Screening of MicroRNAs with Potential Systemic Effects Released from Goose Fatty Liver

Xue Fan[1], Ya Xing[1], Long Liu[1], Chao Zhao[1], Zhenzhen Chen[1], Mawahib K. Khogali[1],
Minmeng Zhao[1], Xuming Hu[1], Hengmi Cui[1], Tuoyu Geng[1,2] and Daoqing Gong[1,2]

[1]College of Animal Science and Technology, Yangzhou University, Yangzhou 225009, Jiangsu Province, P. R. China
[2]Joint International Research Laboratory of Agriculture and Agri-Product Safety of the Ministry of Education of China,
Yangzhou University, Yangzhou 225009, Jiangsu Province, P. R. China

Communication between tissues and organs plays an important role in the maintenance of normal physiological functions as well as the occurrence and development of diseases. Communication molecules act as a bridge for interactions between tissues and organs, playing not only a local role in the tissues and organs where they are secreted but also in exerting systemic effects on the whole body via circulation. In this study, blood microRNA-omics analysis of overfed vs. normally fed (control) Landes geese revealed that the content of each of the 21 microRNAs (miRNAs) in the blood of overfed geese was significantly higher than that in the blood of control geese. These miRNAs may have systematic effects in the development of goose fatty liver as well as being candidate markers for the diagnosis of goose fatty liver. We determined the expression of *miR-143, miR-455-5p, miR-222a-5p, miR-184, miR-1662,* and *miR-129-5p* using quantitative PCR in goose fatty liver vs. that in normal liver. The expression of these miRNAs, except *miR-129-5p*, in goose fatty liver was also significantly higher than that in normal liver ($P < 0.05$), suggesting that these blood miRNAs are released from goose fatty liver. In addition, we found that expression of *IGFBP5*, the predicted target gene of *miR-143*, was significantly decreased in goose fatty liver vs. the normal liver ($P < 0.05$), indicating that *miR-143* may exert both local and systematic effects by inhibiting the expression of *IGFBP5*, thus promoting the development of goose fatty liver. In conclusion, we identified several miRNAs, including those we validated (i.e., *miR-143, miR-455-5p, miR-222a-5p, miR-184, miR-1662,* and *miR-129-5p*) that may serve as candidate markers in the diagnosis of goose fatty liver as well as local and global regulators contributing to the development of goose fatty liver.

Key words: cell communication, diagnostic marker, fatty liver, goose, microRNA

Introduction

Goose fatty liver is an important waterfowl product used as high-grade food. As a physiological fatty liver, it differs from mammalian non-alcoholic fatty liver. Even with severe steatosis, goose fatty liver shows no overt pathological symp-

toms and injuries in geese, suggesting that there is a unique protective mechanism present. This mechanism may have originated over long-term evolution from its ancestor. Recent studies have shown that there are several protective components in goose fatty liver, such as increased expression of fatty acid desaturases and adiponectin receptors and the suppressed expression of endoplasmic reticulum stress and inflammation-related genes (Geng *et al*., 2016). Therefore, goose fatty liver can be considered as a unique model for the study of fatty liver, and elucidating the underlying mechanism of development of goose fatty liver may provide new ideas to promote the production of goose fatty liver and prevent fatty liver disease in other animals.

MicroRNAs (miRNAs) are noncoding small RNAs that are ubiquitous in animals and plants. Mature miRNAs are 19−24 nucleotides in length. Generally, miRNAs inhibit the translation of target genes or promote mRNA degradation of target genes by binding to the 3'UTR of the target mRNA;

thus, they are important regulators of gene expression. The expression of many miRNAs has been indicated to be associated with the development of fatty liver (Correia de Sousa *et al*., 2019). For example, Liu *et al*. reported that 151 differentially expressed miRNAs (114 upregulated) in goose fatty liver vs. normal liver were enriched in carbohydrate, fat, and amino acid metabolism-related pathways, immune-related pathways, and signaling molecules and interaction pathways (Liu *et al*., 2016a). These findings indicated that miRNAs play an important role in the development of fatty liver.

Different tissues or organs in the body secrete several bioactive substances, such as proteins, peptides, bioactive lipids, and nucleic acids. Distribution of these substances via the circulation can therefore affect the structures and functions of other organs and tissues. As molecules involved in communication, they are essential to the maintenance of normal physiological functions or the occurrence of disease. These substances can be directly released into the blood or transported to other parts of the body by exosomes (Hayashi and Hoffman, 2017). Screening such molecules with systemic effects can help reveal the underlying mechanism of the occurrence and development of disease and indicate which molecules could likely be used as candidate markers for disease diagnosis (Hetta *et al*., 2019). miRNAs have small molecular weights, are easily released and absorbed by cells, and are one of the most studied communication molecules and diagnostic markers for disease. Several studies have reported that a number of blood miRNAs can be used as biomarkers for mammalian non-alcoholic fatty liver (Liu *et al*., 2016b; Kim *et al*., 2017; Brandt *et al*., 2018), although there are few studies on miRNAs as communication molecules and their systematic functions in goose fatty liver. Here, we hypothesized that the elevated contents of miRNAs in the blood of geese with fatty liver were mainly released from the fatty liver and that these miRNAs could play a local and global role in the development of goose fatty liver. We used miRNA-omics analysis to screen for miRNAs with significantly higher content in the blood of geese with fatty liver than those in normally fed (control) geese. The expression of several selected miRNAs and predicted target genes was determined in goose fatty liver vs. the normal liver. Thus, we focused on screening microRNAs with potential systemic effects released from the goose fatty liver, which may lay a foundation for elucidating the role of tissue/organ communication in the development of this condition.

Materials and Methods

Experimental Animals

All animal protocols were approved by the Animal Care and Use Committee of Yangzhou University with permission number NSFC2020-DKXY-22. Twenty 65-day-old healthy Landes geese were purchased from Yangzhou Ruinong Technology Co., Ltd and randomly divided into a control group and an overfeeding group (10 per group). Geese in the control group were fed normally and given free access to feed and water, whereas those in the overfeeding group received pre-overfeeding for 5 days, followed by formal overfeeding for 19 days. The overfeeding protocol and feed were described previously (Geng *et al*., 2016). When geese were 89-days-old, blood samples were collected after euthanization using heparin as an anticoagulant. The samples were centrifuged, and plasma samples were subsequently acquired and stored at $-70℃$. Liver samples were also collected, snap-frozen in liquid nitrogen, and stored at $-70℃$.

Blood miRNA-omics Analysis

Plasma samples of three geese per group were sent to Shanghai Personal Biotechnology Co., Ltd for miRNA-omics analysis. The procedures for this analysis were briefly described as follows: small RNAs in the plasma were extracted and purified, followed by quality checking with Bioanalyzer; a cDNA library of qualified small RNAs was constructed and sequenced using the HiSeq system. After removing low-quality reads, clean reads with the size of 15−30 bp were counted and grouped, and each single sequence (unigene) was assembled; alignment and annotation of unigenes were performed using non-coding RNA (ncRNA) databases. In addition, the miRNA database (miRBase, v20.0) comprising 10 known species (i.e., bombyx, chicken, mouse) was used to identify good miRNA homologs. Reads of miRNAs were calculated and are presented as reads per million reads (RPM, which is the ratio of the count of miRNA to the total count of clean reads multiplied by 1,000,000). Using the acquired miRNA readings, miRNAs with significant differences in blood content between the overfeeding and control groups were identified. The screening criteria were that the selected miRNA have at least two reads per million mapped reads (RPM) values >1, the ratio of RPM values of the overfeeding group to the control group of >1.5 or $<2/3$; a *P*-value <0.05 was considered for the miRNAs with differential blood contents.

Purification, Reverse Transcription, and Quantitative PCR (qPCR) Analysis of miRNAs and Predicted Target Genes in Goose Fatty Liver vs. Normal Liver

We used miRDB (http://mirdb.org/miRDB/) and online software programs in the TargetScan (http://www.targetscan.org/) website to predict target genes of differentially expressed miRNAs; we combined this small RNA Group sequencing of target gene prediction results and references to select target genes to be verified in the liver.

miRNAs were isolated from goose fatty liver and normal

Table 1. **Forward sequences of primers for qPCR analysis of the randomly selected miRNAs**

Gene	Forward primer (5′ → 3′)
cli-miR-455-5p	TGTGCCCTTGGACTACATCGTA
ssc-miR-184	TGGACGGAGAACTGATAAGGGT
tgu-miR-1662	TTGACATCATCATACTTGGGAT
efu-miR-143	CTGAGATGAAGCACTGTAGCTAA
pbv-miR-222a-5p	CGCTCAGTAGTCAGTGTAGATTA
gga-miR-129-5p	CTTTTTGCGGTCTGGGCTTGC
oha-miR-191-5p	CAACGGAATCCCAAAAGCAGCT

Table 2. **Primer sequences for qPCR analysis of predicted target genes of miRNA**

Gene	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
IGFBP5	GTGAACATGAGGAGCCGACC	CAAGGGCCCAGCTCAAACTCT
SLC7A2	TGGATGGCACTTGGCTTCTT	TCTCCATCACTGCGATGACC
MAN2A1	ACATTCAACACAGGGCTTGG	GCATAGCGGAAATCATCGCC
PNISR	AACGGTCAGGCTCTGAATCT	GAGGAGGAGTTGTTGACCTTGA
GAPDH	CTGATGCTCCCATGTTCGTG	CCACGATGCCAAAGTTGTCA

liver using miRcute miRNA Isolation Kit (Tiangen Biotechnology Co. Ltd., Beijing, China). First-strand cDNA was synthesized using miRcute Plus miRNA First-Strand cDNA Kit (Tiangen Biotechnology Co. Ltd., Beijing, China), and miRNA expression was determined using qPCR with miRcute Plus miRNA (qPCR) Kit (Tiangen Biotechnology Co. Ltd., Beijing, China). The forward primer for PCR analysis of each miRNA was designed according to the method of tailing miRNA 3' end with Poly A (Table 1), and the reverse primer was provided by miRcute Plus miRNA qPCR Kit (SYBR Green). The internal reference gene was *oha-miR-191-5p* (Zheng *et al*., 2013).

To determine the expression of predicted target genes of miRNA, the total RNA was isolated from goose fatty liver and normal liver using TRIzol (Cat# DP424, Tiangen Biotech (Beijing) Co., Ltd., Beijing, China) according to the manufacturer's instructions. HiScript Q RT SuperMix (Cat# R123-01, Vazyme Biotech Co., Ltd., Nanjing, China) was used for reverse transcription. The expression of target genes and the internal reference gene (*GAPDH*) was determined using fluorescence qPCR with Vazyme AceQ qPCR SYBR Green Master Mix kit (Cat# Q111-02/03, Vazyme Biotech Co., Ltd., Nanjing, China). A previously described protocol was used (Osman *et al*., 2016). The primer sequences are shown in Table 2.

Statistical Analysis

A two-tailed *t*-test was used to determine the significance of the difference in blood content of miRNA or the expression of miRNA and target gene in the liver between the overfeeding group and control group. $P < 0.05$ was considered to be statistically significant. All data are presented as means±SE.

Results

miRNA-omics Screening of miRNAs that Had a Higher Content in the Blood of Overfed Geese Compared to that in the Blood of Normally Fed Geese

Studies have shown that fatty liver in geese can be induced by artificial overfeeding (Liu *et al*., 2016a). Here, body weight (7.57±0.48 kg), liver weight (826.26±182.26 g), and the ratio of liver to body weights (10.86%) in overfed geese were all significantly higher than those of normally fed (control) geese (Supplementary Fig. 1). Using blood samples collected from these geese, miRNA-omics analysis was carried out to screen for miRNAs with a higher content in the blood of overfed geese compared to that in the blood of normally fed geese. Among 1215 annotated miRNAs (Sup-

plementary Table 1), 397 miRNAs met the following criterion: $P < 0.10$ and fold change (overfeeding group vs. control) > 1.5 or < 2/3 (Supplementary Table 2); 274 miRNAs met the following criterion: $P < 0.05$ and fold change > 1.5 or < 2/3 (Supplementary Table 3); and 21 miRNAs met the following criterion: $P < 0.05$ and fold change > 1.5 (Table 3). These selected miRNAs were the candidate miRNAs with potential systemic effects in the development of goose fatty liver and are also potential biomarkers in the diagnosis of goose fatty liver.

The Expression of Selected miRNAs and their Target Genes in Goose Fatty Liver vs. Normal Liver

To study how blood-borne miRNAs are related to goose fatty liver, the expression of several randomly selected miRNAs, including *efu-miR-143, cli-miR-455-5p, pbv-miR-222a-5p, ssc-miR-184, tgu-miR-1662,* and *gga-miR-129-5p* (Table 4) in goose fatty liver vs. the normal liver was determined using qPCR. Compared to that in the normal liver, the expression of *ssc-miR-184, pbv-miR-222a-5p, cli-miR-455-5p, tgu-miR-1662,* and *efu-miR-143* was significantly increased in goose fatty liver ($P < 0.05$), whereas there was no significant difference in the expression of *gga-miR-129-5p* between groups (Fig. 1). For predicted target genes of the miRNA, the data showed that only the expression of *IGFBP5*, the predicted target gene of *efu-miR-143*, was significantly lower in goose fatty liver than in the normal liver ($P < 0.05$), suggesting that *IGFBP5* is the potential target gene of *efu-miR-143*, and that *efu-miR-143* may exert local and global effects via *IGFBP5* (Fig. 2A, 2B). Other predicted target genes including *SLC7A2* for *efu-miR-143* and *MAN2A1* and *PNISR* for *tgu-miR-1662* are probably not their corresponding miRNAs (Fig. 2A, 2B).

Discussion

Recent studies have shown that there is a large amount of communication between different tissues and organs, which mutually affects their structures and functions (Pegtel and Gould, 2019). Since miRNAs can act on multiple target genes and are easily secreted and absorbed by tissues and organs, they may play an important role in systemic regulation of normal physiological function and development of diseases (Mishra *et al*., 2016). For example, Liu *et al*. reported that hepatocyte-derived exosomal *miR-192-5p* can affect the activation of M1 macrophages and hepatitis response (Liu *et al*., 2020); Castaño *et al*. reported that obesity-associated exosomal miRNAs play a central role in the etiopathogeny of glucose intolerance and dyslipidemia (Castaño

Table 3.   **miRNAs with significantly higher content in the blood of overfed geese compared to that in the blood of normally fed geese**

Gene	Sequence (5′ → 3′)	Control	Overfeeding	Fold change
efu-miR-143	CTGAGATGAAGCACTGTAGCT	0.6391	1391	2176.61
cli-miR-455-5p	TGTGCCCTTGGACTACATCGT	5.053	5041	997.41
gga-miR-129-5p	CTTTTTGCGGTCTGGGCTTGC	2.354	1567	665.33
dma-miR-143	TGAGATGAAGCACTGTAGCTCA	0.1818	9.577	52.68
ssc-miR-184	TGGACGGAGAACTGATAAGGGT	90.25	1042	11.54
ssc-miR-122	TGGAGTGTGACAATGGTGTTTGT	10505	110670	10.53
pol-miR-122-5p	TGGAGTGTGACAATGGTGTTTG	19883	200868	10.10
ipu-miR-122	TGGAGTGTGACAATGGTGTTT	16463	147323	8.95
gga-miR-122b	TGGAGTGTGACACTGGTGTTT	1.569	11.66	7.43
mmu-miR-122-3p	AAACGCCATTATCACACTAAT	1.067	7.766	7.28
tni-miR-107	AGCAGCATTGTACAGGGCTATCA	2.2035	9.661	4.38
tgu-miR-454-5p	ACCCTATCAATATTGCCTCTGCT	0.4913	2.056	4.18
mmu-miR-365-2-5p	AGGGACTTTCAGGGGCAGCTGTGT	0.3693	1.330	3.60
gga-let-7a-2-3p	CTGTACAACCTCCTAGCTTTCC	0.9899	2.735	2.76
ssc-miR-130b	CAGTGCAATGATGAAAGGGCGT	0.9547	2.423	2.54
tgu-miR-130b-3p	CAGTGCAATAATGAAAGGGCGT	735.9	1664	2.26
ssc-miR-193a-5p	TGGGTCTTTGCGGGCGAGATG	80.52	162.2	2.01
tgu-miR-1662	TTGACATCATCATACTTGGGAT	109.5	212.4	1.94
tni-miR-222	AGCTACATCTGGCTACTGGGTCTC	1762	3278	1.86
ipu-miR-107a	AGCAGCATTGTACAGGGCTAT	126.8	203.8	1.61
ipu-miR-454b	TAGTGCAATATTGCTTATAGGGT	24.12	37.34	1.55

Note: The values in the columns labeled "control" and "overfeeding" are the average contents of miRNAs in the blood of overfed geese (overfeeding group) and normally fed (control group) geese. Fold change is the ratio of the overfeeding group to the control group. The criteria for selecting the miRNAs are ($P<0.05$ and fold change >1.5). $n=3$.

Table 4.   **miRNAs randomly selected for the determination of their expression in goose fatty liver vs. normal liver**

Gene	Sequence (5′ → 3′)	Control	Overfeeding	Fold change	P-value
efu-miR-143	CTGAGATGAAGCACTGTAGCT	0.6391	1391.1	2176.6	0.031
cli-miR-455-5p	TGTGCCCTTGGACTACATCGT	5.054	5040.6	997.4	0.0017
pbv-miR-222a-5p	CGCTCAGTAGTCAGTGTAGATT	0.7272	1574.0	2165.4	0.061
ssc-miR-184	TGGACGGAGAACTGATAAGGGT	90.25	1041.9	11.54	0.015
tgu-miR-1662	TTGACATCATCATACTTGGGAT	109.5	212.4	1.94	0.041
gga-miR-129-5p	CTTTTTGCGGTCTGGGCTTGC	2.354	1567	665.33	0.047

Note: The values in the columns labeled "control" and "overfeeding" are the average contents of miRNAs in the blood of overfed geese (overfeeding group) and the normally fed (control group) geese. Fold change is the ratio of the overfeeding group to the control group. N=3.

et al., 2018); and Povero *et al*. reported that in diet-induced NAFLD/NASH, *miR-122* and *miR-192* levels were found to be more abundant in the liver and circulating vesicles compared to those in the control (Povero *et al*., 2014). These characteristics of miRNAs have enabled their development as novel therapeutic agents, which has become a key direction in current medical research. The liver is an important site of nutritional metabolism and secretes a large number of bioactive substances, such as *IGF*, *IGFBPs*, and complement (Zheng *et al*., 2009). However, there are only a few reports on the identification of miRNAs secreted by the liver, especially the fatty liver. In this study, goose fatty liver was used to select miRNAs with significantly higher content in the blood of geese with fatty liver compared to that in the blood of geese with a normal liver. On this basis, the differential expression of the randomly selected miRNAs and

their target genes between these two conditions of goose liver was determined. This may help promote further understanding of the role of communication between tissues or organs in the development of goose fatty liver.

Among miRNAs with higher contents in the blood of overfed vs. normally fed geese, several are known to be closely related to obesity and metabolic diseases (*e.g.*, fatty liver and diabetes). For example, it has been reported that *let-7a* (the family to which *let-7a-2-3p* belongs) can regulate glucose metabolism and insulin synthesis and secretion, thereby regulating the occurrence and development of diabetes (Frost and Olson, 2011; Zhu *et al*., 2011). In addition, *miR-143* has been reported to specifically inhibit the insulin-AKT pathway by downregulating oxysterol binding protein-related protein 8 (ORP8), leading to the inhibition of AKT phosphorylation, and ultimately regulating the occurrence
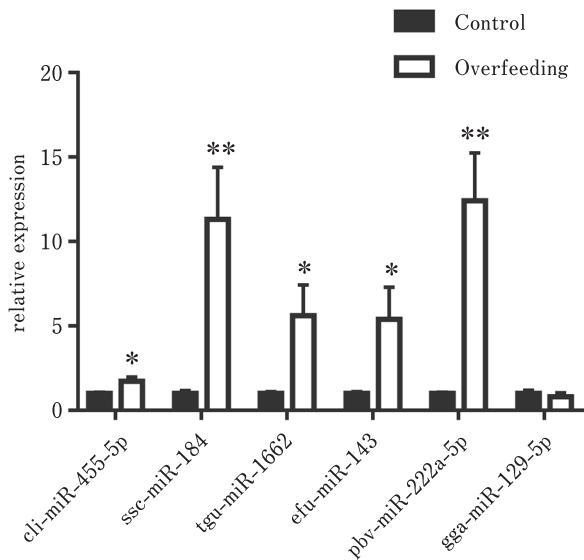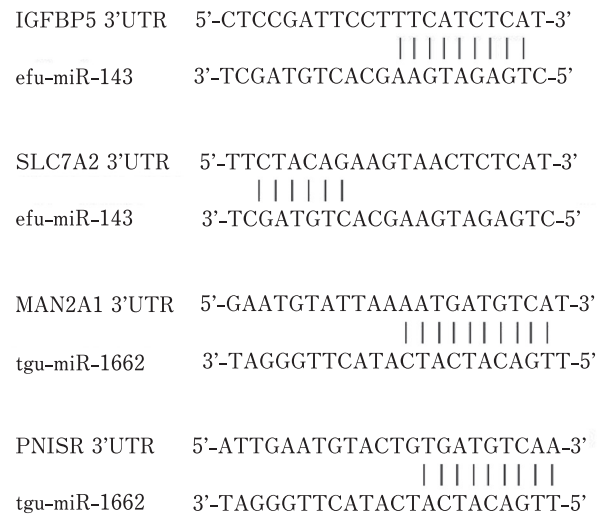
Fig. 1. **Expression of selected miRNAs in goose fatty liver vs. normal liver.** Expression of miRNAs was determined using qPCR from livers of overfed (overfeeding group) vs. normally fed geese (control group) on the 19th day of overfeeding. The relative expression is presented as fold change over control. N＝8. * and ** denote $P<0.05$ and <0.01 vs. control, respectively. The data are expressed as the mean±SE.

(A)

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IGFBP5 3'UTR    5'-CTCCGATTCCTTTCATCTCAT-3'
                     | | | | | | | | | |
efu-miR-143     3'-TCGATGTCACGAAGTAGAGTC-5'


SLC7A2 3'UTR    5'-TTCTACAGAAGTAACTCTCAT-3'
                          | | | | | |
efu-miR-143     3'-TCGATGTCACGAAGTAGAGTC-5'


MAN2A1 3'UTR    5'-GAATGTATTAAAATGATGTCAT-3'
                          | | | | | | | | | |
tgu-miR-1662    3'-TAGGGTTCATACTACTACAGTT-5'


PNISR 3'UTR     5'-ATTGAATGTACTGTGATGTCAA-3'
                         | | | | | | | | | |
tgu-miR-1662    3'-TAGGGTTCATACTACTACAGTT-5'
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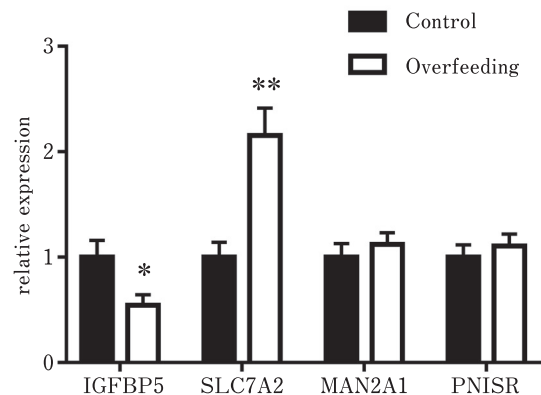
(B)

Fig. 2. **Expression of predicted target genes of miRNA in goose fatty liver vs. normal liver.** (A) Binding sites located within the sequences of mature miRNAs (efu-miR-143 and tgu-miR-1662) of goose and 3′ UTR of their respective predicted target genes of goose. (B) The mRNA expression of the predicted target genes of miRNAs (*efu-miR-143* and *tgu-miR-1662*) was determined using qPCR from the livers of overfed (overfeeding group) vs. normally fed geese (control group) on the 19th day of overfeeding. The relative expression is presented as fold change over control. N＝8. * and ** denote $P<0.05$ and <0.01 vs. control, respectively. The data are expressed as the mean±SE.

and development of diabetes mellitus (Li *et al.*, 2018). Experimental evidence indicates that *miR-184* can regulate the function of pancreatic *β*-cells and its inhibition can promote the release of insulin (Tattikota *et al.*, 2015). Moreover, the expression of *miR-184* in the ovaries of obese mice fed with a high-fat diet has been reported to be significantly higher than that in lean mice (Nteeba *et al.*, 2013). For the *miR-222* family, previous studies have shown that the expression of *miR-222* is upregulated in the liver of mice fed with high-fat and high-glucose diets. Overexpression of *miR-222* in mouse primary hepatocytes can weaken insulin-induced AKT phosphorylation due to *miR-222* binding to the 3'UTR of the *IRS* gene (Ono *et al.*, 2018). Correspondingly, meta-data analysis also shows that the level of *miR-222* in the blood of obese patients with type 2 diabetes is significantly increased compared to that in the healthy cohorts (Villard *et al.*, 2015).

To clarify whether the increased levels of miRNAs in the blood of geese with fatty liver vs. those with normal liver are secreted by the fatty liver, the expression of several randomly selected miRNAs including *miR-455-5p, miR-143, miR-184, miR-222a-5p, miR-1662,* and *miR-129-5p* was determined in the fatty and normal livers of geese. The results indicated that, except for *miR-129-5p*, the expression of other miRNAs in goose fatty liver was significantly higher than that in normal livers, suggesting that the miRNAs identified by blood miRNA-omics analysis (listed in Supplementary Table 3) were mostly secreted by goose fatty liver. These fatty liver-secreted miRNAs are also candidate communication mole-

cules that play a systemic regulatory role in the development of goose fatty liver, although this remains to be confirmed in future studies.

Furthermore, to illustrate how these miRNAs exert their global effects, we determined the expression of predicted target genes of *efu-miR-143* and *tgu-miR-1662* in goose fatty liver vs. normal liver. The results showed that the expression of *IGFBP5* mRNA in goose fatty liver was significantly

lower than that in the normal liver, which is contrary to the expression of *efu-miR-143*, suggesting that *IGFBP5* may be the target gene of *efu-miR-143* mediating the role of this miRNA in the development of goose fatty liver. IGFBP5 is a secreted polypeptide and by binding to IGF-II, it can affect the transport and release of IGF-II and prolong its half-life. Moreover, it can regulate the binding of IGF-II to the receptor and directly control its biological effects (Boisclair *et al*., 2001). The concentration of IGFBP5 is known to be reduced in patients with type I and type II diabetes (Jehle *et al*., 1998). Compared to wild-type mice, those lacking *IGFBP5* exhibited greater weight gain, milder glucose intolerance, and obesity (Gleason *et al*., 2010). In addition, the single nucleotide polymorphism affecting the expression of human *IGFBP5* is also associated with changes in adiponectin concentration (Kallio *et al*., 2009). Since the formation of fatty liver is associated with obesity, diabetes, and adiponectin content, *efu-miR-143* may exert local and global effects by inhibiting the expression of *IGFBP5* in fatty liver and other tissues and promoting the development of goose fatty liver. Blood indicators are often used for disease diagnosis; therefore, the blood-borne miRNAs identified in this study are good candidate biomarkers for the diagnosis of fatty liver.

In this study, target genes were predicted using online programs and databases including miRDB (http://mirdb. org/miRDB/) and TargetScan (http://www.targetscan.org/). However, only IGFBP5 of the four candidate target proteins fitted the prediction. The low accuracy of prediction was probably due to the following reasons: 1) The prediction was based on databases for species (human, rat, mouse, dog, chicken) other than geese. Although the miRNA sequences are quite conserved across different species, there exist differences for some miRNAs among species, including those between chicken and goose. 2) Target mRNAs can be more variable than their miRNAs across different species. 3) The expression of target mRNAs may be regulated by other variables such as transcription factors.

In this study, *miR-191* other than *U6* was used as the reference gene for qPCR analysis of miRNA in the liver. Based on miRNA-omics analysis, *U6* expression in goose fatty liver was significantly different from that in normal liver, making *U6* unsuitable for use as a reference gene. Using literature searches and miRNA-omics data, we chose *miR-191-5p*, *let-7a-5p*, *let-7f-5p*, and *miR-146c-5p* as options for internal reference genes. We used qPCR to verify that the expression of *miR-191* was stable across goose liver samples and that there were few differences in expression between goose fatty liver and normal liver. Therefore, *miR-191* was determined to be the most appropriate reference gene chosen for this study.

Although several miRNAs were identified as candidate markers for the diagnosis of fatty liver, the mechanisms by which they play a systemic role, as well as the targeting relationship between *miR-143* and *IGFBP5* were not addressed or confirmed in this study. These aspects warrant further investigation.

In conclusion, our study lays the foundation for investigat-

ing the role of miRNA-based inter-organ or tissue communication in the development of goose fatty liver.

Conflict of Interest

The authors claim no conflict of interest.

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