PHYSIOLOGY AND REPRODUCTION

The mediation of pigeon egg production by regulating the steroid hormone biosynthesis of pigeon ovarian granulosa cells

Ying Wang,[*] Hai-ming Yang,[*,1] Chen Zi,[†] Jing Gu,[*] and Zhiyue Wang[*]

College of Animal Science and Technology, Yangzhou University, Yangzhou, Jiangsu Province 225009, P. R. China; and †Department of Pathology, Linyi People's Hospital, Linyi 276000, Shandong Province, China

ABSTRACT The aim of this study was to determine the molecular mechanism of miR-205b targeting 11β-hydroxysteroid dehydrogenase type 1 (**HSD11B1**) on the apoptosis and proliferation of granulosa cells (**GC**) of pigeons. Our previous studies suggested that HSD11B1 was the target gene of miR-205b and played a key role in steroid hormone biosynthesis and GC development. The adenovirus-miR-205b recombinant virus and adenovirus-cli-miR-205b-sh recombinant virus were generated, verified, and their characteristics determined. The recombinant viruses were used to infect the GC of pigeons, with real time quantitative PCR used to examine the expressions of HSD11B1 and related genes. The HSD11B1 antibody was obtained and verified, and Western blotting was used to detect the protein level of HSD11B1. The Cell Counting Kit–8 assay kit was used to detect cell viability, and the Annexin V-FITC/PI kit was used for the apoptosis assays. The expression of HSD11B1 was significantly lower in the overexpression (**OE**) than in OE negative control (**OE-NC**) treatments and significantly higher in short hairpin (**SH**) than in SH negative control (**SH-NC**) treatments. The expression levels of cytochrome P4503A5 was significantly higher in SH and lower in OE treatments, and the rhythms of cytochrome P450 aromatase mRNA levels were similar. The mRNA level of cytochrome P450scc in OE was lower than in OE-NC treatments and higher in SH than in SH-NC treatments. The protein expressions of HSD11B1 were decreased in the GC of OE, whereas increased in the SH group. The Cell Counting Kit–8 assay revealed that overexpression of miR-205b significantly suppressed proliferation of the GC of pigeons, whereas interference of miR-205b significantly induced the proliferation of the GC. The overexpression and the interference of miR-205b did not have a significant effect on cell cycle. The overexpression of miR-205b significantly increased the number of apoptotic cells, whereas the interference of miR-205b decreased the number of apoptotic cells. These findings indicated that miR-205b mediated pigeon egg production by regulating the steroid hormone biosynthesis of the pigeon ovarian GC by targeting HSD11B1, which may be useful in increasing pigeon egg production.

Key words: pigeon, miRNA-205b, granulosa cell, HSD11B1, steroid hormones biosynthesis

INTRODUCTION

In recent years, the White King pigeon (*Columba livia domestica*) has been as popular as commercial fowl, as a source of meat and eggs for the human diet (Pomianowski et al., 2009). Pigeon meat, highly digestible, richer in protein, contains all essential amino acids in amounts and proportions required by humans, is considered as medicine in China (Elsayed et al., 1980; Kabir et al., 2013), and pigeon squabs are of crucial importance for meat production (Sales and Janssens, 2003). The average clutch interval of pigeons is nearly 47.44 D (Khargharia et al., 2003), paired pigeons only lay 2 eggs in a laying period (Horseman and Buntin, 1995), and young squabs are fed by their parents and ejected at the age of 23 to 25 D (Hansen, 1966; Silver, 1984). These characteristics affect the reproductive performance of pigeons, which immediately need to be improved to meet consumer demand.

Lighting patterns are widely used to improve the reproductive performance of poultry, and light wavelengths can affect the physiology and reproduction of hens (Foss and White, 1983), turkeys (Pyrzak and Siopes, 1986), and quails (Woodard, 1969). Our previous studies showed that not only did monochromatic light affect the egg production of pigeons, with red light improving egg production, but the reproductive

performance of pigeons was also improved by monochromatic light supplementation in the morning and evening (Wang et al., 2015, 2019). Transcriptome sequencing and small RNA sequencing have been used to investigate the molecular mechanism of monochromatic lights on egg performance (Wang et al., 2018). Network analysis indicated that 11β-hydroxysteroid dehydrogenase type 1 (**HSD11B1**) was a potential target gene of miR-205b and was likely to play a key regulatory role in steroid hormone biosynthesis pathways by catalyzing the interconversion of inactive cortisone and active cortisol, and its activity is stimulated by progesterone (P_4), prostaglandins, and cortisol (Simmons et al., 2010), which participate in follicle differentiation.

The ovarian follicles are the basic functional unit of the ovary and consist of an outer layer of theca interna cells that encircle inner layers of granulosa cells (**GC**) (Hsueh et al., 1984). The process of ovarian follicle maturation in birds is tightly coupled with the functional differentiation of the GC layer (Johnson and Woods, 2009). The transition of GC from an undifferentiated to a differentiated state is directly associated with follicle selection, and follicle viability is largely attributed to the acquired resistance of the GC layer to apoptosis (Fortune et al., 2001). Hernandez and Bahr (2003) concluded that follicular selection is associated with GC, which serve to regulate the process of follicle selection (Hernandez and Bahr, 2003; Ocon-Grove et al., 2012). Huang et al. (1979) demonstrated that GC isolated from both preovulatory and postovulatory follicles of hens synthesize large amounts of progesterone, which is subsequently secreted and used by the theca layer as the precursor for the synthesis of androgens and estrogens (Porter et al., 1989). Robinson and Etches (1986) suggested that as follicles progress through the hierarchy, the granulosa layer became capable of producing an increasing amount of progesterone, which results in the luteinizing hormone surge and initiates ovulation.

Considering that follicle development is closely related to the GC layer and steroid hormones, this study investigated the mechanism of miR-205b overexpression and interference on the expression of HSD11B1 in GC of pigeons to illustrate the molecular mechanism of miR-205b–mediated pigeon egg production by regulating the steroid hormone biosynthesis of pigeon ovarian GC by targeting HSD11B1.

MATERAILS AND METHODS

Ethics Approval

This study was reviewed and approved by the Institutional Animal Care and Use Committee of the Department of Animal Science and Technology, Yangzhou University and was performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (China, 1988). All pigeon procedures were performed in accordance with the Standards for the Administration of Experimental Practices (Jiangsu, China, 2008).

Pigeon Rearing and Cell Collection

Sixty adult female White King pigeons were obtained from the Tangshan Cuigu Pigeon Industry (Nanjing, China). The females used in this study were about 18 mo of age with a normal egg-laying cycle. They were maintained under their usual light regime of 15 h light and 9 h dark; water and feed were given ad libitum. The ovulation time of the female birds were predicted by the egg records. Pigeons were euthanized by cervical dislocation, and the largest follicles (**F1**) and the second largest follicles of the preovulatory ovarian hierarchy were collected and immediately bathed in ice-cold sterile Dulbecco's PBS (Sigma-Aldrich, Saint Luis, MO), which was supplemented with 100 U/mL penicillin and 0.1 mg/mL streptomycin.

Isolation of GC

The granulosa and theca layers were separated in a laminar flow hood under aseptic conditions (Gilbert et al., 1977), and the granulosa layers derived from the given hierarchical follicle position in different pigeons were combined. After the granulosa layers were collected and cut into pieces, the GC were digested in 1 mg/mL collagenase II at 37°C for 10 min with gentle agitation in a 50-mL tube. The cells were washed with the same volume of M199 medium (HyClone, Logan, UT) and were retrieved by centrifugation at 300 g, for 5 min at room temperature. The supernatant was decanted, and the pellet was resuspended in 10 mL M199 medium supplemented with 10% fetal bovine serum (**FBS**; Gibco, Grand Island, NY), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lovell et al., 2002). The suspension was seeded in six-well culture plates and incubated at 39°C, under 5% CO_2. Cell viability was always more than 90%, which was calculated using trypan blue.

Virus and Construction of Recombinant Transfer Plasmids

The Ad5Max adenovirus vector system was obtained from GeneCreate Biological Engineering Company (Wuhan, China). The HEK293A cells were obtained from GeneCreate Biological Engineering Company and were cultured in Dulbecco's Modified Eagle Medium (**DMEM**; Invitrogen, Carlsbad, CA) with 10% heat-inactivated FBS (Gibco). The pre-microRNA sequence of cli-miR-205b was synthesized and subcloned into the pDC316-mCMV-EGFP adenovirus. The pDC316-mCMV-EGFP vector was digested with Not I and Nhe I and then ligated using T4 DNA ligase at 16°C for 1 h. The ligation products were transformed into TOP10 competent cells, and positive clones were confirmed by restriction enzyme digestion and sequencing. Simultaneously, the interference of cli-miR-205b was amplified and sequenced. The pDC316-ZsGreen-shRNA vector was digested with Pst I and BamH I and then ligated using T4 DNA ligase at 16°C for 1 h. The ligation products were transformed into

TOP10 competent cells, and positive clones were confirmed by restriction enzyme digestion and sequencing.

Generation and Characterization of the Adenovirus-cli-miR-205b Recombinant Virus

HEK293A cells were seeded in a 6-cm dish (Corning, Corning, NY), placed in an incubator at 37°C under 5% CO_2, until a 70 to 80% confluent cell monolayer is formed; then, it was transfected with 1.5 μg of the transfer bacmid pDC316-mCMV-EGFP and 6 μg of pBHGloxDeltaE1,3 using the Lipofectamine 2,000 transfection reagent (Invitrogen), with the negative control generated at the same time. After incubation for 6–10 h at 37°C, under 5% CO_2, the fresh growth medium containing DMEM and 10% heat-inactivated FBS was added, and the cell culture continued incubation at 37°C, under 5% CO_2. When more than 90% cells showed cytopathic effects, they were harvested, frozen and thawed 3 times, and centrifuged for the next passage of the adenovirus-miR-205b recombinant virus (**rAd-cli-miR-205b**). After three passages, the third passage rAd-miR-205b was purified by cesium chloride gradient centrifugation, and the titers of the recombinant adenoviruses were tested by fluorescence dilution. Ten thousand HEK293A cells were seeded into 96-well plates and then maintained at 37°C, under 5% CO_2 for 24 h. The harvested recombinant adenoviruses were serially diluted 10 times in DMEM (Invitrogen), from 10^{-1} to 10^{-11} and vortexed thoroughly. After confluence, the monolayer cells were washed with PBS (Gibco), and then, 100 μL of 10-fold serially diluted virus suspensions was inoculated in 2 replicates per dilution. After 24 h of incubation, 100 μL of DMEM (Invitrogen) with 10% heat-inactivated FBS (Gibco) was added. The cells were monitored for 72 h, and fluorescence microscopy was used to detect the viral titers. The expression level of miR-205b in rAD-PEDV-S was determined by real time quantitative PCR (**RT-qPCR**). The adenoviruses were repeatedly harvested and purified to produce the efficient adenovirus-cli-miR-205b-sh recombinant virus (**rAd-cli-miR-205b-sh**) and an empty control adenovirus (Liu et al., 2019).

RNA Extraction and Synthesis of cDNA

The cells were collected by centrifugation at 300 g for 10 min, adding the same volume of cooled PBS buffer, with the supernatant decanted twice. One milliliter TRI-pure solution and 250 μL trichloromethane were added, mixed, and kept on ice for 5 min and then centrifuged at 10,000g, at 4°C for 10 min. Next, 500 μL of the supernatant was added into the 1.5-mL eppendorf tube, and an equal volume of cold isopropanol was added, mixed, and stood at −20°C for 15 min, before centrifugation at 10,000 g, at 4°C for 10 min. The liquid was carefully poured out, and 1 mL cold 75% ethanol was added,

mixed, centrifuged at 10,000 g, 4°C for 5 min, before the liquid was poured out, and dried for several min. Ten microliter of RNase-free water was then added to dissolve the RNA (Chomczynski and Sacchi, 1987). The cDNA synthesis was carried out using an M-MLV Reverse Transcriptase Kit as per the manufacturer's instructions (ELK Biotechnology, Wuhan, China). The cDNA synthesis reactions, using 1 μL of oligo (dT), 1 μL dNTPs, 1 μg RNA, and RNaser-free water was added to 15 μL, were carried out by RT-PCR at 70°C, for 5 min, and then on ice for 2 min. Four microliter of RT buffer and 1 μL M-MLV reverse transcriptase were added, and the procedure was carried out at 42°C for 60 min and then at 95°C for 5 min.

The expressions of genes HSD11B1, cytochrome P4503A5 (**CYP3A5**), cytochrome P450 aromatase (**CYP19A1**), cytochrome P450scc (**CYP11A1**) and glyceraldehyde-3-phosphate dehydrogenase were detected by RT-qPCR, and primers were designed against the coding region of the target gene (Table 1). The EnTurbo SYBR Green PCR SuperMix Kit (ELK Biotechnology) was used to detect the expressions of genes. The ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) was used for the miR-205b (forward: 5′-CTCAACTGGTGTCGTG-GAGTCGGCAATTCAGTTGAGCAGATTCC-3′) and U6 (forward: 5′-GGAACGATACAGAGAAGAT-TAGCA-3′) and the SYBR Green Realtime PCR Master Mix Kit (Toyobo) was used to detect the expressions of miR-205b (forward: 5′-ACACTC-CAGCTGGGCCCTTCATTCCA CCGG-3′) and U6 (forward: 5′-CACATATACTAAAATTGGAACG-3′). Assays were repeated independently 3 times. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase and U6 served as an internal reference, and each sample was analyzed thrice. Relative mRNAs and cli-miR-205b expressions were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittge, 2001).

Western Blotting of HSD11B1

Total proteins were extracted from GC using the total protein extraction kit (Boster Tech, Wuhan, China), and the protein concentrations were measured using the BCA Protein Assay Kit (Solarbio, Beijing, China).

Table 1. Primer sequences of genes and GAPDH gene.

Gene	5'-3'	Length (bp)
HSD11B1	F: TTCCCTTTACGGTTCCCTACTCC	131
	R: AGTATCAATGAAGCCGAGGATGC	
CYP11A1	F: CCACCTCGCCCATGCTCT	171
	R: CCATGTACTCCTTGGTGCTCT	
CYP19A1	F: GGGCTGGTGCGTATGATTGC	86
	R: TTTCCGACTTCAGTGGTTACTTCC	
CYP3A5	F: ATGAGCCCACCAGTAACACA	170
	R: GGTTTCATTCACGGCCATGT	
GAPDH	F: CTCTACTCATGGCCACTTCCG	138
	R: ACAACGTATTCAGCACCAGC	

Abbreviations: CYP19A1, cytochrome P450 aromatase; CYP11A1, cytochrome P450scc; CYP3A5, cytochrome P4503A5; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSD11B1, 11β-hydroxysteroid dehydrogenase type 1.

After culturing the GC of pigeons for 24 h, the cells were harvested by centrifugation and subjected to washing with ice-cold PBS twice. The cell pellet was resuspended in RIPA lysis buffer, and the protein content of cell lysates was determined by the Bradford assay. From each sample, 40 µg of protein was separated by 10–15% SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). The membranes were then treated with tris-buffered saline and exposed to primary antibody at 4°C. The primary antibody was ordered especially for pigeons from GeneCreate Biological Engineering, and the polyclonal antibodies were obtained by immunizing 2 rabbits using the prepared recombinant proteins. This antibody was used for Western blot validation of the recombinant protein, and special recognition was obtained at 42 kDa. Further endogenous protein detection was performed in GC of other pigeons, and the target protein was again found at 42 kDa and control protein (Mahmood and Yang, 2012). The membrane was blocked with casein (1%) blocking buffer for 1 h at room temperature, the blocking solution was removed, and the diluted primary antibody (rabbit polyclonal antibodies, 1:2,000) was added at 4°C and incubated overnight. The membrane was then washed with tris-buffered saline Tween for 5 min, repeated 3 times, and the diluted secondary antibody (horseradish peroxidase-goat anti-rabbit secondary antibody, 1:5,000) was added and incubated at room temperature for 30 min. The membrane was then washed with tris-buffered saline Tween for 5 min and repeated 4 times. All washing antibody incubation steps were performed on a shaker at room temperature, and the enhanced chemiluminescence system was used to detect the results.

Cell Viability Assay

The Cell Counting Kit–8 (**CCK-8**) assay kit (Solarbio) was used to detect cell viability. The GC were seeded in 96-well plates at a rate of 4,000 per plate. The groups included GC of pigeon infected with the recombinant rAd-cli-miR-205b (**OE**) and its negative control (**OE-NC**), rAd-cli-miR-205b-sh (**SH**) and its negative control (**SH-NC**), and wild type (**WT**). Each treatment had triplicates, with triplicates, and was incubated at 37°C, under 5% CO2 for 24 h and then subjected to treatment with 10 µL CCK-8 solution. The cells were subjected to incubation for 2 h at 37°C, under 5% CO2, and the optical density at 450 nm was examined at zero, 24, and 48 h, with the help of a microplate reader.

Apoptosis Assays and Cell Cycle Determination by PI

The ovarian GC with different treatments were seeded in 6-well plates and incubated at 37°C, under 5% CO2 for 24 h. The treated and control cells were harvested and washed with cold PBS. The Annexin V-FITC/PI kit was used for the apoptosis assays (Solarbio). A million cells were collected with 100 µL annexin binding buffer and incubated with 5 µL Annexin V-FITC and 5 µL PI in the dark for 5 min. Finally, 400 µL binding buffer was added into the samples before they were analyzed using the Attune NxT (Thermo Fisher Scientific, Waltham, MA). The tube without Annexin V-FITC and PI was used as negative control.

Cold PBS was used to collect the cells harvested by centrifugation after 3 repeats and were fixed gently by putting cold 70% ethanol (in PBS) in 4°C overnight. The cells were washed by centrifugation thrice to remove residual ethanol and then resuspended in PBS containing 20 µg/mL PI and 0.2 mg/mL RNase and 0.1% Triton X-100 in a dark room. After 15 min at 37°C, the cells were analyzed with a flow cytometer (Becton-Dickinson, San Jose, CA).

Statistical Analysis

Data were expressed as mean ± SD. One-way ANOVA was performed with SPSS 13.0 software (SPSS Inc., Chicago, IL). The statistical significance of differences among the various groups was evaluated by Tukey's honestly significant difference post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Generation and Characterization of the Recombinant rAd-cli-miR-205b and rAd-cli-miR-205b-sh

The recombinant rAd-cli-miR-205b was amplified, colonies were selected and cultured in Luria Bertani medium, and the plasmid DNA was extracted, which was directly used for sequencing to verify the vector construction. The primer of cytomegalovirus promoter of rAd-cli-miR-205 vector of pDC316-mCMV-EGFP
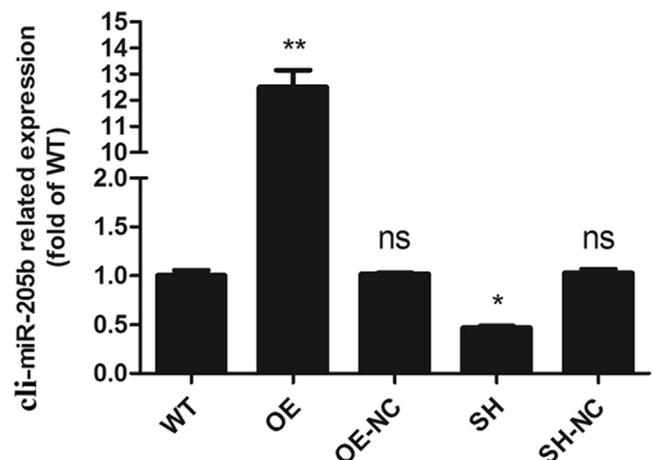
Figure 1. Measurement of expression levels of cli-miR-205b of the recombinant rAd-cli-miR-205b (OE) and its negative control (OE-NC), rAd-cli-miR-205b-sh (SH) and its negative control (SH-NC), and wild type (WT) in infected granulosa cells of pigeon. The * indicates a significant difference, with the P value less than 0.05; ** indicates the P value less than 0.01. Abbreviation: ns, not significant.

(pCMV-F: CGCAAATGGGCGGTAGGCGTG) and the primer of U6 promoter of rAd-cli-miR-205b-sh vector of pDC316-ZsGreen-ShRNA (pU6-F: ATGGAC-TATCATATGCTTACCGTA) were used for sequencing. The sequence of rAd-cli-miR-205b was 5′-CTTGGCACCCTTCATTCCACCG-GAATCTGTCTA TACAGAAACCAGATTTCAGT-GAAATGAAGCCTGTCAGAGAGGCAG-3' (78 bp). The recombinant rAd-cli-miR-205b-sh was also amplified and sequenced as 5′-CCCTTCATTCCACCG-GAATCTG-3' (22 bp). The expression levels of the recombinant rAd-cli-miR-205b and rAd-cli-miR-205b-sh in the GC of pigeons were detected in vitro by RT-qPCR, with the results showing that rAd-cli-miR-205b was significant higher and the rAd-cli-miR-205b-sh significant lower than the WT group of miR-205b ($P < 0.01$) (Figure 1). The results of the fluorescence dilution assay suggested that the recombinant rAd-cli-miR-205b and rAd-cli-miR-205b-sh could be replicated in HEK293A cells and that its titers could reach 10^{11} pfu/mL (Figure 2).

The Expressions of HSD11B1 and Key Genes of Steroid Hormone Biosynthetic Signaling Pathway

The expressions of HSD11B1 in the GC, which were infected by recombinant rAd-cli-miR-205b and rAd-cli-miR-205b-sh, were examined (Figure 3A), and they were significantly lower in the OE treatment than in the OE-NC treatment ($P < 0.05$) and significantly higher in the SH treatment than in the SH-NC treatment ($P < 0.01$). The expressions of CYP3A5, CYP11A1 and CYP19A1 were detected, which are important in the steroid hormone biosynthetic signaling pathway. The expression levels of CYP3A5 were significantly higher in SH and lower in OE treatments ($P < 0.05$), and the rhythms of CYP19A1 mRNA levels were similar (Figures 3B, 3D). The mRNA level of CYP11A1 in OE was lower than in OE-NC treatments ($P < 0.01$) and higher in SH than in SH-NC treatments ($P < 0.01$) (Figures 3C, 3E).

The quality of the customized HSD11B1 antibody was verified, the GC of pigeons were collected, and the protein expression of HSD11B1 was measured. The results showed that HSD11B1 antibody could be detected in pigeon GC (Figure 4A). The protein expressions of HSD11B1 of the GC infected with the recombinant virus were also analyzed and were found to be decreased in the GC of the OE group compared with that of WT, whereas increased in SH treatment (Figure 4B).

Cell Cycle Arrest and Apoptosis in Pigeon GC

The CCK-8 assay revealed that overexpression of miR-205b significantly suppressed cell proliferation of the GC of pigeons, whereas the interference of miR-205b significantly induced proliferation of cells in pigeon GC (Figure 5A). The GC of pigeons were infected with the recombinant rAd-cli-miR-205b and rAd-cli-miR-205b-sh after 48 h, and the cell cycle and apoptosis levels were detected by flow cytometry. The results revealed that the overexpression of miR-205b significantly increased the number of apoptotic cells ($P < 0.001$),
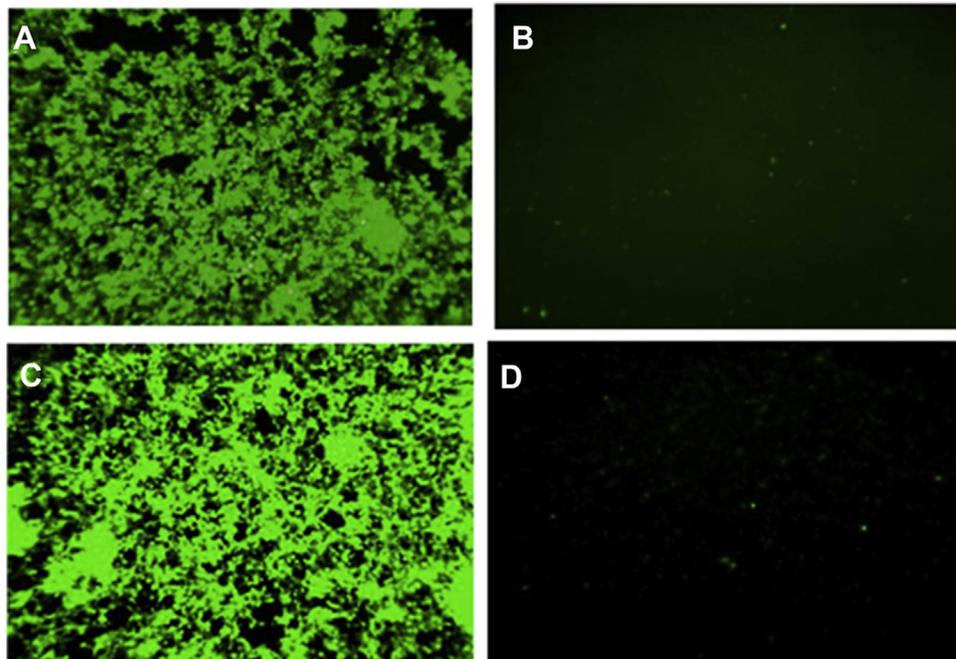
Figure 2. Titer of the virus determined by the rAd-cli-miR-205b (A, B) and rAd-cli-miR-205b-sh (C, D) fluorescence dilution assay. Green fluorescence was emitted by the enhanced green fluorescent protein derived from the vector. The results showed that the recombinant viruses could be well replicated in HEK293A cells and that its titers could reach 10^{11} pfu/mL.
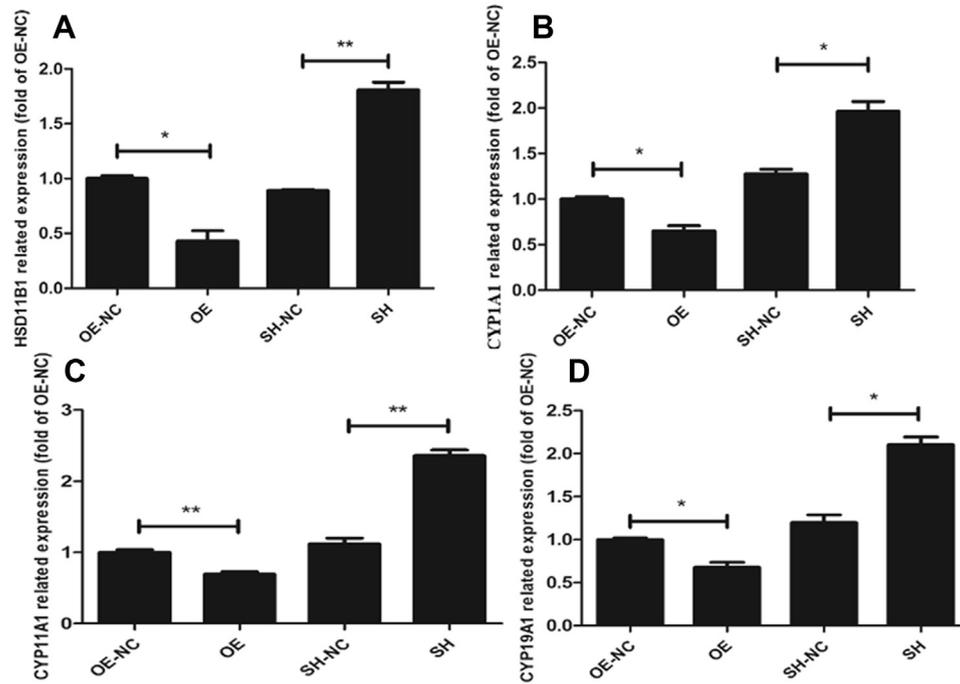
Figure 3. Measurement of mRNA levels of key genes of granulosa cells of pigeon infected with the recombinant rAd-cli-miR-205b (OE) and its negative control (OE-NC) and rAd-cli-miR-205b-sh (SH) and its negative control (SH-NC). Abbreviations: HSD11B1, 11β-hydroxysteroid dehydrogenase type 1; CYP11A1, cytochrome P450scc; CYP3A5, cytochrome P4503A5; CYP19A1, cytochrome P450 aromatase.

whereas the interference of miR-205b decreased the number of apoptotic cells ($P < 0.05$) (Figure 5C). Neither the overexpression nor the inference of miR-205b had a significant effect on the cell cycle ($P > 0.05$) (Figure 5B).

DISCUSSION

In this study, the GC of pigeons were used to demonstrate the molecular mechanism of miR-205b mediating egg production of pigeons by targeting HSD11B1. In pigeons, 40 to 48 h will elapse from the time of ovulation until oviposition (Riddle, 1942). The paired pigeons were selected just 1 to 2 D before laying with the second egg of the clutch being laid 2 D after the first (Birrenkott et al., 1988); however, even with reference to their laying records, the follicles were at different stages, which may be related to the acclimatization of pigeons and their unique reproductive characteristics (Silver, 1984; Khargharia et al., 2003). As a result, this study used F1 and partially second largest follicles, which was also

owing to difficulties finding 2 big follicles even if the pigeon was nearly ovulating as per its record, so the developmental rhythm and changes of follicles before ovulation needs further study to understand it better. The antibody of HSD11B1 of pigeons used for WB was constructed and verified for the first time, which will be helpful for future studies that examine the protein expression of HSD11B1 in pigeons.

The recombinant rAd-cli-miR-205b and rAd-cli-miR-205b-sh were successfully established in this study, the former as overexpression of miR-205b and the latter because of the interference of miR-205b. The miR-205b was screened through deep sequencing on ovaries under different monochromatic lights; Shen et al. (2019) also illustrated that miR-205b binds with circRNA, which was differentially expressed in GC of hens and involved in follicular development. The HSD11B1 had been validated as the potential target of miR-205 by dual-luciferase assay (Wang et al., 2019). It was found that HSD11B1 was highly upregulated in the ovary of the pigeon under red light (Wang et al., 2019), and the mRNA
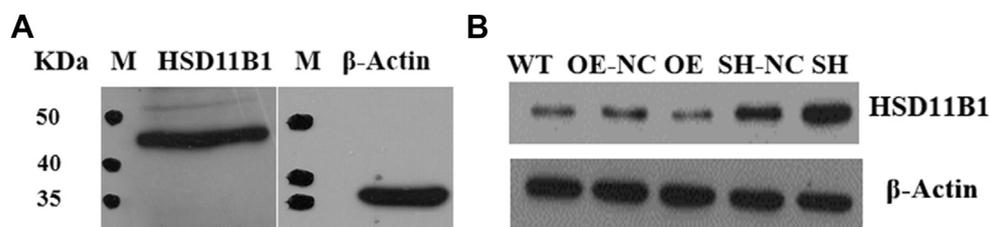
Figure 4. Measurement of protein levels of HSD11B1 of granulosa cells of pigeon infected with the recombinant rAd-cli-miR-205b (OE) and its negative control (OE-NC), rAd-cli-miR-205b-sh (SH) and its negative control (SH-NC) and wild type (WT). Abbreviation: HSD11B1, 11β-hydroxysteroid dehydrogenase type 1.

A

B

C
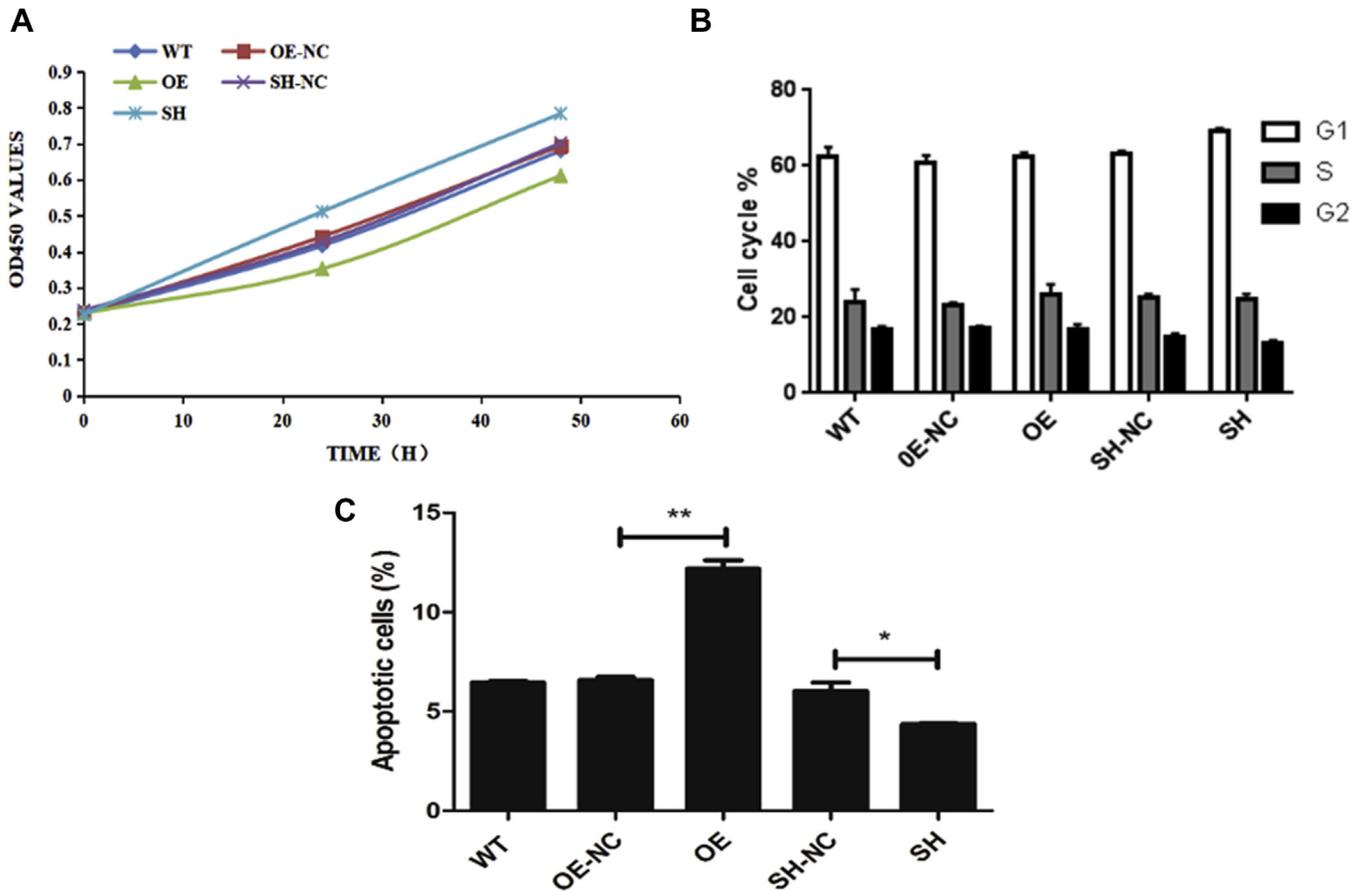
Figure 5. Recombinant miR-205b virus induced apoptosis in granulosa cell of pigeons. (A) CCK-8 assay revealed the proliferation of granulosa cells, at 24 h and 48 h, the OD values of SH was significantly higher than SH-NC, at 24 h and 48 h, the OD values of OE was significantly lower than OE-NC. (B) Cell cycle analysis by flow cytometry. (C) Apoptosis in chondrocytes was measured by Annexin V/PI double-staining assay. Abbreviations: OD, optical density; OE, recombinant rAd-cli-miR-205b; OE-NC, negative control of recombinant rAd-cli-miR-205b; SH, rAd-cli-miR-205b-sh; SH-NC, negative control of rAd-cli-miR-205b-sh.

and protein levels of HSD11B1 were decreased in GC infected with rAd-cli-miR-205b and increased in GC infected with rAd-cli-miR-205b-sh, which confirmed that miR-205b could affect the function of GC through targeting HSD11B1. This result was consistent with that of the study by Xu et al. (2011), indicating that the expression of HSD11B1 increased after an ovulatory stimulus, which promoted maturing follicles. Yong et al. (2000) also confirmed that 11β-HSD activity was inducible in human GC by human chorionic gonadotropin administration. In the final stage of oocyte maturation, cortisol plays a beneficial role, and ovarian type 1 11β-HSD assures its adequate concentration for follicular maturity (Lewicka et al., 2003).

The expressions of CYP3A5, CYP11A1, and CYP19A1 were also detected in this study, with the rhythms of these genes being similar to those of HSD11B1. The expressions of these genes in GC of F1 follicles were high, which is according to Porter et al. (1997) showing that GC were the primary sites of CYP isoform activity. The expression of CYP11A1 increased in F1 follicles, to produce the large amounts of progesterone needed for ovulation in response to the luteinizing hormone surge (Johnson et al., 2002). During the

transition of prehierarchical follicles to a preovulatory hierarchy, the cells of granulosa layer begin to produce CYP11A1 protein and progesterone, the CYP19A1 protein being responsible for estrogen biosynthesis (Meng et al., 2019). The expression of CYP3A5 increases during follicular development and catalyzes the formation of 16α-hydroxy estrone/estradiol (Lee et al., 2003), and it is downregulated in PGF2α-induced corpus luteum (Bishop et al., 2011), which was in accordance with our study. The accumulated evidence indicates that miR-205b affects the expression of HSD11B1 and key genes of steroid hormones biosynthesis signaling pathway in GC. However, further research is required to identify the effect of miR-205b on steroid hormonal biosynthesis of GCs of prehierarchical follicles of pigeons under different monochromatic lights.

CONCLUSION

In conclusion, this study provides the first evidence that miR-205b is expressed in the GC of pigeons, being downregulated there, and its overexpression inhibited their proliferation by triggering apoptosis. The overexpression of miR-205b inhibited the expression of

HSD11B1, CYP3A5, CYP11A1, and CYP19A1, which are important to steroid hormones biosynthesis, whereas miR-205b inference induced their expression. Combined with the previous study, the results suggest that miR-205b mediates pigeon egg production by regulating the steroid hormones biosynthesis of pigeon ovarian GC, by targeting HSD11B1. These findings provide insight into the molecular mechanism of miR-205b on GC and follicular development, which will help to increase egg production of pigeons.

ACKNOWLEDGMENTS

Conflict of Interest Statement: The authors declare that they have no competing interests.

REFERENCES

Birrenkott, G. P., M. A. Shoop, K. Cooper, and M. Wiggins. 1988. Research note: ovarian follicular growth and maturation in the domestic pigeon and Guinea fowl (Numida meleagris). Poult. Sci. 67:1783–1786.

Bishop, C. V., R. L. Bogan, J. D. Hennebold, and R. L. Stouffer. 2011. Analysis of microarray data from the macaque corpus luteum; the search for common themes in primate luteal regression. Mol. Hum. Reprod. 17:143–151.

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156–159.

Elsayed, W. A., A. H. Shehab, F. E. Mourad, F. I. El-Nahry, and A. K. Said. 1980. Biochemical and biological evaluation of pigeon meats: effect of type, age, and sex. Nahrung 24:821–828.

Fortune, J. E., G. M. Rivera, A. C. Evans, and A. M. Turzillo. 2001. Differentiation of dominant versus subordinate follicles in cattle. Biol. Reprod. 65:648–654.

Foss, D. C., and J. L. White. 1983. Early sexual maturity of brown-egg pullets cage-grown in narrow-band light with high nutrient density diets. Poult. Sci. 62:1424.

Gilbert, A. B., A. J. Evans, M. M. Perry, and M. H. Davidson. 1977. A method for separating the granulosa cells, the basal lamina, and the theca of the preovulatory ovarian follicle of the domestic fowl (Gallus domesticus). Reproduction 50:179–181.

Hansen, E. W. 1966. Squab-induced crop growth in ring dove foster parents. J. Comp. Physiol. Psychol. 62:120.

Hernandez, A. G., and J. M. Bahr. 2003. Role of FSH and epidermal growth factor (EGF) in the initiation of steroidogenesis in granulosa cells associated with follicular selection in chicken ovaries. Reproduction 125:683–691.

Horseman, N. D., and J. D. Buntin. 1995. Regulation of pigeon crop milk secretion and parental behaviors by prolactin. Ann. Rev. Nutra. 15:213–218.

Hsueh, A. J., E. Y. A dashi, P. B. Jones, and T.H. Welsh, Jr. 1984. Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr. Rev. 5:76–127.

Huang, E. S. R., K. J. Kao, and A. V. Nalbandov. 1979. Synthesis of sex steroids by cellular components of chicken follicles. Biol. Reprod. 20:454–461.

Johnson, A. L., E. V. Solovieva, and J. T. Bridgham. 2002. Relationship between steroidogenic acute regulatory protein expression and progesterone production in hen granulosa cells during follicle development. Biol. Reprod. 67:1313–1320.

Johnson, A. L., and D. C. Woods. 2009. Dynamics of avian ovarian follicle development: cellular mechanisms of granulosa cell differentiation. Gen. Comp. Endocrinol. 163:12–17.

Kabir, M. A. 2013. Productivity of crossed indigenous pigeon in semi intensive system. Basic Res. J. Agric. Sci. Rev. 2:1–4.

Khargharia, G., R. N. Goswin, and D. Das. 2003. Performance of domestic pigeon of Assam in respect of some traits of reproduction. Indian Vet. J. 80:163–165.

Lee, A. J., M. X. Cai, P. E. Thomas, A. H. Conney, and B. T. Zhu. 2003. Characterization of the oxidative metabolites of 17β-estradiol and estrone formed by 15 selectively expressed human cytochrome P450 isoforms. Endocrinology 14:3382–3398.

Lewicka, S., C. von Hagens, U. Hettinger, K. Grunwald, P. Vecsei, B. Runnebaum, and T. Rabe. 2003. Cortisol and cortisone in human follicular fluid and serum and the outcome of IVF treatment. Hum. Reprod. 18:1613–1617.

Livak, K. J., and T. D. Schmittge. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.

Lovell, T. M., R. T. Gladwell, N. P. Groome, and P. G. Knight. 2002. Modulatory effects of gonadotrophins and insulin-like growth factor on the secretion of inhibin A and progesterone by granulosa cells from chicken preovulatory (F1-F3) follicles. Reproduction 123:649–657.

Liu, X., D. Zhao, P. Zhou, Y. Zhang, and Y. Wang. 2019. Evaluation of the Efficacy of a recombinant adenovirus expressing the spike protein of porcine epidemic diarrhea virus in pigs. Biomed. Res. Int. 2019:1–8.

Mahmood, T., and P. C. Yang. 2012. Western blot: technique, theory, and trouble shooting. North Am. J. Med. Sci. 4:429.

Meng, B., Z. Cao, Y. Gai, M. Liu, M. Gao, M. Chen, Z. L. Ning, and X. Luan. 2019. Effects of recombinant goose adiponectin on steroid hormone secretion in Huoyan geese ovarian granulosa cells. Anim. Reprod. Sci. 205:34–43.

Ocon-Grove, O. M., D. H. Poole, and A. L. Johnson. 2012. Bone morphogenetic protein 6 promotes FSH receptor and anti-Müllerian hormone mRNA expression in granulosa cells from hen prehierarchal follicles. Reproduction 143:825–833.

Pomianowski, J. F., D. Mikulski, K. Pudyszak, R. G. Cooper, M. Angowski, A. Jóźwik, and J. O. Horbańczuk. 2009. Chemical composition, cholesterol content, and fatty acid profile of pigeon meat as influenced by meat-type breeds. Poult. Sci. 88:1306–1309.

Porter, M. B., B. D. Cleaver, M. Peltier, G. Robinson, and D. C. Sharp. 1997. The effect of pulsatile gonadotropin-releasing hormone and estradiol administration on luteinizing hormone and follicle-stimulating hormone concentrations in pituitary stalk-sectioned ovariectomized pony mares. Domest. Anim. Endocrin. 14:275–285.

Porter, T. E., B. M. Hargis, J. L. Silsby, and M. E. E. Halawani. 1989. Differential steroid production between theca interna and theca externa cells: a three-cell model for follicular steroidogenesis in avian species. Endocrinology 125:109–116.

Pyrzak, R., and T. D. Siopes. 1986. Effect of light quality on egg production of caged Turkey hens. Poult. Sci. 65:199–200.

Riddle, O. 1942. Cyclic changes in blood calcium, phosphorus, and fat in relation to egg laying and estrogen production. Endocrinology 31:498–506.

Robinson, F. E., and R. J. Etches. 1986. Ovarian steroidogenesis during follicular maturation in the domestic fowl (Gallus Domesticus). Biol. Reprod. 35:1096–1105.

Sales, J., and G. P. J. Janssens. 2003. Nutrition of the domestic pigeon (Columba livia domestica). Worlds Poult. Sci. J. 59:221–232.

Shen, M., T. Li, G. Zhang, P. Wu, F. Chen, Q. Lou, L. Chen, X. Yin, T. Zhang, and J. Wang. 2019. Dynamic expression and functional analysis of circRNA in granulosa cells during follicular development in chicken. BMC Genomics 20:96.

Simmons, R. M., M.C. Satterfield, Jr, T. H. Welsh, F. W. Bazer, and T. E. Spencer. 2010. HSD11B1, HSD11B2, PTGS2, and NR3C1 expression in the peri-implantation ovine uterus: effects of pregnancy, progesterone, and interferon tau. Biol. Reprod. 82:35–43.

Silver, R. 1984. Prolactin and parenting in the pigeon family. J. Exp. Zool. 232:617–625.

Wang, Y., J. T. Ding, H. M. Yang, W. Cao, and Y. B. Li. 2015. The effect of new monochromatic light regimes on egg production and expression of the circadian gene BMAL1 in pigeons. Poult. Sci. 94:836–840.

Wang, Y., H. M. Yang, W. Cao, Y. B. Li, and Z. Y. Wang. 2018. Deep sequencing identification of miRNAs in pigeon ovaries illuminated with monochromatic light. BMC Genomics 19:446.

Wang, Y., H. Yang, C. Zi, and Z. Wang. 2019. Transcriptomic analysis of the red and green light responses in Columba livia domestica. 3 Biotech 9:20.

Woodard, A. E., J. A. Moore, and W. O. Wilson. 1969. Effect of wavelength of light on growth and reproduction in Japanese quail (Coturnix japonica). Poult. Sci. 48:118–123.

Xu, F., R. L. Stouffer, J. Müller, J. D. Hennebold, J. W. Wright, A. Bahar, G. Leder, M. Peter, M. Thorne, M. Sims, T. Wintermantel, and B. Lindenthal. 2011. Dynamics of the transcriptome in the primate ovulatory follicle. Mol. Hum. Reprod. 17:152–165.

Yong, P. Y., K. J. Thong, R. Andrew, B. R. Walker, and S. G. Hillier. 2000. Development-related increase in cortisol biosynthesis by human granulosa cells. J. Clin. Endocrinol. Metab. 85:4728–4733.