

The Role of MicroRNA in Hepatitis C Virus Replication

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Abstract

Hepatitis C virus (HCV) infection is a major global health problem. There is no effective vaccine and the current treatment regimen with pegylated interferon α and ribavirin is associated with significant adverse events. Therefore, there is an urgent need to identify new antiviral targets for HCV therapy. In recent years, a growing number of microRNAs (miRNAs) have been reported to be able to regulate HCV replication and infection by interacting with the HCV genome directly or by regulating host innate immunity to build a nonspecific antiviral state within cells. In this review, we discuss HCV virology and standard of care followed by miRNA in general, and then give a brief overview of miRNAs involved in HCV infection and discuss their potential application as a therapeutic option for the treatment of HCV infection.

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HCV: virology and treatment

Hepatitis C virus (HCV) is a small, hepatotropic, positive-strand RNA virus with a genome of approximately 9.6 kb in length.^{1,2} The HCV genome consists of a single open reading frame that encodes viral proteins and a 5' and 3' noncoding region (NCR). The 5' NCR contains the internal ribosome entry site that initiates the translation of the HCV genome into a single polypeptide;² the 3' NCR is required for efficient HCV RNA replication,³ as it has a specific tripartite structure: a variable region that is important for efficient RNA replication; a poly(U/UC) tract of variable length; and a highly conserved X tail.^{4–6} Studies have demonstrated that the conserved elements in the 3' NCR, including a minimal poly(U) tract of about 25 bases, are essential for HCV replication in cell culture and *in vivo*.^{7–10} Although the detailed mechanisms by which the 3' NCR elements act on RNA replication are not clear, it is likely that binding of one or more viral or host

proteins to this RNA structure is necessary to establish the replication complex.¹¹ The open reading frame encodes a polyprotein precursor of about 3000 amino acids that is cleaved by host and viral proteases into three structural proteins (core, E1, and E2) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (Fig. 1).^{3,12} HCV has six major genotypes and each genotype contains numerous variants.¹³ Each genotype has its own epidemiologic characteristics. Genotypes 1–3 have worldwide distribution. Genotype 1 is predominant in America^{14,15} and Europe,^{16,17} followed by genotypes 2 and 3;^{18,19} and genotype 4 is the most common genotype in Africa and the Middle East,^{20–22} and is often seen among immigrants or indigenous injection drug users in North America and Europe.^{23,24} Genotype 5 is mainly distributed in South Africa²⁵ and genotype 6 has been found primarily in Asia.^{26–28}

HCV infection is a major cause of liver disease, with a high possibility of chronic infection. If left untreated, chronic HCV infection frequently results in progressive fibrosis, cirrhosis, and an increased risk of hepatocellular carcinoma.² The most recent report from the World Health Organization estimates that about 150 million people worldwide are chronically infected with hepatitis C virus (www.who.int). Unfortunately, there is still no effective vaccine for HCV.²⁹ To date, the standard of care for chronic HCV infection in most countries is combination therapy with pegylated interferon (IFN) α and ribavirin. However, the sustained virologic response (SVR) rate is just 40–50% in patients infected with HCV genotype 1 and 80% in patients infected with HCV genotypes 2 or 3.^{2,30,31} Since 2011, treatment for HCV infection has been improved by adding one of the HCV nonstructural protein NS3/4A serine protease inhibitors, telaprevir or boceprevir, to pegylated IFN α and ribavirin.³² This regimen improves the SVR to 75% in patients infected with HCV genotype 1.³³ NS3/4A serine protease plays at least two roles in the HCV life cycle. First, it is responsible for cleaving the HCV polypeptide into individual viral proteins; it is essential for viral replication and virion assembly.^{32,34,35} Second, NS3/4A protease inhibits the innate immune response to facilitate HCV persistence. Therefore, NS3/4A protease is a good target for inhibitors that inhibit HCV replication and restore the host's innate immunity. Following the successful determination of the crystal structure of NS3/4A protease, small molecules specifically binding to the catalytic site of the NS3/4A protease were developed³⁴ and two protease inhibitors, telaprevir and boceprevir, are now used to treat HCV infection in some countries. Although protease inhibitor-based therapy significantly improves the SVR,^{32,34} the high cost, severe adverse events, and rapid

Keywords: HCV; Innate immunity; Interferon; miRNA; Treatment.

Abbreviations: HCV, hepatitis C virus; DLC, deleted in liver cancer; GAP, GTPase activating protein; IFN, interferon; ISRE, interferon-stimulated response element; miRNA, microRNA; NCR, noncoding region; pre-miRNA, precursor microRNA; RIG, retinoic acid-inducible gene; SVR, sustained virologic response; TLR, toll-like receptor. Received: 17 August 2013; Revised: 15 October 2013; Accepted: 16 October 2013

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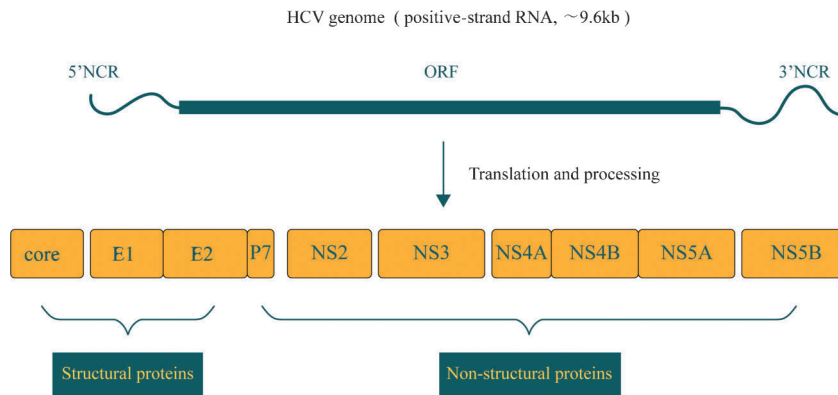


Fig. 1. The hepatitis C virus (HCV) genome and polyprotein. The HCV genome contains a single open reading frame that encodes a polyprotein that is cleaved by viral and host proteases to generate three structural and seven nonstructural proteins. Adapted from Georgel *et al.*³ and Rice¹²

emergence of resistance mutations associated with these drugs limit their use in the developing countries where treatment is needed most.³⁴ Therefore, there is a pressing need to identify new treatment options that are cost- and clinically effective for all HCV genotypes.

MicroRNA: synthesis and functions

MicroRNAs (miRNAs) belong to a noncoding gene family involved in eukaryotic posttranscriptional gene regulation.^{36–39} Transcription of miRNA genes forms primary transcripts (pri-miRNAs) that usually contain a hairpin structure. The stem-loop structure is then cleaved by an RNaseIII-like enzyme called drosha, together with its binding partner DGCR8 (DiGeorge syndrome critical region 8), to yield precursor miRNAs (pre-miRNAs) that are subsequently transferred from the nucleus to the cytoplasm with the help of exportin-5. In the cytoplasm, pre-miRNAs are processed by dicer RNA polymerase III into a duplex structure, from which one strand is separated and functions as the guide strand (functional strand) to be loaded together with Argonaute proteins into an RNA-induced silencing complex that recognizes and binds to the target mRNA. The other strand is degraded and nonfunctional (Fig. 2).^{36,40–44} MiRNA exerts its function by degrading the target mRNA (if the miRNA has perfect base pairing with its target mRNA) or inhibiting mRNA translation (if there is only partial pairing).^{45–47}

The human genome encodes more than 1500 miRNAs (www.mirbase.org, released January 2012). Each miRNA can regulate numerous target genes and each mRNA is likely to be regulated by several miRNAs.^{48,49} The interaction of miRNAs and their target mRNAs results in a complex network that is involved in almost every cell process, including development, differentiation, proliferation, death, disease pathology, and antiviral defence.^{50–53} Most recently, increasing evidence suggests that cellular miRNAs and other components of the miRNA pathway can interact with viruses at multiple levels to influence viral replication.^{54,55} In this review, we summarize the miRNAs involved in HCV infection and their proposed connection with antiviral innate immunity.

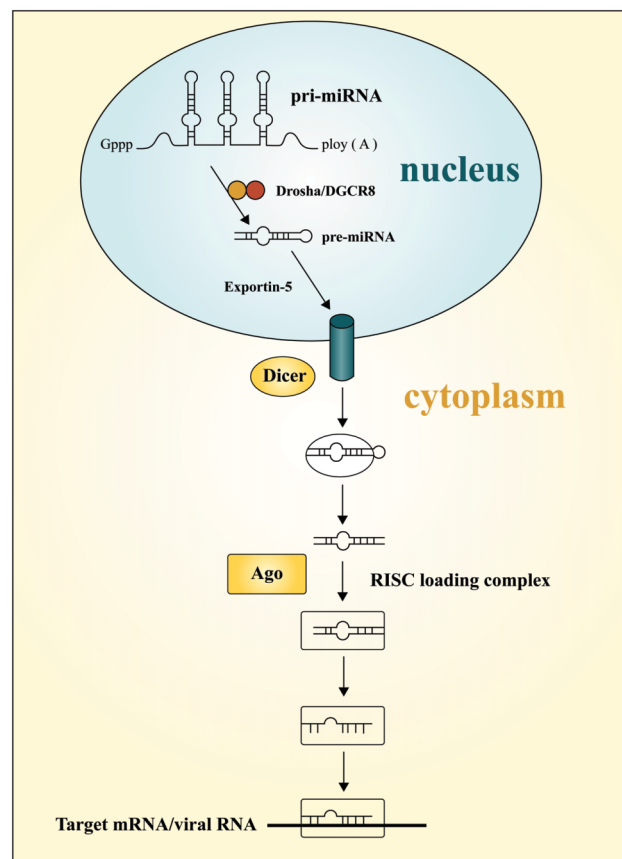


Fig. 2. The biogenesis of miRNA. Primary-miRNA (pri-miRNA) is processed by a drosha–DGCR8 microprocessor complex and forms a 70–100 nucleotide hairpin precursor (pre-miRNA). The pre-miRNA is transported from the nucleus to the cytoplasm by exportin-5 and cleaved by dicer into mature miRNA. Mature miRNA is loaded into an Argonaute (Ago) protein, where one strand of miRNA duplex is discarded. The retained strand mediates target selection by base pairing between miRNA and the 3' untranslated region of the target mRNA. Adapted from Bartel,³⁶ Broderick and Zamore,⁴⁰ Winter *et al.*,⁴¹ Kim,⁴² Thibault and Wilson,⁴³ and Haasnoot and Berkhout⁴⁴

MiRNA and the innate immune response

Innate immunity is the host's first line of defense against invading microbial pathogens. Type I IFN is a major player in the innate immune response, and is induced through pathways mediated by two distinct pathogen-associated molecular-pattern receptors: toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5. The miRNAs miR-155 and miR-146a/b have been shown to be involved in these pathways.⁵⁶ The potential target genes of miR-146a, such as interleukin-1 receptor-associated kinase 1 and 2, TNF receptor-associated factor 6, RIG-I, and IFN-regulatory factor 5, play a critical role in these type I induction pathways.⁵⁷ Inducible expression of miR-155 has been observed in both bacterial and viral infections, and may act as a negative feedback regulator of the TLR pathway to dampen the innate immune response.^{58–60} In addition, miR-155 has been shown to be involved in carcinogenesis.^{61–64} Two oncogenic viruses, Kaposi's sarcoma-associated herpesvirus and the avian α -herpesvirus Marek's disease virus type 1, can encode miR-155 mimics miR-K12-11 and miR-M4, respectively.^{65–67} Both of the virus-encoded miRNAs share high sequence similarity with miR-155 and therefore function as an ortholog of cellular miR-155.^{65–68} Although numerous lines of evidence point to the fact that more and more miRNAs are involved in host innate immunity either directly or indirectly,^{53,55,69,70} we are just at the very start of understanding the diverse roles of miRNA in regulating host immunity. More studies are needed to understand how cellular miRNAs interact with the antiviral innate immune response. Data from these studies may shed light on potential therapeutic targets.

MiRNA and HCV infection

Recent studies demonstrate that miRNAs can affect the replication of some pathogenic viruses. MiRNAs have diverse roles in HCV infection. Some cellular miRNAs inhibit HCV RNA replication, while others stimulate its replication.

MiRNAs that suppress HCV replication

Type I IFN plays an irreplaceable role in anti-HCV defense. Quite interestingly, as the key player in HCV therapy, IFN β treatment can rapidly modulate the expression of numerous cellular miRNAs.⁷¹ Using microarray technology, Pedersen *et al.* analyzed the expression of cellular miRNAs in IFN-stimulated cells and found that the expression levels of about 30 miRNAs were altered (increased or decreased).⁷¹ Furthermore, they analyzed the sequence of these miRNAs and HCV genomic RNA and identified eight IFN β -induced miRNAs (miR-1, -30, -128, -196, -296, -351, -431, and -448) that matched with HCV genome perfectly. Functional assays showed that overexpression of these miRNAs by transfection of the miRNA mimics reproduced the antiviral effect of IFN β in Huh7 cells, while their neutralization with anti-miRNAs dampened the antiviral effects of IFN β against HCV.⁷¹ Similarly, another independent study reported that miR-196 inhibited HCV RNA replication in HCV replicon cells (genotypes 1b and 2a).⁷² Both studies illustrated that one of the mechanisms for IFN inhibition of HCV RNA replication is probably the induction of cellular miRNAs that can directly degrade HCV RNA. In addition to the IFN-inducible miRNAs described above, there are many other miRNAs that can also

inhibit HCV replication. For example, the overexpression of miR-199a has been reported to suppress HCV RNA replication, while inhibition by a specific antisense oligonucleotide upregulated viral replication in two cell lines bearing the replicons HCV-1b or -2a.⁷³

In addition to the direct inhibition, some miRNAs inhibit HCV RNA replication indirectly by activating the IFN pathway. For example, miR-130a has been shown to be able to inhibit HCV replication in both replicon (genotype 1b) and JFH1 infectious models. Li *et al.* transfected miR-130a mimic into cultured Huh7.5.1 cells stably expressing an HCV genome and found that replication of HCV RNA was significantly inhibited.⁷⁴ Further studies showed that the expression levels of type I IFNs (IFN α and IFN β) were significantly increased. As Huh7.5.1 cells are deficient in both TLR3 and RIG-I, two important mediators of type I IFN induction, these cells cannot recognize HCV infection and, as a result, almost no type I IFNs are produced following virus infection. These results imply that miR-130a may inhibit HCV replication indirectly, probably by restoring the host innate immune response in TLR3- and RIG-I-deficient cells.⁷⁴ However, other investigators have demonstrated that HCV infection upregulates miR-130a, and that interferon-induced transmembrane protein 1 (IFITM1) is a direct target for this miRNA: knock-down of miR-130a enhanced IFITM1 expression and reduced HCV replication.⁷⁵ This study demonstrated that HCV evades innate immune attack by decreasing antiviral IFITM1 expression through miR-130a. Since Li *et al.* found miR-130a upregulated the IFN expression, the expression of the IFN-stimulated gene IFITM1 should increase (although not tested in their study). These two studies reached seemingly contradictory results, which may indicate that miR-130a has more than one target, and the interaction of this miRNA with the host innate immune system is complex. In any case, the interaction between miR-130a and the host innate immune system deserves further study.

MiRNAs that stimulate HCV replication

MiR-122 is the most abundant liver-specific miRNA, accounting for around 70% of the total miRNA content in mammalian liver tissue.⁷⁶ It is undetectable in other tissues in mice.⁷⁶ Studies have demonstrated that miR-122 acts as a regulator of fatty-acid metabolism in mouse liver,⁷⁷ and reduced miR-122 levels are associated with hepatocellular carcinoma.^{78–81} In addition, of all the miRNAs, miR-122 is unique in its stimulatory, not inhibitory, role in HCV replication.⁸² The discovery that miR-122 is required for HCV replication linked a host miRNA to a human infectious disease for the first time.⁸³ The role of miR-122 in HCV replication was first reported by Jopling *et al.* in 2005.⁸² They sequestered miR-122 with antisense oligonucleotides and found that HCV RNA accumulation decreased.⁸² In addition, they also identified two target sites in the HCV 5' untranslated region that are necessary for HCV replication. Since then, a large number of elegant studies have been performed to show that the interaction between miR-122 and viral 5'NCR is essential to promote HCV replication.^{84–88}

The role of miR-122 as an important, possibly essential, host factor for HCV production makes it an attractive candidate for antiviral therapy. Modified antisense agents and small-molecule inhibitors have been developed as potential new lead compounds for drug discovery.⁸⁹ Most recently, treatment with miravirsin (SPC3649) efficiently

suppressed HCV genotype 1a and 1b infections in chimpanzees, with no evidence of viral resistance or side effects.⁹⁰ Miravirsen, a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide of miR-122, has also been trialed in patients with chronic HCV genotype 1, with the results showing a dose-dependent reduction in HCV RNA levels without the occurrence of viral resistance.⁹¹

In addition to targeting HCV RNA directly, Yoshikawa *et al.* found that overexpression of miR-122 suppresses the activity of IFN-stimulated response element (ISRE) while significantly silencing miR-122-enhanced IFN-induced ISRE activity.⁹² ISREs are specific nucleotide sequences located in the promoters of IFN-stimulated genes that encode antiviral proteins and can induce transcription of these genes by binding with IFN-stimulated gene factor 3.⁹³ Furthermore, Yoshikawa *et al.* found that silencing miR-122 decreased the expression of suppressor of cytokine signaling, a negative regulator of IFN signaling, in mouse liver, leading to increased IFN anti-HCV activity.⁹² This study indicates that the anti-HCV effect of miR-122 might also be mediated by decreasing the expression of antiviral proteins or increasing negative regulators of cytokine production.

Most recently, it has been reported that miR-141, which can be induced by HCV infection, may also be necessary for efficient HCV replication.⁹⁴ MiR-141 belongs to the miR-200 family, which is believed to play an essential role in tumor suppression by inhibiting epithelial-mesenchymal transition, the initiating step of metastasis.^{95,96} Banaudha *et al.* transfected an miR-141 mimic or antagonist into HCV1a-infected hepatocytes to increase or deplete intracellular miR-141 expression, respectively.⁹⁴ The results showed that overexpression of miR-141 enhanced HCV replication, while depletion of miR-141 inhibited virus replication. Meanwhile, they identified DLC-1 (deleted in liver cancer 1) as one of the target genes.⁹⁴ Increasing miR-141 decreased DLC-1 protein levels without a parallel decrease in DLC-1 mRNA levels, suggesting that miR-141 primarily targets translational inhibition of DLC-1.⁹⁴ DLC-1 encodes a member of the Rho-GTPase activating protein (GAP) family of proteins. The Rho-GAP proteins can specifically catalyze the conversion of the active GTP-bound RhoA protein into the inactive GDP-bound protein.^{97,98} Active RhoA protein is required for Ras-mediated tumorigenic transformation,⁹⁹ and Rho-GAPs may therefore act as important negative regulators in human carcinogenesis. In hepatocellular carcinoma, homozygous deletion or loss of DLC-1 mRNA expression usually occurs *in vivo* and *in vitro*.^{98,100} In addition, restoration of DLC-1 in hepatoma cell

lines lacking DLC-1 results in reduced cell proliferation as well as reduced metastatic activity.¹⁰⁰ Since HCV-infected cells express miR-141, and its overexpression significantly suppresses DLC-1 expression,⁹⁴ the results indicate the presence of a novel mechanism of HCV infection-associated miRNA-mediated regulation of a tumor suppressor protein, which is worth further exploration.

MiRNAs involved in HCV infection and their interactions with the innate immune system are summarized in Table 1.

Future directions

MiRNAs are endogenous, short, noncoding RNAs that function at the posttranscriptional (mRNA) level through mRNA degradation or inhibition of translation. Host miRNAs play various important roles in many cellular processes, including host innate immunity and cell defense. Increasing lines of evidence suggest that many miRNAs are involved in the viral life cycle. For HCV, miRNAs can either stimulate or inhibit viral RNA replication through distinct mechanisms. In the past few years, many studies have focused on identifying differentially expressed miRNAs before and after HCV infection. Identifying their target mRNAs remains a great challenge, as a complex network of interaction exists between miRNAs and mRNAs. Although high-throughput screening methods such as genome-wide association studies and microarrays may reveal the complicated network of regulation and eventually identify targets for intervention, functional studies will have to be performed to validate these targets experimentally.

Remarkably, the advent of anti-miR-122 drugs is opening a new era for HCV therapy. Although many miRNAs have been identified *in vitro*, and the function of many of these has not been verified *in vivo*, miRNAs have great potential as therapeutic targets for viral infection, in addition to many other diseases.

Conflict of interest

None.

Author contributions

Designing the outline of this review (LMC), drafting the manuscript (XQD); collecting related information and editing the manuscript (SLL, YJL, BL, PBZ, CHY).

Table 1. Summary of miRNAs involved in HCV infection and their interactions with the innate immune system

miRNA	Effect on HCV infection	Link to innate immunity	Reference
miR-130a	Inhibits HCV replication <i>in vitro</i>	Restores the IFN signaling pathway	74
	Benefits HCV infection <i>in vitro</i>	Targets interferon-induced transmembrane proteins	75
miR-196	Inhibits HCV expression <i>in vitro</i>	Targets Bach1, which is involved in the inflammatory response	71, 72
miR-199a	Inhibits HCV replication <i>in vitro</i>	Independent of IFN signaling pathway	73
miR-122	Promotes HCV replication	Decreases IFN-induced ISRE activity	92
	Positively regulates the HCV life cycle	Targets the HCV genome directly (independent of the IFN signaling pathway)	82, 86
miR-141	Required for HCV replication <i>in vitro</i>	–	94

HCV, hepatitis C virus; IFN, interferon; ISRE, interferon-stimulated response element; miRNA, microRNA

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