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

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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-
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 euthanasization using heparin as an anticoagulant. The samples were centrifuged, and plasma samples were subsequently acquired and stored at −70°C. Liver samples were also collected, snap-frozen in liquid nitrogen, and stored at −70°C.

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

| Gene     | Forward primer (5’ → 3’ )                           |
|----------|-----------------------------------------------------|
| cli-miR-455-5p | TGTGCCCTTGGACTACATCGTA                           |
| ssc-miR-184  | TGGACGGAGAACCTGAAGGGT                             |
| tgu-miR-1662 | TTGACATCATCATACTGGGAT                             |
| efl-miR-143 | CTGAGATGAGGACTGCTAGCTAA                           |
| pbv-miR-222a-5p | CGCTCAAGTGCTAAGTATTA                        |
| gga-miR-129-5p | CTTTTGCGCTGCTGGCGCTG                             |
| oha-miR-191-5p | CAACGGAATCCCAAAGCAGCT                        |
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 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 *hoa-miR-191-5p* (Zheng *et al*., 2013).

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

| Gene          | Forward primer (5′ → 3′)         | Reverse primer (5′ → 3′)         |
|---------------|----------------------------------|----------------------------------|
| IGFBP5       | GTGAAACATGAGAGGCCGAC            | CAAGGCCCAAGCTCTAATCTC            |
| SLC7A2       | TGGATGCGACTTGGCCCTTTT            | TCTCCACTGCTGAGTACC               |
| MAN2A1       | ACATTCAACACAGGGCTTGG            | GCATAGCGAAATCTACGCCC            |
| PINSR        | AAGGCTAGGGCTGCTGAACTCT          | GAGGGAGAGTGTGGACCTTGA            |
| GAPDH        | CTGTAGTCTCCCATGTITCGTG          | CCACGATGCACAGTGTGCA             |

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*, *tgmu-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*, *tgmu-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 *PINSR* for *tgmu-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 etiopathology of glucose intolerance and dyslipidemia (Castaño...
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 circulatory vessels 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

### 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 | P-value |
|-----------|--------------------|---------|-------------|-------------|---------|
| efu-miR-143 | CTGAGATGAAGACCTGAGCT | 0.6391 | 1391 | 2176.61 | 0.031 |
| cli-miR-455-5p | TGTGCCTTGGGACTCATCGT | 5.053 | 5041 | 997.41 | 0.0017 |
| gga-miR-129-3p | CTTTTTGGCTGCTAGTGGTGC | 2.354 | 1567 | 665.33 | 0.061 |
| dma-miR-143 | TGAAGAGCTGGCTATGCT | 0.1818 | 9.577 | 52.68 | 0.015 |
| ssc-miR-184 | TGGACGGAGAACTGATAAGGGT | 90.25 | 1042 | 11.54 | 0.047 |
| ssc-miR-122 | TGGAGTGACATGGTATGGT | 10505 | 110670 | 10.53 | 0.047 |
| pol-miR-122-5p | TGGAGTGACATGGTATGGTTG | 19883 | 200868 | 10.10 | 0.047 |
| tpu-miR-122 | TGGAGTGACATGGTATGGTT | 16463 | 147323 | 8.95 | 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 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 | CTGAGATGAAGACCTGAGCT | 0.6391 | 1391 | 2176.6 | 0.031 |
| cli-miR-455-5p | TGTGCCTTGGGACTCATCGT | 5.053 | 5041 | 997.4 | 0.0017 |
| pnv-miR-222-5p | CGCCTAGTACACTGTTAGATG | 0.7272 | 1574.0 | 2165.4 | 0.061 |
| ssc-miR-184 | TGGACGGAGAACTGATAAGGGT | 90.25 | 1041.9 | 11.54 | 0.047 |
| tgu-miR-1662 | TTGACATCATCATCTTGGGAT | 109.5 | 212.4 | 1.94 | 0.047 |
| tgu-miR-107a | AGCAGCATTGACAGGCGCATCA | 2.2035 | 9.661 | 4.38 | 0.047 |
| ipu-miR-454b | TAGCAGCTATGCTTAAGGGAT | 24.12 | 37.34 | 1.55 | 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 normally fed (control group) geese. Fold change is the ratio of the overfeeding group to the control group. N=3.
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 molecules 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 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.
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 miRNAs can be more variable than their miRNAs across different species. 3) The expression of target miRNAs may be regulated by other variables such as transcription factors.

In conclusion, our study lays the foundation for investigating the role of miRNA-based inter-organ or tissue communication in the development of goose fatty liver.

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Conflict of Interest

The authors claim no conflict of interest.

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