# Analyses of circRNAs profiles of the lactating and nonlactating crops in pigeon (*Columba livia*)

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**ABSTRACT** Pigeon has the specific biological ability to produce pigeon milk (also known as crop milk) by its crop. Circular RNAs (**circRNA**s) are important noncoding RNAs acting as the sponges of miRNAs, but the molecular mechanism of circRNAs regulating crop milk production has not been reported in pigeon. We compared expression profiles of crops during lactating and nonlactating crops, and networks of competing endogenous RNAs (**ceRNAs**) were constructed. The results showed a total of 8,723 circRNAs were identified, and there were 770 differentially expressed circRNAs (**DEC**s) between these two different periods of crops. The Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway enrichment analysis revealed that the host genes of DECs were enriched in GnRH, MAPK, Insulin, Wnt, and AMPK signaling pathways. Furthermore, gga_circ_0000300 interacted with miR-92-2-5p, which targeted genes participating in lactation and milk composition synthesis. Gga_circ_0003018, gga_circ_0003019 and gga_circ_0003020 could bind with let-7c-5p regulating *SOCS3* in crop milk production. These findings provide the circRNAs expression profiles and facilitate the analysis of molecular mechanism of crop milk production in pigeon.

**Key words:** circRNAs, ceRNAs, crop milk, lactation, pigeon

2023 Poultry Science 102:102464
https://doi.org/10.1016/j.psj.2022.102464

## INTRODUCTION

Both male and female pigeons have the unique biological ability to produce crop milk, which contains protein (60%), fat (32−36%), carbohydrate (1−3%) and minerals (Gillespie et al., 2011). Pigeon crop has the function of food storage and moistening as most avian species, while during lactation it changes obviously in morphological structure and physiological function (Ma et al., 2018). In the lactating period, epithelial cells of crop undergo proliferation, denaturation, shedding, then mixing with other substances of the crop and forming crop milk (Gillespie et al., 2013). Crop milk is essential for growth and development of squabs as the only nourishment of the young (Gillespie et al., 2012). The physiological mechanism of pigeon lactation is different from mammalian for the pigeon crop is not glandular, it is necessary to study the regulatory mechanism of crop milk production (Gillespie et al., 2011).

The production of crop milk including processes of epithelial cells changes, nutrient substances syntheses and accumulation is regulated by hormones like prolactin and insulin (Horseman and Buntin, 1995; Hu et al., 2016). Then hormones combine with the relevant receptors in the cytomembrane and then activate the downstream gene expression through such signaling pathways as IRS1/Akt/TOR and JAK/STAT (Chen et al., 2020). Gillespie et al. (2013) has investigated the molecular mechanism of crop milk production by transcriptome. Cornification-associated genes including *S100-A9, cornulin*, and *A16-like* were up-regulated in the lactating crop, as well as beta-keratin genes (Gillespie et al., 2013). Pathway analysis revealed the up-regulated genes were involved in the proliferation of melanocytes, the adherens junction and the wingless (wnt) signaling pathway (Gillespie et al., 2011). Recently, we have profiled the transcriptome of long noncoding RNA (**lncRNA**s) and mRNAs in lactating and nonlactating pigeon crops, and 6,166 lncRNAs and 6,483 mRNAs were identified differentially expressed (Ma et al., 2020).

Noncoding RNAs, including lncRNAs, microRNAs (**miRNAs**), and circular RNAs (**circRNA**s), play a



Received August 29, 2022.
Accepted December 25, 2022.

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crucial role in regulating lactation (Pamudurti et al., 2017). With potential functions as miRNA sponges, circRNAs contain miRNA response elements (**MRE**s) that lead to derepression of miRNA target and post-transcriptional regulation (Hansen et al., 2013). As reported, the development of mammary gland and lactation are coordinated activities, which including neonate survival, acquisition of passive immunity and early life nutrition (Wang et al., 2021). Many studies have confirmed that they are regulated by a large number of mRNAs and miRNAs. Recently, some circRNAs have been confirmed to participate in these activities. Xu et al. (2017) found that the number of circRNAs in mammary gland (9,665 candidates) was higher than in the adrenal gland (2,311 candidates), the pancreas (1,791 candidates), and the thyroid (3,777 candidates) (Xu et al., 2017). Numbers of circRNA in mammary gland were different in different stages of lactation in rat (Zhang et al., 2015). There have been no reports of circRNAs on the activity of lactation in pigeon crop. In this study, we analyzed the circRNAs profile of lactating and nonlactating crops to identify the differentially expressed circRNAs and reveal the mechanism of crop milk production.

## MATERIALS AND METHODS

### Experimental Design and Sample Collection

We conducted the experiment with White King pigeons from a pigeon breeding farm in Beijing. All animals used in the work were approved by the Animal Care and Use Committee at the Institute of Animal Sciences of the Chinese Academy of Agricultural Sciences (IAS 2018-3). All procedures were conducted in accordance with the institutional animal ethics guidelines set by the Ministry of Agriculture and Rural Affairs of the People's Republic of China. A total of 10 female pigeons, comprising of five nonlactating pigeons and five lactating pigeons (72 h after hatching of their first squabs), were euthanized by $CO_2$ asphyxiation. The crop tissues were frozen rapidly in liquid nitrogen.

### RNA isolation, Library Construction and Sequencing

Total RNA was isolated from 5 lactating and 5 nonlactating crop tissues using Trizol reagent (Invitrogen, Waltham, MA) according to the manufacturer's protocol. RNA purity was checked using a Nanodrop Spectrophotometer (Model kaiaoK5500, Beijing, China). The integrity and concentration of the RNA were measured by the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system with RIN number >7.0 (Agilent Technologies, CA).

After removing ribosomal RNAs, the remaining RNAs were separated by 15% agarose gels to extract the small RNA pieces (18−30 nt). Then approximately 3 $\mu$g of RNA from each sample were used to construct libraries, according to the method and process of Small RNA Sample Preparation Kit (Illumina, RS-200-0048, San Diego, CA). After library construction, miRNA sequencing was performed by using the Illumina HiSeq 2500 platform (Annoroad, Beijing, China) and generating 50 bp single-end reads.

### RNA-seq Reads Mapping, Identification and Differential Expression of circRNAs

The raw reads were filtered using FastQC (0.11.2) to ensure the quality of data. The sequencing data have been submitted to the SRA database with accession no. PRJNA612642 (https://www.ncbi. nlm.nih.gov/sra/PRJNA612642). The 3′ adaptor-polluted reads, low-quality reads and reads with >5% poly-N were discarded as the initial reads to filtered out. The clean reads were mapped onto the reference genome and annotation files (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/ 000/337/935/GCF_000337935.1_ Cliv_1.0/GCF_000337935.1_Cliv_1.0_genomic.gff.gz) using HISAT2 (version 2.0.5, parameter-rna-strandness RF-dta-t-p 4, Kim et al., 2015). StringTie (version 1.3.2d, parameters -G ref.gtf−rf -l, http://ccb.jhu.edu/software/stringtie/) was run to build consensus sets of transcripts (Pertea et al., 2016).

Circular RNA Identifier (**CIRI**) tools were used to predict circRNA. The SAM files, after analyzed with BWA-MEM, were scanned twice to identify and characterize circRNA. The expression level of annotated circRNA was normalized using Spliced Reads per Billion Mapping (**SRPBM**, a normalized unit of transcript expression) approach (Li et al., 2015), and the differentially expressed circRNAs were identified using the DEGseq R package (Love et al., 2014) with the parameters of Q value < 0.05 and $|\log_2$fold change$|\geq 1.5$ as the significance threshold.

### Functional Enrichment Analysis

The DAVID tool (v6.8) was used to analyze the potential functions of the target genes of the differentially expressed circRNAs. The hypergeometric test was applied to map the differentially expressed genes to terms in the Gene Ontology (**GO**) database, and FDR <0.05 was used as the threshold of the significantly enriched GO terms (He and Liu, 2013; Kanehisa et al., 2008). Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway analysis was performed to understand the biological functions of genes. The threshold to find significantly enriched KEGG terms was also FDR <0.05 (He and Liu, 2013; Kanehisa et al., 2008).

### Construction of the circRNA-miRNA-mRNA Network

CircRNA was believed to contribute to the ceRNA network as miRNA sponges to regulate gene expression.

The potential interaction of circRNA-miRNA was predicted by two programs, Targetscan 7.0 (http://www.targetscan.org) and miRanda (http://www.microrna.org). Visualization of ceRNA network used Cytoscape software.

### Quantitative Real-Time PCR Assay

To validate the reliability of the RNA-seq results, 6 differentially expressed circRNAs were analyzed using quantitative real-time PCR (**qRT-PCR**). The RNA samples were reverse transcribed to cDNA using PrimeScript RT Reagent Kit (TaKaRa, Japan), and then qRT-PCR reactions were performed using PrimeScript One Step RT-PCR Kit (TaKaRa, Japan) and ABI QuantStudio 7 Flex Real-Time PCR System (Life Technologies Holdings Pte Ltd., Carlsbad, CA). GAPDH was used as endogenous genes and the relative expression levels were calculated using $2^{-\Delta\Delta Ct}$ methods. The primers, designed using Primer Premier 5 and confirmed by Oligo 6.0, were shown in Table 1.

## RESULTS

### Overview of the Sequencing Data

To identify circRNAs and their different expressions between lactating and nonlactating crops of pigeon, we purified and sequenced RNA using the Illumina HiSeq 2500 platform. We isolated total RNA from 5 nonlactating crop tissues (C1, C2, C3, C4, and C5) and 5 lactating crop tissues (T1, T2, T3, T4, and T5). A total of 578,622,734 and 589,996,902 clean reads were generated by sequencing from nonlactating and lactating crop tissues. The clean reads rate was 90.46% and clean Q30 bases rate was 93.40%. The mapping rates to the pigeon reference genome of C1, C2, C3, C4, C5, T1, T2, T3, T4, and T5 were 98.98%, 99.53%, 99.08%, 98.95%, 99.16%, 99.30%, 99.01%, 99.10%, and 99.03% respectively (Table 2).

### Identification and Characterization of Pigeon circRNAs

We detected 8,723 circRNAs from crop tissues identified by CIRI software (Table S1). Among the identified circRNAs based on mapping of the circRNAs to the pigeon genome (GCF_000337935.1_Cliv_1.0), annotated exons (annot_exons), accounting for 75.98% and 72.57% of the total circRNAs in nonlactating and lactating crops respectively, were the most common sequences. This was followed by intron_exon circRNAs (11.23% and 13.14%) and one_exon circRNAs (10.14% and 11.75%). Remaining circRNAs were smaller amounts of intergenic, intronic and antisense sequences (Figure 1A). CircRNAs identified in the study were widely distributed across the pigeon chromosomes. The majority of circRNAs had odd numbers of exons, and most of the circRNAs contained 3, 5, and 7 exons (Figure 1B).

### Analysis of Differential Expression of circRNAs

The expression levels of circRNAs were normalized by SRPBM. Seven hundred seventy differentially expressed circRNAs (**DEC**s) were identified, including 202 up-regulated and 568 down-regulated circRNAs in crop tissues at lactation comparing with nonlactation (Table S2). The Volcano Plot and heat map were shown in Figure 2A and B. The top 10 differential expressed circRNAs, known source of genes, were shown in Table 3, including 2 up-regulated and 8 down-regulated RNAs.

**Table 1.** Primers used to amplify circRNAs.

| CircRNA | Forward (5′→3′) | Reverse (5′→3′) | Product length (bp) |
|---|---|---|---|
| gga_circ_0002400 | CCTGATGTCACCGAATGG | ATCTGGCTCCACTGGTATA | 295 |
| gga_circ_0004288 | GAGGAGAATGAGGAGAAGAAG | GCACCAGCACTTGAGAAG | 239 |
| gga_circ_0002238 | AGTGCCTGCTCTGTAGAT | GACGACTGCTGCTGTAAT | 180 |
| gga_circ_0003020 | CGACCGAACCATACTTGA | CAGCACCAGAATCCTTGT | 278 |
| gga_circ_0008558 | GTCCATCTTGCCAACCTT | CCTCCACTACCGAACTCT | 184 |
| gga_circ_0002682 | ATCTGGTGAAGACGATGAG | GCTGGTTGTTGGAGGTATA | 210 |

**Table 2.** Basic statistics of the sequencing data.

| Sample | Raw reads | Clean reads | Clean Q30 bases rate, % | Mapped reads | Mapping rate, % | MultiMap reads | MultiMap rate, % |
|---|---|---|---|---|---|---|---|
| C1 | 123,105,520 | 109,577,762 | 93.49 | 108,463,672 | 98.98 | 26,014,654 | 23.74 |
| C2 | 128,175,504 | 119,045,316 | 93.80 | 118,483,297 | 99.53 | 25,551,008 | 21.46 |
| C3 | 131,962,046 | 120,026,894 | 93.37 | 118,922,024 | 99.08 | 27,494,903 | 22.91 |
| C4 | 125,346,964 | 111,498,914 | 93.55 | 110,332,588 | 98.95 | 29,099,303 | 26.10 |
| C5 | 131,369,282 | 118,473,848 | 93.45 | 117,473,587 | 99.16 | 28,663,518 | 24.19 |
| T1 | 130,167,700 | 118,548,640 | 93.46 | 117,553,278 | 99.16 | 36,217,634 | 30.55 |
| T2 | 126,086,574 | 115,719,768 | 93.67 | 114,913,240 | 99.30 | 32,851,361 | 28.39 |
| T3 | 129,766,416 | 118,451,046 | 93.18 | 117,275,687 | 99.01 | 39,097,666 | 33.01 |
| T4 | 131,819,754 | 117,900,806 | 92.68 | 116,836,049 | 99.10 | 35,878,645 | 30.43 |
| T5 | 134,001,318 | 119,376,642 | 93.31 | 118,214,198 | 99.03 | 37,621,098 | 31.51 |

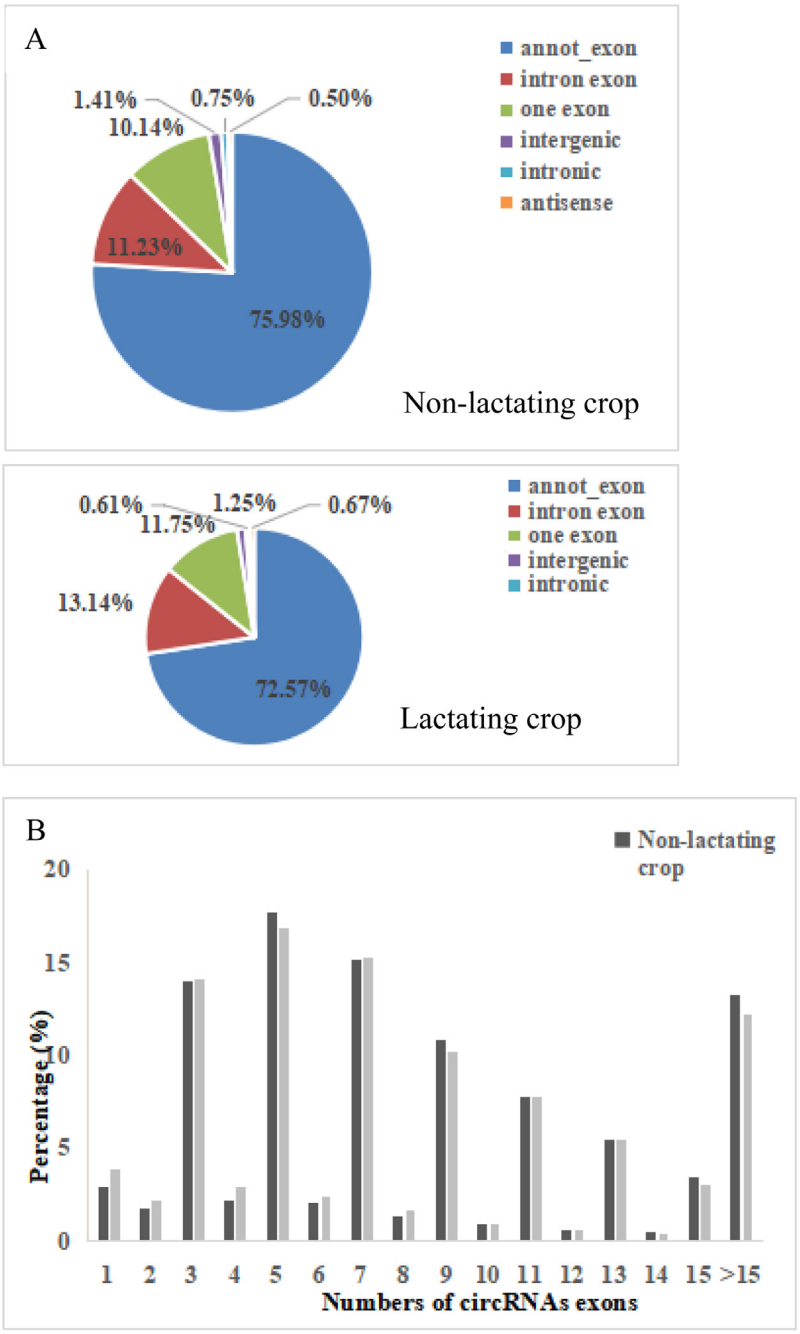


**Figure 1.** Profiling of circRNAs in pigeon crop. (A) Classification of identified circRNAs based on the genomic origin. (B) Numbers of circRNAs exons.

## GO and KEGG Analysis of the circRNA Hosting Genes

In this study, 7,634 circRNAs were annotated to 3,140 circRNA-hosting genes, demonstrating that one gene could produce more than one circRNA, as *RSC4*, *PTN1*, and *PYP1* produced 19, 15, and 17 circRNAs respectively (Table S3). To understand the potential functional pathways of the differentially expressed circRNAs, the circRNAs-hosting genes were analyzed by running queries against the GO database, including molecular function, cellular component and biological process.

The 529 host genes of the 770 DECs were annotated to 6,433 GO terms, among which there were 219 significantly enriched terms, including 96, 57, and 66 terms in biological process, cellular component and molecular function respectively (Table S4). In the biological process, the most significantly enriched GO terms were cytoskeleton organization (GO:0007010), regulation of biological process (GO:0050789), regulation of cellular process (GO:0050794), cellular component organization (GO:0050794) and phosphorus metabolic process (GO:0006793). In the cellular component, the most significantly enriched GO terms were cytosol (GO:0005829), intracellular part (GO:0044424), cytoskeleton (GO:0005856), organelle (GO:0043226) and cytoplasm (GO:0005737). In the molecular function, the most significantly enriched GO terms were protein

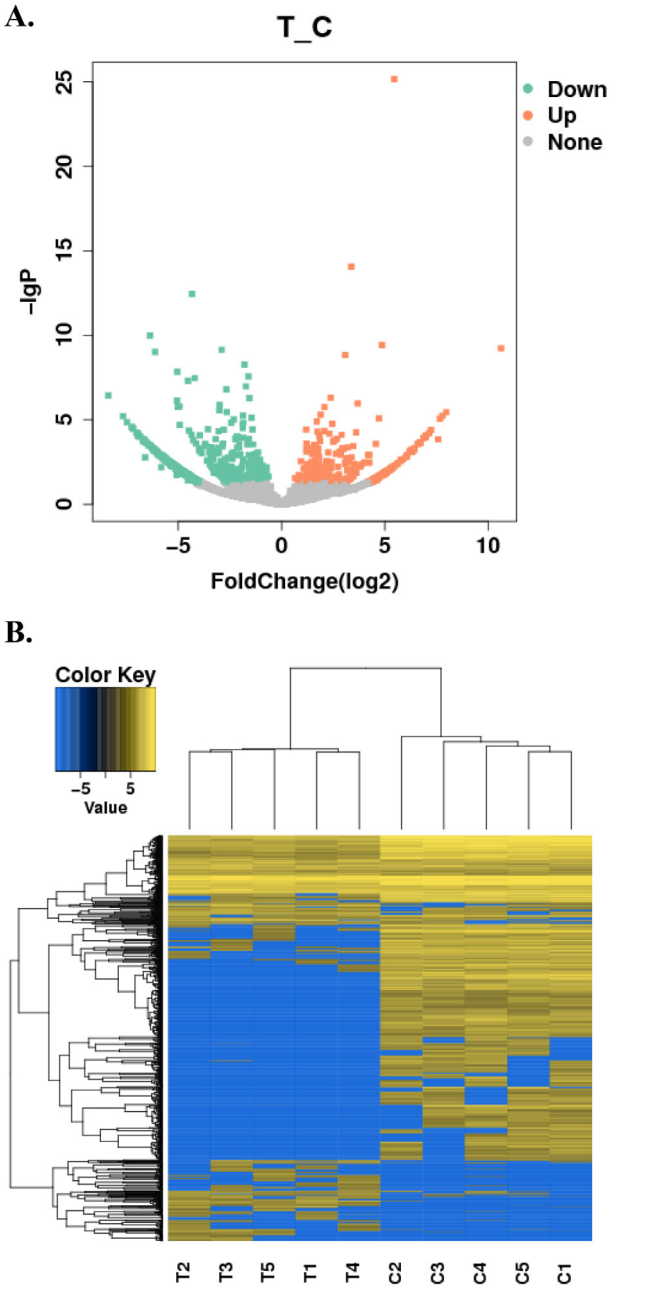


**Figure 2.** The Volcano Plot and heat map of the differential expressed circRNAs between lactating and nonlactating crops. (A) The Volcano Plot. (B) Heat map.

binding (GO:0005515), cytoskeletal protein binding (GO:0008092), ras GTPase binding (GO:0017016), binding (GO:0005488) and small GTPase binding (GO:0031267) (Figure 3). To further understand the biological functions of circRNAs, KEGG enrichment was performed. In this study, 247 KEGG pathways were annotated (Table S5). There were 29 pathways significantly annotated, including GnRH, MAPK, Insulin, Wnt, and AMPK signaling pathway (Figure 4).

## Validation of circRNA with qRT-PCR

The reliability of our RNA-seq results was verified by qRT-PCR. We verified the presence of 6 differentially expressed circRNAs (Figure 5). Results showed that the expressions of gga_circ_0008558, gga_circ_0002682 and gga_circ_0003020 were significantly increased in lactating crops. So the qRT-PCR results validated the reliability of circRNAs expression profile (Figure 5).

**Table 3.** Differentially expressed circRNAs between lactating and nonlactating crops in pigeon.

| ID | Gene name | $Log_2$fold change | *P*-value | Change |
|---|---|---|---|---|
| gga_circ_0001337 | gene3837 | 5.46 | 6.94E−26 | Up |
| gga_circ_0007860 | gene23421 | −4.33 | 3.57E−13 | Down |
| gga_circ_0000095 | gene144 | −2.90 | 7.34E−10 | Down |
| gga_circ_0006272 | gene18348 | −6.13 | 9.67E−10 | Down |
| gga_circ_0002940 | gene8521 | 3.08 | 1.48E−09 | up |
| gga_circ_0001205 | gene3339 | −1.80 | 5.52E−09 | down |
| gga_circ_0006101 | gene17668 | −5.05 | 1.45E−08 | down |
| gga_circ_0004290 | gene12233 | −1.60 | 2.71E−08 | down |
| gga_circ_0000836 | gene2626 | −4.21 | 3.42E−08 | down |
| gga_circ_0000695 | gene1967 | −4.53 | 4.81E−08 | down |

## Target miRNA Predictions of the Differentially Expressed circRNAs

Results from the miRanda analysis revealed a total of 137 target microRNAs identified to the 296 differentially expressed circRNAs (types of annotated exons and antisense). Among these circRNAs, 3 up-regulated (gga_circ_0003018, gga_circ_0008564, gga_circ_0008495) and 3 down-regulated circRNAs (gga_circ_0000300, gga_circ_0001748, gga_circ_0003002) were selected to study the interaction of circRNAs and their miRNA. There were 152 target miRNAs in total for these 6 circRNAs, and the circRNA-miRNA-mRNA networks were constructed (Figure 6).

## DISSCUSION

In the current study, the expression profile of circRNAs in the crop of pigeon was first mapped using RNA sequencing. Recently, the lncRNA and miRNAs profiles of crops in nonlactating and lactating stages were investigated (Ma et al., 2020; Ge et al., 2020). However, there was no information about the roles of circRNA in regulating crop milk production. By sequencing and annotating, a total of 8,723 circRNAs were identified in the lactating and nonlactating crops of pigeons, among which there were 770 differentially expressed. Our results could provide novel insight into the circRNAs profiles of the crops during lactation, thus revealing deep mechanism of pigeon crop lactation.

CircRNAs were spliced from the linear transcript, so the function of circRNAs were associated with their hosting genes (You et al., 2015). Acetyl-CoA carboxylase alpha (***ACACA***), the hosting gene of gga_circ_0002685 and gga_circ_0002688, could encode enzyme that involved in the synthesis of long-chain fatty acids catalyzing the rate-limiting step (Wu et al., 2020). The expression of *ACACA* in lactating crop was significantly higher than that of nonlactating crop, suggesting the large amounts of fatty acid production in the

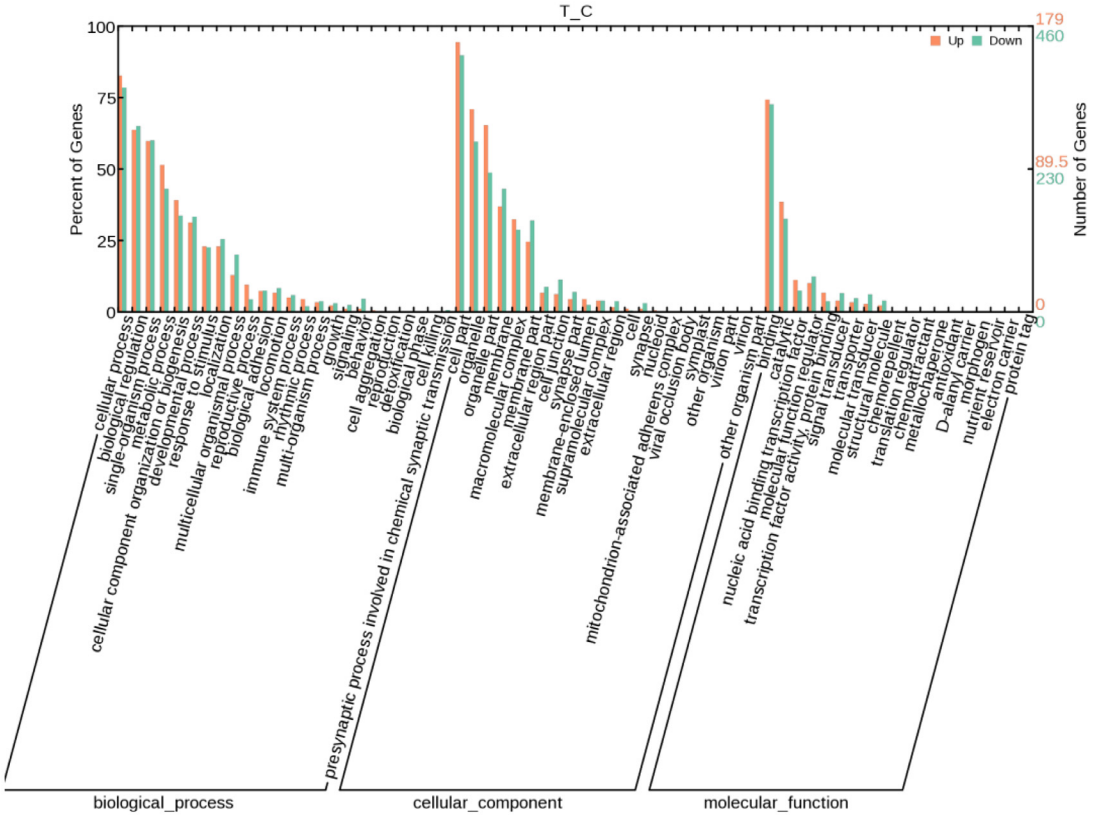


**Figure 3.** The significantly enriched GO terms of the differenially expressed circRNAs between lactating and nonlactating pigeon corps (FDR < 0.05).

lactating crop (Gillespie et al., 2013). Diacylglycerol O-acyltransferase 2 (***DGAT2***), the hosting gene of gga_circ_0001333, gga_circ_0001334 and gga_circ_0001337, could catalyze diacylglycerol and fatty Acyl-CoA condensing to synthesize triacylglycerols and highly expressed in lactating crop in our results (Chitraju et al., 2019). Gillespie et al. (2011) reported two Acyl-CoA synthetase genes were 1.8-fold and 2.5-fold up-regulated in lactating crops. These two genes encoded enzymes regulating he fatty acid oxidation pathway by preceding the synthesis of triglycerides (Gillespie et al., 2011). Besides the fatty acid synthesis, we also found that genes related with protein and glycogen synthesis as *PPM1G*, *PBRM1* and *GSK3B* (hosting genes of gga_circ_0004920, gga_circ_0005598, and gga_circ_0002361) were highly expressed in lactating crop, suggesting lots of nutrients synthesis in the lactating stage as previously reported (Gillespie et al., 2013).

In order to define the biological functions and provide a systematic classification of the differentially expressed circRNAs in these 2 stages of pigeon crops, GO and KEGG pathway enrichment were analyzed. Pathways related with cytoskeleton were significantly annotated in GO terms, that were 'cytoskeleton organization,' 'cytoskeleton,' and 'cytoskeletal protein binding' respectively in biological process (**BP**), cellular component (**CC**), and molecular function (**MF**). Cytoskeleton could regulate milk protein synthesis and secretion by promoting cultured primary epithelial cells differentiation in mouse (Seely and Aggeler, 1991). In pigeon, the synthesis and secretion of milk was also accompanied with large germinal epithelium proliferation of the crop (Gillespie et al., 2011). The highest significantly ranked GO terms in MF category were 'binding' and 'kinase activity' (Fig. S4), suggesting that milk synthesis in crop activated large gene expression participating with metabolic activity. Hou et al. (2020) also reported methionine could stimulate bovine mammary epithelial cells expression genes enriched in 'binding' and 'catalytic activity' (Hou et al., 2020).

KEGG pathway analysis is widely used to understand the interaction between a set of genes in the context of biological functions (Kanehisa et al., 2008). There were 29 KEGG pathways significantly enriched in our results, and all genes included in these pathways were up-regulated. Onset of lactation in the bovine mammary gland was regulated by hormone in the serum. The serum hormone as PRL could also activate crop lactating through the PRLR/JAK2/STAT5 pathway (Chen et al., 2020). In our results, some of the hormone related signaling pathways were significantly enriched as insulin, glucagon and estrogen. Most of the pathways that hormones activated in milk production were significantly enriched, including MAPK signaling pathway, Ras signaling pathway, Wnt signaling pathway, Toll signaling pathway, and AMPK signaling pathway. Wnt signaling participated in intestinal epithelial layer maturation under the stimulation of breast milk nutrients in human (Jong et al., 2021). In pigeon, Gillespie et al. (2013) reported the up-regulated genes in lactating crop were involved in

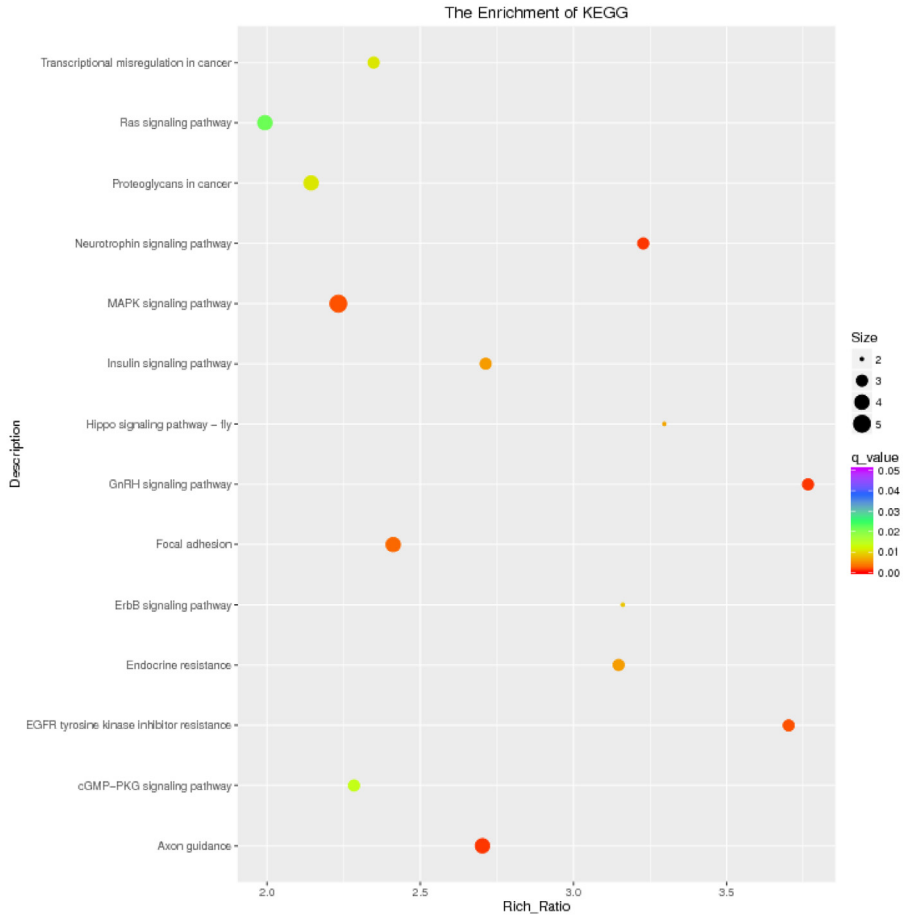


**Figure 4.** The significantly enriched KEGG pathway of the differenially expressed circRNAs between lactating and nonlactating pigeon corps (FDR < 0.05). The Y-axis label represents significant pathways, and the X-axis label represents fold enrichment. The size of the bubbles represents the levels of differentially expressed genes enriched in the pathway. The color of the bubbles represents significance.

the adherens junction and Wnt signaling pathway (Gillespie et al., 2013). Multiple cytokines were identified in milk including MAPK, JAK/STAT, and NF-$\kappa$B. These cytokines were required for proliferation of the epithelial cells in mammary gland and nutrition synthesis (Brenmoehl et al., 2018). AMPK-mTOR pathway was involved in amino acid sensing and utilization in the mammary gland of lactating dairy goats (Cai et al.,

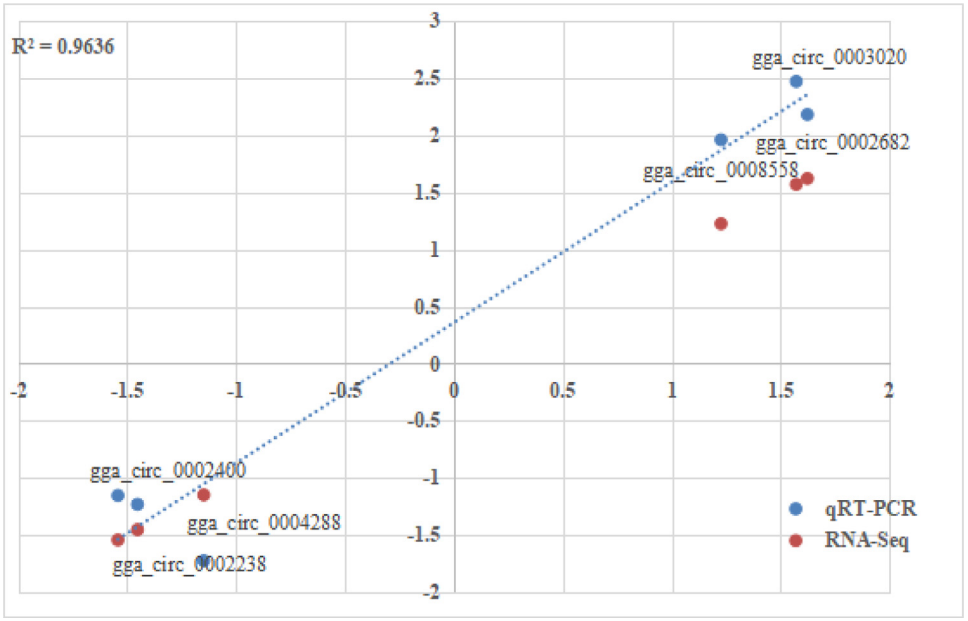


**Figure 5.** A comparison of circRNAs expressions using the RNA-seq results and the qRT-PCR results for 6 differentially expressed circRNAs. Three independent replicates are used for each sample.

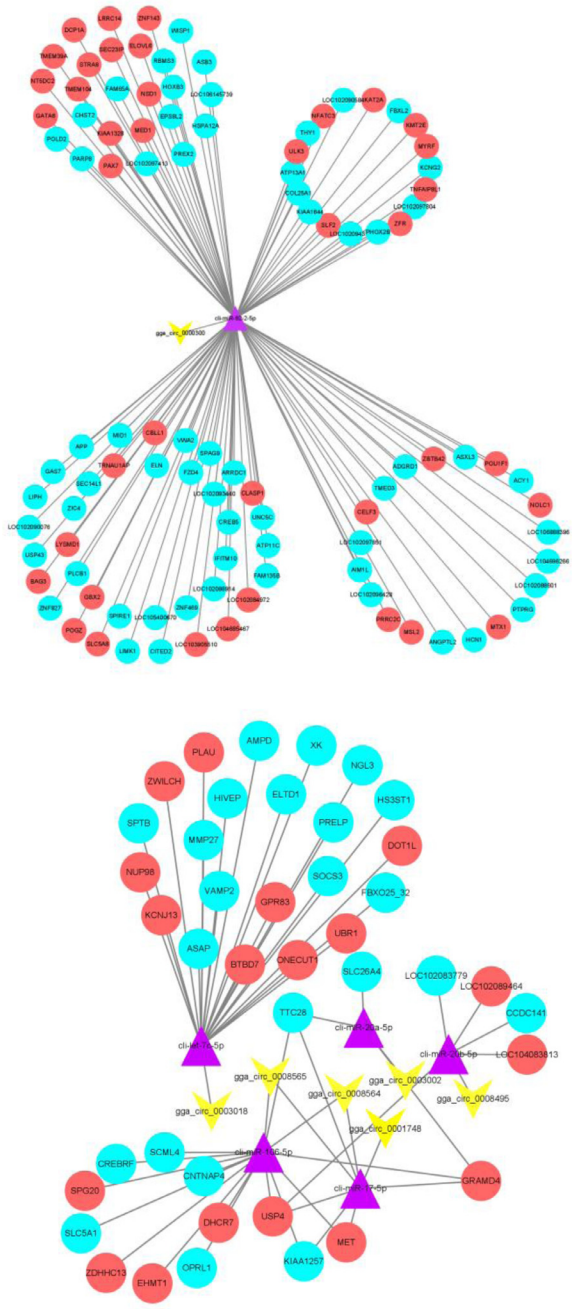


**Figure 6.** The circRNA-miRNA-mRNA interaction network. The inverted triangles and triangles represent circRNAs and the predicted target miRNAs respectively. The circles represent mRNAs, while red represents up-regulated and blue represents down-regulated circRNAs in lactating crops comparing with nonlactating crops.

2020). The important downstream targets of AMPK as PGC-1$\alpha$ and LXR could regulate milk fat synthesis in the mammary gland (Wu et al., 2020).

Studies have confirmed that circRNAs could act as competitive endogenous RNAs by binding miRNAs to exert their biological effects, thus regulating the expression levels of the miRNA target genes (Franco-Zorrilla et al., 2007; Poliseno et al., 2010; Kulcheski et al., 2016). In recent years, many studies have reported that circRNAs played important roles in regulating the lactation of livestock (Ma et al., 2018; Sun et al., 2019; Hao et al., 2020). CircRNAs were related with milk production and milk quality in dairy cows, as circRNAs produced by *CSN1S1* and *CSN2* genes on the 90th day (the prelactation period) was significantly higher than that on the 250th day (the post-lactation period) of lactation (Zhang et al., 2016). *CSN1S1* and *CSN2* were casein-binding genes, and casein was known as an important indicator of milk quality assessment (Cieslak et al., 2019). It was demonstrated that circHIPK3 promoted proliferation of Bovine Mammary Epithelial Cells, while the expression of circHIPK3 was significantly reduced in cells treated with prolactin and STAT5 inhibitors (Wang et al., 2021).

CircRNAs could form ceRNAs (circRNA-miRNA-mRNA) to participate in the regulation of lactation. In goat mammary gland, miR-103 was the target of circRNA_007873, circRNA_010763, and circRNA_015622, and overexpression of miR-103 in mammary epithelial cells increased the transcription of genes related with milk fat synthesis (Lin et al., 2013; Dou et al., 2016). CircRNA_001091 was reported as 43 miRNA sponges including miR-29 in lactating mammary glands of sheep (Hao et al., 2020). Bian et al. (2015) found that miR-29 regulated DNA methylation levels, and inhibitory effect of miR-29 could increase methylation level and promote important lactation related genes in dairy cow. Chen et al. (2020) reported bovine mammary tissue at different stages expressed different levels of circ09863. By constructing the circ09863/miR-27a-3p/FASN network, it was found that overexpression of circ09863 significantly increased TAG and fatty acids content, which was correlated with *FASN* expression (Zhu et al., 2014; Chen et al., 2020).

In our results, gga_circ_0000300/miR-92-2-5p constructed network in the lactation process of pigeon, and miR-92-2-5p targeted 102 genes, including *ADGRD1*, *LRRC14*, *MED1*, and *FZD4*, most of these genes were verified to participate in lactation and milk composition synthesis in other species previously. They constructed ceRNA network as gga_circ_0000300/miR-92-2-5p/*ADGRD1*, gga_circ_0000300/miR-92-2-5p/*LRRC14*, and gga_circ_0000300/miR-92-2-5p/ *MED1* to regulate milk production and traits in pigeon. *ADGRD1* was reported as a candidate gene associated with milk production traits in Egyptian buffalo by genome wide association study (Abdel-Shafy et al., 2020). Ibeagha-Awemu et al. (2016) demonstrated *LRRC14* was candidate gene influencing cow milk traits and mammary gland functions by genome wide association study. *MED1* served as a surrogate of general transcription coactivator complex Mediator to regulate postnatal adipose expansion and the induction of fatty acid synthesis in mice by cell- and gene-specific regulatory roles (Jang et al., 2021). JAK-STAT pathway genes along with SOCS family genes played a crucial role in controlling cytokine signals in the mammary gland development and contributed to the milk production traits (Arun et al., 2015). A gga_circ_0000300/miR-92-2-5p/*ELOVL6* network promoted lipid synthesis in the lactating crops of pigeon. *ELOVL6* was reported as a vital protein for long-chain fatty acids synthesis in mammals

(Fan et al., 2022). Fan et al. (2021) knocked down *INSIG2* in buffalo mammary epithelial cells resulting in marked up-regulation of *ELOVL6* and the content of triacylglycerol significantly increased, suggesting the positive effect of *ELOVL6* on milk fat synthesis in buffalo. In our results, gga_circ_0003018, gga_circ_0003019, and gga_circ_0003020 could all bind with let-7c-5p and regulate the expression of *SOCS3*.

In conclusion, high-quality circRNAs profiles were obtained from the crop tissues of pigeons. We identified 8,723 circRNAs, 770 of which were differentially expressed between lactating and nonlactating crops. The GO, KEGG, and circRNA-miRNA network analysis contribute to understand how circRNAs mediate the regulation of target genes in crop milk production. These findings provide new insights into crop milk regulation during lactation of pigeon.

## ACKNOWLEGMENTS

This study was funded by Beijing Innovation Consortium of Agriculture System (BAIC04); National Natural Science Foundation of China (31802058); Agricultural Science and Technology Innovation Program (ASTIPIAS04).

Data Availability Statement: The datasets presented in this study can be found in the Genome Sequence Archive (GSA) with accession no. CRA001977.

## DISCLOSURES

The authors declare that they have no conflicts of interest to this work.

## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102464.

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