

FIG 10 miR-27a is regulated by the adipocyte differentiation factor C/EBP α . (A) Induction of miR-27a and C/EBP α expression by oleic acid and HCV replication. Huh-7.5 cells were transfected with H77Sv2 Gluc2A RNA or H77Sv2 Gluc2A (AAG) RNA. At 24 h posttransfection, oleic acid (100 μ M) was added to the culture medium. At 72 h after oleic acid treatment, miR-27a (left) and C/EBP α (right) levels were quantified by RTD-PCR ($n = 6$). (B) Induction of miR-27a and C/EBP α expression by tunicamycin. Huh-7.5 cells were treated with different doses of tunicamycin. At 24 h after tunicamycin treatment, miR-27a (left) and C/EBP α (right) levels were quantified by RTD-PCR ($n = 6$). (C) miR-27a promoter luciferase constructs. pGL3-Pri-miR-23a/27a(-736WT) includes -700 to +36 bp relative to the transcription initiation site of pri-miR-23a~27a~24-2. pGL3-Pri-miR-23a/27a(-436) includes -400 to +36 bp relative to the transcription initiation site of pri-miR-23a~27a~24-2, which lacks the consensus C/EBP α binding site. pGL3-Pri-miR-23a/27a(-736MT) has mutations at the -736WT C/EBP α binding site. (D) miR-27a promoter activity in Huh-7.5 cells following HCV infection and oleic acid (left) or tunicamycin (right) treatment. Reporter constructs lacking the C/EBP α binding site did not respond to any of these conditions ($n = 6$). All experiments were performed in duplicate and repeated three times. Values are means \pm standard errors. *, $P < 0.01$; **, $P < 0.005$.

significantly induced the expression of miR-27a and C/EBP α (Fig. 10A and B). To analyze the induction of miR-27a through C/EBP α , we constructed a Luc reporter construct that included the upstream promoter region (-736) of miR-27a [pGL3-Pri-miR-23a/27a(-736WT)] together with a short promoter construct (-436) lacking the C/EBP α binding site [pGL3-Pri-miR-23a/27a(-436)]. In addition, three nucleotide mutations were introduced into the C/EBP α consensus binding site to construct pGL3-Pri-miR-23a/27a(-736MT) (Fig. 10C). The activity of pGL3-Pri-miR-23a/27a(-736WT), but not that of pGL3-Pri-miR-23a/27a(-736MT) or pGL3-Pri-miR-23a/27a(-436), which both lack a C/EBP α binding site, was induced by HCV replication, lipid overload, and tunicamycin treatment (Fig. 10D). These results indicate that the regulation of miR-27a by HCV replication, lipid overload, and ER stress is mediated through C/EBP α .

Pre-miR-27a enhances IFN signaling through the reduction of lipid storage. Finally, we assessed whether miR-27a influences

IFN signaling. IFN- α treatment stimulated IFN signaling in a dose-dependent manner by increasing p-STAT1 expression in Huh-7.5 cells (Fig. 11A). Oleic acid impaired this induction of p-STAT1, while pre-miR-27a restored the expression of p-STAT1 and anti-miR-27a impaired this induction by oleic acid. These findings were observed in both HCV-replicating and non-HCV-replicating cells (Fig. 11A).

HCV replication deduced from Gluc activity is shown in Fig. 11B. IFN sensitivity could be estimated by the relative fold changes in Gluc activity from the baseline activity (in the absence of IFN). The results demonstrated that oleic acid reduced IFN sensitivity, while pre-miR-27a increased IFN sensitivity under either condition with or without oleic acid (Fig. 11B).

These findings were further studied with clinical samples. The expression of miR-27a was evaluated in liver biopsy specimens obtained from 41 patients who received pegylated IFN (Peg-IFN) and ribavirin (RBV) combination therapy (Fig. 12A). Interestingly, the expression of miR-27a was significantly higher

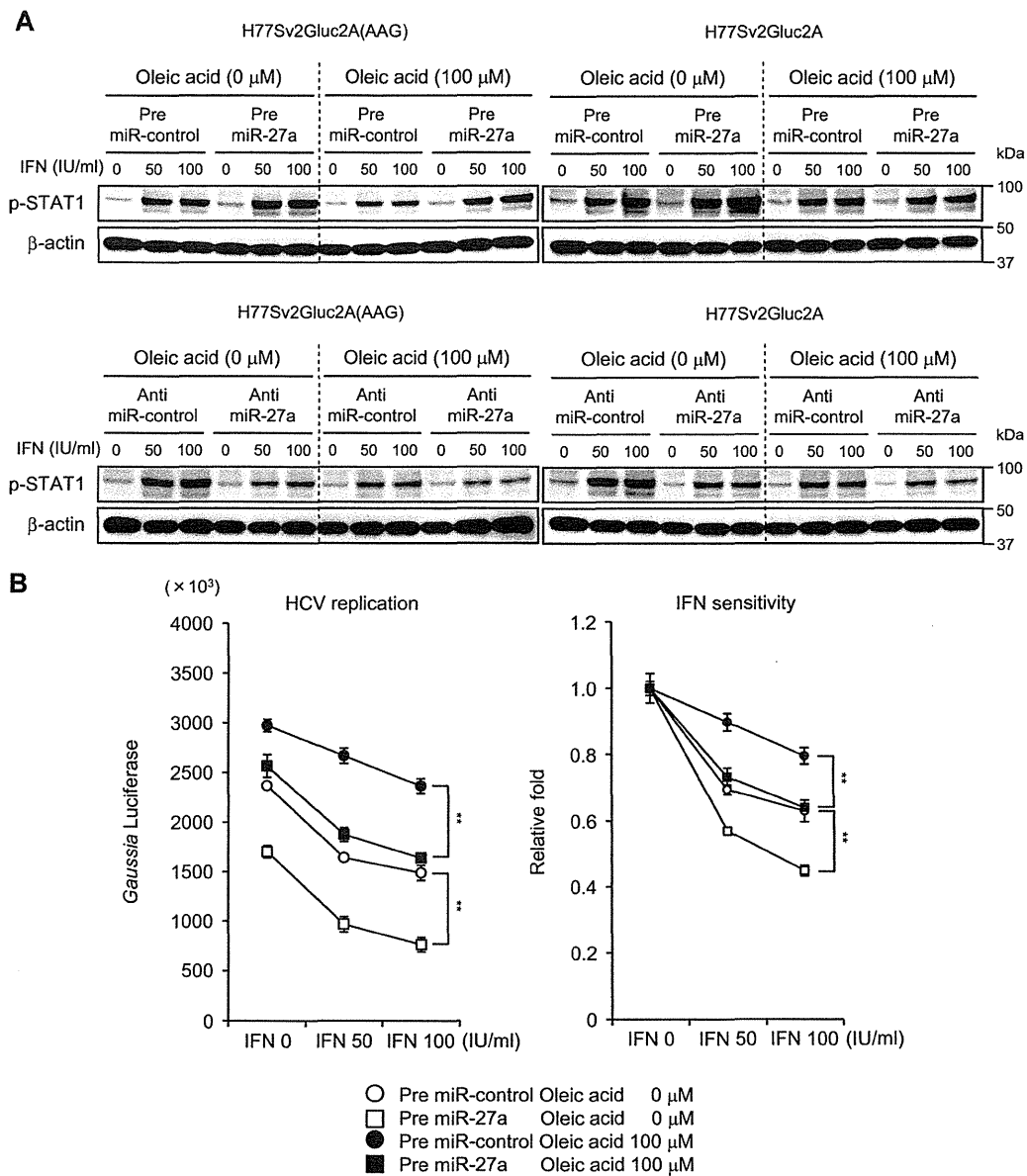


FIG 11 miR-27a restores IFN signaling impaired by lipid overload. (A) Induction of p-STAT1 expression by miR-27a. Huh-7.5 cells were transfected with H77Sv2 Gluc2A RNA or H77Sv2 Gluc2A (AAG) RNA and pre- or anti-miR-control or pre- or anti-miR-27a. At 24 h posttransfection, oleic acid (100 μM) was added to the culture medium. At 48 h after oleic acid treatment, the cells were treated with different doses of IFN-α. At 24 h after IFN treatment, p-STAT1 expression levels were determined by Western blotting. Experiments were repeated three times. (B) Absolute values of Gluc activity (left) and *n*-fold changes in Gluc activity (right) indicate IFN sensitivity (*n* = 6). Experiments were performed in duplicate and repeated three times. Values are means ± standard errors. *, *P* < 0.01; **, *P* < 0.005.

in patients with severe steatosis (grade 3 or 4) than in those with mild steatosis (grade 1 or 2) (Fig. 12B). Importantly, patients with a favorable response to treatment (sustained virological response or transient response) expressed higher miR-27a levels than patients with a poor response (nonresponse) (Fig. 12C). Although there was no significant difference in miR-27a expression according to the interleukin-28B (IL-28B) genotype (Fig. 12D and E), 17 patients had a treatment-resistant IL-28 genotype (TG at rs8099917) (39–41) and 6 of these with a favorable response to treatment expressed significantly higher miR-27a levels than the 11 with a poor response

(Fig. 12E). These data suggest that miR-27a enhances IFN signaling and increases the response to IFN treatment.

DISCUSSION

Previously, we examined miRNA expression in HCC and noncancerous background liver tissue infected with HBV and HCV and showed the presence of infection-specific miRNAs that were differentially expressed according to HBV or HCV infection, but not according to the presence of HCC (2). In this study, we pursued the functional analysis of these miRNAs. Among 19 infection-specific miRNAs, we first focused on 6 that were upregulated by

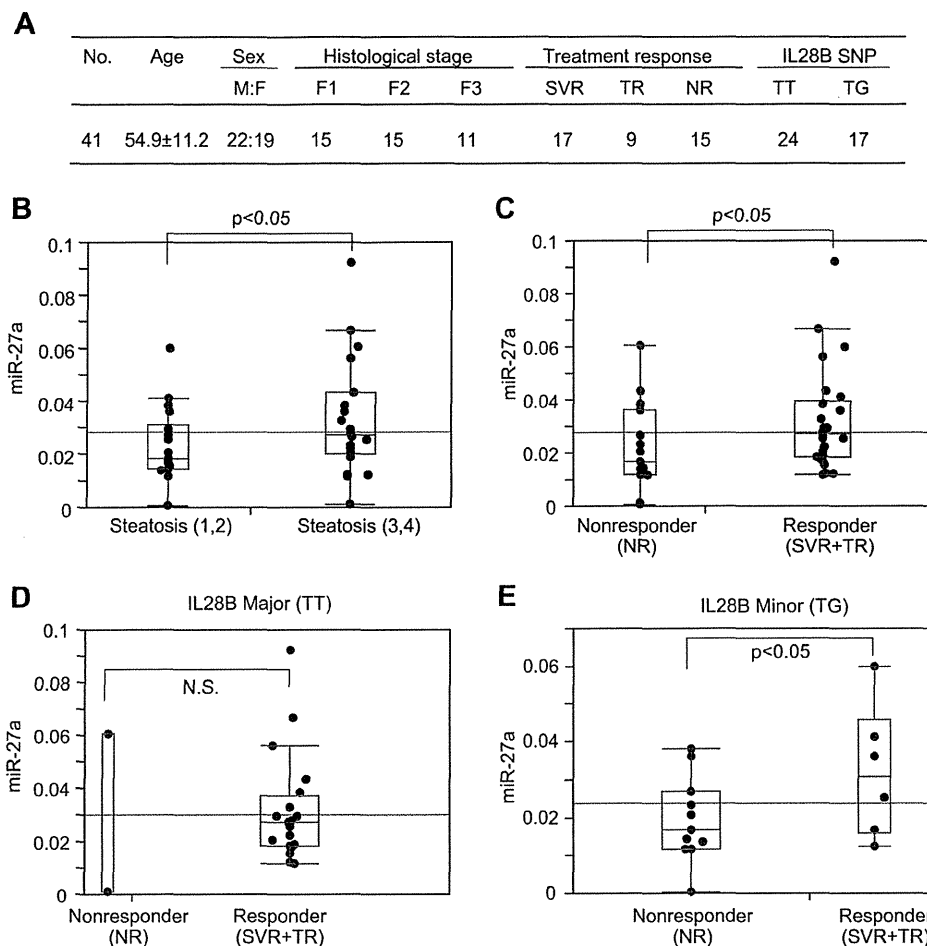


FIG 12 Expression of miR-27a in clinical samples. (A) Clinical characteristics of 41 patients who received Peg-IFN and RBV combination therapy. M:F, male/female ratio; SVR, sustained virological response; TR, transient response; NR, nonresponse; SNP, single nucleotide polymorphism. (B) Significant upregulation of miR-27a expression in the livers of patients with severe steatosis. Steatosis grades 1 and 2, $n = 19$; steatosis grades 3 and 4, $n = 22$. (C) Significant upregulation of miR-27a expression in the livers of patients with a favorable response to treatment (SVR or TR). Nonresponders, $n = 15$; responders, $n = 26$. (D) No significant difference in miR-27a expression between nonresponders and responders of the IL-28B major genotype (treatment-sensitive genotype) was observed. Nonresponders, $n = 3$; responders, $n = 21$. N.S., not significant. (E) Significant upregulation of liver miR-27a was observed in responders of the IL-28B minor genotype (treatment-resistant genotype). Nonresponders, $n = 11$; responders, $n = 6$.

HCV infection, as they were expected to have a positive role in HCV replication. However, inhibition experiments with a series of specific anti-miRNAs showed an unexpected increased in HCV replication. Closer examination clarified that miR-27a had a negative effect on HCV replication. Interestingly, profiling of gene expression in Huh-7.5 cells in which miR-27a was inhibited or overexpressed showed that miR-27a could target lipid metabolism signaling pathways. In support of these findings, the lipid content (TG and TCHO) of Huh-7.5 cells was significantly increased by anti-miR-27a and repressed by pre-miR-27a (Fig. 2 and 3). More importantly, miR-27a was involved in HCV particle formation, as demonstrated by iodixanol gradient centrifugation (Fig. 4). Anti-miR-27a reduced the buoyant density of HCV particles and increased HCV replication and infectivity, while pre-miR-27a decreased HCV replication and dramatically repressed HCV infectivity. In the buoyant-density experiment, the infectious HCV peaks were identical to the RNA peak and the lower infectious virus peak was not observed. We cannot explain this discrep-

ancy from other studies; however, the method used to purify the virus particles could be one reason.

miR-27a regulated many lipid metabolism-related transcription factors, such as RXR α , PPAR α , PPAR γ , FASN, SREBP1, and SREBP2 (Fig. 5 and 6). We also confirmed that miR-27a targets RXR α in human Huh-7.5 cells, which is concordant with a previous study showing that miR-27a targets RXR α in rat hepatic stellate cells (32). Moreover, we newly demonstrated that the gene for the lipid transporter ABCA1 is a target of miR-27a. ABCA1 mediates the efflux of TCHO and phospholipids to the lipid-poor apolipoproteins ApoA1 and ApoE, which then form nascent HDLs (34, 35). It also mediates the transport of lipids between the Golgi apparatus and the cell membrane. Recently, the knockdown of ABCA1 in rat hepatoma cells increased TG secretion to the culture medium and decreased the cellular levels of FFA (29), while liver-specific ABCA1 knockout mice fed a high-fat diet showed increased plasma TG concentrations and decreased TG and TCHO contents in the liver (42). Thus, ABCA1 regulates the lipid content

of hepatocytes, as well as HDL synthesis. In this study, we confirmed that the repression of ABCA1 decreased cellular TG and TCHO levels in Huh-7.5 cells and, importantly, decreased HCV replication and strikingly repressed HCV infection (Fig. 8).

LXR/RXR α was previously shown to activate the ABCA1 promoter (34), but we clearly demonstrated here that miR-27a directly targets ABCA1. Pre-miR-27a repressed the Luc activity of a reporter construct fused with the ABCA1 3' UTR, while anti-miR-27a increased it. We also found that miR-27a regulates the expression of ABCA1 in a 3' UTR sequence-specific manner, as a series of mutations introduced into putative miR-27a binding sites abrogated its regulation (Fig. 7). In addition to these findings, we showed that miR-27a repressed the expression of the apolipoproteins ApoA1, ApoB100, and ApoE3, which were recently shown to play important roles in the production and formation of infectious HCV particles (Fig. 8) (11, 36, 37). Thus, miR-27a may regulate lipid metabolism by reducing lipid synthesis and increasing lipid secretion from cells.

As the expression of miR-27a was upregulated more in CH-C liver than in CH-B liver, it is speculated that miR-27a expression is induced by HCV infection. Indeed, we clearly demonstrated that miR-27a expression was induced by HCV infection, lipid overload, and tunicamycin-induced ER stress (Fig. 9). Furthermore, the adipocyte differentiation-related transcription factor C/EBP α was involved in this regulation. A central role for C/EBP α in the development of adipose tissue has been suggested, as it was found to be sufficient to trigger the differentiation of preadipocytes into mature adipocytes (43). Thus, HCV infection might trigger lipogenesis in hepatocytes by inducing C/EBP α , as shown in this study. Conversely, the induction of C/EBP α expression by miR-27a had a negative effect on lipogenesis and HCV replication. Therefore, miR-27a might play a negative feedback role in HCV infection-induced lipid storage in hepatocytes. Moreover, HCV replication might be hampered by HCV-induced miR-27a, which would partially explain the low HCV titer in CH-C liver.

Besides the anti-HCV effect of miR-27a observed in this study, an antiviral effect against murine cytomegalovirus (MCMV) infection was observed previously (44, 45). MCMV replication was initiated by miR-27a degradation from a viral transcript, while miR-27a had a negative effect on MCMV replication. It was also reported that miR-27a was the target of *Herpesvirus saimiri* U-rich RNAs and was downregulated in transformed T lymphocytes (46). Therefore, the functional relevance of miR-27a in transformed T cells should be explored in a future study. In this study, miR-27a was upregulated by HCV infection, which is in sharp contrast to MCMV and *H. saimiri* infection. Therefore, the differences in antiviral action and host cell interactions also need to be explored further.

Our assessment of miR-27a expression in patients receiving Peg-IFN and RBV combination therapy showed that those with high miR-27a levels had a more favorable treatment response (Fig. 12). Moreover, miR-27a significantly enhanced IFN signaling (Fig. 11), suggesting that it might have therapeutic benefits in combination with IFN therapy, especially in patients with the IFN-resistant IL-28B genotype, who show a more severe steatosis than those with the IFN-sensitive IL-28B genotype (39–41). Further studies should be performed to confirm these findings with more clinical samples.

Although miR-27a has been shown to be upregulated in cancers of the breast, kidney, ovary, and gastric region, its

downregulation has been reported in colorectal cancer, malignant melanoma, oral squamous cell carcinoma, and acute promyelocytic leukemia (47). However, its importance in HCC remains controversial, with one report observing its upregulation compared with the level in normal liver tissue (48), while another showed lower miR-27a expression in HCC than in paired nontumor tissues (49). Moreover, our previous findings on HBV-related and HCV-related HCC showed no miR-27a upregulation compared with the level in the paired background liver (1.14-fold, $P = 0.49$).

In summary, we have revealed the important role of miR-27a in HCV replication for the first time. These findings will be applicable in the improvement of the therapeutic effects of anti-HCV therapy, especially in patients showing treatment resistance and severe hepatic steatosis.

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We have no potential competing interests to declare.

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