MicroRNA 33 Potentially Participates in the Development of Goose Fatty Liver via Its Target Gene *CROT*

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Research

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Abstract

Background

Previous studies indicate that microRNA33 (*miR-33*) and its target gene, *CROT*, are implicated in hepatic lipid metabolism, but it is unclear whether *miR-33* participates in the development of goose fatty liver via *CROT*.

Methods

The expression of *miR-33* in goose fatty liver, muscle and fat tissues, as well as the mRNA and protein expression of CROT in goose fatty liver was determined by q-PCR or Western-blot. The targeting regulatory relationship between *miR-33* and *CROT* in goose liver cells was validated by *miR-33* overexpression and interference assays. The effects of *miR-33* mimic and *CROT* overexpression on lipid deposition and the expression of downstream genes were determined in goose primary hepatocytes. The treatment of high concentrations of glucose and insulin was performed to determine their regulation on the expression of *miR-33* and *CROT* in goose primary hepatocytes.

Results

Here, data showed that *miR-33* expression was significantly increased in the liver, muscle and fat tissues of overfed geese. Consistently, *miR-33* mimic promoted lipid deposition in goose primary hepatocytes. Moreover, the regulatory targeting relationship between *miR-33* and *CROT* was validated in goose primary hepatocytes. Consistently, the mRNA and protein expression of CROT were significantly reduced in goose fatty liver. Interestingly, *CROT* overexpression could induce the expression of fatty acid oxidation associated genes including *CRAT*, *PEX5*, *EHHADH*, *CAT* and *ACOT8* in goose primary hepatocytes, but only the expression of *PEX5* was significantly inhibited in goose fatty liver. However, it seemed conflicting that *CROT* overexpression increased lipid deposition and reduced lipid peroxidation in goose primary hepatocytes. Additionally, high glucose inhibited *miR-33* expression and induced *CROT* expression in goose primary hepatocytes.

Conclusions

These findings suggest that *miR-33* potentially participates in the development of goose fatty liver via *CROT*, and that *miR-33*/ *CROT* may partially mediate the effect of glucose in goose liver cells.

Background

Similar to human nonalcoholic fatty liver disease (NAFLD), a global epidemic disease, goose fatty liver is also characterized by large number of lipid deposited in the liver (or hepatic steatosis) [1]. However,

human NAFLD can progress into steatohepatitis, cirrhosis, even cancer [2], while goose fatty liver maintains simple steatosis without deterioration into other overt pathological symptoms, which suggests goose fatty liver is unique in some aspects [3]. Indeed, recent evidence indicates that goose may have a protective mechanism that prevents occurrence of inflammation in goose fatty liver. For example, most complement genes (e.g., complement *C3* and *C5*, the key genes in complement response) and proinflammatory gene, *TNF-α*, are inhibited in goose fatty liver vs. normal liver, but fatty acid desaturases and adiponectin receptors are induced [4]. This protective mechanism allows goose fatty liver to be fully recovered under certain conditions. Therefore, goose fatty liver, a physiological fatty liver, can be considered as a unique model for NAFLD study.

Compared to human and rodent NAFLD, the mechanism underlying the development of goose fatty liver is much less understood. Previous studies have shown that microRNAs, a class of noncoding RNA molecules with a length of 21–23 nt, play an important role in the development of goose fatty liver and mammalian NAFLD. For example, *miR-29c* can target *COL3A1*, *SGK1* and *INSIG1* genes by binding to the 3'-UTR of their mRNA sequences, thus inhibit their expression contributing to energy homeostasis and cell growth in goose fatty liver [4]. Another example is *miR-128-1*, which can inhibit the expression of *LDLR* and *ABCA1* genes and thus regulate lipid metabolism in human NAFLD [5]. Recent studies show that *miR-33* has a number of target genes including *CROT*, *HADHB* and *NPC1*, and plays a role in hepatic lipid metabolism via *CROT* and *HADHB* genes [6 7]. *CROT* is a member of carnitine acyltransferase family (which includes *CPT1*, *CPT2*, *CROT* and *CRAT*) and plays an important role in fatty acid metabolism by catalyzing the reversible transport of fatty acyl groups between coenzyme A (CoA) and carnitine and providing a cyclic pathway to transport the medium long chain acyl CoA from peroxisome to the cytosol and mitochondria [8–10]. However, it is uncertain whether *miR-33* inhibits fatty acid oxidation and thus promotes the development of goose fatty liver by regulating the expression of *CROT* gene. This study is aimed to validate the targeting regulatory relationship between *miR-33* and *CROT* in goose liver cells, to clarify the association of *miR-33* expression with *CROT* expression in goose fatty liver, to determine the effects of *miR-33* mimic and *CROT* overexpression on lipid deposition in goose primary hepatocytes, as well as to determine the regulation on the expression of *miR-33* and *CROT* by high concentrations of glucose and insulin. The results may provide a foundation for addressing the mechanism by which *miR-33* participates in the development of goose fatty liver via *CROT* gene.

Methods

Animals and sample collection

All animal protocols were approved by the Animal Care and Use Committee of Yangzhou University.A total of 48 male healthy Landes geese (purchased from Yangzhou RuiNong Farm) with similar body weight at 65 days old were randomly divided into a control group and an overfeeding group (24 geese per group). A 5-day-long pre-overfeeding was performed in the overfeeding group before the 19 days of formal overfeeding. The overfeeding procedures and dietary regimes were carried out as previously described [12]. The control group was allowed to feed and water ad libitum. Six geese from each group

were sacrificed on the 7th, 14th and 19th days of overfeeding, respectively. The liver, abdominal fat and pectoral muscle samples were collected, snap frozen in liquid nitrogen and stored at -70°C.

Isolation and treatment of goose primary hepatocytes

Goose primary hepatocytes were isolated from Landes goose embryos on the 23rd day of hatching as previously described [11 12]. The isolated primary hepatocytes were cultured and treated with high level of glucose or insulin, respectively, according to the protocols previously described [4]. In brief, the isolated cells were seeded at the density of 1×10^6 cells/well and cultured in complete culture media (containing high glucose DMEM culture medium plus 10% fetal bovine serum, 1% Penicillin-Streptomycin solution (100 IU/mL), and 10 μL EGF (20 ng/mL)) overnight, followed by treating the cells for 14 h with fresh complete culture media supplemented with or without 100 mmol/L glucose and 100 nmol/L insulin, respectively. For transfection assays, the cells at 70–90% confluent were transfected with *miR-33* mimics (sense: 5'-GUGCAUUGUAGUUGCAUUGC-3'; antisense: 5'-AAUGCAACUACAAUGCACUU-3'), overexpression vector, inhibitor, and their negative controls (sense: 5'-UUCUCCGAACGUGUCACGUTT-3'; antisense: 5'-ACGUGACACGUUCGGAGAATT-3'), respectively, using Lipofectamine 2000 (Cat. No. 11668-027, Invitrogen, USA) according to the manufacturer's instruction. Briefly, for each well of 6-well plate, 4 μL Lipofectamine 2000 was diluted with 96 μL Opti-MEM, followed by dissolving 2 μg mimics, inhibitors, negative controls, or vectors (overexpression vector or empty vector) separately into 100 μL Opti-MEM. After that, the two solutions were mixed and sit for 20 min at room temperature. The mixture was then added to the cultured cells. After 24 h of transfection, the cells were rinsed with phosphate buffer saline (PBS) twice, followed by harvesting the cells with 1 mL TRIzol reagent (Cat. No. 15596026, Life, USA) per well. The transfection of *CROT* overexpression vector or empty vector followed the same procedures. The *miR-33* mimics, overexpression vector, inhibitor, and their controls were provided by GenePharma Co., Ltd. (Shanghai, China). The *miR-33* overexpression vector containing the precursor sequence of goose *miR-33* was constructed by PCR-based amplification of *miR-33* precursor sequence with goose genomic DNA and the pair of primers (Pri-*miR-33*, see Table 1), restriction of the PCR product with HindIII and XhoI enzymes, and cloning into the pcDNA3.1 (+) vector (Cat. No. V79020, Thermo Fisher scientific, Shanghai, China) according to the previously published methods [12]. *CROT* overexpression vector was constructed with pcDNA3.1 (+) vector by Thermo Fisher Scientific Inc. (Cat. No. V79020, Shanghai, China).

Table 1
Primer sequences for quantitative PCR analysis and construction of *CROT* overexpression vector

Gene	GenBank number	Sequence (5'-3')	Product Size (bp)
CROT	XM_013187037.1	F: ACATGGAGAAACAAGTGCAGG	1809
		R: TGTCCTGAAAACACCGGAAT	
CRAT	XM_013198699.1	F: GCAGCCCATCATCAGCG	183
		R: ACGACTGGCAGGCGATA	
PEX5	XM_013199557.1	F: AGTGGCGAATACGAGAA	215
		R: TGGGCACCTAAGTTGAT	
EHHADH	XM_013186160.1	F: TCTGAGTACAGGGACACC	349
		R: CTGAGCACGACATCCAC	
CAT	XM_013194546.1	F: TTATCAGAGGGATGGAC	188
		R: TATAGAAATCTCGCACC	
ACOT8	XM_013198556.1	F: CAGCAGGAGCCGAAGCAA	169
		R: GGTCGAGGGACACCATGAAC	
GAPDH	XM_013199522.1	F: GCCATCAATGATCCCTTCAT	155
		R: CTGGGGTCACGCTCCTG	
Pri-miR-33		F: CCTAAAGCTGGAGCCTTCCT	203
		R: CGGCTCGCTATTTTAGTTGC	
OE-miR-33		F: ggaattcCCTAAAGCTGGAGCCTTCCT	203
		R: ccgctcgagCGGCTCGCTATTTTAGTTGC	

Note: Pri-*miR-33* denotes the pair of primers for PCR-based amplification of precursor sequence of *miR-33*, OE-*miR-33* denote the pair of primers for the construction of *miR-33* overexpression vector.

Oil red O staining

The Oil Red O (ORO) staining of the cultured goose primary hepatocytes was performed with ORO staining solution (Cat. No. G1262, Solarbio, Beijing, China) according to the manufacturer's instruction. Briefly, the procedures were as follows: the cells were rinsed with PBS twice after removing culture medium, followed by fixing the cells with ORO fixative solution for 30 min. The cells were then rinsed with distilled water twice after removing the fixing solution, followed by incubating the cells with 60% isopropanol for 5 min. Next, the cells were stained with ORO staining solution for 15 min after discarding isopropanol. Subsequently, the staining solution was discarded, and the cells were rinsed with distilled

water three times. Finally, the cells were visualized under microscope with 400× magnification after 1 mL distilled water was added.

Lipid peroxidation determined by malondialdehyde (MDA) assay

Lipid peroxidation was determined using MDA Testing Kit (Cat. No. S0131S, Beyotime, Shanghai, China) according to the manufacturer's instruction. Briefly, the treated cells were lysed with RIPA Lysis Buffer (Cat. No. C1053, PPLYGEN, Beijing, China), and the supernatant was harvested by centrifuging the lysates at 8,000 rpm for 10 min. To measure the MDA level, the MDA working solution was made with 150 μL thiobarbituric acid (TBA) diluent, 50 μL TBA stock solution and 3 μL antioxidant, 0.1 mL supernatant was then mixed with 0.2 ml MDA working solution. Subsequently, the mixture was heated at 100 ℃ for 15 min, followed by cooling the mixture to room temperature and centrifuging at 4,500 rpm for 10 min. The OD values of the mixture was determined at 532 nm wavelength. For normalization, the protein content in the supernatant was also determined with Bicinchoninic Acid (BCA) Kit (Cat. No. P0010, Beyotime, Shanghai, China) according to the manufacturer's instruction.

RNA isolation, cDNA synthesis and qPCR

Total RNA were extracted from tissue samples or cells collected using TRIzol according to the manufacturer's instruction. Genomic DNA was cleared by DNase I (D2215, TaKaRa Bio Inc., China) from the purified RNA samples before cDNA synthesis as previously described [13 14]. The first strand of cDNA was synthesized with TaqMan™ MicroRNA Reverse Transcription Kit (Cat. No. 4366596, Thermo Fisher scientific, Shanghai, China) according to the manufacturer's instruction. The mRNA abundance of *CROT* gene was determined by qPCR as previously described [14]. Goose glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as an internal control. The sequences of the primers for qPCR analysis were listed in Table 1. For RT-qPCR analysis of *miR-33*, TaqMan™ MicroRNA Assay Kit (Cat. No. 4427975, Thermo Fisher Scientific, Shanghai, China) was used according to the manufacturer's instruction. The *U6* gene was used as an internal control. The cycle threshold (Ct) was determined with the supplied software. The expression of the target genes was calculated using $2^{-\Delta\Delta Ct}$ [15] and presented as fold change over control.

Protein assay and immunoblot analysis

Protein concentration of each tissue or cell sample was determined as previously described. The protocol for immunoblot analysis was also previously described [16]. The following antibodies were used in this study: CROT antibody (Cat. No. bs-5048R, Beijing Biosynthesis Biotechnology Co., Ltd. Beijing, China) and β-Actin (Cat. No. sc-47778; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Statistical analysis

The data were expressed as the means ± SE. SPSS 18.0 (SPSS China, Shanghai, China) was used to perform the Student's t-test for statistical significance of differences between control and treatment. $P < 0.05$ was considered statistically significant.

Results

MiR-33 was involved in the formation of goose fatty liver

As expected, similar to previous study, on the 19th day of overfeeding, the gain of body weight, the liver weight and the ratio of liver to body weights were much greater in the overfed geese (treatment group) than the normally fed geese (control group) (Tab. S1), and the livers became milk-white in the treatment group while the livers remain normal dark red, suggesting that fatty liver was successfully induced in the overfed geese.

RT-qPCR analysis indicates that the expression of *miR-33* was significantly induced in goose fatty liver vs. normal liver on the 7th,14th and 19th days of overfeeding (Fig. 1A). This induction was also shown in other lipid metabolism related tissues including abdominal fat and pectoral muscle of the overfed geese on the 19th day of overfeeding (Fig. 1A). These results indicated that *miR-33* was involved in the development of goose fatty liver.

To test whether *miR-33* promotes lipid accumulation in goose liver cells, goose primary hepatocytes were transfected with *miR-33* overexpression vectors or empty vectors (as control). ORO staining assay indicated that the cells overexpressing *miR-33* (Fig. 1B) had more lipid deposition than the control (Fig. 1C).

Validation of the targeting regulatory relationship between miR-33 and CROT gene in goose liver cells

Previous study has demonstrated that *miR-33* can target *CROT* gene at two sites of its 3'UTR in CHO cells (Fig. 2A) [6], but this targeting regulatory relationship has not been validated in goose liver cells yet. To address this, *miR-33* mimic, overexpression vector and inhibitor transfection assays were performed. Data showed that *miR-33* mimic could significantly inhibit the expression of *CROT* gene in goose primary hepatocytes compared with the hepatocytes transfected with negative control (Fig. 2B). The inhibitory effect of *miR-33* on the expression of *CROT* was also confirmed by transfecting goose hepatocytes with *miR-33* overexpression vectors vs. empty vectors (Fig. 2B). In contrast, the expression of *CROT* gene in goose hepatocytes was induced by *miR-33* inhibitor vs. negative control (Fig. 2B). These findings indicated that *CROT* expression could be regulated by *miR-33* in goose liver cells.

Induction of CROT gene in goose fatty liver vs. normal liver

In consistent with the targeting regulatory relationship between *miR-33* and *CROT* genes in goose liver cells, the expression of *CROT* gene was significantly inhibited in goose fatty liver vs. normal liver on the 7th, 14th and 19th days of overfeeding (Fig. 3A). In particular, the protein level of *CROT* gene was also significantly lower in goose fatty liver vs. normal liver on the 19th day of overfeeding (Fig. 3B and 3C). The results indicated that there was an association between the induction of *miR-33* and inhibition of *CROT* in goose fatty liver vs. normal liver.

Potential function of CROT in the formation of goose fatty liver

At present, the function of *CROT* in goose liver cells is still unclear. As previous study shows that overexpression of *CROT* in HepG2 cells induces the expression of *CRAT* gene, a gene with known function in β-oxidation of fatty acids, we speculated that *CROT* also could regulate the expression of other genes associated with β-oxidation of fatty acids. To validate this, we overexpressed *CROT* in goose liver cells (Fig. 4A), and indeed, compared to goose primary hepatocytes transfected with empty vector, the expression of *CRAT*,*PEX5*、*EHHADH*, *CAT* and *ACOT8* was significantly increased in the cells transfected with *CROT* overexpression vector (Fig. 4B). The expression of these genes in goose fatty liver vs. normal liver was also determined. Data showed that only the expression of *PEX5* gene in goose fatty liver was significantly lower than that in normal liver on the 19th day of overfeeding (Fig. 4C).

Moreover, to determine the effect of *CROT* gene expression on fatty acid oxidation, ORO staining assay (which determines the deposition of neutral lipid such as triacyglycerols) and MDA assay (which determines the lipid peroxidation mainly due to reactive oxygen species generated by mitochondria) were performed in goose liver cells transfected with *CROT* overexpression vector vs. empty vector. The results showed that the overexpression of *CROT* increased lipid deposition and reduced lipid peroxidation in the cells (Fig. 4D and 4E), which indicated that *CROT* expression was associated with fatty acid oxidation in goose hepatocytes.

Effects of glucose and insulin on the expression of miR-33 and CROT in goose primary hepatocytes

As the development of NAFLD and goose fatty liver is usually accompanied with hyperglycemia and hyperinsulinemia [17 18], goose primary hepatocytes were treated with high levels of glucose (100 mmol/L) and insulin (100 nmol/L), respectively. The data showed that the expression of *miR-33* was significantly inhibited in goose primary hepatocytes treated with 100 nmol/L glucose, and accordingly, the expression of *CROT* was significantly increased (Fig. 5A). However, 100 nmol/L insulin significantly induced the expression of *miR-33* in goose primary hepatocytes but has no effect on the expression of *CROT* (Fig. 5B). The results indicated that the effect of high level of glucose on the expression pattern of *miR-33*/ *CROT* was consistent with that in goose fatty liver.

Discussion

MicroRNAs, as a class of small noncoding RNA molecules, can bind to the 3'UTRs of the mRNAs of their target genes, thus inhibit translation of the genes and/or promote destabilization and degradation of the mRNAs [19]. As a key posttranscriptional repressor of gene expression, microRNAs are implicated in almost all physiological and pathological processes [20]. For example, *miR-33*, embedded in the 16th intron of sterol response element binding transcription factor (*SREBF*) gene [21], is co-transcribed with *SREBF* and can target a number of genes including *CROT*, *HADHB*, *NPC1*, *CPT1A* and *AMPK*, etc. [22]. *CROT* is required for the transport of medium and long chain acyl CoA molecules from the peroxisome to the cytoplasm and mitochondria. It mainly exists in the peroxisome and mitochondria, and functions as an auxiliary enzyme in fatty acid metabolism [23]. *HADHB* encodes the beta subunit of mitochondrial trifunctional protein, which catalyzes the last three steps of long-chain fatty acid β-oxidation in

mitochondria [25]. Therefore, induction of *CROT* and *HADHB* expression may promote fatty acid oxidation, while inhibition of their expression may promote fatty acid accumulation. Indeed, *miR-33* knockdown induces the expression of *CROT*, *CPT1A*, *HADHB* and *AMPK*, and thus promotes fatty acid oxidation [24]. In contrary, overexpression of *miR-33* can inhibit fatty acid oxidation in liver cells [22]. These effects of *miR-33* may be mediated by regulating the expression of its target genes, including *CROT* and *HADHB*.

Although *miR-33* is implicated in fatty acid metabolism, it is unclear whether *miR-33* participates in the development of goose fatty liver via *CROT* gene. Goose fatty liver is characterized by deposition of large amount of fat, which sometimes accounts for about 60% of liver weight. The weight of fatty liver can reach 8–10 times that of normal liver in a short period of time (3–4 wk) [25]. These features reflect the marvelous capacity of goose liver in lipid accumulation. Revealing the mechanism underlying this capacity may help improve the production of goose fatty liver (or foie gras), as well as provide new ideas to develop therapeutic approach to NAFLD in human and other domestic animals. Considering the role of *miR-33* and *CROT* genes in lipid metabolism, we speculate that *miR-33* and *CROT* genes contribute to this unique mechanism that goose liver owns. In this study, data showed that the expression of *miR-33* in the liver, muscle and fat tissues of the overfed geese was significantly upregulated compared with the normally fed control geese. The targeting regulatory relationship between *miR-33* and *CROT* genes was also subsequently validated in goose liver cells. As expected, the mRNA and protein expressions of CROT gene were significantly downregulated in goose fatty liver vs. normal liver, which is contrary to the expression of *miR-33*. These findings suggest that, although *CROT* gene expression could be regulated by multiple factors, *miR-33* is a major factor contributing to the downregulation of *CROT* gene in the development of goose fatty liver. As *miR-33* is an intronic sequence of the major regulator of lipid metabolism, *SREBF*, it may enhance the role of *SREBF* by being co-transcribed with *SREBF* and suppressing its target genes including *CROT*. Interestingly, upregulation of *miR-33* expression also occurred in muscle and fat tissues of the overfed geese vs. control geese, thus lipid accumulation in these tissues may be partially attributed to the upregulation of *miR-33*. Consistently, *miR-33* mimic promoted lipid deposition in goose primary hepatocytes. However, it seemed conflicting that *CROT* overexpression increased lipid deposition and reduced lipid peroxidation in goose primary hepatocytes. One explanation is that the function of *CROT* is to mobilize fatty acids by promoting β-oxidation of medium- and long-chain fatty acids and transport of the product of β-oxidation from peroxisome to the cytosol. Whether *CROT* promotes or inhibits lipid deposition, it depends on the context where other genes join and decide the fate of fatty acids, i.e., entering mitochondria to be further degraded or forming triacylgycerols in the cytosol. In other words, lipid deposition in the cell is not only determined by *CROT*, but also is subjected to regulation by multiple proteins and processes, which may be applicable to the regulation of lipid deposition by *miR-33* in goose fatty liver. In line with this explanation, lipid peroxidation inhibited by *CROT* overexpression in goose primary hepatocytes suggested that fatty acid oxidation in mitochondria was somehow suppressed. Lipid peroxidation is mainly caused by mitochondria-derived reactive oxygen species. Recent studies indicate that *CROT* gene may participate in the regulation of fatty acid composition [27–28], thus it is worthwhile to determine if *CROT* gene has dual functions (i.e.,

involving in β-oxidation of fatty acids, and modulating fatty acid composition) in the development of goose fatty liver.

In addition, previous study shows that *CROT* overexpression in HepG2 cells induces *CRAT* expression, a gene playing a key role in fatty acid oxidation [26]. Consistently, this study showed that *CROT* overexpression in goose primary hepatocytes could induce the expression of *PEX5*,*EHHADH*、*CAT* and *ACOT8* genes in addition to *CRAT* gene. It is known that all the genes are associated with fatty acid oxidation. *PEX5* (peroxisome biogenic factor 5) plays an important role in peroxisome protein input by binding to the C-terminal PTS1 type tripeptide peroxisome targeting signal (SKL type) and thus is necessary for protein assembly of functional peroxisome. *EHHADH* (enol COA hydratase and 3-hydroxyacyl CoA dehydrogenase) is one of the four enzymes involved in peroxisomal β-oxidation pathway as its N-terminal contains enol CoA hydratase activity and C-terminal contains 3-hydroxyacyl CoA dehydrogenase activity [27]. *ACOT8* is a peroxisomal thioesterase, which is involved in the oxidation of fatty acids. As peroxisomes play a key role in fatty acid oxidation and the genes are involved in assembly and function of peroxisome, *CROT* gene may promote fatty acid oxidation via these genes. As upregulation of fatty acid oxidation increases release of reactive oxidative species, it is reasonable that *CROT* gene overexpression induced the expression of *CAT* gene, a key antioxidant enzyme in the cell, which provides an explanation why *CROT* overexpression inhibited lipid peroxidation in goose primary hepatocytes. Similarly, the induction of these genes only indicates mobilization but not degradation of fatty acids as suggested by *CROT* overexpression assay. Induction of *CRAT*, *PEX5*, *EHHADH* and *ACOT8* genes may be due to the increased level of product generated by *CROT*. It is found that significant reduction in *PEX5* expression was concomitant with the inhibition of *CROT* expression in goose fatty liver vs. normal liver. This is probably due to the regulation of other factors on the expression of these genes during the development of goose fatty liver, which needs to be further investigated.

Finally, this study showed that high level of glucose could inhibit *miR-33* expression and induce *CROT* expression in goose primary hepatocytes, whereas high level of insulin could induce *miR-33* expression without changing *CROT* expression. As the development of fatty liver is usually accompanied with hyperglycemia and hyperinsulinemia, the results suggest that hyperglycemia and hyperinsulinemia are involved in the regulation of *miR-33* in the development of goose fatty liver, but only hyperglycemia is involved in the expression of *CROT* gene in goose fatty liver. It is, however, unknown how *miR-33* expression is regulated by glucose and insulin. It is likely that glucose and insulin regulate the expression of *miR-33* by regulating the expression of *SREBP* as previous studies have shown that glucose and insulin can regulate the expression of *SREBF* [18 28]. Moreover, other factors may also regulate the expression of *miR-33* by regulating the expression of *SREBF*, such as thyroid hormone, which is known to be able to stimulate the expression of *SREBF* [29]. These speculations warrant further investigation.

It is noteworthy that, although the regulatory relationship between goose *miR-33* and *CROT* has been published (Zheng et al., 2015), the relationship is demonstrated only in CHO cells (from Chinese hamster ovary) other than goose liver cells. This study provides some new insights into the relationship between *miR-33* and *CROT* and the functions of the genes in goose liver cells, including confirmation of the

reciprocal relationship between *miR-33* and *CROT* in goose fatty liver and primary hepatocytes, the regulation of *miR-33* and *CROT* expression by insulin and glucose, and the induction of lipid deposition by *miR-33* mimics in goose primary hepatocytes.

Conclusions

MiR-33 participates in the lipid accumulation in the development of goose fatty liver, potentially via *CROT* gene, which contributes to the mechanism by which goose fatty liver is quickly developed. This work provides a foundation to further study the role of *miR-33* and the related mechanism in the development of goose fatty liver.

Abbreviations

miR-33
microRNA33; NAFLD:non-alcoholic fatty liver disease.

Declarations

Acknowledgements

Not applicable

Authors' contributions

Conceptualization, L.L. and Y.Z.; methodology, X.L. and Y.X.; data acquisitition, X.L., Y.X., Y.H.Z., B.D., M.M.Z. and J.W.; data analysis, X.L. Y.X., B.D., J.W. and Y.Z.; writing—original draft preparation, X.L., Y.Z. and L.L.; writing—review and editing, T.Y.G.; project administration, T.Y.G. and D.Q.G; funding acquisition, L.L. and D.Q.G. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not pub- licly available due individual privacy information protection but are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal protocols were approved by the animal welfare committee of the Yangzhou University [permission number: SYXK(Su) IACUC 2012–0029], and comply with the associated guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Figures

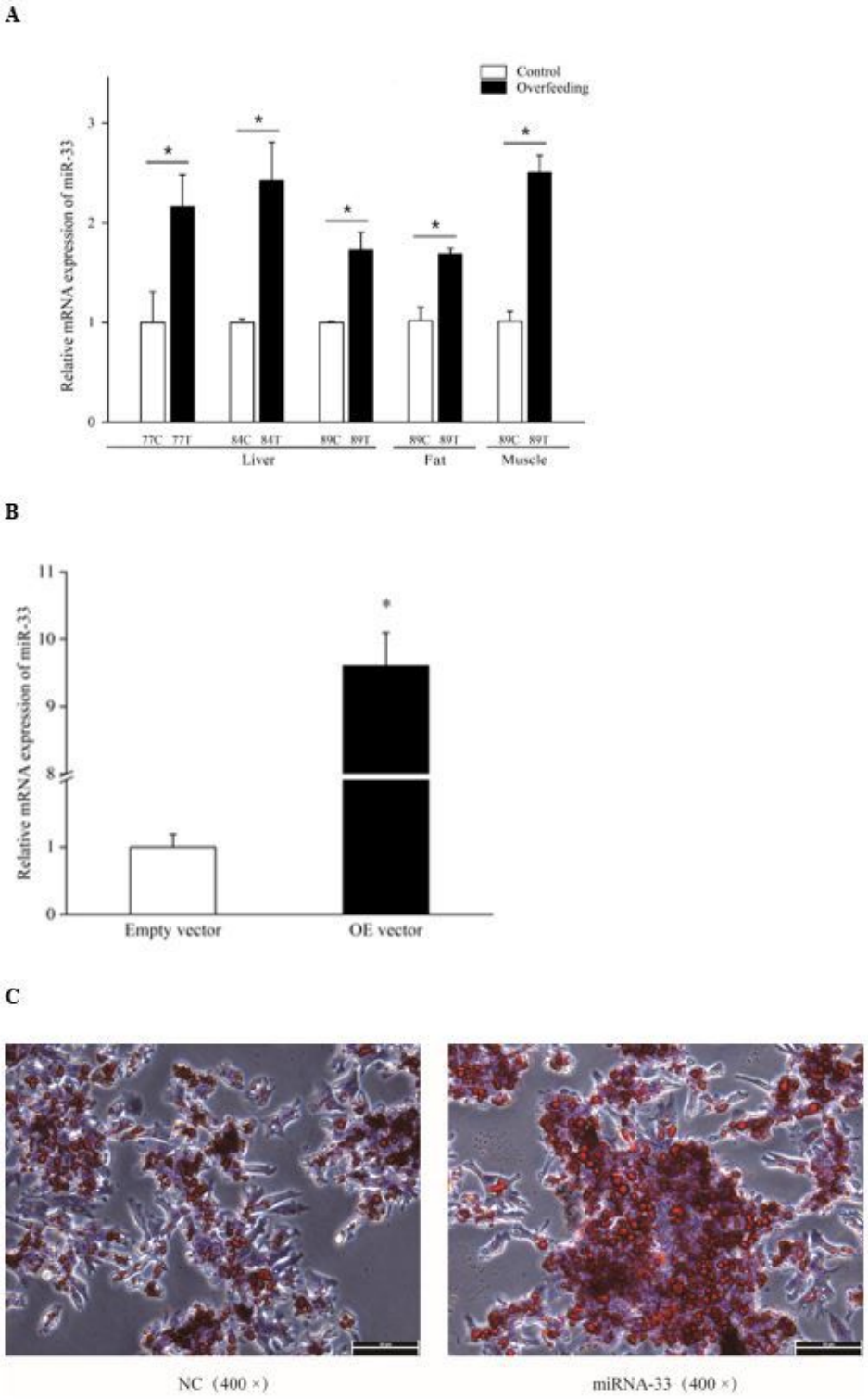

Figure 1

The upregulated expression of miR-33 was associated with lipid accumulation in goose liver cells. Note: (A) The expression level of miR-33 was determined by quantitative PCR in the liver, abdominal fat and pectoral muscle of the overfed vs. control geese on the 7th (77C, 77T), 14th (84C, 84T) and 19th (89C, 89T) days of overfeeding. The expression level is presented as fold change over the control. (B) The expression of miR-33 was determined by qPCR in goose primary hepatocytes transfected with miR-33

overexpression vector (OE) vs. empty vector (NC). (C) The level of lipid deposition in goose primary hepatocytes transfected with miR-33 mimics and negative control (NC) was detected by ORO staining. n=6. * denotes P□0.05 vs. control. The data are expressed as the means ± SE.

A

Gene	Targeting relationship
CROT 3'UTR-B1	5'- CATTTGCAACAGCAATGCAA-3'
	|||||| : |||||||
goose-miR-33	3'- CGUUACGUUGAUGUUACGUG-5'
CROT 3'UTR-B2	5'- GATATGCCTCAACAATGCAA-3'
	| |||| | |||||||
goose-miR-33	3'- CGUUACGUUGAUGUUACGUG-5'

B

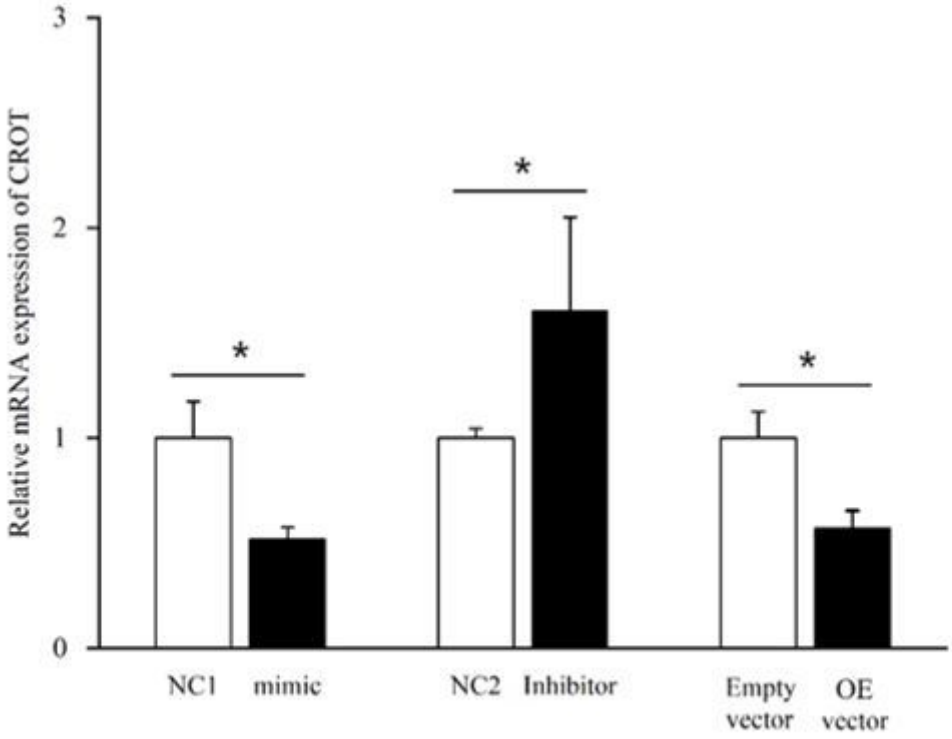

Figure 2

Validation of the regulatory targeting relationship between miR-33 and CROT gene in goose primary hepatocytes. (A) Binding sites between the sequences of mature miR-33 and 3'UTR of CROT genes of goose. (B) The expression of CROT gene was determined in goose primary hepatocytes by qPCR after the cells were transfected with miR-33 mimic (a synthesized mature goose miR-33), miR-33 inhibitor, miR-33

overexpression vector and their respective controls. NC1 and NC2 are negative controls for miR-33 mimic and inhibitor, respectively. The cells transfected with pcDNA3.1 empty vector was used as control for the cells transfected with miR-33 overexpression (OE) vector. The expression level of CROT is presented as fold change over the control. n=3. * denotes P 0.05 vs. control. The data are expressed as the means ± SE.

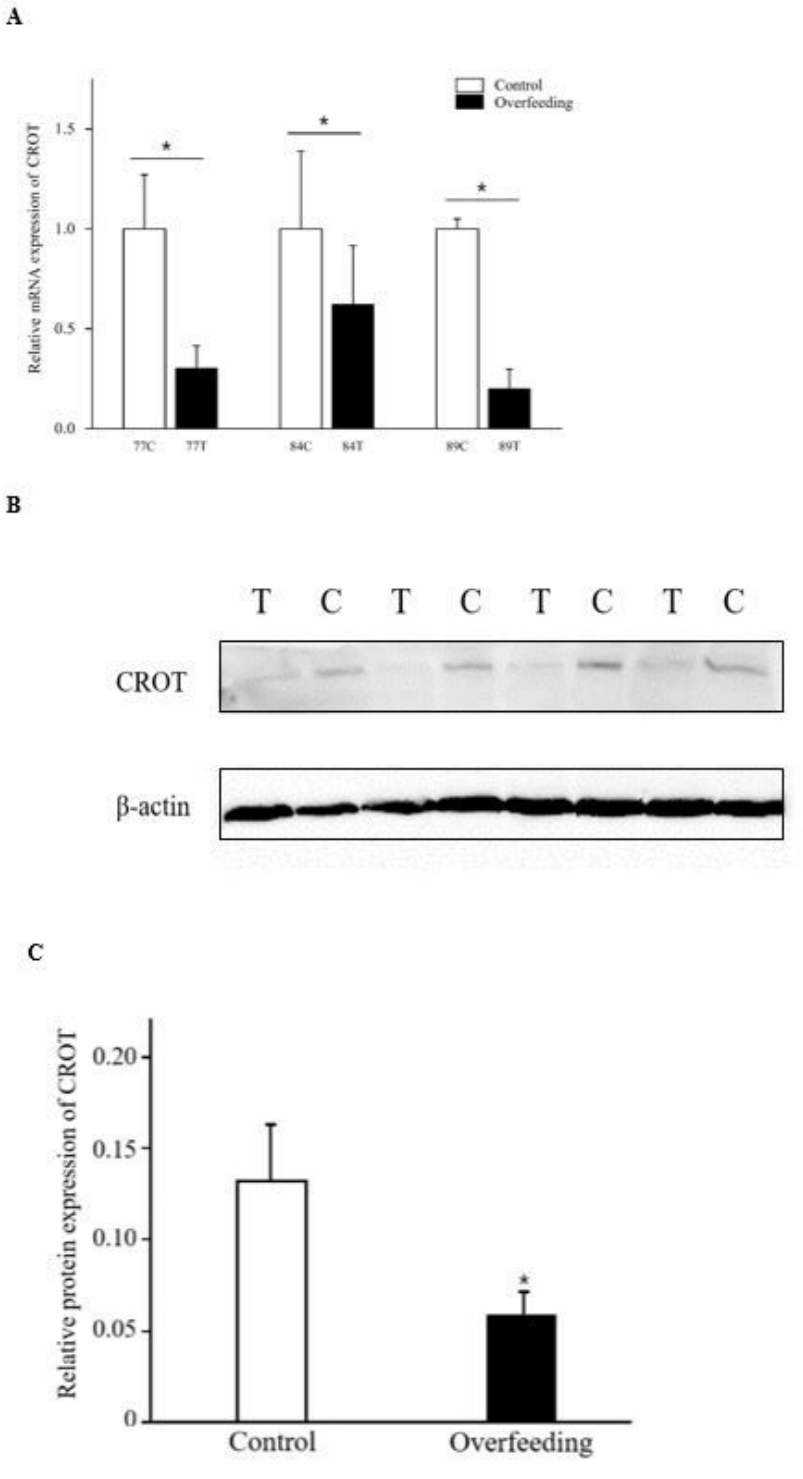

Figure 3

The mRNA and protein expressions of CROT in the liver of the overfed (overfeeding) vs. normally fed geese (control). (A) The mRNA expression of CROT gene was determined by qPCR in the livers of the overfed vs. control geese on the 7th (77C, 77T), 14th (84C, 84T) and 19th (89C, 89T) days of overfeeding. n=6. (B) and (C) show the image and quantification for immunoblot analysis of CROT and β-actin genes in the livers of the overfed vs control geese on the 19th day of overfeeding. n=4. The mRNA and protein expression levels of CROT are presented as fold change over the control. * denotes P□0.05 vs. control. The data are expressed as the means ± SE.

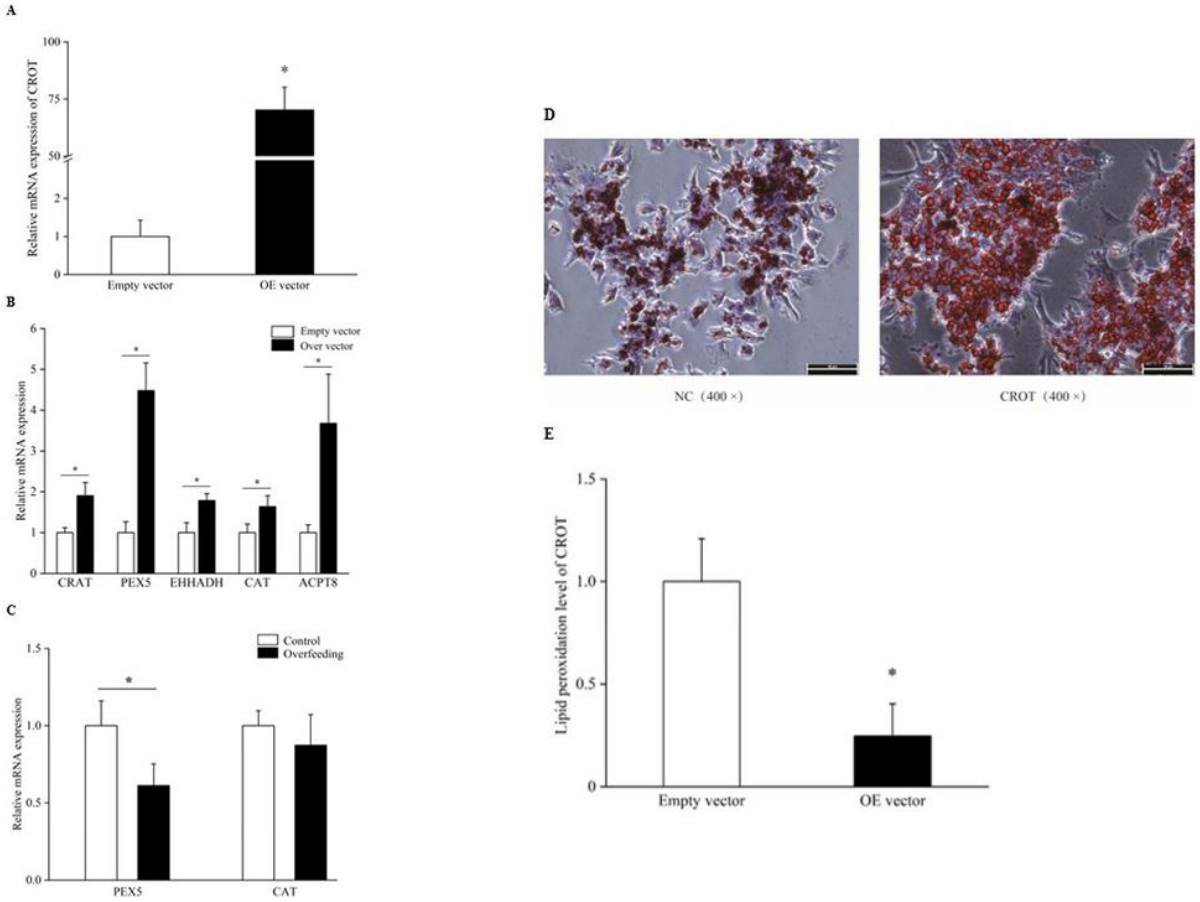

Figure 4

Expression of CROT was associated with fatty acid oxidation in goose primary hepatocytes. The mRNA expression of CROT gene (A) and CRAT□PEX5□EHHADH□CAT and ACOT8 genes (B) was determined by qPCR in goose primary hepatocytes transfected with CROT overexpression vector (OE) vs. empty vector (EM). (C) The mRNA expression of PEX5 and CAT in goose fatty liver vs. normal liver on the 19th (89C, 89T) day of overfeeding. (D) The lipid deposition was determined by ORO staining in goose primary hepatocytes transfected with CROT overexpression vector (OE) vs. empty vector (NC). (E) The level of lipid peroxidation was determined in goose primary hepatocytes transfected with CROT overexpression vector (OE) and empty vector (EM) by MDA assay. n=6. * denotes P□0.05 vs. control. The data are expressed as the means ± SE.

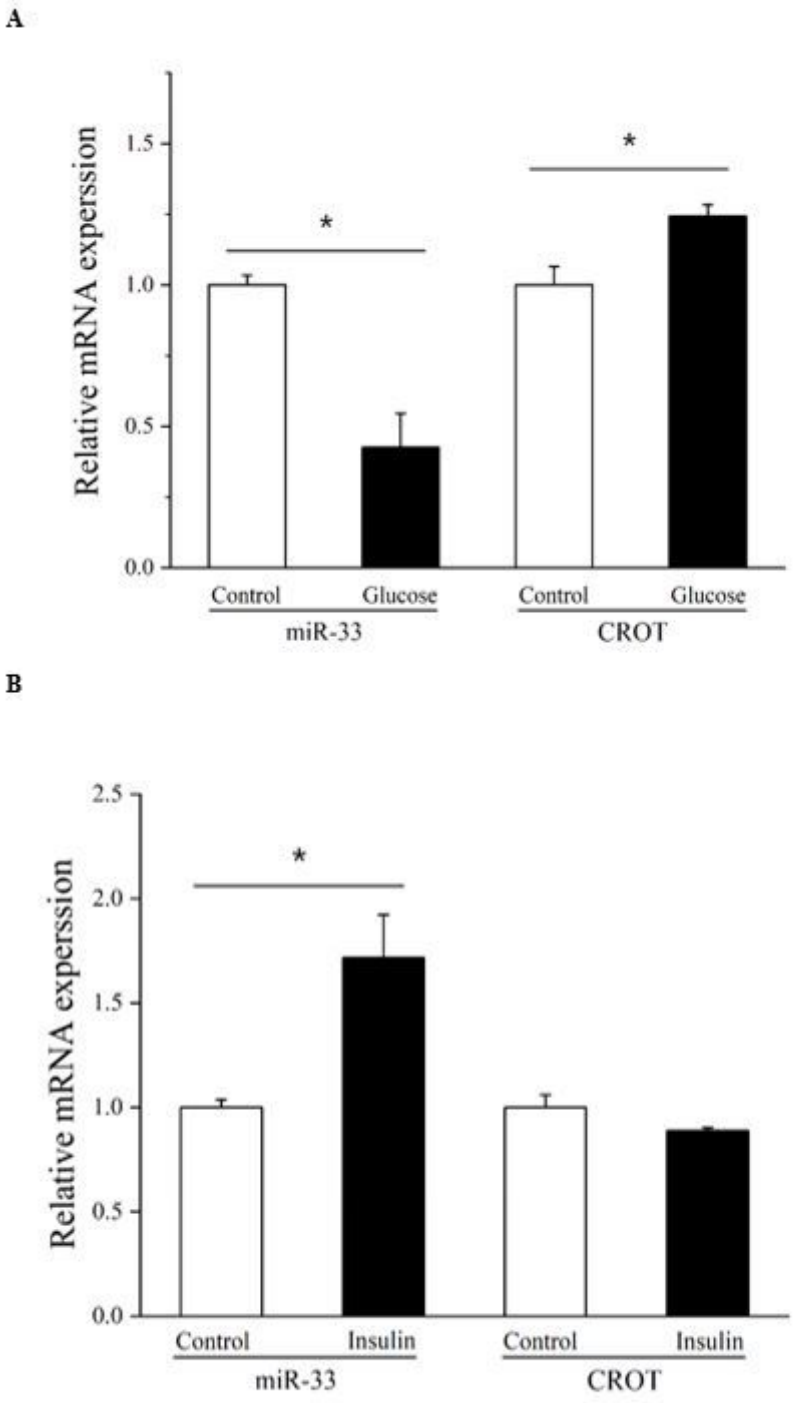

Figure 5

Effects of glucose and insulin on the expression of miR-33 and CROT genes in goose primary hepatocytes. The expression of miR-33 and CROT genes were determined by qPCR in goose primary hepatocytes treated with 100 mmol/L glucose (A) or 100 nmol/L insulin (B), respectively. The controls are the cells with no glucose or insulin treatments. The expression levels of the genes are presented as fold change over the control. n=3. * denotes P□0.05 vs. control. The data are expressed as the means ± SE.

Supplementary Files

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