Expression of miR-33 from an SREBF2 Intron Targets the FTO Gene in the Chicken

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Abstract

The sterol regulatory element binding transcription factor 2 (SREBF2) gene encodes a transcription factor that activates the expression of many genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. Through bioinformatics, we found that intron 16 of the chicken SREBF2 gene might encode the chicken miR-33. Using quantitative RT-PCR, we detected the expression of miR-33 in a variety of chicken tissues including skeletal muscle, adipose tissue, and liver. Three hundred and seventy eight genes were predicted to be potential targets of miR-33 in chickens via miRNA target prediction programs “miRanda” and “TargetScan”. Among these targets, the gene FTO (fat mass and obesity associated) encodes a Fe(II)- and 2-oxoglutarate-dependent nucleic acid demethylase that regulates lipid metabolism, and the possibility that its expression is negatively regulated by miR-33 in the chicken liver was therefore further studied. Co-transfection and dual-luciferase reporter assays showed that the expression of luciferase reporter gene linked to the 3′-untranslated region (3′UTR) of the chicken FTO mRNA was down-regulated by overexpression of the chicken miR-33 in the C2C12 cells (P<0.05). Furthermore, this down-regulation was completely abolished when the predicted miR-33 target site in the FTO 3′UTR was mutated. In contrast, the expression of FTO mRNA in the primary chicken hepatocytes was up-regulated after transfection with the miR-33 inhibitor LNA-anti-miR-33. Using quantitative RT-PCR, we also found that the expression of miR-33 was increased in the chicken liver from day 0 to day 49 of age, whereas that of the FTO mRNA was decreased during the same age period. These data together suggest that miR-33 might play an important role in lipid metabolism in the chicken liver by negatively regulating the expression of the FTO gene.

Introduction

In addition to classical transcription factors, a new class of non-coding RNAs termed microRNAs (miRNAs) has emerged as critical regulators of gene expression acting predominantly at the posttranscriptional level. miRNAs are single-stranded small RNA molecules, with the length of 18–25 nucleotides (nt). They bind to the 3′-untranslated regions (3′UTR) of mRNA transcripts to reduce the translation of these transcripts or to cause their degradation [1]. Bioinformatics predictions and experimental approaches indicate that a single miRNA may target more than 100 miRNAs [2]. In a genome, 20%~30% genes are regulated by miRNAs [3]. miRNAs have been implicated in the regulation of almost all developmental, physiological and pathological processes [4].

microRNA-33 (miR-33) is transcribed from an intronic region within the sterol response element binding transcription factor 2 (SREBF2), also called sterol response element binding protein-2 gene [5], which directly activates the expression of more than 30 genes involved in the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids [6,7]. miR-33 is expressed in numerous mammalian cell types and tissues [8,9]. The expression levels of miR-33 and SREBF2 are closely paralleled in human or mouse hepatocytes and macrophages [5,10], suggesting that they are coregulated at the transcriptional level. Research by multiple groups has shown that miR-33 analogs regulate cholesterol and fatty acid metabolism in mammalian systems, corresponding with the function of its host gene [10,11]. A number of miR-33 targets have been identified, including the ABCA1, ABCG1 and NPC-1 genes, which are involved in cholesterol efflux and high-density lipoprotein metabolism [5,8,11], and the CPT1A, CROT and HADHB genes, which are involved in fatty acid β-oxidation [11]. In addition to regulating cholesterol transport, high-density lipoprotein metabolism and fatty acid β-oxidation, miR-33 was recently reported to regulate cell cycle progression and cellular proliferation [12], inflammatory response [13] and insulin signaling [14].

Genome-wide association studies (GWAS) have initially identified the FTO gene as a gene strongly associated with obesity [15]. Bioinformatics analyses suggest the human FTO is a member of the non-heme dioxygenase (Fe(II)- and 2-oxoglutarate-dependent dioxygenase) superfamily [16,17], that catalyze demethylation of 3-methylthymine and 3-methyluracil in single-stranded DNA and RNA, respectively [18]. Based on its crystal structure FTO has no appreciable activity on double stranded nucleic acids, and it has a substrate preference for methylated RNA over DNA [19].
The objectives of this study were to determine whether miR-33 is expressed in the chicken, and, if so, to identify its target genes. In this paper, we provide computational and experimental evidence demonstrating that miR-33 is expressed in the chicken. We also provide evidence suggesting that miR-33 may regulate the expression of the FTO gene in the chicken liver.

Materials and Methods

Computational Prediction of miR-33 Target Genes

The 3′ UTR sequences of gallus gallus were downloaded from the 3′ UTR database (http://utrdb.ba.ibc.cnr.it/). The miRNA target prediction software miRanda, miRDB (http://mirdb.org/miRDB) and targetscan (http://www.targetscan.org/) were employed to predict miR-33 binding sites in chicken 3′ UTRs.

Construction of Plasmids

A DNA fragment containing the predicted miR-33 and 150 bp upstream and 150 bp downstream sequences was amplified by PCR from chicken genomic DNA. The PCR product was cloned into the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, CA) at the HindIII and XhoI restriction sites to generate the chicken miR-33 over-expression vector pcDNA3.1-miR-33. A negative control vector pcDNA3.1-NC-miRNA was constructed by inserting into pcDNA3.1 a sequence that had no predicted target site in the chicken FTO 3′ UTR. The chicken FTO 3′ UTR encompassing the predicted miR-33 binding site was amplified by PCR and directionally inserted downstream of the luciferase expression cassette of the pMIR-reporter vector (Ambion, Carlsbad, CA) at the SacI and HindIII sites to construct the pMIR-FTO reporter vector. Point mutations in the seed region of the predicted miR-33 binding sequence within the 3′ UTR of chicken FTO were generated using overlap-extension PCR, and the resulting plasmid was named pMIR-FTOmut. All constructs were confirmed by sequencing and prepared to reduce endotoxin by using the PureLinkTM HiPure Plasmid Filter Purification Kits (Invitrogen, Carlsbad, CA).

RNA Isolation and Real-time qRT-PCR

Arbor Acres commercial chickens were used in the present study. Various tissues were collected from 4-week-old chickens and liver samples were taken from 0, 1, 2, 3, 4, 5, 6 and 7-week-old chickens following euthanasia. All procedures involving chickens were approved by the Changshu Institute of Technology Institutional Animal Use and Care Committee. Total RNAs were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturers’ protocol, and RNA concentrations and integrity were determined by NanoDrop ND2000 spectrophotometry (Thermo Scientific, Wilmington, DE) and formaldehyde-agarose gel electrophoresis, respectively. The expression of miR-33 was quantified by real-time qRT-PCR according to the protocol of TaqMan MicroRNA Assay (Applied Biosystems, Foster City, CA). All reactions were performed in duplicate. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Ct values for a miRNA were normalized to that for 18S rRNA. The expression of miRNA was quantified by real-time qRT-PCR using the PrimeScript RT kit, and SYBR Green PCR master mix (Takara, Dalian, China). The Ct values for an mRNA were normalized to those for β-actin mRNA. The sequences of primers for this study are listed in Table 1.

Expression of miR-33 Targets FTO Gene

Hepatocytes were isolated from four-week-old chickens using an improved two-step collagenase method as described before [24]. In brief, chickens were fasted 12 h before being anesthetized by intraperitoneal injection of sodium thiopental (50 mg/kg BW) and anticoagulated by intraperitoneal injection of heparin (1,750 U/kg BW). Livers were first perfused with 250 ml of buffer A (5 mM EDTA, 10 mM of HEPES, 137 mM of NaCl, 3 mM of KCl, 5 mmol/L of Na2HPO4, pH 7.5) and then with 250 ml of buffer B (buffer A without EDTA) until the livers began to pale yellow. Then livers were perfused with 5 ml of buffer C (buffer B containing 0.6 mg/ml of CaCl2 and 0.4 mg/ml of collagenase type IV) and digested for 20 min at 37°C. Digested livers were shredded and continuously incubated in 5 ml of buffer C at 37°C for another 20 min. Digestion was stopped by adding William’s E medium (Gibco, Grand Island, NY) supplemented with 5% chicken serum and 2 mg/ml of BSA. Cells were collected by filtering the digest sequentially through 200, 75 and 30 μm filters. Cells were incubated with red blood cell lysis buffer for 15 min on ice and then washed with William’s E medium containing 100 U/ml of penicillin-streptomycin and 2 mg/ml of BSA to remove cell fragments and erythrocytes. Cell number and viability were verified by the trypan blue exclusion test. Cells were cultured at a density of 6 × 10⁴ cells/ml in 12-well plates in William’s E medium supplemented with 5% chicken serum, 100 U/ml penicillin-streptomycin, 10 μg/ml insulin and 30 mM NaCl at 37°C with 5% CO2 in a humidified incubator.

### Table 1. Primer sequences for plasmid construction and real-time qRT-PCR.

| Primer name | Primer sequences (5′–3′) | Products length(bp) | Tm(°C) | Purpose |
|-------------|-------------------------|---------------------|-------|---------|
| gagmiR33    | F/R ccaaggcttcTCTATTGAGCCAGCATG/cagctcagCAATCCCTTCTCCCATC | 350   | 58   | Cloning |
| gagFTO     | F/R caggtgtcCTAGTAGTGAAATGCAAGG/gcaaggcttcATTCTGAGGCAAGGTA | 288   | 58   | Cloning |
| gagFTOmut  | F/R GTGCTTACCTGAAATCTATTGTTTTCACC/GGTGAGAAACCAATAGAATTTCGAATGAAGCAC | 288   | 58   | Cloning |
| gagrebp2   | F/R AGCGATTCATCAAGGACCTCAGTTGCAATGCAGAAGG | 153   | 58   | qRT-PCR |
| β-actin    | F/R CACGGGATTTCCACCAACTG/ACAGCCTGATGCTCTACATA | 200   | 58   | qRT-PCR |

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Primary chicken hepatocytes were cultured in 12-well plates for approximately 24 h before transfection. Chicken hepatocytes were transfected with 80 nM miRCURY LNA-anti-miR-33 or LNA scramble control (Exiqon, Woburn, USA) utilizing X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany). The expression of miR-33 and FTO mRNA was detected 48 h post-transfection.

Culture and Transfection of C2C12 Cells

C2C12 cells were obtained from Cell Resource Center of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. They were maintained in Dulbecco’s Modified Eagle’s...
Medium (DMEM) containing 2 mM L-Glutamine, 1 mM sodium pyruvate, 100 U/ml of penicillin-streptomycin and 10% fetal bovine serum (FBS) (Gibco) at 37°C with 5% CO₂ in a humidified incubator. To overexpress miR-33, cells were seeded at a density of 1.5 × 10⁵ cells/ml in 6-well plates for 24 h and transfected with pcDNA3.1-miR-33 using the X-tremeGENE 9 DNA Transfection Reagent (Roche) as described previously [25]. After 48 h, total RNA was isolated and used to quantify the expression level of miR-33.

To determine if miR-33 targets the FTO 3'UTR, C2C12 cells were seeded in 24-well plates for 24 h before transfection. pMIR-FTO (Firefly luciferase) or pMIR-mutFTO, pcDNA3.1-miR-33 or pcDNA3.1-NC-miRNA and transfection efficiency control pRL-CMV (Renilla luciferase) were mixed and co-transfected into the cells using X-tremeGENE 9 DNA Transfection Reagent (Roche). Cells were harvested and lysed 48 h after transfection. Luciferase activity was measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI) on a Modulus single tube luminometer (Turner BioSystems, Sunnyvale, CA). Firefly luciferase activity was normalized to Renilla luciferase activity. This transfection experiment was performed in triplicate wells and repeated at least three times.

### Table 2. Computational prediction of partial miR-33 target genes by Targetscan.

| Human ortholog of target gene | Representative transcript | Gene name | Conserved sites | Total context score |
|-------------------------------|---------------------------|-----------|-----------------|--------------------|
| ATP-binding cassette, sub-family A (ABC1), member 1 | NM_005502 | ABCA1 | 8mer | 0.80 |
| Carnitine O-octanoyltransferase | NM_01143935 | CROT | 7mer+1A | 0.90 |
| (Na+/alpha)-acetyltransferase 30, Nat catalytic subunit | NM_01011713 | NAA30 | 7mer+1A | 0.88 |
| Growth factor receptor-bound protein 10 | NM_001001549 | GB10 | 7mer+1A | 0.88 |
| Zinc finger protein 281 | NM_012482 | ZNF281 | 7mer+1A | 0.87 |
| Niemann-Pick disease, type C1 | NM_000271 | NPC1 | 7mer | 0.87 |
| Versican | NM_001126336 | VNAN | 7mer+1A | 0.87 |
| Adenylate cyclase activating polypeptide 1 (pituitary) | NM_01099733 | ACP7 | 7mer+1A | 0.86 |
| Glycine receptor, alpha 1 | NM_000171 | GLR1 | 7mer+1A | 0.86 |
| Solute carrier family 12, member 5 | NM_01134771 | SLC12A5 | 7mer+1A | 0.86 |
| Insulin-like growth factor 1 (somatomedin C) | NM_000618 | IGF1 | 7mer+1A | 0.86 |
| Sodium channel, voltage gated, type VIII, alpha subunit | NM_01177984 | SCN8A | 7mer+1A | 0.85 |
| Mitochondrial ribosomal protein S25 | NM_022497 | MRPS25 | 7mer+1A | 0.85 |
| Pim-3 oncogene | NM_001001852 | PIM3 | 7mer+1A | 0.85 |
| Carnitine palmitoyltransferase 1A (liver) | NM_001876 | CPT1A | 7mer+1A | 0.85 |
| Protein kinase C, epsilon | NM_005400 | PKC1 | 7mer+1A | 0.85 |
| Intestinal cell (MAK-like) kinase | NM_014920 | ICK | 7mer+1A | 0.85 |
| Abhydrolase domain containing 2 | NM_007011 | ABHD2 | 7mer+1A | 0.85 |
| Fibroblast growth factor 7 | NM_002009 | FGF7 | 7mer+1A | 0.85 |
| RAP2A, member of RAS oncogene family | NM_021033 | RAP2A | 7mer+1A | 0.85 |
| Required for meiotic nuclear division 5 homolog A | NM_022780 | RMN5A | 7mer+1A | 0.85 |
| Homeodomain interacting protein kinase 2 | NM_001113239 | HIPK2 | 7mer+1A | 0.85 |
| A kinase (PRKA) anchor protein 2 | NM_001004065 | AKAP2 | 7mer+1A | 0.85 |
| PALM2-AKAP2 readthrough | NM_007203 | PALM2-AKAP2 | 7mer+1A | 0.85 |
| Growth arrest-specific 1 | NM_002048 | GAS1 | 7mer+1A | 0.85 |
| Protocadherin 18 | NM_019035 | PCDH18 | 7mer+1A | 0.85 |
| Tropomyosin 3 | NM_001043351 | TPM3 | 7mer+1A | 0.85 |
| DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked | NM_01193416 | DDX3X | 7mer+1A | 0.85 |
| Zinc finger, MIZ-type containing 1 | NM_020338 | ZMIZ1 | 7mer+1A | 0.85 |
| Ubiquitin-conjugating enzyme E2 variant 2 | NM_003350 | UBE2V2 | 7mer+1A | 0.85 |
| Nucleosome assembly protein 1-like 4 | NM_005969 | NAP1L4 | 7mer+1A | 0.85 |
| Salt-inducible kinase 1 | NM_173354 | SKI | 7mer+1A | 0.85 |
| KIAA1409 | NM_020818 | KIAA1409 | 7mer+1A | 0.85 |
| Glutamate receptor, ionotrophic, AMPA 3 | NM_000828 | GRIA3 | 7mer+1A | 0.85 |

Note: Target genes are listed in the table of that whose total context score is lower than –0.30. Interacting sites with miR-33 in the 3'UTR of predicted target genes are in parentheses. 8 m: An exact match to positions 1–8 of miR-33; 7m+8: An exact match to positions 2–8 of miR-33; 7m+1A: An exact match to positions 2–7 of miR-33 followed by an ‘A’.

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Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). The statistical significance of differences was evaluated with the student’s t-test or one way ANOVA. *P<0.05 was considered significant.

Results

miR-33 is Predicted from Intron 16 of the Chicken SREBF2 Gene

The miR-33 family has been predicted to be present in several mammalian species, including human, rat, mouse, and cow. In some species there is a single member of this family which gives the mature product miR-33. However, primates and a limited number of other species have two members of this family called miR-33a and miR-33b, which are located in the intronic regions of the SREBF2 and SREBF1 genes, respectively. Aligning the chicken SREBF2 and SREBF1 DNA sequences with the corresponding human, mouse, rat, and cow sequences revealed that intron 16 of the chicken SREBF2 gene might encode the chicken miR-33 (Fig. 1). A typical stem-loop pre-miRNA and mature miRNA can be predicted from this region of the chicken genome (Fig. 1).

Expression of miR-33 and SREBF2 Gene in Various Chicken Tissues

The expression of miR-33 in 10 types of tissues from 4 week-old chickens was analyzed using real-time qRT-PCR. miR-33 expression was detected in all 10 chicken tissues with the highest level in the heart (Fig. 2). We also analyzed the expression of the host gene SREBF2 in the same set of chicken tissues. SREBF2 mRNA was also widely expressed in chickens, with the highest level in breast muscle (Fig. 2). The expression levels of miR-33 and SREBF2 mRNA did not parallel in most of the tissues analyzed (Fig. 2). The correlation coefficient (R) between miR-33 and SREBF2 mRNA expression in different chicken different tissues was 0.268 (*P>0.05). This suggests that their expressions are not co-regulated in most chicken tissues.

Computational Prediction of miR-33 Target Genes

To predict the target genes of chicken miR-33, the chicken 3’UTRs were analyzed for potential binding sites of miR-33 by the computational algorithm “miRanda”. Of the 11,891 chicken 3’UTRs in the 3’UTR database, 378 were predicted to be targeted by miR-33. In addition, a variety of online target prediction software was used to predict the targets of miR-33. Top targets of miR-33 (total context score ≥0.30 by TargetScan) are listed in Table 2.

Verification of the Interaction between miR-33 and the FTO 3’UTR

One of the predicted miR-33 targets is the FTO gene. We chose to experimentally validate the physical and functional interaction between miR-33 and FTO because the latter was recently...
discovered to be associated with obesity [15,22,23,26], and because this interaction has not been characterized in any species.

To determine whether the putative miR-33 target sequence in the FTO 3'UTR mediates translational repression by miR-33, we inserted the 3'UTR of the chicken FTO transcript downstream of a luciferase reporter gene to generate the reporter plasmid pMIR-FTO (Fig. 3). We also constructed a similar plasmid, pMIR-FTOmut, in which the putative miR-33 binding site in the FTO 3'UTR was partially mutated, and a chicken miR-33 overexpression vector named pcDNA3.1-miR-33. We transfected C2C12 cells with the pMIR-FTO or pMIR-FTOmut reporter vector, and pcDNA3.1-miR-33 or pcDNA3.1 (empty vector). Successful overexpression of miR-33 was validated by real-time qRT-PCR (Fig. 4A). Co-transfection of pcDNA3.1-miR-33 resulted in a decrease in luciferase activity expressed from pMIR-FTO, compared with co-transfection of pcDNA3.1 (P = 0.05, Fig. 4B). This decrease was abolished by mutation of the miR-33 binding site in the FTO 3'UTR (Fig. 4B). These results indicate that miR-33 can inhibit FTO expression by directly interacting with the predicted target site in the FTO 3'UTR.

miR-33 Knockdown Up-regulated FTO mRNA Expression in Primary Chicken Hepatocytes

The FTO gene appears to play a role in lipid metabolism and energy homeostasis [23,27]. De novo fatty acid synthesis in chickens takes place mainly in the liver [28]. Thus, in chickens, the liver might be the tissue where the FTO gene is involved in lipid metabolism and energy homeostasis. In view of this possibility, we evaluated the interaction between miR-33 and FTO mRNA in primary chicken hepatocytes. Specifically, we determined if knockdown of miR-33 expression by LNA-anti-miR-33 would increase FTO mRNA expression in primary chicken hepatocytes.

Figure 5. Effect of miR-33 knockdown on the expression of miR-33 and FTO mRNA in primary chicken hepatocytes. A: Expression levels of miR-33. Primary chicken hepatocytes were transfected with LNA-anti-miR-33 or LNA scramble control. miR-33 and FTO mRNA were quantified by real-time qRT-PCR 48 h after transfection. Data are means ± SEM (n = 3), P = 0.4. B: Expression levels of FTO mRNA. Data are means ± SEM (n = 3), P = 0.2.

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Figure 6. Expression levels of chicken miR-33 and FTO mRNA in chicken liver at different postnatal ages. A. The expression levels of miR-33 and FTO mRNA in chicken liver from 0 to 49 d of ages were analyzed by qRT-PCR. The former was normalized to 18S rRNA, while the latter to β-actin mRNA. Data are means ± SEM (n = 3). B. Expression levels of chicken miR-33 and FTO mRNA in liver from 0 to 49 d of ages are negatively correlated (P = 0.07), as determined by a regression analysis.

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Transfection of LNA-anti-miR-33 into chicken hepatocytes decreased miR-33 expression by 44% (Fig. 5A). This decrease was associated with a 29% increase in FTO mRNA expression (Fig. 5B). These data suggest the possibility that miR-33 negatively regulates the expression of FTO mRNA in chicken liver.

**Inverse Correlation of miR-33 and FTO mRNA Expression in Chicken Liver at Different Developmental Stages**

To further evaluate the possibility that FTO expression is negatively regulated by miR-33 in chicken liver, we quantified miR-33 and FTO mRNA in chicken liver at 8 different ages using real-time qRT-PCR. We found that the expression of miR-33 was increased, whereas that of FTO mRNA was decreased from 0 to 49 days of age (Fig. 6A). The correlation coefficient between miR-33 and FTO mRNA expression in chicken liver at different developmental stages was –0.669 (P= 0.07)(Fig. 6B). These inverse changes in miR-33 and FTO mRNA expression suggest that miR-33 may be one of the negative regulators of FTO mRNA expression in the chicken liver during development.

**Discussion**

The majority of the characterized miRNA genes are intergenic or oriented antisense to neighboring genes and are therefore suspected to be transcribed as independent units [29]. However, some mammalian miRNAs are located within introns of protein-coding genes or even in exons of long nonprotein-coding transcripts rather than in their own unique transcription units [30]. Intronic miRNAs are typically coordinately expressed and processed with the precursor mRNA in which they reside [31]. miR-33 is an intronic miRNA, and its expression levels paralleled those of its host gene SREBF2 in diverse cell types, including hepatocytes and macrophages in the human and mouse [8,10]. In the present study we predicted computationally and validated experimentally the transcription of miR-33 from intron 16 of the chicken SREBF2 gene. However, our expression data did not support co-regulation of SREBF2 and miR-33 expression across 10 types of chicken tissues examined.

Predicting targets is an important first step to determine the function of a miRNA. Many algorithms and databases for miRNA target predictions have been established, and among them, miRanda [25], TargetScan [25,32], and PicTar [33], appear to be the most widely used miRNA target prediction methods. In this study, 378 genes were predicted as the target genes of miR-33 among the total 11,891 chicken genes within the 5’UTR database using “miRanda”. The “TargetScan” principle was also applied in the prediction procedures: the target site should match to the seed region of miRNA (nucleotides 2–7), the 8th nucleotide of miRNA should be a U, and the first nucleotide of miRNA should be an A [32]. One of the predicted target genes of miR-33 named FTO is a member of the non-heme dioxygenase superfamily, and has been recently implicated in regulation of lipid and energy metabolism [22,23]. Dual-luciferase reporter assays and site mutation analyses validated that chicken FTO was a target gene of miR-33. Because in chickens de novo fatty acid synthesis occurs primarily in the liver, we further studied the possibility that miR-33 targets FTO in the chicken liver. One of the most powerful and straightforward ways to determine the relationship between a miRNA and a mRNA in tissues or cells is to determine the effect of knockdown of the miRNA on the expression of the mRNA of interest. Using LNA-anti-miR-33, we successfully reduced the expression of endogenous miR-33 in primary chicken hepatocytes, and this reduction was associated with an up-regulated expression of FTO mRNA. This association supports that the FTO gene is targeted by miR-33 in chicken hepatocytes. We also observed that miR-33 and FTO mRNA expression were inversely correlated in chicken liver at most of the developmental ages examined. This inverse relationship further supports the possibility that miR-33 negatively regulates FTO expression in chicken liver. At day 35 and day 42 of age, the expressions of miR-33 and FTO mRNA were not inversely correlated. This suggests that the expression of FTO at these two stages may be regulated predominantly by mechanisms other than mi-R-33.

In the chicken, FTO is widely expressed. Expression of FTO in the hypothalamic nuclei involved in energy balance regulation has been shown to respond to nutritional manipulations such as feeding and fasting [34–36]. Fasting has been shown to also increase FTO gene expression in the cerebrum, liver, breast muscle and subcutaneous fat. Alterations in feeding status resulted in significant changes in FTO expression in the liver, but not in other tissues of broiler chickens [37]. In addition to this, hepatic FTO expression changes in response to metabolic states, and glucose reduces hepatic FTO mRNA expression independently of body weight [27]. Since miR-33 inhibits the expression of FTO, it might play a role in mediating the nutritional regulation of FTO expression in chicken liver.

In conclusion, chicken miR-33 is transcribed from intron 16 of the chicken SREBP2 gene and is expressed in various chicken tissues. miR-33 might be involved in lipid metabolism and energy homeostasis in the chicken by negatively regulating the expression of the FTO gene in the liver.

**Author Contributions**

Conceived and designed the experiments: HJ ZG. Performed the experiments: FS XW JY. Analyzed the data: FS ZG. Wrote the paper: FS HJ BZ ZG.

**References**

1. Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.
2. Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian miRNAs are conserved targets of microRNAs. Genome Res 19: 92–103.
3. Enright AJ, John B, Gaul U, Tuschl T, Sander C, et al. (2003) MicroRNA targets in Drosophila. Genome Biol 5: R1.
4. Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. Cell 136: 215–233.
5. Horie T, Ono K, Horiguchi M, Nishii H, Nakamura T, et al. (2010) MicroRNA-33 encoded by an intron of sterol regulatory element-binding protein 2 (Sreb2p) regulates HDL in vivo. Proc Natl Acad Sci U S A 107: 17321–17326.
6. Horton JD, Goldstein JL, Brown MS (2002) SREBP-activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109: 1125–1131.
7. Osborne TF (2000) Sterol regulatory element-binding proteins (SREBPs): key regulators of nutritional homeostasis and insulin action. J Biol Chem 275: 32379–32382.
8. Rayner KJ, Suarez Y, Davalos A, Parathath S, Fitzgerald ML, et al. (2010) MiR-33 contributes to the regulation of cholesterol homeostasis. Science 328: 1570–1573.
9. Najafi-Shoushtari SH, Kristo F, Li Y, Shioda T, Cohen DE, et al. (2010) MiR-33 contributes to the regulation of cholesterol homeostasis. Science 328: 1570–1573.
10. Najad-Shoushtari SH, Kristo F, Li Y, Shioda T, Cohen DE, et al. (2010) MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis. Science 328: 1566–1569.
11. Marquart TJ, Allen RM, Ory DS, Baldan A (2010) MiR-33 links SREBP-2 induction to repression of sterol transporters. Proc Natl Acad Sci U S A 107: 12228–12232.
12. Gerin I, Clerbaux LA, Hamonot O, Lanthier N, Das AK, et al. (2010) Expression of miR-33 from an SREBP2 intron inhibits cholesterol export and fatty acid oxidation. J Biol Chem 285: 33652–33661.
Expression of miR-33 Targets FTO Gene

12. Cirera-Salinas D, Pauta M, Allen RM, Salerno AG, Ramirez CM, et al. (2012) miR-33 regulates cell proliferation and cell cycle progression. Cell Cycle 11: 922–933.

13. Rayner KJ, Sherry FJ, Essau CC, Hussain FN, Temel RE, et al. (2011) Antagonism of miR-33 in mice promotes reverse cholestrol transport and regression of atherosclerosis. J Clin Invest 121: 2921–2931.

14. Davalos A, Goedeke L, Smibert P, Ramirez CM, Warrier NP, et al. (2011) miR-33a/b contribute to the regulation of fatty acid metabolism and insulin signaling. Proc Natl Acad Sci U S A 108: 9232–9237.

15. Freyling TM, Timpon NJ, Weeden MN, Zeggini E, Freathy RM, et al. (2007) A common variant in the FTO gene is associated with body mass index and predispositions to childhood and adult obesity. Science 316: 889–894.

16. Sanchez-Pulido L, Andrade-Navarro MA (2007) The FTO (fat mass and obesity associated) gene codes for a novel member of the non-heme dioxygenase superfamily. BMC Biochem 8: 23.

17. Gerken T, Girard CA, Tong YC, Webbly CJ, Sasuke V, et al. (2007) The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nuclear acid demethylase. Science 318: 1469–1472.

18. Jia G, Yang CG, Yang S, Jian X, Yi C, et al. (2008) Oxidative demethylation of 5-methylcytosine and 5-methyluracil in single-stranded DNA and RNA by mouse and human FTO. FEBS Lett 582: 3313–3319.

19. Han Z, Niu T, Chang J, Lei X, Zhao M, et al. (2010) Crystal structure of the FTO protein reveals basis for its substrate specificity. Nature 464: 1205–1209.

20. Jia G, Fu Y, Zhao X, Dai Q, Zheng G, et al. (2011) N6-methyladenosine in nuclear RNA is a major substrate of the obesity-associated FTO. Nat Chem Biol 7: 883–887.

21. Fredriksson R, Hagglund M, Olzeyrski PK, Stephansson O, Jacobsson JA, et al. (2008) The obesity gene, FTO, is of ancient origin, up-regulated during food deprivation and expressed in neurons of feeding-related nuclei of the brain. Endocrinology 149: 2062–2071.

22. Church C, Moir L, McMurray F, Girard C, Banks GT, et al. (2010) Overexpression of Fto leads to increased food intake and results in obesity. Nat Genet 42: 1096–1099.

23. Church C, Lee S, Bugg EA, McTaggart JS, Deacon R, et al. (2009) A mouse model for the metabolic effects of the human fat mass and obesity associated FTO gene. PLoS Genet 5: e1000599.

24. Douaire M, Belboir B, Guilmot JC, Faridin JM, Langlois P, et al. (1993) Lipogenic enzyme and apoprotein messenger RNAs in long-term primary culture of chicken hepatocytes. J Cell Sci 104 (Pt 3): 713–718.

25. Wang XG, Shao F, Wang HJ, Yang L, Yu JF, et al. (2013) MicroRNA-126 expression is decreased in cultured primary chicken hepatocytes and targets the sproty-related EVH1 domain containing 1 mRNA. PLoS One 9: e89894.

26. Fischer J, Koch L, Emmerling C, Viertkotten J, Peters T, et al. (2009) Inactivation of the Fto gene protects from obesity. Nature 458: 894–898.

27. Portasanos NJ, Lew PS, Mizumo TM (2010) Relationship between blood glucose levels and hepatic Fto mRNA expression in mice. Biochem Biophys Res Commun 402: 713–717.

28. Hermier D (1997) Lipoprotein metabolism and fasting in poultry. J Nutr 127: 8025–8038.

29. Lau NC, Lim LP, Weinstein EG, Bartel DP (2001) An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 294: 838–842.

30. Rodriguez A, Drurovision S, Ashurst JL, Bradley A (2004) Identification of mammalian microRNA host genes and transcription units. Genome Res 14: 1902–1910.

31. Wang D, Lu M, Xiao J, Li T, Wang E, et al. (2009) Cepred: predicting the co-expression patterns of the human intronic microRNAs with their host genes. PLoS One 4: e4421.

32. Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15–20.

33. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, et al. (2005) Combinatorial microRNA target predictions. Nat Genet 37: 495–500.

34. Jia X, Nie Q, Lamont SJ, Zhang X (2012) Variation in sequence and expression of the avian FTO, and association with glucose metabolism, body weight, fatness and body composition in chickens. Int J Obes (Lond) 36: 1054–1061.

35. Wang Y, Rao K, Yuan L, Everaert N, Buyse J, et al. (2012) Chicken FTO gene: tissue-specific expression, brain distribution, breed difference and effect of fasting. Comp Biochem Physiol A Mol Integr Physiol 163: 246–252.

36. Gao X, Shi YH, Li M, Wang F, Tong Q, et al. (2010) The fat mass and obesity associated gene FTO functions in the brain to regulate postnatal growth in mice. PLoS One 5: e1000559.

37. Tiwari A, Kryzys-Walker SM, Ramachandran R (2012) Cloning and characterization of chicken fat mass and obesity associated (Fto) gene: fasting affects Fto expression. Domest Anim Endocrinol 42: 1–10.