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Alcohol Facilitates HCV RNA Replication Via Up-Regulation of miR-122 Expression and Inhibition of Cyclin G1 in Human Hepatoma Cells

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Abstract

Background—Clinical studies demonstrate synergistic liver damage by alcohol and hepatitis C virus (HCV); however, the mechanisms by which alcohol promotes HCV infection remain obscure. The liver-specific microRNA-122 (miR-122) regulates HCV replication and expression of host genes, including Cyclin G1. Here, we hypothesized that alcohol regulates miR-122 expression and thereby modulates HCV RNA replication.

Methods—The J6/JFH/Huh-7.5 model of HCV infection was used in this study. Real-time quantitative polymerase chain reaction, Western blotting, electrophoretic mobility shift assay, and confocal microscopy were used for experimental analysis.

Results—We found that acute alcohol exposure (25 mM) significantly increased intracellular HCV RNA as well as miR-122 levels in Huh-7.5 and Huh-7.5/CYP2E1 expressing cells in the presence and absence of J6/JFH-HCV infection. Expression of the miR-122 target, Cyclin G1, was inhibited by alcohol both in J6/JFH-infected and uninfected Huh-7.5 cells. The use of a miR-122 inhibitor increased Cyclin G1 expression and prevented the alcohol-induced increase in HCV RNA and protein levels, suggesting a mechanistic role for alcohol-induced miR122 in HCV replication. We discovered that siRNA-mediated silencing of Cyclin G1 significantly increased intracellular HCV RNA levels compared with controls, suggesting a mechanistic role for Cyclin G1 in HCV replication. Alcohol-induced increase in miR-122 was associated with increased nuclear translocation and DNA binding of the nuclear regulatory factor-κB and could be prevented by NF-κB inhibition.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Huh-7.5 cells were transfected with Cyclin G1 shRNA or the control shRNA.

Fig. S2. (A) Schematic representation of the plasmid pRFP and pCCNG1-RFP as well as the fusion protein Cyclin G1-RFP. (B) RFP and Cyclin G1-RFP protein expression in Huh-7.5 cells were checked by confocal microscopy 24 hours after plasmid transfection.

Fig. S3. (A–D) C34 (Cyp2E1 deficient) and E47 (CYP2E1 expressing) cells were treated with alcohol for 24 hours or not followed by the analysis of miR-122 and Cyclin G1 expression levels by real time quantitative PCR.

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Conclusions—Our novel data indicate a miR-122-mediated mechanism for alcohol increasing HCV RNA replication. We show for the first time that Cyclin G1, a miR-122 target gene, has regulatory effects on HCV replication and that alcohol increases HCV replication by regulating miR-122 and Cyclin G1.

Keywords

Hepatitis C Virus; MicroRNA; Ethanol; NF-κB; CyP2E1; NS3

HEPATITIS C VIRUS (HCV) infection affects over 170 million people worldwide and most of these individuals consume alcohol occasionally or chronically. Alcohol abuse is a major independent risk factor that exacerbates the progression of liver disease in chronic hepatitis C infection: however, the mechanisms by which alcohol facilitates HCV-related liver damage are still poorly understood (McCartney et al., 2008; Seronello et al., 2010; Szabo et al., 2006, 2010; Zhang et al., 2003). Both in vivo and in vitro studies have demonstrated that alcohol exposure promotes HCV viral replication (Romero-Gomez et al., 2001; Seronello et al., 2010; Zhang et al., 2003). In addition to enhancing HCV replication, alcohol also inhibits antiviral immunity by suppressing type I interferon production (McCartney et al., 2008; Szabo et al., 2006).

HCV, a positive-sense RNA virus of the *Flaviviridae* family, like most viruses, can hijack host factors to facilitate its replication. Of those, microRNA-122 (miR-122), a miRNA representing 70% of all miRNAs in hepatocytes, was recently identified as having a critical role in the HCV life cycle (Jopling et al., 2005) and has been portrayed as a promising target for antiviral drug development (Lanford et al., 2010). It remains unknown if the activity of miR-122 in HCV RNA translation or RNA accumulation requires association with a protein complex similar to the miRNA-induced silencing complex, if the activity of miR-122 involves HCV RNA translocation to mRNA-processing bodies (Beckham and Parker, 2008) or if other miR-122 target genes have an effect on HCV viral levels.

Several groups including ours have demonstrated that ethanol (EtOH) can modulate microRNA expression in the liver (Bala et al., 2011; Dolganiuc et al., 2009; Miranda et al., 2010). In this study, we tested the hypothesis that EtOH facilitates HCV replication through modulation of miR-122. We discovered that at a physiologically relevant dose, EtOH augments HCV replication involving miR-122 induction and its target, Cyclin G1, in human hepatoma cells. Our observation that EtOH modulates the expression of cellular host cofactors provides new insights into the pathomechanisms of alcohol-induced augmentation of HCV replication.

MATERIALS AND METHODS

Cell Cultures

Huh-7.5 cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco) and 1× minimal essential medium (MEM) nonessential amino acids (Gibco) at 37°C in a humidified atmosphere of 5% CO_2. An infectious clone of HCV, J6/JFH (kindly provided by Dr.

Charles Rice), was inoculated into Huh-7.5 cells and the cultures passed as previously described (Blight et al., 2002).

E47 cells (Chen and Cederbaum, 1998), which constitutively express human CYP2E1, and C34 cells (Chen and Cederbaum, 1998), which are HepG2 cells transfected with the empty pCI-neomycin vector, were grown in MEM containing 10% FBS and 0.5 mg/ml G418 supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco cat. #15140-122) in a humidified atmosphere in 5% CO_2 at 37°C.

Alcohol Treatment

For EtOH exposure, cells were placed in incubator culture chambers (C.B.S. Scientific Co., San Diego, CA) with twice the alcohol concentration in the bottom of the chamber to saturate the chamber and maintain a stable alcohol concentration, as described previously (Mandrekar et al., 2009).

Quantification of miRNA Expression

Quantitation of miR-122 was performed using Taqman® micro-RNA assays (Applied Biosystems, Grand Island, NY). RNU6B was used as endogenous control to normalize the expression levels of miR-122.

Real-Time Reverse Transcription Polymerase Chain Reaction

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR)was performed with the CFX96 Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA) and SYBR Green PCR Master Mix (Eurogentec, Fremont, CA) using 18S for normalization of the relative gene expression. Data were analyzed using the comparative ΔCt method. Primers for detection of HCV RNA were described previously (Hou et al., 2008). Specific primers for Cyclin G1 included: CCNG1 sense: 5′-AGCTGCAGTCTCTG TCAAG-3′; CCNG1 antisense: 5′-ATGTCTCTGTGTCAAAGCCA-3.′

Western Blot Analysis

Protein concentration of the whole-cell lysates was quantified by Bio-Rad Protein Assay. Western blot analysis was done with specific antibodies of interest as indicated which included: anti-NS5A (9E10, kindly provided by Dr. Charles Rice), anti-NS3 (ViroStat, Portland, ME and Abcam [Cambridge, MA] cat. # ab65407), anti-RFP Epitope Tag polyclonal antibody (Thermo Scientific [Billerica, MA] cat. # PA1-986), anti-Cyp2E1 (Sigma [St. Louis, MO] cat. # HPA009128), CCCNG1-antibody (Rabbit, polyclonal, sc-7865; Santa Cruz, Santa Cruz, CA), anti–β-actin (Abcam cat. # 6276) were used as primary antibodies, followed by the corresponding species-specific horseradish peroxidase-conjugated secondary antibody and LumiGLO® chemiluminescent as substrate (Cell Signaling Technology, Danvers, MA) to identify the immunoreactive bands.

Cell Cycle Analysis and Cell Viability

Cell cycle analysis was evaluated by flow cytometry. Fixed cells were stained with propidium iodide and analyzed using FACSCalibur (Becton-Dickinson Biosciences, San

Jose, CA) and FlowJo7.6.1 software (Tree Start Inc., Ashland, OR). Cell viability was determined using Cell Proliferation Reagent WST-1; Roche, Branford, CT) according to the manufacturer's recommendation.

Transfection

The hsa-miR-122 Anti-miRTM miRNA Inhibitor (AM11012; Ambion, Austin, TX) and Anti-miRTM Negtive Control #1 (AM17010; Ambion) were transfected into Huh-7.5 cells using siPORTTM NeoFXTM Transfection Agent (Ambion). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfection of plasmid DNA or 27-mer dsRNAs. The sequences for 27-mer dsRNAs were as follows: for Scrambled Negative Control, CUUCCUCUCUUUCUCUCCCUUGUGA (sense) and UCACAAG GGAGAGAAAGAGA GGAAGGA (antisense); for Cyclin G1 dsRNA, GCAAGAGCUUGUAUCCAAAUGUUTA (sense) and UAAACAUUUGGAUACAAGCUCUUGCCA (antisense); for NC1 Negative Control, CGUUAAUCGCGUAU AAUACGCGUAT (sense) and AUACGCGUAUUAUACGC GAUUAACGAC (antisense); and for EGFP-S1 dsRNA, ACCC UGAAGUUCAUCUGCA CCACCG (sense) and CGGUGGUGCAGAUGAAC UUCAGGGUCA (antisense). LipofectamineTM RNAiMAX and LipofectamineTM LTX with PLUSTM reagent (Invitrogen) were used for transfection of siRNA and shRNA/ plasmids, respectively. The siRNA and shRNA (Santa Cruz Biotechnology Inc.) used in this study were as follows: Control siRNA (FITC Conjugate)-A sc-36869; Control siRNA-A sc-37007; Control shRNA Plasmid-A sc-108060; Cyclin G1 shRNA Plasmid (h) sc-35139-SH; Cyclin G1 siRNA (h) sc-35139; CYP2E1 plasmid (h) from Origene (cat. # SC128274; Rockland, MD).

Confocal Microscopy

Intracellular staining was performed as described previously (Hou et al., 2008). Confocal imaging was performed on a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany), and Leica confocal software was used for the acquisition of images. Antibodies used in this study were listed as follows:

Cyclin G1 (H-46) (Rabbit, polyclonal, sc-7865; Santa Cruz);

HCV NS3 (Mouse, monoclonal, 1847; ViroStat);

HCV NS5A (9E10, kindly provided by Dr. Charles Rice);

Nuclear factor-κB (NF-κB) p65 (C22B4) (Rabbit, monoclonal, Cat. #4764; Cell Signaling).

Statistical Analysis

Data are shown as mean ± standard error of the mean and were analyzed using the Student's *t*-test; *p*-values <0.05 were considered statistically significant.

RESULTS

Acute EtOH Treatment Enhances HCV RNA Replication Involving miR-122 Induction

Using the infectious HCV J6/JFH system in Huh-7.5 cells, we found that treatment with a physiologically relevant concentration of EtOH (25 mM) resulted in a significant increase in HCV RNA (Fig. 1*A*) and HCV NS3 protein (Fig. 1*B*) levels compared with the virus infected cells without EtOH exposure. The 25 mM concentration of EtOH in vitro approximates 0.1 g/dl blood alcohol levels that are reached after 4 to 5 drinks in a normal nonalcoholic individual. This concentration of EtOH did not induce cytotoxicity as assessed by cell morphology and WST-1 viability assay (data not shown).

miR-122, a microRNA abundant in hepatocytes, has been shown to modulate HCV replication (Jopling et al., 2005) and we recently found that microRNA expression can be regulated by alcohol in Kupffer cells and in the liver tissue in vivo (Bala et al., 2011). Thus, we hypothesized that EtOH affects miR-122 expression and thereby regulates HCV replication in human hepatoma cells. We found that EtOH treatment resulted in a significant up-regulation of miR-122 levels both in EtOH-treated (Fig. 1*C*) and HCV J6/JFH-infected Huh-7.5 EtOH-treated cells (Fig. 1*D*). The functional role of the EtOH-induced miR-122 increase in HCV replication was further evaluated using an anti-miR-122 inhibitor. Our results show that the anti-miR-122 inhibitor, and not the anti-miR-122 negative control, attenuated HCV replication in EtOH-naïve cells and prevented the EtOH-induced increase in HCV NS3 protein levels (Fig. 1*E*). These observations suggested that alcohol-induced miR-122 induction has a mechanistic role in HCV replication.

EtOH Inhibits Cyclin G1 Via miR-122

MicroRNAs exert their biological effects mostly by silencing target gene expression. As acute alcohol treatment resulted in a significant up-regulation of miR-122 levels in Huh-7.5 cells, we hypothesized that alcohol also affected the expression of miR-122 target genes. Of the miR-122 target genes, CCNG1 (Cyclin G1) (Fornari et al., 2009; Gramantieri et al., 2007) was initially discovered as a novel member of the Cyclin family and a transcriptional target of the tumor suppressor protein p53 (Okamoto and Beach, 1994). We found that EtOH treatment significantly inhibited CCNG1 RNA expression (Fig. 2*A*,*B*) and reduced Cyclin G1 protein levels indicated by microscopy staining and Western blot analysis in HCV-naïve Huh-7.5 cells as well as in J6/JFH-infected Huh-7.5 cells treated with alcohol compared to EtOH-naïve cells (Fig. 2*C*–*E*). The mRNA levels of other miR-122 target genes, including CUX1 and PDRX2 did not change after alcohol treatment (data not shown).

The mechanistic role of miR-122 in Cyclin G1 regulation was further investigated. We found that miR-122 inhibition was associated with an increase in the miR-122 target gene, Cyclin G1 with and without HCV infection in the presence or absence of EtOH (Fig. 2*F*). These observations suggested that alcohol-induced miR-122 induction has a mechanistic role in regulating Cyclin G1 as previously reported (Fornari et al., 2009) as well as HCV in replication in our new findings.

Cyclin G1 Regulates HCV RNA Replication

The observation that EtOH increased HCV RNA and miR-122 levels while the miR-122 target, Cyclin G1, was down-regulated prompted us to evaluate the role of Cyclin G1 in HCV replication. We found that inhibition of Cyclin G1 expression using a transient knockdown approach by a 27-mer dsRNA significantly increased HCV RNA levels (Fig. 3*A*). We also discovered that Huh-7.5 cells were more permissive for J6/JFH infection when Cyclin G1 was stably inhibited using an shRNA approach (Fig. 3*B*,*C*). Confocal microscopy (Fig. 3*D*) as well as Western blotting analysis (Fig. 3*E*) indicated that inhibition of Cyclin G1 led to an increase in HCV NS3 protein expression. We also determined that the enhancement of HCV replication by inhibition of Cyclin G1 was not due to changes in the cell cycle as we found no changes in cell cycle distribution by FACS analysis after Cyclin G1 inhibition (Fig. S1).

To further investigate the regulatory role of Cyclin G1 on HCV replication, we generated a Cyclin G1 overexpression plasmid, pCCNG1-RFP (Fig. S2) and found that overexpression of Cyclin G1 can significantly reduce the intracellular levels of HCV NS3 protein (Fig. 3*F*). Together, these data demonstrate that Cyclin G1 is a novel regulator of HCV expression.

Alcohol Affects miR-122 Directly and Involving CYP2E1

Both alcohol and its metabolites generated during alcohol metabolism by the cytochrome P4502E1 can modulate cellular functions. To investigate the potential role of alcohol metabolites in induction of miR-122 or Cyclin G1 regulation, we tested miR-122 expression, HCV RNA, and/or Cyclin G1 in Huh-7.5 cells with and without CYP2E1 expression (Fig. 4*A*), HepG2-derived C34 cells (deficient in CYP2E1) (Fig. S3A,C), and E47 cell (expressing CYP2E) (Fig. S3B,D). We found that alcohol treatment increased miR-122 and HCV RNA levels in Huh-7.5/CYP2E cells compared with HCV-infected Huh-7.5 cells that had no detectable Cyp2E1 protein expression (Fig. 4*A*–*C*). However, HCV J6/JFH-infected Huh-7.5/CYP2E expressing cells exposed to EtOH showed no significant increase in HCV core protein levels (Fig. 4*C*). Alcohol had the same effect on C34 and E47 cells, indicating that miR-122 up-regulation or Cyclin G1 down-regulation was due to the direct effect of alcohol and not just its metabolites (Fig. S3A,D). Together, these results suggested that alcohol has both direct and indirect, metabolite-meditated, effects on miR-122 and HCV replication.

NF-κB Activation Mediates miR-122 Induction by EtOH

The regulation of the biogenesis of miR-122 is only partially understood. We and other groups have shown that microRNAs are the transactivational targets of NF-κB (Bala et al., 2011; Galardi et al., 2011). This prompted us to investigate whether NF-κB might play a role in modulating miR-122 expression by EtOH. We found that p65/NF-κB translocated into the nucleus in EtOH-treated Huh-7.5 cells or in cells treated with TNF*α* as a positive control (Fig. 5*A*). NF-κB DNA binding was also increased in alcohol-treated cells (Fig. 5*B*). More importantly, the EtOH-induced increase in miR-122 (Fig. 5*C*) and HCV RNA (Fig. 5*D*) levels was prevented by the NF-κB inhibitor, BAY 11-7082, in J6/JFH/Huh-7.5 cells. These results suggested that NF-κB plays a role in alcohol-induced modulation of miR-122 expression.

DISCUSSION

In this study, we report the role of novel host factors involving miR-122 and Cyclin G1 in alcohol-induced up-regulation of HCV replication in human hepatoma cells. We show that alcohol-induced cellular stress via NF-κB activation results inmiR-122 increase and that HCV "highjacks" this cellular protective mechanisms to support its own replication. These observations provide new concepts that underlie host and HCV interactions and the mechanisms for alcohol-induced regulation of HCV replication.

First, we discovered that alcohol increased miR-122 expression in human hepatoma cells with or without HCV infection. Second, we showed that alcohol increased HCV replication in an infectious HCV virus system. This observation is similar to previous reports with the HCV genomic replicon system where alcohol could increase HCV mRNA levels (Seronello et al., 2010). Third, our novel data show that miR-122 modulates HCV replication through its target gene, Cyclin G1. We identified Cyclin G1 as a novel host factor in regulation of HCV infection.

Like most microRNAs, miR-122 targets the 3′ untranslated region (UTR) of mRNA transcripts and suppresses translation. miR-122 also directly binds to 2 adjacent sites of the 5′ UTR of the HCV genome and augments translation and RNA accumulation (Jopling et al., 2005). We found that both miR-122 and Cyclin G1, a miR-122 target, have regulatory effects on HCV replication. Although the role of miR-122 in the HCV life cycle is not well understood, putative mechanisms include altering the conformation of the 5′ UTR of the HCV genome (Diaz-Toledano et al., 2009); masking the 5′ terminal nucleotides of the HCV genome by an unconventional microRNA-target RNA complex (Machlin et al., 2011); enhancement of viral RNA interaction with ribosomes (Henke et al., 2008); translation stimulation via HCV 5′-UTR (Jangra et al., 2010; Jopling et al., 2008; Li et al., 2011; Roberts et al., 2011); having no effect on RNA elongation rates (Villanueva et al., 2010); and the independence of its effect on isoprenoid metabolism (Norman and Sarnow, 2010).

In this study, we demonstrated that Cyclin G1 works as a novel antiviral effector as the knockdown of Cyclin G1 by transient and stable RNAi approaches facilitated HCV replication and viral protein expression, whereas overexpression of Cyclin G1 suppressed HCV replication. Clinical studies have found increased Cyclin G1 levels in livers of patients with chronic HCV infection (Helbig et al., 2005) which may represent a suboptimal host response in antiviral defense against HCV. Cyclin G1 is a transcriptional target of the tumor suppressor p53, and in turn, it negatively regulates p53 family proteins (Ohtsuka et al., 2004). In addition, studies have shown that by modulating Cyclin G1, miR-122 can influence p53 protein stability and transcriptional activity and reduce the invasive capacity of hepatocellular carcinoma cells (Fornari et al., 2009). Recent data also indicate that p53 is a potential contributor to host antiviral defense against HCV (Dharel et al., 2008), thus investigation of the regulation of Cyclin G1 and p53 in HCV replication is of interest for future studies. In HBV infection, loss of miR-122 was shown to increase HBV replication through Cyclin G1-modulated p53 activity (Wang et al., 2012). Our results indicate that in HCV infection, Cyclin G1 overexpression can inhibit viral expression in hepatoma cells.

Our studies also unraveled new mechanisms by which EtOH modulates miR-122 abundance. Among the broad range of transcriptional factors that influence the transcription and production of miRNAs, NF-κB, a transcriptional regulator consisting of reticuloendotheliosis (Rel) protein dimers that bind a DNA sequence motif known as the κB site, regulate a battery of genes that are critical to innate and adaptive immunity, cell proliferation, inflammation, and tumor development. In this study, we show that NF-κB was up-regulated by alcohol administration resulting in translocation of the p65/Rel subunit to the nucleus and resulting in increased DNA binding. Using an inhibitor, we found that alcohol-induced up-regulation of miR-122 was dependent on NF-κB activation suggesting that alcohol increases miR-122 via NF-κB activation. Regulation of NF-κB activation by acute and chronic alcohol has been reported in different cell types including hepatocytes, stellate cells, and a variety of immune cells (Kim et al., 2001; Mandrekar et al., 1997; Szabo and Mandrekar, 2002). In a previous study, we found that alcohol increased NF-κB nuclear levels and DNA binding in HepG2 cells and similar effects were shown by others in primary hepatocytes (Roman et al., 1999; Szabo et al., 2001). Although a broad variety of genes have κB binding sites, NF-κB activation in hepatocytes is generally linked to hepatocytes stress and survival (Dutta et al., 2006; He et al., 2010). The abundance of miR-122 can also be regulated by other miRNAs such as miR-370 (Iliopoulos et al., 2010). However, we found that EtOH exposure had no effects on miR-370 expression indicating that miR-370 might not be involved in this process (data not shown). We speculate that alcohol-induced cellular stress and the resulting NF-κB activation promote HCV replication in a miR-122 and Cyclin G1-dependent manner. Our results also suggest that HCV can “hijack” cellular survival mechanisms (such as NF-κB activation) of the host to promote its own survival.

In summary, our results demonstrate that EtOH facilitates HCV replication involving miR-122 and Cyclin G1 modulation. We show for the first time that Cyclin G1 regulates HCV replication. Our studies provide experimental evidence that the inhibitors of miR-122, NF-κB or overexpression of Cyclin G1 might represent feasible targets of efficient molecular strategy to interfere with HCV replication and the undesirable effects of alcohol use on the HCV infection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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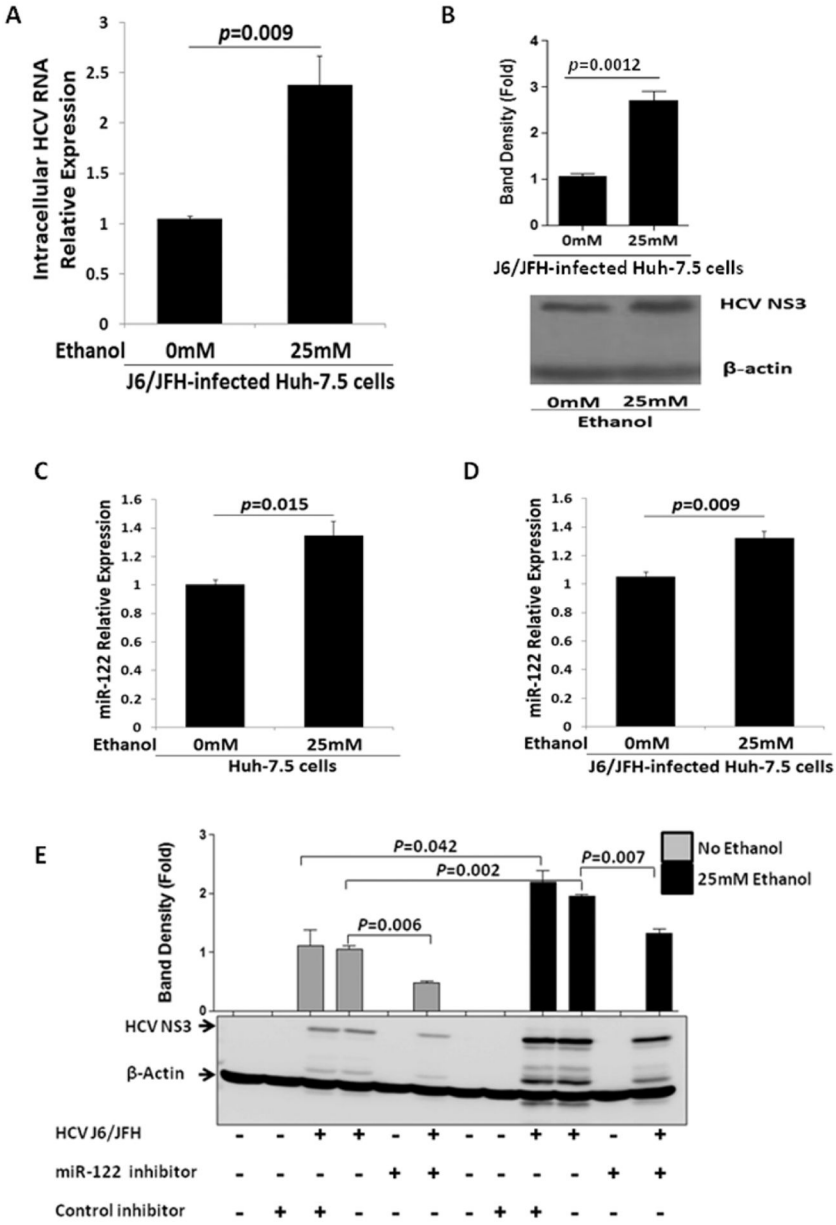

Fig. 1.

Acute ethanol (EtOH) treatment enhanced hepatitis C virus (HCV) RNA replication involving miR-122 induction. (**A**–**D**) J6/JFH-infected or uninfected Huh-7.5 expressing cells were treated with EtOH (0 or 25 mM). (**A**) Intracellular HCV RNA (RT-qPCR) and (**B**) HCV protein expression (Western blot) were evaluated at 24 hours after EtOH exposure. (**C**) HCV-naïve and (**D**) J6/JFH-infected Huh-7.5 cells were treated with and without EtOH (0 or 25 mM), and miR-122 and RNU6B (for normalization) expression were quantified by qPCR 24 hours after EtOH exposure. (**E**) J6/JFH-infected or uninfected Huh-7.5 cells were transfected with a miR-122 inhibitor (50 nM) or negative control (50 nM) prior to EtOH treatment (0 or 25 mM) and intracellular HCV NS3 protein quantified by Western blot 24 hours after EtOH exposure or not. Results presented are representative of 3 to 4 independent

experiments and data expressed as mean ± standard error of the mean, $p < 0.05$ were considered statistically significant (by 2-tailed Student's test). Multiplicity of infection (MOI) of 1 was used for all infections.

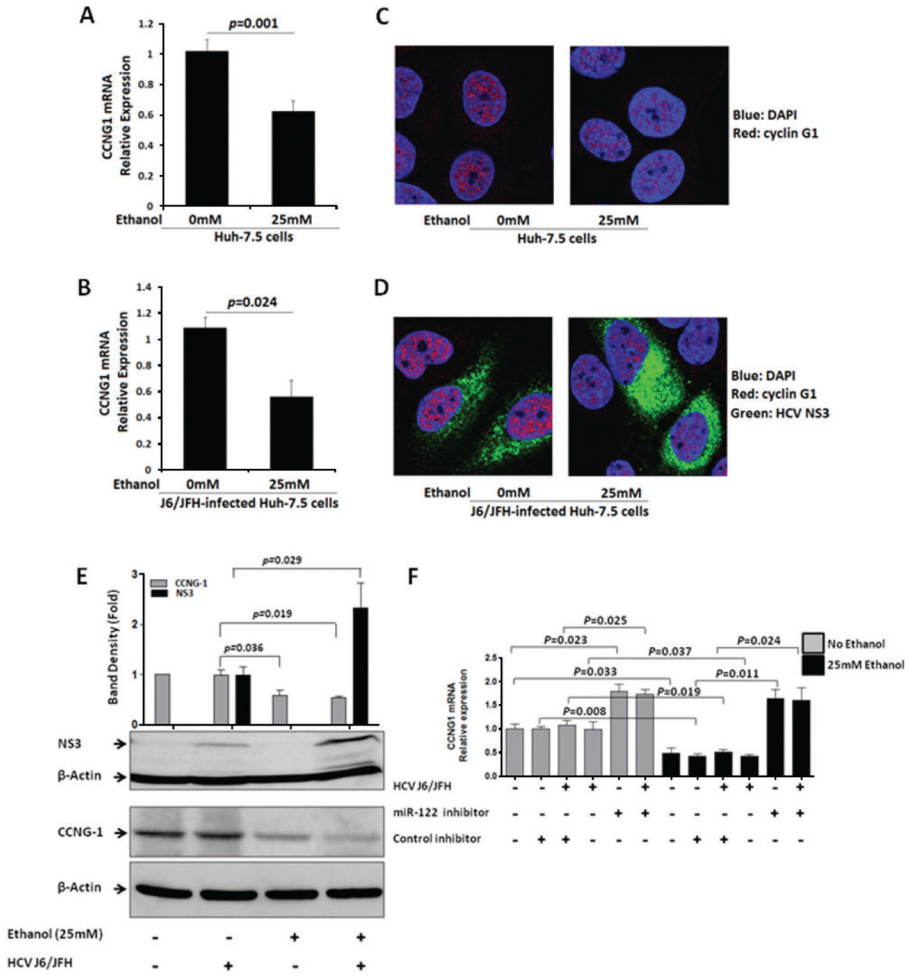

Fig. 2.

Acute ethanol (EtOH) treatment modulated the expression of miR-122 targets. (**A** and **B**) Uninfected and J6/JFH-infected Huh-7.5 cells were treated with EtOH (0 or 25 mM), and CCNG1 mRNA (RT-qPCR) and (**C** and **D**) Cyclin G1 protein expression (confocal microscopy) were evaluated 24 hours after EtOH exposure. (**E**) Uninfected and J6/JFH-infected Huh-7.5 cells were treated with EtOH (0 or 25 mM), and hepatitis C virus (HCV) NS3 expression was determined by Western blot in Huh-7.5 cells HCV J6/JFH infection treated with alcohol or not for 24 hours. (**F**) J6/JFH-infected Huh-7.5 cells were transfected with a miR-122 inhibitor (50 nM) or negative control (50 nM) prior to EtOH treatment (0 or 25 mM) and Cyclin G1 expression determined 24 hours after EtOH exposure or not. Results presented are representative of 3 to 4 independent repeat experiments and data expressed as mean standard error of the mean, $p < 0.05$ were considered statistically significant (by 2-tailed Student's test). (**B** and **D**) One representative image shown of 3 independent repeat experiments with at least 10 fields sequentially analyzed for each microscopy slide to minimize spectral bleed through artifacts. Multiplicity of infection (MOI) of 1 was used for all infections.

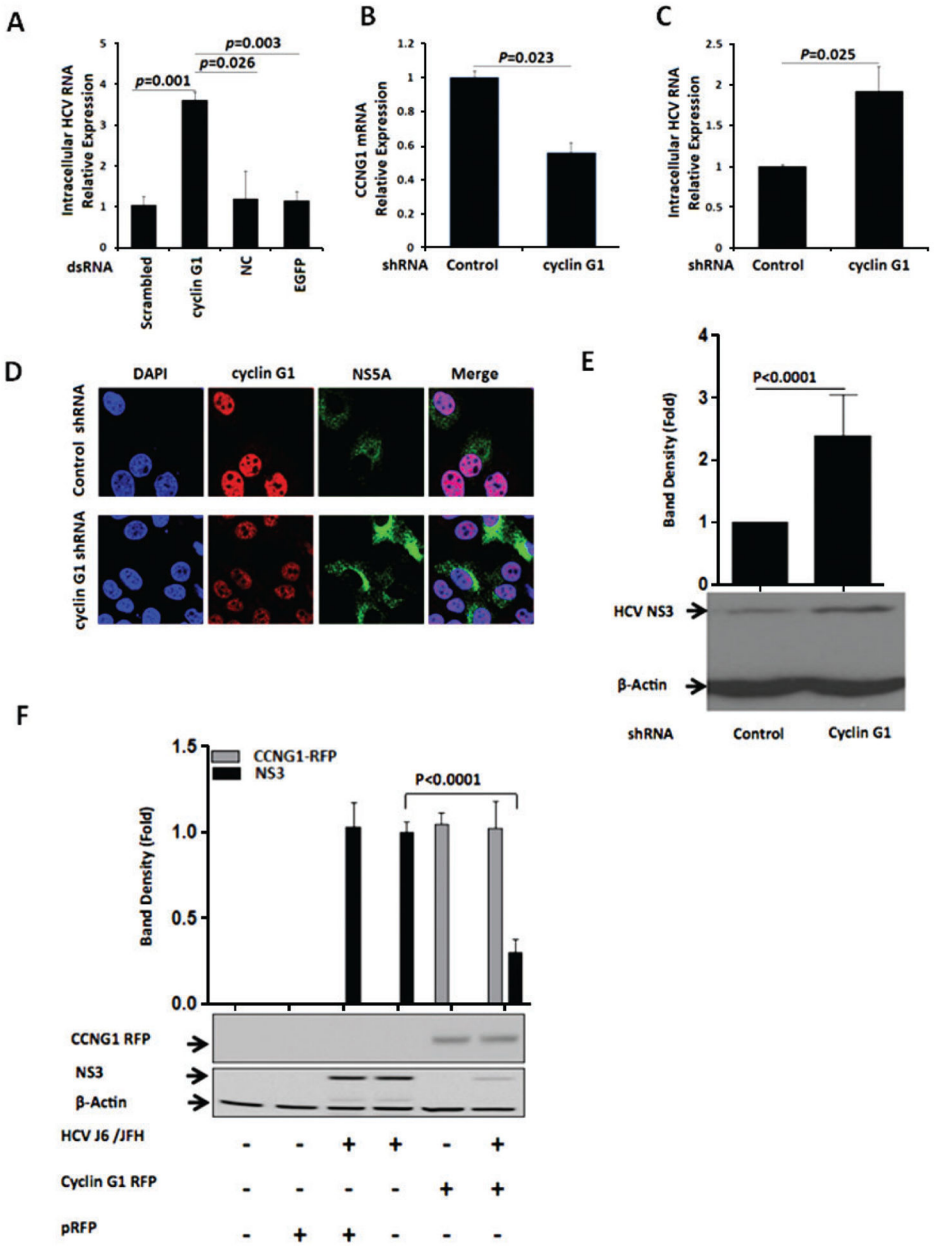

Fig. 3.

Cyclin G1 knockdown or overexpression modulated hepatitis C virus (HCV) gene expression. (**A**) J6/JFH-infected Huh-7.5 cells were transfected with Cyclin G1 dsRNA (5 nM), scrambled dsRNA (5 nM), negative control dsRNA (5 nM), and EGFP dsRNA (5 nM) as controls, respectively. Intracellular HCV RNA was quantified 48 hours after infection. (**B** and **C**) Huh-7.5 cells were transfected with Cyclin G1 shRNA or control shRNA. The Cyclin G1 knockdown Huh-7.5 cells and the control cells were infected with J6/JFH HCV virus for 48 hours and CCNG1 mRNA, HCV RNA were evaluated. (**D**) Cyclin G1 and HCV viral protein expression in the Cyclin G1 knockdown and control Huh-7.5 cells (confocal microscopy) 48 hours after J6/JFH HCV infection. (**E**) HCV protein expression (Western

blot) in Cyclin G1 knockdown and the control Huh-7.5 cells 48 hours after J6/JFH HCV infection. (**F**) J6/JFH-infected Huh-7.5 cells were transfected with Cyclin G1 plasmid, pCCNG1-RFP (Fig. S3) and pRFP, HCV protein expression was evaluated (Western blot) 48 hours after transfection. (**A**–**F**) Results presented are representative of 3 to 4 independent repeat experiments and data expressed as standard error of the mean, $p < 0.05$ were considered statistically significant (by 2-tailed Student's test). (**D**) One representative image shown for 3 independent repeat experiments. Multiplicity of infection (MOI) of 1 was used for all infections.

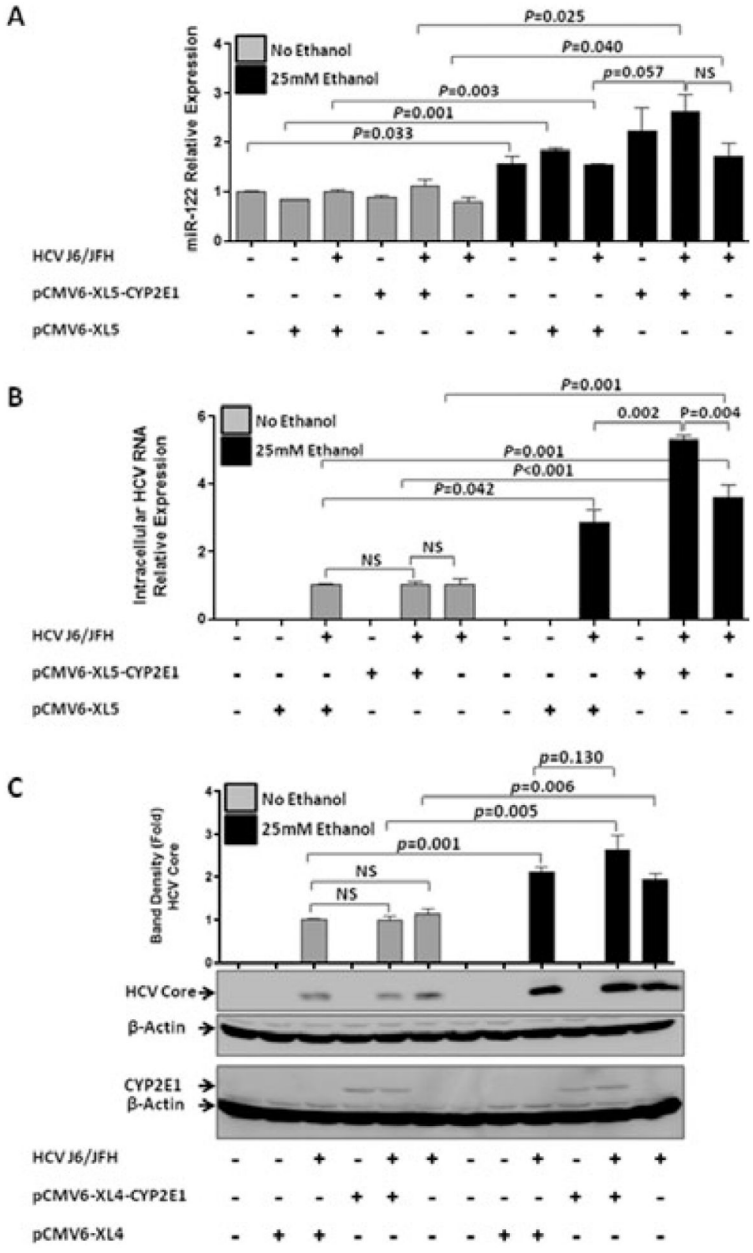

Fig. 4.

Ethanol (EtOH) directly regulates hepatitis C virus (HCV) replication involving CYP2E1. (**A**–**C**) J6/JFH-infected or uninfected Huh-7.5 and Huh-7.5/CYP2E1 expressing cells were treated with EtOH (0 or 25 mM) and (**A**) miR-122 and RNU6B (for normalization), (**B**) intracellular HCV RNA (RT-qPCR) and (**C**) HCV protein expression (Western blot) were evaluated at 24 hours after EtOH exposure. Results presented are representative of 3 independent repeat experiments and data expressed as mean ± standard error of the mean, $p < 0.05$ were considered statistically significant (by 2-tailed Student's test). Multiplicity of infection (MOI) of 1 used was for all infections.

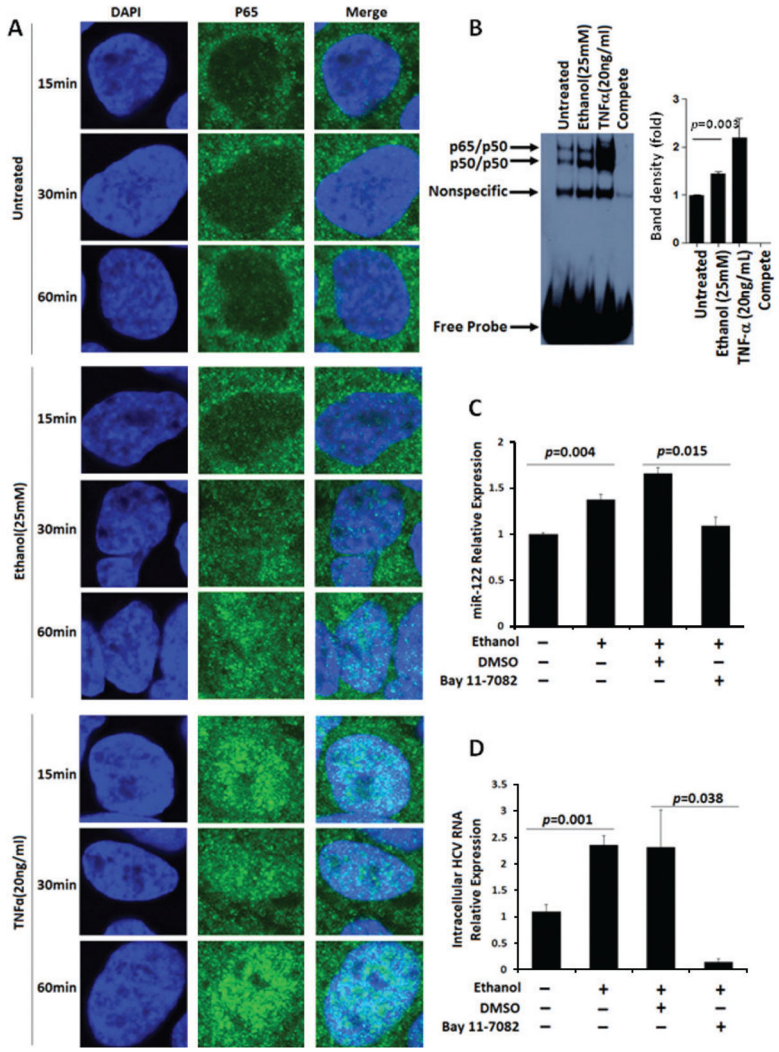

Fig. 5.

Mechanisms of ethanol (EtOH)-induced miR-122. (**A**) Localization of NF-κB p65 protein evaluated by confocal microscopy in Huh-7.5 cells treated with EtOH (0 or 25 mM) or TNF-α (20 ng/ml) for 15 to 60 minutes, respectively. (**B**) Equal amounts of nuclear protein were evaluated in an electrophoretic mobility shift assay using ^{32}P-labeled NF-κB oligonucleotides. A 10-fold excess of nonlabeled oligonucleotide was used as a cold competitor. (**C** and **D**) J6/JFH-infected Huh-7.5 cells were treated with NF-κB inhibitor BAY 11-7082 (0 or 10 uM) or DMSO (10 uM) 30 minutes prior to treatment with EtOH (0 or 25 mM). (**C**) miR-122 expression and (**D**) intracellular hepatitis C virus RNA were quantified 24 hours after EtOH exposure, respectively. Results presented are representative of 3 to 4 independent repeat experiments and data expressed as mean ± standard error of the mean, $p < 0.05$ were considered statistically significant (by 2-tailed Student's test). Multiplicity of infection (MOI) of 1 used was for all infections.