The Thesis Committee for Carolyn Spaniel Certifies that this is the approved version of the following dissertation:

Differences in miR-122 and miR-191 Expression in HBV- versus HCV-associated Hepatocellular Carcinoma

Committee:

Stanley M. Lemon, M.D., Mentor

Steven L. Bachenheimer, Ph.D

MinKyung Yi, Ph.D

Dean, Graduate School

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by

Carolyn Spaniel, B.S.

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Differences in miR-122 and miR-191 Expression in HBV- versus HCV-associated Hepatocellular Carcinoma

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Prior studies have suggested that the expression of miR-122 and miR-191 is decreased and increased, respectively, in hepatocellular carcinoma. However, other evidence suggests miR-122 may be preserved in HCV-associated hepatocellular carcinoma (HCC). The goal of this thesis is to assess miR-122 and miR-191 expression levels in HBV- and HCV-associated HCC, the related non-tumor tissue, and normal liver tissue. Relative quantification of miR-122 and miR-191 in 16 HCV-associated HCC, 10 HBV-associated HCC, the respective neighboring non-tumor tissue, and 9 normal liver tissue samples was performed using RT-PCR. This thesis shows that miR-122 expression levels are maintained in HCV-associated HCC and HBV-non-tumor tissues, but down-regulated in HBV-associated HCC and HCV-non-tumor tissues compared to normal liver tissue. miR-191 was found only to be up-regulated in HBV-associated HCC compared to normal liver expression levels. Furthermore, the miR-122 expression level in HCV-non-tumor tissue was found to have a relationship with rs8099917, a SNP known to predict HCV treatment outcome. miR-122 expression levels were decreased in patients with the TG genotype, unfavorable to HCV treatment, compared to those with the TT genotype, favorable to HCV treatment. A negative correlation of interferon-stimulated gene expression and miR-122 expression was found using Spearman correlation tests. The differences in miR-

122 and miR-191 expression levels in HBV- and HCV-associated HCC hint that there are virus-specific mechanisms that influence liver carcinogenesis during chronic infection.

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BAC	bacterial artificial chromosome
CT	critical threshold
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DNMT	DNA methyltransferase
ER	endoplasmic reticulum
HBsAg	hepatitis B surface antigen
HBV	Hepatitis B virus
HBV-HCC	HBV-associated hepatocellular carcinoma
HBV-NT	HBV-associated non-tumor
HBx	hepatitis B virus X protein
HCC	hepatocellular carcinoma
HCV	Hepatitis C virus
HCV-HCC	HCV-associated hepatocellular carcinoma
HCV-NT	HCV-associated non-tumor
HDAC	histone deacetylase
HNF	hepatocyte nuclear factor
IFN	interferon
ISG	interferon-stimulated gene
miRNA	microRNA
NK	natural killer

NT	non-tumor
Rig-I	retinoic acid induced gene I
RISC	RNA-induced silencing complex
RNS	reactive nitrogen species
ROS	reactive oxygen species
SNP	single nucleotide polymorphism
TACE	transarterial chemoembolism
TAR	Trans-activation responsive
Th	T helper
TLR	toll-like receptor

INTRODUCTION

Chapter 1

HEPATOCELLULAR CARCINOMA AND CURRENT THERAPIES

Globally, liver cancer is the fifth and seventh most common malignancy in men and women, respectively, and the third most deadly cancer [1]. Despite the total cancer death rate decreasing from 2001-2007 by over 1.5%, the liver cancer death rate has increased by 50% in males and by 29% in females in the United States [2]. In fact, the liver cancer death rate increased the most out of all other malignancies in US males and ranked second in US females. Liver cancer is estimated to be the fifth and ninth ranking cause of cancer deaths in males and females, respectively, in the US in the year of 2011. Hepatocellular carcinoma (HCC) comprises the majority, 85-90%, of liver cancer cases [3]. The life expectancy for those unable to qualify for liver resection, the majority of patients, is less than 6 months [4]. Life expectancy, for patients who qualify for liver resection, is a gloomy 25 months. The major risk factors for HCC include hepatitis B virus (HBV) and hepatitis C virus (HCV) infection [5]. HBV infection is the major risk for HCC in the developing countries of Asia while HCV infection is the major risk for HCC in developed countries [2]. Other risk factors for HCC include aflatoxin B1 exposure, persistent heavy alcohol ingestion, and non-alcoholic fatty liver disease [3].

HCC has a positive initial treatment outcome if caught in the very-early-stage. Surgical resection or percutaneous ablation cures over 90% of patients with a very-early-stage HCC tumor of less than 2cm in diameter [6, 7] However, due to the high rate of HCC recurrence the 5-year survival rate for very-early-stage HCC patients is 70% [7].

For early-stage patients with no more than three HCC nodules, each less than 5cm, surgical resection, liver transplantation, or percutaneous ablation are the acceptable

therapies. Percutaneous ablation refers to various localized techniques to destroy the tumor without resection. A probe is inserted into the tumor and ethanol or acetic acid is injected; alternatively, radio waves are emitted to destroy the tumor [7]. The 5-year survival rate is approximately 50-75% [7].

Intermediate-stage patients with large multiple HCC nodules and cirrhotic liver tissue, but without any vascular invasion or metastasis qualify for transarterial chemoembolism (TACE), which increases the 2-year survival rate by approximately 20% [6]. The dual function of the TACE chemotherapy agents, to block the blood supply to the tumor and the delivery of toxic chemotherapy agents, allows for localized destruction of the tumor.

Advance-stage patients have multiple HCC nodules, cirrhosis, vascular invasion, and metastasis. The multiple kinase inhibitor Sorafenib is usually utilized as therapy[6]. Sorafenib, regrettably, can only prolong the life of an advanced stage patient for an average of 3 months [8].

Due to the fact that the life expectancy for those unable to qualify for liver resection, which is the majority of patients, is less than 6 months [9], the 5-year survival rate for US patients with liver cancer is only 14% [10]. Since the majority of patients are diagnosed at a late stage, there is a dire need for improved treatment of HCC.

MIR-122 AND MIR-191 EXPRESSION IN HCC

Mature microRNAs (miRNA) are composed of 20-23 nucleotides and are encoded by miRNA genes or are encoded within the introns of conventional protein-coding genes [11]. Primary transcript miRNAs are processed by Drosha, an RNase, to form a 60-70 nucleotide hairpin pre-miRNA [12]. After transportation from the nucleus to the cytoplasm by exportin-5, they are further processed by Dicer to a mature approximately 21 nucleotide long duplex miRNA. Trans-activation responsive [13] RNA binding protein

guides the Dicer/miRNA duplex complex to Agonaute proteins 1-4 to form the RNA-induced silencing complex (RISC) [13]. During RISC formation one RNA strand of the duplex is discarded. The retained guide strand leads the RISC complex to its complementary RNA target [14]. miRNAs' traditional functions include translation inhibition or mRNA degradation; however, miRNAs under certain circumstances can also up-regulate translation of mRNAs [11].

miR-122, which comprises 70% of all liver microRNAs, may act as a tumor suppressor by regulating ADAM17, ADAM10, serum response factor, IGFR1, and the M2 splice variant of pyruvate kinase [15, 16]. The finding that the down-regulation of miR-122 in HCC leads to a gain of metastic ability and a loss of liver phenotype supports miR-122's capability as a tumor suppressor [17]. Other targets of miR-122 influence iron homeostasis and cholesterol synthesis [18]. Mir-122 has been proposed as a potential therapeutic agent for HCC, due to its ability to decrease cell mobility and growth in HBV-associated HCC (HBV-HCC) derived Hep3b cells [15]. Prior studies investigating miR-122 expression in HCC have been conflicting. Two early studies, which included a predominately HCV-associated HCC (HCV-HCC) study, found miR-122 abundance to be significantly reduced in HCC [19, 20]. However, Hou et al. reported miR-122 expression maintenance in both HBV- and HCV-HCC versus normal tissue [21]. In contrast, Varnholt et al. [22] found that miR-122 levels were increased significantly in HCV-HCC versus normal tissue. Suggesting a virus-specific difference in miR-122 expression levels, Coulouarn et al. [17] reported higher miR-122 expression levels in HCV- versus HBV-HCC. No study thus far has reported miR-122 expression level differences in HCV- and HBV-HCC, the respective surrounding non-tumor, and normal liver tissue. Important to note, miR-122 stabilizes and promotes translation of HCV RNA [23-25], but inhibits HBV replication [26-29].

miR-191 has been found to be up-regulated in liver and lung cancers [30, 31]. Mir-191 up-regulation may promote carcinogenesis by repressing expression of Mxi-1, a c-

myc antagonist [32, 33]. Although miR-191 has been found to be up-regulated in HCC, the patients in the study were predominately diagnosed with HBV-HCC [31]. miR-191 has been proposed as a potential therapeutic target due to anti-miR-191's ability to increase caspase-3/7 activity and decrease proliferation in Hep3b cells. This study is the first to distinguish miR-191 expression levels in HCV- and HBV-HCC.

HCV/HBV Life Cycle, Immune Response, Evasion, and Mechanisms of Carcinogenesis

The major risk factor for HCC in the US and the world is HCV and HBV, respectively [3]. HCV replication and translation is enhanced by mir-122 [23-25, 34]. In contrast to HCV, miR-122 inhibits HBV replication [26-29]. If results show miR-122 and miR-191 is decreased and increased, respectively, in HBV-HCC yet maintained in HCV-HCC it is an indication that viral mechanisms could influence carcinogenesis. A brief introduction into HCV and HBV, the resultant immune response to infection, how persistent infection is achieved, and how the viruses promote carcinogenesis follows.

HCV belongs to the *Flaviviridae* virus family. See Figure 1 for an overview of the HCV life cycle [35]. It is an enveloped virus with a positive, single-stranded RNA genome. Various receptors (CD81, SR-B1, claudin, occludin, Niemann-Pick C1-like 1 cholesterol absorption receptor, low density lipoprotein receptor, glycoaminoglycans) are recognized and target the virus to the hepatocytes [36-38]. IRES-mediated translation yields a single polyprotein that is inserted into the endoplasmic reticulum (ER). Upon cleavage by cellular and viral proteases, 3 structural proteins (core, E1, and E2), an ion channel (p7), and 6 nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are produced [39]. RNA replication occurs at the NS4B-induced membranous web [40]. Viral assembly and budding occurs at the ER and lipid droplet[41]. Endosomal-release and cell

to cell transmission have been implicated in HCV release, but the mechanisms are not fully understood [42].

HBV belongs to the *Hepadnaviridae* family. HBV is enveloped with a partially double-stranded DNA genome, which encodes 4 open reading frames. HBV proteins include core, precore, envelope protein L, envelope protein M, envelope protein S, Hepatitis B virus X protein (HBx), and reverse transcriptase [43]. HBV takes a stealth approach and translocates the genome to the nucleus where it is converted to a covalently closed circular DNA. Transcription by RNA polymerase II yields polyadenylated and capped viral RNAs. After translation of viral mRNAs, the pre-genome RNA is packaged, reverse-transcribed, and the resulting single-stranded DNA is copied to produce the partially double-stranded HBV genome in the nucleocapsid. Budding is believed to occur into the ER lumen. [43]. Virus is released through exosome secretion [43]. See figure 2, for a depiction of the HBV life cycle.

HCV is detected by the innate immune response through toll-like receptor (TLR)3 and retinoic acid inducible gene I (Rig-I) resulting in interferon-stimulated genes (ISGs) expression in the hepatocytes [44]. Upon TLR3 and TLR7 activation, myeloid dendritic cells (DCs) and plasmacytoid DCs, respectively, secrete IFN-α, TNF-α, IL-12, and IL-10 [45]. Natural Killer (NK) cells are further activated by IL-12 secreting DCs [45]. IFN-γ producing NK cells promote antigen presentation and the development of an adaptive immune response [44]. A strong Th1 immune response is necessary for spontaneous clearance of HCV [46]. CD4 positive T cells secrete IL-2, IFN-γ, and TNF-α which promote the killing action of CD8 positive cytotoxic lymphocytes (CTLs) [45]. CTLs promote HCV clearance by killing infected cells through specific antigen recognition accompanied by the release of granzymes and perforins, promote apoptosis by expressing Fas ligand, and through non-specific, non-cytolytic release of IFN-γ [45].

Due to HBV's stealth approach of replication, the virus is able to infect 100% of all hepatocytes without inducing an innate immune response [47]. By sequestering

replication to the nucleocapsid the HBV avoids early detection by pathogen recognition receptors [47]. CD8 T cells, through cytopathic action and IFN-γ secretion, are the major players in HBV clearance. A late T-helper (Th)2 response and neutralizing antibodies are important for sustained viral clearance after HBV resolution [47]. See figure 3 for an overview of the HBV and HCV immune response.

HCV has evolved multiple mechanisms to dampen the innate and adaptive immune responses mounted against it and as a result 70% of infected individuals become chronically infected [46, 48]. NS3/4a inhibits Rig-I and TLR3 pathogen-recognition receptor signaling [49]. NS5a and core proteins inhibit the IFN Jak-Stat pathway [49]. Immunodominant viral epitopes are frequently changed due to HCV's high mutation rate [46]. Furthermore, immunodominant viral epitopes that promote a Th2 response may be selected to enhance viral persistence [46]. HCV infection inhibits the ability of DCs to mature and interact with T-cells [46]. High ISG expression is detected in non-responders to peg-IFN-α and ribavirin treatment [50]; however, high IFN I and III expression levels may inhibit a strong CTL response by over-activating NK cells, which in turn kill immature DCs in an effort to down-regulate the response [46]. HCV's error-prone polymerase would then allow escape mutants to evade any cumbered adaptive immune response [46].

HBV is not as successful in evading the adaptive immune response compared to HCV, which contributes to the low percentage, 1%, of infected immunocompetent adults developing chronic infection [51]. However, in high risk areas such as sub-Saharan Africa and eastern Asia the majority of HBV transmissions occur during birth [3]. 90% of infected newborns develop chronic infection [3]. HBV's RNA-dependent DNA polymerase mutation rate is 100-fold lower than HCV although relatively high in comparison to other DNA viruses, which results in adaptive immunity escape mutants [52]. Induction of liver tolerance can occur and last for decades, especially if infection occurred at birth or early childhood [53]. HBV surface and pre-core proteins are believed to contribute to liver tolerance. [52].

Once chronic HCV infection is established, the patient is increasingly prone to HCC through viral-specific mechanisms as well as through chronic inflammation and regeneration of the liver. HCV proteins directly manipulate and compromise the function of cell cycle regulators, tumor suppressors, and signaling pathways that influence proliferation. NS5a interaction with p53 circumvents apoptosis and promotes survival of infected cells [54]. NS5b interacts with Rb promoting its degradation while promoting E2F-mediated G1 to S cell cycle transition [54]. Core interacts with SMAD3 preventing TGF-β from promoting apoptosis and blocking mitosis [54]. HCV core and NS5a proteins have been implicated in increased mitochondria calcium ion concentration which may cause an increase in DNA-damaging reactive oxygen species production [55]. Indirectly, chronic HCV infection results in persistent inflammation that damages the DNA of proliferating hepatocytes. Activated neutrophils and Kupffer cells continually produce reactive oxygen species and nitrogen species (ROS and RNS) [55]. RNS and NOS oxidize nucleotides and induce single-stranded breaks. Faulty DNA repair can lead to unstable intermediates, base insertions, base deletions, and base pair substitutions [56]. Perforins and granzymes released by NK cells and CTLs induce hepatocyte apoptosis as inflammatory cytokines, such as TNFα and IL-6, promote proliferation in a DNA damaging environment. The persistent inflammation leads to cirrhosis in 15% of those chronically infected [3].

The known HBV-induced carcinogenesis mechanisms are similar to HCV, through chronic liver injury, ROS production, and viral-mediated deregulation of tumor suppressors and activation of oncogenes [47]. Chronic HBV infection promotes carcinogenesis through persistent inflammation, apoptosis, and regeneration similarly to HCV chronic inflammation [57]. ER stress due to accumulation of HBV S, M, and L envelope proteins increases ROS production [57]. The HBx protein binds to a multitude of host cell proteins modulating and compromising their activity which promotes carcinogenesis. HBx protein binds and inhibits p53 preventing p53-dependent cell

growth arrest and apoptosis [58]. HBx interaction with DNA damage-binding proteins may be responsible for HBx interference on the nucleotide excision repair pathway [59]. HBx ability to bind to transcription factors and signaling pathway proteins hinders proper cell regulation [57]. HBx may induce epigenetic transcriptome changes by up-regulating DNA methyltransferases which may silence various tumor suppressor genes [60]. Another mechanism of HBV carcinogenesis is viral DNA integration into the host genome. Integration can occur over a hundred times per clonal tumor and is associated with host genome instability [61, 62]. HBV DNA has a tendency to integrate near the catalytic subunit of the telomerase gene and up-regulates its expression and promotes immortality [60]. See figure 4 for an overview of HCV and HBV carcinogenesis mechanisms.

RS8099917 AND HCV TREATMENT OUTCOME

HCV IFN-α and ribavirin treatment has been surrounded by the enigma of single nucleotide polymorphisms (SNPs) in the IL28b gene which predict treatment outcome [63, 64]. IL28b encodes for IFN-λ3, a type III IFN. A genotype of TT at rs8099917, which is approximately 8.9 kb upstream of the IL28b gene, indicates the patient is more likely to respond to treatment than those patients with a TG or GG genotype at rs8099917 [63]. MiR-122 expression levels have been reported to be higher in HCV treatment complete early responders compared to primary non-responders [65]. This thesis investigates whether the same pattern can be seen according to the rs8099917 genotype in the HCV-associated non-tumor samples.

GOAL AND IMPORTANCE

The goal of this thesis is to investigate miR-122 and miR-191 expression levels in HBV- and HCV-HCC, HBV- and HCV-associated non-tumor (HBV- and HCV-NT), and

normal liver tissue. mir-122 and mir-191 are considered to be a potential therapeutic agent and target, respectively [15, 31]. It is important to determine if miR-122 and miR-191 expression deviates between HBV-HCC and HCV-HCC in the quest to develop therapeutics for HCC and to understand differences in etiologic HCC carcinogenesis.

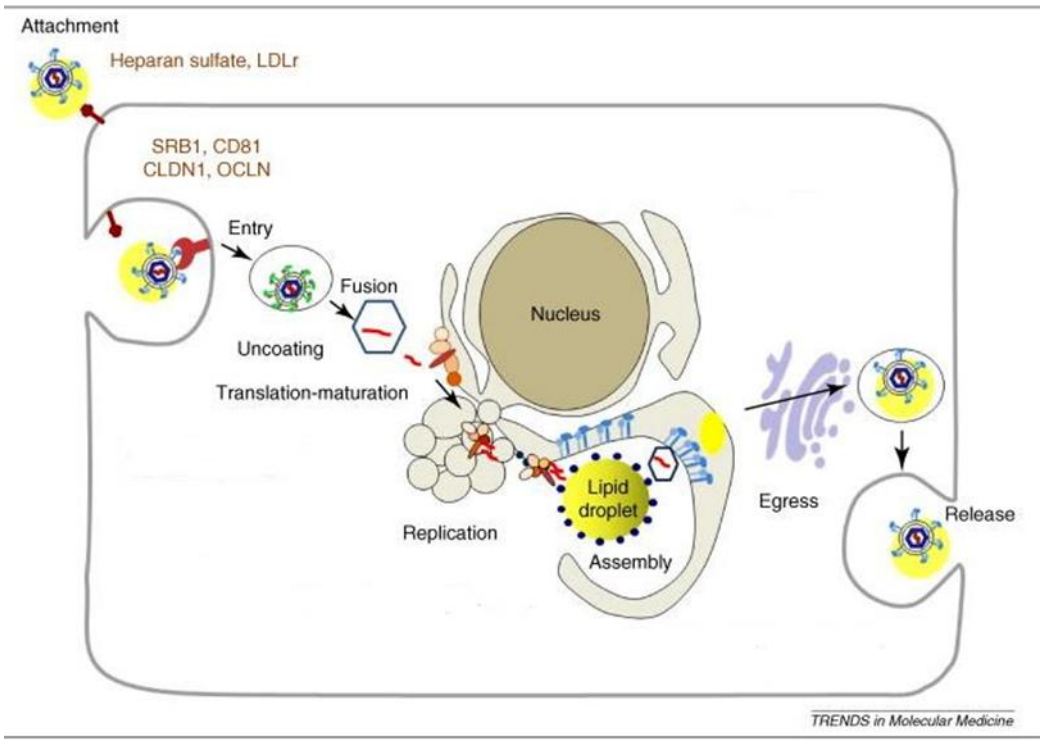


Figure 1. HCV Life Cycle. HCV binds to receptors expressed on hepatocytes promoting endocytosis. Viral membrane fusion with the endosome releases the viral nucleocapsid. Viral RNA translation results in an ER-inserted HCV polyprotein that is processed into functional proteins by host and viral proteases. RNA replication occurs at the virus-induced membranous web. Viral assembly occurs in association with lipid droplets at the ER membrane. Virus is transported through the golgi apparatus and released through exocytosis.

Adapted from "Virus–host interactions in hepatitis C virus infection: implications for molecular pathogenesis and antiviral strategies" by P. Georgel, C. Schuster, M. B. Zeisel, F. Stoll-Keller, T. Berg, S. Bahram, and T. F. Baumert, 2010, *Trends in Molecular Medicine, 16*, p. 280. Copyright © 2010 by Elsevier. Adapted with permission.

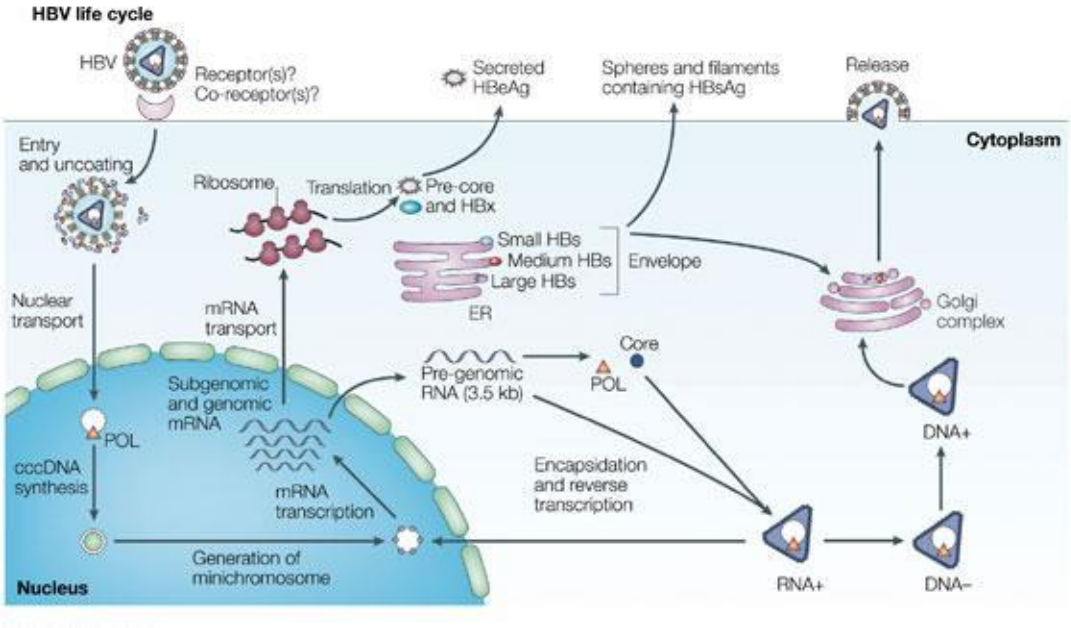


Figure 2. HBV Life Cycle. After viral entry into the hepatocyte, the viral genome is translocated to the nucleus and is processed to form a closed circular DNA. Viral mRNAs and the pregenome are transcribed in the nucleus, transported to the cytoplasm, and translated. Envelope proteins associate with the ER. Viral genome replication occurs within preformed core capsids. Budding may occur at the ER or the golgi apparatus resulting in exosome-mediated viral release.

Adapted from "Immunology of hepatitis B virus and hepatitis C virus infection" by B. Rehermann, and M. Nascimbeni, 2005, *Nature Reviews: Immunology, 5*, p. 220. Copyright© 2005 by Nature Publishing Group. Adapted with permission.

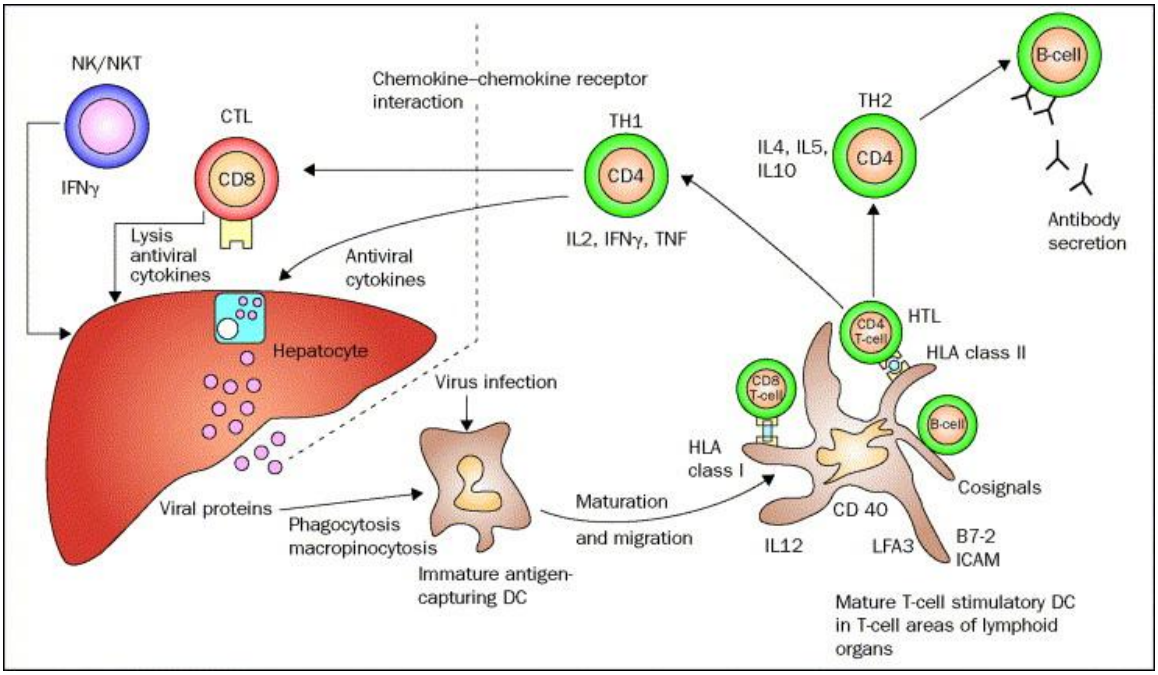


Figure 3. HCV and HBV clearance is mediated by Th1 and Th2 immune responses. HBV and HCV differ in immunology detection, but many of the resultant immune responses are the same. In HCV, initial detection is most likely to occur through TLR3 or Rig-I in the infected hepatocyte. pDCs may also be stimulated by TLR7 to secrete IFN-α, TNF-α, IL-12, and IL-10. In HBV, initial detection may occur by IFNγ secreting NK cells which promote antigen presentation and the development of an adaptive immune response. Th1 cells promote cytotoxic T lymphocytes by secreting IL-2, IFNγ, and TNF-α. The Th2 response helps neutralize circulating virus.

Adapted from "Immunology of hepatitis B infection" by M.-C. Jung, and G. R. Pape, 2002, *The Lancet Infectious Diseases, 2*, p. 44. Copyright© 2002 by Elsevier. Adapted with permission.

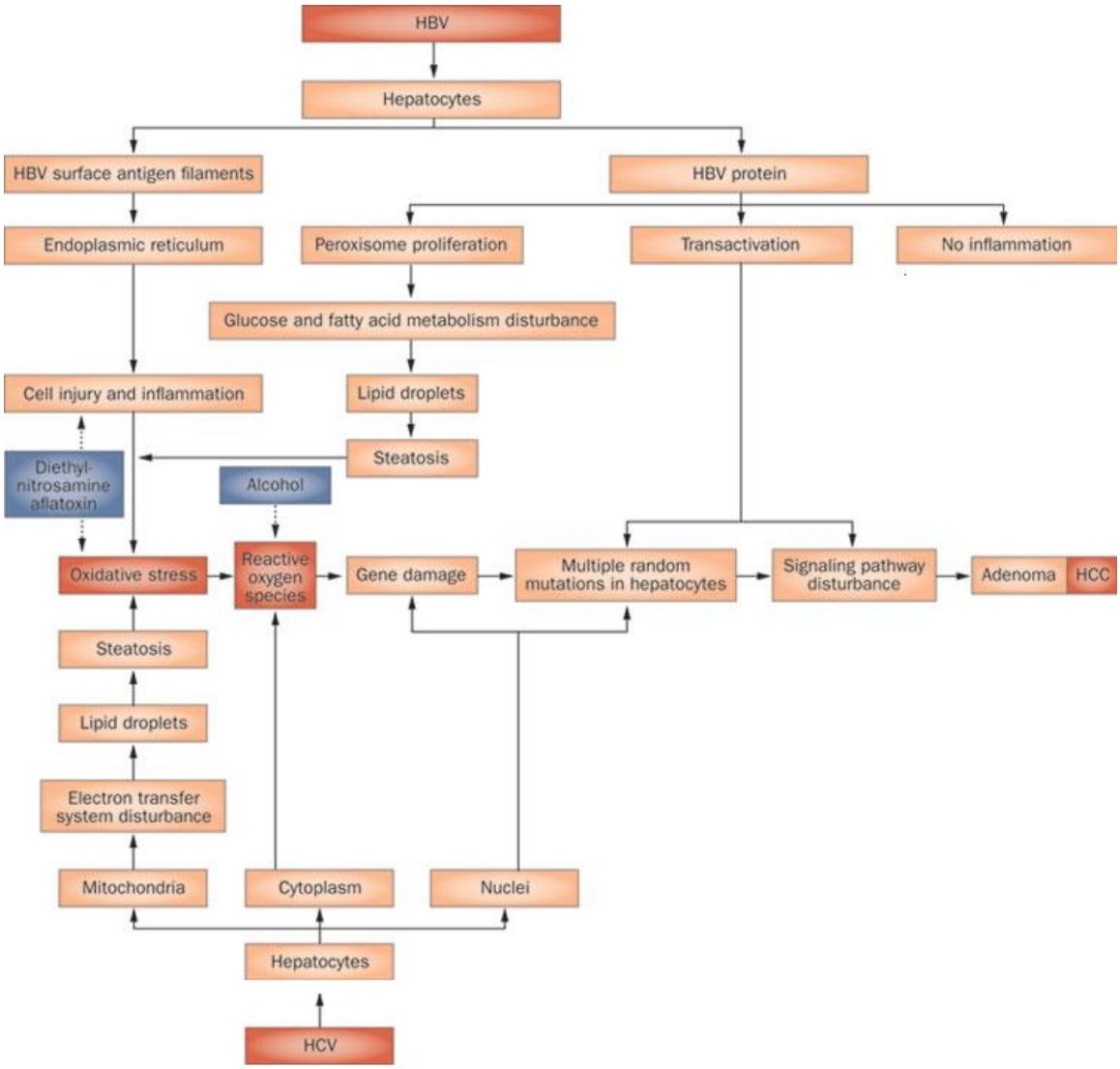


Figure 4. Overview of HBV- and HCV- induced carcinogenesis. HCV and HBV share over-lapping mechanisms of carcinogenesis. ER stress induced by viral proteins promote DNA damaging ROS production. In a mutation-favorable environment, chronic hepatitis induces a continuous cycle of liver injury and regeneration which promotes carcinogenesis.

Adapted from "Hepatocellular carcinoma: insight from animal models" by Y. Li, Z.-Y. Tang and J.-X. Hou, 2011, *Nature Reviews: Hepatology and Gastroenterology, 9*, p. 34. Copyright© 2011 by Nature Publishing Group. Adapted with permission.

METHODS

Chapter 2

STUDY SUBJECTS AND TISSUE SAMPLES

Paired samples of HCC and non-malignant liver tissue were collected at surgery from Japanese patients undergoing resection of HCC at Kanazawa University Hospital in Kanazawa, Japan. Normal liver tissue was similarly collected from patients undergoing resection of metastases of non-hepatic primary cancers. Patients were categorized as HCV-infected by the presence of HCV RNA (COBAS Ampli-Prep/COBAS TaqMan System) and absence of hepatitis B surface antigen (HBsAg) in serum or plasma at the time of surgery, while HBV infection was defined by the presence of HBsAg and absence of anti-HCV antibodies. HCC was scored according to the degree of cellular differentiation, while fibrosis and inflammation in non-tumor tissue from HBV- and HCV-infected patients was scored according to the Metavir system. The IL28b genotype of study subjects with HCV infection was defined at the rs8099917 locus as described previously [66].

MIRNA QUANTIFICATION

All tissue samples were stored in liquid nitrogen until processed for RNA extraction. Approximately 1 mg of tissue was ground using a tissue homogenizer and total RNA isolated using the mirVana miRNA isolation kit (Ambion). Liver RNA samples were subsequently stored at -80ºC or on dry ice during shipment. Quantification of miR-122, miR-191, Let-7a, miR-24, and the small nuclear RNA (snRNA) U6 was carried out by quantitative reverse-transcription, polymerase chain reaction (qRT-PCR) in

a two-step process. First, 12.5 ng RNA was reverse-transcribed in a 10 ul reaction mix using primers included in the Universal cDNA Synthesis kit (Exiqon) and the manufacturer's recommended procedure. Quantitative PCR was carried out subsequently using the SYBR Green Master Mix Kit (Exiqon), mixed locked-nucleic acid primer sets specific for each miRNA or snRNA (Exiqon), and the CFX96 PCR System (Bio-Rad).

AFFYMETRIX ARRAY ANALYSIS

RNA samples were subjected to high-density oligonucleotide microarray analysis using Affymetrix U133 Plus 2.0 GeneChip arrays as described previously [66]. The quality of the isolated RNA was estimated using an Agilent 2001 Bioanalyzer (Palo Alto, CA, USA). Aliquots of total RNA (50 ng) were subjected to amplification with the WT-Ovation Pico RNA Amplification System (NuGen, San Carlos, CA, USA) according to the manufacturer's instructions. Approximately 10 ug of cDNA was amplified from 50 ng of total RNA, and 5 ug of cDNA was used for fragmentation and biotin labeling using the FL-Ovation cDNA Biotin Module V2 (NuGen) according to the manufacturer's instructions. Biotin-labeled cDNA was suspended in 220 ul of hybridization cocktail (NuGen), and 200 ul was used for hybridization. After stringent washing, the microarray chips were labeled with streptavidin-phycoerythrin. Probe hybridization was determined using a GeneChip Scanner 3000 (Affymetrix). Data files [67] were generated with the GeneChip Operating Software 1.4 (Affymetrix).

STATISTICAL ANALYSIS

Statistical analyses were carried out using Prism V software (Graphpad Software, Inc). The paired t test was used for comparison of results arising from groups of paired tissue specimens (HCC versus non-tumor tissue), while the unpaired t test was used for

comparisons between groups of unrelated tissues (e.g., HBV versus HCV infection). Nonparametric analysis of the correlation between miR-122 and ISG expression levels used the Spearman method.

RESULTS

Chapter 3

PATIENT CHARACTERISTICS

The age, histological classification of HCC, and Metavir score of non-tumor tissues are shown in Fig. 5. HCV-infected patients were approximately one decade older than those with HBV infection (66.6 ± 8.0 s.d. versus 54.3 ± 9.1 s.d. years, p=0.001, Fig. 5A), consistent with previous studies indicating that HCC is generally diagnosed at an earlier age in HBV-infected Japanese patients [68]. There were no significant differences in the histological classification of HCC or Metavir scores for fibrosis or inflammatory activity in non-tumor tissues between the two groups (Figs. 5B and C). There were more females among those with HCV infection (11 male and 5 female) than HBV (9 male and 1 female), but this difference did not achieve statistical significance (Chi square test with Yate's correction).

NORMALIZATION

There is no universal microRNA normalizer to account for differences in degradation or reverse transcriptase efficiency between samples. U6 is a small nuclear RNA commonly used for microRNA normalization; however, there was a significant difference in expression between sample groups indicating it was a poor normalizer for the sample set. (Fig. 6A) The standard deviation of U6's critical threshold was 1.34. To find better normalizers for the sample set, microRNAs that were stably expressed between the sample groups with a <1.34 standard deviation of the CT were sought. The standard deviation of the CT was 0.79 and 1.27 for miR-24 and let-7a, respectively, and

the expression level between sample groups had no significant difference. (Figures 6B and 6C) To assess the relationship between expression levels of these small RNAs, HCC and NT miR-24 expression levels were plotted against U6, let-7a, and miR-122. (Figures 6D-6F). There was no correlation between miR-24 and U6 or miR-122, which indicates that U6 and miR-122 are regulated independently of miR-24. There was a highly significant strong correlation, Spearman rs=0.7959 and p<0.0001, between miR-24 and let-7a, figure 6E. This supports the use of miR-24 and let-7a as normalizers. MiR-24's standard deviation of the CT was less than let-7a; therefore, in this study miR-24 and total RNA were used as normalizers for the sample sets.

MIR-122 EXPRESSION IS INFLUENCED BY VIRAL ETIOLOGY

MiR-122's expression levels were measured in paired tumor and non-tumor tissues from 16 HCV and 10 HBV patients undergoing HCC surgical resection. qRT-PCR was used to measure miR-122 and was normalized to total RNA and miR-24. (Figure 7A-B) Results were similar no matter how normalized, which indicates the RNA samples between subgroups were of similar quality and total RNA is an acceptable normalizer. Normalized to total RNA, miR-122 abundance was significantly lower in HBV-HCC versus HCV-HCC (p=0.006). In contrast, the miR-122 abundance in HBV-NT was significantly greater HCV-NT (p<0.0001). In HBV-infected patients, the mean miR-122 abundance in HCC tissue was less than half that in NT tissue (p=0.003 by paired t test). In contrast, HCV-infected patients miR-122 expression was near twice that of NT tissue (p=0.008 by paired t test). There was no significant difference in miR-122 expression between HBV-NT and HCV-HCC. To compare our findings to normal liver tissue, miR-122 overexpression was measured in 9 normal liver samples collected by surgical resection of hepatic metastases along with the NT samples (Fig. 8A). As expected, HBV-NT expression levels were similar to normal tissue. In contrast, HCV-

NT expression levels were considerably decreased compared to normal, p=0.0009. The normalized composite of three experiments shows miR-122 expression levels are decreased in HBV-HCC, maintained near normal in HBV-NT and HCV-HCC, and down-regulated in HCV-NT compared to normal liver tissue expression. (Fig. 9) These results show differential miR-122 expression not only between HBV- and HCV-HCC tissues but also between the HBV- and HCV-NT tissues as well. This is a strong indication that the viral and immunological factors that induce HBV and HCV carcinogenesis differ, and result in differential expression patterns within the HCCs.

METAVIR SCORE AND MIR-122

To further investigate whether down-regulation of miR-122 expression levels in HCV-NT is influenced by fibrosis or inflammation, HCV-NT samples were plotted according to their Metavir fibrosis and activity score. We were concerned that high numbers of inflammatory cells might dilute the liver-specific miR-122 and cause false significance in miR-122 expression levels. The Metavir system scores liver fibrosis at 5 degrees, ranging from F0-F4. A score of F0 equates no fibrosis while a score of F4 equates liver cirrhosis. As seen in figure 8B, there was no significant difference or down-regulation of miR-122 from F1-3 to F4 miR-122 expression levels. Inflammation is scored from A0-A3. A0 is defined by no inflammation activity as A3 is defined as severe inflammation activity. Our samples had A1, mild inflammation activity, or A2, moderate inflammation activity. Interestingly, A2 fibrosis had a significantly higher miR-122 expression level, p=0.031, than A1in HCV-NT. (Fig. 8B) This result is attributed not to the degree of inflammation as increased numbers of inflammatory cells would be expected to reduce tissue miR-122 expression levels due to the dilution effect, but rather the difference in patient immune response. Six out of the seven A2 samples were derived

from patients with the rs8099917 TT responder genotype. Patients with the TT genotype are prone to a higher inflammation response than those with the TG or GG [69]. In contrast, non-responder prone patients have a higher ISG expression level. The higher interferon response in the TG patients could possibly restrain transcription of miR-122 in HCV-NT tissue. [50]

TUMOR DIFFERENTIATION AND MIR-122

To rule out any possible skewing of the data due to differences in the tumor differentiation between HBV- and HCV-HCC, we separately analyzed data from only the moderately differentiated HCC and the corresponding NT samples. (Fig. 10A-B) MiR-122 down-regulation in HBV-HCC, its maintenance at normal levels in HBV-NT and HCV-HCC, and its down-regulation in HCV-NT was still evident.

IL28B GENOTYPE INFLUENCES HCV NON-TUMOR EXPRESSION

miR-122 has previously been found to be reduced in primary non-responders in relation to complete early virological responders to pegylated IFN-α and ribavirin treatment [65]. We wondered if this pattern could be seen according to rs8099917 genotype. A TT genotype at rs8099917 indicates the HCV patient is more likely to respond to therapy than the GT genotype [63]. Indeed, the results showed that those patients with the GT genotype had a lower miR-122 expression level in the NT tissue compared to the TT patients, $p=0.011$. (Fig. 11A)

Patients who are non-responsive to a regimen of pegylated-IFN and ribavirin, or who have IL28B genotypes predictive of a poor response to therapy, are likely to have increased pre-treatment ISG transcript levels compared to those who respond well to

treatment [50, 70, 71]. We next investigated if there was a correlation between miR-122 expression and ISG expression in HCV-NT. The Affymetrix 133U Plus 2.0 array was used to determine ISG expression levels. For this analysis, we selected ISGs shown previously to be correlated with treatment response to pegylated-IFN and ribavirin [50] (Fig. 11B). We also included MS1 and OAS1, both well-known ISGs. Overall, the Affymetrix signals for these genes showed a strong trend toward negative correlations with miR-122 abundance (14 of 24 genes having a Spearman rank-order coefficient, r_s, $\leq$ -0.300) that was significant ($p<0.05$) by one-tailed t test for 7 ISGs (Fig. 11B). This is consistent with the notion that reductions in miR-122 abundance are associated with strong intrahepatic IFN-mediated responses to the virus.

MIR-191 EXPRESSION IS UP-REGULATED IN HBV-ASSOCIATED HCC

MiR-191 has been found to be up-regulated in HCC and was proposed as a therapeutic target for HCC [31]. However, the majority of HCC samples in prior studies were HBV-associated. Our results indicate that miR-191 is only up-regulated in HBV-HCC. Normalized to total RNA, HBV-HCC miR-191 expression levels were increased ($p=0.049$ by two-sided, paired t test) compared to HBV-NT (Fig. 12A) No difference was detected between HCV-HCC and NT samples. The significant difference is diminished ($p=0.045$ by one-sided, paired t test, Fig. 12B) but the upward trend remains upon normalization to miR-24. However, miR-24 expression median is slightly higher in HBV-HCC compared to all other sample sets and may account for the diminished p-value (Fig. 6B). Non-tumor and normal liver miR-191 expression levels were found to have no significant difference (Fig. 12C).

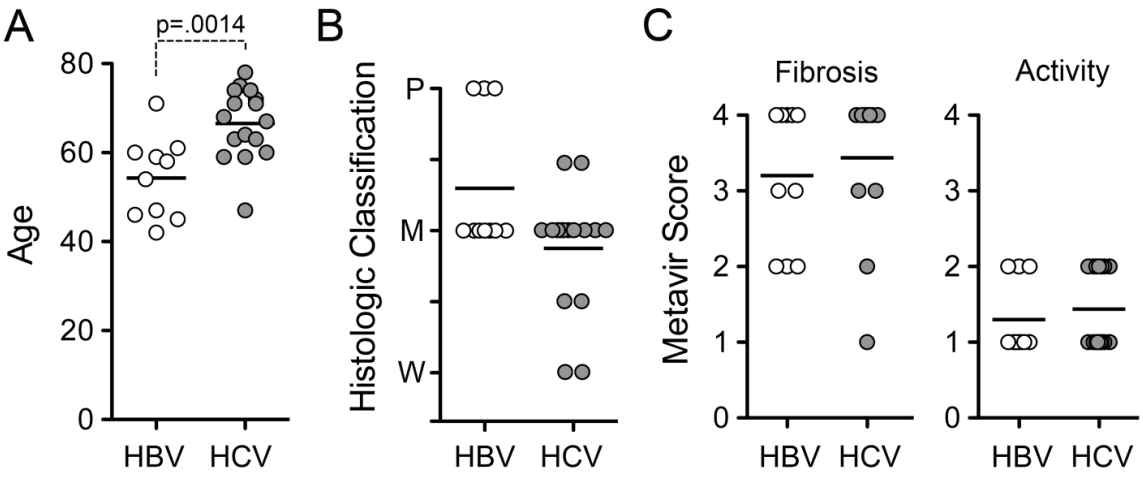


Figure 5. Age, histological classification tumors, and Metavir score of non-tumor tissues. (A) Age of subjects from whom HBV- and HCV-associated HCC and paired non-tumor samples were obtained. **(B)** Histological classification of tumors: W = well differentiated, M = moderately differentiated, P = poorly differentiated. **(C)** Metavir scores for fibrosis and inflammatory activity in non-tumor tissue. Bars represent mean values.

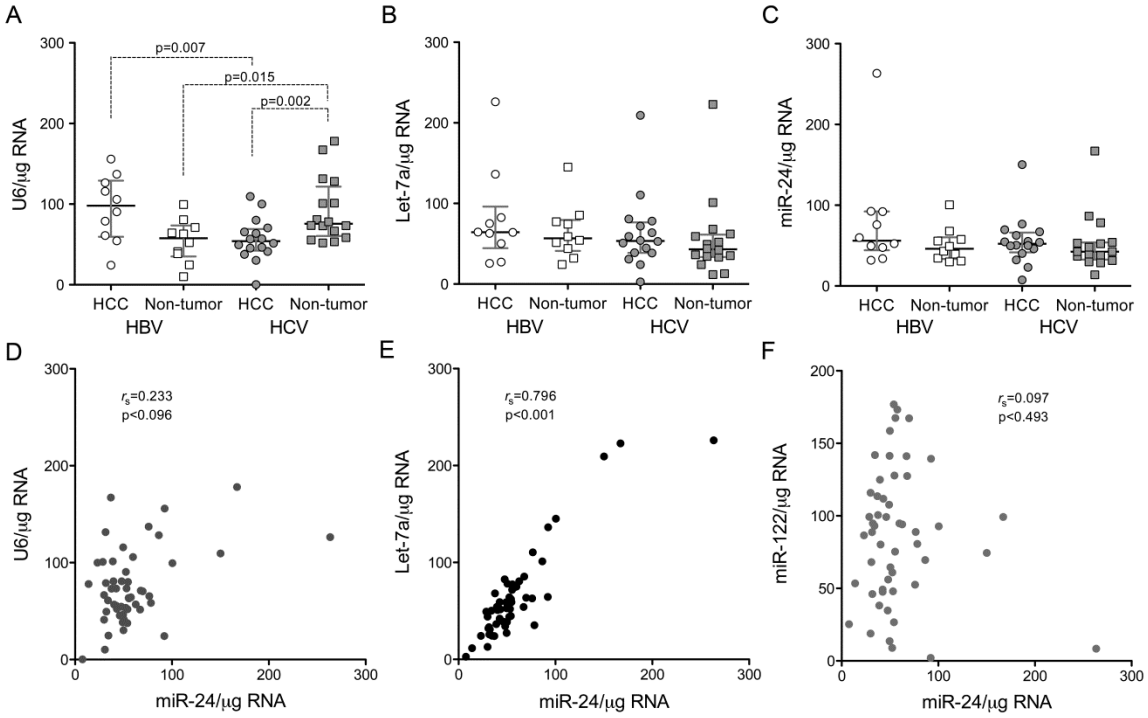


Figure 6. Potential small RNA normalizers for comparison of miR-122 abundance. In the panels at the top, the relative abundance of **(A)** U6 snRNA, **(B)** Let-7a, and **(C)** miR-24 miRNAs is shown normalized to total RNA. Bars represent median and quartiles for each subgroup. In the lower set of panels, relative miR-24 abundance is plotted against **(D)** U6, **(E)** Let-7a, and **(F)** miR-122 abundance. r_s = Spearman rank-order correlation coefficient. The y-axis in figures 6A-C, and subsequent figures showing PCR results, indicates relative expression units. In this and all subsequent figures, statistical significance was assessed by two-sided paired t test for comparisons between groups of paired tissue samples, and by non-paired two-sided t test for comparisons between virus infections.

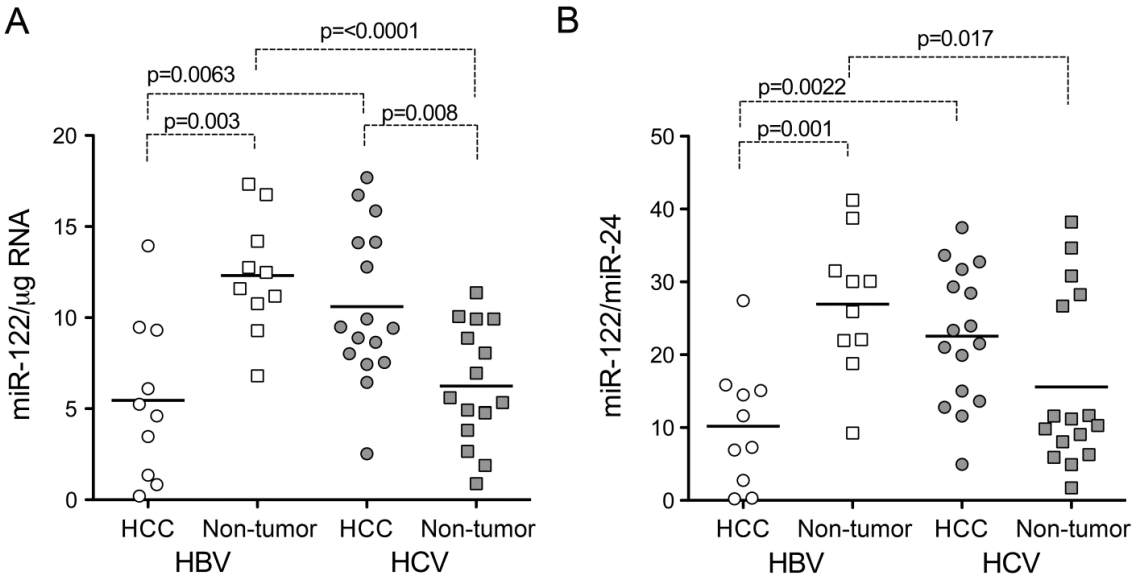


Figure 7. Quantification of miR-122 in HCC and non-tumor tissue. (A) Relative miR-122 abundance quantified by qRT-PCR in paired tumor and non-tumor tissues, normalized to total RNA. **(B)** miR-122 abundance normalized to miR-24 abundance in the same tissues. Bars represent mean values in this and all subsequent figures.

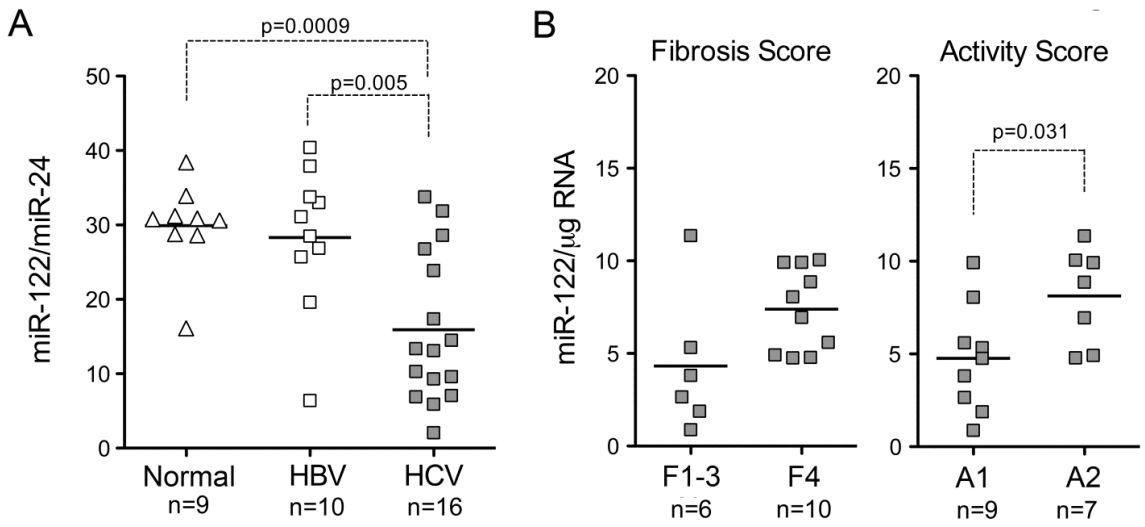


Figure 8. miR-122 expression in normal and non-tumor liver samples. (A) miR-122 abundance relative to miR-24 abundance in normal and non-malignant liver tissue from HBV- and HCV-infected subjects. **(B)** miR-122 expression levels in non-tumor tissue from HCV-infected subjects categorized according to Metavir score for (left) fibrosis and inflammatory activity.

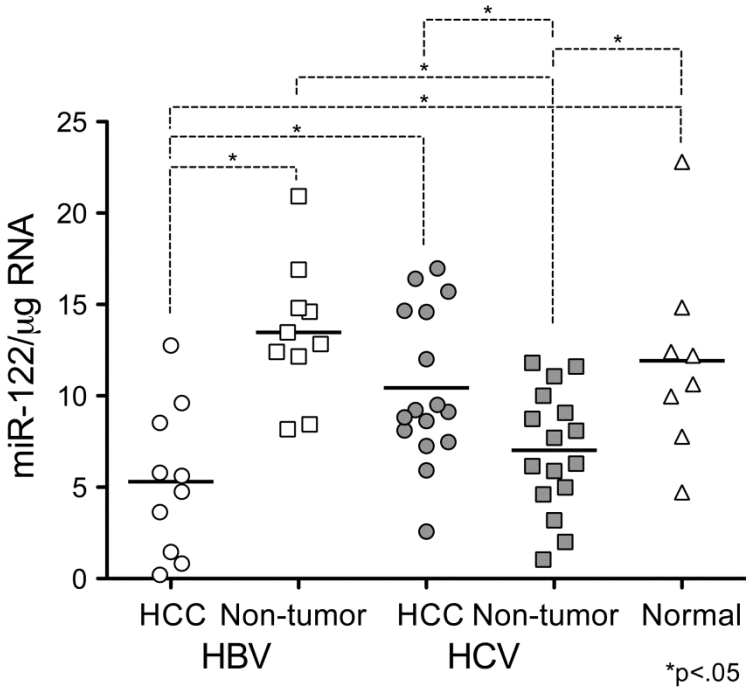


Figure 9. Relative abundance of miR-122 in HCC, paired non-tumor tissues and normal liver tissue. The figure is a composite of data from multiple assays, the results of which were normalized to a sample with high miR-122 expression that was tested in all assays and demonstrated a small variance (value set arbitrarily to 100). Statistical significance was determined by two-sided paired (paired tissue samples) or unpaired (non-paired tissues) t tests.

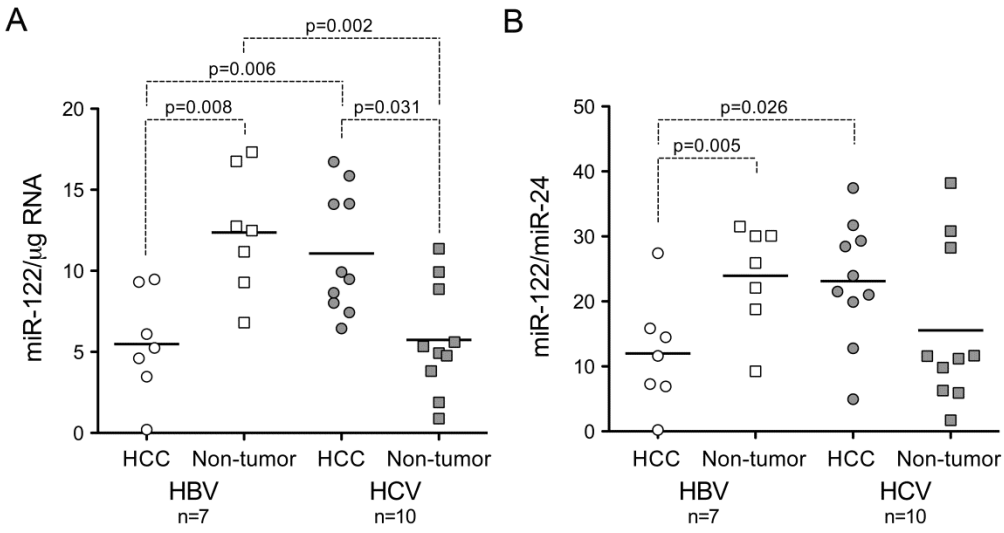


Figure 10. miR-122 expression in subjects with moderately-differentiated HCC. (A) Relative miR-122 abundance in HCC and paired non-tumor tissues from patients whose tumors were classified histologically as "moderately differentiated" HCC. **(B)** miR-122 abundance in this subset of tissues, normalized to miR-24 abundance.

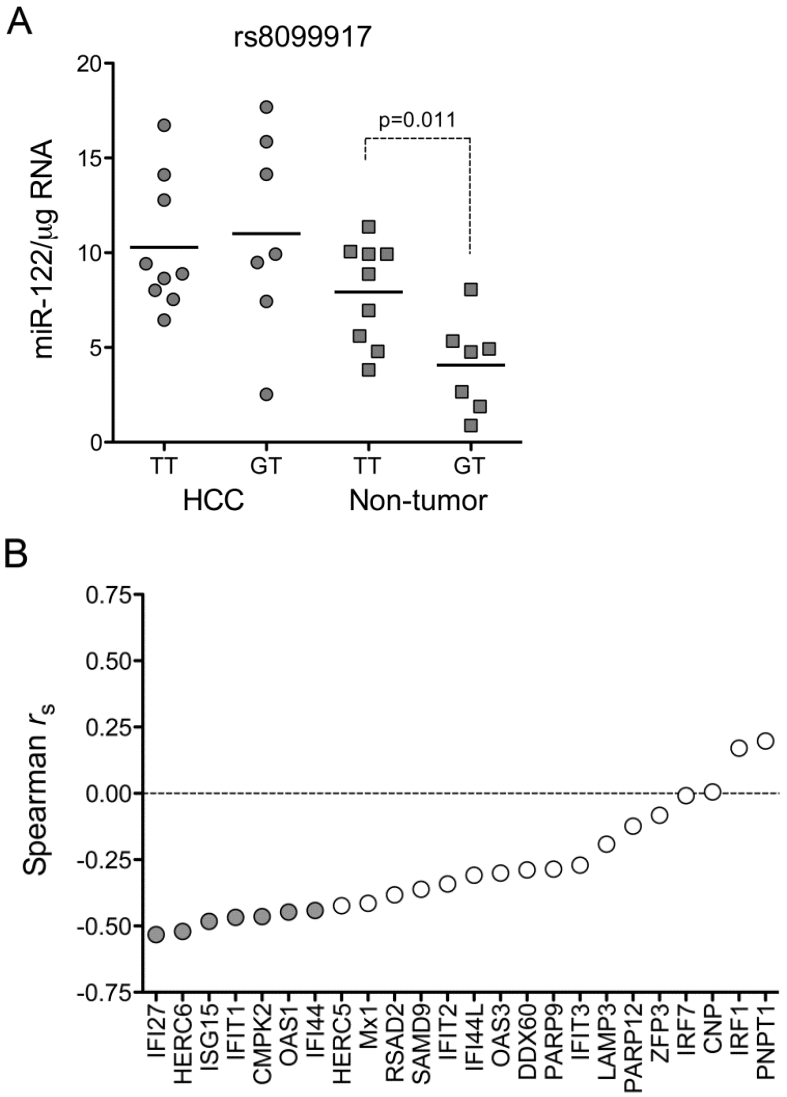


Figure 11. miR-122 expression, IL28B genotype, and ISG transcript levels. (A) Relative miR-122 expression in HCC and paired non-tumor samples from subjects with HCV infection, grouped according to rs8099917 genotype (TT or GT). **(B)** Correlation between relative miR-122 abundance and expression levels of selected ISGs determined by Affymetrix U133 Plus 2.0 Array analysis. With the exception of OAS1 and MX1, intrahepatic transcript levels of these ISGs are predictive of Peg-IFN/RBV treatment outcome [50]. "r_s" = Spearman rank-order correlation coefficient. Filled symbols indicate a statistically significant negative correlation ($p<0.05$ by one-sided t test).

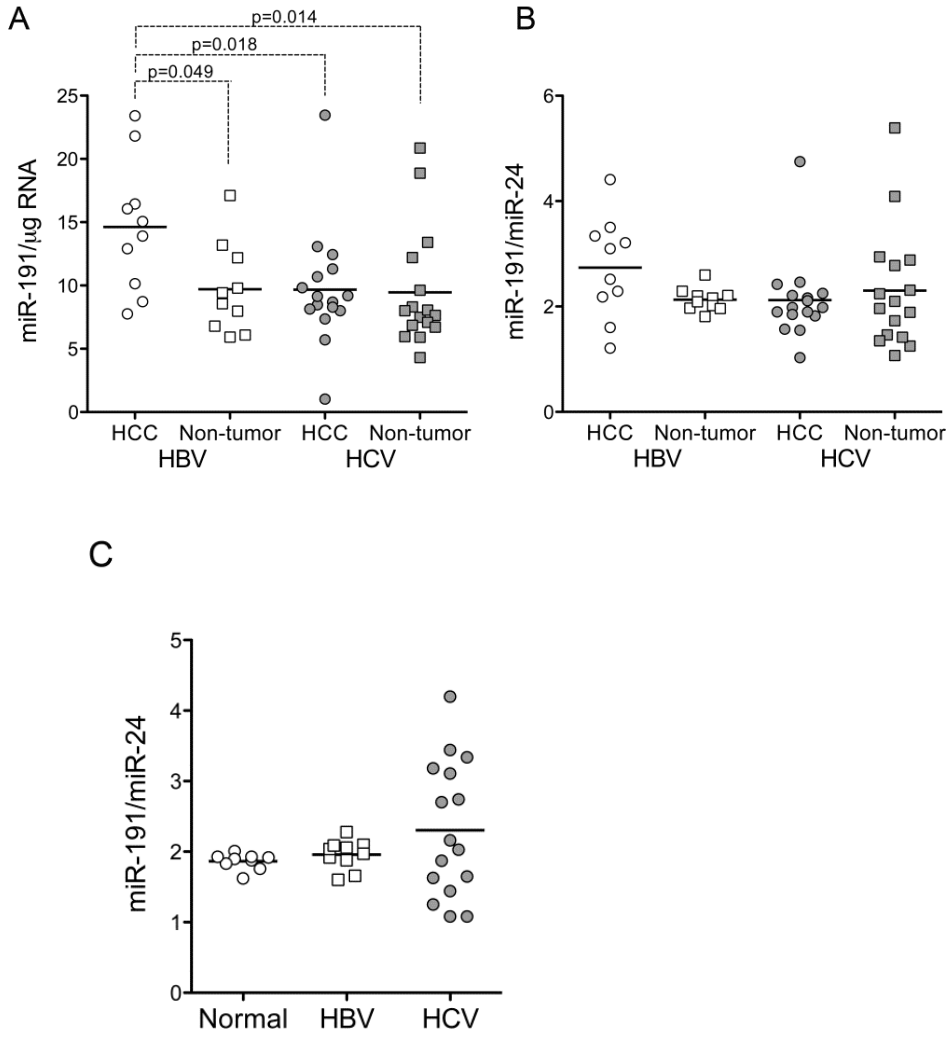


Figure 12. Relative abundance of miR-191 in tumor and non-tumor tissue. (A) miR-191 abundance normalized to total RNA in tumor and paired non-tumor samples from HBV- and HCV-infected subjects. **(B)** miR-191 abundance normalized to miR-24 levels in the same tissue samples. **(C)** miR-191 expression level in normal liver tissue and non-tumor tissues from HBV- and HCV-infected subjects.

DISCUSSION

Chapter 4

This thesis provides confirming evidence that miR-122 expression is down-regulated in HBV-HCC while maintained at near normal levels in HCV-HCC. A previous report suggested miR-191 was up-regulated in HCC in general [31], but this thesis reports miR-191 is up-regulated only in HBV-HCC samples. These variances in expression level are believed to be attributed to differential virus-mediated carcinogenesis. Either directly, indirectly through constant insult by the immune system, or a combination of these factors, HBV and HCV infection leads to a unique accumulation of mutations and epigenetic alterations that promote carcinogenesis. Also reported is a relationship between rs8099917 genotype and miR-122 expression levels as well as a negative correlation between ISG expression and miR-122 expression levels.

WNT/B-CATENIN MUTATIONS MAY AFFECT MIR-122 EXPRESSION

Persistent HBV or HCV infection promotes carcinogenesis through a myriad of mechanisms [73, 74]. The Wnt/β-catenin pathway is a very important 'hit' for HCC development [75]. Although sustained activation of the Wnt/β-catenin pathway alone cannot induce carcinogenesis, mutation or deregulation of this pathway promotes carcinogenesis.

Evidence suggests that activation of the Wnt/β-catenin pathway and the resultant inhibition of GSK3-β activity decrease miR-122 expression levels. The restoration of adenomatosis polyposis coli (APC), needed for constitutive GSK3-β activity, increased miR-122 expression levels by 2-fold in gastrointestinal cancer cells [76]. GSK-3β activates

CCAAT/enhancer binding protein alpha (C/EBPα), a transactivator of miR-122 [77]. Therefore, any mutation or deregulation that negatively affects GSK3-β and/or C/EBPα will decrease miR-122 expression. Activation of the Wnt/β-catenin pathway results in LEF1 interaction and modulation of HNF4α's, a miR-122 transcription factor, transcriptional activity [78]. How this interaction effects miR-122's expression level is currently unknown.

Another important consideration is that miR-122 is a host factor for HCV RNA replication while evidence suggests miR-122 limits HBV replication [25-29]. Interestingly, of the major known mutations to affect the Wnt/β-catenin pathway, the Axin1 mutation which deregulates GSK3-β function is predominant and present in 10% of HBV-associated HCC, while the CTNNB1 β-catenin stabilization mutation, which has no effect on miR-122 expression levels, is predominant and present in 40% of HCV-associated HCC [74, 79, 80]. It appears mutations that benefit both viral infection and carcinogenesis are preferred. This is in agreement with the hypothesis that virus-infected cells are prone to undergo carcinogenesis. See figure 13A and 13B for a depiction of the Wnt/β-catenin pathway and the above described mutations.

DNA Methylation and Histone Modifications May Affect Mir-122 Expression

The previous mutations described above cannot by themselves account for the differences in miR-122 expresssion between the two etiologic classes of HCC. The putative miR-122 promoter is hyper-methylated in HBV-HCC and hepatoblastoma-derived, Hep3b and HepG2 cell lines, respectively [81]. It remains to be seen if hyper-methylation of the putative miR-122 promoter occurs *in vivo* in HBV-HCC. Bacterial artificial chromosome array-based methylated CpG island amplification (BAMCA) revealed that the methylation status pattern of HBV- and HCV-HCC BAC clones differed

significantly although the deviating BAC clone number did not [82]. Differences in the DNA methylation pattern between HBV- and HCV-HCC could be responsible for miR-122 and miR-191 differential expression.

The loss of miR-122 in HBV-HCC could result from viral influence on the host. HBV infection results in the down-regulation of miR-122 *in vitro*[26-29]. This may be due to epigenetic changes promoted by the virus. HBx can increase the abundance of DNA methyltransferase (DNMT) 1, DNMT3A1, and DNMT3A2 as well as histone deacetylase 1 (HDAC1)[83]. TLR9 stimulation by CpG rich DNA also triggers an up-regulation of DNMT1 expression[84, 85]. The HBV-induced up-regulation of DNMTs and HDAC1 may promote the down-regulation of miR-122.

Activation of p53 may promote silencing of miR-122 during chronic viral hepatitis. Inflammation, HBx, and HCV core protein produce reactive oxygen and nitrogen species (ROS and RNS) [86-88], which increase activated p53 [89]. It has been proposed that p53 inhibits HNF4α and HNF6 transcriptional activity by binding to HNF6, recruiting an HDAC to the HNF4α promoter, and promoting the subsequent deacetylation of HNF4α [90]. HNF4α and HNF6 are important transcription factors in maintaining a hepatocyte phenotype and miR-122 expression [91]. HCV, however, needs miR-122 to sustain replication and translation [23, 25]. HCV may circumvent the loss of miR-122 through multiple viral protein mediated mechanisms of p53 inhibition. NS5B interacts and relocalizes p68, an important p53 co-activator[92]. Both NS3 and NS5A bind and inhibit p53 directly [93-95]. See figure 6 for a depiction of viral-mediated suppression HNF4α expression.

HBV-ASSOCIATED HCC MAY ARISE FROM OVAL CELLS

Differences in miR-122 expression between HBV-HCC and HCV-HCC may arise due to the differentiation status of the original cell that undergoes carcinogenesis. HBV may be associated with the transformation of oval cells. Oval cells or human progenitor cells are liver stem cells that can differentiate into hepatocytes or cholangiocytes. HBV not only has the ability to infect 100% of hepatocytes during acute infection [96], but also has the potential to infect progenitor liver, a.k.a. oval cells [97]. In HBV endemic countries, HBV infection and aflatoxin are believed to synergistically induce carcinogenesis [98]. Carcinogenic diets are known to induce oval cell proliferation and are often used in rat studies [99]. Furthermore, oval cell expression of HBx and subsequent aflatoxin exposure induces hepatocellular carcinoma in rats [100]. Progenitor liver cell markers have also been found to be expressed in 80% of HBV-HCC [101]. The expression of miR-122 positively correlates with the expression level of liver-enriched transcription factors [17], which are reduced prior to hepatocyte differentiation [91]. It is therefore possible that the majority of HBV-HCC arises from a liver progenitor cell that fails to differentiate. HCV's aforementioned requirement for miR-122 likely restricts replication in mature hepatocytes preventing HCV-mediated oval cell transformation.

TYPE I IFN EFFECT ON MIR-122 EXPRESSION IN NON-TUMOR TISSUE

The results show that miR-122 expression levels are higher in HBV-NT tissue compared to HCV-NT tissue. We propose that this phenomenon can be explained by differences in the type I IFN response. The fact that interferon-stimulated gene expression is enhanced and miR-122 is decreased in non-responders has caused some to question whether IFN inhibits miR-122 expression. Indeed, several studies have shown that IFN-β inhibits

miR-122 expression *in vitro* [65, 102]. HBV manages to gain entry into the hepatocyte and replicate without inducing TLR and evading a type I IFN response [67]. HCV, in contrast, is recognized by Rig-I and TLR3 early in acute infection, produces a robust type I IFN response, and remains detected throughout chronic infection [103]. Although, HCV-HCC is presumably exposed to type I IFN, its miR-122 expression levels could be maintained because the accumulation of epigenetic changes and mutations under IFN selection allows the development of resistance to the liver's type I IFN response.

MIR-122 EXPRESSION IS INFLUENCED BY RS8099917 GENOTYPE IN HCV NON-TUMOR TISSUE

A patient's probability to respond to HCV treatment can be predicted by genotyping several SNPs, including rs8099917, upstream of the IL28b gene, which encodes IFN-λ3 [63]. Patients with the TT genotype at rs8099917 are more likely to respond to pegylated IFN-α and ribavirin than those with the TG and GG genotypes. Poor responders to pegylated IFN-α and ribavirin have a higher ISG expression in comparison to patients that respond to therapy [50]. This indicates poor responders, TG and GG genotypes of rs8099917, have a stronger IFN response. miR-122 is down-regulated in liver tissue of primary non-responders compared to complete early virological responders to HCV treatment [65]. We believe type I IFN down-regulates miR-122 expression levels. The 16 HCV samples provided for this study included 9 with the TT genotype and 7 with the TG genotype. Our results were in complete agreement with the hypothesis that miR-122 is down-regulated in the NT tissue of HCV patients with the unfavorable GT genotype in comparison to patients with the favorable TT genotype. Using Spearman correlation tests, we also found a negative association between miR-122 and ISG expression levels. These findings seem to indicate that responders and non-

responders differ in immune response and possibly cellular state, i.e. difference in proportion of cells proliferating vs. arrested or differences in cell arrest phase.

MIR-191 UP-REGULATION IN HBV-HCC

This study revealed that miR-191 expression was up-regulated in HBV-HCC and maintained at normal levels in HCV-HCC. Normal liver tissue expression levels were similar to fibrotic, NT liver expression. A previous study found miR-191 was up-regulated in HCC. However, 70 percent of the samples were from HBV patients, 11 percent from HCV patients, and 19 percent from non-viral etiologies [31]. Anti-miR-191 showed potential as a therapeutic agent; however, tests were conducted on Hep3B and SNU423 cells which are both derived from HBV-HCC. Therefore, the data thus far only supports that miR-191 is up-regulated in HBV-HCC, which the results in this thesis confirm.

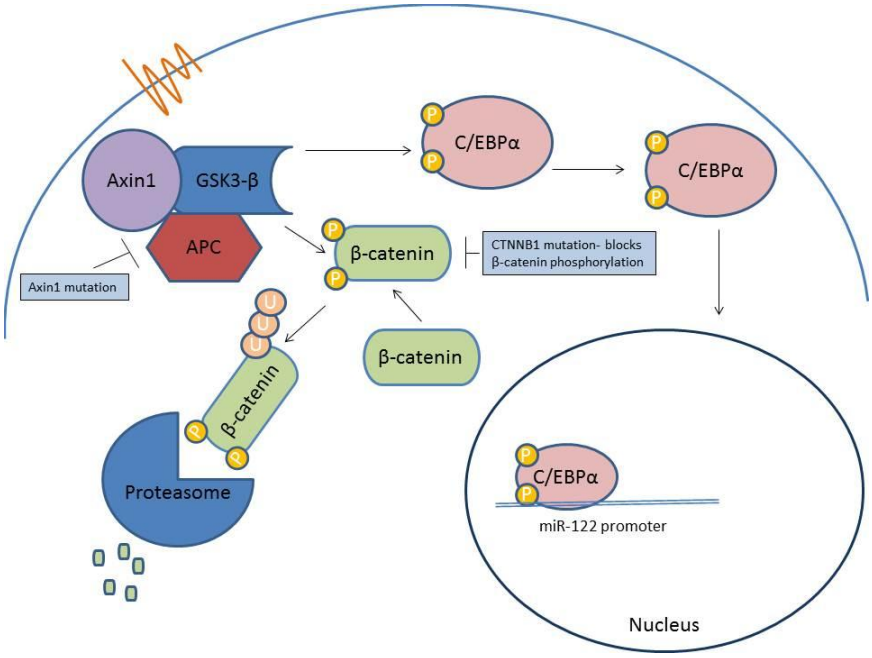
SUMMARY AND FUTURE DIRECTIONS

In summary, miR-122 is down-regulated in HBV-HCC and HCV-NT, but maintained in HCV-HCC and HBV-NT at normal liver expression levels. Patients with a less favorable genotype at rs8099917, alleles TG, have lower miR-122 expression levels in the non-tumor tissue compared to those with the favorable genotype, TT. There was no down-regulation of HCV-NT miR-122 expression due to increased degrees of fibrosis or inflammation. The evidence supports the hypothesis that HCV-NT miR-122 is down-regulated by type I IFN. miR-191 is up-regulated in HBV-HCC, but not in HCV-HCC. These findings indicate HBV-HCC and HCV-HCC arise via different virus-specific

mechanisms of carcinogenesis and this needs to be taken into account when designing or assessing the effect of a potential therapy.

The mechanisms behind miR-122 down-regulation in non HCV-associated liver cancer should be sought. I believe miR-122 regulation to be complex and that it is down-regulated upon various stimuli that brings the hepatocyte out of a functional Go cell cycle phase. An understanding of how LEF interaction with HNF4α effects miR-122 upon Wnt/β-catenin signaling should be pursued. I believe an investigation of p53 mediated down-regulation of miR-122 is warranted since p53 has been shown to down-regulate HNF4α [90]. Proving HBV-HCC and HCV-HCC arises from progenitor or mature hepatocytes is extremely challenging as there is no widely available animal model for these viruses. Defining which cell undergoes carcinogenesis may have to wait till a suitable animal model arises. The mechanism responsible for IFN's influence on miR-122 expression levels should also be investigated.

A



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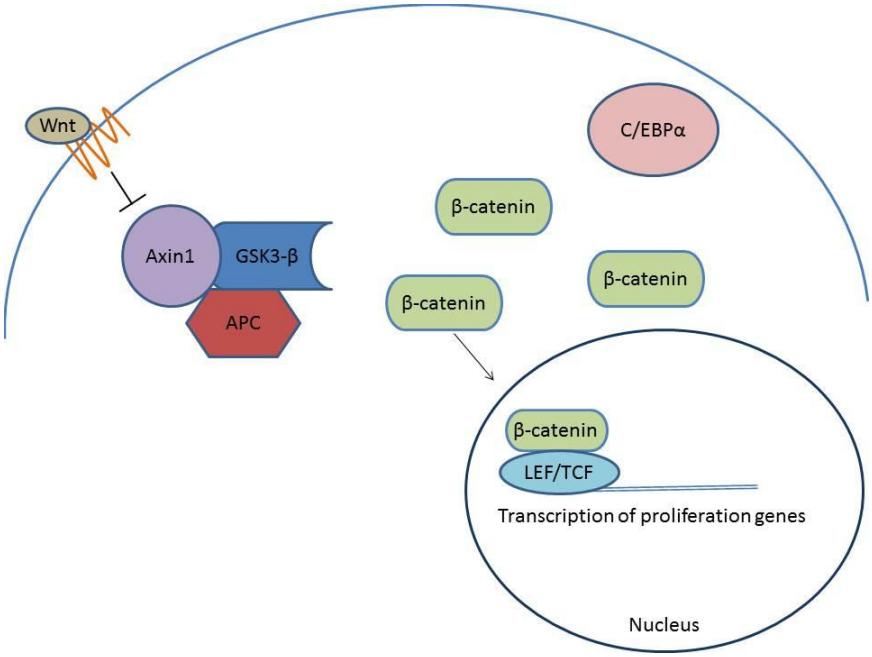

Figure 13. The Wnt/β-catenin pathway. Constitutively activated GSK3-β phosphorylates β-catenin and C/PEBα resulting in degradation and

activation, respectively. C/PEBα translocates to the nucleus and allows miR-122 expression **(A)**. Upon Wnt ligand binding, GSK3-β is inactivated resulting in β-catenin accumulation in the cytoplasm, β-catenin translocation to the nucleus, and transcription of proliferation genes **(B)**. The CTNNB1 mutation, occurring more frequently in HCV-HCC, prevents phosphorylation of β-catenin resulting in stabilization and accumulation without affecting miR-122 expression levels. An Axin1 mutation, which is associated with HBV-HCC, prevents GSK3-β activity resulting in β-catenin accumulation and preventing C/EBPα phosphorylation **(A)**.

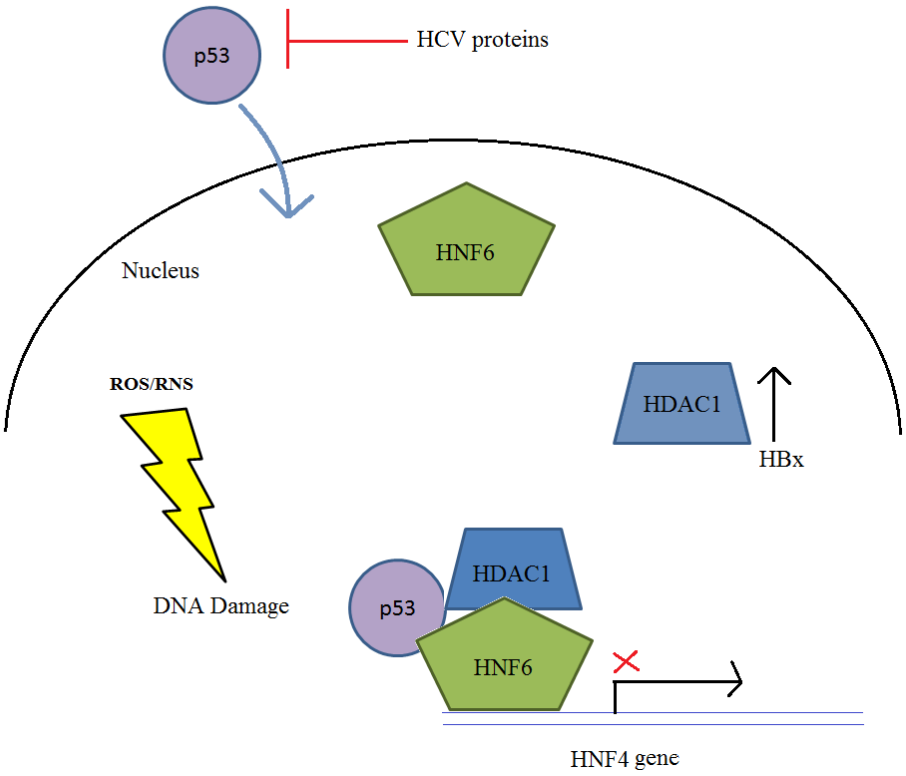

Figure 14. Proposed p53 mechanism that indirectly down-regulates miR-122 by suppressing HNF4α expression. Viral-induced ROS/RNA cause DNA damage which leads to the activation and translocation of p53 to the nucleus. P53 binds HNF6 and recruits a HDAC, possibly HDAC1, to the HNF4 gene suppressing its expression. HNF4α expression is important for maintaining a liver phenotype and miR-122 expression. HBV X protein may promote this interaction by up-regulating HDAC1. HCV may circumvent this fate by inhibiting p53 function.

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Vita

Carolyn Spaniel was born in Waxahachie, TX to farmers Edwin and Ruth Spaniel in February of 1981. She graduated from Waxahachie High School in 1998. She obtained a bachelor degree in biology from Texas A&M-Corpus Christi in 2003. She worked as a research assistant at UT Southwestern from 2005-2008 under the supervision of Benjamin Chen, Ph.D. She is currently pursuing a master degree in microbiology and immunology from the University of Texas Medical Branch.

Permanent Address: 1059 Old Church Rd, Waxahachie, TX 75165

This thesis was typed by Carolyn Spaniel.