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Hepatitis C virus and human miR-122: insights from the bench to the clinic

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MicroRNAs (miRNAs) are small non-coding RNAs that function as part of RNA-induced silencing complexes that repress the expression of target genes. Over the past few years, miRNAs have been found to mediate complex regulation of a wide variety of mammalian viral infections, including Hepatitis C virus (HCV) infection. Here, we focus on a highly abundant, liver-specific miRNA, miR-122. In a unique and unusual interaction, miR-122 binds to two sites in the 5′ untranslated region (UTR) of the HCV genome and *promotes* viral RNA accumulation. We will discuss what has been learned about this important interaction to date, provide insights into how miR-122 is able to modulate HCV RNA accumulation, and how miR-122 might be exploited for antiviral intervention.

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Introduction

MicroRNAs (miRNAs) belong to a class of endogenous small non-coding RNAs that are expressed in a developmentally regulated and tissue-specific manner. With over 2500 human miRNAs identified to date, they are predicted to regulate at least 60% of all human genes [1,2]. Mammalian miRNAs typically recognize partially complementary sequences in the 3′ untranslated region (UTR) of mRNAs and direct translational repression or accelerated deadenylation of their targets [3,4]. Since miRNAs participate in regulation of almost every cellular process investigated, it is not surprising that human miRNAs have been implicated in the life cycles of numerous mammalian viruses, including Hepatitis C virus (HCV).

HCV infection is a global health concern with 2–3% of the world's population infected. Infected individuals typically develop a persistent infection that can progress to chronic liver disease, steatosis (fatty liver), cirrhosis (liver fibrosis) and hepatocellular carcinoma (HCC). HCV is a hepatotropic, positive-sense single-stranded RNA virus of the family *Flaviviridae*. The ~9.6 kb genome contains a single open reading frame that is subsequently cleaved into 10 mature viral proteins. The open reading frame is flanked by 5′ and 3′ UTRs, which direct translation through an internal ribosomal entry site and replication through conserved secondary and tertiary RNA structures.

Over the past few years, HCV has been found to have important interactions with numerous miRNAs that modulate HCV replication, pathogenesis, and disease and/or treatment outcome. A summary of these miRNAs identified to date, their targets and effects on HCV replication, liver pathology and carcinogenesis is found in Table 1 (and is reviewed in Singaravelu *et al.* [5] on page 1 of this issue). For many of these miRNAs, we are just beginning to understand their complex regulation and role(s) in modulating disease pathogenesis [6]. In this review, we focus on the interaction between human miR-122 and the HCV genome, an interaction that has direct effects on viral RNA accumulation *in vitro* and *in vivo* [7••,8,9].

miR-122 directly promotes the life cycle of HCV

In contrast to most miRNAs, miR-122 *promotes* HCV accumulation through direct interactions with the viral genomic RNA [8,10]. While miR-122 is normally involved in stimulation of cholesterol biosynthesis through canonical miRNA-target RNA interactions, miR-122 modulates HCV RNA abundance independently of its effects on cholesterol and lipid metabolism [11,12]. miR-122 binds to two target sites in the 5′ UTR of the HCV genome by imperfect base-pairing, and annealing to both the seed sequence (nucleotides 2–7 of the miRNA), and auxiliary sequences (nucleotides 14–17 of the miRNA), are essential for enhanced HCV RNA accumulation (Figure 1a) [13,14••]. This relationship is unlike that seen for any other miRNA–mRNA complex, and is unique to HCV and the closely related *hepacivirus*, GB virus-B [15•]. The host miRNA pathway protein Argonaute 2 (Ago2) is essential for miR-122-mediated viral RNA accumulation but the mechanism of action remains unclear [16••,17,18]. Association with miR-122

Table 1

miRNAs implicated in HCV replication and pathogenesis

miRNA	Target(s)	Effect(s)	Reference
miRNAs that affect HCV replication			
let-7b	HCV	Inhibits HCV replication	[67]
miR-21	MyD88, IRAK1, NF-κB	Negatively regulates innate immune response	[46,68]
miR-24		Inhibits HCV replication	[69]
miR-27a/b	RXRα, PPARα, ABCA1, ANGPTL3, Lipid metabolism	Modulates lipid metabolism; inhibits HCV replication and infectivity	[70,71]
miR-30(a-d)	SOCS1/3?	Expression induced by IFNα; inhibits HCV production	[72]
miR-122	HCV	Promotes HCV replication; may suppress inflammation	[8,40]
miR-130a		Inhibits HCV replication	[73]
miR-149*		Promotes HCV production	[69]
miR-192		Expression induced by IFNα; promotes HCV replication	[72]
miR-196b	HCV	Expression induced by IFNβ; inhibits HCV replication; may suppress inflammation	[62•,74,75]
miR-215		Promotes HCV replication	[76]
miR-296		Expression induced by IFNβ; inhibits HCV replication	[62•]
miR-320c	PI3K/Akt, MAPK, NF-κB	Negatively regulates innate immune response	[77]
miR-351		Expression induced by IFNβ; inhibits HCV replication	[62•]
miR-431		Expression induced by IFNβ; inhibits HCV replication	[62•]
miR-448	HCV	Expression induced by IFNβ; inhibits HCV replication	[62•]
miR-483-5p	PI3K/Akt, MAPK, NF-κB	Negatively regulates innate immune response	[77]
miR-491		Modulates PI3K/Akt signaling; promotes HCV replication	[76]
miR-638		Promotes HCV production	[69]
miRNAs that affect liver pathology and carcinogenesis			
miR-29	COX-2?, IFNλ?	Inhibits HCV replication; modulates hepatic stellate cell activation and liver fibrosis	[69,78–80]
miR-124		Modulates tumor progressiveness	[81,82]
miR-155	Wnt Signaling	Modulates inflammation and promotes HCC	[83–85]
miR-199a*	HCV	Correlates with liver fibrosis; may inhibit HCV replication; tissue tropism?	[86,87]
miR-221/222	CDKN1C/p57, CDKN1B/p27	Correlates with liver fibrosis; correlates with HSC activation; may modulate carcinogenesis	[87–90]
miR-449a		Modulates the inflammatory response via NOTCH signaling	[91]

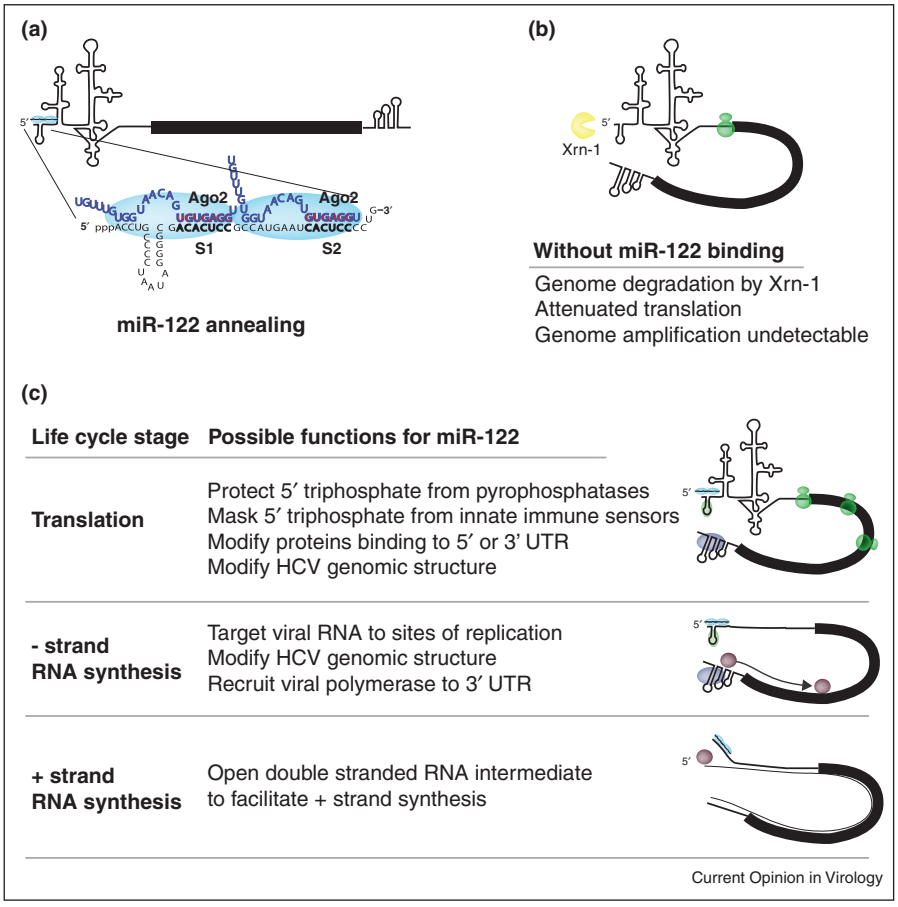
HCV, hepatitis C virus; MyD88, myeloid differentiation primary response protein 88; IRAK1, interleukin-1 receptor-associated kinase 1; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RXRα, retinoid X receptor-α; PPARα, peroxisome proliferator activated receptor-α; ABCA1, ATP-binding cassette transporter 1; ANGPTL3, Angiopoietin-like 3; SOCS, suppressor of cytokine signaling; interferon (IFN); PI3K, phosphatidylinositol-3-kinase; Akt, v-Akt murine thymoma viral oncogene; MAPK, mitogen activated protein kinase; COX, cyclooxygenase, Wnt, wingless-related integration, hepatocellular carcinoma (HCC); CDKN, cyclin-dependent kinase inhibitor; HSC, hepatic stellate cell.

stabilizes the viral RNA by protecting the 5′ terminus of the HCV genome from degradation by the host 5′–3′ exonuclease, Xrn-1 [19••,20•] and has also been reported to modestly stimulate HCV translation (Figure 1b) [21]. However, while genome stabilization may be important for miR-122 promotion of HCV RNA accumulation there appears to be an as yet unidentified function for miR-122 in promoting HCV RNA accumulation beyond protection from Xrn-1, since depletion of Xrn-1 restores stability to viral RNAs where miR-122 binding has been disrupted, but does not restore viral RNA replication in the absence of miR-122 [19••]. Furthermore, binding of mutant miR-122 molecules that protect HCV genomes from degradation by Xrn-1 *in vitro* fail to support genome replication *in vivo* [20•]. These results suggest that protecting the genomic RNA from degradation by Xrn-1 is not the only role for miR-122 in the HCV life cycle.

Hypothetical models for the mechanism of action of miR-122

Many questions therefore remain regarding the mechanism by which miR-122 promotes HCV RNA accumulation and there are several potential roles for miR-122 based on our current understanding (Figure 1c). These include masking the viral 5′ end from additional enzymes and sensors. The HCV genome has a triphosphate on its 5′ terminus [19••]; however, we do not know the mechanism by which the triphosphate is converted into a 5′ monophosphate, the substrate of Xrn-1 [22]. In addition, viral RNAs bearing 5′ triphosphates could be recognized and activate host innate antiviral proteins such as IFIT-1 and/or IFIT-5 [23]. Thus, miR-122 binding could mask the 5′ end from pyrophosphatases and/or innate immune sensors. In addition, miR-122 binding could modify protein binding to the HCV genome, or modify the viral genomic

Figure 1



Model for association of miR-122 with the HCV genome and hypothetical roles for miR-122 in HCV RNA accumulation. **(a)** Proposed annealing between miR-122 and the two binding sites on the 5′UTR of the HCV genome. **(b)** Observed phenotype of HCV genomic RNA to which miR-122 binding has been abolished. **(c)** Hypothetical functions for miR-122 binding during different phases of the HCV life cycle. These functions are not mutually exclusive.

RNA structure. Finally, miR-122 may be part of a mechanism by which viral RNA is localized to sites of viral replication or virion assembly. However, in spite of the strong impact of miR-122 in promoting HCV RNA accumulation, evidence suggests miR-122 binding is not essential for HCV replication, but potently enhances it [24••,25•].

Thus far, the individual contributions of each miR-122 binding site to genome stability and viral RNA accumulation are still unclear. For example, if the 5′ triphosphate of the HCV genome is masked by the 3′ overhang generated by site 1-bound miR-122, then binding to site 1 on the HCV genome would be primarily responsible for genome stabilization. Alternatively, binding to both miR-122 sites would be required if stabilization involves modification of HCV RNA structures or perhaps recruitment or displacement of RNA-binding proteins on the 5′ UTR. Studies on HCV genome stability, replication, and the influence of Xrn-1 on HCV RNA accumulation where one or both of the miR-122 binding sites are disrupted will provide insight into the role(s) of each miR-122 site and the relative impact of Xrn-1 in limiting HCV replication.

The Ago2 protein is known to interact with the HCV 5′UTR and is required for miR-122-mediated viral RNA accumulation, but it is still unclear whether Ago2 remains associated or simply delivers mature miR-122 to the HCV genome [16••,17]. Similarly, miR-122 and Ago2 association may recruit or displace other Ago2 or RNA-binding proteins, or alter the secondary or tertiary structure of 5′ end of the viral genome. Analysis of host and/or viral proteins binding to the HCV 5′ UTR, and changes to HCV RNA conformations in the presence or absence of miR-122 will provide further insight into the mechanism of HCV regulation by miR-122.

Despite all that we have learned about HCV:miR-122 interactions, it is still unclear at what stage(s) of the viral life cycle miR-122 associates with the HCV genome. It

seems likely that miR-122 associates with the viral genome during translation since it has been demonstrated to stabilize the genome and enhance viral translation [17,19••,21], but this has yet to be shown directly. In addition, it is unknown whether miR-122 associates with the HCV genome during viral replication or packaging, and whether miR-122 is incorporated into mature HCV virions. It has been speculated that miR-122 may regulate the switch between translation and viral RNA replication but data to support this notion are also lacking [10,19••,26,27].miR-122 also seems to play a role in HCV tropism [25•,28–30]. Considering the role of miR-122 in the life cycle of other *hepaciviruses*, one wonders if these viruses have evolved tropism for the liver based on their reliance on the liver-specific miR-122. HCV and GBV-B are both hepatotropic viruses, which have two active miR-122 binding sites in their 5′ UTRs [13,15•]. By contrast, examination of the sequence of recently identified equine and rodent *hepaciviruses* suggest a single miR-122 binding site in their 5′ UTR, but their tropisms have yet to be confirmed as they were initially isolated from the lungs or serum of infected animals [31•,32•,33]. It will be interesting to determine the tropism of these novel *hepaciviruses* and determine whether they are also subject to regulation by miR-122. However, these studies await the development of appropriate model replication systems.

miR-122 as a target for antiviral therapy

Expression of miR-122 is liver specific, where it comprises over 70% of the total miRNA population [34]. miR-122 is normally involved in regulation of fatty acid and cholesterol biosynthesis, metabolism, and transport [35,36]. Sequestration of miR-122 results in decreased gene expression of cholesterol biosynthesis pathway genes [35,36]. Furthermore, miR-122 knockout mice develop steatosis due to global impairment of lipid metabolism as well as lipoprotein assembly and secretion [36]. Furthermore, miR-122 is thought to be a tumor-suppressor since miR-122 knockout mice also develop HCC [37–39].

In spite of an incomplete understanding of miR-122-mediated viral RNA accumulation, agents that target miR-122 have been used to treat HCV infection [7••,9]. In a recent Phase II clinical trial, miravirsen, an antisense locked nucleic acid molecule that binds to and sequesters miR-122, reduced serum HCV titers in treatment-naïve HCV-infected patients [7••]. At the highest doses used, HCV RNA became undetectable, but rebounded following completion of the 4-week course of miravirsen mono-therapy [7••]. A 12-week course of treatment is currently being tested to determine if patients can achieve sustained viral clearance [7••]. Importantly, there was no evidence of virus resistance by deep sequencing [7••,9], and while there were no harmful side effects of the treatment, it decreased serum cholesterol levels due to de-repression of cellular miR-122 targets [9]. However, since miR-122 is also a tumor suppressor [36,40], treatment with miR-122 antagonists should be carried out with caution and remain short in duration.

miR-122 in HCV-infected patients

In spite of evidence that miR-122 promotes HCV RNA accumulation in cell culture and in infected patients, serum and hepatic miR-122 levels correlate poorly with HCV titer, and suggest that the relationship between HCV and miR-122 is more complicated *in vivo* than *in vitro*. Serum miR-122 abundance, while being a possible marker of liver damage, appears to be a poor measure of HCV-induced liver disease [41–43]. In addition, hepatic miR-122 levels do not correlate well with viral RNA titer, except in acute infection. During acute HCV infection, hepatic miR-122 levels increase and correlate with HCV RNA titer [44,45]. However, in chronically infected patients, hepatic miR-122 levels decrease and are inversely correlated with HCV RNA titer [44,46,47•]. Consistent with a reduction in miR-122 as the disease progresses, miR-122 levels remain low in fibrotic liver tissue [42]. In accordance with the reported tumor suppressor activity, miR-122 is typically lost in HCCs; however, miR-122 expression is conserved in HCV-induced HCC consistent with a requirement for miR-122 in HCV RNA accumulation *in vivo* [47•].

miR-122 levels and outcomes of IFN-based therapy

Although there is poor correlation between miR-122 abundance and HCV titers in infected patients, patients with low pretreatment miR-122 levels are less likely to achieve viral clearance through PEG-IFN-α plus ribavirin combination therapy [48••,49]. Recent evidence suggests a link between miR-122 expression and the human genetic polymorphism rs8099917 in the IL28B locus (also known as IFNλ3) that is predictive of the response to IFN-based therapy. This link may suggest a complex relationship between IFN, miR-122 expression *in vivo* and treatment outcomes [47•]. High endogenous pre-treatment expression of IFN-stimulated genes is associated with poor treatment outcome [50–53]. In addition, it has been established that unfavorable genotypes at two loci of the IL28B gene region are linked to both high pre-treatment IFN-stimulated genes and poor treatment outcome [49,54–59]. Further, evidence suggests that serum and hepatic miR-122 levels may be related to the IL28B genotype [47•,60••]. In two studies, low pre-treatment miR-122 levels in the liver or serum correlated with a genotype (TG or TT at rs8099917) predictive of poor response to IFN-based therapy [47•,60••]. This suggests that miR-122 levels might also be related to the IL28B genotype. However, a similar study found no correlation between serum miR-122 levels and treatment outcome, although this might

be reflective of the different analytical methods used [61]. Interestingly, IFN has been demonstrated to down-regulate the expression of miR-122 [62•,63,64], and downstream effectors of IFN may themselves be subject to miR-122-mediated regulation [63,65]. Thus, low pre-treatment miR-122 levels may be linked to the high endogenous expression of IFN stimulated genes observed in patients having the unfavorable IL28B genotype. While these data suggest a correlation between miR-122, IL28B genotype, and patient response to IFN-based therapy, it does not reveal a mechanistic link between treatment outcome and miR-122 levels. Whether changes in miR-122 levels are a bystander effect of IL28B genotype and whether the modest changes in miR-122 abundance affect HCV RNA accumulation *in vivo* remain to be seen. Future studies are likely to clarify whether differences in patient miR-122 levels are driven by IL28B genotype and/or IFN and ultimately whether treatment outcomes are related to changes in miR-122 levels in HCV patients.

Conclusion/significance

HCV is one of the most important infectious diseases affecting the world today. The development of effective, cost efficient and easily tolerated treatments is essential to control the burden of infection. The standard of care is combination IFN/ribavirin, although many patients do not benefit from this treatment. It is widely expected that in future small molecule drugs that target specific viral proteins that play essential roles in the viral life cycle (a.k.a. direct-acting antivirals) will replace IFN-based therapies. The approvals of two protease inhibitors (2011) and polymerase inhibitors (2013) were significant milestones in this regard [66]. Despite the remarkable progress in drug discovery and development, the clinical use of direct-acting antivirals is associated with their own challenges. Poor adherence to drug regimens, combined with the error-prone nature of the viral polymerase can rapidly select for drug resistance. Targeting host molecules, like miR-122, has numerous potential advantages such as a higher barrier to resistance, pan-genotypic activity and a wide range of druggable targets where viral targets are limiting. Thus, a better understanding of the mechanism by which miR-122 mediates HCV RNA accumulation will reveal novel targets for therapeutic intervention. In addition, while omission of IFN from therapeutic strategies is a likely goal for many patients, IFN-based therapies will probably persist due to the prohibitively high cost of new drug regimens. Thus, strategies to predict or enhance the success rate of IFN therapy will remain an important goal in HCV patient stratification and treatment. Finally, since the mechanism of action of miR-122 appears to be unique from that of canonical miRNAs, identification of the mechanism of action of miR-122-mediated viral RNA accumulation may lead to the discovery of novel forms of miRNA-mediated gene regulation.

Conflict of interest

The authors declare no conflict of interest.

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