP1B1* in goose granulosa cells. (B) Effects of interference of *CYP1B1* by using si-542 on E_2 secretion in goose granulosa cells. (C) Effects of interference of *CYP1B1* by using si-542 on the mRNA expression of several key genes involved in steroid hormone synthesis. (D) Effects of interference of *CYP1B1* by using si-542 on the mRNA expression of several key genes involved in the AMPK signaling pathway. $^{*}P < 0.05$; $^{**}P < 0.01$.

that miR-27b-3p plays a negative role in regulating E_2 secretion of goose F4-F2 granulosa cells.

The major steroidogenic pathways have been well established in avian ovarian follicles and are involved in a series of steroidogenic-related genes, including *StAR, CYP11A1, CYP19A1*, and *3β-HSD*. Specifically, *StAR* plays a critical role in regulation of the rate-limiting step in steroid hormone synthesis, the transport of cholesterol from the outer to the inner mitochondrial membrane (Johnson and Bridgham, 2001), where *CYP11A1* initiates its conversion to pregnenolone, the precursor of all steroid hormones (Tilly et al., 1991). The pregnenolone is subsequently converted to P_4 by *3β-HSD*, and P_4 is converted to androstenedione by cytochrome P450 17α-hydroxysteroid dehydrogenase (*CYP17A1*) (Lee et al., 1998). The derivative androstenedione is then converted to T by 17β-hydroxysteroid dehydrogenase (*17β-HSD*), and T is finally aromatized to E_2 by *CYP19A1* (Lee et al., 1998). Regarding the expression of steroidogenic-related genes, we found that the miR-27b-3p mimic significantly decreased while its inhibitor significantly increased the mRNA levels of *STAR*. Meanwhile, the miR-27b-3p mimic significantly decreased the *CYP19A1* levels but increased the *CYP11A1* and *3β-HSD* levels. These results suggested that the negative effects of miR-27b-3p on E_2 secretion by goose F4-F2 granulosa cells could be exerted by inhibiting both the incorporation of cholesterol into inner mitochondrial membranes and the conversion of P_4 into T or E_2. Noticeably, several studies have demonstrated that both E_2 and P_4 can modulate the expression of miR-27b-3p. In MCF-7 cells, the miR-27b-3p expression levels were enhanced at 6 h but inhibited at 48 h after E_2 treatment (Klinge, 2009; Maillot et al., 2009), and both E_2 and P_4 were shown to suppress the miR-27b-3p expression in human endometrial stromal cells (Reed et al., 2018). These data altogether indicate that there may be a feedback mechanism between the miR-27b-3p expression and steroid hormone secretion, which requires to be further investigated.

Furthermore, through both the bioinformatic prediction and experimental verification, the *CYP1B1* gene

whose 3′-UTR contains the miR-27b-3p binding site was confirmed as a downstream target of miR-27b-3p. As a key member of *CYP* superfamily, *CYP1B1* has been reported to be involved in endogenous metabolic pathways of many important physiological compounds such as E_2, arachidonic acid, melatonin, and retinoids (Li et al., 2017). In the chicken ovary, *CYP1B1* was localized into granulosa cell layers, implying its potential role in regulating granulosa cell steroidogenesis (Zhuge et al., 2009). Moreover, an inverse relationship between the miR-27b-3p and *CYP1B1* expression levels were observed in breast cancerous cells, and miR-27b-3p was also found to regulate cancer cell metabolism by targeting *CYP1B1* (Tsuchiya et al., 2006). These observations were in good accordance with our results, indicating that miR-27b-3p regulates goose granulosa cell steroidogenesis, at least in part, by targeting *CYP1B1*. Indeed, although the siRNA-mediated downregulation of *CYP1B1* showed no significant effects on E_2 secretion, it significantly inhibited the mRNA levels of *STAR* and *CYP19A1* while enhanced those of *3βHSD* in granulosa cells of goose F4-F2 follicles, which were consistent with the miR-27b-3p mimic treatment, supporting the negative role of miR-27b-3p in E_2 synthesis by goose F4-F2 granulosa cells.

Among the KEGG pathways significantly enriched by our predicted target genes of miR-27b-3p, the AMPK signaling pathway is known as a sensor in cellular lipid, glucose, and cholesterol metabolism (Yan et al., 2018). The AMPK signaling has also been previously reported to be involved in regulating ovarian cell steroidogenesis. In rat granulosa cells, increased expression or phosphorylation of AMPK stimulated P_4 secretion (Tosca et al., 2005), while in bovine granulosa cells, phosphorylation of AMPK*α* inhibited the secretion of both P_4 and E_2 (Tosca et al., 2007). In chicken hierarchical granulosa cells, activation of the AMPK signaling significantly increased the secretion of P_4 and expression of several steroidogenic-related genes (Tosca et al., 2006a). Moreover, a recent study showed that knockdown of *CYP1B1* changed cellular energy homeostasis via activation of the AMPK signaling in adult C57BL/6J mice (Liu et al., 2015). In the present study, we found that the siRNA-mediated downregulation of *CYP1B1* significantly reduced the mRNA expression levels of *LKB1* and *AMPKα* while enhanced those of *CAMKK2* and *TAK1* in granulosa cells of goose F4-F2 follicles, suggesting that the steroidogenic actions of *CYP1B1* in goose granulosa cells were mediated by the AMPK signaling pathway.

In conclusion, our results suggest that miR-27b-3p is ubiquitously expressed in granulosa cell layers during goose ovarian follicle development and plays a more important role in hierarchical- than in prehierarchical follicles. Furthermore, the inhibitory actions of miR-27b-3p on goose F4-F2 granulosa cell steroidogenesis are evidenced to be exerted, at least in part, by targeting *CYP1B1* via the AMPK signaling pathway. These data provide novel insights into the miR-27b-3p-mediated post-transcription regulatory mechanisms of granulosa cell steroidogenesis in the goose ovary.

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AUTHOR CONTRIBUTIONS

Conceptualization, Shenqiang Hu, Yujing Rong, and Jiwen Wang; Data curation, Shenqiang Hu, Yujing Rong, Yan Deng, and Li Li; Funding acquisition, Shenqiang Hu, Xin Yuan, and Jiwen Wang; Investigation, Shenqiang Hu, Yujing Rong, Yan Deng, Li Li, Jiwei Hu, Xin Yuan, Hua He, and Liang Li; Methodology, Shenqiang Hu, Yujing Rong, Yan Deng, Li Li, and Xin Yuan; Project administration, Shenqiang Hu, Liang Li, and Jiwen Wang; Resources, Shenqiang Hu, Jiwei Hu, and Jiwen Wang; Supervision, Shenqiang Hu and Jiwen Wang; Visualization, Shenqiang Hu, Yujing Rong, and Yan Deng. Writing − original draft, Shenqiang Hu, Yujing Rong, and Yan Deng; Writing - review & editing, Liang Li and Jiwen Wang.

DISCLOSURES

The authors declare no conflicts of interest.

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