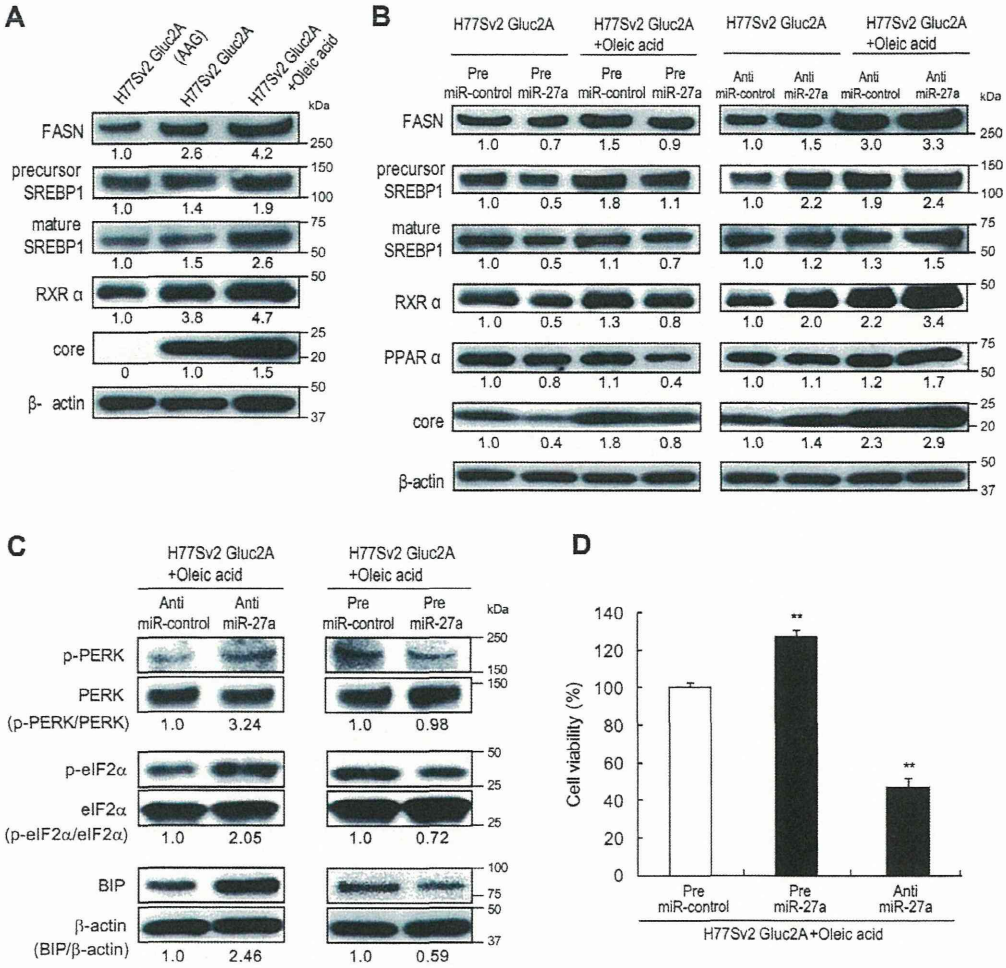

FIG 6 Expression of lipid metabolism-related transcription factors and ER stress-related factors. Huh-7.5 cells were transfected with H77Sv2 Gluc2A RNA or H77Sv2 Gluc2A (AAG) RNA and pre- or anti-miR-27a. At 24 h posttransfection, oleic acid (100 μM) was added to the culture medium. At 72 h after oleic acid treatment, the cells were harvested. (A) Western blotting of lipid metabolism-related transcription factors changed by HCV infection and oleic acid. Experiments were repeated three times. (B) Western blotting of lipid metabolism-related transcription factors changed by pre- or anti-miR-27a. Experiments were repeated three times. (C) Western blotting of ER stress-related transcription factors changed by pre- or anti-miR-27a. Experiments were repeated three times. (D) Cell viability in the same experiments was determined by MTS assay ($n = 9$). Experiments were performed in triplicate and repeated three times. Values are means ± standard errors. *, $P < 0.01$; **, $P < 0.005$.

miR-27a targets RXRα and the ATP-binding cassette transporter ABCA1. We next analyzed the expression of miR-27a target genes. A previous report showed that miR-27a targets RXRα in rat hepatic stellate cells (32), and we confirmed that miR-27a targets the 3′ UTR of human RXRα in Huh-7.5 cells (data not shown). Although the primary sequence of the human RXRα 3′ UTR shares approximately 60% homology with the corresponding rat sequence, the putative miR-27a binding site (ACUGUGAA) is conserved among several different species. Therefore, we constructed an expression vector containing a luciferase (Luc) reporter gene fused to the human RXRα 3′ UTR (pmirGLO-RXRα 3′ UTR) and reevaluated Luc activity (data not shown). Pre-miR-27a repressed Luc activity, while anti-miR-27a significantly increased Luc activity. The introduction of three nucleotide mutations into the conserved miR-27a binding site was shown to abolish these changes in Luc activity. These results confirmed previous findings that miR-27a targets RXRα (32). RXRα interacts with liver X receptor (LXR) and regulates many lipid synthetic genes such as *SREBP1* and *FASN*. We found that the expression of *SREBP1*, *FASN*, and *SREBP2* was regulated by miR-27a (Fig. 6B) and confirmed that *PPARγ* was also regulated by miR-27a, as reported previously (Fig. 5) (33). In addition, *PPARα* was shown to be regulated by miR-27a (Fig. 6B).

We next evaluated the expression of lipid transporter genes. The ATP-binding cassette transporter ABCA1 is mutated in Tangier's disease (34) and plays an important role in the efflux of TCHO for high-density lipoprotein (HDL) synthesis (35). A recent report demonstrated a functional role for ABCA1 in hepatocyte TG secretion to the plasma and in the reduction of cellular TG levels (29). Here we found that pre-miR-27a significantly repressed ABCA1 and, conversely, that anti-miR-27a increased the mRNA and protein levels of ABCA1 (Fig. 7A and B). We identified two miR-27a binding sites (sites 1 and 2) in the 3′ UTR of ABCA1 (Fig. 7C) that were conserved between species (Fig. 7C). An expression vector containing the *luc* reporter gene fused to the human ABCA1 3′ UTR (wild type [WT]) was constructed, and a

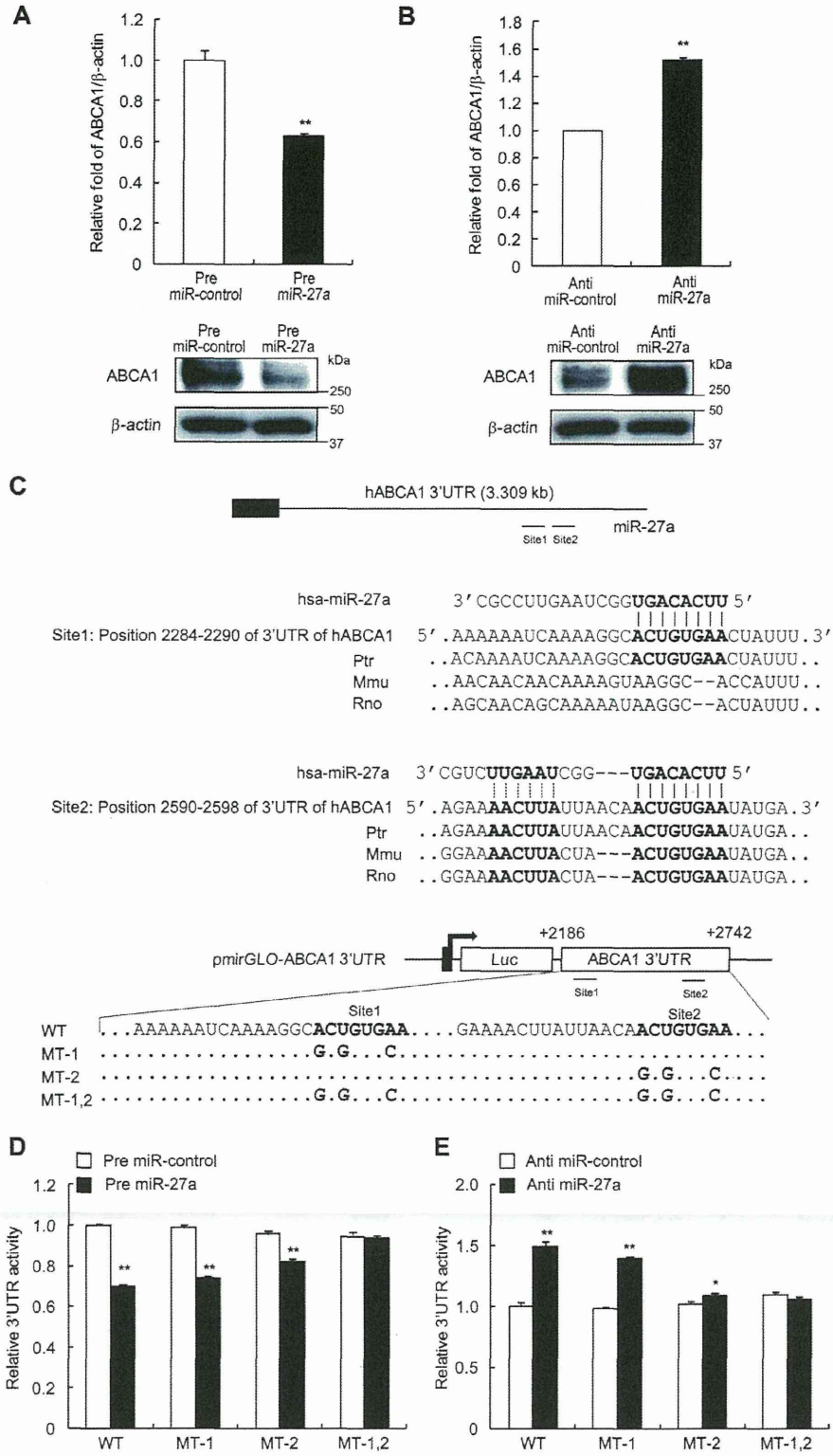

FIG 7 miR-27a targets ABCA1. (A) Regulation of ABCA1 by pre-miR-27a. RTD-PCR and Western blotting of ABCA1 in Huh-7.5 cells at 72 h posttransfection with pre-miR-control and pre-miR-27a ($n = 6$). **, $P < 0.005$. (B) Regulation of ABCA1 by anti-miR-27a. RTD-PCR and Western blotting of ABCA1 in Huh-7.5 cells at 72 h posttransfection with pre-miR-control and anti-miR-27a ($n = 6$). **, $P < 0.005$. (C) Sequence alignment of the ABCA1 3′ UTR and the construction of the luciferase (Luc) expression vector fused to the ABCA1 3′ UTR. Ptr, *Pan troglodytes*; Mmu, *Mus musculus*; Rno, *Ruttus norvegicus*; WT, Luc reporter vector with the WT ABCA1 3′ UTR (2186 to 2742); MT-1, Luc reporter vector with the site 1 mutation of the WT; MT-2, Luc reporter vector with the site 2 mutation of the WT; MT-1,2, Luc reporter vector with mutations at sites 1 and 2 of the WT. (D, E) Suppression (D) or induction (E) of Luc activity with the various mutations of the ABCA1 3′ UTR by pre-miR-27a. Huh-7.5 cells were transfected with pre- or anti-miR-control or pre- or anti-miR-27a, and WT, MT-1, MT-2, and MT-1,2. Luc activities were measured at 24 h posttransfection ($n = 6$). All experiments were performed in duplicate and repeated three times. Values are means ± standard errors. *, $P < 0.01$; **, $P < 0.005$.

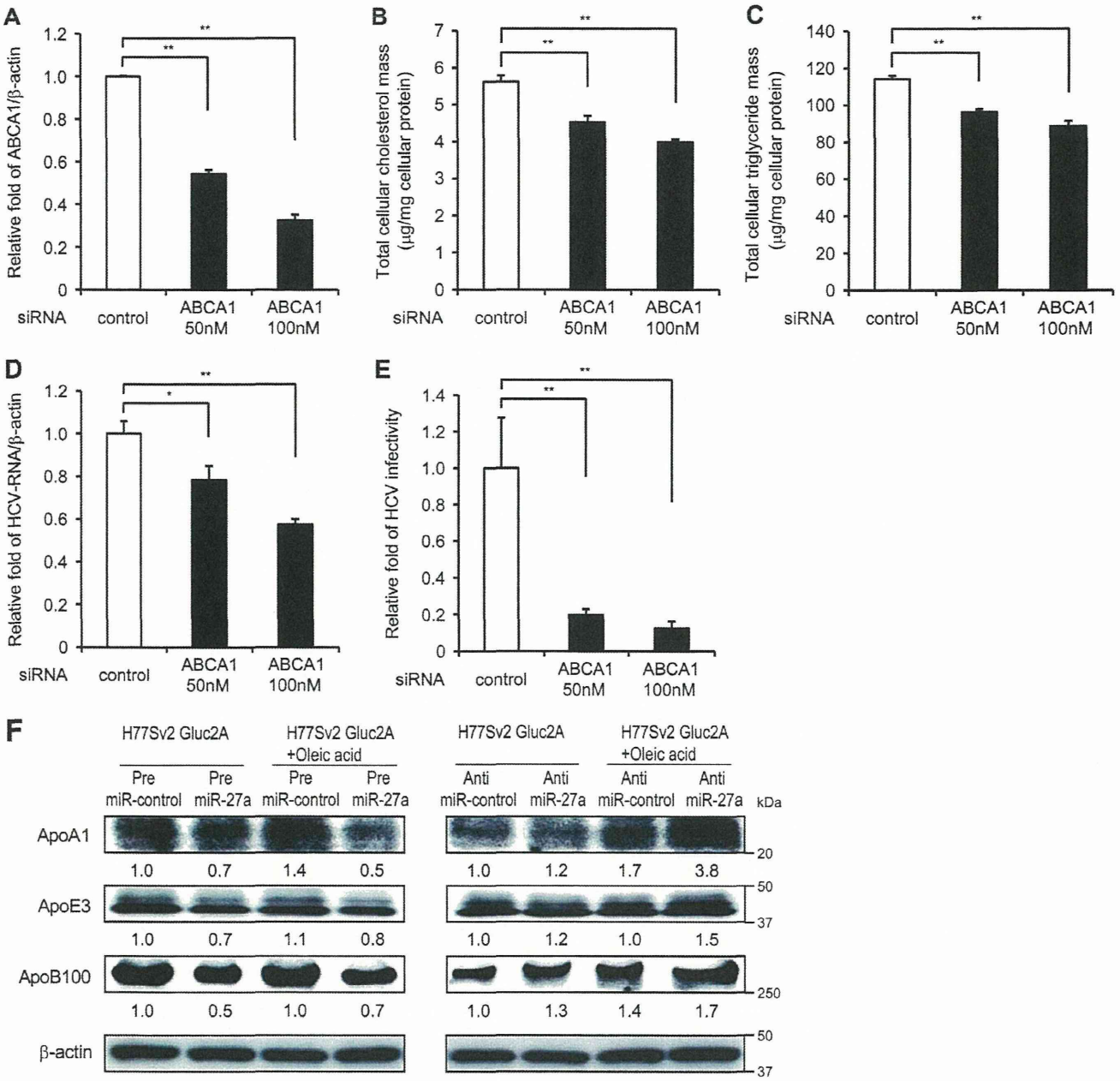

FIG 8 Suppression of ABCA1 inhibits HCV replication and infection. Huh-7.5 cells were transfected with H77Sv2 Gluc2A RNA and siRNA to ABCA1 or control siRNA. ABCA1 expression was quantified at 72 h posttransfection by RTD-PCR ($n = 6$). (A) Knockdown efficiency of ABCA1 in Huh-7.5 cells by siRNA. (B) TG concentration in cells ($n = 6$). (C) TCHO concentrations in cells ($n = 6$). (D) HCV RNA assay by RTD-PCR ($n = 6$). (E) HCV infectivity. Huh-7.5 cells were infected with HCVcc derived from ABCA1 knockdown Huh-7.5 cells. HCV RNA was quantified at 72 h postinfection by RTD-PCR ($n = 6$). Experiments were performed in duplicate and repeated three times. Values are means ± standard errors. *, $P < 0.01$; **, $P < 0.005$. (F) Regulation of ApoA1, ApoE2, and ApoB100 by miR-27a. Experiments were performed under the same conditions as Fig. 6B and C and repeated three times.

series of mutations were introduced into the putative miR-27a binding sites (MT-1, MT-2, and MT-1,2). The Luc activity of the WT was significantly repressed by pre-miR-27a and increased by anti-miR-27a. However, there was a smaller change in Luc activity caused by pre- and anti-miR-27a in the single mutants (MT-1 and MT-2) and no change in Luc activity in the double mutant (MT-1,2) (Fig. 7D and E). These results show that miR-27a targets ABCA1 to decrease the lipid content of cells.

The functional relevance of ABCA1 in lipid metabolism and HCV replication in Huh-7.5 cells was examined by inhibiting ABCA1 with an siRNA (Fig. 8). siRNA to ABCA1 repressed the expression of ABCA1 in a dose-dependent manner (Fig. 8A). Under this condition, the cellular TG and TCHO levels decreased significantly (Fig. 8B and C) and HCV RNA levels also decreased to 57% of the control. More strikingly, HCV infectivity decreased to 12% of the control (Fig. 8D and E).

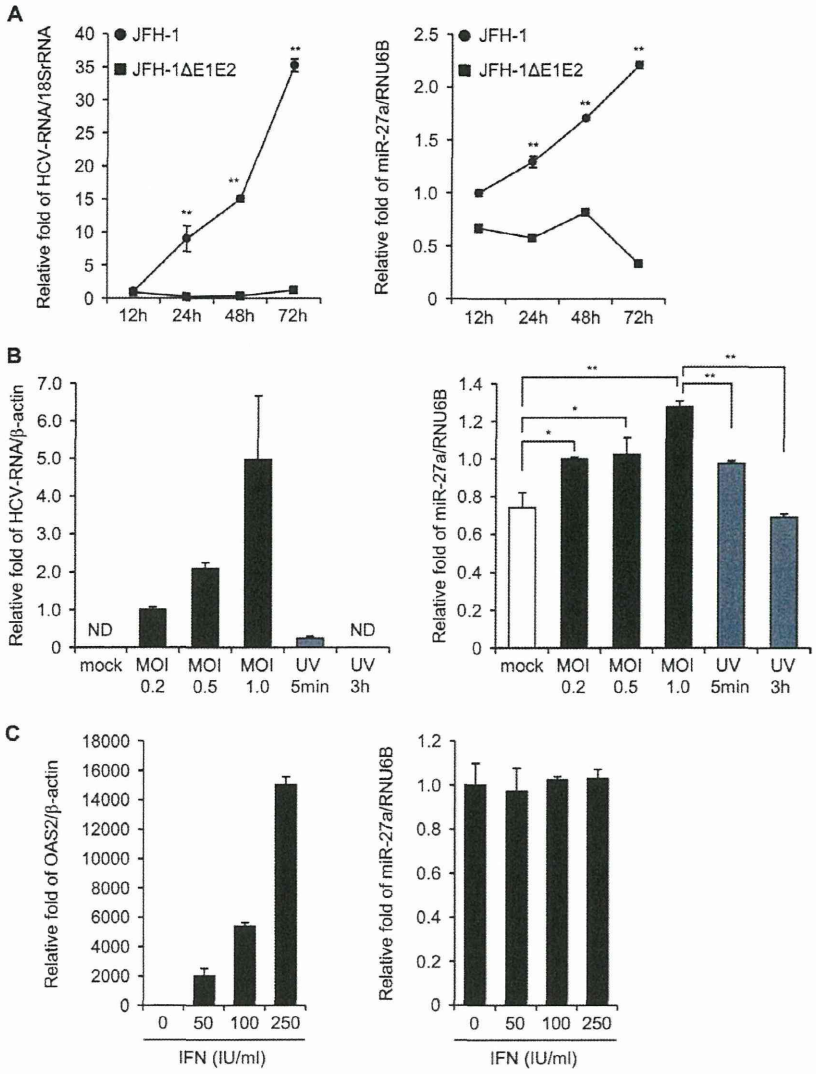

FIG 9 miR-27a is upregulated by HCV infection. (A) Kinetics of HCV replication and induction of miR-27a. Huh-7.5 cells were transfected with JFH-1 RNA or infection-incompetent JFH-1ΔE1E2 RNA (20). At 12, 24, 48, and 72 h posttransfection, HCV RNA (left) and miR-27a (right) levels were quantified by RTD-PCR ($n = 6$). (B) Induction of miR-27a and UV-irradiated HCV particles. Huh-7.5 cells were infected with infectious HCV (multiplicity of infection [MOI] of 0.2, 0.5, or 1) or UV-inactivated HCV. At 72 h postinfection, HCV RNA (left) and miR-27a (right) were quantified by RTD-PCR ($n = 6$). *, $P < 0.01$; **, $P < 0.005$; ND, not detected. (C) Induction of miR-27a and IFN-α treatment. Huh-7.5 cells were treated with different doses of IFN-α. At 24 h posttreatment, OAS2 (left) and miR-27a (right) were quantified by RTD-PCR ($n = 6$). All experiments were performed in duplicate and repeated three times. Values are means ± standard errors.

Several reports have demonstrated the importance of apolipoproteins, including the major components of VLDL and LDL apoE3 (36) and apoB100 (11), in the production of infectious HCV particles. More recently, the functional relevance of ApoA1 in HCV replication and particle production has been reported (37). Here the expression of apoA1, apoB100, and apoE3 was repressed by pre-miR-27a and increased by anti-miR-27a, suggesting that miR-27a regulates the expression of apolipoproteins to reduce the production of infectious HCV particles (Fig. 8F).

Regulation of miR-27a expression through C/EBPα. miR-27a forms a gene cluster with miR-23a and miR-24-2, and both of these miRNAs are regulated by the same promoter (38). However, no detailed analysis of the regulation of this promoter has been carried out. Because the expression of miR-27a was upregulated more in CH-C liver than CH-B liver, it could be speculated that HCV infection induces the expression of miR-27a. To examine this, we evaluated the expression of miR-27a during HCV infection (Fig. 9). The expression of miR-27a increased, correlating with the increase in JFH-1 RNA, while infection-incompetent JFH-1ΔE1E2 did not induce miR-27a expression (Fig. 9A). In addition, UV-irradiated HCV particles did not induce miR-27a expression (Fig. 9B). However, IFN-α treatment did not induce the expression of miR-27a (Fig. 9C). Thus, HCV infection was essential for induction of miR-27a expression.

We identified a C/EBPα binding site (−614 to −606), a key regulator of adipocyte differentiation, in the promoter region of miR-27a. Interestingly, H77Sv2 Gluc2A and tunicamycin

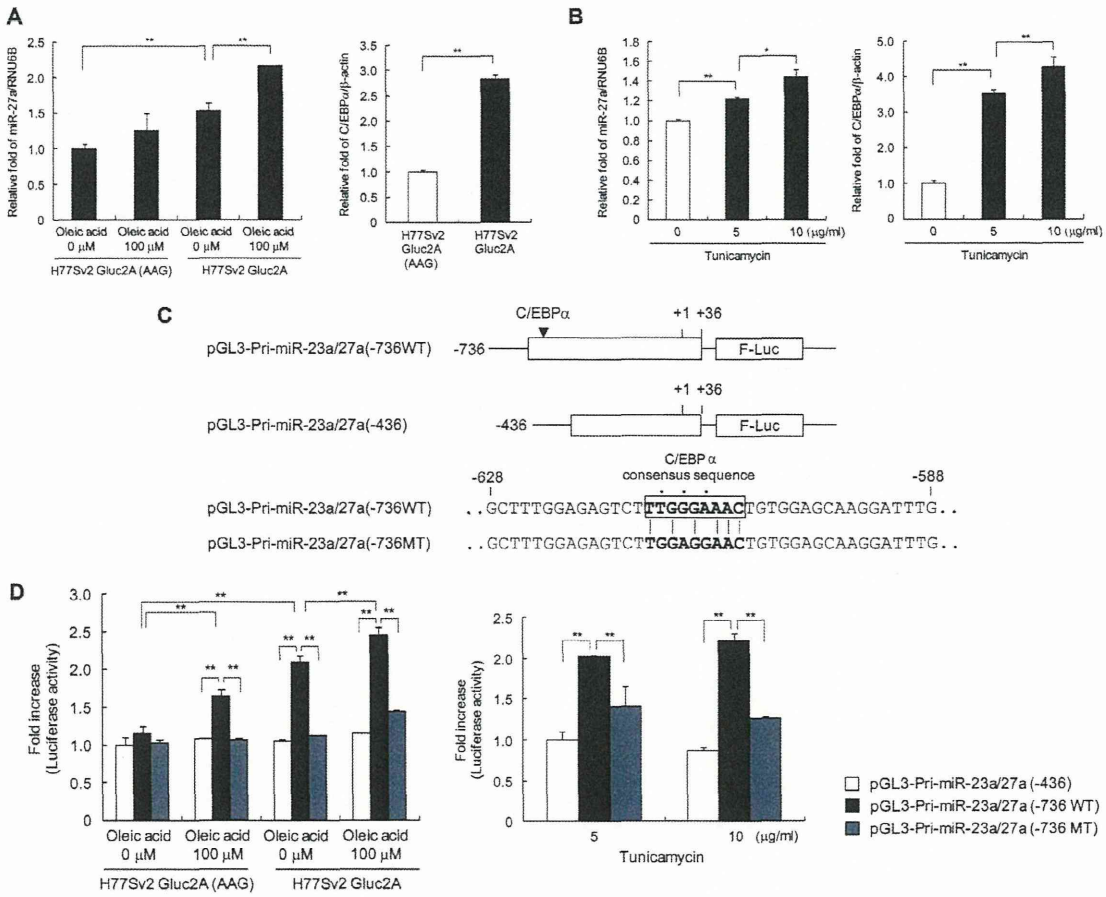

FIG 10 miR-27a is regulated by the adipocyte differentiation factor C/EBPα. (A) Induction of miR-27a and C/EBPα expression by oleic acid and HCV replication. Huh-7.5 cells were transfected with H77Sv2 Gluc2A RNA or H77Sv2 Gluc2A (AAG) RNA. At 24 h posttransfection, oleic acid (100 μM) was added to the culture medium. At 72 h after oleic acid treatment, miR-27a (left) and C/EBPα (right) levels were quantified by RTD-PCR ($n = 6$). (B) Induction of miR-27a and C/EBPα 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/EBPα (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/EBPα binding site. pGL3-Pri-miR-23a/27a(−736MT) has mutations at the −736WT C/EBPα 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/EBPα 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 ± standard errors. *, $P < 0.01$; **, $P < 0.005$.

significantly induced the expression of miR-27a and C/EBPα (Fig. 10A and B). To analyze the induction of miR-27a through C/EBPα, 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/EBPα binding site [pGL3-Pri-miR-23a/27a(−436)]. In addition, three nucleotide mutations were introduced into the C/EBPα 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/EBPα 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/EBPα.

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

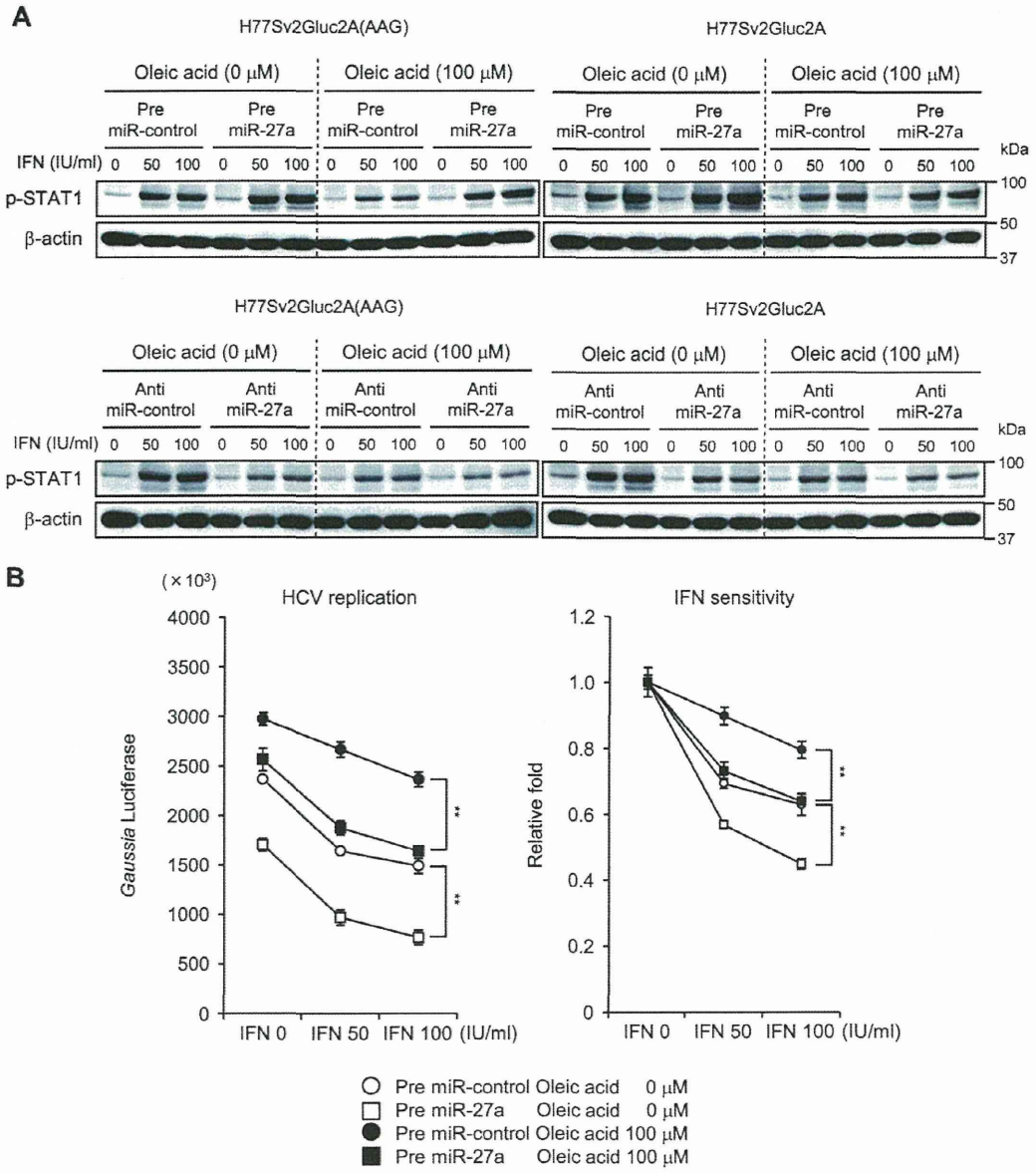

FIG 11 miR-27a restores IFN signaling impaired by lipid overload. (A) Induction of p-STAT1 expression by miR-27a. Huh-7.5 cells were transfected with H77Sv2 Gluc2A RNA or H77Sv2 Gluc2A (AAG) RNA and pre- or anti-miR-control or pre- or anti-miR-27a. At 24 h posttransfection, oleic acid (100 μM) was added 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

A

No.	Age	Sex	Histological stage			Treatment response			IL28B SNP	
		M:F	F1	F2	F3	SVR	TR	NR	TT	TG
41	54.9±11.2	22:19	15	15	11	17	9	15	24	17

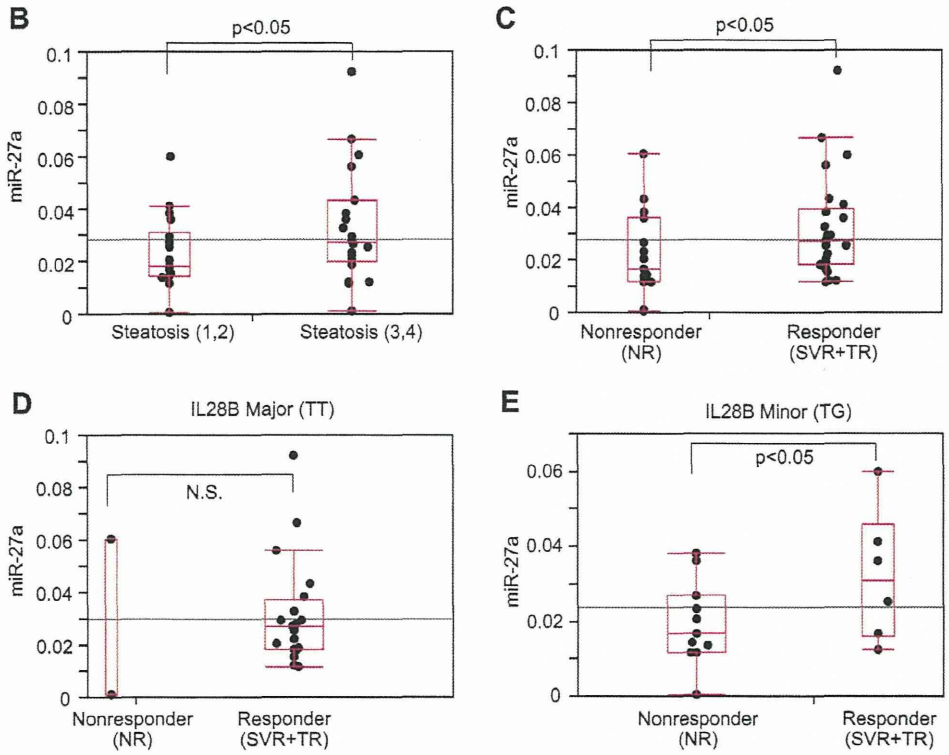

FIG 12 Expression of miR-27a in clinical samples. (A) Clinical characteristics of 41 patients who received Peg-IFN and RBV combination therapy. M:F, male/female ratio; SVR, sustained virological response; TR, transient response; NR, nonresponse; SNP, single nucleotide polymorphism. (B) Significant upregulation 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 discrepancy 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 RXRα, PPARα, PPARγ, FASN, SREBP1, and SREBP2 (Fig. 5 and 6). We also confirmed that miR-27a targets RXRα in human Huh-7.5 cells, which is concordant with a previous study showing that miR-27a targets RXRα 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/RXRα 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/EBPα was involved in this regulation. A central role for C/EBPα 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/EBPα, as shown in this study. Conversely, the induction of C/EBPα 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 antivirus 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.

ACKNOWLEDGMENTS

We thank Mina Nishiyama and Masayo Baba for their excellent technical assistance.

We have no potential competing interests to declare.

REFERENCES

1. Esteller M. 2011. Non-coding RNAs in human disease. Nat. Rev. Genet. 12:861–874.
2. Ura S, Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, Sunakozaka H, Sakai Y, Horimoto K, Kaneko S. 2009. Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma. Hepatology 49:1098–1112.
3. André P, Komurian-Pradel F, Deforges S, Perret M, Berland JL, Sodoyer M, Pol S, Brechot C, Paranhos-Baccala G, Lotteau V. 2002. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J. Virol. 76:6919–6928.
4. Thomssen R, Bonk S, Propfe C, Heermann KH, Kochel HG, Uy A. 1992. Association of hepatitis C virus in human sera with beta-lipoprotein. Med. Microbiol. Immunol. 181:293–300.
5. Thomssen R, Bonk S, Thiele A. 1993. Density heterogeneities of hepatitis C virus in human sera due to the binding of beta-lipoproteins and immunoglobulins. Med. Microbiol. Immunol. 182:329–334.
6. Agnello V, Abel G, Elfahal M, Knight GB, Zhang QX. 1999. Hepatitis C virus and other Flaviviridae viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. U. S. A. 96:12766–12771.
7. Germi R, Crance JM, Garin D, Guimet J, Lortat-Jacob H, Ruigrok RW, Zarski JP, Drouet E. 2002. Cellular glycosaminoglycans and low density lipoprotein receptor are involved in hepatitis C virus adsorption. J. Med. Virol. 68:206–215.
8. Triyatni M, Saunier B, Maruvada P, Davis AR, Ulianich L, Heller T, Patel A, Kohn LD, Liang TJ. 2002. Interaction of hepatitis C virus-like particles and cells: a model system for studying viral binding and entry. J. Virol. 76:9335–9344.
9. Shi ST, Lee KJ, Aizaki H, Hwang SB, Lai MM. 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J. Virol. 77:4160–4168.
10. Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K. 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. 9:1089–1097.
11. Huang H, Sun F, Owen DM, Li W, Chen Y, Gale M, Jr, Ye J. 2007. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. Proc. Natl. Acad. Sci. U. S. A. 104:5848–5853.
12. Bressler BL, Guindi M, Tomlinson G, Heathcote J. 2003. High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. Hepatology 38:639–644.
13. Poynard T, Ratziu V, McHutchison J, Manns M, Goodman Z, Zeuzem S, Younossi Z, Albrecht J. 2003. Effect of treatment with peginterferon or interferon alfa-2b and ribavirin on steatosis in patients infected with hepatitis C. Hepatology 38:75–85.

14. Jopling CL, Yi M, Lancaster AM, Lemon SM, Sarnow P. 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. Science 309:1577–1581.
15. Murakami Y, Aly HH, Tajima A, Inoue I, Shimotohno K. 2009. Regulation of the hepatitis C virus genome replication by miR-199a. J. Hepatol. 50:453–460.
16. Hou W, Tian Q, Zheng J, Bonkovsky HL. 2010. MicroRNA-196 represses Bach1 protein and hepatitis C virus gene expression in human hepatoma cells expressing hepatitis C viral proteins. Hepatology 51:1494–1504.
17. Bandyopadhyay S, Friedman RC, Marquez RT, Keck K, Kong B, Icardi MS, Brown KE, Burge CB, Schmidt WN, Wang Y, McCaffrey AP. 2011. Hepatitis C virus infection and hepatic stellate cell activation downregulate miR-29: miR-29 overexpression reduces hepatitis C viral abundance in culture. J. Infect. Dis. 203:1753–1762.
18. Cheng JC, Yeh YJ, Tseng CP, Hsu SD, Chang YL, Sakamoto N, Huang HD. 2012. Let-7b is a novel regulator of hepatitis C virus replication. Cell. Mol. Life Sci. 69:2621–2633.
19. Bhanja Chowdhury J, Shrivastava S, Steele R, Di Bisceglie AM, Ray R, Ray RB. 2012. Hepatitis C virus infection modulates expression of interferon stimulatory gene IFITM1 by upregulating miR-130A. J. Virol. 86: 10221–10225.
20. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11:791–796.
21. Shetty S, Kim S, Shimakami T, Lemon SM, Mihailescu MR. 2010. Hepatitis C virus genomic RNA dimerization is mediated via a kissing complex intermediate. RNA. 16:913–925.
22. Che ML, Yan YC, Zhang Y, Gu Y, Wang NS, Chen N, Mao PJ, Zhang JY, Ding XQ, Yuan WJ, Mei CL, Yao J, Fan YL, Zhou Y, Zhang W, Zhu HW, Liu M, Jin HM, Qian JQ. 2009. Analysis of drug-induced acute renal failure in Shanghai. Zhonghua Yi Xue Za Zhi 89:744–749. (In Chinese.)
23. Shimakami T, Welsch C, Yamane D, McGivern DR, Yi M, Zeuzem S, Lemon SM. 2011. Protease inhibitor-resistant hepatitis C virus mutants with reduced fitness from impaired production of infectious virus. Gastroenterology 140:667–675.
24. Yi M, Villanueva RA, Thomas DL, Wakita T, Lemon SM. 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. U. S. A. 103:2310–2315.
25. Honda M, Takehana K, Sakai A, Tagata Y, Shirasaki T, Nishitani S, Muramatsu T, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, Nakamura M, Shimakami T, Yi M, Lemon SM, Suzuki T, Wakita T, Kaneko S. 2011. Malnutrition impairs interferon signaling through mTOR and FoxO pathways in patients with chronic hepatitis C. Gastroenterology 141:128–140.
26. Malhi H, Barreyro FJ, Isomoto H, Bronk SF, Gores GJ. 2007. Free fatty acids sensitise hepatocytes to TRAIL mediated cytotoxicity. Gut 56:1124–1131.
27. Honda M, Nakamura M, Tateno M, Sakai A, Shimakami T, Shirasaki T, Yamashita T, Arai K, Sakai Y, Kaneko S. 2010. Differential interferon signaling in liver lobule and portal area cells under treatment for chronic hepatitis C. J. Hepatol. 53:817–826.
28. Shirasaki T, Honda M, Mizuno H, Shimakami T, Okada H, Sakai Y, Murakami S, Wakita T, Kaneko S. 2010. La protein required for internal ribosome entry site-directed translation is a potential therapeutic target for hepatitis C virus replication. J. Infect. Dis. 202:75–85.
29. Chung S, Gebre AK, Seo J, Shelness GS, Parks JS. 2010. A novel role for ABCA1-generated large pre-beta migrating nascent HDL in the regulation of hepatic VLDL triglyceride secretion. J. Lipid Res. 51:729–742.
30. Lewis BP, Burge CB, Bartel DP. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120:15–20.
31. Lee JS, Mendez R, Heng HH, Yang ZQ, Zhang K. 2012. Pharmacological ER stress promotes hepatic lipogenesis and lipid droplet formation. Am. J. Transl. Res. 4:102–113.
32. Ji J, Zhang J, Huang G, Qian J, Wang X, Mei S. 2009. Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation during rat hepatic stellate cell activation. FEBS Lett. 583:759–766.
33. Kim SY, Kim AY, Lee HW, Son YH, Lee GY, Lee JW, Lee YS, Kim JB. 2010. miR-27a is a negative regulator of adipocyte differentiation via suppressing PPARgamma expression. Biochem. Biophys. Res. Commun. 392: 323–328.
34. Schmitz G, Langmann T. 2005. Transcriptional regulatory networks in lipid metabolism control ABCA1 expression. Biochim. Biophys. Acta 1735:1–19.
35. Liu M, Chung S, Shelness GS, Parks JS. 2012. Hepatic ABCA1 and VLDL triglyceride production. Biochim. Biophys. Acta 1821:770–777.
36. Hishiki T, Shimizu Y, Tobita R, Sugiyama K, Ogawa K, Funami K, Ohsaki Y, Fujimoto T, Takaku H, Wakita T, Baumert TF, Miyanari Y, Shimotohno K. 2010. Infectivity of hepatitis C virus is influenced by association with apolipoprotein E isoforms. J. Virol. 84:12048–12057.
37. Mancone C, Steindler C, Santangelo L, Simonte G, Vlassi C, Longo MA, D'Offizi G, Di Giacomo C, Pucillo LP, Amicone L, Tripodi M, Alonzi T. 2011. Hepatitis C virus production requires apolipoprotein A-I and affects its association with nascent low-density lipoproteins. Gut 60:378–386.
38. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN. 2004. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 23: 4051–4060.
39. O'Brien TR. 2009. Interferon-alfa, interferon-lambda and hepatitis C. Nat. Genet. 41:1048–1050.
40. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J. 2009. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat. Genet. 41:1100–1104.
41. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat. Genet. 41:1105–1109.
42. Chung S, Timmins JM, Duong M, Degirolamo C, Rong S, Sawyer JK, Singaraja RR, Hayden MR, Maeda N, Rudel LL, Shelness GS, Parks JS. 2010. Targeted deletion of hepatocyte ABCA1 leads to very low density lipoprotein triglyceride overproduction and low density lipoprotein hypercatabolism. J. Biol. Chem. 285:12197–12209.
43. Porse BT, Pedersen TA, Xu X, Lindberg B, Wewer UM, Friis-Hansen L, Nerlov C. 2001. E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis in vivo. Cell 107:247–258.
44. Libri V, Helwak A, Miesen P, Santhakumar D, Borger JG, Kudla G, Grey F, Tollervey D, Buck AH. 2012. Murine cytomegalovirus encodes a miR-27 inhibitor disguised as a target. Proc. Natl. Acad. Sci. U. S. A. 109:279–284.
45. Marcinowski L, Tanguy M, Krmpotic A, Radle B, Lisnic VJ, Tuddenham L, Chane-Woon-Ming B, Ruzsics Z, Erhard F, Benkartek C, Babic M, Zimmer R, Trgovcich J, Koszinowski UH, Jonjic S, Pfeffer S, Dolken L. 2012. Degradation of cellular mir-27 by a novel, highly abundant viral transcript is important for efficient virus replication in vivo. PLoS Pathog. 8:e1002510. doi:10.1371/journal.ppat.1002510.
46. Cazalla D, Yario T, Steitz JA. 2010. Down-regulation of a host microRNA by a Herpesvirus saimiri noncoding RNA. Science 328:1563–1566.
47. Chhabra R, Dubey R, Saini N. 2010. Cooperative and individualistic functions of the microRNAs in the miR-23a~27a~24-2 cluster and its implication in human diseases. Mol. Cancer 9:232.
48. Huang S, He X, Ding J, Liang L, Zhao Y, Zhang Z, Yao X, Pan Z, Zhang P, Li J, Wan D, Gu J. 2008. Upregulation of miR-23a approximately 27a approximately 24 decreases transforming growth factor-beta-induced tumor-suppressive activities in human hepatocellular carcinoma cells. Int. J. Cancer 123:972–978.
49. Wang W, Zhao LJ, Tan YX, Ren H, Qi ZT. 2012. MiR-138 induces cell cycle arrest by targeting cyclin D3 in hepatocellular carcinoma. Carcinogenesis 33:1113–1120.

HEPATOLOGY
Official Journal of the American Association for the Study of Liver Diseases

Discrete Nature of EpCAM$^+$ and CD90$^+$ Cancer Stem Cells in Human Hepatocellular Carcinoma

Taro Yamashita,[1] Masao Honda,[1] Yasunari Nakamoto,[1] Masayo Baba,[1] Kouki Nio,[1] Yasumasa Hara,[1] Sha Sha Zeng,[1] Takehiro Hayashi,[1] Mitsumasa Kondo,[1] Hajime Takatori,[1] Tatsuya Yamashita,[1] Eishiro Mizukoshi,[1] Hiroko Ikeda,[1] Yoh Zen,[1] Hiroyuki Takamura,[1] Xin Wei Wang,[2] and Shuichi Kaneko[1]

Recent evidence suggests that hepatocellular carcinoma (HCC) is organized by a subset of cells with stem cell features (cancer stem cells; CSCs). CSCs are considered a pivotal target for the eradication of cancer, and liver CSCs have been identified by the use of various stem cell markers. However, little information is known about the expression patterns and characteristics of marker-positive CSCs, hampering the development of personalized CSC-targeted therapy. Here, we show that CSC markers EpCAM and CD90 are independently expressed in liver cancer. In primary HCC, EpCAM$^+$ and CD90$^+$ cells resided distinctively, and gene-expression analysis of sorted cells suggested that EpCAM$^+$ cells had features of epithelial cells, whereas CD90$^+$ cells had those of vascular endothelial cells. Clinicopathological analysis indicated that the presence of EpCAM$^+$ cells was associated with poorly differentiated morphology and high serum alpha-fetoprotein (AFP), whereas the presence of CD90$^+$ cells was associated with a high incidence of distant organ metastasis. Serial xenotransplantation of EpCAM$^+$/CD90$^+$ cells from primary HCCs in immunedeficient mice revealed rapid growth of EpCAM$^+$ cells in the subcutaneous lesion and a highly metastatic capacity of CD90$^+$ cells in the lung. In cell lines, CD90$^+$ cells showed abundant expression of c-Kit and *in vitro* chemosensitivity to imatinib mesylate. Furthermore, CD90$^+$ cells enhanced the motility of EpCAM$^+$ cells when cocultured *in vitro* through the activation of transforming growth factor beta (TGF-β) signaling, whereas imatinib mesylate suppressed *TGFB1* expression in CD90$^+$ cells as well as CD90$^+$ cell-induced motility of EpCAM$^+$ cells. *Conclusion*: Our data suggest the discrete nature and potential interaction of EpCAM$^+$ and CD90$^+$ CSCs with specific gene-expression patterns and chemosensitivity to molecular targeted therapy. The presence of distinct CSCs may determine the clinical outcome of HCC. (HEPATOLOGY 2013;57:1484-1497)

The cancer stem cell (CSC) hypothesis, which suggests that a subset of cells bearing stem-cell–like features is indispensable for tumor development, has recently been put forward subsequent to advances in molecular and stem cell biology. Liver cancer, including hepatocellular carcinoma (HCC), is a leading cause of cancer death worldwide.[1] Recent studies have shown the existence of CSCs in liver cancer cell lines and primary HCC specimens using various stem cell markers.[2-7] Independently, we have identified novel HCC subtypes defined by the hepatic stem/progenitor cell markers,

Abbreviations: 5-FU, fluorouracil; Abs, antibodies; AFP, alpha-fetoprotein; CK-19, cytokeratin-19; CSC, cancer stem cell; DNs, dysplastic nodules; EMT, epithelial mesenchymal transition; EpCAM: epithelial cell adhesion molecule; FACS, fluorescent-activated cell sorting; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HSCs, hepatic stem cells; IF, immunofluorescence; IHC, immunohistochemistry; IR, immunoreactivity; MDS, multidimensional scaling; NBNC, non-B, non-C hepatitis; NOD/SCID, nonobese diabetic, severe combined immunodeficient; NT, nontumor; OV-1, ovalbumin 1; qPCR, quantitative real-time polymerase chain reaction; SC, subcutaneous; Smad3, Mothers against decapentaplegic homolog 3; TECs, tumor epithelial cells; TGF-β, transforming growth factor beta; T/N, tumor/nontumor; VECs, vascular endothelial cells; VM, vasculogenic mimicry; VEGFR, vascular endothelial growth factor receptor.

From the [1]Liver Center, Kanazawa University Hospital, Kanazawa, Ishikawa, Japan; and [2]Laboratory of Human Carcinogenesis, Center for Cancer Research, National Cancer Institute, Bethesda, MD.

Received July 9, 2012; revised October 22, 2012; accepted November 6, 2012.

This study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (23590967), a grant from the Japanese Society of Gastroenterology, a grant from the Ministry of Health, Labor, and Welfare, and a grant from the National Cancer Center Research and Development Fund (23-B-5) of Japan. X.W.W. is supported by the Intramural Research Program of the Center for Cancer Research, U.S. National Cancer Institute.