NOVEL ROLES OF MICRORNAS IN HEPATITIS C

by

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ABSTRACT

HOSSEIN SENDI. Novel roles of microRNAs in hepatitis C.
(Under the direction of MARK CLEMENS and HERBERT BONKOVSKY)

Approximately, 170 million people are infected by hepatitis C virus (HCV) worldwide, and of these, nearly 85% will develop chronic hepatitis C (CHC). Despite finding new anti-viral treatment that increase response rate from 45 % to 65-70 %, investigations continue to find more effective treatments for hepatitis C because of side effects and limitations of current treatment. It is known that miR-122 enhances HCV replication by binding to two closely spaced target sites in the 5'-UTR of the viral genome, which leads to an increase in abundance of HCV RNA. We found that miR-122 down- regulates Occludin (OCLN), one of the key HCV receptors, by directly targeting 3'-UTR of OCLN mRNA. We also found that interaction of miR-122 with 3'-UTR of OCLN mRNA eventually results in a decrease in HCV entry. In accordance with our *in vitro* study, we found an inverse correlation between pre-treatment levels of miR-122 and HCV RNA levels in patients with CHC. This is a new finding of our study which is consonant with our hypothesis that miR-122 may play an antiviral role in uninfected hepatocytes and early stages of HCV infection. Protein Kinase R (PKR), a double-stranded RNA-dependent protein kinase, is among the well-known members of cellular antiviral proteins transcriptionally induced by IFNs in response to viral infection. We found that miR-122 down- regulates PRKRA expression by targeting 3'-UTR of PRKRA mRNA in uninfected Huh7.5 cells. This down-regulation led to decrease in phosphorylation of PKR. Our results are consonant with the notion that, in infected hepatocytes, miR-122 preferentially binds to 5'-UTR of HCV RNA rather than to the

3'-UTR of PRKRA, and this is the main factor that increases HCV replication. Based on our findings, both *in vitro* and in CHC patients, we speculate that miR-122 could play a dual role in HCV infection; in uninfected hepatocytes miR-122 plays an antiviral role through down-regulation of OCLN while, in infected hepatocytes, miR-122 increases HCV replication through binding to the 5'-UTR of HCV RNA. Our results suggest that miR-122 mimics may be more beneficial than miR-122 inhibitors in the earlier stages of infection or as a prophylactic approach when few or no hepatocytes are infected with HCV.

Both responses to treatment as well as spontaneous outcome of HCV infection are critically affected by host genetic factors. We found that pre-treatment levels of hepatic miR-29b were significantly lower in CHC patients with early viral response (EVR) than those without EVR. This novel finding could be very important both for predicting the outcome of disease as well as suggesting new treatment approaches for chronic hepatitis C. Low levels of miR-29b in early responders to HCV therapy might potentially benefit future therapeutic interventions involving the use of miR-29 antagomirs. We also showed that miR-29b level serves as an independent factor for predicting advanced stages of fibrosis in patients with CHC. These findings are unexpected, because miR-29b has been shown to exhibit anti-fibrotic effects *in vitro*. Hence, caution should be exercised in extrapolating *in vitro* observations to subjects with CHC. Higher levels of mir-29b in these patients may suggest a role of over-expression of miR-29b as an anti-fibrotic factor in advanced degree of liver fibrosis as a healing process for liver. Broader translational and *in vitro* studies are needed to unravel the importance of miR-29b in prognosis and treatment of hepatitis C.

DEDICATION

To my beautiful lovely wife Marjan and to my little angel Daria

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I sincerely thank my mentor, Dr. Herbert Bonkovsky, for providing me with the opportunity to work in his lab and giving me the freedom to develop new ideas and test every single one of those. Having the honor to work with this great physician-scientist, not only I learned lessons of science and medicine but also I learned more important lessons of life like honesty, kindness, and patience. I also appreciate all the support and guidance of Dr. Mark Clemens, my co-advisor, who has been one of the best teachers in my entire academic life. I also would like to thank Dr. Laura Schrum for all her support and advices during my PhD research. I also acknowledge Dr. Valery Grdzelishvili, and Dr. Kent Curran for their guidance on my proposal and thesis. I also need to thank Drs. Charles Rice (Rockefeller University) and Stanley Lemon (UNC Chapel Hill) for providing us with the materials of HCVpp and HCVcc systems. I would like to thank all members of LDMD group at CMC. I also appreciate all the help and advice from Drs. David Foureau, and Eugene Sokolov. I would also like to thank my parents for always supporting me in difficult periods of life. Lastly, I am most grateful to the only love of my life; my beautiful lovely wife Marjan; who not only collaborated with me in the lab, but also took all the responsibilities of our family life on her shoulders these past years. I wish I could have found a much more appropriate word than grateful or thankful, but there are no words in any language that can adequately express my gratitude and love for her.. I also sincerely thank the love and patience of my little angel Daria who was always waiting for daddy to come back home at midnight. This study was supported by a grant (DK388525) and contract (DK 065193) from NIH to Dr. Herbert Bonkovsky and by institutional funds from Carolinas Medical Center.

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LIST OF ABBREVIATIONS

Ago2	argonaute2
CHC	chronic hepatitis C
CLDN	claudin
CMC	Carolinas Medical Center
DMEM	Dulbecco's modified Eagle's medium
E-1	envelope-1
ECL	enhanced chemiluminescence
eIF2	eukaryotic initiation factor 2
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
EVR	early viral response
GAG	glucosaminoglycan
GAPDH	glyceraldehyde- 3-phosphate dehydrogenase
GFP	green fluorescent protein
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C Virus
HCVcc	HCV in cell culture
HCVpp	HCV pseudoparticles
HEK	human embryonic kidney
HNFs	hepatocyte nuclear factors
HSCs	hepatic stellate cells

Huh7	human hepatoma cells
IFN	Interferon
IFN-R	IFN receptors
IL	interleukin
IRES	internal ribosome entry site
IRF3	interferon regulatory factor 3
ISGs	IFN-stimulated genes
JAK	Janus kinase
KO	knock out
LDLR	low-density lipoprotein receptor
LT	liver transplant
LTR	long terminal repeat
miRNAs	microRNAs
MMNC	miRNA mimic negative control
MOI	multiplicity of infection
NCR	noncoding region[s]
NS,	non-structural
OCLN	occludin
ORF	open reading frame
PCR	polymerase chain reaction
peg-IFN	pegylated interferon
PKR	protein Kinase R
PRKRA	protein kinase R activator

qRT-PCR	real-time quantitative PCR
RBV	ribavirin
RCs	replicase complexes
RdRp	RNA-dependent RNA polymerase
RIG-I	retinoic acid inducible gene-I
RISC	RNA-induced silencing complex
SBN	Silybin
SR-BI	scavenger receptor class B type I
STAT	signal transducer and activator of transcription
TAR	trans-activation response element
TGFb	transforming growth factor b
TLR3	toll like receptor 3
TNF-α	tumor necrosis factor- α
TRBP	TAR RNA binding protein
UTR	untranslated region
VSV-G	vesicular stomatitis virus glycoprotein

CHAPTER 1: INTRODUCTION

Hepatitis C is a global health problem [1]. Approximately, 170 million people are infected by HCV worldwide, and of these, nearly 85% will develop chronic hepatitis C (CHC). CHC progresses in approximately 30-40 % of individuals, which could potentially lead to fibrosis, cirrhosis, end-stage liver disease and hepatocellular carcinoma (HCC) [2]. Until recently, the standard of care for individuals with CHC was therapy with pegylated Interferon (peg-IFN) alpha plus ribavirin (RBV), but the therapy is effective only in 41 to 52% of those with HCV genotype 1 infection - the most common strain of HCV in the United States [3,4]. In addition to their high cost and relatively low rates of sustained viral response (SVR) in patients with genotype 1 infection, peg-IFN and RBV are often poorly tolerated because of side effects that include influenza-like symptoms, cytopenias, and neuropsychiatric symptoms [5]. The viral targets which received the most attention are the Non-Structural 3-4A (NS3-4A) serine protease, the NS5B RNA dependent RNA polymerase, and more recently the viral nonstructural protein NS5A [5]. Accordingly, two NS3-4A protease inhibitors, boceprevir and telaprevir, have recently been approved by the US FDA and the European Drug Agency for the treatment of genotype 1 chronic HCV infection. Phase 3 clinical trials of these agents in combination with peg-IFN/RBV demonstrated a significant improvement in SVR rates in treatment-naive individuals, from 45% for peg-IFN/RBV alone to 67%–75% for protease inhibitor-containing regimens [6, 7]. However, these protease inhibitors have important limitations. First, they cannot be given as mono-therapy because of the

rapid emergence of viral resistance. To reduce the rates of viral resistance, boceprevir and telaprevir must be given in combination with peg-IFN and ribavirin. Patients who are intolerant of either peg-IFN or ribavirin, as a result, cannot receive protease inhibitor therapy. Second, the protease inhibitors are associated with significant side effects of their own, in addition to the considerable side effects of peg-IFN and RBV. Third, boceprevir and telaprevir are not currently approved for the treatment of HCV genotypes other than 1 and, therefore, are not options for patients with non-genotype 1 infections [5]. Inhibitors of NS5A block viral production at an early stage of assembly, so that no viral RNA or nucleocapsid protein is released. Agents that block NS5B activity inhibit the virus RNA dependent RNA polymerase (RdRp) and are referred to as polymerase inhibitors. Both nucleoside and non-nucleoside RdRp inhibitors have been synthesized. Nucleoside inhibitors (NIs) bind to RdRp's active site, whereas the non-nucleoside inhibitors (NNIs) bind to the enzyme outside the active site, inducing conformational changes that down-regulate RdRp's activity [7]. Milk thistle (Silybum marianum) has been used since ancient times as a liver tonic. Silymarin (SI), a purified extract of polyphenolic flavonoids isolated from milk thistle is composed mainly of silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B. Legalon-SIL (LS) is a form of Silybin (SBN) which is a waters soluble formulation of the dihydro-succinate sodium salt of SBN A and SBN B in equal proportion. In a recent study, we found that treatment with LS down-regulates HCV core and NS5A expression in CON1 cells, which express full length HCV genotype 1b, suggesting that LS may prove to be a valuable alternative or adjunctive therapy for the treatment of HCV infection [8].

A complementary strategy is to target the host cellular factors that support the HCV life cycle. Several studies, including RNA interference screens, demonstrated that HCV depends on dozens, if not hundreds, of cellular proteins to complete its life cycle. A better understanding of the interactions between HCV proteins and host factors may help to identify host targets for antiviral therapy [5]. Despite the progress in finding novel and alternative treatments for hepatitis C, investigations continue to find more efficient treatment for hepatitis C with fewer side effects and limitations.

HCV

HCV is a single-stranded positive-sense RNA flavivirus. The viral genome is comprised of about 9600 nucleotides and includes 5′ and 3′ noncoding regions (NCR) and a single open reading frame (ORF) that encodes a polyprotein. The poly-protein is cleaved by viral and host proteases into three structural (core, Envelope 1(E1), E2) and seven non-structural (NS) (p7, NS2-NS5B) peptides (Fig1).

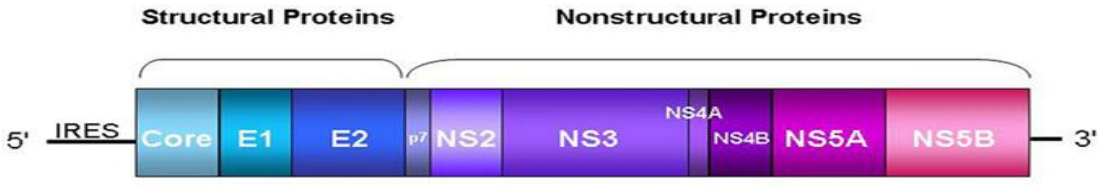

Figure 1 Schematic presentation of HCV genome and proteins. E indicates Envelope; NS, Non-structural .Figure from: http://www.bioscience.org/

Following translation and processing of the 10 viral proteins, all of the gene products remain associated with intracellular membranes (Figure 2). This is a feature common to all positive-strand RNA viruses and is not yet well understood. The membrane-associated replicase works by copying incoming viral genomic RNA into a

negative-strand intermediate, which is then used to generate additional positive-strand RNAs for subsequent rounds of translation and packaging into virus particles. The replication factories use microtubules to move around the cell as they function, coalescing into vesicular structures termed the membranous web [9].

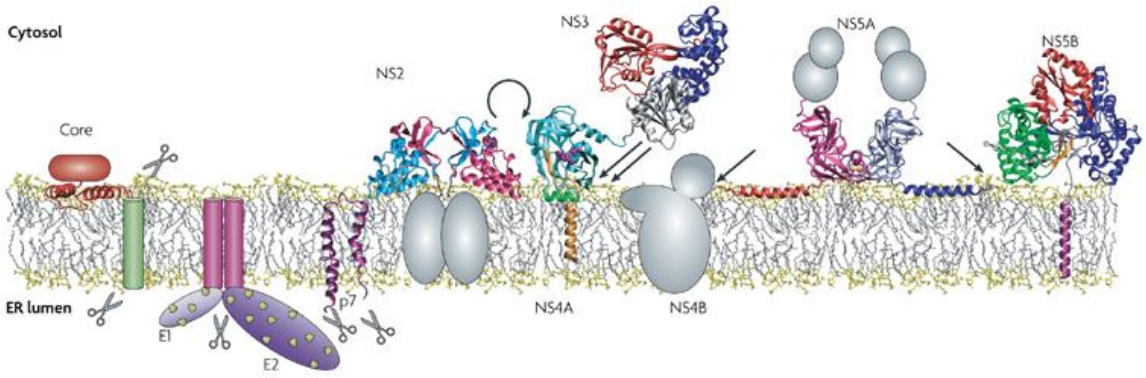

Figure 2. Hepatitis C virus proteins and functions. E indicates envelope; ER, endoplasmic reticulum; NS, non-structural. Scissors indicate cleavage by host enzymes, arrows represent processing by viral proteases. Figure from Reference [10] with permission

The NS2 protein is a trans-membrane protein of 21–23 kDa. NS3–4A is a complex bi-functional molecule essential for viral polyprotein processing and RNA replication. NS3 is a fairly hydrophobic protein of 69 kDa with a serine protease encoded by its N-terminal one-third region that non-covalently binds the NS4A cofactor. NS4A is a 54-aa polypeptide [11]. NS5A does not contain any trans-membrane domains; instead, it is tethered to the intracellular membranes by an N-terminal helix that inserts itself horizontally into the lipid bilayer [12,13]. The amphipathic nature and the in-plane membrane association of the N-terminal helix are conserved features of NS5As encoded by several related members of Flaviviridae [14]. The crystal structure of the largest and most conserved domain, domain I, revealed a dimeric structure that contains a putative RNA-binding groove [15], consistent with ability of NS5A to bind RNA *in vitro*. Multiple lines of evidence point to an important role of the phosphorylation status ofNS5A in viral replication. Adaptive mutations in NS5A and other NSs that enhance replication abolish NS5A hyper-phosphorylation [16], and selective inhibitors of NS5A

phosphorylation stimulated the replication of wild-type HCV RNA [17]. NS5A is important for the assembly of infectious viral particles in cell culture as well [18].NS5A also interacts with many cellular proteins that are implicated in HCV replication. NS5B is the RdRp responsible for synthesizing the complementary negative strand of the genome as the replication intermediate and then genomic RNA using the negative strand as the template [9].

HCV entry into cells

Identifying the mechanisms of HCV entry into cells was not possible for a long time due to lack of an appropriate animal model or an efficient *in vitro* cell culture system supporting the complete HCV life cycle. Progress in delineating the key mechanisms for HCV entry has been made since the development of *in vitro* models that support all steps of the HCV replication cycle, including cell entry [19,20]. HCV entry factors include tetraspanin [CD81], scavenger receptor class B type I (SR-BI), and the tight-junction protein claudin-1 (CLDN1). Despite identification of these factors for HCV entry, several human cell lines, as well as all cell lines of non-primate origin remained resistant for HCV entry, even when cells were manipulated to express all of these factors together [21]. Recently, Ploss and colleagues have identified occludin (OCLN), an essential component of tight junctions, as a host-cell protein essential for HCV entry [22]. They showed that whenever human CD81, SR-BI, claudin-1 and OCLN were present, all cell types tested were able to be infected with HCV. Although the detailed mechanism of HCV entry has yet to be elucidated, It has been suggested that HCV might use CD81, and SR-BI for binding to the cell surface and movement to tight junctions, where OCLN, and CLDN facilitate HCV entry into the hepatocytes (Figure 3) [23].

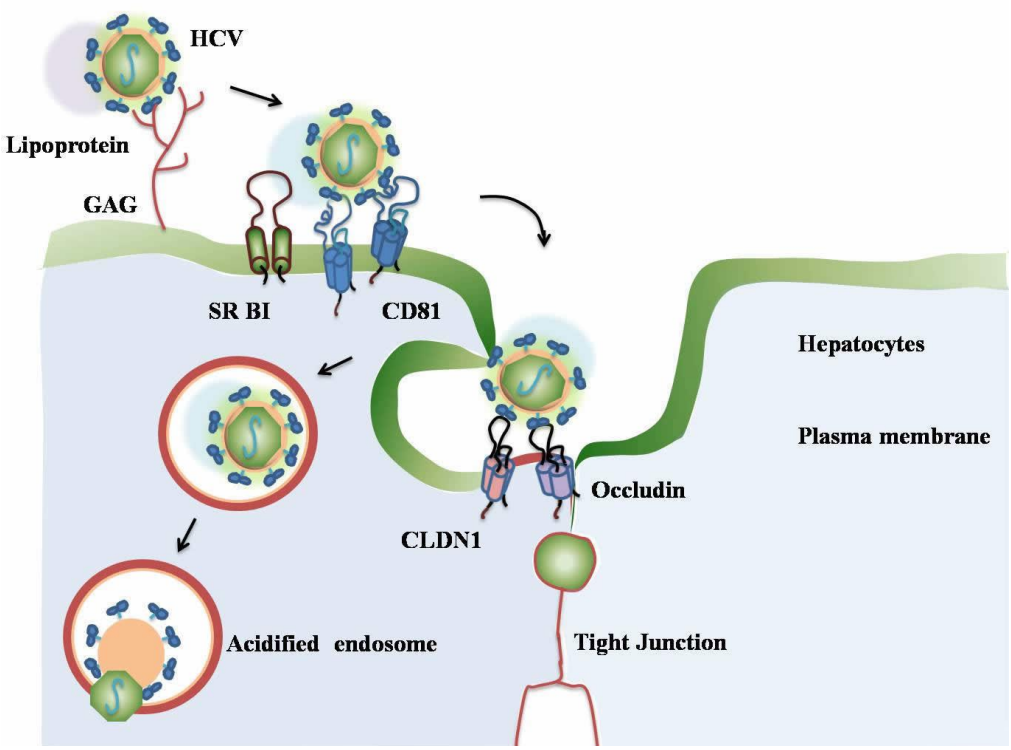

Figure 3. Current model of hepatitis C virus entry into the hepatocyte. GAG indicates glucosaminoglycan; SR-BI, scavenger receptor type BI; CLDN-1, claudin-1. Figure from http://chemeng.iisc.ernet.in

HCV Life cycle

HCV mainly infects liver in vivo, and this tropism is recapitulated *in vitro*, as both HCV in cell culture (HCVcc) and HCV pseudoparticles (HCVpp)-HIV particles pseudo-typed with HCV envelopes- mostly infect cell lines of liver origin. The virus uses specific attachment and entry factors to cross the plasma membrane of hepatocytes and release its genomic RNA. Once released, the RNA serves as a template for replication and translation of the polyprotein. The polyprotein must then be directed to specific cellular locations where it is co-translationally and post-translationally modified. Mature viral proteins are then able to assemble into new virions, which leave the host cell using the secretory pathway used by lipoproteins [18] (Fig 4).

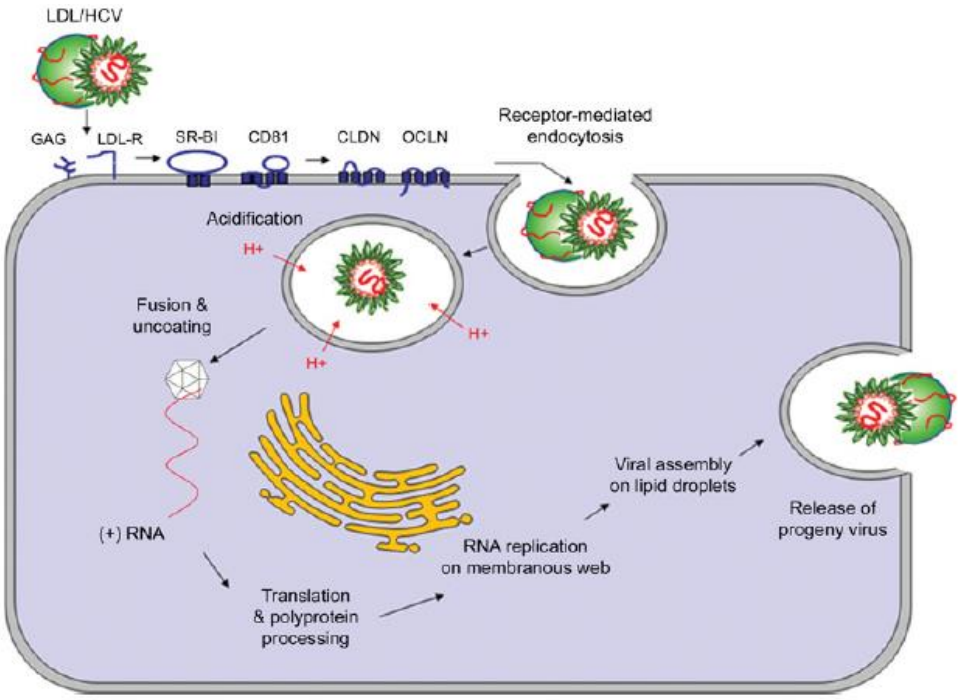

Figure 4. Schematic of life cycle of HCV. Entry into target cells initiates HCV infection. The virus–receptor complex translocates to the tight junctions where CLDN and OCLN act as cofactors and induce receptor-mediated endocytosis. The virus-containing endosome is acidified in the cytoplasm, a process that triggers the envelope glycoproteins to initiate fusion and to release the nucleocapsid. HCV genomic RNA is then released and translated into polyprotein. Negative strnads of viral RNA are also synthesized by the viral RNA-dpendent polymerase. The presence of negative strands is a marker of active viral replication within hepatocytes. The polyprotein is co- and post-translationally modified to produce mature viral proteins which can form replicase complexes (RCs), which are sites where new positive strands of viral RNA must be synthesized from the negative strand templates, in order for complete virions to be assembled. These progeny virions bud into the lumen of the ER and leave the host cell through the secretory pathway, completing the viral life cycle. Figure from reference [18] with permission

IFN response to HCV

In HCV-infected cells, Induction of IFN type I is initiated after binding of dsRNA structures to Toll like receptor 3 (TLR3) or retinoic acid inducible gene-I (RIG-I) (Fig 5), which in turn activate the transcription factors such as interferon regulatory factor 3 (IRF3) [24]. The coordinated action of these transcription factors leads to induction of IFN and IFN-stimulated genes (ISGs). Type I IFNs are secreted proteins that exert their

function after binding to specific IFN receptors (IFN-R) and activate Janus kinase (JAK)/signal transducers and activator of transcription (STAT) signaling pathway [25] inducing over 300 genes, collectively referred to as ISGs [26]

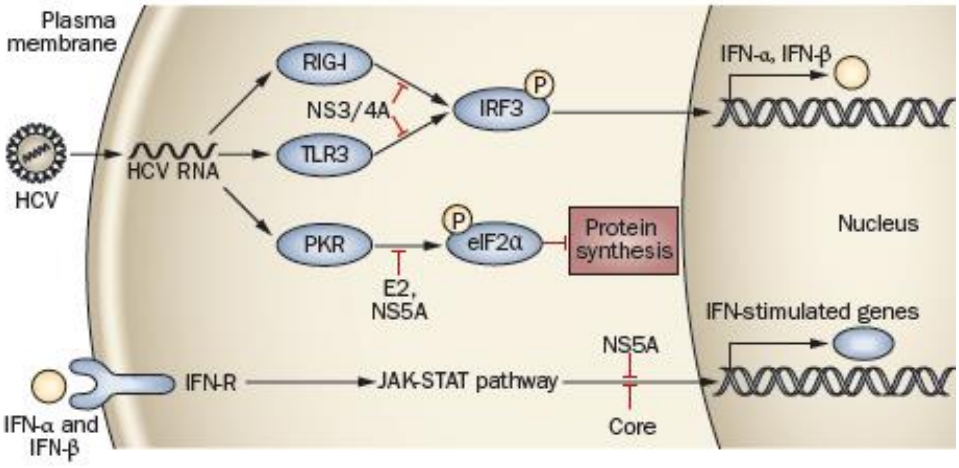

Figure 5. Innate immune signaling pathways interdicted by HCV. HCV interferes with numerous defense mechanisms, including virus detection, IFN signaling, and antiviral effectors. TLR3 and RIG-I are pathogen recognition receptors whose downstream signaling pathways are blocked by the HCV protease NS3/4A. HCV proteins NS5A and core block IFN signaling via the JAK-STAT pathway. HCV proteins E2 and NS5A inhibit PKR, an antiviral effector that leads to inhibition of protein synthesis in infected cells. Figure from reference [27] with permission

Protein Kinase R (PKR), a double-stranded RNA-dependent serine/threonine protein kinase, is among the well-known members of cellular antiviral proteins transcriptionally induced by IFNs in response to viral infection [28]. Viral dsRNA binds and activates PKR through dimerization and phosphorylation [29]. Activated PKR phosphorylates eukaryotic initiation factor 2 (eIF2) to limit mRNA translation. As a result, viral replication is blocked at the level of protein synthesis (Fig 5). PKR is suggested as an important mediator of IFN action against HCV. Elevated PKR expression was reported in acute HCV infection in chimpanzees [30], as well as in chronic HCV infection [31] and also in HCC from patients with HCV infection [32]. PKR-knockout

mice support replication of HCV sub-genomic RNA [33]. The HCV phosphoprotein NS5A has been also known to bind to PKR and to confer resistance to the virus against IFN [34].

MicroRNAs and Liver Diseases

MicroRNAs (miRNAs) are approximately 22 nucleotide non-coding RNAs that can down-regulate various gene products by inducing either cleavage or a reduction in the translational efficiency of the target mRNAs [35]. In the nucleus, pri-miRNA, which are double stranded RNA segments of approximately 60-70 nucleotides, are cleaved by the RNAse III enzyme, Drosha. The resulting short, hair-pin shaped double-stranded RNAs, which are called pre-miRNAs, are transported by exportins to the cytoplasm, where Dicer, which is also an RNAse III enzyme, cuts them further into mature miRNAs. Translational repression or degradation of mRNA occurs when miRNA binds to the RNA-induced silencing complex (RISC) [35].

The importance of miRNAs in liver diseases has been the focus of many recent investigations. The physiological importance of miRNA in the liver has been recognized in regulation of metabolic pathways, immunity, viral hepatitis, cancer, and liver fibrosis [36]. In depth investigation of miR-150 and miR-194 revealed significantly reduced levels in fibrosis and showed that both of these miRNAs inhibit stellate cell activation. In another study, expression of miR-199a, 199a′, 200a, and 200b was found to be correlated with the degree of progression of liver fibrosis in chronic hepatitis C patients [37].

miR-29 family, including miR-29a, miR-29b and miR-29c, can regulate extracellular matrix genes and affect numerous signal pathways that relate to fibrosis through targeting the corresponding factors involved in these pathways, such as

TGFβ/Smads, Wnt/β-catenin and MAPK pathway [38,39]. It is known that family of miRNA-29 plays a role in murine models of fibrosis as well as in humans [40]. It was shown that treatment of hepatic stellate cells with transforming growth factor β (TGFβ) suppressed miR-29 expression suggesting that part of the fibrogenic effects of TGFβ is mediated via miR-29 down-regulation [40].

The miRNA-29 family was also recently reported to be aberrantly expressed in multiple cancers. Increasing evidence shows that the abnormal expression of miR-29 family is associated with carcinogenesis and cancer progression, making miR-29s a well-analyzed group of miRNAs in cancer research [41]. Besides the regulatory role of miR-29 in fibrosis and cancer progression, the miR-29 family has been identified to have significant effect on immune cell proliferation and cytokine production by helper T cells, especially IFN- γ. Ma et al. showed miR-29 suppressed IFN- γ production by directly targeting IFN- γ mRNA. They also showed mice with endogenous inhibition of miR-29 had enhanced T helper type 1 (TH1) responses and greater resistance to infection with Mycobacterium tuberculosis. Therefore they suggested that miR-29 suppresses immune responses to intracellular pathogens by targeting IFN-g [42].

MiR-122

MiR-122, is a 22 nucleotide miRNA that accounts for 70% of the total miRNA population in normal adult hepatocytes with approximately 66000 copies per cell [43]. There is now compelling evidence that miR-122, as a regulator of gene networks and pathways in hepatocytes, plays a central role in diverse aspects of hepatic function and in the progress of liver diseases. Interestingly, the pri- and pre-miRs of miR-122 are regulated in a circadian manner [44]. This is a very surprising observation given the

finding that the turnover of mature miR-122 is several weeks in the liver, pointing to possible functional roles for the precursor molecules of miR-122 [36].

The human miR-122 locus is in a noncoding RNA exon and is not part of a cluster [45]. The pri-miR-122 sequence shows only short regions of evolutionary conservation, of which the pre-miR-122 hairpin is the strongest [44]. The promoter region was isolated, and showed liver-specific activity when tested in a reporter vector. The core promoter is highly conserved and possesses elements typical of a pol II promoter. It contains a conserved target site for the liver-enriched transcription factor hepatocyte nuclear factor 4a (HNF-4a), which stimulates miR-122 expression [46]. In another study, Xu et al. found that miR-122 functions as an effector of liver-enriched transcription factors and contributes to liver development by regulating the balance between proliferation and differentiation of hepatocytes, at least by targeting CUTL1 [47].

The most well-known function of miR-122 in the mammalian liver is to regulate lipid and cholesterol metabolism. The knockdown of miR-122 expression in mice and monkeys down-regulates cholesterol and lipid metabolizing enzymes and reduces plasma cholesterol levels [48-50]. Mir122a-knock out (KO) animals developed steatohepatitis and liver fibrosis [51, 52] phenotypes that were not observed in prior studies using antisense oligonucleotide–mediated miR-122a knockdown. Both groups also demonstrated an increase in infiltrating inflammatory cells in miR-122–deficient liver [51,52]. They produce high levels of interleukin-6 (IL-6) and tumor necrosis factor- α (TNF-α), and these cells are known to promote fibrosis in the injured liver [53].

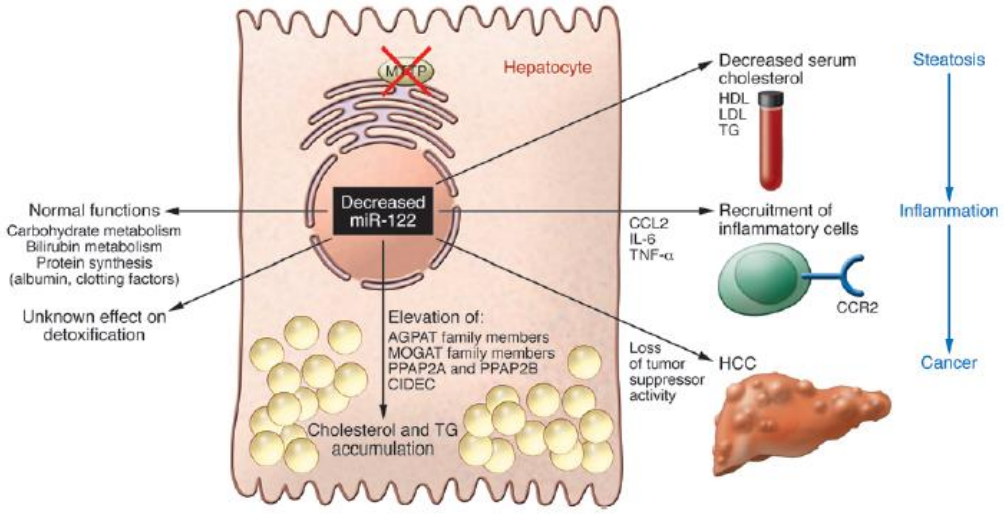

Figure 6. Overview of the consequences of miR-122 loss on hepatocyte function. The normal functions of the hepatocyte include carbohydrate and lipid metabolism, bilirubin excretion, and detoxification of endogenous compounds and xenobiotics. The loss of miR-122 results in increased lipid synthesis and decreased lipid export, but other hepatocyte functions are unaltered. Loss of miR-122 also led to increased inflammation and fibrosis, and eventually the development of HCC, suggesting the miR-122 plays a tumor-suppressive role. AGPAT; 1-acylglycerol-3-phosphate O-acyltransferase. CIDEC; cell death-inducing DNA fragmentation factor-α-like effector. MOGAT; monoacylglycerol transferases. PPAP2A; phosphatidic acid phosphatase 2a. Figure from reference [54] with permission

MiR-122 levels are reduced in experimental models and human samples of HCC, and loss of miR-122 is associated with tumor invasiveness and cancer progression [55]. Therefore, it has been speculated that this microRNA acts as a tumor suppressor. In the models described by Tsai et al. and Hsu et al., HCC develops in KO mice. To further understand the involvement of miR-122 in carcinogenesis, Hsu et al. made use of an HCC experimental model wherein transgenic mice harbor both a tetracycline-repressible MYC gene and a liver activator promoter–driven tet-transactivator protein, resulting in hepatic tumors in the absence of liver damage or inflammation [51]. Interestingly, miR-122 levels are strongly reduced in this model. Remarkably, administration of a recombinant adeno-associated viral vector expressing miR-122 (after the establishment of

small tumors) resulted in a reduction in tumor burden from 40% to 7.7% of liver mass. Thus, miR-122 appears to act as a tumor suppressor in a manner that is independent of its roles in fat metabolism or inflammation (Fig 6).

In another recent study, deletion of mouse mir122 resulted in hepatosteatosis, hepatitis, and the development of tumors resembling HCC. These pathologic manifestations were associated with hyperactivity of oncogenic pathways and hepatic infiltration of inflammatory cells that produce pro-tumorigenic cytokines, including IL-6 and TNF [56]. They also showed that delivery of miR-122 to a MYC-driven mouse model of HCC strongly inhibited carcinogenesis, further supporting the tumor suppressor activity of this miRNA. Taken together, these findings reveal critical functions for miR-122 in the maintenance of liver homeostasis and have important therapeutic implications, including the potential utility of increase in miR-122 levels for selected patients with HCC.

MiR-122 and hepatitis B

In a recent study, cyclin G1 was found to be a direct target of miR-122 [57]. They suggested that miR-122 down-regulation, consequent to HBV infection, leads to up-regulation of cyclin G1, which initiates formation of a cyclin G1-p53 complex. They speculated that the subsequent release of p53 from binding to HBV enhancers facilitates HBV mRNA transcription [57]. The result of this study would be more plausible if it will be combined with the results of another recent study which shows that over-expression of cyclin G1 increases AKT activation [58]. This, in turn leads to subsequent phosphorylation of GSK-3β and stabilization of snail, a critical epithelial-mesenchymal transition (EMT) mediator. They also found a significant correlation between expression

of cyclin G1 and p-Akt levels in a cohort of patients with HCC. Therefore, they suggested that cyclin-G1 may serve as a novel prognostic biomarker and therapeutic target [58]. Taken together, down-regulation of miR-122 consequent to HBV infection can lead to over-expression of cyclin-G1. This, in turn not only leads to increase in HBV replication, but also leads to increase in AKT activation and subsequent initiation of epithelial-mesenchymal transition. These additive/synergistic outcomes of miR-122 suppression in the context of chronic hepatitis B virus (HBV) infection may predispose patients to an increased risk of HCC development and progression. Therefore, miR-122 as well as cyclin-G1 [58] may serve as a novel prognostic biomarker and have some therapeutic applications in HBV-induced HCC. It is known that miR-122, as an unusual host factor, enhances HCV replication by binding to two closely spaced target sites in the 5'-untranslated region (5'-UTR) of the viral genome, which leads to increased abundance of HCV RNA [59, 60]. In contrast, new findings highlight an anti-viral as well as tumor-suppressive role for miR-122. The anti-proliferative properties of miR-122 have been reported in other recent studies as well [61, 62]. Although, miR-122 was found to be suppressed in chronic HBV infected patients, the mechanism is still unclear. The antiviral role of miR-122 is suggested by inhibitory effect of p53 on HBV transcription through blocking of binding of transcription factors like hepatocyte nuclear factors (HNFs) to HBV enhancers [57]. Worthy of note is that it has been shown that hepatocyte nuclear factors like HNF3 and HNF4α regulate miR-122 expression in hepatocytes [46, 63]. Hence both HBV and miR-122 need HNFs for enhancement of transcription of their genome. It is possible that in chronic HBV infection HBV utilizes HNFs for its own transcription rather than being used for transcription enhancement of miR-122, and this in

turn leads to suppression of miR-122. It is also interesting to mention that we have identified a novel double HBV core promoter mutation, which is specific and common in genotype D of HBV isolates of patients with chronic hepatitis B and which creates a new binding site for HNF3 [64]. This suggests that mutation variability in different genotypes of HBV might be explained by different levels of expression in miR-122 in selected contexts of host genetic factors and HBV genotypes. The role of hepatocyte nuclear factors (HNFs) in co-regulation of HBV and miR-122 is in need of further clarification.

MiR-122 and HCV

Jopling et al. found that miR-122 was expressed in human hepatoma cells (Huh7), which are permissive for HCV replication, but not in a different human liver cell line, HepG2 which does not support HCV replication. HCV RNA was reduced by about 80% when miR-122 was silenced in Huh7 cells stably expressing genotype 1b HCV replicon [59]. The effect of miR-122 on HCV is due to binding to the 5'-UTR of HCV RNA [51]. This identified miR-122–binding site is in an unstructured region of the 5'-UTR, upstream of the HCV internal ribosome entry site (IRES), and is conserved across all six genotypes of HCV [60]. This finding showed that miR-122 is important for efficient replication of HCV RNA *in vitro*. Because these authors did not observe any effect of miR-122 binding on HCV translation or RNA stability, they concluded that miR-122 positively regulates HCV at the level of viral replication. A second binding site for miR-122 was identified adjacent to, and downstream of, the first one. It was shown that miR-122 binds to both sites within the same HCV molecule to increase HCV RNA abundance [60]. However, it is not yet known whether this is due to a requirement for overlapping binding to the two sites, or for a sequential binding process [60].

It was also reported that miR-122 expression was decreased after IFN treatment, and over-expression of miR-122 partially alleviated the antiviral effect of IFN [65]. Later, Lanford et al. showed that silencing of miR-122 in chronically HCV-infected chimpanzees led to long-lasting suppression of HCV viremia [66]. These observations roused much interest in the role of miR-122 in HCV infection and its potential as a therapeutic target. Interestingly, in another recent study, Shimakami et al. showed that miR-122 binds HCV RNA in association with Argonaute2 (Ago2), and that this slows decay of the HCV RNA in infected cells [67] These observations roused much interest in the role of miR-122 in HCV infection and its potential as a therapeutic target.

In a very recent study, Li et al elaborated more on the RNA degradation pathways against which miR-122 provides protection [68]. They found that transfected HCV RNA is degraded by both the 5′ exonuclease Xrn1 and 3′ exonuclease exosome complex, whereas replicating RNA within infected cells is degraded primarily by Xrn1 with no contribution from the exosome (Fig 7). They found that Xrn1 knockdown enhanced HCV replication, indicating that Xrn1 decay and the viral replicase compete to set RNA abundance within infected cells [68]. They also showed that Xrn1 knockdown and miR-122 supplementation had equal, redundant, and non-additive effects on the rate of viral RNA decay. These findings indicate that miR-122 protects HCV RNA from 5′ decay. Nevertheless, they observed that Xrn1 knockdown does not rescue replication of a viral mutant defective in miR-122 binding, indicating that miR-122 has additional yet uncharacterized function(s) in the viral life cycle [68].

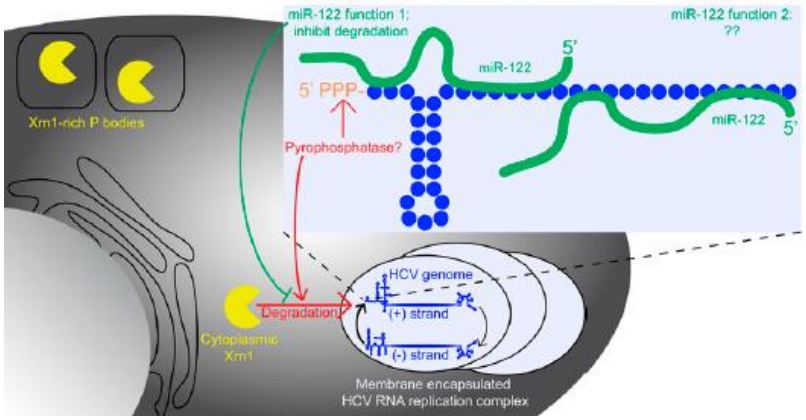

Figure 7. MiR-122 impacts HCV RNA stability and HCV RNA replication. Illustration of HCV RNA replication within membrane bound vesicles in the host cell cytoplasm. Also shown is a zoomed-in view of the 5′ end of the HCV genomic RNA with two bound miR-122 molecules. Cytoplasmic, but not P body-associated, Xrn1 degrades the HCV genomic RNA, perhaps after the 5′ triphosphate is modified by a pyrophosphatase. Although binding of miR-122 to the HCV RNA inhibits Xrn1 degradation, miR-122 also exerts another effect to enhance HCV RNA replication. Figure from reference [69] with permission

MiR-122 in CHC

As a conserved host factor that can be effectively inhibited without associated toxicity, and would not be expected to evolve resistance mutations, miR-122 presents a highly appealing antiviral target [44].Accordingly; Lanford et al. showed that silencing of miR-122 in chronically HCV-infected chimpanzees led to long-lasting suppression of HCV viremia [66]. However, when hepatic mir-122 levels were measured in patients with CHC, different results were obtained. Initially Sarasin-Flipowicz studied liver biopsies from patients with CHC who were undergoing IFN therapy and found no correlation of miR-122 expression with HCV RNA levels [70]. They also detected markedly decreased pretreatment miR-122 levels in subjects who had no virological response during later IFN therapy. In another study [71], it was found that, although hepatic miR-122 expression was weakly and positively correlated with the serum HCV

RNA levels, it was not correlated with the hepatic HCV RNA levels [71]. It was also found that hepatic miR-122 expression in patients seronegative for HCV RNA was significantly higher than that in patients seropositive for HCV RNA. The level of hepatic miR-122 expression was inversely correlated with the severity of functional and histopathological liver damage, and levels of aminotransferases; therefore it was concluded that unlike *in vitro* findings, hepatic miR-122 expression is not correlated with HCV RNA levels in the human liver [71]. In another study, sera from patients with CHC contained higher levels of miR-122 than sera from healthy controls, and the investigators showed that serum miR-122 levels correlated well with markers of liver inflammatory activity like ALT but not with fibrosis stage and functional capacity of the liver [72]. A recent study on 53 HCV-positive serum samples suggested that circulating miR-122 levels might also serve as a biomarker in the evaluation of severity of fibrosis in CHC [73]. In this study, hepatic levels of miR-122 decreased significantly with the severity of fibrosis. It was also found that circulating miR-122 levels correlated negatively with increasing stages of fibrosis, although the inverse correlation was moderate due to a two phase miR-122 pattern during fibrosis progression [74]. The investigators observed that circulating miR-122 levels decreased in patients with severe fibrosis, while at early stages with distinct fibrotic structures and high inflammatory activity, miR-122 serum levels were elevated [74]. Therefore they concluded that during progression of fibrosis less miR-122 is released into the blood stream due to the loss of liver cells and the decrease of hepatic miR-122 levels.

miR-122 and PKR

PKR, a double-stranded RNA-dependent protein kinase, is among the well-known members of cellular antiviral proteins transcriptionally induced by IFNs in response to viral infection. Also, it is known that human Dicer performs this function in cooperation with its protein partners protein kinase R activator (PRKRA), Argonaute 2 (AGO2), and TAR RNA binding protein (TRBP). The exact role of these accessory proteins in Dicer activity is still poorly understood. In a study by Koscianska, inhibition of either Dicer protein partner, including PRKRA, substantially affected not only miRNA levels but also pre-miRNA levels, and it had a rather minor effect on the specificity of Dicer cleavage [75]. They demonstrated that PRKRA, AGO2, and TRBP were required for the efficient functioning of Dicer in cells, and speculated that one of the roles of these proteins is to assure better synchronization of cleavages triggered by two RNase III domains of Dicer [75].

It is known that activated PKR phosphorylates eIF2, and thereby blocks viral replication at the level of protein synthesis (Fig 5). It is also reported that miR-122 expression was decreased after IFN treatment, whereas over-expression of miR-122 partially alleviated the antiviral effect of IFN [65]. Based on these results and based on the fact that PKR is an important mediator of IFN actions in HCV infection, and due to the finding of a conserved binding site for miR-122 in the 3'-UTR of PRKAR mRNA, we hypothesize that miR-122 up-regulates HCV proteins through inhibition of PRKRA.

Liver transplant (LT)

Liver disease caused by the hepatitis C virus is the main indication for liver transplantation in Western countries [76]. Yet for HCV-infected patients, LT does not

result in disease resolution, as viral reservoirs remain to infect the donor liver. Infection occurs with reperfusion of the transplanted organ and, although a temporary drop in serum HCV RNA is observed during the first 24 hours after liver reperfusion, an increase in viral load is virtually always seen over subsequent weeks to months as the infection is reestablished [77]. Progression of HCV-mediated liver disease can cause reduced graft survival, leading to the possible need for re-transplantation and a reduction in patient survival at 3–5 years post-transplantation [77]. Because of the impact of HCV recurrence on graft and patient survival, several treatment strategies have been evaluated. The ideal is for antiviral therapy to be administered before transplantation to achieve a cure of HCV infection [76]. However, this approach is applicable in relatively few patients with decompensated cirrhosis due to intolerance to and adverse effects of antiviral therapy, particularly in patients with decompensated cirrhosis. Pre-emptive therapy in the early post-transplant period is limited by the high rate of side effects. Frequently, antiviral therapy is initiated when active hepatitis recurs. The goal of the therapy is to obtain viral eradication and/or reduce disease progression. Treatment of established graft lesions with PEG-IFN and RBV combination therapy results in a SVR in about 30% of patients [76]. There is always the possibility that different factors contributing to an individual's failure to resolve primary HCV infection might also contribute to the severity of its recurrence. For instance, by the time a patient receives a new liver allograft treatment has already failed and viral adaptations in response to T-cell or antibody-mediated response have already taken place. Therefore, the potential interplay between factors such as preexisting immunity, viral load, quasispecies diversity and complexity pre- and post-transplant and

immune suppression combine to make the circumstance of HCV recurrence after LT different than primary acute or chronic infection [77].

Several recent studies have sought to find molecular determinants both of HCV recurrence after liver transplant as well as biomarkers of LT rejection., Farid et al. showed that liver injury is associated with the release of hepatocyte-derived microRNAs, including miR-122, into the circulation; and they suggested these miRNAs could work as promising candidates as early, stable, and sensitive biomarkers of rejection and hepatic injury after liver transplantation [78]. In another recent study performed in HCV-infected LT recipients, Mensa et al. found that there was a significant correlation between the levels of CLDN and OCLN and the slope of HCV-RNA increase during the first week after LT [79].

Response to treatment in CHC

As mentioned above, miR-122 plays an important role in HCV pathogenesis. Based on the fact that miR-122 expression was associated with higher RNA abundance in HCV infected cells, it might be inferred that miR-122 levels should be higher in those who do not respond to combination therapy. Although it is known that miR-122 can act as a positive regulator for HCV RNA *in vitro*, yet, in a conflicting finding, miR-122 levels were markedly decreased pretreatment in subjects who had no virological response during IFN therapy [70]. We found OCLN and PRKRA as potential targets for miR-122. Accordingly we hypothesize that over-expression of miR-122 can lead to down-regulation of OCLN, and PRKRA. Therefore, we hypothesized that miR-122 plays a dual role in HCV infection; in uninfected hepatocytes, miR-122 can bind to the 3'-UTR of OCLN and this will lead to decrease of HCV entry into hepatoctyes, while, in infected

hepatocytes, miR-122 can bind to both 3'-UTR of PRKRA and 5'-UTR of HCV RNA and both of these will lead to increase in HCV replication (Figure 8).

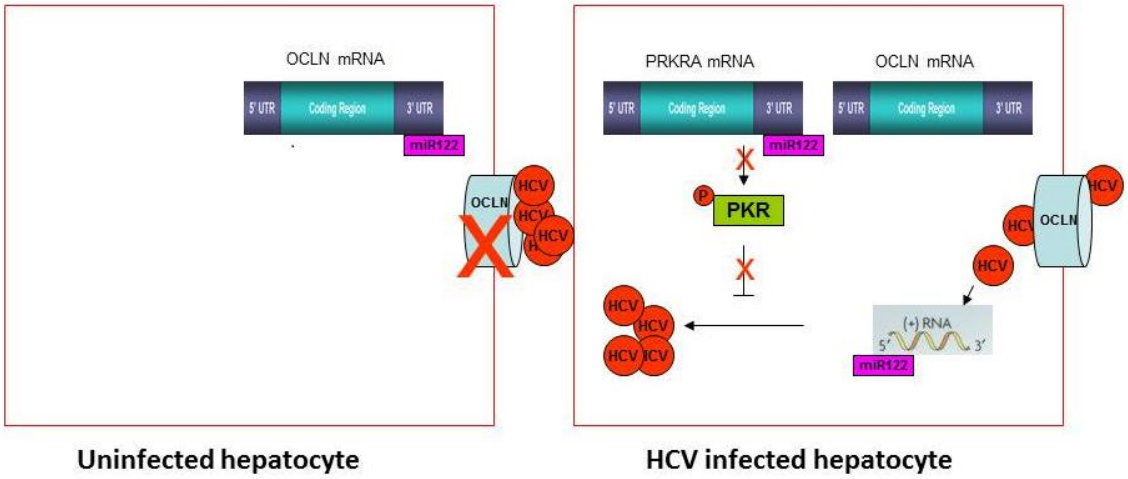

Figure 8 . Main hypothesis: Dual roles for miR-122 in HCV infection. In uninfected hepatocytes, miR-122 decrease HCV entry into hepatocytes through down-regulation of OCLN, while in infected hepatocytes, miR-122 increases HCV replication through both binding to the 5'-UTR of HCV RNA and 3'-UTR of PRKRA. Binding of miR-122 to the 3'-UTR of PRKRA could lead to a decrease in PRKRA levels and consequently to a decrease in phosphorylation of PKR and this will result in removing the inhibitory effect of PKR on HCV replication.

On the other hand, the ability to determine the outcome of hepatitis C may be useful in terms of implementing treatment strategies; however, to date, the predictive associations in the literature are limited. We hypothesized that gene expression prior to or during the early phase of anti-HCV therapy may elucidate important molecular pathways for achieving virological response. Based on our findings and previous studies, we chose miR-122, miR-29b as well as CLDN, OCLN, PKR, and PRKRA, to determine if there was any association between pre-treatment levels of any of these molecular factors with HCV RNA levels, response to treatment and/or stage of liver disease.

CHAPTER 2: MATERIALS AND METHODS

Chemicals and antibodies

Mouse monoclonal antibodies against OCLN and glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) and goat polyclonal antibody against PRKRA were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibody against CLDN-1 was purchased from Invitrogen (Grand Island, NY, USA). Rabbit polyclonal antibody against PKR was purchased from Cell Signaling Technology (Danvers, MA, USA).Enhanced chemiluminescence (ECL)-Plus Western blotting detection reagent was obtained from Amersham Biosciences (Piscataway, NJ, USA).

Cell Cultures

Huh-7.5 subgenomic genotype 1b HCV replicon cell lines were from Apath LLC (St. Louis, MO). Huh-7.5 is a highly permissive, interferon-–cured Huh-7 human HCC cell line derivative. The 293T/17 cell line is a derivative of the 293T cell line that was purchased from ATCC. 293T is a highly transfectable derivative of the Human Embryonic Kidney (HEK) 293 cell line into which the temperature sensitive gene for SV40 T-antigen was inserted. Huh-7.5 and 293 T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Additionally 750 µg/mL of G418 was used for Huh7.5 cells.

miRNAs, and Reporter Construct

The miRIDIAN miRNA mimics for hsa-miR-122, hsa-miR-200b, customized hsa-miR-122 mutants, miRNA mimic negative control (MMNC) were obtained from Dharmacon (Lafayette, CO). pLuc-OCLN, and pLuc-PRKRA were obtained from Genecopoiea (Rockville, MD, USA). Both vectors contain firefly luciferase reporter gene as well as complementary DNA (Rluc) encoding Renilla luciferase as an internal control reporter (Fig 9). The pLuc-OCLN also contained the full-length 3'-UTR of OCLN mRNA down-stream of the firefly luciferase gene, while pLuc-PRKRA contained the full-length 3'-UTR of PRKRA mRNA down-stream of the firefly luciferase gene. These constructs were confirmed by way of restriction enzyme digestion and sequencing (Genecopoiea).

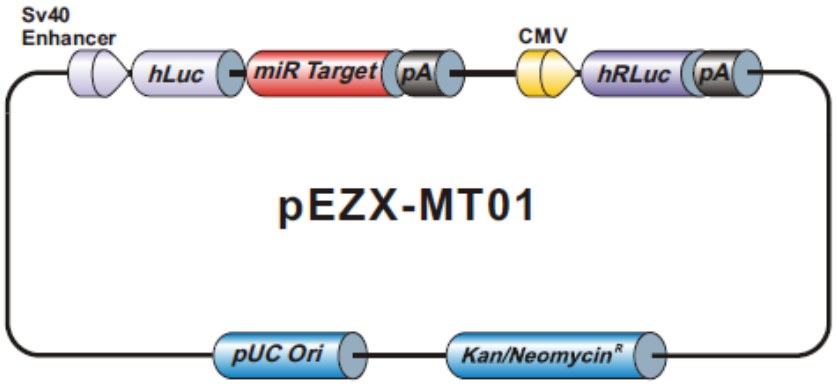

Figure 9. Schematic representation of pEZX-MT01 (Genecopoeia). This vector was used as a template for insertion of 3'-UTR of OCLN and PRKRA mRNA. Figure from http://www.genecopoeia.com/

Transfection and Luciferase Activity Assays

Transfection of miR-122 mimic or miR-200b was performed using Lipofectamine 2000 from Invitrogen (Carlsbad, CA) according to the manufacturer's protocol. Co-transfection of miRNA mimics and reporters was also performed using Lipofectamine 2000. Briefly, cells were co-transfected with 0.4 μg/mL of pLuc-OCLN with 0-50 nM tested miRNAs or 50 nM of micro RNA mimic negative control (MMNC). Forty-eight hours after transfection, cells were harvested and lysed, and the luciferase reporter activities were measured using the LucPair™ miR Dual Luciferase Assay Kit (Genecopoiea) on Synergy HT luminometer from Biotek (Burlington, VT, USA). Firefly luciferase activity was normalized to Renilla luciferase activity and total protein, determined using the bicinchoninic acid protein assay kit.

RT-PCR

Total RNA from tested cells was extracted, and complementary DNA was synthesized, and qRT-PCR was performed as previously described [8]. GAPDH primers were designed as described [8]. The following primers were used for OCLN, and PRKRA. OCLN: forward primer 5'CTCCCGTTTGGATAAAGA3'; OCLN Reverse primer 5'TGATGTGTGACAATTTGCTC3'; PRKRA forward primer 5'AAGAAGCTGGCGAAACATAG3'; PRKRA reverse primer 5'GCCAATTCCTGTAATGAACC3'

RNA isolation and miRNA RT-PCR from liver biopsy samples

Total RNA was isolated from liver biopsy samples using Trizol Reagent from Invitrogen Corp. (Carlsbad, CA, USA) per manufacturer's instructions. The integrity of

the RNA was verified by an Agilent 2100 Bioanalyzer profile from Agilent Technologies Inc. (Santa Clara, CA, USA).

For miRNA, first-strand complementary DNA synthesis was performed using TaqMan® MicroRNA Reverse Transcription Kit primed with miR-specific primer from Applied Biosystems (Grand Island, NY, USA). Real-time quantitative RT-PCR (qRT-PCR) was performed using TaqMan® MicroRNA Assays (Applied Biosystems), following the manufacturer's recommendations, with an ABI Prism 7500 Sequence Detection System using TaqMan® Universal Master Mix (Applied Biosystems). Fold change values were calculated by comparative Ct analysis and normalized to SNORD44 concentrations. SNORD44 was used as an invariant control. [RNA isolation and both RNA and miRNA RT-PCR from liver biopsy samples were done by molecular core laboratory at Cannon Research Center at Carolinas Medical Center (CMC).]

Western Blotting

Cells were grown to near confluence and washed with phosphate-buffered saline (PBS), lysed in a buffer containing 1% (v/v)Triton X-100 with PBS and Halt Protease Inhibitor Cocktail from Pierce Chemicals (Rockford, IL, USA). Protein concentrations were measured using the bicinchoninic acid method. Total proteins were separated on 4%-12% gradient sodium dodecyl sulphate-polyacrylamide gel (Invitrogen) and electrophoretically transferred onto an Immuno-Blot. The membranes were blocked for 1 h in PBS containing 5% nonfat dry milk (v/v), and then incubated for 1 h with the primary antibody at room temperature. The dilutions of the primary antibodies were as follows: 1:500 for anti-OCLN, anti-PKR, anti-PRKRA and 1:2000 for anti-GAPDH antibody. After 4 washes with 0.1% (v/v) Tween 20 in PBS (PBS-T), the membranes were incubated for 1 h with the appropriate secondary antibody. Finally, the membranes

were washed 4 times with PBS-T, and the bound antibodies were visualized with the ECL-Plus chemiluminescence system. A computer based imaging system, LAS 3000 (Fuji Film, USA) was used to measure the relative optical density of each specific band obtained after Western blotting. In densitometry, all band intensities were within linear range of the dynamic range of the membrane exposure.

Immunohistochemistry (IHC)

Four micron sections of formalin fixed paraffin embedded liver biopsies were stained with PACT from Abcam (Cambridge, MA, USA), OCLN or CLDN from Santa Cruz Biotechnology Inc. (CA, USA) mouse monoclonal antibodies. Slides were deparaffinized and rehydrated through several changes of xylene, graded alcohol and distilled water. Antigen retrieval was done using citrate buffer pH 6.0 from Polyscientific (Bay Shore, NY, US). Tissue sections were blocked with 2.5% normal horse serum and incubated with antibodies OCLN (1:50), CLDN (1:50) or PACT at 5µg/ml overnight at 4 degrees. A mouse IgG ImmPRESS polymerized reporter enzyme staining system from Vector Labs (Burlingame,CA, US) was used to detect bound antibody. We used 3, 3'-diaminobenzidine (DAB) from Vector to visualize antibody staining. Nuclei were counter stained with Mayer's hematoxylin. Slides were cleared and dehydrated then cover-slipped with Permount. Images were taken at 40x on an Olympus BX40 microscope with a DP72 camera and CellSense software [IHC was performed by the histology core facility at Cannon Research Center.]

Immunofluorescence (IF)

Treated cells were fixed with either cold methanol or 4% (v/v) paraformaldehyde in phosphate-buffered saline and then permeabilized in 0.2% Tween 20. Primary

antibodies used were rabbit polyclonal anti-CLDN-1 and mouse monoclonal anti-OCLN. Slides were also incubated in the absence of primary antibody and or with control immunoglobulin as negative controls. Goat anti-mouse/rabbit antibodies conjugated with Alexa 488, 555 and or 594 were used as secondary reagent. Nuclear staining was performed by using premixed DAPI with proLong gold antifade mounting media. Images were captured by a Zeiss LSM710 confocal microscope from Carl Zeiss GMBh (Jena, Germany) with 63X objective using Z stack optical sectioning. Image processing and analysis were performed using different image analysis software that includes the Zeiss ZEN LE and our self-developed analyzing applications. The same settings for parameters were applied to capture all the images to allow direct comparisons. The results were correlated well and supported by the numerical quantification of co-localization data. The quantification of co-localization between OCLN and CLDN was performed using overlap correlation coefficient.

HCV pseudo particle generation and infection

HCVpp is recognized as the best model currently available for studying HCV entry. HCVpp are recombinant viral particles containing a retroviral core surrounded by an envelope, bearing HCV glycoproteins E1 and E2 (Fig 10) [80]. To generate luciferase reporter HCVpp and controls, plasmids encoding a provirus containing luciferase as reporter gene, HIV gag-pol and either HCV E1-E2, or vesicular stomatitis virus glycoprotein (VSV-G) were co-transfected giving rise to HCVpp, VSVGpp. These plasmids were given to us as a generous gift by Dr. Charles Rice from Rockefeller University (NY, USA). In the infection assay, pseudo-particles assembled in 293T cells

and released into the culture medium were subsequently used for infecting Huh7 cells (Fig 10).

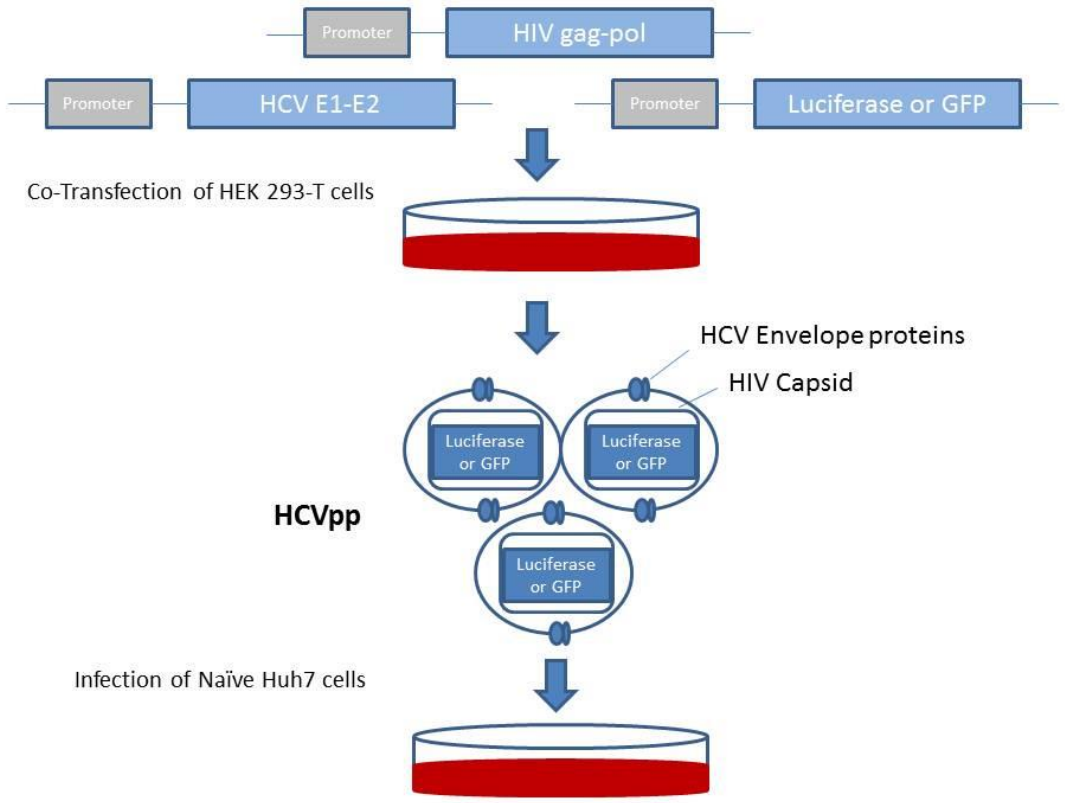

Figure 10. *In vitro* HCVpp generation and infection. Human embryonic kidney (HEK) 293T cells are co-transfected with three different vectors expressing HCV E1-E2 glycoproteins, HIV gag-pol, and a protein works as a reporter (GFP or luciferase). Supernatant containing HCVpp are used for infecting naïve Huh7 cells.

One day prior to transfection, 293T cells were seeded in a p150 mm^2 dish. A total of 32 ug DNA was transfected using FuGENE 6 from Roche Applied Science (IN, USA) and media were replaced after 6 h. Supernatants were harvested at 48 h and 72 h after transfection, pooled and filtered (0.45 um pore size). The following plasmid

combinations and ratios (by weight) were used: To generate luciferase reporter HCVpp and controls, equal amounts of pNL43.luc.R-.E-(encoding a provirus containing luciferase and HIV gag-pol) and either HCV E1E2 or VSV-G were co-transfected giving rise to HCVpp and VSVGpp respectively. To generate GFP reporter HCVpp and VSVpp, the following plasmids were used: (1) a provirus encoding the respective reporter gene (CSGW), (2) HIV gag-pol and (3) either HCV E1E2 or VSV-G were transfected at a 1:1:4 ratio.

All infection assays were performed in 6-well format using $3x10^5$ Huh7.5 cells per well. Cells were infected with pseudovirus supernatants diluted in fresh media (1:2 for HCVpp and 1:100 for VSVGpp) and polybrene was added to a final concentration of 4 μg/mL. After 6-18 h, the medium was changed. For pseudoparticles with luciferase activity, luciferase assays were performed 72 h after infection. Briefly, cells were lysed with 1X luciferase lysis buffer from Promega (WI, USA) and the expression of the luciferase reporter was measured after the addition of 100 μl luciferase substrate (Promega) on a Synergy HT luminometer from Biotek. The HCVpp luciferase activity was then normalized to VSVpp luciferase activity. Results of infection experiments were the mean of three independent infections. Errors bars represent the standard deviation of the mean.

Lenti-miRNA Expression System

The Lenti-miRNA Expression System from Applied Biological Materials Inc. (BC, Canada) allows production of replication-incompetent lentivirus that can transduce Huh7.5 cells. These lentivirus particles are made by transfection of both pLenti-III-mir-GFP (Fig 11) and a packaging mix.

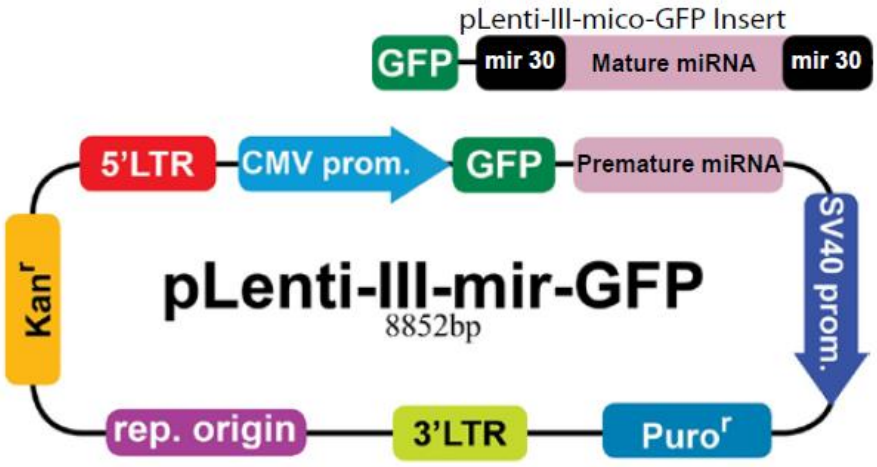

Figure 11. Maps of miRNA lentiviral plasmid. The mature mi-122 sequence was subcloned within two mir-30 backbones (pLenti-IIImico-GFP) downstream of GFP reporter. Figure from www.abmgood.com

Viral particle production

Transfection of vectors and packaging mix into 293 T cells was already done by ABM, Canada. (Fig 12).

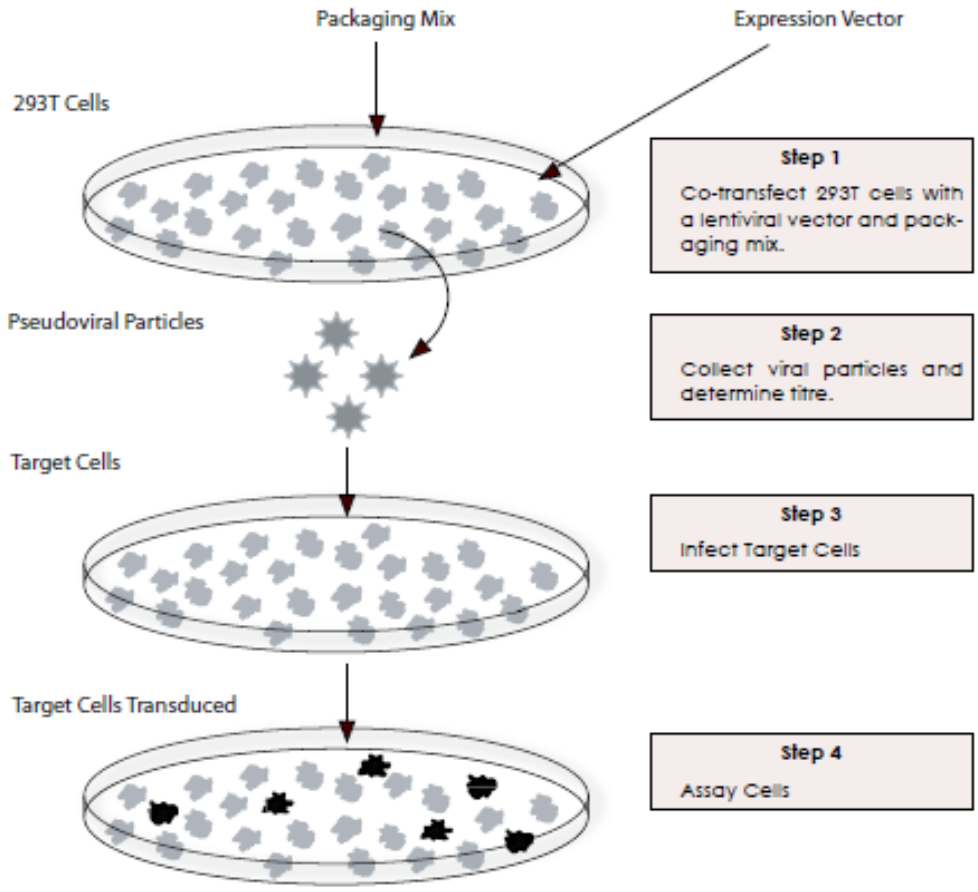

Figure 12. Procedure for transient production of pseudoviral particles and transduction of target cells based on lentivirus expression system (ABM, Canada). Figure from www.abmgood.com

A Brief description of transfection protocol follows:

1-One day before transfection (Day 1), 293T cells were plated into a 10cm tissue culture plate so that they were 90-95% confluent on the day of transfection.

2- On the day of transfection (Day 2), transfection mix was set up:

15µg of Lenti-Combo Mix and 10µg of pLenti expression plasmid DNA was diluted in 1.0 mL of medium without serum and was gently mixed. In a separate tube, 80 µL of Lentifectin was diluted in 1.0 mL of medium without serum and incubated for 5 minutes

at room temperature. After 5 minutes of incubation, the diluted DNA was combined with the diluted Lentifectin. This was Incubated for 20 minutes at room temperature to allow the Lentifectin/DNA complexes to form. 4.5 mL serum-free medium was added to the complexes followed by gentle mixing. The medium was removed from the cells, and Lentifectin/DNA complexes was carefully added to culture dishes without dislodging cells. The cells were incubated for 5-8 hours at 37°C in a humidified 5% CO2 incubator.0.65 mL serum was added to each transfected culture dish and was incubated overnight.

3. The following day (Day 3), the medium containing the Lentifectin/ DNA complexes was removed and replaced with 10 mL complete culture medium, and incubated at 37°C in a humidified 5% CO2 incubator.

4. Virus-containing supernatants were harvested 48-72 hrs post-transfection (Day 4-5) by collecting media into 15 mL sterile, capped, conical tube.

5. Supernatants were centrifuged at 3000rpm for 15 min. at 4°C to pellet debris.

6. Viral supernatants were aliquot and virus titer was assayed according to the manufacturer's protocol (ABM, Canada).

Lenti-virus infection

Huh7.5 cells were plated in a 24-well plate 24 hours prior to viral infection at a density of 0.5×10^5 cells per well and were incubated at 37°C with 5% CO2 overnight. The following day, media were removed from the wells and replaced with 0.5ml of the polybrene/media mixture per well. Huh7.5 cells were infected by adding 5 μL [multiplicity of infection (MOI) of 1] of viral particles containing lenti-miR122 genome

or the same amount of a control virus with the same genetic backbone but miR-122 genome. Both viral particles contained GFP reporter within their genome.

Cells were incubated at 37°C with 5% CO2 overnight. Culture medium was removed, replaced with 1 mL of complete medium, and cells were incubated at 37°C with 5% CO2 overnight. The following day, cells were split 1:3 and incubated for 48 h in complete DMEM. After 48 h GFP expression was assessed by flow cytometery and cells were infected with supernatants diluted in fresh media (1:2 for for HCVpp) and (1:100 for VSVpp), and in presence polybrene for 6-18 h. The media was changed and cells were further cultured for 72 h prior to harvesting. Firefly luciferase activity was measured after harvesting (Promega), and HCVpp luciferase activity was normalized to VSVpp luciferase activity. To determine GFP expression, cells were fixed with 1X BD FACS lysing solution (BD Bioscience), diluted 1/10 in PBS, green fluorescence protein (GFP) expression was quantified using FACS Aria II flow cytometer (BD Bioscience). Data were analyzed using FlowJo analysis software version 9.5.3 from Treestar Inc. (OR, USA).

HCVcc system

For producing infectious HCV virions in cell culture, the first step is the transfection of *in vitro* transcribed JFH-1 into Huh-7-derived cells (Fig 13). Transcripts from the cDNA derived from the viral RNA induce infection when introduced into a permissive cell [81]. This was based on the observation that *in vitro* transcribed HCV RNA is infectious when transfected into the liver of chimpanzees [82]. Infectious viruses are obtained from cell culture supernatants and infectivity is determined by indirect immunofluorescent staining of infected cells for the viral NS5A protein. This system

yields viral titers of 10^4–10^6 infectious units per ml of culture supernatant. Infection spreads throughout the culture within a few days after inoculation at low multiplicity of infection (moi) and the virus can be serially passaged without loss of infectivity [19, 20]. The replication of HCV is the most efficient in the highly permissive Huh7.5 cells derived from an HCV replicon-harboring Huh7 cell line selected for the highest HCV replication efficiency [83].

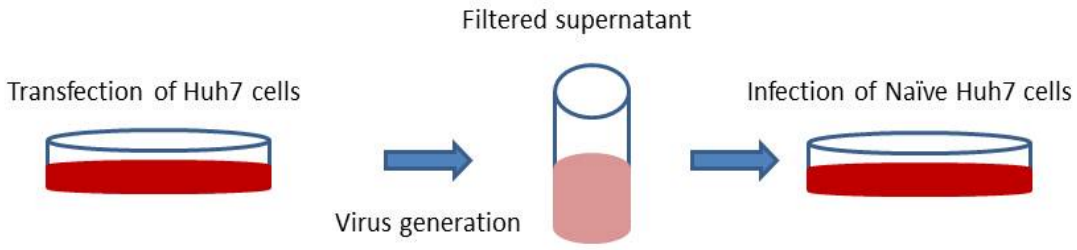

Figure 13. *In vitro* generation of infectious HCV. Huh7 cells are transfected with *in vitro* transcribed HCV RNA . After 72 h incubation, the supernatant containing HCV particles are collected and used for infection of new Huh7 cells.

In vitro transcription of HCV plasmids

pJH3-5 and S1-S2-p6m plasmids are respectively wild-type and mutant JFH1 based vectors. These plasmids were given to us as a generous gift by Dr. Stanley Lemon from UNC Chapel Hill (NC, USA). Briefly, Two µg of each of pJH3-5 and S1-S2-p6m plasmids were linerized in a total of 10 µL reaction containing 0.5 ul XbaI enzyme, 1 ul Buffer 4 and 0.1 µL 100xBSA. For *in vitro* transcription, T7 MEGAscript kit was used, supplemented with 1 ul RNase Inhibitor (promega) in the reaction mix (total 20 µL) according to manufacturer's protocol, and was incubated for 4-6 hrs. 1.5-2 µg DNA/reaction was used of each linearized pJH3-5 and S1-S2-p6m. After the reaction,

2 µL of TurboDNase was added to each plasmid and incubated for 15 min at 37 C. *In vitro* transcripted RNA was purified with RNeasy mini kit (Qiagen) using the cleanup method according to the manufacturer's protocol. Concentration of RNA was measured and transfection was done by TransIT mRNA transfection kit by Mirus (MIR 2250).

Transfection of Huh7.5 cells with HCV RNAs

Briefly, 18–24 hours before transfection, Huh 7.5 cells were plated in 2.5 mL complete growth medium per well in a 6-well plate. Cells were about 60–90% confluent prior to transfection. 250 µL of Opti-MEM I Reduced-Serum Medium was mixed with 2.5 µg of HCV RNA and 5 µL of each mRNA Boost Reagent plus and TransIT-mRNA Reagent was added. This mix was incubated at room temperature for 2–5 minutes to allow sufficient time for complexes to form. Complexes were added drop-wise to different areas of the wells, and culture vessel was gently rocked back-and-forth and from side-to-side to evenly distribute the TransIT-mRNA Reagent:mRNA Boost:RNA complexes. The media was changed 6 h after transfection and every 24 h afterward. Supernatant was collected 72 and 96 hours after infection, pooled, and filtered for infection step.

Infection of Naïve Huh7.5 cells with HCV particles

Briefly, 18–24 hours before transfection, Huh 7.5 cells were plated in 2.5 mL complete growth medium per well in a 6-well plate. Cells were about 60–90% confluent prior to infection. The following day, cells were infected using supernatant containing HCV particles. After 6-12 hours, media was changed and cells were transfected with selected miRNAs using lipofectamie 2000. After 72 hours of incubation, cells were

harvested for detecting protein levels of HCV core and NS3 as well as PRKRA and GAPDH.

Statistical Analysis

The Student t test (for comparison of two conditions) or analysis of variance (for comparisons among more than two) was used to analyze the differences among means. Bonferroni correction was used as post-hoc test for analysis of variance. A non-parametric test was used for comparing the means if the data was not normally distributed. Values of $P<0.05$ were considered statistically significant. Experiments were repeated at least three times with similar results. All experiments included at least triplicate samples (n=3) for each treatment group. Representative results from single experiments are presented.

CHAPTER 3: MIR-122 DECREASES HCV ENTRY INTO HEPATOCYTES THROUGH BINDING TO THE 3'-UTR OF OCLN MRNA

Introduction

MicroRNAs (miRNAs) are approximately 22 nucleotide non-coding RNAs that can down-regulate various gene products by inducing either cleavage or a reduction in translational efficiency of the target mRNAs [35]. MiRNA-122 (miR-122), is a 22 nucleotide miRNA that accounts for 70% of the total miRNA population in normal adult hepatocytes with approximately 66,000 copies per cell [43]. One function of miR-122 in the mammalian liver is to help regulating lipid and cholesterol metabolism. The knockdown of miR-122 expression in mice and monkeys down-regulates cholesterol and lipid metabolizing enzymes and reduces plasma cholesterol levels [48-50]. Jopling *et al.* found that miR-122 was expressed in human hepatoma cells (Huh7), which are permissive to hepatitis C virus (HCV) replication, but not in a different human liver cell line, HepG2 which does not support HCV replication. HCV RNA was reduced by about 80% when miR-122 was silenced in Huh7 cells stably expressing genotype 1b HCV replicon [59]. The effect of miR-122 on HCV is due to binding to the 5'-UTR of HCV RNA [59]. This identified miR-122–binding site is in an unstructured region of the 5'-UTR, upstream of the HCV internal ribosome entry site (IRES), and is conserved across all six genotypes of HCV [59]. This finding showed that miR-122 is important for efficient replication of HCV RNA *in vitro*. Because the authors did not observe any effect of miR-122 binding on HCV translation or RNA stability, they concluded that miR-122

positively regulates HCV at the level of viral replication [59]. A second binding site for miR-122 was identified adjacent to, and downstream of, the first one. It was shown that miR-122 binds to both sites within the same HCV molecule to increase HCV RNA abundance [60]. However, it is not yet known whether this is due to a requirement for overlapping binding to the two sites, or to a sequential binding process [60]. It was also reported that miR-122 expression was decreased after Interferon (IFN) treatment, and over-expression of miR-122 partially alleviated the antiviral effect of IFN [65]. Lanford *et al.* showed that silencing of miR-122 in chronically HCV-infected chimpanzees led to long-lasting suppression of HCV viremia [59]. These observations roused much interest in the role of miR-122 in HCV infection and its potential as a therapeutic target.

HCV entry factors include tetraspanin CD81, scavenger receptor class B type I (SR-BI), and the tight-junction protein claudin-1 (CLDN1). Despite identification of these factors for HCV entry, several human cell lines, as well as all cell lines of non-primate origin remained resistant for HCV entry, even when cells are manipulated to express all of these factors together [21]. Recently, Ploss and colleagues have identified Occludin (OCLN), an essential component of tight junctions, as a host-cell protein essential for HCV entry [22]. They showed that, whenever human CD81, SR-BI, claudin-1 and OCLN were present, all cell types tested could be infected with HCV. Although the detailed mechanism of HCV entry has yet to be elucidated, It has been suggested that HCV might use CD81, and SR-BI for binding to the cell surface and movement to tight junctions, where OCLN, and CLDN facilitate HCV entry into the hepatocytes [23].

As mentioned above, the effect of miR-122 on HCV is unusual in that it enhances HCV replication rather than down-regulating viral mRNA or poly-protein. However, new

findings highlight an anti-viral as well as tumor-suppressive role for miR-122 [84]. An online search of the TargetScan 6.2 database demonstrated at least one putative miR-122 "seed" sequence match in the 3'-UTR of OCLN mRNA. This 8-mer binding site is highly conserved across human, other primate, mouse, rat, guinea pig, horse, cow, and elephant mRNA. Because miR-122 plays an important role in HCV replication, and because OCLN is an HCV entry molecule, and based on the finding of a conserved binding site in 3'-UTR of OCLN mRNA, we aimed to determine if miR-122 decreases HCV entry into human hepatoma cells through down-regulation of OCLN. Members of the miR-200 miRNA family are down-regulated in human cancer cells and tumors due to aberrant epigenetic gene silencing and play a critical role in the suppression of epithelial-to-mesenchymal transition [85]. In a recent study at CMC, it was found that miR-200b are among several miRNAs which are down-regulated in HCV infected cells [86]. We also found miR-200b as a candidate miRNA which targets 3'-UTR of OCLN *in silico.* Therefore, we aimed to determine if miR-200b could bind 3'-UTR of OCLN of decrease its expression in vitro.

Results

In Silico analysis predicts seed region matches for miR-122 and miR-200b on the 3'-UTR of OCLN mRNA.

An online search of the TargetScan 5.2 database (http://www.targetscan.org/vert_50/) demonstrated that at least one separate putative seed sequence match site was found in the 3'-UTR of OCLN mRNA for miR-122 (8 mer seed-match), and miR-200b (7mer-8m) respectively. As shown in Figure 1, the predicted seed sequence binding site for miR-122 within OCLN mRNA (184-192 nt) was perfectly

conserved in human, chimpanzee, monkey, mouse, rat, guinea pig, horse, cow, and elephant (Fig 14).

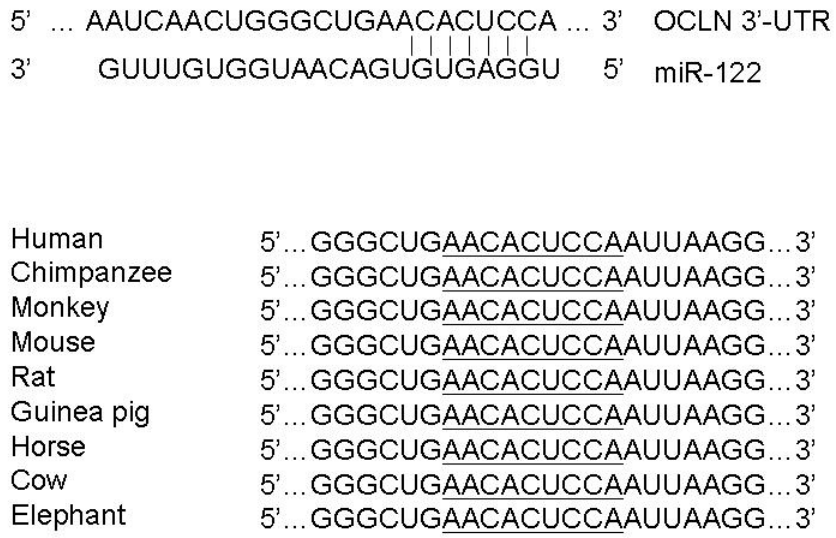

Figure 14. *In silico* prediction of putative binding sites for miR-122 within the 3'-UTR of OCLN. A schematic of seed match region between miR-122 and 3'-UTR of OCLN mRNAs as putative target. The seed match (underlined) is perfectly conserved in the human, chimpanzee, monkey, mouse, rat, guinea pig, horse, cow, and elephant.

miR-122 down-regulates OCLN protein expression in Huh7.5 cells

To experimentally verify that the putative miR-122 binding site is functional, Huh7.5 cells were transfected with miR-122 mimic and OCLN mRNA and protein levels were measured by qRT-PCR and Western blotting, respectively. We did not observe any decrease in OCLN mRNA levels unless when high amount (100 nM) of miR-122 was used (Fig 15A). However, Huh7.5 cells transfected with miR-122 mimic showed a significant reduction in the expression of OCLN protein levels (80% after 48 hours) compared with negative control (Fig 15 B-C).

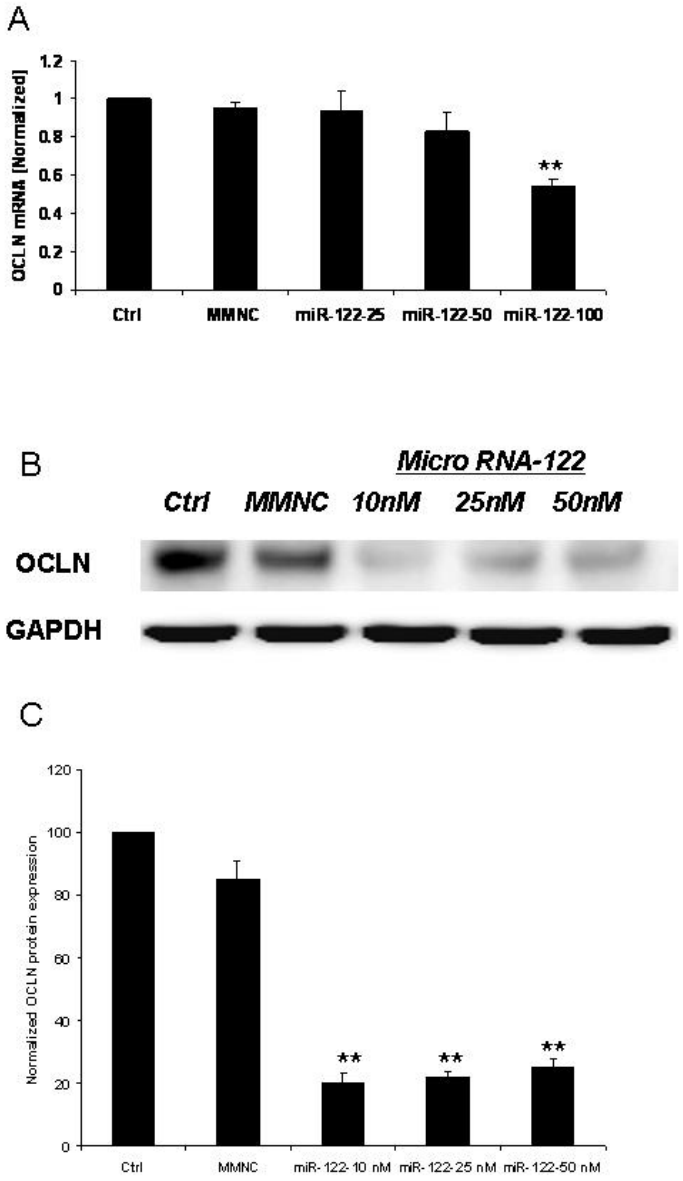

Figure 15. MiR-122 down-regulates OCLN protein levels. (A) Huh 7.5 cells were transfected with 0, 25, 50 or 100 nM miR-122 mimic or 100 nM MMNC in Lipofectamine 2000. Twenty-four hours after transfection, the cells were harvested. The levels of OCLN mRNA were quantified using qRT-PCR. The amounts of OCLN mRNA were normalized to those of GAPDH. Values for cells with a mock transfection were set equal to 1 (B) Huh 7.5 cells were transfected with 0, 10, 25 or 50 nM miR-122 mimic or 50 nM MMNC by Lipofectamine 2000. Forty-eight hours after transfection, the cells were harvested. OCLN and GAPDH protein levels were assessed by Western blotting. (C) The amounts of OCLN protein were normalized to those of GAPDH. Values for cells with a mock transfection were set equal to 100. Data are presented as the mean ± standard error (n = 3). $^{**}P < 0.01$ versus mock transfected control.

MiR-122 interacts with the 3'-UTR of OCLN mRNA and down-regulates its expression in Huh7.5 cells

To further establish that miR-122 targets the 3'-UTR of OCLN mRNA , a reporter construct, pLuc-OCLN, was constructed with the OCLN 3'-UTR inserted downstream of the firefly luciferase open reading frame driven by SV40 promoter (pLuc-OCLN). It also contained complementary DNA (Rluc) encoding Renilla luciferase driven by CMV promoter as an internal control. Huh7.5 cells were infected with pLuc-OCLN together with increasing amount of miR-122 mimic or non-specific miRNA (MMNC), as a negative control. Forty-eight hours after transfection, the luciferase reporter activity was assayed. miR-122 mimic transfection significantly decreased reporter activity [by 55% with 50 nM of miR-122 ($P<0.01$)], while MMNC was without a significant effect (Fig 16A). To further establish the interaction between miR-122 and OCLN 3'-UTR, a mutant miR-122 was created in which the seed match site for the 3'-UTR of OCLN mRNA was abolished (Fig 16B). Huh 7.5 cells were cotransfected with pLuc-OCLN, and with increasing concentrations of miR-122 as well as MMNC as negative control and luciferase reporter activity was assayed. As anticipated, miR-122 mutant co-transfection did not affect luciferase activity opposite to what observed with wild-type miR-122 (Fig 16C). This further indicated that miR-122 binds 3'-UTR of OCLN mRNA

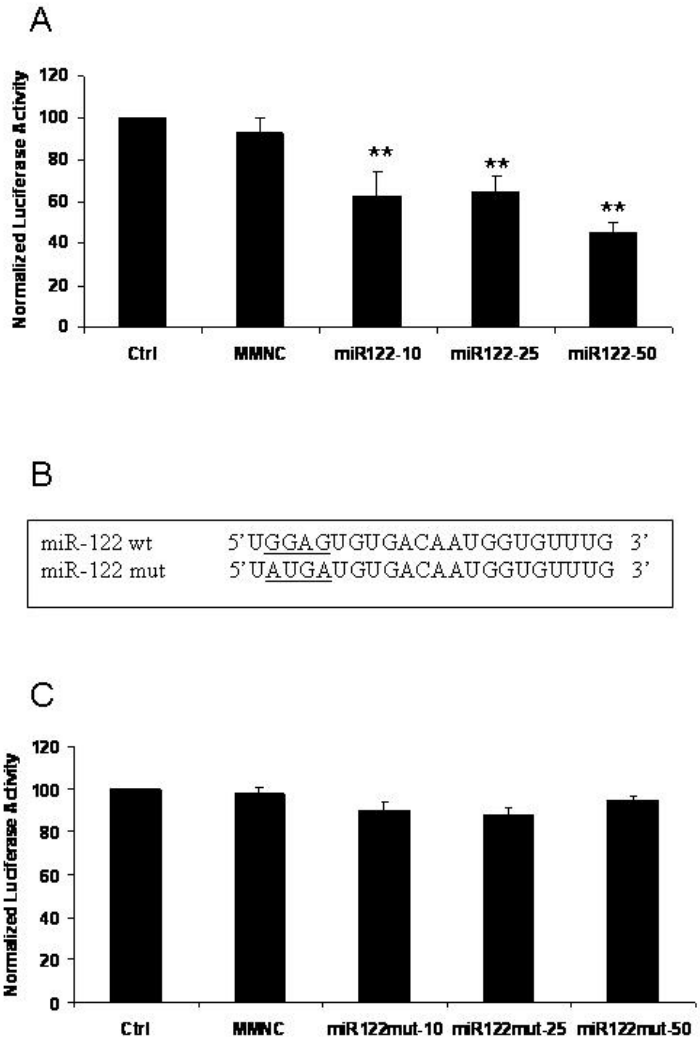

Figure 16. MiR-122 binds to the 3'-UTR of OCLN and down-regulates its expression. (A) Huh7.5 cells were cotransfected with 0.4 µg/mL of pLuc-OCLN and with 0, 10, 25 or 50 nM miR-122 mimic or 50 nM MMNC in Lipofectamine 2000. Forty-eight hours after transfection, the luciferase reporter activities were measured using LucPair™ miR Dual Luciferase Assay Kit (Genecopoiea). Firefly luciferase activity was normalized to Renilla luciferase activity and total protein. Values for cells with a mock transfection were set equal to 100. Data are presented as the mean ± standard error (n = 3). **P<0.01 versus negative control. (B) Schematic of miR-122 wild-type (wt) and miR-122 mutant (mut) sequences. Substituted nucleotides have been underlined. (C) Huh7.5 cells were co-transfected with 0.4 ug/mL of pLuc-OCLN and with 0, 10, 25 or 50 nM miR-122 mutant or MMNC by Lipofectamine 2000 and luciferase activity was measured as described above.

MiR-200b does not interact with the 3'-UTR of OCLN mRNA

In Silico analysis also identified miR-200b as a candidate miRNA that targets OCLN mRNA. Therefore, we transfected Huh7.5 cells with miR-200b mimic and measured OCLN protein levels by Western blotting. In addition, we also co-infected Huh7.5 cells with pLuc-OCLN together with increasing amount of miR-200b mimic as well as MMNC. Although miR-200b down-regulated OCLN protein expression (Fig 17A), the luciferase activity was not affected with increasing concentration of miR-200b (Fig 17B). This shows that unlike the effects of miR-122, miR-200b down-regulatory effects on OCLN is not exerted through interaction with the 3'-UTR of OCLN mRNA despite harboring an *in silico* target within its 3'-UTR.

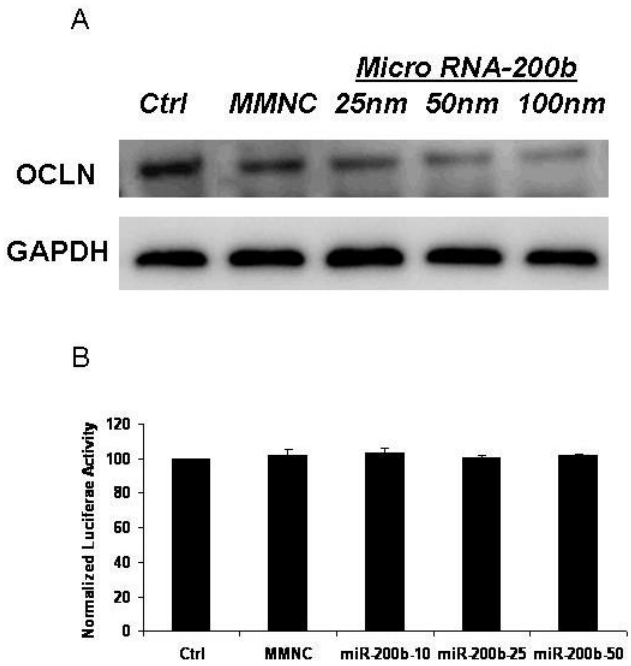

Figure 17. MiR-200b does not bind to the 3'-UTR of OCLN.(A) Huh 7.5 cells were transfected with 0, 25, 50 or100 nM miR-200b mimic or 100 nM MMNC by Lipofectamine 2000 as indicated. 48 hours after transfection, the cells were harvested. OCLN protein levels were assessed by Western blotting with anti-OCLN or GAPDH-specific antibodies. (B) Huh7.5 cells were co-transfected with 0.4 µg/mL of pLuc-OCLN and with 0, 10, 25, 50 nM miR-200b mimic or 50 nM MMNC by Lipofectamine 2000 as indicated. Forty-eight hours after transfection, the luciferase reporter activities were measured using LucPair™ miR Dual Luciferase Assay Kit (Genecopoiea). Firefly luciferase activity was normalized to Renilla luciferase activity and total protein. Values for cells with a mock transfection were set equal to 100. Data are presented as the mean ± standard error (n = 3).

MiR-122 decreases OCLN and CLDN co-localization in HCV infection

Effects of miR-122 on co-localization of OCLN and CLDN were determined using IF. We investigated co-localization of OCLN and CLDN immediately before and 6 h after pseudo-particle infection of miRNA-transfected Huh 7.5 cells. Huh7.5 cells transfected with miR-122 showed lower co-localization of OCLN and CLDN following HCV infection (Fig 18F vs Fig 18D; 0.47 vs 0.57, p <0.01), while the cells transfected with nonspecific miRNA (MMNC) did not show any significant change in co-localization of OCLN and CLDN following HCV infection (Fig 18C vs Fig 18A; 0.63 vs 0.60, p >0.05). The co-localization was not also changed following VSVpp infection (Fig 18E vs Fig 18D; 0.58 vs 0.57, p >0.05).

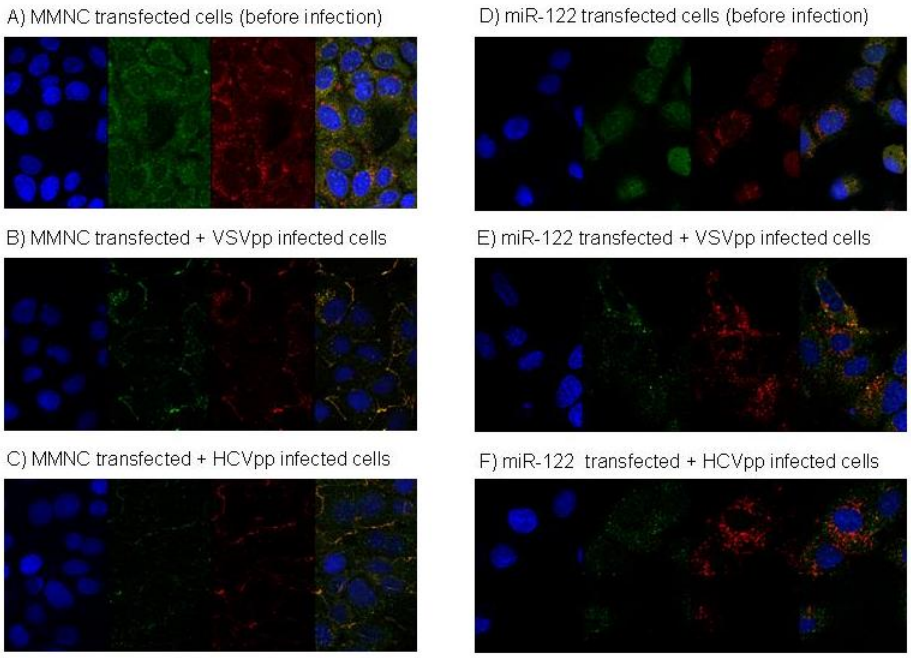

Figure 18. MiR-122 decreases co-localization of OCLN and CLDN in HCV infection. To investigate co-localization of OCLN and CLDN immediately before and 6 h after pseudo-particle infection, Huh 7.5 cells were transfected with 50 nM of either miR-122 or MMNC as negative control. After 48 h, transfected cells were infected either with HCVpp (1:2) or VSVpp (1:100). Six hours after infection, cells were fixed as described in M& M (chapter 2). Primary antibodies used were rabbit polyclonal antibody, anti-CLDN-1 and mouse monoclonal anti-OCLN. Goat anti-mouse/rabbit antibodies conjugated with Alexa 488 (green for OCLN) or 594 (red for CLDN) were used as secondary antibodies. Nuclear staining was performed by using premixed DAPI with proLong gold antifade mounting media. Images were captured with a 63X objective using Z stack optical sectioning. Yellow color represents co-localization of OCLN and CLDN. The results were correlated well and supported by the numerical quantification of co-localization data. The quantification of co-localization between OCLN and CLDN was performed using overlap correlation coefficient. Correlation coefficients were compared with Anova using Bonferroni correction as a post-hoc test. Figures 18 (A-C) show Huh7.5 cells transtfected with MMNC as negative control; A) MMNC-transfected Huh7.5 cells before infection. B) MMNC-transfected Huh 7.5 cells after 6 h of infection with VSVpp. C) MMNC-transfected Huh 7.5 cells after 6 h of infection with HCVpp. Figures 18 (E-D) show Huh7.5 cells transtfected with miR-122; D) miR-122-transfected Huh7.5 cells before infection. E) miR-122-transfected Huh 7.5 cells after 6 h of infection with VSVpp. F) miR-122-transfected Huh 7.5 cells after 6 h of infection with HCVpp.

MiR-122 decreases HCV entry into Huh7.5 cells

As described above, we showed that miR-122 targets OCLN 3'-UTR mRNA and decreases OCLN expression. We also showed that miR-122 transfection can lead to a decrease in co-localization of OCLN and CLDN. Therefore, we aimed to investigate if miR-122 over-expression in Huh7.5 cells could lead to a decrease in HCV entry. To further establish this idea, we used a lenti-miRNA system (ABM, Canada) for over-expression of miR-122 in Huh7.5 cells. To determine infection efficiency, green fluorescent protein (GFP) was quantified by flow cytometry in both cells infected with lentiviral particles containing miR-122 or negative control particles lacking miR-122 seed match sequence within viral genome. The results showed that similar proportions of Huh7.5 cells were infected with miR-122 expressing pseudo-particles compared with negative control pseudo-particles (59% vs 58%; Fig 19A-B). After 48 h, both lenti-miR-122 expressing cells and control cells were infected with HCVpp or VSVpp. Luciferase activity was measured after 72 h, and HCVpp activity was normalized to VSVpp activity. HCV entry was decreased 42% in lenti-miR-122 expressing cells compared with control cells.

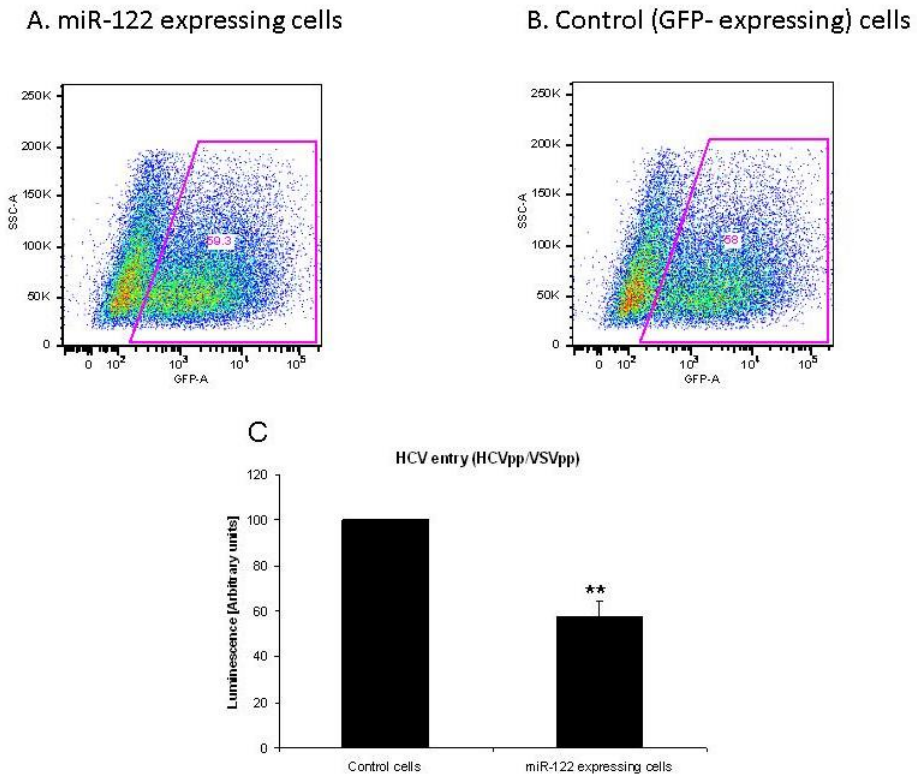

Figure 19. MiR-122 decreases HCV entry into Huh7.5 cells. Huh7.5 cells were plated in a 24-well plate 24 hours prior to viral infection at a density of 0.5×10^5 cells per well and were incubated overnight. The following day, Huh7.5 cells were infected by adding 5µL (MOI=1) of viral particles containing lenti-miR122 genome or same amount of a control virus. After 48 h cells were split 1:3 and incubated for another 48 h. (A-B) GFP expression was assessed by flow cytometery (C) The cells were infected with supernatants diluted in fresh media (1:2 for for HCVpp) and (1:100 for VSVpp), and in presence polybrene for 6-18 h. Firefly luciferase activity was measured after 72 h (Promega), and HCVpp activity was normalized to VSVpp activity. Values for cells with control virus infection were set equal to 100. Data are presented as the mean ± standard error (n = 3). **P<0.01 versus negative control.

Discussion

We found that miR-122 down- regulates OCLN expression by targeting 3'-UTR of OCLN mRNA as we had predicted based upon *in silico* analysis. The down-regulatory effect was more pronounced at the protein level, which shows a post-transcriptional effect of miR-122 on OCLN mRNA. This inhibitory effect was confirmed by luciferase assay and mutagenic analysis. The wild-type miR-122 decreased luciferase activity when co-infected with a construct containing 3'-UTR of OCLN down-stream of the firefly luciferase gene, while a mutant miR-122 which harbors a few point mutations within its seed match (Fig 16B) did not have any effect on the 3'-UTR-dependent luciferase activity. We also showed that miR-122 decreases co-localization of OCLN and CLDN in HCV infection (Fig 18). This effect could be partly explained by a down-regulatory effect of miR-122 on OCLN. The further decrease in co-localization of tight junction components in the presence of HCV pseudo-particles may be attributed to yet unknown anti-viral effects of miR-122 on interaction between OCLN and CLDN. We found that interaction of miR-122 with 3'-UTR of OCLN mRNA eventually results in a decrease in HCV entry. It is noteworthy to mention that we are not aware of the exact levels of miR-122 in Huh7.5 cells before and after over-expressing miR-122; therefore in future studies measurement of levels of miR-122 after over-expression in HepG2 cells, which do not express endogenous miR-122 are recommended for further comparison with Huh7.5 cells. This is particularly important in light of the fact that we know that OCLN plays an important role as an HCV receptor; it has been shown that knocking down of OCLN with siRNA leads to 80% decrease in HCV entry into hepatocytes [22]. It is also suggested that interaction of OCLN and CLDN plays an important role in later phases of HCV entry

[23]. Therefore a decrease in co-localization between OCLN and CLDN in the presence of miR-122 could further prevent entry of HCV into hepatocytes. On the other hand, it has been shown by us and others that miR-122 increases the abundance of HCV RNA in HCV-infected cells [59,67, 87-88]. However this seems to be unexpected from a host factor unless it is being utilized by a virus for its own sake. Therefore, we believe that, in uninfected hepatocytes, miR-122 could play an anti-viral role and decreases HCV entry into these cells. Accordingly, new findings highlight an anti-viral as well as tumor-suppressive role for miR-122. The anti-proliferative properties of miR-122 have been reported in several studies [61-62, 89]. In a recent study, targeted deletion of mir122a in mice mimicked several key features of the phenotypes of human liver diseases like non-alcoholic fatty liver disease (NAFLD) and HCC [44]. In another recent study, delivery of miR-122 to a MYC-driven mouse model of HCC strongly inhibited carcinogenesis [51]. Although, miR-122 was found to be suppressed in chronic HBV infected patients, the mechanism of this effect is still unclear. The antiviral role of miR-122 is suggested by the inhibitory effect of p53 on HBV transcription through blocking of the binding of transcription factors like HNFs to HBV enhancers [57]. Considering the above mentioned anti-viral and anti-proliferative roles for miR-122, it is tempting to speculate that miR-122 may play a dual role in HCV molecular pathogenesis. There is an initial effect of miR-122 to diminish HCV entry into uninfected hepatocytes, but a positive effect of miR-122 on HCV replication within infected hepatocytes. The latter effects will ensue after HCV entry into the cells in which instance the virus utilizes miR-122 for its own replication rather than being used to prevent HCV from entry into hepatocytes.

Therefore, while silencing of miR-122 has been suggested as a novel therapeutic approach in combating HCV infections, indeed, miR-122 mimics may be considered as a novel therapeutic candidate for HBV infection and HBV-induced HCC [84]. The results of our study also suggest that miR-122 mimics may be more beneficial than miR-122 inhibitors in the earlier stages of infection or as a prophylactic approach when few or no hepatocytes are infected with HCV. This might have its highest application in preventing HCV re-infection in patients with CHC who are candidates for liver transplant. Taken together, while the advantages of using miR-122 inhibitors include decreasing plasma cholesterol or HCV RNA levels in the context of hyperlipidemia or hepatitis C, the putative benefits of using miR-122 mimics include tumor suppressive effects and decreasing in HBV replication and HCV entry into hepatocytes. Thus either of miR-122 inhibitors or mimics needs to have further careful evaluation considering the potential risks of oncogenesis or hyperlipidemia, respectively [84]. The results of these recent studies coupled with further studies will doubtless help us to unravel the still largely unknown mechanisms of the actions of miR-122, the most abundant and still mysterious hepatic micro-RNA.

CHAPTER 4: MIR-122 DECREASES PKR PHOSPHORYLATION THROUGH BINDING TO THE 3'-UTR OF PRKRA MRNA

Introduction

PKR, a double-stranded RNA-dependent protein kinase, is among the well-known members of cellular antiviral proteins transcriptionally induced by IFNs in response to viral infection. The activated PKR phosphorylates eukaryotic initiation factor 2 (eIF2), and thereby blocks viral replication at the level of protein synthesis [27]. It is also reported that miR-122 expression was decreased after IFN treatment, whereas over-expression of miR-122 partially alleviated the antiviral effect of IFN.

Also, it is known that human Dicer performs its function in cooperation with its protein partners PRKRA, AGO2, and TRBP [75]. The exact role of these accessory proteins in Dicer activity is still poorly understood. It is shown that inhibition of either Dicer protein partner, including PRKRA, substantially affected not only miRNA levels but also pre-miRNA levels, and it had a rather minor effect on the specificity of Dicer cleavage [75]. They demonstrated that PRKRA, AGO2, and TRBP were required for the efficient functioning of Dicer in cells, and speculated that one of the roles of these proteins is to assure better synchronization of cleavages triggered by two RNase III domains of Dicer [75].

On the other hand, an online search of the TargetScan 5.1 database demonstrated at least one putative miR-122 seed match in the 3'-UTR of PRKRA mRNA which is highly conserved among different species. We Based on the fact that PKR is an important

mediator of IFN actions in HCV infection, and also based on the importance of PRKRA within dicer complex, and due to the finding of a conserved binding site for miR-122 in the 3'-UTR of PRKAR mRNA, we aimed to determine first if miR-122 can bind and down-regulate PRKRA and consequently inactivate PKR through decreasing its phosphorylation in uninfected hepatocytes, and secondly we aimed to determine if the interaction of miR-122 with PRKRA RNA can lead to increase in HCV replication in infected hepatocytes.

In Silico analysis predicts seed region matches for miR-122 on the 3'-UTR of PRKRA mRNA

An online search of the TargetScan 5.2 database (http://www.targetscan.org/vert_50/) demonstrated that at least one separate putative seed match site was found in the 3'-UTR of PRKRA mRNA for miR-122 (8 mer seed-match). As shown in Figure 1, the predicted seed sequence binding site for miR-122 within PRKRA mRNA was perfectly conserved in human, chimpanzee, tree shrew, rabbit, dog, horse, and cow (Fig 20).

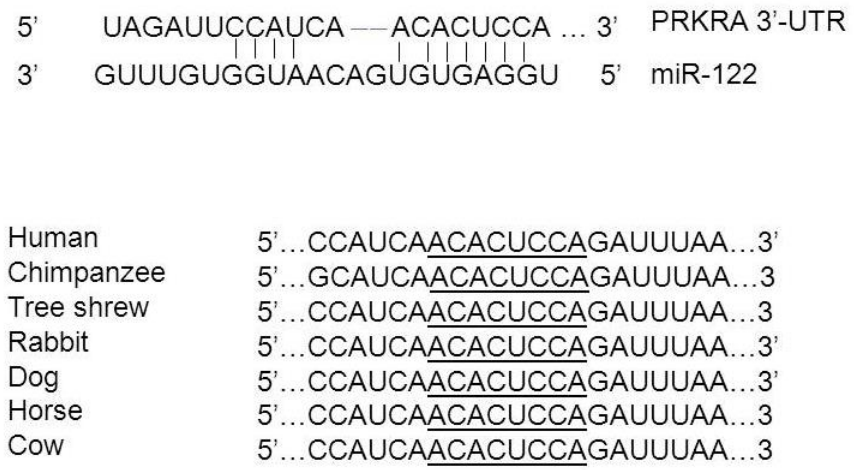

Figure 20. *In silico* prediction of putative binding sites for miR-122 within the 3'-UTR of PRKRA. A schematic of seed match region between miR-122 and 3'-UTR of PRKRA mRNA as putative target. The seed match (underlined) is perfectly conserved in the human, chimpanzee, tree shrew, rabbit, dog, horse, and cow.

MiR-122 does not change PRKRA mRNA levels in Huh7.5 cells

Huh7.5 cells were transfected with miR-122 mimic and PRKRA mRNA levels were measured by qRT-PCR. We did not see any significant difference in the PRKRA mRNA levels after miR-122 overexpression (Fig 21).

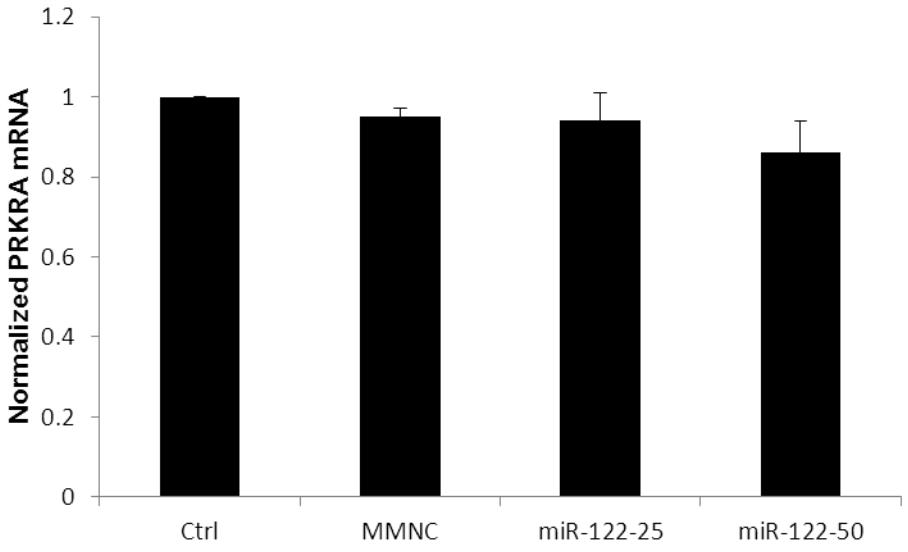

Figure 21. MiR-122 does no change PRKRA mRNA levels significantly (A) Huh 7.5 cells were transfected with 0, 25 or 50 nM miR-122 mimic or 50 nM MMNC in Lipofectamine 2000. Twenty-four hours after transfection, the cells were harvested. The levels of PRKRA mRNA were quantified using qRT-PCR. The amounts of PRKRA mRNA were normalized to those of GAPDH. Values for cells with a mock transfection were set equal to one.

MiR-122 down-regulates PRKRA protein expression in Huh7.5 cells

To experimentally verify that the putative miR-122 binding site is functional, Huh7.5 cells were transfected with miR-122 mimic and PRKRA protein levels were measured by Western blotting. Huh7.5 cells transfected with miR-122 mimic showed a significant reduction in the expression of PRKRA protein levels after 48 hours (56%, $p<0.01$) compared with negative control (Fig 22 A-B).

MiR-122 down-regulates p-PKR protein expression in Huh7.5 cells

We showed that miR-122 can down-regulate PRKRA expression. As PRKRA phosphorylate PKR and activates PKR by this phosphorylation, we decided to see if miR-122 can decrease p-PKR levels. Huh7.5 cells were transfected with miR-122 mimics and PKR and p-PKR levels were measured by Western blotting.

We did not observe any significant difference in level of PKR after miR-122 over-expression (Fig 23 A-B) while p-PKR levels were significantly down-regulated by miR-122 (45%, $p<0.01$; Fig 23 C).

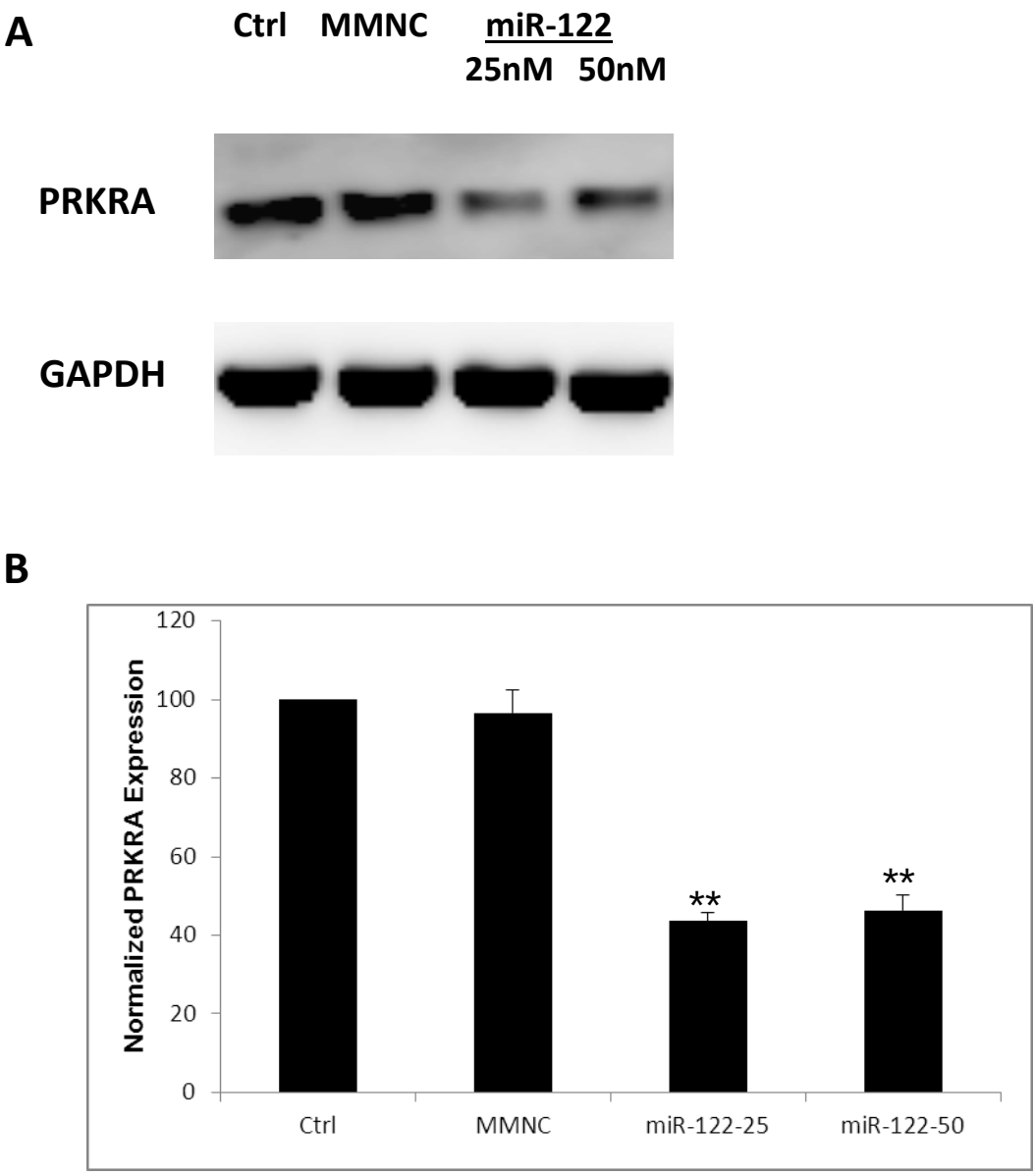

Figure 22. MiR-122 significantly down-regulates PRKRA protein level (A) Huh 7.5 cells were transfected with 0, 25 or 50 nM miR-122 mimic or 50 nM MMNC by Lipofectamine 2000. Forty-eight hours after transfection, the cells were harvested. PRKRA and GAPDH protein levels were assessed by Western blotting. (B) The amounts of PRKRA protein were normalized to those of GAPDH. Values for cells with a mock transfection were set equal to 100. Data are presented as the mean ± standard error (n = 3). ***P* <0.01 versus negative control.

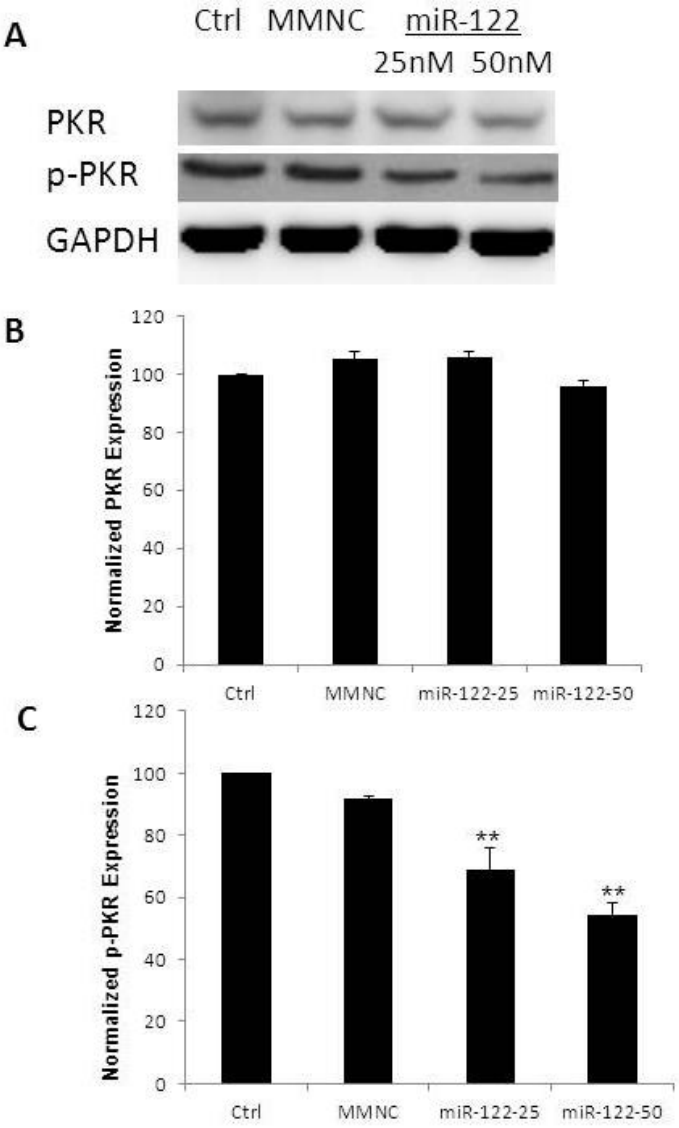

Figure 23. MiR-122 significantly down-regulates p-PKR level while does not change PKR levels(A) Huh 7.5 cells were transfected with 0, 25 or 50 nM miR-122 mimic or 50 nM MMNC by Lipofectamine 2000. Forty-eight hours after transfection, the cells were harvested. PKR, p-PKR and GAPDH protein levels were assessed by Western blotting. The amounts of PKR protein (B), and p-PKR (C) were normalized to those of GAPDH. Values for cells with a mock transfection were set equal to 100. Data are presented as the mean ± standard error (n = 3). ***P* <0.01 versus negative control.

MiR-122 binds to the 3'-UTR of PRKRA mRNA and down-regulates its expression in Huh7.5 cells

To further establish that miR-122 targets the 3'-UTR of PRKRA mRNA , a reporter construct, pLuc-PRKRA, was constructed with PRKRA 3'-UTR inserted downstream of the firefly luciferase open reading frame driven by SV40 promoter (pLuc-PRKRA). It also contained complementary DNA (Rluc) encoding Renilla luciferase driven by CMV promoter as an internal control. Huh7.5 cells were infected with pLuc-PRKRA together with increasing amount of miR-122 mimic or non-specific miRNA, as a negative control (MMNC). Forty-eight hours after transfection, the luciferase reporter activity was assayed. miR-122 mimic transfection significantly decreased reporter activity [by 55% with 50 nM of miR-122 ($P<0.01$)], while MMNC was without a significant effect (Fig 24A). To further establish the interaction between miR-122 and PRKRA 3'-UTR, a mutant miR-122 was created in which the seed match site for the 3'-UTR of OCLN mRNA was abolished (Fig 24B). Huh 7.5 cells were co-transfected with pLuc-PRKRA, and with increasing concentrations of miR-122 as well as MMNC as negative control and luciferase reporter activity was assayed. As anticipated, miR-122 mutant co-transfection did not affect luciferase activity opposite to what observed with wild-type miR-122 (Fig 24C). This further indicated interaction between miR-122 and the 3'-UTR of PRKRA mRNA.

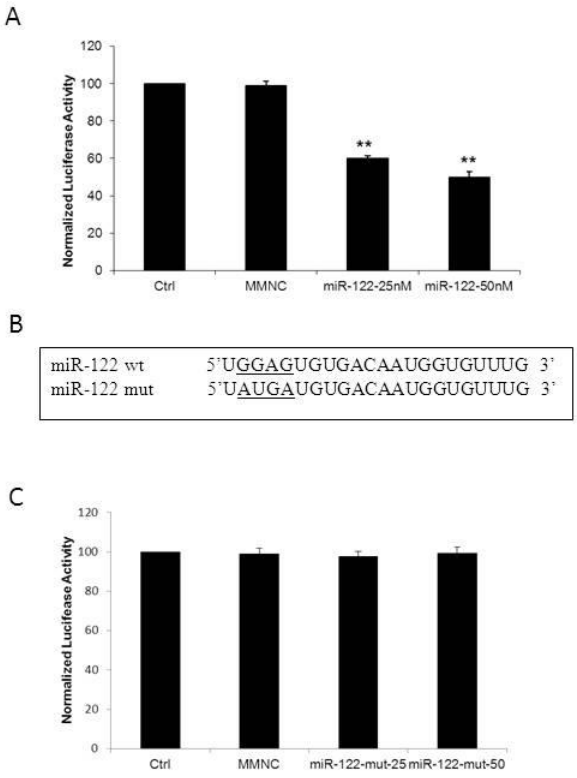

Figure 24. MiR-122 binds to the 3'-UTR of PRKRA and down-regulates its expression.(A) Huh7.5 cells were cotransfected with 0.4 µg/mL of pLuc-PRKRA and with 0, 25 or 50 nM miR-122 mimic or 50 nM MMNC in Lipofectamine 2000. Forty-eight hours after transfection, the luciferase reporter activities were measured as described in M & M. Firefly luciferase activity was normalized to Renilla luciferase activity and total protein. Values for cells with a mock transfection were set equal to 100. Data are presented as the mean ± standard error (n = 3). **P<0.01 versus negative control. (B) Schematic of miR-122 wild-type and miR-122 mutant sequences. Substituted nucleotides have been underlined. (C) Huh7.5 cells were co-transfected with 0.4 ug/mL of pLuc-PRKRA and with 0-50 nM miR-122 mutant or MMNC by Lipofectamine 2000 and luciferase activity was measured as described above.

HCV replication requires miR-122 binding to 5'-UTR of HCV RNA and this binding dominates the interaction of miR-122 and PRKRA RNA in infected Huh7.5 cells

We showed that in uninfected Huh7.5 cells, miR-122 can bind to the 3'-UTR of PRKRA and this leads to decrease in PRKRA protein levels as well as decrease in phosphorylation of PKR. Therefore, we determined to see if this binding also happens in an infected hepatocyte in presence of both miR-122 and HCV RNA. We used HJ3-5 virus which was packaged after transfection of *in vitro*-transcribed HJ3-5 RNA into Huh7.5 cells. Naïve Huh7.5 cells were infected with supernatant containing HJ3-5 virus, and either wild-type miR-122 or mutant miR-122 (miR-122p6m; Fig. 25A, Upper) was over-expressed in infected cells. After 3 days when we sought to determine the levels of HCV core and NS3 proteins, it was found that both HCV proteins were up-regulated in the presence of wild-type miR-122 (Fig 25B). On the other hand, we did not see any difference in the levels of PRKRA protein (Fig 25B) with either wild-type miR-122 or mutant miR-122 (miR-122p6m).

For further confirming these results we used an HCV mutant defective in miR-122 binding at both sites (S1-S2-p6m; Fig 24A, Lower) and also the complementary miR-122 mutant (miR-122p6m) which is able to bind S1-S2-p6m RNA. S1-S2-p6m virus was packaged after transfection of *in vitro*-transcribed S1-S2-p6m RNA into Huh7.5 cells. Naïve Huh7.5 cells were infected with supernatant containing S1-S2-p6m, and either non-specific miRNA (MMNC) or miR-122 wt or miR-122 mutant (miR-122p6m) or a combination of miR-122 and miR-122p6m was over-expressed in infected cells. After 3 days when we sought to determine the levels of HCV proteins, it was found that

HCV proteins were almost undetectable in the presence of either MMNC or miR-122 (lane 1 or lane 2, Fig 25C).

On the other hand, both HCV core and NS3 proteins were detectable (lane 3; Fig 25 C) after we transfected these cells with complementary mutant miR-122 (miR-122p6m). Both HCV proteins were slightly further up-regulated when a combination of wild-type miR-122 and miR-122p6m was transfected (compare lane 4 with lane 3; Fig 25C). PRKRA protein level was slightly down-regulated when a combination of wild-type miR-122 and miR-122p6m was transfected.

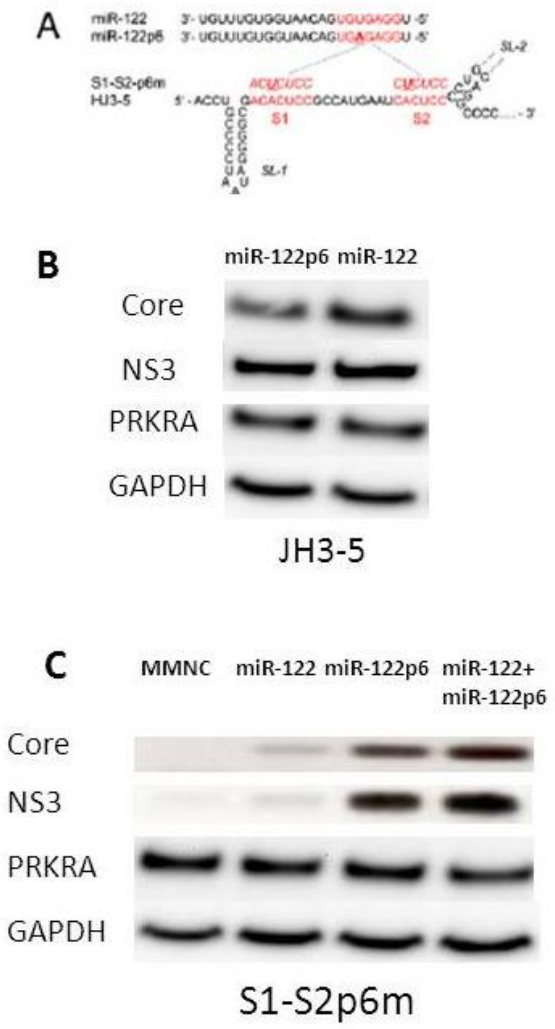

Figure 25. HCV replication requires miR-122 binding to 5'-UTR of HCV RNA and it dominates the interaction of miR-122 and PRKRA RNA in infected Huh7.5 cells. (A) Upper: miR-122 and mutant miR-122p6 strand sequences. Lower: 5' terminal sequence of HCV (HJ3-5 virus), with S1 and S2 binding sites shown in red. Point mutations (underlined) in the related S1-S2-p6m mutant are shown above. SL-1 and SL-2 are putative stem-loop structures in the 5'-UTR (Figure from Ref [67] with permission.). (B) Naïve Huh7.5 cells were infected with supernatant containing HJ3-5 virus, and after 6 hours 50 nM of either miR-122 wt or miR-122 mutant (miR-122p6m; Fig 24A, Upper) was over-expressed in infected cells. After, 72 hours, cells were harvested and levels of HCV core and NS3, as well as PRKRA and GAPDH were determined by western blotting. (C) Naïve Huh7.5 cells were infected with supernatant containing S1-S2-p6m, and after 6 hours 50 nM of either non-specific miRNA (MMNC), miR-122 wt, miR-122 mutant (miR-122p6m) or a combination of miR-122 and miR-122p6m was over-expressed in infected cells. After, 72 hours, cells were harvested and levels of HCV core and NS3, as well as PRKRA and GAPDH were determined by western blotting.

Discussion

We found that miR-122 down- regulates PRKRA expression by targeting 3'-UTR of PRKRA mRNA. The down-regulatory effect was more pronounced at the protein level, which shows a post-transcriptional effect of miR-122 on PRKRA mRNA. We also showed that p-PKR levels were significantly down-regulated by miR-122 while PKR protein levels were not significantly changed. This confirmed that the inhibitory effect of miR-122 on PRKRA RNA will lead to a down-stream effect of decrease in phosphorylation of PKR and its inactivation. This is of particular importance because PKR is an antiviral protein and its inactivation by miR-122 should lead to a consequent decrease in HCV replication. The inhibitory effect of miR-122 on PRKRA RNA was also confirmed by luciferase assay and mutagenic analysis. The wild-type miR-122 decreased luciferase activity when co-infected with a construct containing 3'-UTR of PRKRA down-stream of the firefly luciferase gene, while mutant miR-122 which harbored a few point mutations within its seed match did not have any effect on the 3'-UTR-dependent luciferase activity.

However, based on previous extensive studies, it is already known that there are two adjacent binding site within 5'-UTR of HCV RNA for miR-122 binding, and interaction of miR-122 with these binding sites lead to increase of abundance of HCV RNA. Therefore, after we confirmed that PRKRA is one of the targets of miR-122 in uninfected Huh7.5 cells, we decided to determine if the interaction of miR-122 and PRKRA RNA has any effect on HCV replication in a setting of infected hepatocytes where all of these molecular elements including HCV RNA, PRKRA RNA, and miR-122 exist.

After infecting naïve Huh7.5 cells with supernatant containing HJ3-5 virus, we found that both HCV core and NS3 proteins were up-regulated in the presence of wild-type miR-122 compared with mutant miR-122. This result confirms that miR-122 is necessary for efficient replication of HCV. On the other hand, we did not see any difference in the levels of PRKRA protein (Fig 25B) with either wild-type or mutant miR-122. As there is a functional miR-122 binding site in the 3'-UTR of PRKRA, this results are consonant with the notion that in infected cells, miR-122 preferentially binds to its binding sites in 5'-UTR of HCV RNA than 3'-UTR of PRKRA RNA.

For further confirming these results and to show that the two adjacent binding sites are necessary for this binding, we used an HCV mutant defective in both of these binding sites. It has been shown that mutations in S1 and S2 that ablate miR-122 binding are lethal to replication of HCV that has been adapted to growth in cell culture (HJ3-5 virus). Therefore, we used an HCV mutant defective in miR-122 binding at both sites (S1-S2-p6m) and also we used the complementary miR-122 mutant (miR-122p6m) which is able to bind S1-S2-p6m RNA. After naïve Huh7.5 cells were infected with supernatant containing S1-S2-p6m, and transfected with respective miRNA, we found that HCV protein was almost undetectable in the presence of either MMNC or miR-122. On the other hand, both HCV core and NS3 proteins were detectable after we transfected these cells with complementary mutant miR-122 (miR-122p6m). This confirms that both of these binding sites are essential for HCV replication. Interestingly, both HCV core and NS3 proteins were further up-regulated when a combination of miR-122 wt and miR-122p6m was transfected. We also found that PRKRA protein level was slightly down-

regulated when a combination of wild-type miR-122 and mutant miR-122 was transfected. The latter finding could indicate that when both adjacent binding sites within mutant HCV RNA are occupied with complementary mutant miR-122, wild-type miR-122 can freely bind to its different targets in infected Huh 7.5 cells including PRKRA, and down-regulates its expression. Down-regulation of PRKRA, and consequent decrease in phosphorylation and activation of PKR, an antiviral protein, leads to increase in HCV protein. However, this latter effect only was observed in case that we used mutant HCV RNA, and does not usually happen in HCV infection when wild-type HCV RNA predominating as shown in Fig 25B.

It is important to mention that we used infected Huh7.5 cells which are deficient in Rig-I pathway, one of the signaling pathways that induces IFN type-I. We used Huh7.5 cells mainly because of more efficient HCV replication in these cells than Huh7 cells. Although in Huh7.5 cells, two other major immune pathways, namely, TLR3 and PKR are unaffected, partial decrease in IFN induction through deficiency in Rig-I pathway, might alter response of other ISGs including PKR and PRKRA. Therefore, future studies which compare the effect of miR-122 on PKR in infected Huh7 cells vs infected Huh7.5 cells are recommended.

In conclusion, we showed that miR-122 down- regulates PRKRA expression by targeting 3'-UTR of PRKRA mRNA. The down-regulatory effect was more pronounced at the protein level, which shows a post-transcriptional effect of miR-122 on PRKRA mRNA. We also showed that p-PKR levels were significantly down-regulated by miR-122 while PKR protein levels were not significantly changed. However, it seems that in infected hepatocytes, miR-122 preferentially binds to 5'-UTR of HCV RNA than 3'-UTR

of PRKRA, and this is the main factor that increases HCV replication. The probable effect of miR-122 on PRKRA, as a component of dicer complex, and any consequent effect on HCV needs to be determined.

CHAPTER 5: PRE-TREATMENT LEVELS OF MIR-29B ARE ASSOCIATED WITH RESPONSE TO TREATMENT AND STAGE OF LIVER DISEASE IN CHC PATIENTS

Introduction

The ability to determine the outcome of hepatitis C may be useful in terms of implementing treatment strategies; however, to date, the predictive associations in the literature are limited. HCV genotype and HCV RNA levels are considered major determinants of response to treatment in HCV infection [90]. However, increasing data clearly indicate that host genetics also critically influence response to treatment [91]. A better understanding of these genetic factors may enable the development of individualized treatment algorithms leading to increased cure rates and better quality and safety of care, and may also permit novel therapeutic approaches [92]. Although recent studies show that IL28B genotype [93] or in the newly discovered IFN lambda 4 gene [94] is an independent association factor for pegylated IFN-α2b/ribavirin treatment response, much still needs to be accomplished to understand the molecular mechanisms that underlie these associations. Other yet unknown genetic factors with response to treatment or severity of liver disease likely remain to be uncovered.

As a conserved host factor that can be effectively inhibited, miR-122 presents a highly appealing antiviral target [44]. Accordingly, Lanford et al. showed that silencing of miR-122 in chronically HCV-infected chimpanzees led to long-lasting suppression of HCV viremia [66]. However, when hepatic miR-122 levels were measured in patients with CHC, various results were obtained.

As it was already discussed, CHC progresses and may lead to fibrosis, cirrhosis, end-stage liver disease and HCC [2]. Hepatic fibrosis is part of a dynamic process associated with the continuous deposition and resorption of extracellular matrix, mainly fibrillar collagen [95]. The end stage of liver fibrosis, cirrhosis, is histologically characterized by increased deposition and altered composition of the ECM and the appearance of regenerative nodules. The destruction of the normal architecture and the loss of hepatocytes prevent the liver from its normal synthetic and metabolic function [96]. Thus, the fibrogenic evolution progresses to cirrhosis, liver failure, and HCC. Studies of fibrogenesis conducted in many organs including the liver demonstrate that the primary source of the extracellular matrix in fibrosis is the myofibroblast [95]. Hepatic stellate cells, the resident peri-sinusoidal cell type that store vitamin A, are the major source of ECM during diseases that injure hepatocytes through their activation into contractile myo-fibroblasts, and a similar transition to myofibroblasts from portal fibroblasts drives the fibrogenic response when biliary cells rather than hepatocytes are injured [95]. In the activation process, transforming growth factor beta is thought to be the main mediator of fibrogenesis and platelet-derived growth factor is the major inducer of HSC proliferation [97].

The family of miRNA-29 also plays a role in modulating severity of liver disease [36]. It was shown that treatment of hepatic stellate cells with TGFβ suppressed miR-29 expression suggesting that part of the fibrogenic effects of TGFβ is mediated via miR-29 down-regulation [36]. It was also shown that fibrosis and mortality were enhanced in miR-29 knockout mice in response to carbon tetrachloride [98]. MiR-29 was previously shown to have antiviral activity against human immunodeficiency virus [99, 100]. In

another recent study, it was shown that HCV infection down-regulates miR-29 in hepatocytes and may potentiate collagen synthesis by reducing miR-29 levels in activated HSCs [101]. Besides the regulatory role of miR-29 in fibrosis and cancer progression, the miR-29 family has been identified to have significant effect on immune cell proliferation and cytokine production by helper T cells, especially IFN- γ [42].We sought potential target genes for miR-29b and found that the tight junction component, CLDN-an important player in the HCV entry into hepatocytes is among candidate targets of miR-29. Thus, we were motivated to determine if there is an association between levels of miR-29 and response to treatment or stage of liver disease in patients with CHC.

We hypothesized that gene expression prior to or during the early phase of anti-HCV therapy may elucidate important molecular pathways for achieving virological response. Based on our findings and previous studies, we chose miR-122, miR-29b as well as CLDN, OCLN, PKR, and PRKRA, to determine if there was any association between pre-treatment levels of any of these molecular factors with HCV RNA levels, response to treatment and/or stage of liver disease.

Patients and Methods

Patients

The study included 25 patients with CHC (HCV genotype 1) followed in the liver-Biliary-Pancreatic of Carolinas Medical Center (CMC) who agreed to participate in this study between 2008 and 2012. To be eligible for this study, patients had not been treated before study, and had been considered candidates for treatment after liver biopsies was performed. Patients with autoimmune hepatitis, or alcohol-induced liver injury, or hepatitis B virus-associated antigen/antibody or anti-human immunodeficiency virus

antibody were excluded. Patients with a history of prior IFN therapy or immune-modulatory therapy before enrollment in the study were excluded. Levels of HCV RNA in serum were quantified before starting of treatment. Liver biopsy specimens (obtained for routine clinical evaluation and care) were collected from patients not later than one week prior to receiving combination therapy. Histological grading and staging of liver biopsy specimens from the CHC patients was examined by an expert hepato-pathologist at CMC who was blinded as to other clinical or laboratory features. Pretreatment blood tests were conducted to determine each patient's level of liver function test, and liver chemistries.

Treatment protocol

All enrolled patients are treated for 48 weeks (Figure). Pegylated IFNa-2a was administered at a dose of 1800 mcg/ week at the starting point. RBV was administered following the dose recommended by the manufacturer (1000 mg/d if weight<70 Kg, 1200 mg/d if weight>70 Kg). Telaprevir was administered 750 mg q8hr.

Definitions of drug response to therapy

Drug response is defined according to how rapidly and to what extent HCV RNA levels decreased in each patient's serum (Fig 26). The patients were classified into the following two groups according to virological responses after 12 weeks of drug administration: early viral response (EVR): patients whose HCV RNA levels at 12 weeks were reduced by 2-$\log_{10}$ or more compared to baseline (week 0). Non-EVR (n-EVR): patients whose HCV RNA levels were not reduced or reduced less than 2- $\log_{10}$ of HCV RNA before drug administration (week 0).

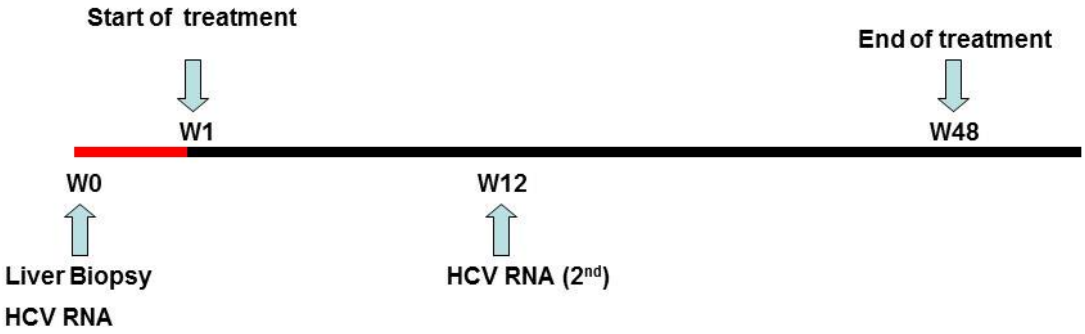

Figure 26. Study design and treatment protocol for enrolled patients with CHC. The goal os for all patients who agreed to be treated are treated for a total of 48 weeks. A liver biopsy specimen as well as HCV RNA levels was taken from all treated patients before starting the treatment. A second HCV RNA levels were determined in all treated patients at 12 weeks after starting the treatment. Patients were classified based on their response to anti-viral therapy into two categories of patients with EVR, and patients without EVR. W: Week.

Laboratory methods

About, five mg (range 3-10) portions of liver biopsy specimens were flash frozen in liquid N2 and also immediately placed to the RNA later. Total RNA was extracted and real time PCR was done for the quantification of miR-122, miR-29b levels as well as OCLN, CLDN and PRKRA mRNA as described in chapter 2. CLDN, OCLN and PRKRA protein levels were also quantified by Immunohistochemistry as described in chapter 2.

Statistical analysis

Statistical analysis was performed with Chi-square and Fisher's exact tests for comparing demographic among different groups of patients. Independent t test was used to compare levels of miR-122, miR-29b, CLDN RNA, OCLN RNA, PRKRA RNA, or HCV RNA among different groups of patients. Non-parametric test was used for

comparing the means of two groups if the data was not normally distributed.(SPSS Inc., Chicago, IL). Pearson's correlation coefficient (cc) was used to calculate the correlation between levels of HCV RNA and other quantitative variables respectively. We used a general linear model using regression analysis and analysis of variance for HCV RNA levels as single dependent variable. Age and sex were considered as factors and miR-122, CLDN RNA, PRKRA RNA and stage of fibrosis as covariates. We also employed a multivariate logistic regression analysis in order to find the association between advanced stage of fibrosis and a set of independent variables (SPSS Inc., Chicago, IL).

Results

Demographic, biochemical and virological data of the patients are shown in Table 1. A total of 25 patients with CHC were included in this study among them 11 went under treatment and 14 remained treatment naïve at the time of this study. There was no significant difference in mean age or race ratio between patients under treatment and treatment naïve subjects. There was more females who underwent treatment compared to males ($p<0.05$). Serum levels of HCV RNA and liver transaminase levels were not significantly different between two groups.

Table 1. Demographic, biochemical data and HCV RNA levels at baseline in all 25 patients with CHC in the study. AA indicates African-American. Results are mean±SEM

Characteristics	Treated	Not treated	All patients
Total (n)	11	14	25
Age	53.3±1.5	53.4±2.1	53.3±1.3
Male; Female	5;6	13;1	18;7
Caucasian; AA	8;3	8;6	16;9
ALT(IU/L)	49.9±10	96.7±24.8	76.1±15.1
AST(IU/L)	49.6±9.3	66.3±13	59±8.4
HCV RNA [Copy/ml]	3,753,000± 1,295,000	4,067,000± 1,237,000	3,929,000± 879,000

Pre-treatment levels of miR-29b are higher in CHC patients who do not respond to treatment early

Among 11 patients who were treated, six patients received a combination of peg-IFN and ribavirin. Four patients received a triple therapy of peg-IFN, ribavirin plus Telaprevir, and one patient received vertex-114 in a trial study. Among these 11 patients who underwent treatment, seven patients achieved reduction of HCV RNA levels by 2-$\log_{10}$ or more (EVR) while four patients did not (n-EVR).

Demographic characteristics of the two groups of treated CHC patients (with EVR and n-EVR) are summarized in Figure 27. Six of seven (86 %) patients with EVR were younger than 56, while 2/4 (50%) of patients with n-EVR were younger than 56 (Fig 27 A). This difference in age between two groups of EVR and n-EVR was not statistically significant. Four of seven (57%) of patients with EVR were female while half (50%) of patients with n-EVR were female (Fig 27 B). This slight difference in sex ratio also was not statistically significant. Two of seven (28%) patients with EVR were African-American while rest of them (72%) was Caucasians. On the other hand, one of four (15%) patients without EVR was African-American while 5/7 (75%) were Caucasians (Fig 27 C). This difference also was not statistically significant.

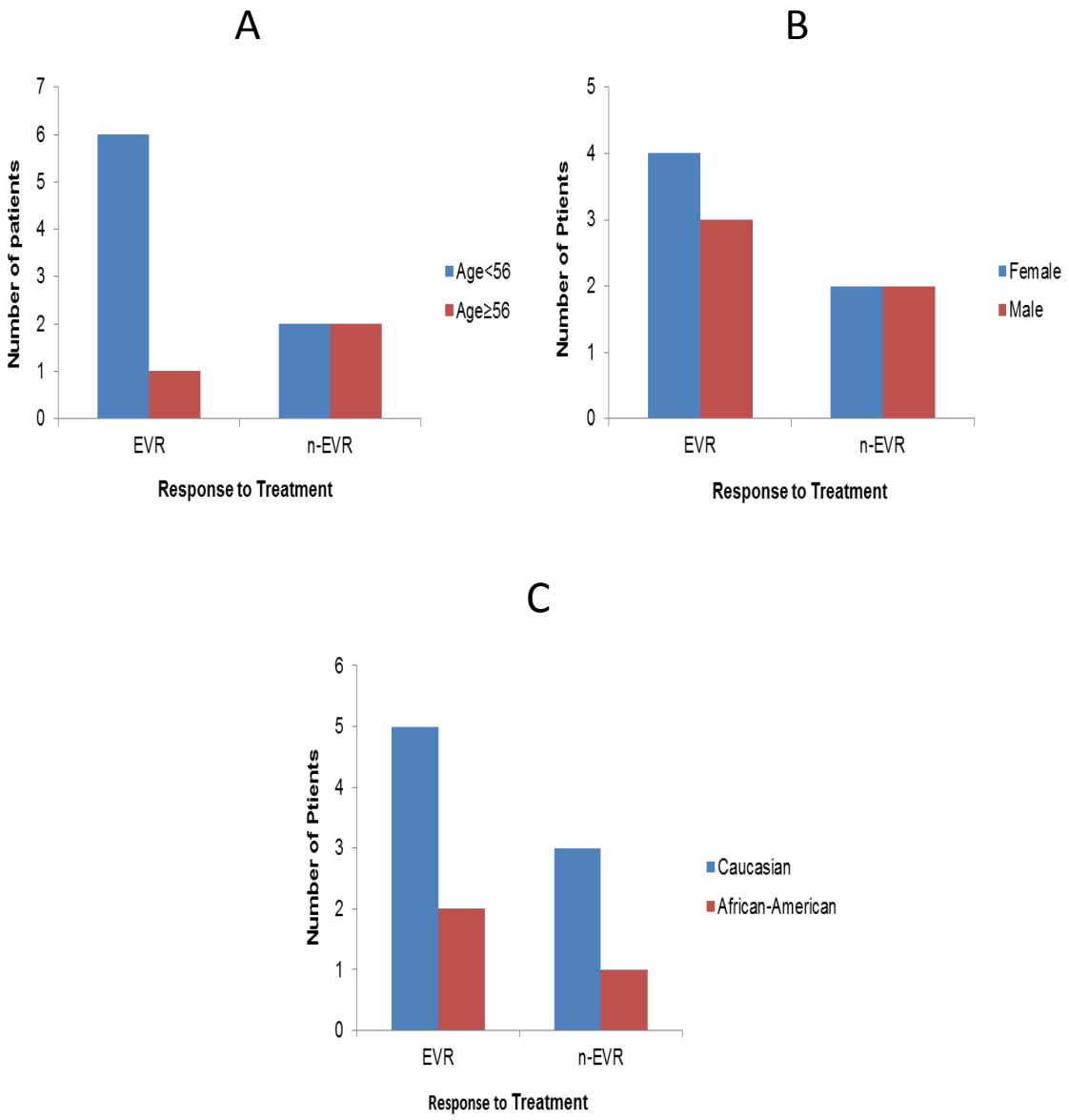

Figure 27. Comparison of demographic characteristics between two groups of treated CHC patients with EVR and without EVR (n-EVR). A) Age of patients with EVR and n-EVR. B) Genders of patients with EVR and n-EVR. C) Racial composition of patients with EVR and n-EVR.

We also compared pre-treatment levels of hepatic miR-122, and miR-29b based on response to treatment. We found that pre-treatment miR-122 levels were slightly lower in patients with EVR compared to those with n-EVR (49 vs 59, $p>0.05$; Fig 28) which was not statistically significant.

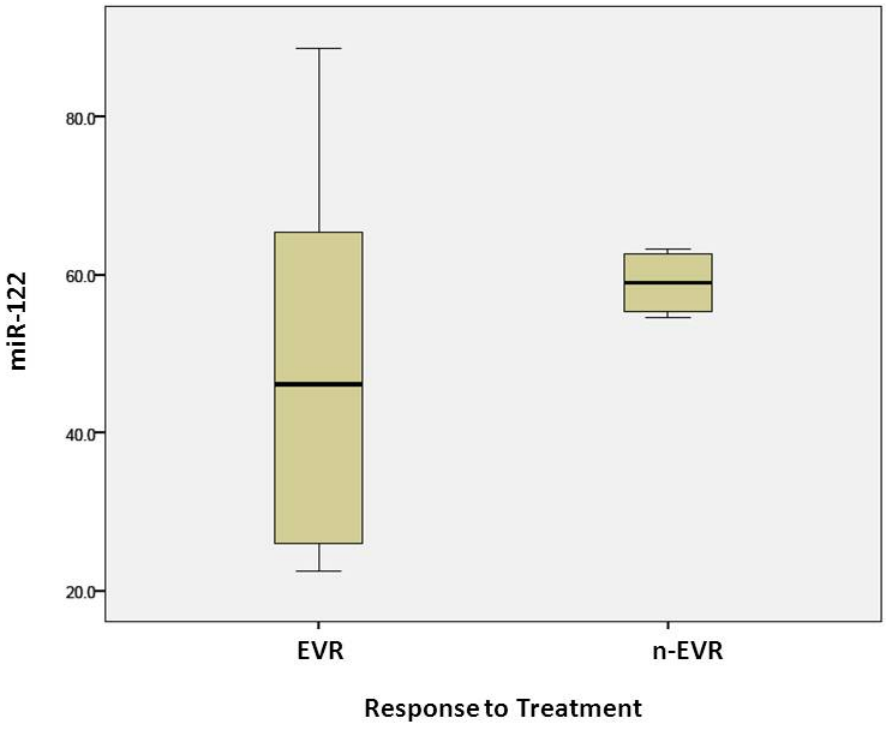

Figure 28. Pre-treatment levels of miR-122 are slightly higher in CHC patients with EVR than those without EVR (n-EVR). Boxplots summarize relative miR-122 [normalized to SNORD44] levels in each group. Differences were calculated according to mean mir-122 levels of patients in each group (7 patients with EVR vs 4 patients with n-EVR]. The heavy horizontal lines are the median values; the shaded boxes represent interquartile ranges, and the whiskers' span the ranges of miR-122 levels of patients within each group. Pre-treatment miR-122 levels were slightly higher in patients with n-EVR compared to those with EVR (59 vs 49, $p>0.05$)

In contrast, pre-treatment levels of miR-29b (normalized to SNORD44) were significantly lower in patients with EVR vs those with n-EVR (0.015 vs 0.130, $p<0.05$; Fig 29). We did not find any significant difference between RNA levels of OCLN, CLDN, PKR and PRKRA based on response to treatment (Results not shown).

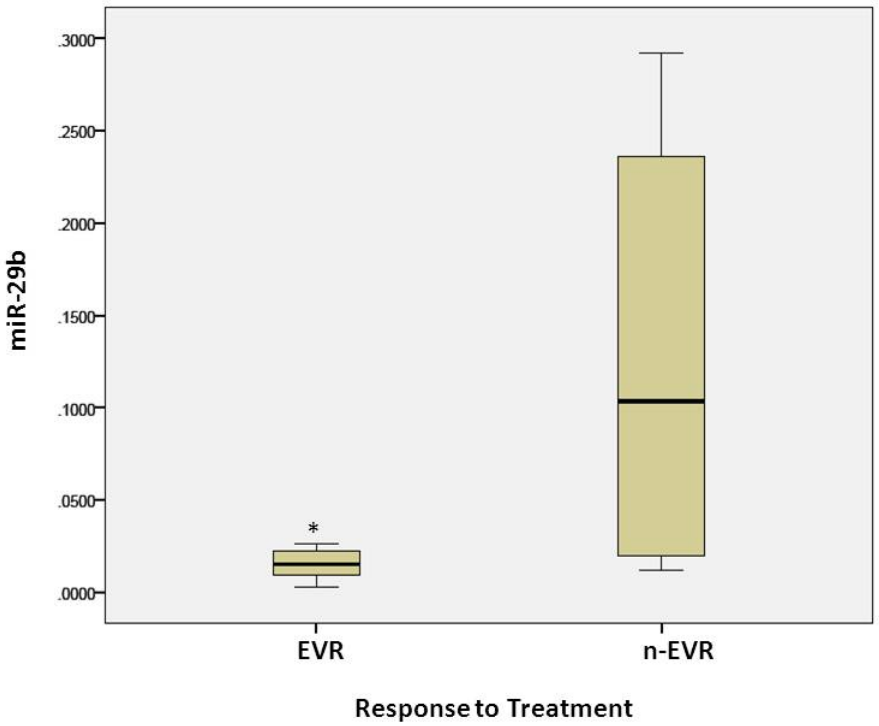

Figure 29. Pre-treatment levels of hepatic miR-29b are significantly lower in CHC patients with EVR than those with n-EVR. Boxplot represents relative miR-29b [normalized to SNORD44] levels in each group. Differences were calculated according to mean mir-29b levels of patients in each group (7 patients with EVR vs 4 patients with n-EVR]. The heavy horizontal lines are the median values; the shaded boxes represent interquartile ranges, and the whiskers' span the ranges of miR-29b levels of patients within each group. Pre-treatment levels of miR-29b were significantly higher in patients with n-EVR compared to those with EVR (0.130 vs 0.015, $p<0.05$).

Pre-treatment level of miR-29b is an independent predictor of stage of liver disease (fibrosis)

We also compared different factors in CHC patients with different stages of hepatic fibrosis. For this, we divided CHC patients into two categories; those with Metavir stages 0- 2 of fibrosis on their pre-treatment biopsies and those with Metavir stages of 3-4 and higher on their pre-treatment liver biopsies. There was not a significant difference in mean age of patients between these two groups (54 vs 52, $p>0.05$). We found that only 3 out of 17 (18%) CHC patients with lower stages of fibrosis were female, while 4 out of 8 (50%) CHC patients with higher stages of fibrosis were female, though this difference was not statistically significant ($p=0.11$). We also found that 8 out of 17 (47%) CHC patients with lower stages of fibrosis were African-American while only 1 out of 8 (12%) CHC patient with higher stages of fibrosis was African-American. This difference also was not significant ($p=0.11$). Then, we compared pre-treatment levels of miR-122, and miR-29b between these two groups of CHC patients. Levels of hepatic miR-122 were slightly higher in patients with lower stages of fibrosis (55 vs 50, $p>0.05$) which was not statistically significant. The weak inverse correlation between miR-122 and stage of liver disease ($r= -0.31$, $p=0.13$) was not statistically significant. Finally, we compared levels of miR-29b between these two groups, it was found that CHC patients with higher stages of fibrosis had strong trend of higher hepatic miR-29b levels than patients with lower stages of fibrosis (0.215 vs 0.120, $p=0.057$).

A multi-variable logistic regression analysis was done with age, sex, race, and pre-treatment levels of miR-122, and miR-29b considered as different factors and covariates while high stage of fibrosis (Stage3-4) was considered as a single dependent

variable. Multivariate analysis showed miR-29b levels as the only independent factor for predicting advanced stage of fibrosis (p=0.028, Table 2).

Table 2. Factors correlated with advanced hepatic fibrosis in subjects studied. Univriate and multivariate analysis for advanced stage of fibrosis (Stage3-4) as dependent variable. Hepatic micro RNA levels were measured before the start of treatment (at week 0). Note that, among several variables, only levels of miR-29b in the liver were significantly inversely correlated with stage of fibrosis. * ($p<0.05$)

Variable	Univariate *p*-value	Multivariate *p*-value
Age	0.487	0.701
Sex	0.116	0.102
Race	0.107	0.275
miR-122 levels	0.644	0.286
miR-29b levels	0.057	0.028*

Pre-treatment levels of CLDN RNA are positively correlated with HCV RNA levels at baseline

We also investigate whether there is any correlation between pre-treatment levels of miR-122, or miR-29b with HCV RNA levels in serum before start of treatment (week 0). There was no correlation between pre-treatment levels of miR-29b and HCV RNA levels, while we found that miR-122 is negatively correlated with HCV RNA levels before starting the treatment (r= -0.43, p=0.03; Fig 30A)

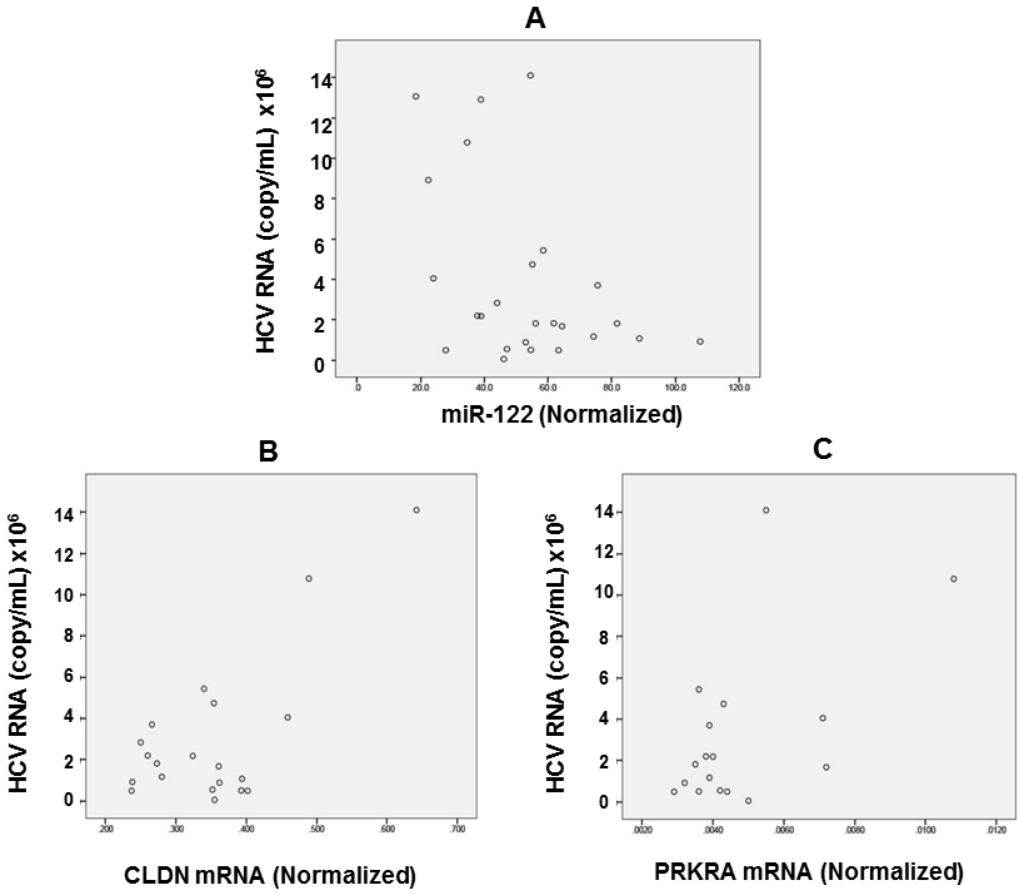

Figure 30. Levels of HCV RNA at baseline are negatively correlated with miR-122 levels while positively correlated with both CLDN, and PRKRA RNA levels. Scatter plots correlate HCV RNA levels on Y axis with hepatic miR-122 levels (Fig 30A), CLDN mRNA (Fig 30B), or PRKRA RNA (Fig 30C) on X axis. Pearson correlation coefficients show significant correlations of each of miR-122 (r= -0.43, p=0.03; Fig 30A), CLDN RNA (r= 0.71, p<0.001; Fig 30B), and PRKRA RNA (r= 0.55, p=0.02; Fig 30C) with HCV RNA levels respectively.

We found that pre-treatment levels of hepatic CLDN RNA are positively and strongly correlated with HCV RNA before starting the treatment (r= 0.71, $p<0.001$; Fig 30B). There was also a positive correlation between pre-treatment levels of PRKRA RNA and HCV RNA levels (r= 0.55, $p=0.02$; Fig 30C). Because of the appearance of the scatter plots shown in Fig 30 B and C, we tested whether the two highest values were outliers [Grubb's test –SPSS]. They were not outliers. Similarly, in Fig 30A none of the points was an outlier.

We constructed a general linear model using regression analysis and analysis of variance for HCV RNA levels (week 0) as single dependent variable (Table 3). Age and sex were considered as factors and miR-122, CLDN RNA, PRKRA RNA and stage of fibrosis as covariates. The whole model was statistically significant ($p<0.01$). Hepatic CLDN RNA was found as the single independent factor which positively correlated with HCV RNA levels (F=15.7, $p=0.003$) while hepatic miR-122 levels showed a strong trend or borderline negative correlation with HCV RNA levels (F=4.8, $p=0.056$).

Table 3. General inear model (GLM) analysis for HCV RNA (week 0) as dependent variable. All biochemical, molecular, and viral data measured before the start of treatment (at week 0) were included in the model. GLM: General Linear Model, CC: Correlation Coefficient * represents significant ($p<0.05$) ** represents significant ($p<0.01$)

Variable	CC	*p*-value	GLM F-value	GLM *p*-value
Age	0.05	0.820	0.02	0.882
Sex	0.11	0.613	0.11	0.753
Fibrosis stage	0.04	0.860	2.82	0.127
miR-122 level	-0.47	0.030*	4.8	0.056
CLDN RNA	0.71	0.001**	15.71	0.003**
PRKRA RNA	0.55	0.020*	0.53	0.458

CHC Patients with higher levels of miR-122 have lower hepatic expression of OCLN

The sections of paraffin embedded liver biopsies were stained with either PRKRA, OCLN, or CLDN. We did not see any significant difference in expression of either CLDN or PRKRA in patients with different levels of miR-122. Mir-122 levels were slightly higher in patients with lower expression of OCLN than those with higher expression of OCLN though this difference was not statistically significant (56 vs 50; $p>0.05$). However, when we divided CHC patients into two categories of miR-122 levels (higher than average vs lower than average), we found that 80 % of patients with high miR-122 levels have low expression of OCLN while 67 % of patients with low expression of miR-122 have high expression of OCLN ($p<0.05$; Figure 31).

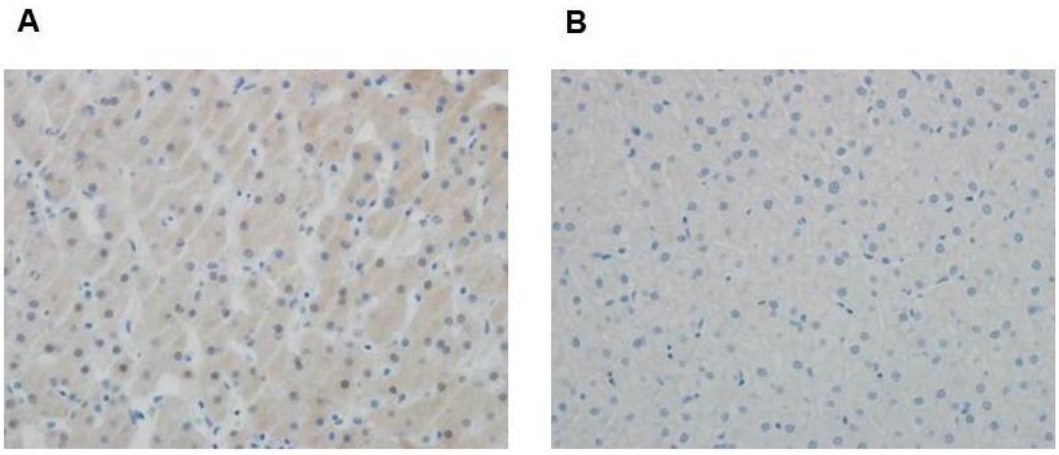

Figure 31. Immunohistochemistry of liver biopsies from patients with CHC. OCLN expression was detected by DAB while nuclei were counter stained with hematoxylin. A) Immunohistochemistry of liver biopsies from a patient with low levels of miR-122. B) Immunohistochemistry of liver biopsies from a patient with high levels of miR-122.

Discussion

We divided our treated patients into two categories based on their response to treatment. We did not find any statistically significant difference in age, sex and race between CHC patients with EVR and those with n-EVR. When we looked at pre-treatment levels of miR-122 in two groups of patients, we found that patients with n-EVR had a higher pre-treatment levels of miR-122 compared with those with EVR, although this difference was not statistically significant. Interestingly, when we did a correlation analysis between initial HCV RNA levels and different molecular factors in all 25 CHC patients, we also found an inverse correlation between pre-treatment levels of miR-122 and HCV RNA levels which was statistically significant (p=0.03, Fig 30A). This negative correlation was almost preserved to a high extent (p=0.056, table 2) in a GLM analysis considering all factors that might correlate with pre-treatment HCV RNA levels. Finally, to determine if there is a correlation between pre-treatment levels of miR-122 and advanced stages of fibrosis, we did a multivariate regression which revealed that miR-122 is not associated with severity of hepatic fibrosis in CHC patients. Taken together, our results show that pre-treatment hepatic miR-122 levels are inversely correlated with HCV RNA levels and are higher in those who are not early responders to HCV treatment. These findings are in contrast with what Sarasin-Filipowicz et al. found [70]. They studied liver biopsies from patients with CHC who were undergoing IFN therapy and detected markedly decreased pretreatment miR-122 levels in subjects who had no virological response during later IFN therapy. They also found no correlation of miR-122 expression with HCV RNA levels [70]. In contrast, Morita et al., found that, although hepatic miR-122 expression was weakly and positively correlated with the serum HCV

RNA levels, it was not correlated with the hepatic HCV RNA levels [71]. They also found that the level of hepatic miR-122 expression was inversely correlated with the levels of serum aminotransferases and severity of histopathological liver damage [71]. In a study by Bihrer et al. sera from patients with CHC contained higher levels of miR-122 than sera from healthy controls, and they showed that serum miR-122 levels correlated well with markers of liver inflammatory activity like ALT levels but not with fibrosis stage which latter finding was in accordance with our multivariate regression analysis [72]. Thus, in summary there has been no general agreement on the association of hepatic levels of miR-122 with HCV RNA, liver fibrosis and response to treatment in patients with CHC. Then, too, hepatic levels of miR-122 could be quite different from and not mirrored by levels of serum miR-122 adding further complexity to this puzzle. In our study, with a multivariable analysis, we showed that pre-treatment levels of hepatic miR-122 are not associated with advanced stages of fibrosis. The inverse correlation between pre-treatment hepatic miR-122 and HCV RNA is a new finding of our study which is consonant with our hypothesis that miR-122 may play an antiviral role in uninfected hepatocytes and early stages of HCV infection. We also found that patients with high levels of miR-122 have low expression of OCLN in their hepatocytes. This result was also in accordance with our *in vitro* finding which showed miR-122 can down-regulate expression of OCLN. In chapter 3, we showed that miR-122 targets the 3'-UTR of OCLN and decreases HCV entry into hepatocytes by down-regulation of OCLN. Based on this we hypothesize that in uninfected hepatocytes or in early stages of HCV infection, miR-122 could play an antiviral role and therefore we anticipate an inverse correlation between miR-122 and HCV RNA levels. On the other hand there is a positive effect of

miR-122 on HCV replication within infected hepatocytes [59]. The latter effects will ensue after HCV entry into the cells while virus utilizes miR-122 for its own replication-as already shown by several *in vitro* studies-rather than being used to prevent HCV from entry into hepatocytes. It is not surprising that this negative correlation lost some part of its statistical significance in a GLM analysis as the positive effect of mIR-122 on HCV replication should be added up when HCV enters into hepatocytes.

We also found that pre-treatment levels of CLDN RNA are positively correlated with HCV RNA levels before starting the treatment (r= 0.71, $p<0.001$; Fig 30B), while there was also a positive correlation between pre-treatment levels of PRKRA RNA and HCV RNA levels (r= 0.55, $p=0.02$; Fig 30C). In a GLM analysis, CLDN RNA was found as the single independent factor, which positively correlated with HCV RNA levels.

CLDN has been identified as a key HCV entry receptor [21] and it was suggested that it works together with OCLN in late steps of HCV entry into hepatocytes. The monoclonal antibodies against CLDN efficiently inhibited infection by HCV of all major genotypes as well as highly variable HCV quasispecies isolated from individual patients. Furthermore, antibodies efficiently blocked cell entry of highly infectious escape variants of HCV that were resistant to neutralizing antibodies [102].

In accordance with our results and in a study performed in HCV-infected Liver transplant (LT) recipients, Mensa et al. found that there was a significant correlation between the levels of CLDN and OCLN and the slope of HCV-RNA increase during the first week after LT (r = 0.63, P = 0.005; [79]) . Therefore our results, together with previous studies, highlight the role of CLDN expression as the single most important

indicator of HCV RNA levels both in the native livers of patients with CHC, and in livers transplanted into patients with CHC, which rapidly are infected with HCV.

Amongst the miRNAs with deregulated hepatic expression, miR-29 has emerged as an important miRNA capable of modulating both carcinogenesis and fibrosis [36,103]. The 3'-UTR of the collagen-1 and 24 subtypes were identified to bind miR-29. Hence, miR-29a/b overexpression in HSC resulted in a marked reduction of collagen-I and -IV synthesis [98]. Conversely, a decrease in miR-29 levels is observed during collagen accumulation in models of experimental fibrosis, in vivo, and after TGF-b stimulation of HSC, *in vitro*. Therefore they concluded that up-regulation of miRNA-29 by HGF and down-regulation by TGF-b take part in the anti- or pro-fibrogenic response of HSC, respectively [98]. The levels of hepatic miR-29b have not been extensively studied in patients with CHC. To our knowledge, there has been only one study examined hepatic levels of miR-29 in CHC patients and showed that miR-29 was low in most HCV-infected patients compared to healthy subjects [101]. *In vitro*, they also showed that HCV infection down-regulated miR-29b, and that miR-29b overexpression reduced HCV RNA abundance. In addition to these effects on hepatic fibrosis, miR-29 has also been implicated in several other models of fibrosis, including systemic sclerosis, pulmonary fibrosis and cardiac fibrosis [104, 105].

More interestingly, when we compared levels of miR-29b in CHC patients with EVR, and n-EVR, we found that pre-treatment levels of hepatic miR-29b were significantly lower in patients with EVR than those without EVR (0.015 vs 0.130, $p<0.05$). This novel finding could be very important in both predicting outcome of disease as well as new treatment approaches for hepatitis C. In contrast to what *in vitro*

studies indicate, namely that miR-29b is indeed required for anti-fibrotic properties of host cells; its low level in early responders to HCV therapy might potentially benefit future therapeutic interventions involving the use of miR-29 antagomirs. It is possible that CHC patients with EVR would be able to induce an increase in expression levels of miR-29b during the course of treatment which leads to eradication of virus, while CHC patients with n-EVR would not because their pre-treatment levels are already at or near maximum range and it cannot be increased any further. A second probable explanation is that miR-29b plays yet unknown antiviral HCV function similar to what has been shown in HIV infection.

We also found that CHC patients with higher stages of fibrosis had higher miR-29b levels than patients with lower stages of fibrosis (0.215 vs 0.120, p=0.057). A multivariate regression analysis showed only miR-29b levels as independent factor for predicting advanced stage of fibrosis (p=0.028, Table 3).These findings are unexpected, because as just described, miR-29b has been shown to exhibit anti-fibrotic effects *in vitro*, and one might expect to see an increase in miR-29b levels in patients with lower stages of liver diseases. Hence, caution should be exercised in extrapolating *in vitro* observations to subjects with CHC. We should emphasize that despite increase of hepatic levels of miR-29b in patients with advanced stages (3-4) of fibrosis, we did not find any positive correlation between miR-29b and stage of fibrosis in general. Higher levels of mir-29b in these patients may indicate the necessity of over-expression of miR-29b as an anti-fibrotic factor in advanced degree of liver fibrosis, while underlies the possibility of having a defective response down-stream of miR-29b.

It is important to mention that there are some limitations for this study. The first limitation is the small number of total patients with CHC (n=25), including low number of treated patients (n=14), which decreases the power of study. The second limitation is lack of evaluation of serum biomarkers like serum micro RNAs. The serum levels are important to be evaluated to determine if the serum levels of micro RNAs have similar correlation with response to treatment, degree of fibrosis and HCV RNA as do hepatic levels of micro RNAs. It is, perhaps, worthy of mention that previous studies show that hepatic and serum micro RNA levels may not be correlated [74].

In conclusion, we found that hepatic miR-122 levels were inversely correlated with HCV RNA levels, while hepatic CLDN RNA was positively correlated with HCV RNA levels. We also found that pre-treatment levels of hepatic miR-29b were significantly lower in patients with EVR than those without EVR. Finally, we found that CHC patients with higher stages of fibrosis had higher miR-29b levels than patients with lower stages of fibrosis. Broader translational and *in vitro* studies are needed to unravel the importance of miR-29b in prognosis, and treatment of hepatitis C

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

It is known that miR-122 enhances HCV replication by binding to two closely spaced target sites in the 5'-UTR of the viral genome, which leads to an increased abundance of HCV RNA [59, 60]. Finding two miR-122–binding sites in an unstructured region of the 5'-UTR, and their conservation across all six HCV genotypes suggested that miR-122 could be important for the efficient replication of HCV RNA *in vitro*. However, later it was shown that miR-122 does not directly stimulate HCV RNA synthesis [106, 107]. The fact that binding sites of miR-122 were located directly upstream of an internal ribosome entry site (IRES) suggested the second theory, namely, that miR-122 can stimulate viral protein translation. However, translation enhancement only partially explains the role of miR-122 in the HCV life cycle [67, 87]. Interestingly, in another recent study, Shimakami *et al.* showed that miR-122 binds HCV RNA in association with Argonaute2 (Ago2), and that this slows decay of the HCV RNA in infected cells [67].

We found that miR-122 down- regulates OCLN expression by directly targeting 3'-UTR of OCLN mRNA. The down-regulatory effect was more pronounced at the protein level, which shows a post-transcriptional effect of miR-122 on OCLN mRNA. This inhibitory effect was confirmed by luciferase assay and mutagenic analysis. We also showed that miR-122 decreases co-localization of OCLN and CLDN in HCV infection. Finally, we found that interaction of miR-122 with 3'-UTR of OCLN mRNA eventually results in a decrease in HCV entry. On the other hand; it has been shown by us and others

that miR-122 increases the abundance of HCV RNA in HCV-infected cells [59,67, 87-88]. However this seems to be unexpected from a host factor unless it is being utilized by a virus for its own sake. Therefore, we believe that, in uninfected hepatocytes, miR-122 could play an anti-viral role by decreasing HCV entry into these cells. Accordingly, new findings highlight an anti-viral as well as tumor-suppressive role for miR-122. The anti-proliferative properties of miR-122 have been reported in several studies [61-62, 89]. Considering the above mentioned anti-viral and anti-proliferative roles for miR-122, it is plausible to speculate that miR-122 may play a dual role in HCV molecular pathogenesis; there is an initial effect of miR-122 to diminish HCV entry into uninfected hepatocytes, but a positive effect of miR-122 on HCV replication within infected hepatocytes. It is also noteworthy to mention that, for this particular study, we used HCVpp system to determine the effects of miR-122 on HCV entry into hepatocytes. Although HCVpp system is the best known system to evaluate the viral entry into the cells, it has some limitations: it is structurally different from real HCV virions. Using HCVcc systems, which are replication deficient, and determining kinetics of viral entry into hepatocytes in early hours after infection will be recommended for future studies.

In accordance with our *in vitro* study, we found an inverse correlation between pre-treatment levels of miR-122 and HCV RNA levels in patients with CHC. We also did not find any association between hepatic levels of miR-122 and stages of hepatic fibrosis. The inverse correlation between pre-treatment hepatic miR-122 and HCV RNA is a new finding of our study which is consonant with our hypothesis that miR-122 may play an antiviral role in uninfected hepatocytes and early stages of HCV infection. Based on our findings, both *in vitro* and in CHC patients, we speculate that in uninfected hepatocytes

or in early stages of HCV infection, miR-122 could play an antiviral role and therefore we anticipate an inverse correlation between miR-122 and HCV RNA levels. On the other hand there is a positive effect of miR-122 on HCV replication within infected hepatocytes [59]. The latter effects will ensue after HCV entry into the cells while virus utilizes miR-122 for its own replication- as already shown by several *in vitro* studies- rather than being used to prevent HCV from entry into hepatocytes. Our results suggest that miR-122 mimics may be more beneficial than miR-122 inhibitors in the earlier stages of infection or as a prophylactic approach when few or no hepatocytes are infected with HCV. This might have its highest application in preventing HCV re-infection in those patients who are candidates for liver transplant. Taken together, while the advantages of using miR-122 inhibitors include decreasing plasma cholesterol or HCV RNA levels in the context of hyperlipidemia or CHC, the benefits of using miR-122 mimics include tumor suppressive effects and decreasing in HBV replication and HCV entry into hepatocytes. Using either miR-122 inhibitors or mimics needs to have further careful evaluation considering the potential risks of oncogenesis or hyperlipidemia, respectively [84]. The results of these recent studies coupled with further studies will doubtless help us to unravel the still largely unknown mechanisms of the actions of miR-122, the most abundant and still mysterious hepatic micro-RNA.

PKR, a double-stranded RNA-dependent protein kinase, is among the well-known members of cellular antiviral proteins transcriptionally induced by IFNs in response to viral infection. The activated PKR phosphorylates eukaryotic initiation factor 2 (eIF2), and thereby blocks viral replication at the level of protein synthesis [27]. It is also reported that miR-122 expression was decreased after IFN treatment, whereas over-

expression of miR-122 partially alleviated the antiviral effect of IFN [65]. We found that miR-122 down- regulates PRKRA expression by targeting 3'-UTR of PRKRA mRNA. We also showed that p-PKR levels were significantly down-regulated by miR-122 while PKR protein levels were not significantly changed. The inhibitory effect of miR-122 on PRKRA RNA was also confirmed by luciferase assay and mutagenic analysis. We know that there are two adjacent binding sites within 5'-UTR of HCV RNA for miR-122 binding, and interaction of miR-122 with these binding sites leads to increase of abundance of HCV RNA. Therefore, after we confirmed that PRKRA is one of the targets of miR-122 in uninfected Huh7.5 cells, we decided to determine if the interaction of miR-122 and PRKRA RNA has any effect on HCV replication within the context of infected hepatocytes where all of these molecular elements including HCV RNA, PRKRA RNA, and miR-122 exist. When we used an HCVcc system, we found that HCV proteins were significantly up-regulated in the presence of miR-122. This result confirms that miR-122 is necessary for efficient replication of HCV, while we did not see any difference in the levels of PRKRA protein with miR-122. As there is a functional miR-122 binding site in the 3'-UTR of PRKRA, this result is in accordance with the notion that, in infected cells, miR-122 preferentially binds to its binding sites in 5'-UTR of HCV RNA rather than to the 3'-UTR of PRKRA RNA. We furthered confirmed these results and showed that the two adjacent binding sites are necessary for this binding. Using a virus defective in 5'-UTR binding sites for miR-122, we found out that HCV protein was almost undetectable in the presence of either non-specific miRNA or miR-122. On the other hand, HCV proteins were detectable after we transfected these cells with complementary mutant miR-122. Interestingly, HCV proteins were further up-regulated

when a combination of wild-type and mutant miR-122 was used. We also found that PRKRA protein level was slightly down-regulated when a combination of miRNAs was used. These findings are in accordance with the notion that when both adjacent binding sites within mutant HCV RNA are occupied, wild-type miR-122 can freely bind to its different targets in infected Huh 7.5 cells including PRKRA, and down-regulates its expression. Therefore we propose that miR-122 preferentially binds to 5'-UTR of HCV RNA rather than to the 3'-UTR of PRKRA, and this is the main factor that increases HCV replication. The probable effect of miR-122 on PRKRA, as a component of dicer complex, and any consequent effect on HCV needs to be determined.

Both responses to treatment as well as spontaneous outcome of hepatitis C virus infection are critically affected by host genetic factors. However, most of the identified association genes could not be confirmed in subsequent studies and almost none of the identified risk factors had a noticeable impact on clinical decisions [92].There has subsequently been rapidly increasing data regarding the significance of the IL28B polymorphism not only in response to therapy but also in spontaneous clearance of acute HCV infection. However, much needs to be accomplished regarding exploring the mechanism of this association. Therefore, investigating continues to find new host predictor of response to treatment with therapeutic applications. Investigating different molecular factors in 25 patients with CHC, we found hepatic CLDN RNA as the single independent factor, which positively correlated with HCV RNA levels in CHC patients. Besides, we found that pre-treatment levels of miR-29b may serve as an independent predictor of stage of liver disease (fibrosis) and response to treatment in patients with CHC.

The family of miRNA-29 plays a role in modulating severity of liver disease. It was also shown that fibrosis and mortality were enhanced in miR29 knockout mice in response to carbon tetrachloride [98]. In another recent study, it was shown that HCV infection down-regulates miR-29 in hepatocytes and may potentiate collagen synthesis by reducing miR-29 levels in activated HSCs [101]. We found that pre-treatment levels of hepatic miR-29b were significantly lower in patients with EVR than those without EVR. This novel finding could be very important in both predicting outcome of disease as well as new treatment approaches for hepatitis C. Low levels of miR-29b in early responders to HCV therapy might potentially benefit future therapeutic interventions involving the use of miR-29 antagomirs. We also showed that miR-29b level serves as an independent factor for predicting advanced stage of fibrosis .These findings are unexpected, because, as just described, miR-29b has been shown to exhibit anti-fibrotic effects *in vitro*. Hence, caution should be exercised in extrapolating *in vitro* observations to subjects with CHC. Higher levels of mir-29b in these patients may indicate the value of over-expression of miR-29b as an anti-fibrotic factor in chronic hepatic fibrosis while highlighting the possibility of having a defective response down-stream of miR-29b.

It is possible that miR-29b plays yet unknown antiviral HCV function similar to what has been shown in HIV infection [108, 109]. It is also known that miR-29 family plays some immunomodulatory role by targeting IFN-γ [42].We found Xrn1 among candidate targets of miR-29b.Recent findings show that Xrn1 knockdown enhances HCV replication, indicating that Xrn1 decay and the viral replicase compete to set RNA abundance within infected cells [68]. Future research to find if miR-29b can directly bind to Xrn1 and decrease its expression is of crucial importance. Translational research to

determine the levels of Xrn1 in CHC patients with EVR and without EVR and its correlation with miR-29b in different stages of liver disease would have both prognostic and therapeutic implication. Broader translational and *in vitro* studies are needed to unravel the importance of miR-29b in prognosis, and treatment of hepatitis C.

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