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Interaction of Host Cell microRNAs with the HCV RNA Genome during Infection of Liver Cells

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Abstract

It has remained an enigma how hepatitis C viral (HCV) RNA can persist in the liver of infected patients for many decades. With the recent discovery of roles for microRNAs in gene expression, it was reported that the HCV RNA genome subverts liver-specific microRNA miR-122 to protect its 5' end from degradation by host cell exoribonucleases. Sequestration of miR-122 in cultured liver cells and in the liver of chimpanzees by small, modified antisense RNAs resulted in dramatic loss of HCV RNA and viral yield. This finding led to the first successful human trial in which subcutaneous administration of antisense molecules against miR-122 lowered viral yield in HCV patients, without the emergence of resistant virus. In this review, the authors summarize the molecular mechanism by which miR-122 protects the HCV RNA genome from degradation by exoribonucleases Xrn1 and Xrn2 and discuss the application of miR-122 antisense molecules in the clinic.

Keywords

hepatitis C virus; microRNA; exoribonucleases

Hepatitis C virus (HCV) is a hepatotropic, single-stranded, positive-sense, enveloped RNA virus that belongs to the family *Flaviviridae* and the genus *Hepacivirus*. The liver is the primary target organ, with the hepatocyte being its primary target cell. Hepatitis C virus can establish persistent infections that lead to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.¹ With an estimated 170 million people chronically infected worldwide,² and more than 350,000 people dying from HCV-related liver disease each year, HCV infection is a serious global health problem.

Current therapies, based on the protease inhibitors boceprevir and telaprevir, in combination with pegylated interferon and ribavirin, have been ineffective and result in the emergence of resistant viruses (reviewed in³). However, the recently developed compound Sofosbuvir (Gilead, Foster City, CA), which is a nucleoside analog inhibitor of the viral RNA-dependent RNA polymerase NS5B, has shown a phenomenal sustained virological response

rate of over 95% and is clearly the most effective treatment to combat HCV in the absence of a vaccine.

The HCV RNA genome is 9.6 kilobases in size, and it contains conserved 5' and 3' untranslated regions (UTRs) that are important for viral replication and translation (Fig. 1). An internal ribosome entry site directs the synthesis of a large, 3010 amino acid viral polyprotein, which is co- and posttranslationally cleaved by host and viral proteases to yield 10 mature structural and nonstructural (NS) proteins, each with distinct functions (Fig. 1). The structural proteins consist of two envelope glycoproteins (E1 and E2), both of which are targets of host antibody responses, and the core protein, which interacts with progeny viral genomes for assembly of the virus. The nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B form a complex with viral RNA to initiate replication on specialized endoplasmic reticulum-derived membranes, the so-called membranous web.⁴ Viral assembly initiates at the interface of the replication complex and cytoplasmic lipid droplets, which are cellular lipid storage organelles important for lipid homeostasis. The assembly complex then follows the cellular host pathway for lipoprotein production to achieve the secretion of very low-density lipoprotein- like lipoviral particles.

A large number of virus-host interactions take place in virus-infected cells. For example, viruses usurp certain host factors to aid in the translation and replication of the viral genome. On the other hand, many viruses counteract the host innate immune system by encoding factors that mimic host cell ligands to inhibit host cell signaling, or proteinases that degrade factors of the innate immune response. In this review, we describe the subversion of the liver-specific micro-RNA miR-122 by HCV to protect its RNA genome from degradation by host 5'-3' exonucleases. Furthermore, we discuss the findings that overexpression of specific micro-RNAs reduce the intracellular abundance of HCV RNA, suggesting a novel antiviral approach to lower HCV viral yield.

The Intricate Biogenesis of microRNAs

MicroRNAs (miRNAs) comprise a large class of endogenous, noncoding, single-stranded RNAs of 19 to 25 nucleotides in length. These short RNA molecules function in posttranscriptional gene silencing by base pairing with their target mRNAs to promote mRNA target cleavage and translational repression. This is accomplished in most cases by interaction of the seed sequences in miRNAs (nucleotides 2–8) with complementary seed match sequences, located in 3' noncoding regions of target messenger RNAs (mRNAs), to inhibit mRNA function.^{5,6} MiRNAs are one of the largest gene families, accounting for approximately 1% of the human genome and modulating the expression of at least one third of all human mRNAs.⁷ MiRNAs can function in a tissue-specific manner, and are important for diverse biological processes such as development, cell proliferation, apoptosis, cancer, and viral infection.

Genomic positions of miRNA genes indicate that they can reside as distinct transcriptional units or in polycistronic clusters from where they can be transcribed as distinct miRNAs.⁸ Unlike most small RNAs that are transcribed by RNA polymerase III (Pol III), the transcription of miRNA genes is mediated by Pol II. Long primary transcripts (pri-miRNAs)

that contain 5' cap structures and 3' poly(A) tails, and a local RNA hairpin structure are generated by Pol II (Fig. 2).⁹ The subsequent processing of the pri-miRNA occurs in two steps, first in the nucleus to precursor miRNA (pre-miRNA), and then in the cytoplasm to mature miRNA. In the nucleus, the RNase III-type enzyme Drosha, aided by its cofactor DGCR8, cleaves the stem-loop structure of the pri-miRNA to release a pre-miRNA of approximately 70 nucleotides (Fig. 2). The pre-miRNA hairpin is exported to the cytoplasm by the nuclear transport receptor exportin-5. In the cytoplasm, the pre-miRNA is subjected to a second processing step by another RNase III enzyme called Dicer, aided by its cofactor TRBP, to give rise to an approximately 22-nucleotide miRNA duplex (Fig. 2).¹⁰ The less stable of the two strands in the duplex RNA is incorporated into an effector complex, known as the miRNA-containing RNA-induced silencing complex (miRISC), that contains one of the four Argonaute (Ago) proteins and factor GW182. The miRISC causes translational repression by inhibition of ribosome recruitment to the capped 5' end of the mRNA and, subsequently, degradation of the target mRNA by activation of the CNOT1/CCR4 deadenylase complex (Fig. 2).^{11–13}

Remarkable progress has been made in our understanding of miRNA biogenesis and function in the last few years. In-depth studies of the mechanisms that are used by miRNAs to control viral infection and how viruses subvert potential inhibitory miRNAs are currently under intense scrutiny.¹⁴ In the next section, we will discuss how the HCV RNA genome subverts the liver-specific miR-122 to protect its RNA genome.

Subversion of Liver-Specific miR-122 by the HCV RNA Genome

The nucleotide sequences of the highly abundant, liver-specific miRNA-122 are highly conserved from zebrafish to humans.¹⁵ With approximately 66,000 copies per cell, miR-122 constitutes over 70% of the total miRNA population of the human liver.¹⁶ Extensive studies have revealed the important functions of miR-122 in lipid metabolism. Specifically, inhibition of miR-122 by antisense miR-122 oligonucleotides dramatically reduced fatty acid and sterol biosynthesis rates in mice and humans.^{17–19} Thus, miR-122 normally upregulates fatty acid and sterol biosynthesis, likely through the downregulation of an inhibitor of these pathways.

Curiously, miR-122 plays a crucial role in the HCV life cycle, as it positively modulates HCV RNA abundance. It was noted that HCV RNA and protein abundances dramatically decreased when miR-122 was sequestered by antisense miR-122 oligonucleotides.²⁰ The effects of miR-122 on HCV RNA abundance were studied by Machlin et al²¹ using a miR-122 mutational analysis approach. It was discovered that miR-122 forms an extensive oligomeric complex with the 5' end sequences of HCV RNA. This unique oligomeric complex likely protects the viral genome from nucleolytic degradation or sensors of the immune response²¹(Fig. 3).

Li et al²² reported that miR-122 can protect the viral RNA genome from degradation by the 5'-3' exoribonuclease Xrn1. However, depletion of Xrn1 did not allow replication of the viral RNA in the absence of miR-122. We discovered that Xrn2, the Xrn1 paralogue, is responsible for the degradation of the viral RNA genome, and that depletion of Xrn2 by

siRNAs allowed the viral RNA to replicate in the absence of miR-122.²³ To reconcile these differences, it is important to understand the roles of these two exoribonucleases in cellular and viral metabolism.

Roles of Exoribonucleases Xrn1 and Xrn2 in Cellular and Viral RNA

Turnover

Exoribonucleases 1 (Xrn1) and 2 (Xrn2) are evolutionarily conserved 5'-3' exoribonucleases that were first identified in *Saccharomyces cerevisiae*.^{24–27} Xrn1 (175 kDa) and Xrn2 (108 kDa) show extensive conservation within their N-terminal regions and share an enzymatic site.²⁸ Sequence conservation is much lower outside the XRN active site domain. For example, the C-terminus of Xrn1 extends much further than the C-terminus of Xrn2. Xrn1 is localized in the cytoplasm,²⁹ whereas Xrn2 is predominantly localized in the nucleus.³⁰ Orthologs of the exoribonucleases have been identified in most model organisms, suggesting important functions for these nucleases.^{31–36}

Xrn1 is responsible for the degradation of a wide range of cytoplasmic RNAs, including mRNAs that have undergone attack by RNAi or nonsense-mediated decay. On the other hand, Xrn2 plays important functions in RNA metabolism in the nucleus, such as processing of ribosomal RNAs and small nucleolar RNAs,³⁷ and it is crucial for the transcription termination of Pol II.^{38–41} Although Xrn1 and Xrn2 show redundancy in the processing of some of their substrates RNAs, only XRN2 is essential in *S. cerevisiae*.²⁴

These exoribonucleases have also been found to play roles in the degradation of the HCV RNA genome. Xrn1 was identified as a restriction factor for HCV by Jones et al.⁴² Li et al.²² found that siRNA-mediated depletion of Xrn1 slowed the decay rate of HCV RNA in liver Huh7.5 cells that were stably infected with a genotype 1a virus (H77S.3), whereas depletion of PM/Scf-100, a component of the exosome, had no effects on the decay rates of viral RNA. Xrn1 depletion also enhanced viral RNA abundance and production of infectious virus. However, depletion of Xrn1 was reported by others to have no effects on HCV abundance.^{43–45} We reported that Xrn2 modulates HCV RNA abundance. Specifically, depletion of Xrn2 did not alter translation or replication rates of HCV RNA, but affected RNA stability. Importantly, during sequestration of miR-122, Xrn2 depletion restored HCV RNA abundance, arguing that Xrn2 depletion eliminates the miR-122 requirement for viral RNA stability.²³ The cause for the different effects seen by different investigators has not been sorted out yet. It is possible that different HCV RNA complexes exist in infected cells, only some of which may contain bound miR-122, or some complexes may be specially localized because of their engagement in translation, replication, or packaging. Thus, Xrn1 and Xrn2 seem to target distinct complexes that display different requirements for miR-122.

Roles for Components of the MicroRNA Pathway during HCV Infection

Because miR-122 plays an essential role in the life cycle of HCV, it was of interest to examine whether factors of the microRNA pathway that generate mature miR-122 affect HCV RNA abundance. Wilson et al.⁴⁶ investigated the role of RISC proteins during HCV infection and reported that Ago2 was required for miR-122 to enhance HCV RNA

accumulation and translation. Ago2 has also been implicated in miR-122-mediated stabilization of HCV RNA.^{47,48} In addition, Dicer and TRBP were also found to be required for efficient HCV replication.⁴⁹ Dicer and TRBP are essential for pre-miR-122 processing and activity, arguing that processing of miR-122 by Dicer and TRBP is required for miR-122 function on HCV RNA abundance. In an interesting twist, Cox et al⁵⁰ also noted that pre-miR-122 can fulfill the functions of mature miR-122 in stabilization of the HCV RNA genome, suggesting that Dicer/TRBP complexes are needed for the generation of additional microRNAs, other than miR-122, to aid in HCV RNA amplification.

Effects of Non-miR-122 Host Cell miRNAs on HCV RNA Amplification

Binding sites for several miRNAs, other than miR-122, have been detected on the HCV RNA genome. For example, Pedersen et al⁵¹ reported that miR-448 and miR-196 directly target the RNA genome of HCV. MiR-448 binds to the coding sequence of core, whereas miR-196 interacts with a sequence within NS5A (Table 1). Overexpression of these miRNAs reduced HCV RNA replication.⁵¹ Interestingly, miR-448 and miR-196 are positively modulated by interferon alpha/beta (IFN α/β), suggesting that the IFN network can use miRNAs to combat viral infections.

In addition, Murakami et al⁵² used a miRNA target-search algorithm to identify miRNAs that target the HCV RNA genome. MiR-199a* was identified and its binding site was located in stem loop (SL) II of the 5' UTR of HCV, downstream of the binding sites for miR-122. The miR-199a* binding sites on the HCV genome are conserved across genotypes. It was found that miR-199a* overexpression inhibited HCV RNA replication in Huh7-1b or -2a HCV replicons cells, whereas miRNA sequestration using antisense oligonucleotides caused an increase in viral RNA replication. This work suggests that miRNAs can negatively regulate HCV RNA replication. The antiviral activity of miR-199a* is dependent on its direct interaction with the viral genome because mutations that altered the binding site eliminated the effects of miR-199a* on HCV RNA abundance. Because the secondary structure of SLII is important for both translation and replication of the viral genome,⁵³ the exact mechanism by which miR-199a* reduces HCV RNA abundance remains unknown.

Let7-b, which is known to function as a tumor suppressor miRNA in lung cancer^{7,49,54} and in high-risk prostate cancer,⁵⁵ and to contribute to the epithelial immune response against *Helicobacter pylori* infection,⁵⁶ has also been shown to be a regulator of HCV replication.⁵⁷ Cheng et al⁵⁷ showed that let-7b suppresses HCV replication, but has no effect on HCV RNA translation. Their mutational analysis also showed that let-7b directly interacts with the HCV RNA genome at different positions to modulate viral RNA abundance. Two let-7b binding sites are located within the coding region of NS5B, and in one in SLIV of the 5' UTR (Table 1). All of these sites are conserved among HCV genotypes and were shown to bind let-7b. Another potential target site was found in SL1 at the 3' UTR and does not appear to be functional. The main caveat with effects on non-miR-122 molecules on HCV RNA amplification is that all experimental protocols involve overexpression of the examined microRNAs. In contrast to miR-122, these miRNAs are much less abundant in liver cells than miR-122, and it is currently unknown whether they affect HCV gene expression at physiological concentrations.

Loss of HCV in Patients Treated with Locked Nucleic Acids that Sequester miR-122 in the Liver

The loss of HCV RNA abundance and virus yield by antisense oligonucleotides directed against miR-122 in cultured cells encouraged Santaris Pharma (Copenhagen, Denmark) to conduct preclinical trials in chimpanzees infected with HCV. It was found that a locked nucleic acid directed against miR-122, termed *miravirsen*, reduced viral load by 2.6 logs in the animals without displaying adverse effects, and importantly, without the emergence of drug resistance.⁵⁸ Pharmacokinetic analysis showed that miravirsen was secreted from the animals after 7 weeks of administration. Subsequently, in phase 2 clinical trials, patients who had not been treated before with any antiviral compound, were subcutaneously injected with miravirsen once a week for 5 weeks.¹⁸ At the highest dose of 7 mg/kg, the viral load in treated patients declined by 3 logs and was undetectable in four out of nine patients.¹⁸ Again, there was no evidence of the appearance of escape mutations, at least in the viral noncoding regions that include the binding sites for miR-122.¹⁸ Follow-up studies with these patients will reveal whether miravirsen is efficacious and safe in the long term. It is important to note that mice that lack the gene for miR-122 do have a higher risk of developing a fatty liver and hepatocellular carcinoma.^{59,60} Thus, miR-122 functions as a tumor-suppressor gene and should only be sequestered short term. Despite this caution, short-term application of an antagomir directed against miR-122 together with the application of a long-term powerful and novel therapeutic agent may quickly lower viral yield by several logs. This could allow the innate and humoral immune responses to take over the infection.

Concluding Remarks

The past few years have revealed that mature miRNAs and pre-miRNAs can display astonishing diverse functions. In infected liver, HCV can subvert both pre-miR-122 and mature miR-122 to aid in the protection of the viral RNA genome from degradation by host cell 5'-3' exoribonucleases. Because the liver is amenable to targeting miRNAs with small anti-sense molecules, sequestration of miR-122 presents an Achilles' heel for HCV. Long-term phase 2b and phase 3 clinical trials with antisense molecules directed against miR-122 will reveal whether sequestration of miR-122 is an additional antiviral therapeutic venue in addition to the classical application of compounds that inactivate virus-encoded enzymes.

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Abbreviations

Ago2	Argonaute 2
HCV	hepatitis C virus
mRNAs	messenger RNAs

miRNAs	microRNAs
miRISC	microRNA-induced silencing complex
NS	nonstructural
pol II, III	RNA polymerase II, III
pre-miRNA	precursor microRNA
pri-miR	primary microRNA
SL	stem loop
UTR	untranslated region

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Fig. 1.

Hepatitis C virus (HCV) RNA genome. The HCV RNA genome consists of a single-stranded, positive-sense RNA molecule. It contains one long open reading frame (ORF) that is flanked by 5' and 3' untranslated regions (UTRs). Internal ribosomal entry site- (IRES-) mediated translation of the ORF gives rise to a polyprotein that is co- and posttranslationally processed into structural (C, E1, E2) and nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5B) viral proteins.

Fig. 2. MicroRNA (miRNA) processing pathway. Transcription of the primary miRNA transcript (pri-miRNA) by RNA polymerase II and pri-miRNA cleavage by the Drosha-DGCR8 complex occurs in the nucleus. The resulting precursor hairpin (pre-miRNA) of 50 to 70 nucleotides is exported from the nucleus to the cytoplasm. In the cytoplasm, the RNase Dicer, in complex with the double stranded RNA-binding protein TRBP, cleaves the pre-miRNA hairpin to its mature length. The miRNA duplex is loaded into the RNA-induced silencing complex miRISC (Ago1–4, GW182 in mammals). One of the duplex strands is preferentially retained in miRISC, where it guides miRISC to silence target mRNAs by mRNA translational repression and deadenylation by CNOT1/CCR4.

Fig. 3. Hepatitis C virus– (HCV–) miR-122 interactions. Two molecules of miR-122 form extensive base-pairing interactions with the 5' untranslated region (5' UTR) of the HCV RNA genome to protect it from Xrn2-mediated 5'-3' degradation. The viral 5' UTR and open reading frame (ORF) are indicated.

Table 1

Summary of microRNAs that interact with the HCV RNA genome

miRNA	Target	Function	Reference
miR-199a*	Domain II of HCV IRES	Inhibits HCV RNA replication	52
miR-448	HCV core coding region	Inhibits HCV RNA replication	51
miR-196	HCV NS5A coding region	Inhibits HCV RNA replication	51
let-7b	Stem loop IV of 5' UTR HCV NS5B coding region	Inhibits HCV RNA replication	57
miR-122	HCV 5' UTR	Enhances HCV RNA replication and translation	22
			48
			47
			21
			46
			61
			20

Abbreviations: HCV, hepatitis C virus; IRES, internal ribosomal entry site; UTR, untranslated region.