MicroRNA's in chronic hepatitis B and C virus infection
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MICRORNA’S

The role of microRNAs (miRNAs) has been extensively studied since their discovery almost 25 years ago. miRNAs are small (19-24 nucleotides), non-coding RNA molecules involved in various cellular processes of post-transcriptional regulation of gene expression. The first identified miRNAs, lin-4 and let-7, were discovered as being involved in the timing of the larval development of Caenorhabditis elegans.1,2 These miRNAs were found to downregulate gene expression by base pairing with the 3’ untranslated regions (3’ UTRs) of their target messenger RNAs (mRNAs).1,2 Since the discovery of lin-4 and let-7, hundreds of miRNAs have been identified.3-5 All identified miRNAs are registered in a database, which contains more than 35,000 mature miRNA products in 223 different species.6 miRNAs are now recognized as important players in the pathogenesis of a number of liver diseases, and they are promising targets for therapeutic interventions. In 2008, it was discovered that circulating miRNAs were detectable in the serum of lymphoma patients.7 Circulating miRNAs are stable and easy to detect suggesting their potential as miRNA-based blood biomarkers.8,9 It was shown that circulating miRNAs can serve as biomarkers in several liver diseases such as chronic viral hepatitis, liver fibrosis and hepatocellular carcinoma (HCC).10-13

BIOGENESIS AND FUNCTION OF MIRNA’S

The biogenesis of miRNAs is a complex, and multi-step process (FIGURE 1).14 Briefly, primary miRNA (pri-miRNA) is transcribed via RNA polymerase II and III in the nucleus.15,16 Pri-miRNA is cleaved into smaller segments by the Drosha-DGCR8 complex to produce a hairpin precursor (pre-miRNA) in the nucleus.17,18 Pre-miRNA is transported from the nucleus into the cytoplasm by Ran-GTP and receptor exportin-5.19 In the cytoplasm, the ribonuclease Dicer cleaves pre-miRNA to form a duplex of miRNA.20 Argonaute 2 (Ago2) will select a guide strand of miRNA, incorporate this into a RNA-induced Silencing Complex (RISC) which can pair to an mRNA and thereby regulate the expression of the specific mRNA.14,21 miRNAs regulate gene expression by binding to the 3’ UTR of target mRNAs, but functional base-pairing can also occur in the 5’ UTR.22,23 The binding between the miRNA and target mRNA is almost never perfectly complementary and therefore, a single miRNA can regulate the expression of multiple mRNAs.22,24 In addition, a single UTR of a given mRNA may be targeted by numerous miRNAs or may have multiple binding sites for a single miRNA.22 It is estimated that more than 30% of human genes are conserved miRNA targets,25 and more than 45,000 miRNA target sites are conserved in the 3’ UTR, making this the most important target site of miRNAs.26 The main mode of action of miRNAs is the inhibition of mRNAs. The first identified miRNAs, lin-4 and let-7, act by translational repression of the target mRNA.1,27 Further research showed that miRNAs can block translational initiation followed by deadenylation and degradation of target mRNAs.28,29 Mature miRNAs can also be released by the cell and transferred to
other cells (FIGURE 1). These circulating miRNAs exist in different conditions: associated with RNA-binding proteins or lipoprotein complexes, but also packaged in microvesicles and exosomes.30

HEPATITIS C VIRUS

HCV is an enveloped, positive, single-stranded RNA virus of the Flaviviridae family. The HCV genome, flanked by the 5’ and 3’ UTR, contains a single open reading frame with a length of 9.6 kb which is translated into a polyprotein of about 3000 amino acids.31 This large protein is cleaved into ten proteins by cellular and viral proteases: three structural proteins (core, E1, and E2), an ion channel (p7), and six non-structural proteins (NS2, NS3A, NS4A, NS4B, NS5A, and NS5B). HCV replication is characterized by a high turnover rate with a production of around $10^{10}$ to $10^{12}$ virions per day. Lack of proof-reading activity of the HCV RNA dependent RNA-polymerase results in a high viral mutation rate. This will lead to the emergence of a viral population consisting of so called quasispecies, which are highly

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**Figure 1.** Biogenesis of miRNAs In the nucleus, primary microRNA (pri-miRNA) is transcribed from microRNA (miRNA) genes via polymerase II or III. Following, pri-miRNA is cleaved by the Drosha DGCR8 complex into precursor microRNA (pre-miRNA). Pre-miRNA is exported to the cytoplasm via the exportin-5 complex. In the cytoplasm, Dicer processes pre-miRNA to mature miRNA. Mature miRNA is loaded into the RNA-inducing silencing complex (RISC) together with argonaute 2 (Ago2). RISC can bind to a target messenger RNA (mRNA) and, depending on the complementarity of the binding, this can lead to translational repression or mRNA abundance. Mature miRNAs can also bind to RNA-binding proteins, such as Ago2 or to lipoproteins, and be released by the cells. Alternatively, miRNAs can be loaded into microvesicles or exosomes and be released into the circulation.
related but include a spectrum of mutated viruses within an infected person. In total, seven HCV genotypes have been identified, which differ by 30-35% over the complete genome, and which are further subdivided into various subtypes. HCV is a major causative agent of chronic liver disease throughout the world. Acute HCV infection results in a chronic infection in approximately 75% of the patients. Worldwide, it is estimated that 170 million people have a chronic HCV infection and are thereby at risk of developing liver-related complications. Over a period of 10 to 30 years approximately 10–20% of HCV-infected patients develops cirrhosis and 1-5% of patients with cirrhosis will develop hepatocellular carcinoma. The goal of antiviral treatment is to achieve a sustained virological response (SVR), defined as undetectable HCV RNA in the blood 12 to 24 weeks after completing a course of antiviral treatment, which is associated with a reduced occurrence of liver-related complications and a prolonged survival. Until 2014, the standard of care for chronic hepatitis C patients was pegylated-interferon (peg-IFN) therapy combined with ribavirin (RBV) with or without an HCV protease inhibitor (depending on the HCV genotype). Such a course of 24 to 48 weeks of peg-IFN based treatment was accompanied by severe side-effects and was successful in approximately 50% of CHC patients. With new insights in the HCV replication cycle, many highly potential direct-acting antiviral (DAA) agents have been developed in the last decade. The vast majority of new anti-HCV drugs target specific viral proteins, such as the NS3A protease, NS5A protein, and NS5B polymerase. Current IFN-free therapy consisting of treatment with a combination of DAAs for 12 to 24 weeks results in high SVR rates in more than 90% of CHC patients. An alternative treatment strategy is interference with host cell components that are essential for replication of HCV, such as cyclophilin A and microRNA-122 (miR-122).

**MIRNA-122 AND HCV**

HCV interacts with host cellular miRNAs during its lifecycle. miR-122, encoded at a single genomic locus in chromosome 18, accounts for 70% of the total miRNA population in the liver, and is an important host-factor for HCV replication. The first link between miR-122 and HCV was revealed in 2005 when three highly conserved binding sites for miR-122 in the HCV viral genome were identified. Two binding sites (S1 and S2) are located between stem loops I and II of the 5′-UTR (FIGURE 2) and one is located in the variable region of the viral 3′-UTR. miR-122 binds to S1 and S2 in association with Ago2 and this interaction is required for HCV RNA stability and accumulation. High-throughput sequencing and crosslinking immunoprecipitation (HITS-CLIP) using an antibody against Ago protein has confirmed binding of miR-122 to both S1 and S2 in the 5′UTR viral genome. miR-122 forms an oligomeric complex with HCV RNA wherein one miR-122 molecule binds to the 5′-terminus of the HCV RNA with 3′ overhanging nucleotides, masking the 5′-terminal sequences of the HCV genome. It has been postulated that miR-122 protects the HCV 5′-terminal sequences from degradation by cellular exoribonucleases XRN1 and/or XRN1. Although it is probably not the primary...
function of miR-122, the miR-122-Ago2 complex could also mask the presence of a 5’ triphosphate and thereby limit the recognition of HCV RNA by the immune sensor RIG-1.\textsuperscript{53} Furthermore, it was shown that interaction of miR-122 with the HCV genome may induce an increase in the rate of viral RNA synthesis.\textsuperscript{54} Another study demonstrated moderate effects of miR-122 on the HCV internal ribosome entry site (IRES) mediated translation.\textsuperscript{55} The relevance of miR-122 in HCV replication was shown in cell lines that normally do not support HCV replication such as HepG2 cells. HepG2 cells do not express endogenous miR-122 and are normally insufficient for efficient viral assembly.\textsuperscript{56} When miR-122 was expressed in HepG2 cells, efficient production of HCV RNA and release of infectious virions was observed.\textsuperscript{57} Even in non-hepatic cells, the expression of miR-122 facilitated viral replication.\textsuperscript{58} A mutation of S1 and S2 to a seed match for another miRNA (miR-21) failed to rescue regulation of HCV replication by miR-21, suggesting that HCV replication is specifically regulated by its interaction with miR-122.\textsuperscript{59}

Silencing of miR-122 using an oligonucleotide resulted in a reduced level (approximately 80\%) of HCV viral RNA in Huh7 liver cells.\textsuperscript{43} In HCV infected chimpanzees, silencing miR-122 by a locked nucleic acid (LNA)-modified phosphorothioate oligonucleotide resulted in a potent and sustained inhibition of HCV replication.\textsuperscript{44} Two chimpanzees were treated with intravenous injections of 1 mg/kg and two other chimpanzees with 5 mg/kg on a weekly basis for 12 weeks, followed by a period of 17 weeks. In the 5 mg/kg group this resulted in

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\caption{Interaction between miR-122 and HCV genome (A) Sequence of microRNA-122 (miR-122). Boxes indicate the miR-122 binding sites to the HCV genome. (B) Schematic representation of the HCV genome: genes encoding the structural and non-structural proteins are flanked by 5’- and 3’-untranslated regions (UTRs). The 5’-UTR of the HCV genome with the miR-122 binding sites is expanded. Seed site 1 (S1) interacts with nucleotides 2-8 of miR-122 and seed site 2 (S2) interacts with nucleotides 2-7 of miR-122. Nucleotides 14-16 of miR-122 have an additional base pair interaction with the HCV genome.}
\end{figure}
a maximal decrease of HCV RNA levels in the serum and the liver of 2.6 and 2.3 orders of magnitude 2 weeks after the end of treatment. Decreased HCV RNA levels were associated with improved liver histology. No apparent viral resistance was observed during treatment with the miR-122 antagonim as shown by the lack of adaptive mutations by deep sequencing of the seed sites of miR-122 in the HCV 5’-UTR.\textsuperscript{44} In 2009-2010, a phase 2a study, using an LNA-modified antisense oligonucleotide (miravirsen), was performed in 36 therapy-naive, genotype 1 chronic hepatitis C patients.\textsuperscript{40} Patients were randomized to receive either placebo or miravirsen (in doses of 3, 5, or 7 mg/kg of body weight) in five weekly doses over a 29-day period. Five injections of miravirsen resulted in a dose-dependent and prolonged decrease of HCV RNA levels. The mean of the maximal reduction in HCV RNA levels (log 10 IU/ml) from baseline was 1.2 (P=0.01) for patients receiving 3 mg/kg, 2.9 (P=0.003) for those receiving 5 mg/kg, and 3.0 (P=0.002) for those receiving 7 mg/kg, compared with a decline of 0.4 in the placebo arm. HCV RNA became undetectable in 5 of the 27 actively-treated patients.\textsuperscript{40} No serious adverse events were reported. In one patient HCV RNA was undetectable at the end of follow-up, 14 weeks after the last dose of miravirsen. Population based sequencing revealed the emergence of a single mutation (C3U) in the 5’UTR of HCV genotype 1 infected patients with virological rebound following miravirsen dosing.\textsuperscript{60} This was the first clinical trial wherein the therapeutic effect of host miRNA antagonimirs was demonstrated. In summary, miR-122 binds to two binding sites in the 5’-UTR of the HCV genome and thereby promotes HCV RNA accumulation, mostly by stabilizing the HCV genome and protecting the HCV genome from degradation. Treatment with an antisense inhibitor of miR-122 in HCV-infected chimpanzees and HCV-infected patients resulted in robust virological responses.

OTHER MIRNA’S AND HCV

It has been demonstrated that, in addition to miR-122, other miRNAs are involved in the regulation of HCV replication, either in a direct or indirect manner.\textsuperscript{61} The presence of miR-199a, which can bind to a seed site in stem-loop II of the HCV RNA, reduced the efficiency of HCV replication.\textsuperscript{62} Let-7b, which can bind to several seed sites in the viral genome, inhibits HCV replication and seems to have a synergistic inhibitory effect with IFN-α on HCV.\textsuperscript{63} Overexpression of miR-196, which is induced by IFN-β and contains a binding site in the NS5A sequence, also has an inhibitory effect on HCV replication. HCV-infected cells treated with mimics of miR-196 exhibited a reduced HCV replication efficiency.\textsuperscript{64,65} miR-491 was demonstrated to stimulate HCV replication in Huh-7 cells by inhibiting the phosphoinositol-3 (PI3) kinase/Akt pathway that enhances the entry of HCV into cells.\textsuperscript{66,67} Furthermore, miR-27a and miR-27b, which are involved in the regulation of lipid metabolism, have been reported to affect HCV replication; however their exact role needs to be further investigated.\textsuperscript{68,69} It was shown that HCV induces the expression of miR-208b and miR-499a-5p which mediate degradation of the IFN-λ3 (IFNL3) mRNA, and may evade the immune system in this way.\textsuperscript{70} These data suggest that several miRNAs
could be therapeutic targets for restoring the host antiviral response. Further research needs to be performed to determine the exact roles of these miRNAs in HCV infection.

HEPATITIS B VIRUS

HBV is a member of the Hepadnaviridae family and has a circular partially double stranded DNA genome. Its viral genome contains overlapping reading frames, encoding for the following proteins: viral polymerase, HBV surface antigen (HBsAg), HBV core and e antigen (HBeAg and HBeAg), and hepatitis B virus X (HBx) protein. Viral DNA, viral proteins, and antibodies directed against the different viral proteins can be detected during different stages of HBV infection. The HBV genome persists in the nucleus of the hepatocyte as covalently closed circular DNA (cccDNA) (FIGURE 3). The cccDNA is used as a template for transcription of pregenomic RNA (pgRNA) and mRNAs encoding the viral proteins. The pgRNA together with viral polymerase is assembled into core particles in which reverse transcription of the pgRNA into viral DNA takes place. The DNA containing core particles are either re-imported into the nucleus to form new cccDNA or enveloped in the endoplasmic reticulum before release as viral particles. In addition to infectious virus particles, HBV produces large amounts of virus like particles containing HBsAg. HBV infection can lead to wide range of liver complications such as fulminant hepatic failure, liver cirrhosis and ultimately HCC.\textsuperscript{71} Although children are at high risk to develop chronic HBV infection, less than 5\% of adults who encounter HBV will develop chronic infection. Despite the availability of an effective vaccine, chronic HBV infection is a major health problem, affecting an estimated 240 million people worldwide. The main goal of antiviral therapy is halting the progression of liver disease and achievement of functional cure (defined as HBsAg loss with or without the formation of anti-HBs antibodies). Current treatment options for CHB patients include peg-IFN and nucleos(t)ide analogues (NUCs). While NUCs very efficiently inhibit viral replication, treatment rarely leads to a functional cure.\textsuperscript{72} In peg-IFN treated patients a functional cure is only achieved in 3-7\% of patients.\textsuperscript{73,74} Combination treatment with peg-IFN and NUCs have resulted in functional cure rates of 9-17\% in HBeAg-positive and –negative patients.\textsuperscript{75–77} There is a need for new therapeutic options to treat chronic hepatitis B patients. If miRNAs are shown to have a direct or indirect role in the regulation of HBV replication, these miRNAs could be attractive novel targets for antiviral treatment (FIGURE 3).

MIRNA’S AND HBV

Contrary to in HCV, miR-122 inhibits HBV replication. Overexpression of miR-122 in hepatoma cells inhibited HBV expression, whereas antisense inhibition of miR-122 resulted in increased HBsAg and HBeAg levels.\textsuperscript{78–80} It has been postulated that miR-122 can bind directly to the polymerase region of HBV and thereby directly suppresses HBV replication.\textsuperscript{78}
In addition, loss of miR-122 expression enhances HBV replication through cyclin G1-modulated p53 activity, suggesting that miR-122 also has an indirect role in the regulation of HBV replication. HBV has also been reported to modulate expression levels of cellular miRNAs, such as decreased expression of miR-122 in the presence of HBV in hepatoma cells. Another interesting finding was that treatment with IFN-α seems to inhibit miR-122 by inducing an IFN-stimulated gene, NT5C3, whose mRNA binds endogenous miR-122 and, thus, serves as a sponge to diminish the intracellular pool of miR-122. This may negatively affect the anti-HBV efficiency of IFN-α. Similar observations for direct targeting of HBV transcripts were made for other miRNAs. Expression of miR-199-a-3p and miR-210, which bind to the HBsAg region and pre-S1 region of the viral transcript, efficiently reduced HBsAg levels in HepG2 cells. In addition, miR-125a-5p was found to interact with the viral RNA, and thereby downregulate the expression of HBsAg. Several miRNAs are indirectly involved in up- or down-regulation of HBV replication. miR-372 and miR-373, which are up-regulated in HBV-infected liver tissue, were shown to promote HBV gene expression through a pathway involving the transcription factor I/B. miR-141 was demonstrated to indirectly suppress HBV replication by reducing HBV promoter activities through the down-regulation of peroxisome proliferator-activated receptor α in HepG2 cells. miR-155, which enhances innate antiviral immunity, mildly inhibited HBV infection in human hepatoma cells. To summarise, although the exact role of miR-122 in HBV infection needs to be further studied, miR-122 seems to have an (either direct or indirect)
inhibiting role in HBV replication. HBV can modulate the expression of several cellular miRNAs in order to promote a favorable environment for its replication. They provide the basis for the development of novel therapeutic strategies for HBV.

CIRCULATING MIRNA’S IN VIRAL HEPATITIS

Elevations of serum miR-122 levels have been shown to be a sensitive marker for inflammatory activity in the liver and are strongly correlated with serum alanine aminotransferase (ALT) activity in chronic hepatitis C patients. No correlations were found between serum HCV viral load and hepatic or serum levels of miR-122. Low pre-treatment levels of serum and hepatic miR-122 were shown to be associated with a poor virological response to peg-IFN and RBV therapy in chronic hepatitis C patients, but another study did not confirm this finding. Circulating levels of miRNAs have also been linked to multiple aspects of HBV infection. miR-122 serum levels are elevated in chronic hepatitis B patients and are correlated with markers of HBV infection such as ALT level, HBV DNA and HBsAg levels. Furthermore, it has been demonstrated that miR-122 levels can discriminate between chronic hepatitis B patients with a high or low risk for disease progression. In addition, it has been observed that various miRNAs were upregulated in plasma of CHB patients, and that miRNAs could be used as markers predicting treatment response in CHB patients.

NON-VIRAL FUNCTIONS OF MIR-122

miR-122 is involved in cholesterol and triglyceride metabolism, development of liver inflammation, steatosis and fibrosis, and may act as a tumor suppressor. The role of miR-122 in liver metabolism was discovered when silencing of miR-122 with an antisense oligonucleotide for 4 weeks in mice resulted in a reduction of total cholesterol and triglycerides levels in plasma. Microarray analysis following miR-122 inhibition indicated that under these conditions the expression of genes involved in the regulation of cholesterol metabolism and fatty-acid synthesis was reduced. Similar to what was observed in mice, treatment of nonhuman primates and chronic hepatitis C patients with an antagonim of miR-122 resulted in a decrease of total plasma cholesterol. The reduction in serum cholesterol and triglyceride levels was much greater in mice totally lacking the gene encoding for miR-122. In a high fat induced obesity mouse model miR-122 inhibition resulted in a reduction of steatosis. However, mice with a knock-out or liver-specific knock-out of miR-122 developed microsteatosis and inflammation of the liver that progressed to steatohepatitis and HCC later on in life. It was observed that chemokine (C-C motif) ligand 2 (CCL2) was upregulated in these mice, which resulted in intrahepatic recruitment of inflammatory cells as well as interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α) production. Previously, it was shown that these cytokines
could promote HCC development. Several identified targets of miR-122 were previously associated with HCC such as cyclin G1, ADAM-17 (disintegrin and metalloprotease family 17), BCL-W, and insulinlike growth factor 1. In addition, it was found that miR-122 levels are reduced in human HCC cells as compared with normal hepatocytes, and that the loss of miR-122 expression is correlated with HCC metastases and poor prognosis. Restoration of miR-122 in HCC cells reversed their malignant phenotype and tumorigenic properties. Temporary inhibition of miR-122 using an antisense oligonucleotide was well tolerated in adult mice, and these mice did not develop HCC – suggesting that carcinogenesis is accelerated in miR-122 knock-out mice but not in mice that were treated short-term with miR-122 antagonirs. Recently, it was shown that HCV RNA functionally reduces miR-122 binding on endogenous mRNA targets, acting like a “sponge”, which results in de-repression of host miR-122 targets that may contribute to the long-term oncogenic potential of HCV. In addition, it was shown that HBV viral transcripts contain miR-122 binding sites as well and therefore also could act as a “sponge” to sequester miR-122. This virus-induced suppression of miR-122 may contribute to the development of HCC in chronically infected patients. The potential and safety of inhibition of miR-122 as a therapeutic target for HCV eradication needs to be further examined by long-term follow up analysis of patients treated with an antisense inhibitor of miR-122.
OUTLINE OF THIS THESIS

The main focus of this thesis is to evaluate therapeutic miR-122 targeting in chronic HCV patients and to improve our understanding of the role of miRNAs and viral factors in chronic hepatitis B infection.

I. Targeting microRNA-122 in chronic hepatitis C patients

The first part of this thesis describes the effects of silencing miR-122 with an antisense oligonucleotide in chronic hepatitis C patients. The aim of the study presented in chapter 2 is to assess changes in circulating miRNA levels in HCV genotype 1 chronic hepatitis C patients before, during and after dosing with an antagomir that targets miR-122 (miravirsen). In chapter 3 we present safety and clinical efficacy data up to 35 months following miravirsen dosing in the cohort of chronic hepatitis C patients described in chapter 2. Chapter 4 presents safety, pharmacokinetics, and efficacy of a single dose of a N-acetylgalactosamine conjugated oligonucleotide against miR-122 (RG-101) in patients with chronic HCV genotype 1, 3 and 4 infection. The N-acetylgalactosamine structure binds to the asialoglycoprotein receptor on hepatocytes and thereby increases the intracellular potency of RG-101 by up to 10-20 fold compared to a non-conjugated oligonucleotide. In chapter 5 we aimed to assess whether treatment with RG-101 changes important immune effectors in chronic hepatitis C patients and whether restored antiviral immunity could play a role in the long-term antiviral effect of RG-101 observed in a number of patients in chapter 4. Finally, chapter 6 describes circulating miR-122 levels and treatment success following DAA therapy in chronic hepatitis C patients with and without prior RG-101 dosing. Furthermore, we assess whether inhibition of viral replication by DAA treatment results in less immune activation and restoration of HCV-specific T cell function.

II. Role of microRNA’s and viral factors in chronic hepatitis B virus infection

In the second part of this thesis we describe the role of miRNAs and viral factors in a cohort of 92 chronic hepatitis B patients who were treated with a combination of peg-IFN and adefovir for 48 weeks. In this previously described cohort of HBeAg-positive and –negative patients a high rate of HBsAg loss was observed (11-17% at year 2 of treatment-free follow-up). In chapter 7 we aim to identify plasma miRNAs that are associated with HBeAg status, HBV DNA and HBsAg levels, as well as therapy response in this cohort of chronic hepatitis B patients. Chapter 8 describes HBV core protein signatures identified by next-generation sequencing associated with HBeAg status and treatment response in the same cohort of patients. Lastly, in chapter 9 we aim to assess the sustainability of the outcome of peg-IFN and adefovir combination treatment in the 92 chronic hepatitis B patients at 5 years of follow-up.
REFERENCES


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