


miR-193-5p negatively regulates *PIK3CD* to promote crop fibrocyte proliferation in pigeon (*Columba livia*)

Hui Ma,¹ Pingzhuang Ge,¹ Shixiong Bian, Yunlei Li, Aixin Ni, Ran Zhang, Yuanmei Wang, Jinmeng Zhao, Yunhe Zong, Jingwei Yuan, Yanyan Sun, and Jilan Chen ²

Key Laboratory of Animal (Poultry) Genetics Breeding and Reproduction, Ministry of Agriculture and Rural Affairs, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100193, China

ABSTRACT The crop of pigeon has specific characteristics as producing crop milk in the lactating period. However, the exact mechanisms underlying the regulation of crop lactation remain unclear. miRNAs, the essential regulators of gene expression, are implicated in various physiological and biological activities. In this study, we discovered a new miRNA that regulated phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta (*PIK3CD*) and crop fibrocyte proliferation. Results of the luciferase

reporter assay suggested that miR-193-5p suppressed *PIK3CD* expression by targeting a conserved binding site in the 3'-untranslated region (**UTR**) of *PIK3CD* mRNA. MiR-193-5p promoted crop fibrocyte proliferation and migration, whereas *PIK3CD* inhibited these effects. These findings suggested an important regulatory role of miR-193-5p in crop fibrocyte proliferation, suggesting that miR-193-5p and *PIK3CD* might be important regulators of crop milk production.

Key words: *PIK3CD*, miR-193-5p, crop fibrocyte, cell proliferation, pigeon

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INTRODUCTION

Both male and female pigeons could produce a curd-like substance in their crops called crop milk (Gillespie et al., 2011). Similarly, male and female flamingos and male emperor penguins could produce crop milk, whereas in other birds, crops only had the function of food storage (Gillespie et al., 2011). Crop produced crop milk 2 d before the first egg hatches following the regulation of hormone as prolactin (Dumont, 1965). Studies of the synthesis of crop milk by crop have been conducted since 1786, and prolactin has been shown to play an important role in lipid accumulation, fatty acid transportation and de novo synthesis at the hormone level (Dumont, 1965; Wan et al., 2019). In the lactation period, the germinal cell layer of the crop proliferated quickly and formed the nutritive cell layer in response to prolactin (Dumont, 1965; Wan et al., 2019). With the consist proliferation of crop fibrocyte, it began to be cornified and sloughed off to produce milk (Gillespie et al.,

2013). But the physiological mechanism of lactation in pigeon is not similar to that in mammals. Mammalian mammary glands are glandular tissues, but the crop of pigeon is not glandular (Gillespie et al., 2011). Thus, it is necessary to reveal the molecular mechanism regulating crop milk protein synthesis, fatty acid synthesis, fatty acid transporters, and the differentially expressed gene enriched pathways (Xie et al., 2017; Chen et al., 2019; Xie et al., 2019; Chen et al., 2020).

Phosphoinositide 3-kinase (*PI3K*) plays a key role in physiological activities as metabolic regulations by activating serine-threonine protein kinase AKT and its downstream effector (Du et al., 2018). *PI3K* can be divided into 3 classes according to their substrate specificity and structures. Class I *PI3K* can be divided into subclasses IA and IB. The catalytic isoforms of Class IA including p110 α , p110 β , and p110 δ are encoded by *PIK3CA*, *PIK3CB*, and *PIK3CD*, respectively (Thorpe et al., 2015; Singh et al., 2016; Bilanges et al., 2019). When the growth factors or ligand bind to tyrosine kinases receptor, phosphatidylinositol 4,5-bisphosphate (**PIP2**) is phosphorylated by PI3Ks, then phosphatidylinositol 3,4,5-trisphosphate (**PIP3**) is generated (Raimondi and Falasca, 2012). Then the pleckstrin homology (**PH**) domain-containing proteins such as *AKT* could be recruited by PIP3 to the plasma membrane. These events activate the signaling cascade to promote metabolic activities (Kriplani et al., 2015).

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¹These authors contributed equally to this work.

²Corresponding author: chen.jilan@163.com

MicroRNAs (miRNAs) are noncoding RNAs made up of approximately 21 to 25 nucleotides, which can regulate gene expression post-transcriptionally and control diverse biological processes including cell proliferation, division, migration, and apoptosis (Bartel, 2004; He and Hannon, 2004; Bartel, 2009; Pennisi, 2014). miRNAs inhibit gene expression by targeting the 3'-untranslated region (UTR) of mRNA to induce mRNA degradation and suppress of translation (Bartel, 2004; Bartel, 2009). Many studies have showed that miRNAs played important roles in regulating milk production and milk composition synthesis. As report, miR-103, miR-17-5p, and miR-148a expressed in goat mammary epithelial cells enhanced milk fat synthesis by facilitating lipid droplet formation, whereas miR-181a, miR-34b, and miR-130a expressed in bovine mammary epithelial cells inhibited the synthesis of milk fat by downregulating the genes participated in lipid synthesis (Lin et al., 2013; Chen et al., 2017; Wang et al., 2019). However, few studies have examined the crop milk synthesis regulated by miRNAs. We identified the miRNAs associated ceRNA networks regulating crop milk production by transcriptome sequencing, and we found miR-193-5p was an important miRNAs in lactation which targeted *PIK3CD* (Ge et al., 2020; Ma et al., 2020).

MATERIALS AND METHODS

Fibrocytes Isolation, Culture, and Identification

Fibrocytes were obtained from the crop of pigeon. Crops were isolated from the incubated pigeon eggs at 17 d under sterile conditions. The tissues were rinsed with PBS and minced to 1 mm³ of pieces. The minced tissues were added to centrifuge tubes containing 0.25% pancreatic enzymes, then the tubes were put in a 37°C waterbath and digested for 30 min. The tissue digests were mixed with DMEM (Gibco, Grand island, NY) and centrifuged 2 times (5 min, 1,000 r/min), then a 200- μ m sterile nylon mesh filter was used to prepare cellular suspensions (Zhang et al., 2021). Cells were cultured in DMEM (Gibco) containing 100 IU/mL penicillin, 100 μ g/mL streptomycin, and 10% fetal bovine serum (Gibco), incubated in a humidified incubator at 37°C in 5% CO₂, and identified by immunofluorescence (Galligan and Fish, 2017; Zhou et al., 2020).

Plasmid Construction

To construct dual-luciferase miRNA target reporter vector, the segment sequences of *PIK3CD* 3'-UTR that contained the putative miR-193-5p binding sequence were amplified by PCR, and then subcloned into XhoI and NotI restriction sites in the psi-check-2 dual luciferase reporter vector. The wild-type and mutant *PIK3CD* 3'-UTRs were named as psi-check-wt and psi-check-mut, respectively. MiR-193-5p mimic and miR-193-5p inhibitor were designed and synthesized by

Shanghai Generay (Shanghai, China). Interfering RNA (siRNA) against *PIK3CD* was synthesized by Shanghai Gima (Shanghai, China).

Cell Transfection and Dual-Luciferase Activity Assay

Lipofectamine LTX Reagent (Invitrogen Ligies, Carlsbad, CA) was used for cell transfection according to manufacturer's directions. Dual-luciferase reporter assays were performed as previously described (Cai et al., 2017; Ma et al., 2018). HEK293T cells were cotransfected with 100 ng of luciferase-*PIK3CD* mRNA 3'-UTRs constructs and with 50 nM of either miR-193-5p mimic or mimic NC together with the Renilla luciferase construct using Lipofectamine 3,000 (Invitrogen). After 48 h, HEK293T cells were collected, and the luciferase and Renilla luciferase activities were determined by Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer's instructions (Hou et al., 2020).

Cell Proliferation Assay

The cell proliferation assay was performed using the Cell Counting Kit-8 according to the manufacturer's instructions. A total of 2×10^4 fibrocytes cells per well were seeded into 96-well plates, and transfected with miR-193-5p mimic, mimic NC, miR-193-5p inhibitor, inhibitor NC, siRNA NC, siRNA *PIK3CD*, and siRNA FITC, respectively. The CCK-8 was added to each well (10 μ L/well) at 24 h, 36 h, 48 h, 60 h, and 72 h post-transfection, and incubated at 37°C for 2 h. The absorbance at 450 nm was measured using the microplate reader. All experiments were performed in triplicate (Han et al., 2016; Yang et al., 2020).

Migration Assay

A total of 1.5×10^5 cells/well were seeded in the 24-well plates. The migration ability of fibrocytes was detected at 24 h after transfection using wound healing assay as previous description (Jonkman et al., 2014). The gap was created by scratching a confluent monolayer with a pipette tip. Then the plates were put into the incubator at 37°C for 24 h. Microscopy was used to monitor the status of migration into the cell-free area.

Statistical Analysis

Data were expressed as the means \pm SD. All experiments were repeated at least 3 times. Statistical differences between 2 groups were determined by Student's *t* test and 1-way ANOVA was used to compare 3 or more groups. The value of *P* < 0.05 was considered to be statistically significant.

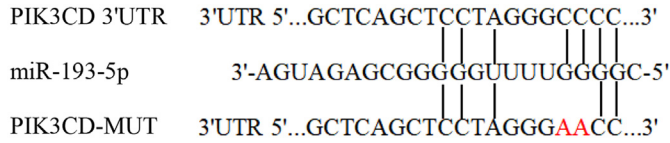


Figure 1. miR-193-5p binding sites. The specific binding sites of miR-193-5p in *PIK3CD* 3'-UTR, mutation sites were designed according to this sequence.

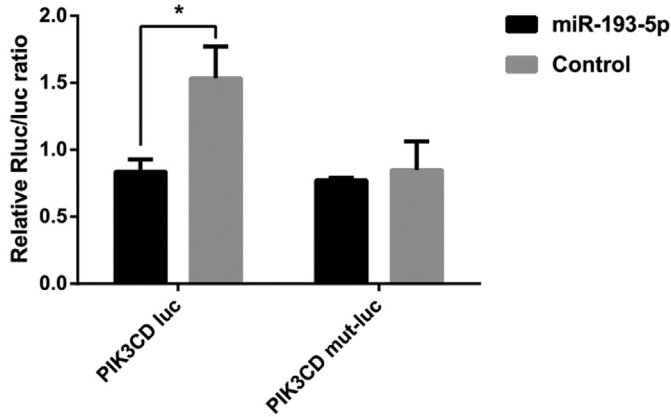


Figure 2. The *PIK3CD* 3'-UTR was a target of miR-193-5p. miR-193-5p targeted *PIK3CD* directly as measured by luciferase reporter assays. Luciferase activities of *PIK3CD*-luc were markedly decreased in cells transfected with miR-193-5p compared to control. The values were means \pm SD. * $P < 0.05$, ** $P < 0.01$.

RESULTS

MiR-193-5p Targets *PIK3CD* and Inhibits Its Expression

By using TargetScan6.2 software prediction, we found the presence of miR-193-5p targeted at 3814-3833 nt at *PIK3CD* 3'-UTR (Figure 1). To clarify the relationship between miR-193-5p and *PIK3CD* gene, the luciferase reporter system was used. We constructed the wild-type and mutant plasmids. The wild-type *PIK3CD* reporter vector (*PIK3CD*-3'-UTR-wt), the mutant plasmids (*PIK3CD*-3'-UTR-mut), miR-193-5p mimic and mimic NC were co-transfected into HEK-293T cells.

The luciferase assay showed that miR-193-5p significantly inhibited the relative luciferase activity of the *PIK3CD*-3'-UTR-wt, whereas the relative luciferase activity of the *PIK3CD*-3'-UTR-mut was not altered. Therefore, miR-193-5p could negatively regulated *PIK3CD* expression by targeted binding to the site in 3'-UTR region of *PIK3CD* (Figure 2).

MiR-193-5p Promotes Proliferation of Pigeon Fibrocyte

To evaluate the biological function of miR-193-5p, we used the CCK-8 assay to assess the effects of miR-193-5p on proliferation of pigeon fibrocyte. We transfected miR-193-5p mimic, a negative control (mimic NC), miR-193-5p inhibitor and a negative control (inhibitor NC) to the pigeon fibrocyte, then cell proliferation was measured. As shown in Figure 3, cells proliferation increased significantly in miR-193-5p mimic group compared with mimic NC group at 36 h and 60 h after transfection ($P < 0.05$), whereas cells proliferation decreased significantly in miR-193-5p inhibitor group compared with inhibitor NC group at 24 h, 36 h, 60 h, and 72 h after transfection ($P < 0.05$). The results suggested that miR-193-5p overexpression promoted cell proliferation, and miR-193-5p inhibition decreased cell proliferation.

We used Annexin V-FITC/PI apoptosis kit to test apoptosis of pigeon fibrocyte transfected with miR-193-5p mimic, mimic NC, miR-193-5p inhibitor, and inhibitor NC. Cells transfected with miR-193-5p mimic and mimic NC didn't show apoptosis signal both in early and late detection, whereas cells transfected with miR-193-5p inhibitor showed significant apoptosis both in early and late detection (Figure 4).

Migration of pigeon fibrocyte transfected with these 4 vectors was tested by scratch assays in vitro. Compared with cells transfected with mimic NC, cells transfected with miR-193-5p mimic completely repaired after 24 h of scratch (Figure 5), whereas cells transfected with miR-193-5p inhibitor repaired slowly than that transfected with inhibitor NC, which was completely repaired after 24 h of scratch (Figure 5).

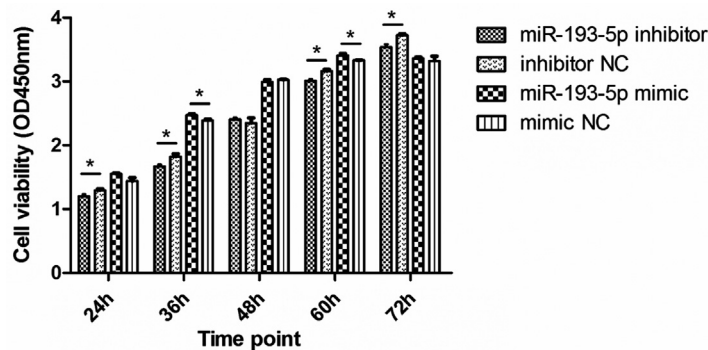


Figure 3. Effects of miR-193-5p on proliferation of pigeon crop fibroblasts. The cell proliferation assay was performed using the Cell Counting Kit-8. The fibrocytes cells were transfected with miR-193-5p mimic, mimic NC, miR-193-5p inhibitor, and inhibitor NC. The values were means \pm SD. * $P < 0.05$, ** $P < 0.01$.

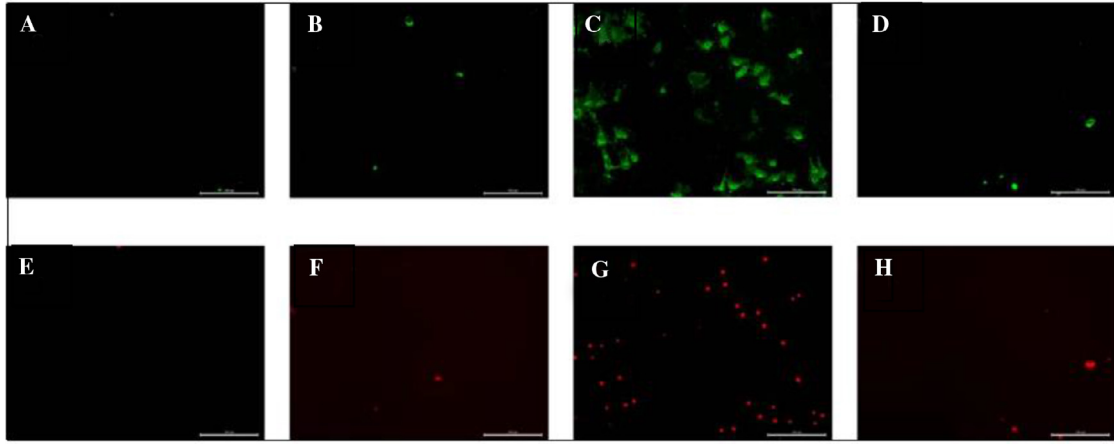


Figure 4. Effects of miR-193-5p on apoptosis of pigeon crop fibroblasts. (A) Transfected with mimic in 0 h; (B) transfected with mimic NC in 0 h; (C) transfected with inhibitor in 0 h; (D) transfected with inhibitor NC in 0 h; (E) transfected with mimic in 24 h; (F) transfected with mimic NC in 24 h; (G) transfected with inhibitor in 24 h; (H) transfected with inhibitor NC in 24 h.

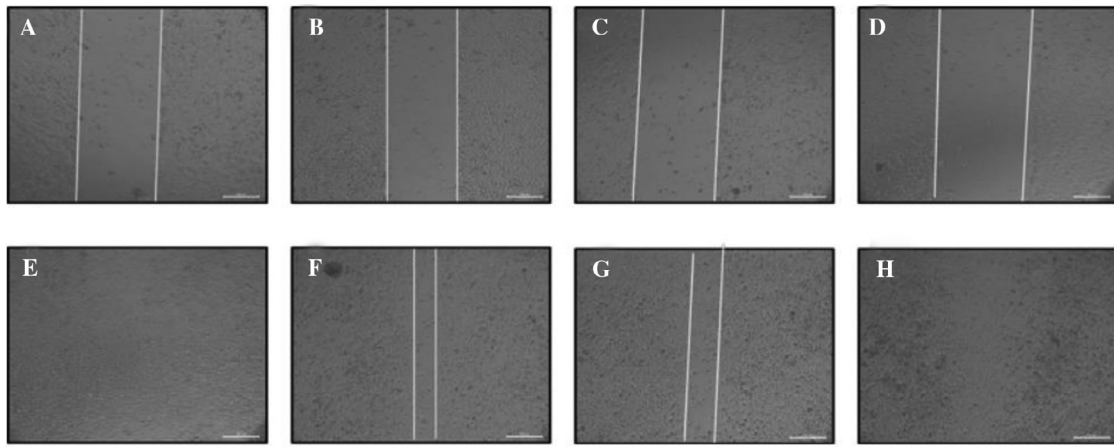


Figure 5. Effects of miR-193-5p on wound healing repair of pigeon crop fibroblasts. (A) Transfected with mimic in 0 h; (B) transfected with mimic NC in 0 h; (C) transfected with inhibitor in 0 h; (D) transfected with inhibitor NC in 0 h; (E) transfected with mimic in 24 h; (F) transfected with mimic NC in 24 h; (G) transfected with inhibitor in 24 h; (H) transfected with inhibitor NC in 24 h.

***PIK3CD* Inhibits Proliferation of Pigeon Fibroblast Cell**

To investigate the role of *PIK3CD* in proliferation of pigeon fibroblast cell, *PIK3CD* siRNA and a negative control (siRNA NC) were transfected in the pigeon

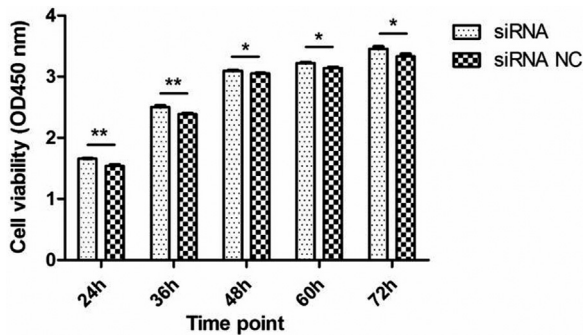


Figure 6. Effects of *PIK3CD* on proliferation of crop fibroblasts in pigeons. The cell proliferation assay was performed using the Cell Counting Kit-8. The fibrocytes cells were transfected with *PIK3CD*-siRNA and siRNA NC. The values were means \pm SD. * $P < 0.05$, ** $P < 0.01$.

fibroblast cell. The CCK-8 assay showed cells transfected with *PIK3CD* siRNA increased significantly than that transfected with siRNA NC at 24 h, 36 h, 48 h, 60 h, and 72 h after transfection (Figure 6). The scratch assays showed cells transfected with *PIK3CD* siRNA proliferated much quicker than that transfected with siRNA NC (Figure 7). All these results showed that *PIK3CD* could inhibit the proliferation of pigeon fibroblast cell.

DISCUSSION

Although much progress has been made in investigating the regulatory mechanism of crop milk production (Bartel, 2004; Lin et al., 2013; Chen et al., 2017; Zhang et al., 2020), the role of gene regulation is still poorly understood. miRNA plays an important role in regulating tissue growth and metabolism acting as an endogenous post-transcriptional gene regulator, and it always negatively regulates the translation of specific target genes by binding to their 3'-UTRs (Bartel, 2009). However, compared to other species, little is known

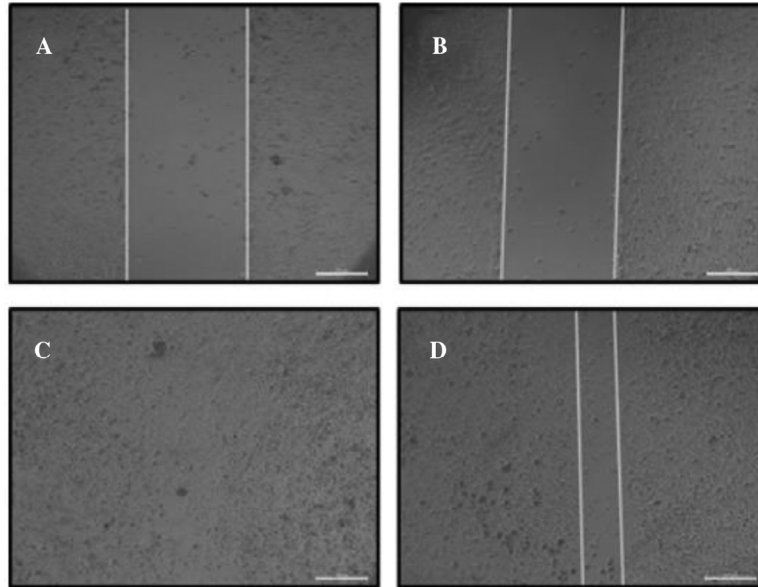


Figure 7. Effects of *PIK3CD* on wound healing repair of pigeon crop fibroblasts. (A) Transfected with *PIK3CD*-siRNA in 0 h; (B) transfected with siRNA NC in 0 h; (C) transfected with *PIK3CD*-siRNA in 24 h; (D) transfected with siRNA NC in 24 h.

about the function of miRNAs in crop milk production. In previous study, we identified miRNA associated ceRNA networks regulating crop milk production, and 71 miRNAs were identified differentially expressed. MiR-193-5p was predicted as an important ceRNA located in the central position of many regulatory axes affecting lactation, and *PIK3CD* gene was predicted as a potential target of miR-193-5p (Ge et al., 2020). Lactating crops showed a significant high-level expression of miR-193-5p compared with non-lactating crops by small RNA sequencing ($P < 0.01$), whereas in lactating crops, the expression of *PIK3CD* was significantly lower than in non-lactating crops ($P < 0.01$, Ge et al., 2020).

In the present study, we researched the targeting relationship of miR-193-5p and *PIK3CD*, and assessed the function of miR-193-5p in fibrocyte proliferation and crop milk production. miRNAs regulate post-transcriptional gene expression by binding to the 3'-UTR of target genes (Rashidi et al., 2022). miR-193a generate 2 arms including 3P and 5P, also known as dominant arm and passenger arm, respectively (Wang et al., 2019; Azar et al., 2021). Dysregulation of miR-193-3P and miR-193-5P could cause many diseases such as cancer (Lv et al., 2014; Wang et al., 2019), Parkinson and chronic myeloid leukaemia (Dong et al., 2016; Prinsloo et al., 2017). However, few reports described their functions in nutrition synthesis, and especially the function of miR-193-5p in regulation of crop milk production has remained unknown. Here, we first analyzed the expression of miR-193-5p and *PIK3CD* in lactating and non-lactating crops, and their regulation of pigeon crop fibrocyte in vitro.

We found that expression of miR-193-5p was upregulated in the lactating crop compared with the non-lactating crop, and miR-193-5p promoted crop fibrocyte proliferation in vitro. Similarly, Fan et al. (2021) reported that miR-193a-5P could promote the synthesis of polyunsaturated fatty acids (PUFAs) by targeting

fatty acid desaturase 1 (*FADS1*) in bovine mammary epithelial cells, and miR-193a-5P expression was positively correlated with the expression of genes associated with milk fatty acid metabolism, including RLOVL fatty acid elongase (*ELOVL6*) and diacylglycerol O-acyltransferase 2 (*DGAT2*) (Fan et al., 2021). Lactation is a complex activity regulated by endocrine regulation and changes in gene activity, and the activity is accompanied with various cellular processes as proliferation, differentiation, survival, and apoptosis (Dysin et al., 2021). As previous report, many studies have shown that mammary gland development was controlled by miRNAs by regulating proliferation and apoptosis of mammary epithelial cells, as well as the formation of mammary ducts and acinus (Avril-Sassen et al., 2009; Shimono et al., 2009). The number of epithelial cells influenced the dairy yield of milk (Boutinaud et al., 2004), and in pigeon the lactating crop was also accompanied by extensive proliferation and folding of the germinal epithelium (Gillespie et al., 2011). So our results verified that miR-193-5p could accelerate the proliferation of crop fibrocyte and process crop milk production.

To further validate the relationship of miR-193-5p and its predicted target gene *PIK3CD*, the luciferase activity assay was used. The results suggested that miR-193-5p negatively impacted *PIK3CD* by targeting the 3'-UTR sequence of *PIK3CD*. PI3Ks are a family of lipid kinases regulating variety of biological responses in different cells (Li et al., 2015). *PIK3CD*, a subunit of PI3K, has mostly been found to regulate progression of cancer and various metabolic disease (Fang et al., 2012; Tzenaki and Papakonstanti, 2013; Shi et al., 2014). The reports of *PIK3CD* functions were not consistent. Most of the results showed it could promote cell proliferation, whereas only few reported it suppressed cell proliferation. Xie et al. (2020) showed miR-224 could suppress diffuse large B-cell lymphoma (DLBCL) cells by

targeted inhibition of *PIK3CD*, which could promote proliferation of HBL1, a DLBCL cell line (Xie et al., 2020). Chen et al. (2019) showed that *PIK3CD* induced cell growth and invasion in colorectal cancer by activating AKT/GSK-3 β / β -catenin signaling, but the studies of Song et al. (2019) suggested that overexpression of *PIK3CD-AS1* inhibited proliferation, colony formation, and cell cycle progression of hepatocellular carcinoma (HCC) cells (Chen et al., 2019). However, little is known about the function of *PIK3CD* in mammary glands development and metabolism of milk. We first reported *PIK3CD* suppressed crop fibrocyte proliferation as the targeted gene of miR-193-5p.

In conclusion, we found miR-193-5p was upregulated in lactating pigeon crop and it promoted crop fibrocytes proliferation and migration, which indicated its promotion of crop milk production. *PIK3CD* was proved to be a functional target of miR-193-5p, which was negatively regulated by miR-193-5p. *PIK3CD* was downregulated in lactating pigeon crop and it inhibited cell crop fibrocytes proliferation and migration. The results provided novel insights into the regulation of crop milk production by miRNAs.

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DISCLOSURES

The authors declare that there is no conflict of interest.

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