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MicroRNA-122 targets genes related to liver metabolism in chickens

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression by targeting mRNAs. MicroRNA-122 (miR-122) has important functions in mammalian and fish livers, but its functions in the poultry liver are largely unknown. In this study, we determined the expression patterns of miR-122 in the chicken and identified its target genes in the chicken liver. We found that chicken miR-122 was highly expressed in the liver and that its expression in the liver was up-regulated during the early posthatch life. By bioinformatics and reporter gene analyses, we identified *PKM2*, *TGFB3*, *FABP5* and *ARCN1* as miR-122 target genes in the chicken liver. miR-122 knockdown in primary chicken hepatocytes and expression analysis of miR-122 and predicted target mRNAs in the chicken liver suggested that the expression of *PKM2* and *FABP5* in the chicken liver is regulated by miR-122. Knockdown of miR-122 affected the expression of 123 genes in cultured chicken hepatocytes. Among these genes, the largest cluster, which consisted of 21 genes, was involved in liver metabolism. These findings suggest that miR-122 plays a role in liver metabolism in the chicken by directly or indirectly regulating the expression of genes involved in liver metabolism.

1. Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs of about 22 nucleotides in length, and they enhance the degradation or repress the translation of mRNAs by binding to the 3′ untranslated regions (UTRs) of target mRNAs (Lee et al., 1993; Bartel, 2004; Yekta et al., 2004). miRNAs have been shown to be involved in almost every biological process, including growth, development and metabolism (Bartel, 2004; Wienholds and Plasterk, 2005).

The liver is a key metabolic organ. Several miRNAs, including miR-33, miR-370, miR-335, miR-216, miR-302a, and miR-168, have been shown to play important roles in liver metabolism in the mouse and human (Nakanishi et al., 2009; Gerin et al., 2010; Iliopoulos et al., 2010; Rayner et al., 2010; Hoekstra et al., 2012; Zhang et al., 2012). miR-33 regulates cholesterol metabolism and lipid homeostasis in the liver by targeting the genes involved in cholesterol transport (Rayner et al., 2010), and reduces fatty acid degradation in the liver by targeting the genes related to fatty acid β-oxidation (Gerin et al., 2010). miR-370 represses fatty acid β oxidation in the liver by targeting carnitine palmitoyltransferase 1A and increases triglyceride and cholesterol accumulation in the liver by up-regulating the expression of genes fatty acid synthase (*FASN*), acetyl-CoA carboxylase alpha (*ACACA*) and diacylglycerol O-acyltransferase 2 (*DGAT2*) which are known to be involved in lipid metabolism (Iliopoulos et al., 2010). miR-335 plays a similar role in hepatic content of triglyceride and cholesterol (Nakanishi et al., 2009).

miR-122 is specifically and abundantly expressed in human, woodchuck and mouse livers (Lagos-Quintana et al., 2002; Chang et al., 2003), and is processed from the transcript of the non-coding gene *hcr*, which is conserved in various animals (Chang et al., 2004; Li et al., 2011). In mammals, miR-122 regulates metabolism and several other processes in the liver (Chang et al., 2004; Krutzfeldt et al., 2005; Esau et al., 2006; Xu et al., 2010; Laudadio et al., 2012). miR-122 affects liver protein metabolism in the mouse by regulating the expression of *CAT-1*, an important amino acid metabolic regulator (Chang et al., 2004). When liver miR-122 expression is inhibited in the mouse, or a non-human primate, its plasma cholesterol level decreases (Krutzfeldt et al., 2005; Esau et al., 2006; Elmen et al., 2008a, 2008b). When miR-122 is knocked down, hepatic fatty acid and cholesterol synthesis rate decrease, and fatty acid oxidation increases in mouse hepatocytes (Esau et al., 2006). These findings show that miR-122 plays important roles in lipid metabolism in the mammalian liver. Furthermore, miR-122 plays roles in hepatocyte differentiation (Laudadio et al., 2012), liver development (Xu et al., 2010), iron homeostasis (Castoldi et al., 2011), hepatocellular carcinoma proliferation (Jung et al., 2011), hepatocarcinogenesis (Tsai et al., 2012), intrahepatic metastasis of hepatocellular carcinoma (Tsai et al., 2009), and inflammation (Hsu et al., 2012), in mammals. In fish like rainbow trout, inhibition of

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miR-122 increases postprandial glucose concentration, and decreases lipogenesis and plasma cholesterol concentration by repressing hepatic FAS protein expression and increasing the expression of genes involved in cholesterol degradation and excretion (Mennigen et al., 2014). While the miRNA itself is highly conserved in the vertebrate world, the targets are not, as the 3′ UTR is highly variable between species, and individuals as well, so it is necessary to investigate the miRNA targets in other vertebrate species.

Little is known about the expression or function of miR-122 in the chicken. The objective of the current study was to determine whether chicken miR-122 is expressed only in the liver and its potential functions.

2. Material and methods

2.1. Animal experiment

Various tissues were collected from 4-week-old Arbor Acres (AA) commercial chickens and the livers were collected from 0, 1, 2, 3, 4, 5, 6 and 7-week-old AA commercial chickens. Tissues including the chest muscle, thigh muscle, adipose tissue, heart, liver, spleen, lung, kidney, glandular stomach, muscular stomach, intestine and brain were snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. All procedures involving animals were approved by Changshu Institute of Technology Institutional Animal Care and Use Committee.

2.2. RNA isolation and real-time qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and its concentrations and quality were determined by NanoDrop ND2000 spectrophotometry (Thermo Scientific, Wilmington, DE) and formaldehyde-agarose gel electrophoresis. The expression of miRNAs was detected by stem-loop real-time qRT-PCR following the protocol of TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA) (Chen et al., 2005). The RT reaction and the PCR were performed using the TaqMan MicroRNA Reverse Transcription Kit, TaqMan Universal Master Mix II, no UNG, and an Applied Biosystems 7500 Thermocycler (Applied Biosystems). All reactions were run in duplicate. miRNA expression data were analyzed using the $2^{-\Delta\Delta CT}$ method and normalized to *18S* rRNA (Livak and Schmittgen, 2001). The expression of mRNAs was detected using a PrimeScript RT reagent kit and the SYBR Premix Ex Taq (Takara, Dalian, China). mRNA data were analyzed also using *18S* rRNA as the internal control. The sequences of primers for this study are listed in Table S1.

2.3. Computational prediction of genes targeted by miR-122

The chicken 3′ UTR sequences were downloaded from the 3′ UTR databases at http://utrdb.ba.itb.cnr.it/ (Mignone et al., 2005). The target genes of miR-122 and the 3′ UTR sequences bound by miR-122 were predicted using the computational algorithm "miRanda" with the "TargetScan" principle by homology, free energy and seed region as previously described (Wang et al., 2013). Sequence homology was analyzed by a BLAST search in GenBank (http://www.ncbi.nlm.nih.gov).

2.4. Plasmid construction

The DNA sequence encoding the miR-122 precursor was PCR amplified from chicken genomic DNA. The PCR product was cloned into the pcDNA3.1 (+) vector (Invitrogen) using the HindIII and XhoI restriction sites to construct the miR-122 overexpression vector pcDNA3.1/miR-122. The 3′ UTRs predicted to contain miR-122 binding sites were amplified from chicken genomic DNA and inserted into the pMIR-REPORT vector (Ambion, Carlsbad, CA) between the SacI or MluI and HindIII sites to construct the pMIR-3′ UTR vectors. Binding site-mutated pMIR-mut3′ UTR vectors were generated from pMIR-3′ UTRs using overlap-extension PCR (Fig. 1A). All constructed vectors were verified by sequencing.

2.5. Cell culture, transfection and luciferase reporter assay

Chinese hamster ovary (CHO) cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and 100 U/ml penicillin–streptomycin (Gibco) at 37 °C with 5% CO_2 in a humidified incubator. For the miRNA overexpression assay, cells were seeded in 6-well plates for 24 h and transfected with pcDNA3.1/miR-122 using X-tremeGENE 9 DNA Transfection Reagent (Roche, Mannheim, Germany) as previously described (Wang et al., 2013). In this transfection, pcDNA3.1 was used as the control for pcDNA3.1/miR-122. At 48 h after transfection, total RNA was isolated from cells and used to quantify the expression levels of miR-122 and mRNAs predicted to be targeted by miR-122. For the luciferase reporter assay, the CHO cells were seeded in 24-well plates for 24 h and the transfection was performed as described previously (Wang et al., 2013). Cells were harvested and lysed in passive lysis buffer (PLB) 48 h post-transfection. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) on a Modulus single tube luminometer (Turner BioSystems, Sunnyvale, CA) according to the manufacturer's instructions. Transfection efficiency was controlled by normalizing the firefly luciferase activity to the *Renilla* luciferase activity in the same cell lysates.

2.6. Isolation and culture of primary chicken hepatocytes

Chicken hepatocytes were isolated using an improved two-step collagenase method as previously described (Wang et al., 2013). The isolated hepatocytes were cultured at a density of 6×10^5 cells/ml in William's E medium supplemented with 5% chicken serum, 100 U/ml penicillin–streptomycin, 10 μg/ml insulin and 30 mmol/l NaCl at 37 °C with 5% CO_2 in a humidified incubator.

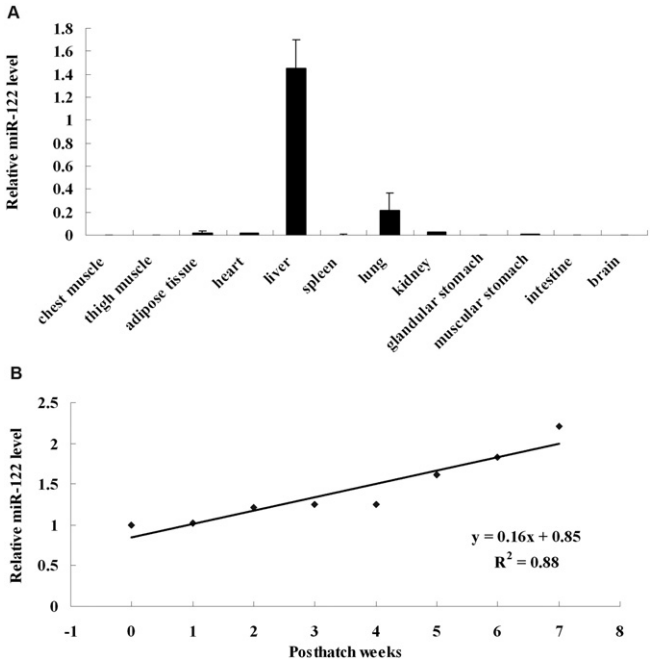

Fig. 1. Expression of miR-122 in chicken tissues and liver at different posthatch days. (A) The expression levels of miR-122 in 12 chicken tissues were analyzed by real-time qRT-PCR and normalized to *18S* rRNA. Three 4-week-old chickens were used. (B) The expression levels of miR-122 in livers at 8 posthatch stages. Three chickens at every stage were used. Data are means ± SEM and analyzed by ANOVA.