miR-29a Promotes Lipid Droplet and Triglyceride Formation in HCV Infection by Inducing Expression of SREBP-1c and CAV1

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Abstract

Aims: To examine the regulation of SREBP-1c and CAV1 by microRNA-29a (miR-29a) in cells infected with hepatitis C virus (HCV) in an attempt to control HCV-induced non-alcoholic fatty liver disease. Methods: In order to examine the manipulation of SREBP-1c and CAV1 by miR-29a, oleic acid (OA)-treated JFH-1-infected Huh-7 cells were used. OA was added 24 h post-transfection and gene expression was investigated by qRT-PCR at 48 h post treatment. The functional impact of the observed alteration in SREBP-1c and CAV1 expression was analyzed by examining lipid droplet (LD) and triglyceride (TG) content at 72 h post-OA treatment using light microscopy and spectrophotometry, respectively. Viral load was quantified by qRT-PCR at 72 h post-transfection.

Results: OA treatment induced the expression of miR-29a and SREBP-1c, as compared to untreated cells. Forced miR-29a expression led to a significant up-regulation of SREBP-1c as well as CAV1 compared to mock untransfected cells. Ectopic expression of miR-29a resulted in a marked increase in LDs and their respective TGs, while miR-29a antagonirs decreased both the LD and TG content compared to mock untransfected cells. Moreover, forcing the expression of miR-29a in JFH-1 HCV-infected Huh-7 cells resulted in 53% reduction in viral titers compared to mock uninfected Huh-7 cells.

Conclusion: Inducing miR-29a expression significantly induces SREBP-1c and CAV1 expression, thereby increasing LDs as well as their respective TGs. Nonetheless, forcing the expression of miR-29a resulted in reduction of HCV RNA levels in Huh-7 cells.

Keywords: Caveolin-1; HCV; Lipid droplets; MicroRNA-29a; SREBP-1c.

Abbreviations: ANGPTL3, angiotensin-like protein 3; CAVs, Caveolins; HCV, hepatitis C virus; LD, lipid droplet; miRNA, microRNA; miR-29a, microRNA-29a; NAFLD, Non-alcoholic fatty liver disease; NSSA, non-structural protein 5A; ORO, oil-red-O solution; OA, oleic-acid; (PPAR)-a, peroxisome proliferator activated receptor-a; SREBP, sterol regulatory element binding protein; TG, triglyceride.

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Introduction

Hepatitis C virus (HCV) is a major causative agent of non-alcoholic fatty liver disease (NAFLD), where 40% to 80% of HCV-infected patients develop steatosis depending on several factors, such as presence of diabetes and obesity as well as other risk factors, making the occurrence of steatosis in HCV infection 2-fold higher than in other chronic liver diseases, including infection with hepatitis B virus.1,2 Although little is known about the mechanism leading to lipid accumulation in HCV-infected hepatocytes, some studies showed that several HCV proteins, specifically the structural core and the non-structural protein 5A (NSSA), can lead to hepatic steatosis.3 In addition, overexpression of the HCV proteins core and NS4B independently activates miR-27a expression, leading to larger and more abundant lipid droplets (LDs) as the overexpression represses peroxisome proliferator-activated receptor-α (PPAR)-α and angioteni-like protein 3 (ANGPTL3), known regulators of triglyceride (TG) homeostasis in hepatocytes, which is considered a novel mechanism of HCV-induced steatosis.4 It has also been shown that Huh-7 hepatoma cells infected with a mutant HCV core protein that does not target the LDs had extremely lower amounts of intracellular lipids compared to cells infected with the wild-type core protein. Thus, HCV core protein expression appears to increase the amount of LDs, and an increased LD content results in increased HCV core protein expression, which is favorable for HCV replication.5

LDs are mono-layered organelles with a hydrophobic core of TG and cholesteryl esters surrounded by phospholipids. Their size ranges from nanometres to several micrometres in diameter. Several proteins associate with the phospholipid monolayer, including perilipin and RAB family members, which play a role in the structure, formation and function of LDs.6,7 Other proteins associate with the surface of LDs in special conditions, including the caveolins (CAVs). CAVs constitute the structural framework of caveolae, which are small
invaginations of the plasma membrane. The caveolin family consists of three members: CAV1, CAV2 and CAV3. CAV1, in particular, has been reported as important for LD stability.

Among the regulators of LDs are the family of transcriptional regulators of the lipid synthetic genes, known as the sterol regulatory element binding proteins (SREBP). To date, three SREBP isoforms have been identified: SREBP-1a, SREBP-1c and SREBP-2. It was reported that perturbed expression of SREBP-1c, in particular, leads to an increase in LD proteins in fatty liver dystrophic mice. Several studies have also demonstrated that there is a positive correlation between the amount of LDs and SREBP-1c expression.

SREBP-1 is known to bind to the sterol regulatory elements (SREs) in the CAV1 gene promoter and to subsequently regulate CAV1 expression, either positively or negatively, depending on the cell type as well as the upstream signal. Many research groups have investigated the relation between CAV1 levels and LDs in different cell types, including adipocytes, fibroblasts and hepatocytes. In a study of hepatocytes, NAFLD was shown to be associated with up-regulation of the hepatic CAV1 gene and protein expression as well as an increase in its localization on LDs. Furthermore, CAV1 was found to play a role during liver regeneration. However, neither its expression nor its relation with SREBP-1c have been investigated in HCV-infected hepatocytes.

Several microRNAs (miRNAs) can inhibit SREBP-1 expression in hepatocytes, including miR-449, miR-122 and miR-613, thus down-regulating its target lipogenic genes and the related LD content. Bioinformatics have previously shown that miR-29a targets SREBP-1c. miR-29a has been reported to alleviate cholestatic liver injury as well as fibrosis. It has also been reported to be down-regulated in the liver of chronic HCV-infected patients as well as in genotypes 2a HCV-infected cell models. Furthermore, overexpression of miR-29a led to decreased HCV RNA abundance in HCV genotypes 2a-infected cell models. To date, very limited data is available concerning the role of miRNAs in controlling LDs in HCV infection. Hence, this study aimed at uncovering the role of miR-29a in regulating LD formation of SREBP-1c and the subsequent CAV1, in an attempt to control LD formation as well as their respective TGs in HCV infection.

Methods

Cell culture

Adherent human hepatoma cells (Huh-7 cells) were incubated at 37 °C and 5% CO2 following culturing in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 4.5g/L glucose and L-glutamine.

In vitro transcription and production of HCV cell culture (HCVcc)

HCV construct (pJFH-1) harboring the full-length HCV genotype 2a (kindly provided by Prof. Wakita, National Institute of Infectious Diseases, Tokyo, Japan) was linearized using XbaI, and then purified using phenol/chloroform. In vitro transcription was carried out with 1 μg of the purified DNA using the MEGAscript® T7 Transcription Kit (AM1330: Ambion, Austin, TX, USA). To produce the HCVcc, liposome-mediated transfection of 10 μg of the in vitro transcribed HCV genomic RNA into Huh-7 cells was performed using the Superfect® Transfection Reagent (Qiagen, Hilden, Germany). At 72 hrs post-transfection, supernatants were collected, filtered using 0.45-μm filters, and stored at −80°C for further infection of naïve Huh-7 cells.

RNA extraction

Total RNA, mRNA and miRNA, was extracted using the Biozol Reagent (Bioer Technology, Hangzhou, China). Huh-7 cells were first lysed in Biozol then chloroform was added on top of the cell lysate. Following centrifugation at 12,000 rpm at 4 °C for 15 min, RNA in the aqueous layer was precipitated using isopropanol. Followed additional centrifugation at 12,000 rpm at 4 °C for 15 min, the precipitated RNA was then washed using 75% ethanol and dissolved in DPEC water.

Reverse transcription and RNA quantification using qRT-PCR

miR-29a and the housekeeping gene RNU6B were reverse transcribed into complementary DNA (cDNA) using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s instructions and using TaqMan microRNA assays (Applied Biosystems). Total cellular mRNA was reverse transcribed into cDNA using the high-capacity CDNA reverse transcription kit (Qiagen) according to the manufacturer’s instructions.

The mRNA and miRNA expression levels were quantified using StepOne™ real-time quantitative polymerase chain reaction (qRT-PCR) (SN: 271000301; Applied Biosystems). The amount of miR-29a was then calculated relative to the amount of the housekeeping gene RNU6B, and the mRNA expression level of SREBP-1c and CAV1 was then normalized to the housekeeping gene B2-microglobulin (B2M) using TaqMan sterol regulatory element binding factor-1 (SREBF1), CAV1 and B2M assays (Applied Biosystems).

Transfection of oligonucleotides

In order to perform mRNA quantification and intracellular LD imaging, Huh-7 cells were transfected with 25 nM of miR-29a mimics or antagonims, or SREBP-1 siRNAs (used as a positive control in this study) in 96-well plates by means of the Hiperfect transfection reagent (Qiagen) using reverse transfection method according to the manufacturer’s instructions. In order to quantify TGs as well as viral load, Huh-7 cells were transfected in 24-well plates by the Hiperfect transfection reagent (Qiagen) using the traditional transfection method according to the manufacturer’s instructions. Cells were cultured under normal conditions (37°C and 5% CO2).

Fatty acid treatment

In order to develop a model for hepatic steatosis in Huh-7 cells, induction of LD formation was achieved by incubating cells with OA. The Huh-7 cells were incubated with 200 μM bovine serum albumin-coupled with OA at 24 hrs post-transfection with oligonucleotides, then at 48 hrs post-OA treatment the gene expression profiling was performed using qRT-PCR. LD visualization and TG quantification were performed at 72 hrs post-OA treatment.

Lipid droplet staining and imaging

A stock solution of 0.35% oil-red-O solution (ORO; Serva, Heidelberg, Germany) was prepared and filtered through a 0.22-μm filter. A working solution of ORO was freshly prepared
by diluting the stock solution with double-distilled water at a ratio of 6:4. The working solution was left to stand for 20 min and filtered through a 0.22-μm filter. Fixation of cultured cells was performed using 4% paraformaldehyde in phosphate buffer saline (PBS; pH = 7.4) for 10 min at room temperature. Fixed cells were then washed with 3 changes of PBS, 10 min each. Permeabilization was performed by incubating the cells with 0.05% (Tween 20 in PBS) for 15 min followed by 3 washes with PBS then washing once with 60% isopropanol. Permeabilized cells were then incubated with ORO stain for 10 min followed by 6 washes with distilled water, 5 min each. LDs were visualized using Axiom Zeiss Light microscope with 100x magnification (SN 3832001601: Carl Zeiss, Jena, Germany) as described in other research papers.39,40 Images were captured using Zen2011 software (Carl Zeiss).

**Triglyceride extraction and quantification**

Seventy-two hrs after the OA treatment, TGs were extracted from the JFH-1-infected Huh-7 cells using a triglyceride colorimetric assay kit (Item no. 10010303: Cayman Chemical, Ann-Arbor, MI, USA) and then quantified using the Triglycerides-LQ Kit (Spinreact, Girona, Spain) according to the manufacturer’s instructions.

TGs were quantified using a spectrophotometric-based TG kit, whereby the TGs are chemically derivatized to quinone and measured at 505 nm. Before calculating the TG concentration, the average of the standard concentrations of different experiments was calculated. Concentration of the unknown TGs was calculated using the following 1-point calibration equation: absorbance of sample/absorbance of standard*TG concentration (200 mg/dL-1).

**Viral RNA extraction and quantification**

Seventy-two hrs post-transfection, viral RNA was extracted from HCV-infected Huh-7 cells using the InvisorbSpin Virus RNA Mini Kit (Invitek, Hayward, CA, USA) according to the manufacturer’s protocol. The extracted RNA was stored at −80°C until further use. Viral nucleic acid was quantified using a viral nucleic acid detection kit (Genesig; PrimerDesign, Chandler’s Ford, UK), and following the manufacturer’s protocol using real-time PCR.

**Statistical analysis**

Data were expressed as mean ± standard error of the mean (SEM). Differences between samples were analyzed using Student’s t-test. A p-value lower than 0.05 was considered significant. ***p < 0.001, **p < 0.01, *p < 0.05 and ns indicated not statistically significant. All the data were statistically analyzed using GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA).

**Results**

**Impact of OA treatment on miR-29a, SREBP-1c and CAV1 mRNA expression and on LD and TG content in JFH-1-infected cells**

OA treatment of JFH-1-infected Huh-7 cells resulted in up-regulation of miR-29a (p = 0.0002) and SREBP-1c (p = 0.0443) mRNA expression, with no significant change in CAV1 mRNA expression, as compared to untreated JFH-1-infected cells (Fig. 1A, 1B, 1C). LDs were markedly increased in the OA treated cells (Fig. 1D). TG concentration is significantly increased upon OA treatment (102.3 ± 3.068, p = 0.0305*, n = 5) compared to untreated JFH-1-infected cells (84.58 ± 6.573). Results are expressed as a mean ± SEM. Student’s t-test was used for the statistical analysis.
OA-treated JFH-I-infected Huh-7 cells, and the intracellular TG content was also significantly increased ($p = 0.0305$) compared to the untreated JFH-1-infected Huh-7 cells (Fig. 1D, 1E).

Impact of manipulating miR-29a on SREBP-1c and CAV1 mRNA expression and on cellular lipid droplet (LD) and triglyceride (TG) content in JFH-I-infected Huh-7 cells.

At 3 days post-infection with JFH-I, miR-29a oligonucleotides were transfected into the cells, and at 24 hrs post-transfection the cells were treated with 200 μM OA. Forty-eight hrs following the OA treatment, qRT-PCR was used to assess the mRNA expression of SREBP-1c and CAV1. Before assessing the expression of both genes, it was important to confirm the efficiency of transfection of the oligonucleotides; mimicking of miR-29a significantly increased its expression ($p = 0.0035$), by a mean of 1157.018-fold compared to untransfected cells ($n = 4$) (Fig. 2A). Mimicking with miR-29a lead to up-regulation of SREBP-1c mRNA expression (1.369 ± 0.1292, $p = 0.0276$, $n = 9$) with no significant change observed for antagonizing miR-29a expression (1.067 ± 0.1949, $n = 9$) compared to untransfected cells (1.043 ± 0.07413). C) miR-29a mimics induced the mRNA expression of CAV1 (1.606 ± 0.1704, $p = 0.0337$) compared to untransfected cells (1.081 ± 0.1350). D) Mimicking of miR-29a resulted in increased amount of LDs, as shown by increase in the red coloration due to the oil red O staining, while miR-29a antagonir showed a slight decrease in the amount of LDs compared to untransfected cells, $n = 3$. Scale bars of 10 μm are shown on the images. E) Mimicking of miR-29a also significantly increased the concentration of TGs (114.8 ± 2.578, $p = 0.0038$, $n = 6$) and antagonizing miR-29a significantly decreased TG concentration (80.73 ± 4.382, $p = 0.0125$, $n = 2$) compared to untransfected cells (99.87 ± 2.352). All results are expressed as a mean ± SEM. Student’s t-test was used for the statistical analysis.

Impact of SREBP-1c siRNA on LD formation and CAV1 mRNA expression

To investigate the association between level of SREBP-1c and LD, OA-treated JFH-I-infected Huh-7 cells were transfected with SREBP-1c siRNAs. SREBP-1c level was measured to ensure efficient repression of SREBP-1c mRNA levels ($p < 0.0001$) (Fig. 3A). LD formation markedly decreased (Fig. 3B). CAV1 mRNA expression significantly increased following SREBP-1c siRNA transfection ($p = 0.0030$) (Fig. 3C).

Impact of miR-29a on viral replication

Mimicking of miR-29a in JFH-I-infected Huh-7 cells resulted in 53% reduction ($p = 0.0499$) in viral titers compared to untransfected Huh-7 cells (Fig. 4).
miR-29a is reported to have anti-viral and anti-fibrotic activities. It was shown to decrease the HCV RNA abundance in Huh-7.5 cells infected with the J6 strain of genotype 2a (in a HCVcc system).\(^3\) miR-29a was also reported to inhibit hepatic stellate cells and thus fibrosis.\(^4\) The regulation of LDs by miR-29a in HCV infection has not yet been investigated. However, given the aforementioned reports, miR-29a was of particular interest in this study. In silico analysis revealed that miR-29a targets the 3'UTR of SREBP-1c mRNA. Moreover, a recent study supported that inhibition of miR-29a decreased the SREBP-1 levels in the livers of chow diet-fed C57BL/6 J female mice.\(^35\)

SREBP-1c is an important pro-lipogenic transcription factor, which is induced by the NS2 protein of HCV.\(^42\) Activation of SREBP-1c by the liver X receptor/retinoid X receptor (LXR/RXR) leads to activation of its target lipogenic genes and thus increases the amount of LDs.\(^3\) Since SREBP-1c is the predominant hepatic isoform among the SREBP family and CAV1 is regulated by SREBP-1 and is one of the LD proteins responsible for its stability, they were chosen to be the main focus of this study.\(^11,44\) In this context, this study aimed at uncovering the impact of miR-29a on SREBP-1c and its downstream target, CAV1, in order to better elucidate their roles in LD formation.

In order to simulate fatty liver conditions, OA treatment was performed for LD induction in JFH-1-infected Huh-7 cells. miR-29a, SREBP-1c and CAV1 expression were then screened. Both miR-29a and SREBP-1c showed a significant up-regulation, whereas no significant change in the expression of CAV1 was observed after OA treatment (Fig. 1A, 1B, 1C). The LD and TG content also significantly increased following LD induction (Fig. 1D, 1E), indicating that induction of LDs in cells could alter the level of miRNAs and hence their impact on their downstream target genes. Thus, the next experiments were all carried out in OA-treated JFH-1-infected Huh-7 cells.

miR-29a expression was manipulated using oligos and its impact on SREBP-1c, CAV1, LDs and TGs was analyzed. SREBP-1c showed a significant increase at the transcriptional level following transfection of miR-29a mimics (Fig. 2B). This finding is in line with those reported by Kurtz et al.\(^35\) who recently measured mRNA expression of 883 genes in fatty liver mouse models that were injected with locked nucleic acids (LNAs) against miR-29a. Among the lipogenic genes measured was the SREBF-1 gene which was found to be down-regulated.\(^35\) miR-29a mimics also induced CAV1 expression, and thus it was important to investigate whether this induction was mediated by SREBP-1c, especially since SREBP-1 is known to bind to SREs in the CAV1 gene promoter. Binding of SREBP-1c to the SRE subsequently regulates CAV1 expression, either positively or negatively, depending on the cell type as well as the upstream signal.\(^20,21\) Thus, it was important to investigate this relationship in OA-treated JFH-1-infected Huh-7 cells. SREBP-1c was knocked down using siRNAs, which resulted in a significant increase in CAV1 mRNA level (Fig. 3C); this result suggests that miR-29a might have mediated its effect on CAV1 indirectly and not through SREBP1c. Moreover, forcing miR-29a expression in OA-treated JFH-1-infected cells led to a marked increase in intracellular LD as well as TG content (Fig. 3E). In accordance with our results, it has been recently demonstrated that miR-29a LNAs reduced fatty acid production by 70% in Huh-7 cells.\(^35\) However, it was shown in another study that miR-29a might protect the liver from development of steatosis.\(^45\) Moreover, the study of Whitaker and colleagues indicated that miR-29a decreases LD content in naive Huh-7 cells, yet this
contradiction with our obtained results might be due to the HCV infection.\textsuperscript{46}

It was tempting then to investigate whether the impact of miR-29a on LDs was mediated by SREBP-1 or not. Knockdown of SREBP-1c using specific siRNAs resulted in a marked decrease in LD content, which might confirm that this decrease in the LDs was mediated via SREBP-1c (Fig. 3B).

miR-29a inhibitors resulted in a slight decrease in LD formation, even though they had no impact on SREBP1 and CAV1 mRNA levels (Fig. 2D). Similar to our observations, a recent study showed that miR-182 inhibitors can lead to a reduction in the viral load without affecting its respective target gene.\textsuperscript{47} This finding might reflect the fact that the antagonists were not able to completely repress the miRNA levels, and thus the levels of SREBP-1c and CAV1 were not completely suppressed; however, this minor repression of the miRNA levels was sufficient to repress LDs as well as their respective TGs.

Finally, it was interesting to study the sole effect of miR-29a on viral replication. Transfection of miR-29a mimics lead to a respective TGs. miRNA levels was sufficient to repress LDs as well as the levels of SREBP-1c and CAV1 were not completely suppressed; however, this minor repression of the miRNA levels, and thus the levels of SREBP-1c and CAV1 were not completely suppressed; however, this minor repression of the miRNA levels was sufficient to repress LDs as well as their respective TGs.

Our study shows a controversial role of miR-29a in HCV-induced steatosis, where it was shown to inhibit HCV viral replication, while promoting LD formation. This dual function of miRNAs has been previously observed in several cases, such as the liver-specific miR-122, where it resulted in an increase in HCV viral replication, while it was shown to act as a tumor suppressor miRNA in hepatocellular carcinoma, repressing liver cancer development and progression.\textsuperscript{48,49} Nonetheless, the contradicting role of miR-182 was also recently reported by our group; specifically, it was shown to promote HCV viral replication, while resulting in a significant augmentation of primary normal killer cell cytotoxicity.\textsuperscript{46}

Conclusions

In conclusion, although miR-29a has an anti-HCV action, forcing its expression paradoxically induced LD formation, as well as its respective TGs, through inducing the expression of the transcription factor SREBP-1c. Thus, miR-29a can be considered to have a potential role in the pathogenesis of steatosis as well as the course of HCV infection.

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Conflict of interest

None

Author contributions

Performed experiments (MMM, RAS, RSH), analyzed and interpreted data (MMM, NME, RMH, AIA), performed statistical analysis (MMM), wrote all drafts of the manuscript (MMM, NME, HMEA, AIA), designed the experiments (NME, RMH, AIA), provided materials (HMEA). All authors read, discussed and approved the manuscript.

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