

Original Article

Mutation of miR-21 targets endogenous lipoprotein receptor-related protein 6 and nonalcoholic fatty liver disease

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Abstract: Nonalcoholic fatty liver disease (NAFLD) is a chronic disorder characterized by hepatic fat accumulation and abnormal lipid metabolism. Although miR-21 has been implicated in nonalcoholic fatty liver disease, it is unknown whether miR-21 could function as a therapeutic target. Here, we perform transfection analysis of miR-21 mimic or control mimic to evaluate the effects of miR-21 expression levels on human HepG2 nonalcoholic fatty liver cells. We used siRNA techniques to knock down miR-21 in HepG2 and control 293T cell lines, and then monitored lipid production and the expression levels of genes involved in lipid metabolism. The effects of miR-21 expression levels on LDL receptor-related protein 6 (LRP6) expression were evaluated using qRT-PCR and western blot analyses. Luciferase reporter assays were conducted to confirm the effects of miR-21 expression levels on LRP6. The results indicated that transfection of miR-21 mimic induced changes in the expression levels of lipogenic enzymes, including acetyl-CoA carboxylase 1 (ACC1), stearoyl CoA desaturase (1SCD1), sterol regulatory element-binding protein 1 (SREBP1), and liver X receptor alpha (LXR α). Transfection of miR-21 mimic suppressed the transcription and translation of LRP6 at the mRNA and protein levels, whereas miR-21 knockdown increased the expression levels of LRP6. Transfection of miR-21 mimic in HepG2 cells also induced lipid production and triggered the expression of critical lipid metabolic enzymes. These data suggest that mutation of miR-21 may be a new therapeutic strategy to treat nonalcoholic fatty liver diseases by targeting endogenous LRP6.

Keywords: Nonalcoholic fatty liver disease, miR-21, low-density lipoprotein (LDL), LDL receptor-related protein 6 (LRP6)

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a major chronic liver dysfunction disease globally, and its prevalence is increasing along with epidemics of obesity, diabetes, and metabolic syndrome. The NAFLD spectrum spans from steatosis to nonalcoholic steatohepatitis (NASH), which can lead to cirrhosis and hepatocellular cancer. Currently, there is a lack of therapeutic strategies to alleviate NAFLD, especially those that remove fatty liver cells without causing severe tissue damage.

MicroRNAs (miRs) are short, non-coding RNAs that act as post-translational regulators of gene expression. MiRs are conserved, small (20 to 25 nucleotide), non-coding RNAs that negative-

ly regulate the expression of messenger RNAs (mRNAs) at the post-transcriptional level [1, 2]. MiR-21 has been implicated in the development of NAFLD, and the association between nonalcoholic fatty liver and circulating miR-21, miR-34a, miR-122, and miR-451 has been documented [3]. MiR-21 and miR-155 are significantly upregulated in mice fed with a choline- and amino acid-deficient diet (CDAA), which promoted the development of NASH and hepatocellular carcinoma [4]. MiR21 targets and represses the grainy head like 3 (GRHL3) transcription factor, which leads to dephosphorylation of endothelial nitric oxide synthase (NOS3). NOS3 is a crucial mediator of endothelial function [5, 6]. The low-density lipoprotein (LDL) receptor gene family encodes for at least 13 structurally related cell surface receptors that

MiR-21 promotes lipogenesis via LRP6 pathway

Table 1. Human primer sequences for qRT-PCR analysis

Target Gene	Reverse	Forward	Accession
LRP6	Forward	5'-CAGTTGGAGTGGTGCTGAAAGG-3'	NM_002336
	Reverse	5'-CCATCCAAAGCAGCCCGTTCAA-3'	
ACC1	Forward	5'-TTCACCTCCACCTTGTCAGCGGA-3'	NM_000664
	Reverse	5'-GTCAGAGAAGCAGCCCATCACT-3'	
FASN	Forward	5'-TTCTACGGCTCCACGCTCTCC-3'	NM_004104
	Reverse	5'-GAAGAGTCTTCGTGAGCCAGGA-3'	
SCD1	Forward	5'-CCTGGTTTCACTTGGAGCTGTG-3'	NM_005063
	Reverse	5'-TGTGGTGAAGTTGATGTGCCAGC-3'	
LXR α	Forward	5'-TGGACACCTACATGCGTCGCA-3'	NM_001130101
	Reverse	5'-CAAGGATGTGGCATGAGCCTGT-3'	
SREBP1	Forward	5'-ACTTCTGGAGGCATCGCAAGCA-3'	NM_001005291
	Reverse	5'-AGGTTCCAGAGGAGGCTACAAG-3'	

ACC1, acetyl-CoA carboxylase 1; FASN, fatty acid synthase; SCD1, stearoyl CoA desaturase 1; LXR α , liver X receptor; SREBP1, sterol regulatory element-binding protein 1; LRP6, low-density lipoprotein related receptor 6.

perform diverse biological functions in different organs, tissues, and cell types [7]. LDL receptors are transmembrane cell surface proteins involved in receptor-mediated endocytosis of lipoprotein and protein ligands. The Wnt signaling co-receptor LRP6 (low-density lipoprotein receptor-related protein 6) functions as a receptor or co-receptor (with Frizzled) for Wnt, and therefore functions in the canonical Wnt/ β -catenin signaling cascade for endocytosis [8, 9]. Several studies have highlighted the key roles of Wnt, LRP6, and its transcriptional co-factor, T-cell transcription factor 4 (TCF4), in the regulation of plasma LDL, triglyceride (TG)/very low-density lipoprotein (VLDL) synthesis, glucose homeostasis, and vascular cell proliferation [8-10]. *Ex vivo* examination of primary cells from mutant LRP6 carriers showed that an arginine to cysteine mutation at residue 611 (R611C) impaired LRP6 phosphorylation by Wnt and inhibited the nuclear localization of both β -catenin and TCF4 [10].

In this study, we investigated how miR-21 affects lipogenesis. We report that miR-21 inhibits LRP6 expression in HepG2 cells, thereby inducing lipid production.

Materials and methods

Cell lines and reagents

The human kidney epithelial cell line 293T (ATCC CRL-3216) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplement-

ed with 5% penicillin, 5% streptomycin, and 10% heat-inactivated fetal bovine serum (Gibco, USA). The human fatty liver cancer cell line HepG2 was maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 5% penicillin, 5% streptomycin, and 10% heat-inactivated bovine calf serum (Gibco, USA).

DNA constructs

The 3'-UTR and mutated 3'-UTR of LRP6 (mRNA residue positions 5,042-5,048) were synthesized and sub-cloned into the 3' end of the pGL3-Luc vector (Promega, USA).

Quantitative real-time PCR

MicroRNAs were isolated using mirVana™ miRNA Isolation Kit (Life Technologies). Mature miR-21 was quantified with the TaqMan miRNA assay kit for mmu-miR-21 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Other qRT-PCR analyses were performed using the SYBR® Green kit (Bio-Rad). Primers used in this study are listed in **Table 1** [8].

Western blotting

HepG2 cells were lysed on ice using lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, and 1 mM DTT) supplemented with a protease inhibitor mixture (Sigma). Then, 20 μ g lysates were separated on 9% SDS-polyacrylamide gels (SDS-PAGE). Proteins were transferred for western blotting. The blots were incubated with anti-LRP6 antibody (1:500; ab24-386, Abcam, UK) or anti-b-actin antibody (ab82-27, Abcam) overnight at 4°C. After washing, blots were incubated with the secondary antibody, which was a 1:2,000 dilution of HRP-linked anti-IgG, followed by detection using ECL reagents (Pierce, USA).

MiR-21 mimic and control mimic

The miR-21 mimic (dsRNA oligonucleotides) and control mimic were purchased from Gene-

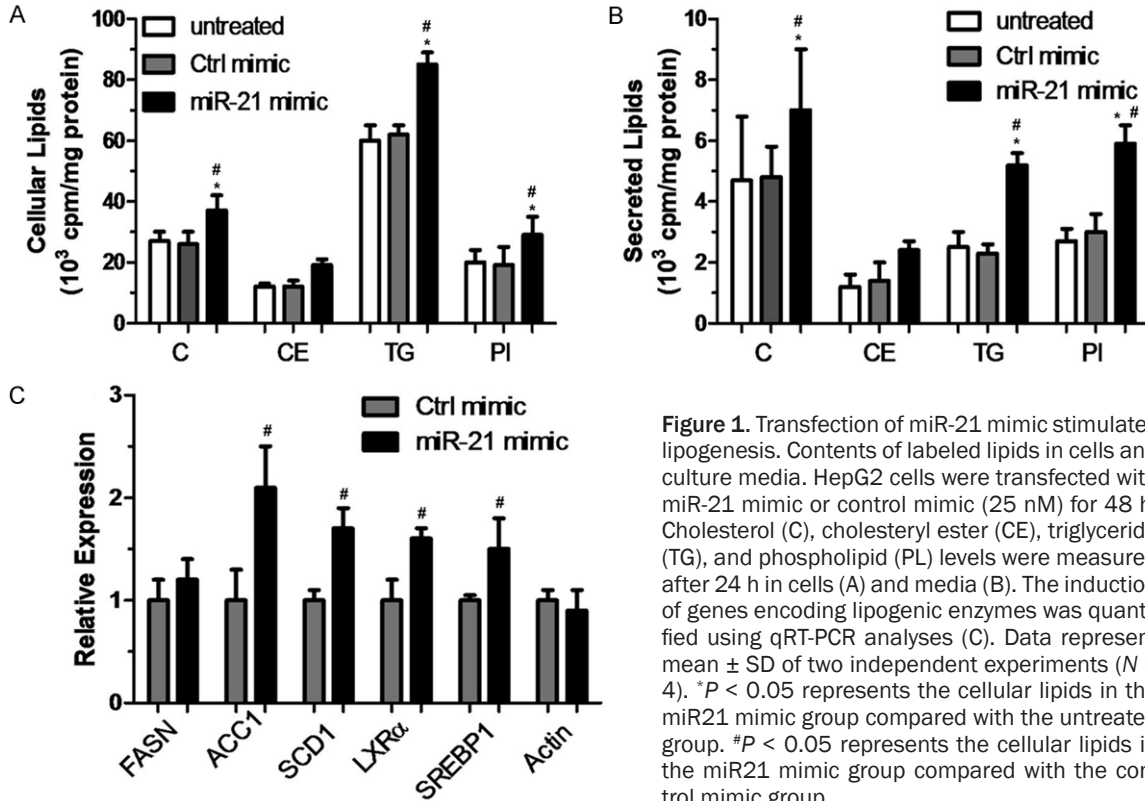


Figure 1. Transfection of miR-21 mimic stimulates lipogenesis. Contents of labeled lipids in cells and culture media. HepG2 cells were transfected with miR-21 mimic or control mimic (25 nM) for 48 h. Cholesterol (C), cholesteryl ester (CE), triglyceride (TG), and phospholipid (PL) levels were measured after 24 h in cells (A) and media (B). The induction of genes encoding lipogenic enzymes was quantified using qRT-PCR analyses (C). Data represent mean \pm SD of two independent experiments ($N = 4$). * $P < 0.05$ represents the cellular lipids in the miR21 mimic group compared with the untreated group. # $P < 0.05$ represents the cellular lipids in the miR21 mimic group compared with the control mimic group.

Pharma (Shanghai, China). HepG2 cells were transfected with miR-21 mimic or control mimic (5, 10, or 25 nM) using Lipofectamine RNAiMAX (Invitrogen, USA) according to the manufacturer's instructions.

Lipid synthesis and secretion assays

We adapted a detailed protocol from Skoog *et al.* [11] to determine both the cellular and secreted lipids, and evaluated the levels of cholesterol (C), triglyceride (TG), phospholipid (PL), and cholesteryl ester (CE).

Statistical analysis

Data were analyzed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). All data were analyzed with Student's *t*-test; a *P* value less than 0.05 was considered statistically significant.

Results

Transfection with miR-21 mimic increases lipogenesis

We transfected miR-21 mimic and control mimic into HepG2 cells, and investigated the

effects on lipogenesis. The miR-21 mimic, but not the control mimic, increased the levels of both cellular and secreted cholesterol, triglyceride, and phospholipid, whereas the cholesteryl ester levels did not change significantly (**Figure 1A** and **1B**). Quantitative RT-PCR analysis indicated that transfection of miR-21 mimic induced the expression of genes encoding lipogenic enzymes, including ACC1, SCD1, SREBP1, and LXR α , but not fatty acid synthase (FASN) (**Figure 1C**).

LRP6 is downregulated in transfected HepG2 cells expressing miR-21 mimic

To identify the target of miR-21 that is responsible for the observed increase in lipid production, we employed the Target Scan program (MIT). The results indicated that LRP6 was a potential target of miR-21. The 3'-UTR region of human LRP6 mRNA contains the seed sequence that may be recognized by miR-21 (**Figure 2A**). We performed qRT-PCR and western blot analyses to experimentally verify this result, and found that miR-21 transfection into HepG2 cells suppressed LRP6 expression at both the transcriptional (mRNA) and translational (pro-

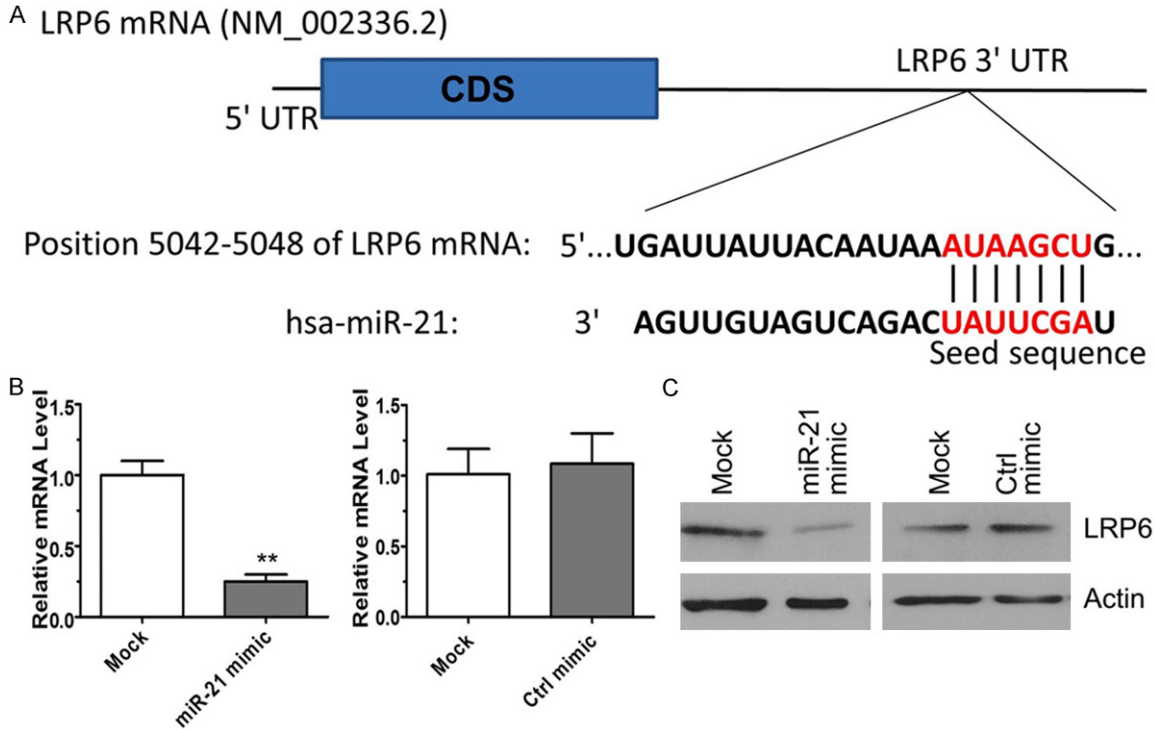


Figure 2. MiR-21 directly targets the 3'-UTR of LRP6. (A) Putative binding site of miR-21 in the 3'-UTR of LRP6 mRNA. HepG2 cells were transfected with 25 nM miR-21 mimic or control mimic. Cellular RNA or total protein was isolated at 48 h post-transfection for qRT-PCR (B) or western blot (C) analysis. ** $P < 0.01$ represents the relative mRNA levels in the miR-21 mimic group compared with that in the mock transfected group.

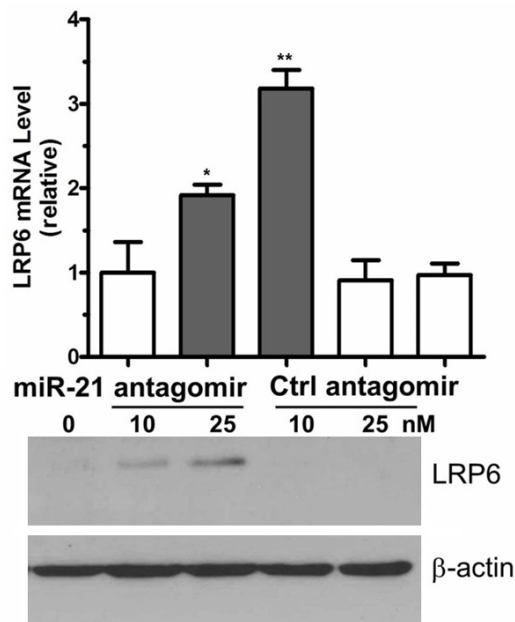


Figure 3. MiR-21 antagonism induces LRP6 expression. HepG2 cells were transfected with the indicated amount of miR-21 antagonism or control for 48 h. LRP6 mRNA and protein were quantified by qRT-PCR and western blotting, respectively. * $P < 0.05$ and ** $P < 0.01$ represent the LRP6 mRNA levels in the miR-21 antagonism group compared with the miR-21 mimic group.

tein) levels (Figure 2B and 2C). These combined results indicate that LRP6 is a cellular target of miR-21.

Blocking miR-21 expression increases LRP6 expression

To corroborate the results of the miR-21 mimic experiments, we transfected miR-21 antagonism or control antagonism into HepG2 cells and measured LRP6 expression using qRT-PCR and western blot analyses. The results clearly show that blocking miR-21 expression induced LRP6 expression (Figure 3). These combined results confirm that miR-21 levels are inversely correlated with LRP6 levels (Figures 2 and 3). Therefore, miR-21 appears to be a novel regulator of endogenous LRP6 expression.

LRP6 is a direct target of miR-21

To mechanistically verify the regulation of LRP6 expression by miR-21, we sub-cloned the LRP6 3'-UTR into a firefly luciferase reporter construct. Similarly, we cloned a mutant LRP6 3'-UTR with a mutation in the miR-21 seed sequence. The LXRA 3'-UTR was generated as a negative control (Figure 4A). These con-

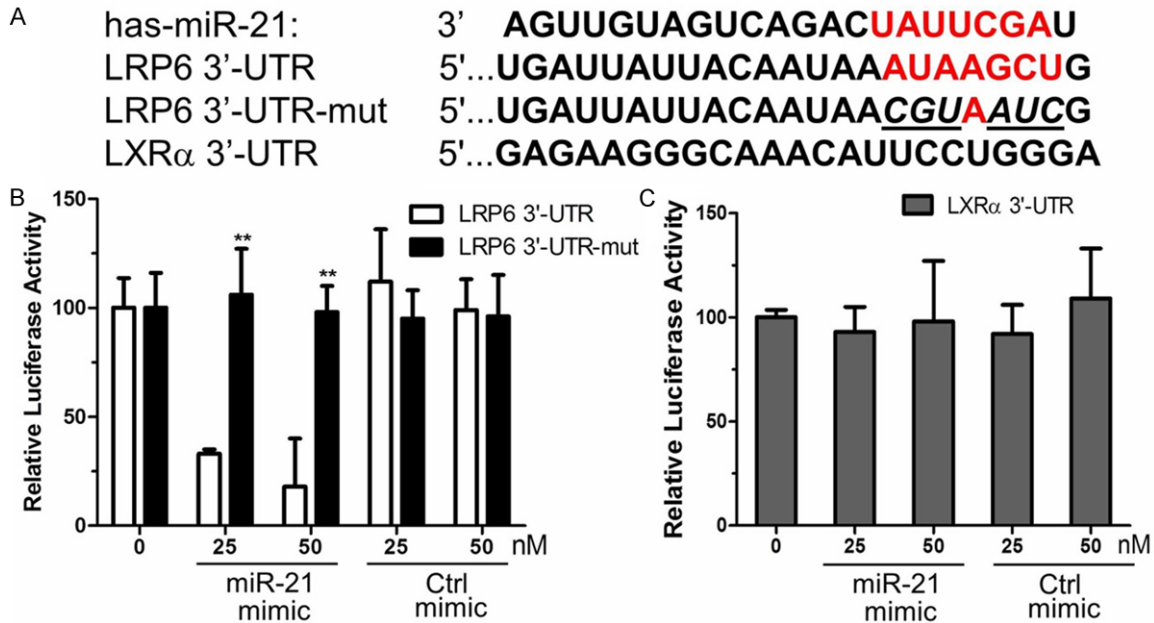


Figure 4. MiR-21 specifically inhibits LRP6 expression. A. Putative binding site of miR-21 in the 3'-UTR of *LRP6* mRNA and the mutated nucleotide residues are shown in red and italics, respectively. B. HepG2 cells were co-transfected with 0.2 μ g LRP6 3'-UTR-Luc or LRP6 3'-UTR-mut-Luc and 25 nM miR-21 mimic or control. C. HepG2 cells were transiently co-transfected with LXR α 3'-UTR-Luc and 25 nM of the indicated RNA oligonucleotides. Luciferase assays were conducted 48 h post-transfection. Data represent the firefly luciferase activities normalized with respect to the β -galactosidase activities. ** $P < 0.01$ represents the relative luciferase activity of the LRP6 3'-UTR-mut group compared with the LRP6 3'-UTR group.

structs were transfected into HepG2 cells along with miR-21 mimic or control mimic. The results indicate that miR-21 mimic specifically inhibited luciferase expression, but the mutated miR-21 mimic did not (**Figure 4B** and **4C**). The miR-21 mimic had no effect when luciferase was fused to the LXR α 3'-UTR.

Discussion

Previous studies report that microRNAs play important roles in adipogenesis and osteogenesis [12]. In this study, we investigated the function of miR-21 in lipogenesis. Wu *et al.* [13] reported that miR-21 may function as a link between nonalcoholic fatty liver disease and the development of hepatocellular carcinoma. MicroRNAs also have been associated with lipoprotein metabolism, including miR-30, miR-21, and miR-221 [14]. These miRNAs were identified using gene chip assays [15].

Our study demonstrated for the first time that miR-21 may be a new therapeutic target for the treatment of nonalcoholic fatty liver disease. We also explored the specific mechanism of the

potential therapeutic effects. Transfection of miR-21 mimic into HepG2 cells increased the expression of lipogenic enzymes and suppressed LRP6 expression at the transcriptional (mRNA) and translational (protein) levels. We investigated the mechanism of miR-21-mediated inhibition of LRP6 expression. Wang *et al.* [16] reported that miR-183 could downregulate LRP6 expression via a specific target site, the first binding site in the 3'-UTR of LRP6 mRNA. It is possible that miR-21 could have suppressed LRP6 expression via the same mechanism. Serum miR-21 levels were significantly higher in hepatocellular carcinoma patients who carried the hepatitis B or C virus compared with those with chronic hepatitis and healthy individuals [17-19]. Based on previous reports and the present study, we speculate that miR-21 may be involved in lipogenesis by activation of the LRP6 signaling pathway.

LRP6 plays important roles in metabolic regulation, specifically in the nutrient-sensing pathway, which has recently garnered considerable interest [10]. Patients carrying an *LRP6* mutation exhibit elevated levels of LDL cholesterol,

triglycerides, and fasting glucose, which cooperatively constitute the risk factors of metabolic syndrome and atherosclerosis [20]. LRP6 is one of the co-receptors of the Wnt/ β -catenin pathway, and is essential for its activation. LRP6 is linked with several disease processes via its potential role in suppressing lipogenesis. Kawakita *et al.* [21] reported that miR-21 promoted oral cancer invasion via the Wnt/ β -catenin signaling pathway. Lan *et al.* [22] found that miR-21 linked the Wnt/ β -catenin signaling pathway with glioblastomas. Chen *et al.* [23] reported that miR-183 regulated adipogenesis by inhibiting the canonical Wnt/ β -catenin signaling pathway via its targeting of LRP6. Our future studies will investigate the function of miR-21 in lipogenesis and the Wnt/ β -catenin signaling pathway.

In conclusion, this study determined that LRP6 is a novel target for miR-21, suggesting that miR-21-mediated lipogenesis may proceed via its inhibition of LRP6 expression. Future studies will determine if this regulatory pathway can be therapeutically targeted to reduce lipogenesis.

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Disclosure of conflict of interest

None.

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