

The Effect of miRNA-122 in Regulating Fat Deposition in a Cell Line Model

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ABSTRACT

Accumulating evidence supports the role of miR-122 in fatty liver disease. We investigated miR-122 expression in a steatotic hepatocyte model, the effect of miR-122 over-expression and inhibition in the pathogenesis. Human hepatic cell line L02 was induced with oleic acid to establish the steatotic hepatocyte model. Intracellular lipid content was observed with laser scanning confocal microscope (LSCM), and triglyceride content was determined with kits. Total RNA was extracted and reversely transcribed into cDNA. miR-122 expression was measured using qRT-PCR. Subsequently, miR-122 mimic and miR-122 inhibitor were transfected into steatotic hepatocytes to observe their effect on intracellular lipid content. The lipid fluorescence intensity and triglyceride content within the steatotic hepatocytes were significantly higher than those in normal control (860.01 ± 26.52 vs. 257.77 ± 29.69 and 3.47 ± 0.12 vs. 1.85 ± 0.02 at 24 h) ($P < 0.01$). miR-122 expression in steatotic hepatocytes was down-regulated compared with that in control ($2^{-\Delta C_t}$ value: 0.0286 ± 0.0078 vs. 0.0075 ± 0.0012) ($P \ll 0.01$). After transfection, miR-122 expression ($2^{-\Delta C_t}$ value) in the miR-122 mimic group increased 2.96-fold compared with that in control, and its lipid fluorescence intensity was significantly lower than that in control (790.92 ± 46.72 vs. $1,022.16 \pm 49.66$) ($P < 0.01$). Nevertheless, miR-122 expression decreased 3.45-fold in the miR-122 inhibitor group compared with that in control, and its fluorescence intensity was significantly higher than that in control ($1,386.49 \pm 40.34$ vs. $1,022.16 \pm 49.66$) ($P \ll 0.01$). We concluded that miR-122 was down-regulated in steatotic hepatocytes model. The pathogenesis of hepatocyte steatosis was enhanced by miR-122 mimic and reduced with miR-122 inhibitor. *J. Cell. Biochem.* 115: 839–846, 2014. © 2013 Wiley Periodicals, Inc.

KEY WORDS: miR-122; HEPATOCYTE; STEATOSIS; FATTY LIVER DISEASE

Fatty liver disease (FLD), which includes alcoholic fatty liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD), is one of the most common forms of chronic liver diseases and a cause of elevated serum aminotransferases worldwide. FLD is a clinical syndrome defined by lipid accumulation exceeding 5% of the normal range for liver weight or having more than 5% of liver cells displayed increased adiposity under the microscope. NAFLD refers to a spectrum of histological findings ranging from simple fatty liver (SFL) to non-alcoholic steatohepatitis (NASH) and NASH-related cirrhosis, which can progress to hepatocellular carcinoma (HCC) [Argo and Caldwell, 2009; Bellentani et al., 2010; Vernon et al., 2011]. Due to improved living standards and altered dietary habits, the prevalence of NAFLD has been increasing steadily in recent years in the Asia-Pacific region [Fan et al., 2005; Amarapurkar et al., 2007]. Our survey

conducted in China revealed a prevalence of 15% in a general population, which was close to the incidence in developed countries (20–30%) [Zhou et al., 2007, 2012].

As the pathogenesis of FLD is still unclear, the options for its treatment are limited. MicroRNAs (miRNAs or miRs), which are post-transcriptional regulators, can potentially be used to treat fatty liver. They play important roles in modification of cellular processes, for example, proliferation, differentiation, and apoptosis by degrading or inhibiting the translation of target mRNA via RNA interference, so as to alter target protein expression. Disruption of the balance will lead to the development of a wide range of disorders. miRs have been studied more extensively in cancers and immune-mediated diseases than in metabolic disease [Feinberg, 2007; Kozomara and Griffiths-Jones, 2011; Tomankova et al., 2011; Tili et al., 2013]. Recently, some

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studies have demonstrated that miR-122 is crucial in metabolic-related disorders, but the data concerning miR-122 in NAFLD are not as conclusive [Cheung and Sanyal, 2010; Fernandez-Hernando et al., 2011; Ceccarelli et al., 2013]. In order to explore the roles of miR-122 in the pathogenesis of hepatic steatosis, we examined the expression of miR-122 and the effect of miR-122 over-expression and inhibition on steatotic hepatocytes.

MATERIAL AND METHODS

ESTABLISHMENT OF STEATOTIC HEPATOCYTE MODEL

Normal hepatocyte cell line L02 (provided by Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, China) [Yang and Chen, 2007; Wang et al., 2009, 2013; Chen et al., 2013] was cultured in RPMI 1640 media containing 10% fetal bovine serum. As high dose of unsaturated fatty acid including oleic acid might and palmitic acid might cause cell death or decreased cell proliferation [Gomez-Lechon et al., 2007; Cui et al., 2010; Wu et al., 2010], we tried to screen its optimal concentration of culture. Oleic acid at concentrations of 0, 5 μg (17.70 μM), 10 μg (35.40 μM), 20 μg (70.81 μM), and 40 μg (141.62 μM)/ml dissolved in dimethyl sulfoxide (DMSO) was supplemented to RPMI 1640 media containing 10% fetal bovine serum in order to induce fat-overloading. The methods were referred to literature [Malhi et al., 2006, 2007; De Gottardi et al., 2007; Zhao et al., 2013]. After 24, 48, and 72 h, the cells were harvested to determine the optimal oleic acid concentration for further steatotic hepatocyte culture. The cell morphology and viability were observed under a microscope (Nicon Co., Japan) with and without trypan blue staining. The cell proliferation was evaluated by MTT assay (Sigma Co.). The optimal oleic acid concentration for culture was determined from steatotic hepatocytes survival rate and the least destruction. By comparing the results from different groups, the oleic acid concentration of 20 μg (70.81 μM)/ml was established to be suitable for the rest of the study. Intracellular fat content was determined by Nile Red staining and a triglyceride quantification kit (Biovision Co.). The methods were referred to literature [Yang and Chen, 2007; Wang et al., 2009]. The cells were stained with Nile Red and viewed under a laser scanning confocal microscope (LSCM) (Olympus FV-1000, Japan). Three steatotic hepatocytes in each of 10 microscopic fields (10 \times 40) were selected randomly. The fluorescence intensity of intracellular lipid droplets was quantified with the software (Olympus FV-3.0 viewer, Japan). The assay was repeated five times to obtain an average value. To quantify triglyceride content, hepatocytes (10^7) cultured in optimal condition harvested at 24, 48, and 72 h were used. The OD570 value was determined after ELISA. Normal hepatocytes were used as controls. The intracellular triglyceride concentration (C) was obtained from the equation: $C = T_s/S_v \text{ nmol}/\mu\text{l}$ (T_s represents triglyceride content in the standard curve, and S_v represents sample volume before dilution).

TOTAL RNA EXTRACTION AND cDNA Synthesis

Total RNA containing miR was extracted using TRIZOL Reagent (Invitrogen Life Technologies). The A260/A280 ratio was determined with a spectrometer. RNA integrity was viewed with formaldehyde degeneration gel electrophoresis. RNA was reverse transcribed into complementary DNA (cDNA) with miRCURYRNATM Universal cDNA Synthesis Kit (Exiqon, Denmark).

DETERMINATION OF MIR-122 EXPRESSION

The expression of miR-122 in hepatocytes with and without fat was quantified using a PCR assay kit (Exiqon, Denmark). Briefly, miR-122 expression was measured with a 20 μl final reaction volume, which contained 8 μl of cDNA dilution, 10 μl of SYBR[®] Green master mix, and 2 μl of miR-122 primer or U6 snRNA (as an internal control). Initial denaturation at 95°C for 10 min was followed by 40 cycles of denaturation at 95°C for 10 s and then 60°C for 1 min. The products were measured with the PCR software Opticon Monitor 2 (MJ Research, Inc.). Each sample was tested three times. The miR level of each sample was calculated by $2^{-\Delta C_t}$, then $2^{-\Delta\Delta C_t}$, which represented the fold change between two groups (steatotic hepatocyte/normal hepatocyte). $\Delta C_t = C_t$ miR value of target gene – C_t value of internal referee U6RNA, $\Delta\Delta C_t = \Delta C_t$ steatotic hepatocyte – ΔC_t normal hepatocyte [Schmittgen and Livak, 2008].

TRANSFECTIONS OF MIR-122 MIMIC AND INHIBITOR

Transient transfection of the oligonucleotides (provided by Dharmacon Co.) into steatotic hepatocytes was conducted 24 h after oleic acid induction. In miR-122 mimic group, steatotic hepatocytes were transfected with synthetic pre-miR-122 (miRIDIAN mimic, has-miR-122, UGGAGUGUGACAAUGGUGUUUG); in mimic control group, transfected with pre-miR-122 control (miRIDIAN miR mimic transfection control with Dy547); in inhibitor group, transfected with synthetic anti-miR-122 (miRIDIAN miR-122 Hairpin inhibitor); in inhibitor control group, transfected with anti-miR-122 control (miRIDIAN miR hairpin inhibitor transfection control with Dy547). The sequences of pre-miR-122 control, anti-miR-122 and anti-miR-122 control were all kept confidential by Dharmacon Co. In the blank control group, steatotic hepatocytes were co-incubated with a liposome (LipofectamineTM 2000 provided by Invitrogen Co.).

For transfection, miR/lipofectamine compound was prepared. Five microliters of Lipofectamine-2000 and 245 μl opti-MEM culture media were incubated at 25°C for 5 min to produce lipofectamine dilution. To produce miR dilution, 5 μl (100 pmol) of miRIDIAN miR-122 mimic, miRIDIAN miR-122 hairpin inhibitor, or transfection control were diluted with 245 μl opti-MEM culture media respectively. Then, 500 μl of lipofectamine dilution and 500 μl of each miR dilution were mixed and incubated at 25°C for 20 min to generate miR/lipofectamine compound. Before transfection, the hepatocytes were cultured in RPMI 1640 medium containing 10% fetal bovine serum in a CO₂ incubator at 37°C overnight. The cells were rinsed twice with phosphate buffered saline (PBS), added into 2 ml of opti-MEM culture media, then mixed with 500 μl of miR/lipofectamine compound, incubated in CO₂, at 37°C for 6 h. Afterwards, RPMI 1640 media containing 10% fetal bovine serum were used to replace the compound. Transfection efficiency was evaluated at 6 h after transfection with a fluorescence microscope. miR-122 expression was determined at 72 h with qRT-PCR. Intracellular lipid droplets were observed before and 72 h after transfection using LSCM. The methods were described in previous sections.

STATISTICAL ANALYSIS

The data were analyzed with the SPSS 17.0 for Windows statistical package (Chicago, IL, USA). Continuous data with normal distribution were expressed as mean \pm standard deviation and examined using

the Student's *t*-test. Continuous data with skewed distribution were examined using rank sum test. Categorical variables were expressed as a percentage and examined using the Chi-square and Fisher's exact tests. Statistical significance was set at $P < 0.05$ (two-tailed).

RESULT

ESTABLISHMENT OF A STEATOTIC HEPATOCYTE MODEL

Under light microscope, the hepatocyte cells (L02) had a spindle-shaped with clear cytoplasm. The optional time for cell passage was on Day 3 after oleic acid treatment when the cells have become round with blurred margins. More than 95% of cells survived after the treatments. The OD values derived from MTT assay, which reflected cell proliferation, in different oleic acid concentration groups were shown in Table I. The optional culture condition for this steatotic hepatocyte model was treating with the highest oleic acid concentration since these hepatocytes had the highest cell survival rate and showed proliferation. Oleic acid treatment did not change cell proliferation with the exception of when 40 μg (141.62 μM)/ml was used. Thus, 20 μg (70.81 μM)/ml oleic acid was used for subsequent experiments. Before and after oleic acid induction, hepatocyte lipid droplets stained by Nile Red fluorescence were clearly seen under light microscope and LSCM (Fig. 1). Both the intracellular mean lipid fluorescence intensity tested by LSCM and the intracellular triglyceride content measured by triglyceride kit increased gradually with prolonged treatment (Fig. 2). Because data from the fluorescence intensity correlated well with the triglyceride content ($R^2 = 0.89$, $P < 0.01$) (Fig. 2), fluorescence intensity test alone was used for the rest of the studies. This method was reported in literature [Malhi et al., 2006; Gomez-Lechon et al., 2007; Wu et al., 2010].

EXPRESSION OF MIR-122 IN STEATOTIC HEPATOCYTES

The miR-122 expression in steatotic hepatocytes expressed with $2^{-\Delta C_t}$ (0.0075 ± 0.0012) tested by qRT-PCR was significantly decreased than that (0.0286 ± 0.0078) in control of normal hepatocytes ($P < 0.01$).

THE RELATIONSHIP BETWEEN MIR-122 AND FAT CONTENT IN HEPATOCYTES

miR-122 level decreased significantly (0.0286 ± 0.0078) after 3 day oleic acid treatment. The miR-122 mimic (pre-miR-122), mimic control (pre-miR-122 control), miR-122 inhibitor (anti-miR-122), and inhibitor control (anti-miR-122 control) were transfected into

steatotic hepatocytes, respectively. High transfection rate ($>70\%$) determined by oligonucleotides (marked by Dy-547) confirmed successful transfections. Intracellular lipid fluorescence intensity of the steatotic hepatocytes was observed by LSCM (Fig. 3). After transfection with miR-122 mimic, intracellular lipid fluorescence intensity decreased significantly (Fig. 3B) compared with that before transfection (Fig. 3A). In contrast, the fluorescence intensity was increased significantly after transfection with miR-122 inhibitor (Fig. 3C). The miR-122 expression ($2^{-\Delta C_t}$ value) had a 2.96-fold increase in the miR-122 mimic group (0.0222 ± 0.0048) compared with that (0.0075 ± 0.0012) in the steatotic hepatocytes before transfection (control) ($P < 0.01$). Its intracellular lipid fluorescence intensity (790.92 ± 46.72) was significantly lower than that ($1,022.16 \pm 49.66$) in control ($P < 0.01$). The miR-122 expression ($2^{-\Delta C_t}$ value) decreased 3.75-fold in the miR-122 inhibitor group (0.0002 ± 0.0001) compared with that (0.0075 ± 0.0012) in control ($P < 0.01$), and its intracellular lipid droplet fluorescence intensity ($1,386.49 \pm 403.44$) was significantly higher than that ($1,022.16 \pm 49.66$) in control ($P < 0.01$). The miR-122 expression and fluorescence intensity in miR-122 mimic control and inhibitor control groups did not differ from those in control ($P > 0.05$). The miR-122 expression and intracellular lipid levels before and after transfection with miR-122 mimic, miR-122 inhibitor and their controls were shown in Table II and Figure 4.

DISCUSSION

During the past decade, the role of epigenetic mechanisms in the pathogenesis of diseases has been increasingly recognized. Epigenetic modification, mainly including miRs, DNA methylation and histone modification, refers to phenotypic changes caused by the mechanisms unrelated to changes in the underlying DNA sequence. Among epigenetic modifications, miRs are studied most extensively in liver diseases. miRs are a class of endogenously expressed small regulatory noncoding RNAs regulating mRNA degradation or translation inhibition by binding with imperfect complementarity in their 3' untranslated region (UTR), subsequently altering protein expression of target genes [Ambros, 2004; Bartel, 2004; Fabian et al., 2010].

Since the first discovery in 1993, many miRs in a variety of organisms have been determined. In 2011, more than 1,420 miRs have been identified in humans and miR-122 expression has been detected in 18 vertebrates so far (miRBase v17). (<http://www.mirbase.org/>) [Tomankova et al., 2011]. miR-122 was first identified in 2002. By using cloning approaches in various mouse tissues, the study

TABLE I. Steatotic Hepatocyte Proliferation in Culture Media With Different Oleic Acid Concentrations

	24 h		48 h		72 h	
	$\bar{x} \pm s^a$	<i>P</i>	$\bar{x} \pm s^a$	<i>P</i>	$\bar{x} \pm s^a$	<i>P</i>
0 $\mu\text{g}/\text{ml}$ (0 μM)	0.528 ± 0.029		0.660 ± 0.020		0.925 ± 0.022	
5 $\mu\text{g}/\text{ml}$ (17.70 μM)	0.542 ± 0.016	0.229	0.657 ± 0.017	0.704	0.922 ± 0.017	0.755
10 $\mu\text{g}/\text{ml}$ (35.40 μM)	0.546 ± 0.049	0.352	0.669 ± 0.025	0.437	0.923 ± 0.017	0.776
20 $\mu\text{g}/\text{ml}$ (70.81 μM)	0.544 ± 0.036	0.343	0.653 ± 0.022	0.497	0.925 ± 0.008	0.946
40 $\mu\text{g}/\text{ml}$ (141.62 μM)	0.232 ± 0.025	<0.01	0.261 ± 0.022	<0.01	0.305 ± 0.049	<0.01

^aExpressed as OD values.

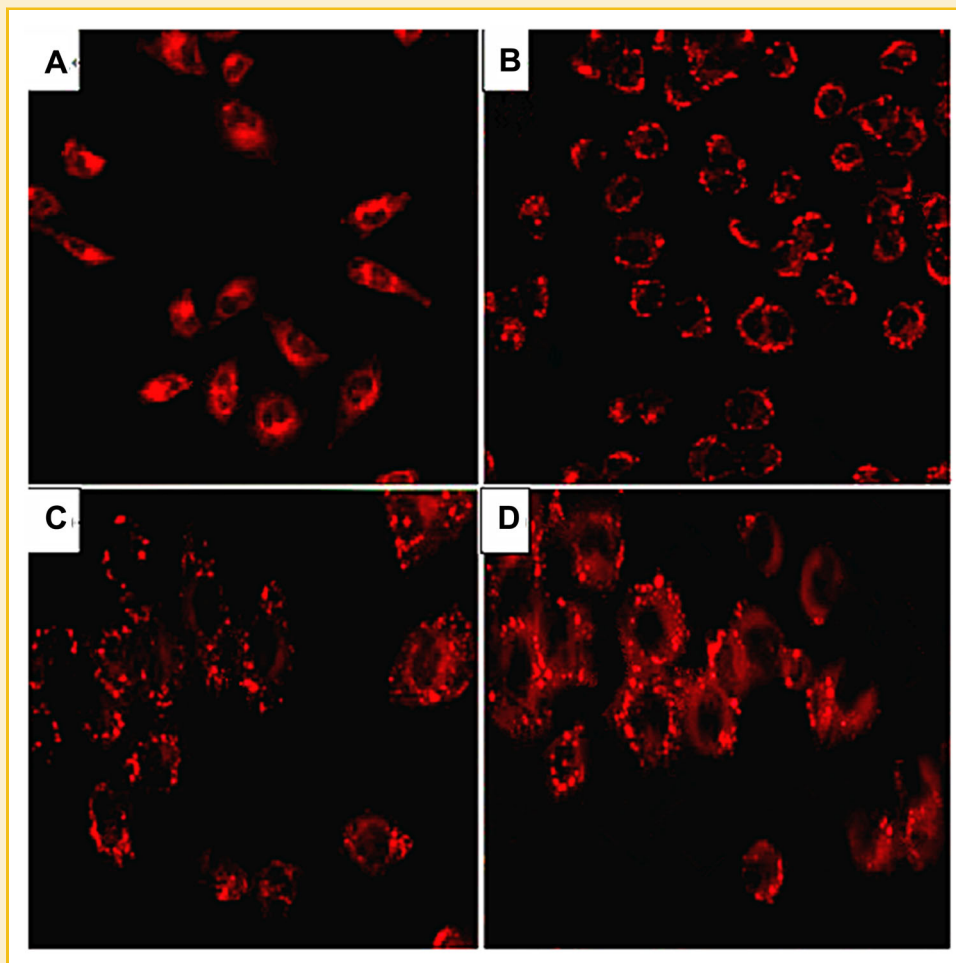


Figure 1. Hepatocytes stained with Nile Red under LSCM (10×40) at different time points after oleic acid induction. A: Normal hepatocytes (0 h), (B) Steatotic hepatocytes (24 h), (C) Steatotic hepatocytes (48 h), (D) Steatotic hepatocytes (72 h).

uncovered a number of novel miRs including miR-122, which was highly enriched in the liver but absent from other tissues [Lagos-Quintana et al., 2002]. The precise molecular function of miR-122 in humans is largely unknown. It is one of many tissue-specific miRs important for establishing patterns of gene expression that may be responsible for maintaining the differentiated state of a tissue [Lagos-Quintana et al., 2002; Lim et al., 2005; Jopling, 2012]. Accumulating evidence supports the effects of miR-122 in lipid and cholesterol metabolism, and adipocyte differentiation [Krutzfeldt et al., 2005]. In humans, miR-122 is expressed in the developing liver and at high levels in the adult liver, where it makes up 70% of all miRs. RNase protection analysis indicated that miR-122 was present at approximately 66,000 copies per cell in adult liver [Chang et al., 2004]. In NASH patients, miR-122 was significantly under-expressed (63%) compared to normal controls [Chang et al., 2004; Cheung et al., 2008]. In the first clinical study regarding miR-122 with NASH, the miR profiles of 15 patients with biopsy proven NASH and 15 normal controls were investigated. Out of a total of 474 tested miRs, 46 were differentially expressed in NASH with 23 being down-regulated (in particular, miR-122), and 23 being up-regulated (in particular, miR-

34a and miR-146b). These differentially expressed miRs were further validated by quantitative real-time PCR [Cheung et al., 2008]. Serum levels of miR-122 were significantly elevated in NAFLD patients than in controls, and positively correlated with disease severity from simple steatosis to steatohepatitis. miR-122 was also associated with liver enzyme levels, fibrosis stages, and inflammation activities [Cermelli et al., 2011]. Inhibition studies have further our understanding of the critical role played by miR-122 in lipid synthesis modulation and NAFLD. In normal mice, miR-122 inhibition with antisense oligonucleotide (ASO) resulted in reduced plasma cholesterol level, increased hepatic fatty-acid oxidation, and decreased rates of hepatic fatty-acid and cholesterol synthesis. Activation of the central metabolic sensor AMPK was also increased due to the inhibition of miR-122. In a diet-induced obesity mouse model, miR-122 inhibition with ASO caused decreased plasma cholesterol levels, significant improvement of liver steatosis and reduced expression of several lipogenic genes [Esau et al., 2006]. All these findings strongly suggested the significance of miR-122 in the regulation of lipid metabolism and the contribution to NAFLD development. However, the phenotypes of miR-122 in hepatic and

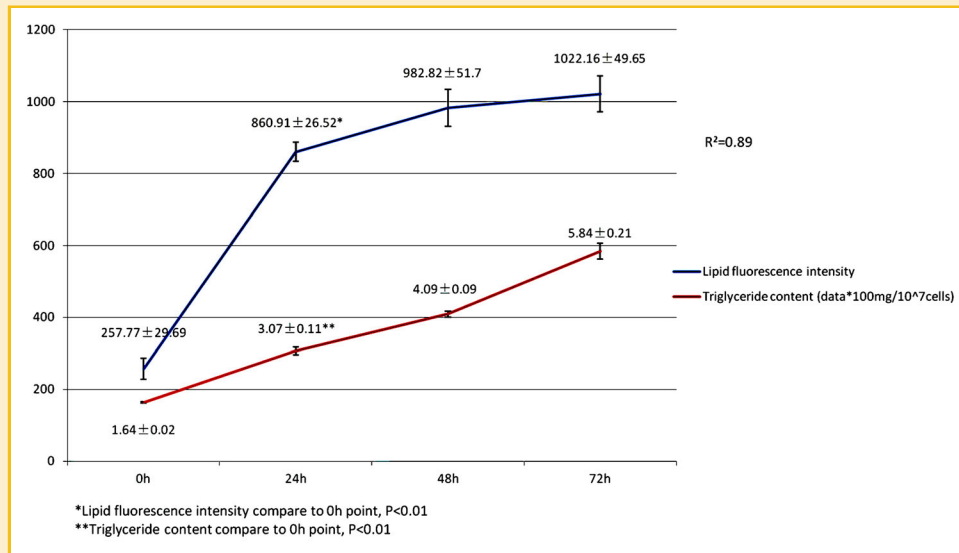


Figure 2. Intracellular lipid fluorescence intensity and triglyceride content in steatotic hepatocyte model after oleic acid induction.

non-hepatic tissues might not always be concordant. In liver-specific knockouts (LKO) and germline knockouts (KO) mice, miR-122 inhibition was shown to reduce 30% serum total cholesterol via down-regulation of genes involved in cholesterol biosynthesis such as the rate-limiting enzyme 3-hydroxy-3-methylglutaryl coenzyme

A (HMGCoA) reductase [Esau et al., 2006], but the livers developed progressive steatohepatitis [Tsai et al., 2012; Hsu et al., 2012]. In animal study, genes involved in lipid synthesis in the liver, for example, *Agpat1*, *Mogat1*, *Agpat3*, *Agpat9*, *Ppap2a*, *Ppap2c* and *Cidec* were found to be the direct targets of miR-122 [Kim et al., 2008].

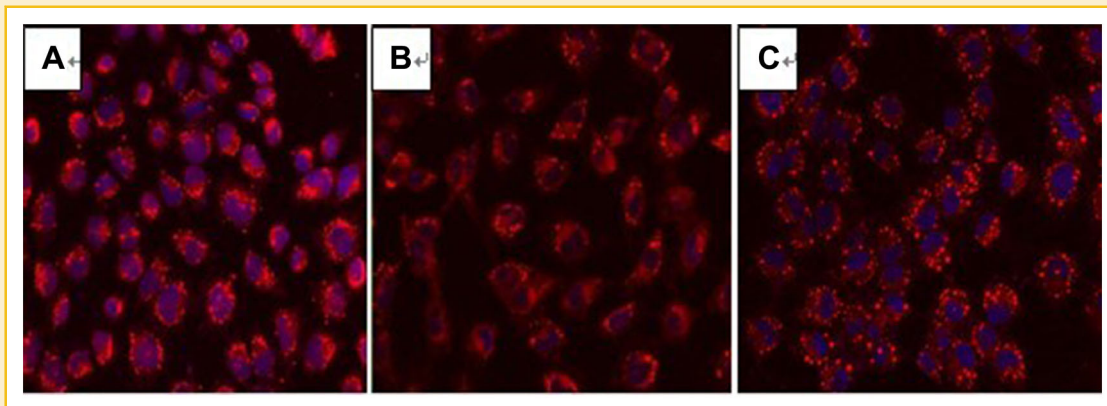


Figure 3. Hepatocytes stained with Nile Red under LSCM (10 × 40) before and after transfection. A: Steatotic hepatocytes before transfection (control), (B) Steatotic hepatocytes transfected with miR-122 mimic, (C) Steatotic hepatocytes transfected with miR-122 inhibitor.

TABLE II. miR-122 Expression and Intracellular Lipid Levels After Transfection With miR-122 Mimic, miR-122 Inhibitor and Their Controls

	miR-122 expression	Intracellular lipid level (fluorescence intensity)	P-Value
miR-122 mimic	0.0222 ± 0.0048	790.92 ± 46.72	<0.01
miR-122 inhibitor	0.0002 ± 0.0001	1386.49 ± 40.34	<0.01
Mimic control	0.0063 ± 0.0009	1015.65 ± 47.34	>0.05
Inhibitor control	0.0088 ± 0.0007	1025.12 ± 47.52	>0.05
Steatotic hepatocyte before transfection	0.0075 ± 0.0012	1022.16 ± 49.65	-

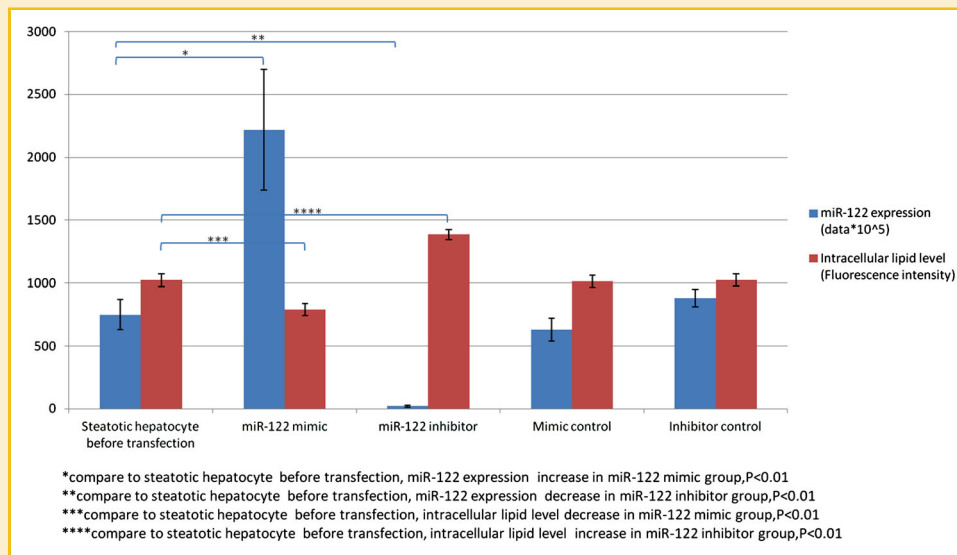


Figure 4. miR-122 expression and intracellular lipid levels after transfection with miR-122 mimic, miR-122 inhibitor and their controls.

miR-122 was shown to link to the output system of the circadian clock by regulating circadianly expressed genes [Gatfield et al., 2009].

The roles of miR-122 in other liver diseases have also been documented. miR-122 is required for hepatitis C virus (HCV) replication in cultured human hepatic cell line Huh7 [Jopling et al., 2005, 2008]. In patients with chronic hepatitis C (CHC), serum levels of miR-122 were correlated with liver enzyme levels, fibrosis stage and inflammatory activity. miR-122 enhanced the replication of HCV and influenced the efficiency of interferon therapy [Cermelli et al., 2011]. miR-122 levels were frequently reduced in hepatocellular carcinoma (HCC) compared with those in normal liver [Kutay et al., 2006] and were correlated with poor prognosis [Coulouarn et al., 2009]. Over-expression of miR-122 reduced tumorigenic properties of HCC cell lines [Bai et al., 2009].

Besides miR-122, other miRs have also been demonstrated to be involved in NAFLD development. miR-34a and miR-146b were shown to be significantly over-expressed (99% and 80%, respectively) in human NASH [Cheung et al., 2008]. The expression of miR-335 in the liver was up-regulated in mice. The increased miR-335 expression was associated with increased body, liver and white adipose tissue weight, as well as elevated hepatic triglyceride and cholesterol levels. Furthermore, hepatic miR-335 level was closely correlated with the expression of adipocyte differentiation markers, that is, PPAR- α and FAS in adipocyte [Nakanishi et al., 2009]. The presence of miR-181d significantly decreased lipid droplets in the liver (60%), and subsequently reduced cellular triglyceride and cholesterol [Whittaker et al., 2010]. miR-10b regulated steatosis level through PPAR- α pathway in a steatotic hepatocyte (L02 cell line) model. Post-transcriptional regulation of PPAR- α by miR-10b was maintained by a single binding site [Zheng et al., 2010]. In a smaller sample clinical study (n = 12/group), many miRs (-132, -150, -433, -28, -511, -517a, -671) were found to be differentially expressed in patients with NAFLD [Estep et al., 2010]. Other miRs involved in

NAFLD included miR-16, -29c, -33 (up-regulated) and miR-99b, -150 (down-regulated) [Ceccarelli et al., 2013]. So far, there is insufficient evidence to support the relation of these miRs with NAFLD.

To our knowledge, this is the first attempt to observe the effect miR-122 transfection on steatotic hepatocytes in vitro. We find that the miR-122 expression increased, but lipid content decreased significantly after miR-122 mimic transfection. On the contrary, the miR-122 expression decreased, but the lipid content increased significantly after miR-122 inhibitor transfection. These findings were consistent with the data observed in animal studies with ASO inhibition, and demonstrated the importance of miR-122 in the pathogenesis of fatty liver disease. miR-122 may be an attractive target for NAFLD diagnosis and treatment.

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