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miR-148a and miR-30a Limit HCV-Dependent Suppression of the Lipid Droplet Protein, ADRP, in HCV Infected Cell Models

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Hepatitis C Virus (HCV) promotes lipid droplet (LD) formation and perturbs the expression of the LD associated PAT proteins ADRP and TIP47, to promote its own lifecycle. HCV enhances TIP47 and suppresses ADRP by displacing it from LD surface in infected cell models. We have previously shown that suppression of TIP47 by miR-148a and miR-30a decreased intracellular LDs and HCV RNA. Thus, this study aimed at examining whether this microRNA-mediated suppression of HCV would limit HCV-dependent displacement of ADRP from LDs. ADRP expression was examined in 21 HCV-infected liver biopsies and 9 healthy donor liver tissues as well as in HCV-infected Huh7 cells using qRT-PCR. miR-148a and miR-30a expression was manipulated using specific oligos in JFH-1 infected, oleic acid treated cells, to study their impact on ADRP expression using qRT-PCR, and immunofluorescence microscopy. Intracellular HCV RNA was assessed using qRT-PCR. ADRP is down regulated in patients as well as HCVcc-JFH-I infected cell models. Forcing the expression of both miRNAs induced ADRP on the mRNA and protein levels. This study shows that HCV suppresses hepatic ADRP expression in infected patients and cell lines. Forcing the expression of miR-148a and miR-30a limits the suppressive effect of HCV on ADRP. ***J. Med. Virol. 89:653–659, 2017***.

KEY WORDS: microRNAs; liver; viral infection; PAT proteins; lipids

INTRODUCTION

Over the past decades Hepatitis C Virus (HCV), a member of the family *Flaviviridae*, genus *Hepacivirus*, has proven to be an extremely persistent microorganism that is capable of manipulating various systems of its host's cell machinery for its own benefit. One of the most important host systems affected by HCV is the lipid machinery, where around 55% of HCV-infected patients develop steatosis [Asselah and Rubbia-Brandt, 2006]. HCV promotes host lipid droplet (LD) accumulation by various mechanisms, one of which is the activation of sterol regulatory element binding protein (SREBP) pathway [Waris et al., 2007], which in turn activates cholesterol and fatty acid biosynthetic pathways, leading to the accumulation of LDs [Sato, 2010; Li et al., 2013]. Several protein families are associated to LDs, including PAT proteins family, named after its first three identified members, namely Perilipin, Adipose differentiation-related protein (ADRP), and Tail-interacting protein of 47 kDa (TIP47) [Bickel, 2009]. The virus then utilizes the formed LDs and their associated proteins to promote its own lifecycle, where HCV proteins, such as core, NS5A as well as the viral RNA associate to the formed lipid droplets promoting HCV assembly [Gosert et al., 2003; Sato

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et al., 2006; Boulant et al., 2007; Miyanari et al., 2007]. Moreover, PAT proteins were also found to be affected by HCV, where cells expressing HCV core protein showed higher TIP47 expression compared to cells that are not expressing the core protein [Sato et al., 2006]. Overexpression of TIP47 was reported to increase replication and release of HCV particles [Ploen et al., 2013; Vogt et al., 2013]. In addition, HCV core expressing cells as well as JFH-I infected Huh7 cells show lower ADRP protein expression [Sato et al., 2006]. The reduction in ADRP was reported to be due to the displacement of ADRP from LD surface by HCV core protein, and since unbound ADRP is unstable, and undergoes proteomic degradation, its protein expression was lower in infected cells [Xu et al., 2005; Boulant et al., 2008]. We have previously shown that HCV infection enhances the expression of TIP47 and that epigenetic regulation of TIP47 by miR-148a and miR-30a suppressed TIP47 in infected cells, and hence decreased cellular LDs, and subsequently HCV RNA levels [El-Ekiaby et al., 2015]. These data triggered our interest to examine whether the microRNA-dependent suppression of HCV via decreasing TIP47, will limit the HCV-induced displacement and suppression of ADRP. Hence, this study aimed at examining the impact of miR-148a and miR-30a on ADRP expression in HCV infected cell models.

PATIENTS AND METHODS

Patients

Twenty-one patients (14 males and 7 females) chronically infected with HCV were recruited in this study and liver needle biopsies were taken from them. All patients were treatment naïve and HBV negative, and were recruited from Al Kasr Al Aini Hospital, Cairo University Medical School. Nine healthy liver biopsies were obtained from donors during liver transplantation. The study followed the ethical guidelines of the 1975 Declaration of Helsinki. All patients gave their written informed consent. Clinical parameters of the patients are presented in Table I.

Cell Culture

Dulbecco's modified Eagle's medium (DMEM), which was supplemented with 10% Fetal Bovine Serum (FBS), 4.5 g/L Glucose, L-Glutamine and 1% Penicillin/Streptomycin was used to culture Human Hepatocellular Carcinoma cells (Huh-7). Cells were incubated in a CO_2 incubator adjusted to 5% CO_2 at 37°C.

In Vitro Transcription and Transfection of HCV RNA

HCV replicon cells were generated using pJFH-I harboring the full-length HCV genotype 2a genome (kindly provided by Prof. Wakita). Briefly, Xba1 restriction enzyme was used to linearize pJFH-I. Linearized DNA was purified with phenol/chloroform. Full length HCV RNA was obtained by in vitro transcription of 1 μg of purified DNA using MEGAscript® T7 Transcription Kit (Ambion, Austin, TX). Ten micro grams of HCV RNA were transfected into Huh7 cells. Cell culture supernatant containing cell culture derived HCV particles (HCVcc) was collected 3 days post transfection, filtered using 0.45 μm filter then stored at −80°C for later infection of naïve Huh7 cells.

Infection of Naïve Huh7 Cells

Naive Huh7 cells cultured in 10 cm plates were inoculated overnight with 3 ml of the cell culture supernatant obtained from HCV RNA transfected

TABLE I. **Patients' Clinical Data**

Patient #	Age	Gender	VL	ALT	AST	Metavir score	Genotype
P1	43	M	1,17,744	28	30	F1	4
P2	37	M	11,80,492	74	74	F1	4
P3	36	M	2,15,500	80	62	F1	4
P4	28	F	1,69,000	32	25	F1	4
P5	28	M	1,06,000	58	37	F1	4
P6	50	M	10,60,000	56	60	F2	4
P7	35	F	16,50,000	18	21	F3	4
P8	25	M	45,190	105	55	F1	4
P9	47	M	32,30,000	26	28	F3	4
P10	28	F	1,19,000	33	26	F4	4
P11	46	M	16,54,000	78	101	F3	4
P12	55	M	1,79,000	76	80	F3	4
P13	32	M	31,20,000	52	47	F1	4
P14	35	M	40,20,000	74	65	F1	4
P15	34	M	17,90,000	62	53	F1	4
P16	52	M	11,20,000	34	53	F4	4
P17	68	F	1,54,32,000	54	43	F2	4
P18	34	M	4,03,200	42	55	F1	4
P19	40	F	5,32,00000	60	44	F1	4
P20	38	F	76,70,000	15	18	F1	4
P21	56	F	56,300	67	53	F4	4

cells. Cells were then washed and fresh media was added. Three days post infection cells were utilized for microRNA transfection experiments.

RNA Extraction and Quantification

Biozol reagent was used to extract total RNA (Bioer Technology, Hangzhou, China). Briefly, liver biopsies or Huh7 cells were lysed in biozol reagent. RNA was extracted by addition of chloroform at a ratio of 1:5 to biozol reagent and centrifugation at $12{,}000 \times g$ 4˚C for 15 min. The aqueous layer containing the RNA was then harvested and RNA was precipitated using equal volume of Isopropanol followed by centrifugation at $12{,}000 \times g$ 4˚C for 15 min. RNA was then washed using 75% ethanol and dissolved in DPEC water.

mRNA was reverse transcribed into complimentary DNA (cDNA) using High-capacity cDNA reverse transcription kit (Qiagen, Hilden, Germany). The mRNA expression level of ADRP was quantified using TaqMan real-time quantitative polymerase chain reaction (PCR) (StepOne, Applied Biosystems, Foster City, CA) normalized to the housekeeping gene β2-microglobulin (B2M) using TaqMan PLIN2 Assay and TaqMan β-microglobulin Assay (B2M), respectively, (Applied Biosystems,USA).

RNA expression was quantified using the relative quantitation method as follows:

ΔCT is the difference in threshold cycles (CT) for target and endogenous control (ΔCT=CT target − CT B2M). ΔΔCT is the difference in ΔCT for target and reference, that is healthy controls or mock cells (ΔΔCT = ΔCT target- ΔCT reference). The relative quantitation (RQ) of gene expression is determined by the following equation ($RQ = 2^{-\Delta\Delta CT}$).

Transfection of microRNA Mimics, Inhibitors and siRNAs

Huh7 cells were transfected with 25nM miR-148a or miR-30a mimics or antagomirs or siRNAs against ADRP (Qiagen, Germany) in 96-well plates using the reverse transfection method using HiPerFect transfection reagent according to manufacturer's instructions. Cells were incubated under normal growth conditions (typically 37˚C and 5% CO_2), and ADRP mRNA and protein expression, LDs or viral RNA levels were monitored after an appropriate time.

Fatty Acid Treatment

Induction of lipid droplet formation in Huh7 cells was performed as a model for hepatic steatosis by incubating cells with the fatty acid oleic acid. Twenty-four hours post transfection with oligos Huh7 cells were incubated with 600 μM bovine serum albumin-coupled oleic acid (OA) to induce lipid droplet formation. Cells were either harvested 48 hr after incubation with OA for gene expression profiling using qRT-PCR or after 72 hr for LD and protein imaging using immunofluorescence microscopy.

Immunofluorescence Microscopy and Lipid Staining

A stock solution of 0.35% of oil-red-O (ORO) (Serva, Heidelberg, Germany) was prepared and then filtered using a 0.22 μm filter. The stock solution was diluted with double distilled water at a ratio of 6:4 to prepare the ORO working solution. The working solution was left to stand for 20 min and filtered using a 0.22 μm filter.

Cultured cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were then washed with three changes of PBS, 10 min each. Cells were permeabilized to allow for the influx of antibodies into the cells. Permeabiliztion was performed by incubating with 0.05% Tween-20 in PBS for 15 min followed by three washes with PBS then once with 60% isopropanol. Intracellular lipid droplets were then stained by incubating permeabelized cells with ORO for 10 min followed by four washes with distilled water, 5 min each. To block non-specific binding of antibodies, cells were then incubated for one hour in blocking buffer (1% BSA in PBS). After a single wash with PBS, primary mouse monoclonal antibodies against either ADRP or HCV core protein were added to the cells and kept overnight at 4˚C. The following day cells were washed in three changes of PBS (5 min each), and incubated with FITC-conjugated-goat-anti-mouse antibodies for one hour. After three washes with PBS, cells were mounted with 90% glycerol in PBS to prevent fading of fluorescence. Lipid droplets and immunolabeled proteins were visualized using Axiom Zeiss immunofluorescence microscope. Images were captured using Zen software.

Viral Nucleic Acid Extraction and Quantification

Invisorb Spin Virus RNA Mini Kit (Invitek, Glasgow, UK) was used to extract viral RNA from Huh7 cells according to the manufacturers' instruction. Viral nucleic acid quantification was performed using HCV viral nucleic acid detection kit (Genesig, Primer design, Chandlers Ford, UK) according to manufacturer's protocol.

Statistical Analysis

Data are expressed as mean ± standard error of the mean. Differences between samples were analyzed using Student *T* test, Graph Pad Prism 5. *P* values lower than 0.05 were considered statistically significant.

RESULTS

Expression Profile of ADRP in Liver Biopsies of Healthy Controls Compared to HCV Infected Patients

Expression profile of ADRP was investigated in liver biopsies of HCV infected patients in comparison with liver tissues of healthy donors taken during

liver transplantation. ADRP mRNA expression was quantified using qRT-PCR and normalized to the housekeeping gene B2M. ADRP was found to be down regulated in HCV patients compared to healthy controls ($P = 0.0261^*$) (Fig. 1).

Impact of Infection of Huh7 Cells with HCVcc-JFH-I on ADRP Expression

Huh7 cells were infected with cell culture derived JFH-I. Twenty-four hours post infection ADRP mRNA was quantified using qRT-PCR. Infection of Huh7 cell lines with JFH-I resulted in suppression of ADRP mRNA expression compared to uninfected cells ($P = 0.0167^*$) (Fig. 2).

Influence of Manipulating miR-148a and miR-30a on ADRP Expression

miR-148a and miR-30a mimics or antagomirs were transfected in HCV JFH-I infected Huh7 cells. siRNAs against ADRP were also transfected as a positive control. Cells were treated with oleic acid-BSA (OA) 24 hr post transfection. ADRP mRNA expression was then examined using qRT-PCR 48 hr after oleic acid treatment. LDs and ADRP protein were visualized 72 hr post OA treatment using immunofluorescence microscopy. To confirm efficient HCV replication, HCV core protein was visualized in mock cells using immunofluorescence microscopy, which confirmed the presence of intracellular core proteins, indicating efficient viral replication and translation (Fig. 3A).

Mimics of miR-148a and miR-30a induced ADRP mRNA ($P = 0.0103^*$ and $P = 0.02^*$, respectively), and protein expression but reduced cellular LDs compared to mock cells. Both miR-148a and miR-30a antagomirs neither affected ADRP mRNA or protein expression nor LDs. siRNAs against ADRP efficiently knocked down its expression ($P = 0.032^*$) (Fig. 3B and C).

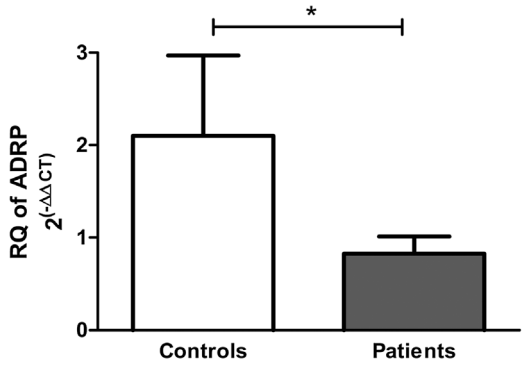

Figure. 1. Expression profile of ADRP in liver biopsies of HCV infected patients. ADRP is down regulated(0.8270 ± 0.1878 N=21) in HCV liver biopsies compared to healthy controls (2.100 ± 0.8715 N=9) ($P = 0.0261^*$). Results are expressed as mean ± SEM.

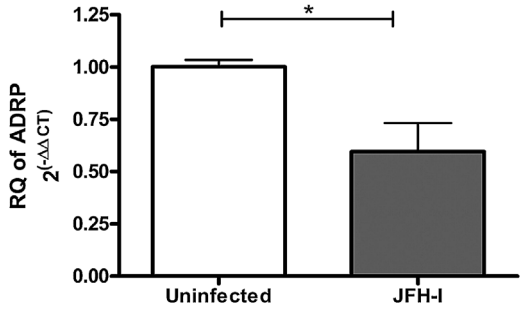

Figure. 2. Impact of infection of Huh7 cells with HCVcc-JFH-I on ADRP expression. ADRP expression is suppressed in JFH-I infected Huh7 cells (0.5960 ± 0.1361) compared to uninfected cells (1.001 ± 0.03356) ($P = 0.0167^*$). Results are expressed as mean ± SEM.

Impact of ADRP Knockdown on Intracellular HCV RNA

The impact of knocking down ADRP on HCV was examined by transfecting JFH-I infected Huh7 cells with siRNAs against ADRP. Twenty-four hours post transfection, cells were treated with OA. Forty-eight hours after OA treatment viral nucleic acid was extracted and quantified using qRT-PCR. Knockdown of ADRP did not affect intracellular HCV RNA levels (Fig. 4).

DISCUSSION

Hepatitis C virus interacts with many of the liver's organelles and machinery to promote its own survival. Among these organelles are the lipid droplets and their surface proteins. HCV infection was shown to enhance the expression of the LD protein TIP47, which in turn promotes HCV replication and assembly [Sato et al., 2006; Ploen et al., 2013; Vogt et al., 2013]. MicroRNA regulation of lipid droplets and their associated proteins represent a novel mechanism by which HCV infection as well as HCV-induced steatosis can be controlled. We have previously shown that miR-148a and miR-30a, which are both down regulated in HCV infection, directly target and suppress the expression of TIP47 in HCV infected Huh7 cells. The suppression of TIP47 subsequently led to a decrease in cellular lipid droplets as well as HCV RNA [El-Ekiaby et al., 2015]. Since lipid droplets are coated with other proteins, it was interesting to examine how these proteins were affected by this microRNA manipulation. A couple of studies have previously reported that HCV infection suppresses the expression of ADRP in cell lines, by displacing it from the surface of lipid droplets [Sato et al., 2006; Boulant et al., 2008]. Therefore, this study aimed at examining the expression pattern of ADRP for the first time in HCV infected patients, and investigating whether the suppression of HCV by miR-148a and miR-30a through repressing

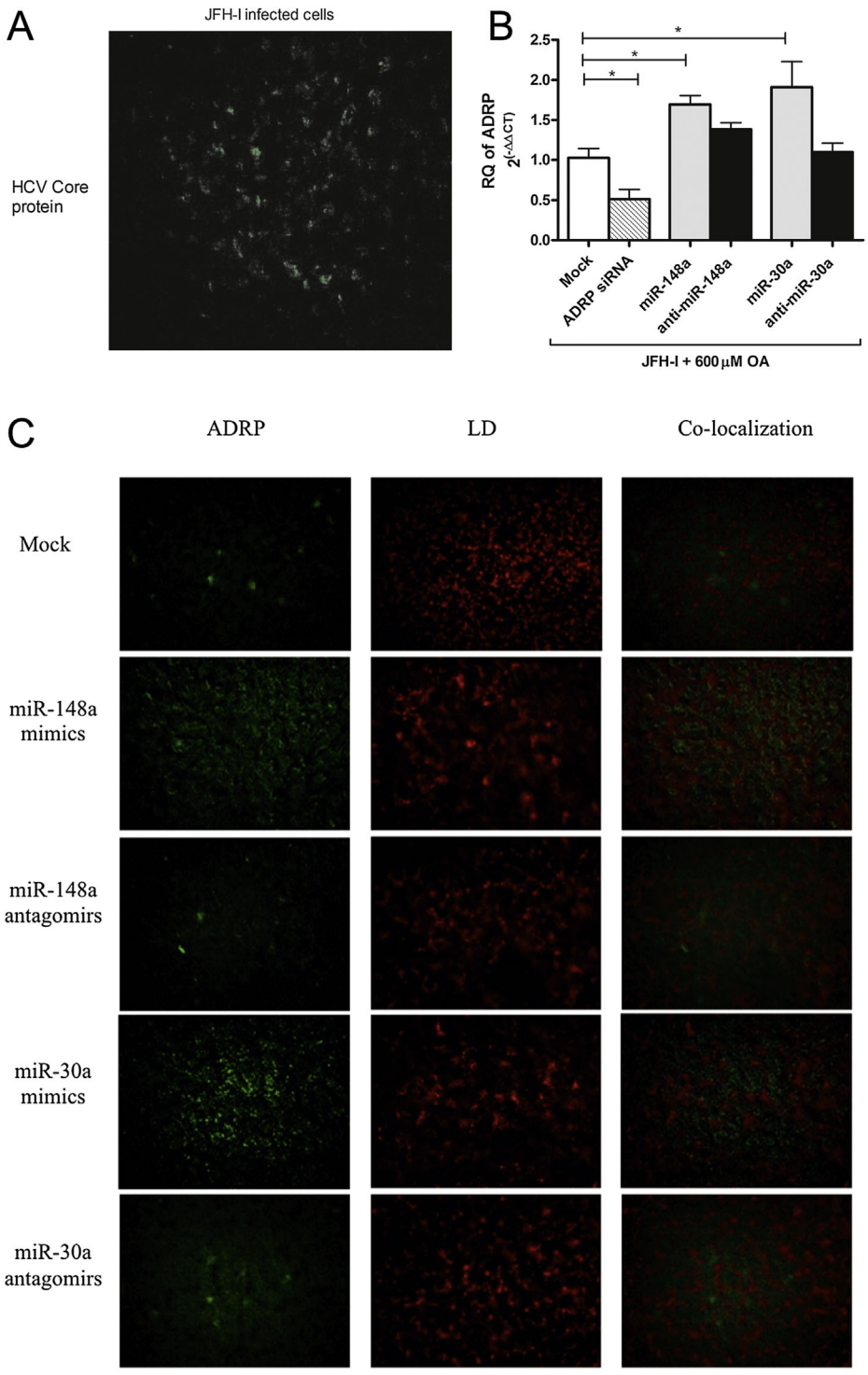

Figure. 3. Impact of manipulating miR-148a and miR-30a on ADRP expression in JFH-I infected Huh7 cells. **A**: JFH1 infected Huh7 cells showing the expression of HCV core protein (green fluorescence) visualized by anti-HCV core antibodies using immunofluorescence microscopy 72 hrs post infection for confirmation of efficient infection and viral replication. **B**: miR-148a and miR-30a mimics induced ADRP expression (1.693 $\pm$ 0.1118, $P = 0.0103^{*}$) and (1.912 $\pm$ 0.3161, $P = 0.02^{*}$), respectively, compared to mock cells (1.026 $\pm$ 0.1213). Both miR-148a and miR-30a antagomirs did not affect ADRP mRNA expression (1.383 $\pm$ 0.08190) and (1.099 $\pm$ 0.1119), respectively. siRNAs against ADRP efficiently knocked down its expression (0.5144 $\pm$ 0.1207, $P = 0.032^{*}$). **C**: miR-148a and miR-30a increased ADRP protein expression (green fluorescence) but decreased LDs (red fluorescence) in Huh7, OA treated cells, compared to mock cells. Co-localization images show the increase of ADRP (green) relative to LDs (red) in miR-148a and miR-30a transfected cells compared to mock cells. miR-148a and miR-30a antagomirs did not affect LDs or ADRP expression.

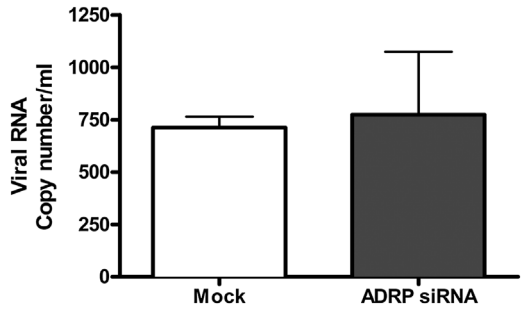

Figure. 4. Impact of ADRP knockdown on intracellular HCV RNA Knockdown of ADRP using specific siRNAs did not affect HCV RNA levels (774.5 ± 172.3) compared to mockcells (711.5 ± 37.50) ($P = 0.7968$). 89×51mm.

TIP47 would alleviate the HCV-mediated suppression of ADRP in HCV infected cell models.

The impact of HCV infection on ADRP mRNA expression was investigated by measuring its relative quantity in liver biopsies of 21 HCV infected patients and nine healthy controls using qRT-PCR. ADRP was found to be down regulated in patients compared to controls (Fig. 1). In order to assess whether this down regulation is a result of HCV infection, Huh7 cells were infected with HCVcc-JFH-I and ADRP expression was compared to uninfected cells. Interestingly, infection of Huh7 cells suppressed ADRP expression as was observed in liver biopsies of HCV infected patients (Fig. 2). These results go in accordance with a couple of studies, where one study reported that JFH-I infected cells show lower ADRP protein expression levels and that ADRP could not be detected on the surface of LDs of infected cells [Boulant et al., 2008]. And the other study showed that ADRP in HepG2 cell lines that constitutively expressed HCV core protein was significantly lower compared to cells not expressing the core protein [Sato et al., 2006]. The impact of miR-30a and miR-148a on the expression of ADRP was then analyzed. JFH-I infected Huh7 cells were either left untransfected or transfected with miR-148a or miR-30a mimics/antagmoirs or ADRP-specific siRNAs. Cells were treated with oleic acid (OA), an inducer of LDs, 24 hr after transfection. Forty-eight hours post OA treatment, mRNA expression of ADRP was examined using qRT-PCR, while its protein expression was investigated 72 hr post OA treatment using immunofluorescence microscopy. Interestingly, PCR analysis showed that both miR-148a and miR-30a mimics induced the mRNA expression of ADRP (Fig. 3A). Immunofluorescence microscopy showed that untransfected Huh7 cells harbored relatively low ADRP protein levels. Upon mimicking with miR-148a and miR-30a ADRP protein expression markedly increased, even though LD levels decreased (Fig. 3B). This finding contradicts the results of Whittaker and colleagues, which stated that miR-148a overexpression decreases the expression of ADRP in Huh7 cells [Whittaker et al., 2010]. However, the Whittaker study was performed on naïve cells and since HCV infection alters ADRP expression, the impact of microRNAs on ADRP might differ between infected and naïve cells. We have previously shown that miR-148a and miR-30a suppress HCV infection [El-Ekiaby et al., 2015]. Since HCV core protein displaces ADRP from LD surface, and since unbound ADRP is unstable and undergoes proteomic degradation, its protein expression was lower in infected compared to uninfected cells [Boulant et al., 2008]. Thus, this could explain the microRNA-mediated induction of ADRP, where the suppression of HCV by both microRNAs possibly minimizes the displacement of ADRP from the surface of LDs and thus protects it from proteomic degradation. This could be further supported by our previous data that showed that knockdown of TIP47 decreases HCV RNA and increases ADRP (data not shown), indicating that the observed induction in ADRP in response to miR-148a and miR-30a is rather an indirect effect.

In order to assess whether the interaction between ADRP and HCV is a two-way interaction, the impact of knocking down ADRP on intracellular HCV RNA was examined. Interestingly, ADRP knockdown had no effect on HCV RNA levels (Fig. 4), suggesting that ADRP is affected by HCV but has itself no impact on the virus.

In conclusion, this study shows that HCV infection decreases the expression of the lipid droplet associated protein, ADRP, in liver biopsies as well as Huh7 cell lines. The HCV mediated suppression of ADRP is alleviated by miR-148a and miR-30a. This alleviation is suggested to be a compensatory response to the suppression of HCV infection by both microRNAs.

MicroRNAs have been in clinical trials for some time now and some were approved for HCV therapy as miR-122 antagomirs. Therefore, that drove our interest to study the role of other miRNAs in HCV. In our hand we showed a couple of microRNAs that not only suppress HCV in vitro but also decrease the lipid droplet formation and restore PAT protein expression. This represents a promising platform for further investigations, which pave the way for a couple of miRNAs with dual therapeutic effect.

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