Association between MicroRNA-373 and Long Noncoding RNA NORAD in Hepatitis C Virus-Infected Hepatocytes Impairs Wee1 Expression for Growth Promotion

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ABSTRACT Chronic hepatitis C virus (HCV) infection may lead to end-stage liver disease, including hepatocellular carcinoma (HCC). We have shown previously that microRNA-373 (miR-373) is upregulated in HCV-infected human liver biopsy specimens. To gain insight into the role of miR-373 in HCV-mediated pathogenesis, we investigated its interacting partner for hepatocyte growth regulation. Transcriptome sequencing (RNA-seq) data revealed that Wee1 is associated with miR-373 and is a direct target. Interestingly, higher expression of Wee1 was noted in HCV-infected hepatocytes than in uninfected hepatocytes, suggesting that other factors may block miR-373-mediated Wee1 inhibition. We subsequently found an association between the long noncoding RNA NORAD (LINC00657) and miR-373, and we demonstrated that NORAD binds to miR-373 and Wee1 independently. However, the high level of Wee1 expression in HCV-infected hepatocytes suggested that miR-373 forms a complex with NORAD. Depletion of miR-373 or the inhibitor Wee1 reduces the growth of Huh7.5 cells harboring the HCV genome as well as reducing Wee1 expression. Taken together, our data demonstrate a novel mechanism of hepatocyte growth promotion during HCV infection involving a miR-373–NORAD–Wee1 axis, which may be a target for future therapy against HCV-associated HCC.

IMPORTANCE The mechanism of HCV-mediated liver pathogenesis is poorly understood. In this study, we observed that HCV infection upregulates miR-373 and Wee1, a pivotal player in the G₂ checkpoint in the cell cycle, although Wee1 is a direct target for miR-373. Subsequent investigation demonstrated that miR-373 forms a complex with the long noncoding RNA NORAD, resulting in the release of their common target, Wee1, in HCV-infected cells, which, in turn, favors uncontrolled cell growth. Our study suggested a previously unknown mechanism for hepatocyte growth promotion following HCV infection, and this pathway can be targeted for future therapy against HCV-mediated liver pathogenesis.

KEYWORDS hepatitis C virus, miRNA-373, Wee1, NORAD

The hepatitis C virus (HCV) genome is a single-stranded positive-sense RNA and belongs to the family *Flaviviridae*. HCV is an important factor in the etiology of fibrosis/cirrhosis, as well as hepatocellular carcinoma (HCC). Several HCV proteins exhibit oncogenic potential (1–3), although the HCV genome does not integrate with the host genome, and the mechanisms by which HCV promotes hepatocyte growth leading to HCC are poorly understood.

Transcriptome studies and genome-tiling arrays have suggested that a large proportion of the genome (more than 90%) transcribes noncoding RNAs (ncRNAs) (4–6). MicroRNAs (miRNAs) constitute a class of ~18- to 22-nucleotide RNAs and bind to the 3' untranslated regions (3' UTRs) of mRNAs, thereby inhibiting mRNA translation or

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promoting mRNA degradation. miRNAs play crucial roles in a variety of diseases, including liver disease. Long noncoding RNAs (lncRNAs) have >200 nucleotides and no significant protein-coding potential. Like miRNAs, lncRNAs have many specific functional features and are involved in many diverse biological processes in cells. Further, lncRNAs can act as competing endogenous RNA. However, very little is known about the coordinated functions of miRNAs and lncRNAs in regulating hepatocyte growth during HCV infection. We have demonstrated that HCV-induced miR-373 blocks the interferon signaling pathway at multiple steps (7). miR-373 is also upregulated in HCV-infected liver biopsy specimens.

In this study, we investigated the role of miR-373 in hepatocyte growth regulation. We observed that miR-373 targets *Wee1* mRNA and inhibits its expression. *Wee1* is a serine-threonine kinase that regulates the G₂ checkpoint for DNA damage repair through the inhibitory phosphorylation of Cdc2 (8, 9). Interestingly, *Wee1* expression is higher in HCV-infected hepatocytes than in uninfected hepatocytes. In-depth analysis showed that the lncRNA NORAD (noncoding RNA activated by DNA damage) is associated with miR-373 and binds to *Wee1*. The mechanistic study revealed that miR-373 interacts with NORAD in HCV replicating hepatocytes, resulting in higher expression of *Wee1*. Together, our results suggest a novel mechanism of hepatocyte growth regulation during HCV infection.

RESULTS

Wee1 is a direct target of miR-373. We have shown previously that HCV infection upregulates miR-373 expression in primary human hepatocytes and HCV-infected liver biopsy specimens (7). Modulation of miR-373 has been demonstrated in several cancers (10); however, its role in hepatocyte growth regulation following HCV infection has yet to be understood. To examine the miR-373-interacting partners for cell growth regulation, we immunoprecipitated the miR-373-Ago2 RNA-induced silencing complex (RISC) with an Ago2 antibody or isotype control IgG and performed transcriptome-sequencing (RNA-seq) analysis. Data analysis revealed that the *Wee1* cell cycle-regulatory gene coprecipitates with miR-373 with elevated expression in the complex (log fold change, 2.1; $P = 6.6 \times 10^{-8}$). We validated the expression of *Wee1* mRNA by reverse transcription-quantitative PCR (RT-qPCR). The expression of miR-373-bound *Wee1* mRNA was significantly higher in Ago2 immunoprecipitates than with the isotype control (Fig. 1A), suggesting an association between these two molecules. *In silico* analysis also suggested a potential binding site of miR-373 in the *Wee1* 3' UTR (Fig. 1B, top). To verify binding, the 3' UTR of *Wee1*, including a potential binding site for miR-373, was cloned into the pMIR-Report luciferase vector and was cotransfected with miR-373 or miR-10b (as a negative control) into immortalized human hepatocytes (IHH), and luciferase activity was measured. miR-19a targets *Wee1* in leukemia (11) and was included as a positive control. The results demonstrated inhibition of *Wee1* 3' UTR expression by the miR-373 mimic (Fig. 1B). miR-10b did not exhibit inhibition of luciferase activity, and miR-19a displayed inhibition of luciferase activity, as expected. A negative correlation has been reported between the relative expression of miR-19a and *Wee1* in leukemic cell lines (11). Subsequently, Huh7.5 cells transfected with a control, a miR-373 mimic, or anti-miR-373 exhibited down- or upregulation of *Wee1* mRNA expression (Fig. 1C). Further, overexpression of miR-373 in hepatocytes significantly inhibited *Wee1* expression at the protein level (Fig. 1D). *Wee1* plays a pivotal role by phosphorylating Cdc2 on the G₂ checkpoint to repair DNA damage. Huh7.5 cells transfected with a control or a miR-373 mimic also displayed inhibition of phosphorylated Cdc2 (p-Cdc2) expression (Fig. 1E). RNA was isolated from cells transfected with miR-373 or a control miR and was examined for miR-373 expression by qRT-PCR. As expected, higher expression of miR-373 than of the control was noted (Fig. 1F). Results after normalization with U6 as an internal control are presented.

HCV infection enhances *Wee1* expression. Since miR-373 is upregulated in HCV-infected hepatocytes, we next examined the status of *Wee1* expression. We observed significant upregulation of *Wee1* mRNA expression in HCV-infected

FIG 1 miR-373 targets Wee1. (A) miR-373-transfected cell lysates were immunoprecipitated with an Ago2-specific monoclonal antibody or an unrelated IgG2a isotype control. RNA was isolated from immunoprecipitates by using an RNeasy kit, and Wee1 expression was analyzed by quantitative RT-PCR. (B) IHH were cotransfected with the 3' UTR of Wee1 cloned into the pMIR-Report luciferase vector (500 ng) and either a control miR (mock), a miR-373 mimic, a miR-19a mimic (positive control), or a miR-10b mimic (negative control), each at 25 nM. Relative luciferase activity was measured after 48 h of transfection. *In silico* analysis also suggested a potential binding site of miR-373 in the Wee1 3' UTR (shown above the graph). (C) RNA was isolated from Huh7.5 cells transfected with either a control miR, a miR-373 mimic, or anti-miR-373 (25 nM each), and relative mRNA expression was analyzed by qRT-PCR using specific primers. The 18S rRNA gene was used as an internal control. Data are presented as the means and standard deviations from three independent experiments. (D) Huh7.5 cell lysates transfected with a control miR or a miR-373 mimic were prepared after 48 h of transfection. (Left) Wee1 expression was analyzed by Western blotting using a specific antibody. The blot was reprobed with an antibody to actin for normalization. (Right) Densitometric analysis was performed using ImageJ software. (E) (Left) Cell lysates were also analyzed for p-Cdc2 and total-Cdc2 expression by Western blotting using specific antibodies, and blots were reprobed with an antibody to GAPDH for normalization of the protein load. (Right) Densitometric analysis was performed using ImageJ software. (F) miR-373 expression was analyzed by qRT-PCR of RNA from mimic-transfected cells. Results after normalization with U6 as an internal control are presented. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Huh7.5 cells (Fig. 2A). Lysates from HCV-infected Huh7.5 cells also exhibited higher levels of Wee1 protein expression (Fig. 2B). Wee1 inactivates Cdc2 by phosphorylating its tyrosine-15 residue, resulting in G₂/M cell cycle checkpoint arrest in response to DNA damage (9). This restriction was found to be crucial for cell survival by preventing cellular apoptosis in the context of viral pathogenesis as well as tumorigenesis (12). We observed a significant increase in phospho-Cdc2 expression over that in the control (Fig. 2C).

The lncRNA NORAD associates with miR-373 in HCV-infected hepatocytes.

Since we observed upregulation of miR-373 and its target Wee1, we hypothesized that the miR-373–Wee1 interaction is interrupted in HCV-infected hepatocytes. We then examined the other miR-373-associated molecules in our RNA-seq data. We observed significant enrichment of the long noncoding RNA NORAD (log fold change, 1.09; $P = 0.001$). We validated NORAD expression in the miR-373–Ago2 complex by qRT-PCR and observed significant enhancement (Fig. 3A). NORAD is a cytoplasmic lncRNA and is ubiquitously expressed in human tissues (13, 14). *In silico* analysis suggested the presence of a miR-373 binding site in NORAD (<http://starbase.sysu.edu.cn>) (Fig. 3B). To confirm this, the miR-373 binding site of NORAD was cloned into the pMIR-Report luciferase reporter plasmid. Cells were transfected with the miR-373 binding site of NORAD plasmid DNA and a miR-373 mimic or a control miR, and luciferase activity was measured. The results demonstrated dose-dependent inhibition of luciferase activities by the miR-373 mimic (Fig. 3B), suggesting an association between miR-373 and NORAD. On the other hand, miR-130a (without a NORAD binding site) does not have an effect on NORAD sensor luciferase activity (Fig. 3C), suggesting specificity. We also observed enhancement of NORAD expression in HCV-infected hepatocytes over that in the mock-treated control (Fig. 3D).

FIG 2 Increased Wee1 expression in HCV-infected hepatocytes. (A) Huh7.5 cells were either mock treated or infected with HCV (multiplicity of infection, 1), and RNA was isolated after 48 h. The relative expression of Wee1 mRNA was analyzed by qRT-PCR. 18S rRNA was used as an endogenous control and for target gene normalization. (B) (Left) Mock-treated or HCV-infected Huh7.5 cell lysates were subjected to Western blot analysis for Wee1 expression using a specific antibody. The blot was reprobed with an antibody to actin for normalization. (Right) Densitometry analysis was performed using ImageJ software. (C) (Left) Lysates from mock-treated or HCV-infected cells were subjected to Western blot analysis for p-Cdc2, total Cdc2, or an actin antibody. (Right) Densitometry analysis was performed using ImageJ software. Bands in panels B and C were spliced from the same gel for labeling purposes. Data are presented as the means and standard deviations from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

The miR-373–NORAD association regulates Wee1 expression. Next, we examined Wee1 status in hepatocytes in which NORAD is exogenously expressed. We observed significant downregulation of Wee1 mRNA (Fig. 4A). Similarly, Wee1 protein expression was decreased significantly in NORAD-overexpressing hepatocytes (Fig. 4B). Sequence analysis suggested a potential binding site for the Wee1 3' UTR in NORAD (http://rtools.cbrc.jp/cgi-bin/RNARNA/_detailPageGenerator.pl?ncRNA=LINC00657&mRNA=Wee1&submit=submit).

To verify the association, we cotransfected Wee1 3' UTR reporter plasmid DNA with control or NORAD plasmid DNA into IHH, and luciferase activity was measured. NORAD overexpression significantly downregulated luciferase activity in a dose-dependent manner (Fig. 4C), suggesting an association between Wee1 and NORAD. Next, we examined whether coexpression of miR-373 and NORAD can interfere with Wee1 expression. NORAD and miR-373 independently downregulated Wee1 luciferase activity, while coexpression of miR-373 and NORAD with a Wee1 luciferase construct rescued luciferase activity (Fig. 4D). NORAD mRNA expression in IHH transfected with control or NORAD plasmid DNA was determined. Results are presented after normalization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control (Fig. 4E). As expected, higher NORAD expression was observed after transfection.

The miR-373–NORAD complex regulates PUM1 expression in HCV-infected hepatocytes. NORAD has been shown to bind with the RNA binding protein Pumilio-1/2 (PUM1/2) (13, 14). Depletion of NORAD resulted in chromosomal instability. A recent study suggested that the interaction between NORAD and the RNA-binding protein SAM68 antagonizes the function of Pumilio. Our RNA-seq data showed the presence of PUM1 in the miR-373–Ago2 complex. *In silico* analysis suggested the presence of a potential binding site for miR-373-5p (other arm of stem loop) in the PUM1 3' UTR (data not shown). As expected, overexpression of

FIG 3 miR-373 interacts with the lncRNA NORAD. (A) miR-373-transfected cell lysates were immunoprecipitated with an Ago2-specific monoclonal antibody or an unrelated IgG2a isotype control. RNA was isolated from immunoprecipitates by using an RNeasy kit, and the expression of NORAD mRNA was analyzed by quantitative RT-PCR. (B) Predicted binding site for miR-373 in NORAD. IHH were cotransfected with a NORAD luciferase reporter plasmid contacting the miR-373 binding site (200 ng) and control miR or miR-373 mimic (25 or 50 nM [+ or ++, respectively]). The relative luciferase activity of NORAD was measured as described for Wee1 in the legend to Fig. 1B. (C) Luciferase activity was measured from IHH cotransfected with a NORAD luciferase reporter plasmid contacting the miR-373 binding site (200 ng) and a control miR or a miR-130a mimic (50 nM). (D) Huh 7.5 cells were either mock treated or infected with HCV, and RNA was isolated after 48 h of infection. The relative expression of NORAD mRNA was analyzed by qRT-PCR. GAPDH was used as an internal control. Data are presented as the means and standard deviations from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

NORAD in hepatocytes inhibited PUM1 expression (Fig. 5A). Huh7.5 cells transfected with miR-373 also inhibited PUM1 expression (Fig. 5B). We further validated PUM1 expression in the miR-373–Ago2 complex by qRT-PCR and observed significant enhancement (Fig. 5C). We also examined the status of PUM1 in HCV-infected Huh7.5 cells and observed that PUM1 expression was elevated over that in mock-treated cells (Fig. 5D). Therefore, it is possible that, as with Wee1, the association of miR-373 and NORAD interrupts PUM1 binding in HCV-infected hepatocytes.

Depletion of miR-373 reduces the growth of hepatocytes harboring HCV. Next, we examined the growth of hepatocytes harboring replicating HCV. Knockdown of miR-373 using anti-miR in HCV-harboring hepatocytes reduced cell proliferation (Fig. 6A). HCV-harboring Huh7.5 cells were treated with a predetermined dose of a Wee1 inhibitor (MK1775), and reduced cell proliferation was observed. On the other hand, HCV-infected Huh7.5 cells displayed better growth than uninfected cells (data not shown). It should be mentioned here that a dramatic difference in proliferation is generally difficult to observe for any fully transformed cell line, such as Huh7.5 cells. We next examined the expression of Wee1 in anti-miR-373-treated Huh7.5 cells harboring the HCV genome and observed reduced expression (Fig. 6B). Subsequently, we observed that the HCV-mediated upregulation of Wee1 and p-Cdc2 can be inhibited by anti-miR-373 (Fig. 6C). This result explains a novel mechanism for Wee1 upregulation in HCV-infected hepatocytes.

FIG 4 NORAD regulates Wee1 expression. (A and B) IHH were transfected with a control plasmid or a plasmid containing full-length NORAD. After 48 h of transfection, RNA was isolated, and cell lysates were prepared. (A) The relative expression of Wee1 mRNA was analyzed by qRT-PCR. The 18S rRNA gene was used as an endogenous control. (B) (Left) Wee1 protein expression was analyzed by Western blotting using a specific antibody, and the membrane was re probed with an antibody to actin for normalization. The bands were spliced from the same gel for labeling purposes. (Right) Densitometry analysis was performed using ImageJ software. (C) IHH were cotransfected with NORAD plasmid DNA (250 ng [+]) or 500 ng [++], and the 3' UTR of a Wee1 reporter construct (250 ng), and the relative luciferase activity of the Wee1 3' UTR was measured as described above. (D) Wee1 3' UTR luciferase reporter plasmid DNA (250 ng) and either a miR-373 mimic (25 nM) or full-length NORAD plasmid DNA (250 ng), or both, were transfected into hepatocytes, and luciferase activity was measured. (E) NORAD mRNA expression in IHH transfected with control or NORAD plasmid DNA was determined by qRT-PCR. Results after normalization with GAPDH as an internal control are presented. Data are presented as the means and standard deviations from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

DISCUSSION

Our transcriptome analysis of the miR-373-Ago2 complex revealed that Wee1 is associated with miR-373. Since chronic HCV infection leads to end-stage liver disease, including HCC, we examined the status of Wee1 and observed a higher expression level. In fact, Wee1 is upregulated in HCC irrespective of etiology (15). Our study demonstrated several important findings: (i) Wee1 is a common target of both miR-373 and NORAD; (ii) Wee1 expression and NORAD expression are significantly upregulated in HCV-infected hepatocytes; and (iii) the association of miR-373 and NORAD in HCV-infected cells enhances Wee1 expression.

HCV is an important etiologic agent for the development of HCC, although the mechanism for cellular changes is poorly understood. miRNAs and lncRNAs play critical roles in multistep cell growth-regulatory processes (16, 17). miRNAs are responsible for regulating many cellular processes, and viruses modulate the miRNA milieu in different ways to facilitate pathogenesis. Several lncRNAs are involved in HCV replication and modulation of the antiviral response (18); however, their role in pathogenesis is poorly understood. miR-373 has been implicated in the regulation of several cellular functions, including cell proliferation, invasion, and DNA damage repair in different cancers (10). We showed that miR-373 targets the Wee1 gene, which regulates the G₂/M cell cycle checkpoint and is crucial for cell survival by preventing cellular apoptosis (9, 12). Normal cells repair damaged DNA during the G₁/S cell cycle checkpoint; however, defects in the G₁/S checkpoint lead to damaged cells for arrest at the G₂/M checkpoint (12, 19–21). Defects in the G₁/S checkpoint are seen in many cancers; thus, a functional G₂/M checkpoint is necessary for the survival of these cells. Further, in normal cells,

FIG 5 miR-373 and NORAD regulate PUM1 expression. (A and B) Huh7.5 cells were transfected with either NORAD plasmid DNA (250 ng), or 25 nM miR-373 mimic, or control plasmid DNA. Western blot analysis for PUM1 expression was performed. The membranes were reprobed with an antibody to actin for normalization. The control lane is same for panels A and B. (C) miR-373-transfected cell lysates were immunoprecipitated with an Ago2-specific monoclonal antibody or an unrelated IgG2a isotype control. RNA was isolated from immunoprecipitates, and PUM1 expression was analyzed by qRT-PCR. (D) PUM1 expression in mock-infected or HCV-infected Huh7.5 cells was analyzed using a specific antibody. Densitometry analysis was performed using ImageJ software. Data are presented as the means and standard deviations from three independent experiments. **, $P < 0.01$.

wild-type p53 enforces both the G_1/S and G_2/M cell cycle checkpoints to allow all cells to repair DNA before entering the S and M phases. Cells with defective p53 function rely heavily for survival on the G_2 checkpoint, which is maintained by alternative signaling pathways in the absence of wild-type p53. However, p53 is deregulated by HCV, and p53 mutation is one of the common mutations in HCC. Higher expression of Wee1 may enhance aberrant mitosis. Targeting Wee1 with a specific inhibitor shows promising antitumor effects in preclinical studies. In fact, a Wee1 inhibitor is in clinical trials for solid tumors, and its use may be a successful strategy in p53-mutated cancer cells.

Interestingly, Wee1 expression is elevated in HCV-infected hepatocytes. Treatment of HCV-infected cells with anti-miR-373 displays inhibition of Wee1 expression, suggesting the possibility that NORAD contributes to the regulation of Wee1 expression. *In silico* analysis does not predict an association of the HCV genome with miR-373. How miR-373 is regulated in HCV-infected hepatocytes remains unclear. In pancreatic cancer, miR-373 is regulated by the cyclic AMP (cAMP)-responsive element-binding protein (CREB) (22). HCV infection also induces CREB phosphorylation (23). It is possible that miR-373 is regulated by HCV through CREB, a possibility that will be addressed in future studies. To protect the integrity of the genome, normal cells depend on various mechanisms for DNA repair; however, due to genetic changes, most tumor cells are disabled for repairing DNA damage. Further, p53 is either mutated or underexpressed in human HCC, including available cell lines. An important caveat with regard to HCV-infected hepatocytes for *in vitro* studies is the use of transformed cell lines. In IHH, p53 is downregulated, and Huh7 cells express high levels of mutant p53. Cell cycle regulation in normal uninfected hepatocytes may differ from that of HCV-infected primary human hepatocytes, although miR-373 was originally identified in HCV-infected primary human hepatocytes (7). During HIV infection, Vpr induces G_2/M cell cycle arrest by inactivating Cdc2, while a Wee1 inhibitor abrogates this restriction by acting as a Vpr suppressor (24). Thus, functional activation of the G_2/M checkpoint by

FIG 6 Silencing miR-373 reduces the growth of hepatocytes harboring HCV. (A) (Left) Huh7.5 cells harboring genome-length HCV were transfected with 25 nM control or anti-miR-373. Viable cells were counted using trypan blue at the indicated time points. (Right) HCV-harboring Huh7.5 cells were treated with a vehicle (control) or 300 nM MK1775, and viable cells were counted at the indicated time points. Data are presented as means and standard deviations from at least three independent experiments. (B) HCV-infected Huh7.5 cells were transfected with 25 nM control or anti-miR-373, and RNA was isolated after 48 h. The relative expression of Wee1 mRNA was analyzed by qRT-PCR. 18S rRNA was used as an endogenous control and for target gene normalization. (C) (Left) HCV-infected cells were transfected with 25 nM control or anti-miR-373, and cell lysates were subjected to Western blot analysis using the indicated antibodies. The membrane was reprobbed with an antibody to actin for normalization. (Center and right) Densitometry analysis was performed using ImageJ software. Data are presented as the means and standard deviations from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

the modulation for Wee1 expression may be critical for the survival of HCV-infected cells.

We observed enriched expression of NORAD in the miR-373-Ago2 complex. Cytoplasmic expression of NORAD suggested that this lncRNA is not directly involved in transcriptional regulation. NORAD is elevated in HCV-infected hepatocytes. Aberrant expression of NORAD has been reported in several cancers, including esophageal, breast, colorectal, and pancreatic cancers (25–28). On the other hand, differential expression of NORAD has been reported in HCC, although higher expression of NORAD was correlated with poor survival (25). Abnormal expression of the Pumilio 1 gene is associated with chromosomal aberration and cancer, neurological disorder, and cardiovascular disease (29, 30). We noted the presence of PUM1 in the miR-373-Ago-2 complex, and a subsequent target search revealed a miR-373 binding site in the PUM1 3' UTR. However, PUM1 is upregulated in HCV-infected cells. We postulate that the enhancement of PUM1 may be due to the interaction between NORAD and miR-373, resulting in the rescue of PUM1 in HCV-infected hepatocytes. However, the role of PUM1 in HCV-infected hepatocytes remains unknown and will be examined in future studies.

A link among miR-373, NORAD, and Wee1 was identified in this study, and their relationship was established in HCV-infected hepatocytes. The ratio of miR-373 to NORAD is difficult to calculate. NORAD is almost 5 kb long, and miR-373 is only 22

FIG 7 Schematic diagram of miR-373 and NORAD in the regulation of Wee1 in HCV-infected cells.

nucleotides long. Although several miRNAs are predicted to bind NORAD, the nucleotide components of their miRNA response elements may be different. Further, miRNAs are detected with variable binding affinities due to binding strength rather than target site frequency (31).

This is the first study dissecting the functions of miR-373 and NORAD in the context of HCV infection and hepatocyte growth regulation. We suggest that two noncoding RNAs, NORAD and miR-373, form a complex resulting in the release of their common target, Wee1, in HCV-infected cells, which, in part, favors uncontrolled cell growth (Fig. 7). It is conceivable that NORAD acts as a miRNA sponge enabling miR-373 to regulate Wee1 in HCV infection, and this regulation provides a novel mechanism of HCV-mediated hepatocyte growth regulation. Our study not only suggests a previously unknown mechanism for hepatocyte growth promotion following HCV infection but also provides a potential target for future therapy against HCV-mediated liver pathogenesis.

MATERIALS AND METHODS

Cells, HCV infection, and transfection of noncoding RNAs. Immortalized human hepatocytes (IHH) and hepatoma Huh7.5 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, MO) containing 10% fetal bovine serum (FBS), 100 U of penicillin/ml, and 100 mg of streptomycin/ml. The generation of IHH has been described previously (7). All cells were maintained at 37°C under a humidified 5% CO₂ atmosphere. HCV genotype 2a (clone JFH1) was grown in Huh7.5 cells. The virus released into the cell culture supernatant was filtered through a 0.45- μ m-pore-size cellulose acetate membrane and was quantitated in standard international units (IU) per milliliter.

Hepatocytes were incubated with HCV genotype 2a (multiplicity of infection, 1) for 48 h. Hepatocytes were transfected with a control miR, a miR-373 mimic, miR-19a, or miR-10b (Ambion) using EndoFectin (GeneCopoeia) according to the manufacturer's instructions and were incubated for 48 h. The plasmid DNA of full-length NORAD (pcDNA3_PI_LOC647979) was a kind gift from Joshua Mendell, University of Texas Southwestern Medical Center. Hepatocytes were transfected with control pcDNA3 or pcDNA3-NORAD using EndoFectin.

Ago2 RNA coimmunoprecipitation and RNA sequencing. miR-373-transfected hepatocytes were lysed with lysis buffer (150 mM KCl, 25 mM Tris-HCl [pH 7.4], 5 mM EDTA, 1% Triton X-100, 5 mM dithiothreitol [DTT], a protease inhibitor mixture, and 100 U/ml RNaseOUT [Invitrogen]). Lysates were clarified, incubated with an anti-Ago2 monoclonal antibody (MAb) (11A9; Sigma) or isotype control IgG2a at 4°C overnight, and mixed with protein G-Sepharose (GE Healthcare) for 2 h. The beads were washed

four times, and RNA was isolated by using the RNeasy minikit (Qiagen) and was sequenced on an Illumina HiSeq-2500 system using single reads extending for 50 bases.

RNA isolation and reverse transcription-quantitative PCR. Total RNA was isolated from cells using TRIzol reagent (Invitrogen, CA). cDNA was synthesized using miR-373- or U6-specific primers with a TaqMan microRNA reverse transcription kit and a random hexamer with SuperScript III reverse transcriptase. Real-time PCR was performed for the quantitation of gene expression using TaqMan Universal PCR master mix and 6-carboxyfluorescein (FAM)-MGB probes for miR-373 (assay ID 000561) and Wee1 (assay ID Hs01119384_g1) according to the manufacturer's protocol (Thermo Fisher Scientific). U6 (assay ID 001973) or 18S rRNA (assay ID Hs03928985_g1) was used as an endogenous control for microRNA or gene expression, respectively. Gene expression analysis of NORAD (forward primer [FP], 5'-AGCGAAGTCCCGAACGACGA-3'; reverse primer [RP], 5'-TGGGCATTTCACACGGGCCAA-3') was carried out by a SYBR green-based detection system as per standard procedure. GAPDH (FP, 5'-CATGTTTCGTCATGGGTGTGAA CCA-3'; RP, 5'-AGTGATGGCATGGACTGTGGTCAT-3') was used as an endogenous control. The relative gene expression was analyzed by using the $2^{-\Delta\Delta C_T}$ formula ($\Delta\Delta C_T = \Delta C_T$ of the sample $- \Delta C_T$ of the untreated control).

Western blot analysis. Cell lysates were prepared by using 2× SDS sample buffer, and Western blot analysis was performed using a specific antibody to Wee1 (dilution, 1:500; SC-325; Santa Cruz Biotechnology), phospho-Cdc2 (Tyr 15) (1:1,000; catalog no. 9111; Cell Signaling Technology), total Cdc2 (1:1,000; catalog no. 9116; Cell Signaling Technology), or Pumilio 1 (1:1,000; catalog no. 12322; Cell Signaling Technology). The blot was reprobed with a β -actin antibody (1:5,000; catalog no. 12620; Cell Signaling Technology) to compare protein loads in each lane. Densitometry analysis was done by using ImageJ software.

Luciferase reporter assay. The Wee1 3' UTR luciferase reporter plasmid (pMIR-Report) was a gift from Nancy Zeleznik-Le, Loyola University Medical Center. The miR-373 binding site in NORAD mRNA was generated by cloning the PCR-amplified human NORAD sequence into the MluI/HindIII sites of the pMIR-Report luciferase reporter plasmid. The primers used were 5'-ATTCAATGCTACGCGTGATATAATG GAA-3' (FP) and 5'-CCATGTTGGCCAAGCTTGGTCCT-3' (RP). For the luciferase assay, cells were cotransfected with the luciferase reporter plasmid and a control miR, mimic miRNAs, or lncRNA. In all experiments, a green fluorescent protein (GFP)-tagged cytomegalovirus (CMV) plasmid was used as an internal control for determination of the transfection efficiency. Cell extracts were prepared after 48 h of transfection, and the relative luciferase activity was determined.

Statistical analysis. All the experiments were carried out at least in triplicate. The results are presented as means $\pm$ standard deviations. Data were analyzed by Student's *t* test. A *P* value of <0.05 was considered statistically significant.

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