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**Hepatitis B virus and microRNAs: Complex interactions affecting hepatitis B virus replication and hepatitis B virus-associated diseases**

Lamontagne J *et al*. Interplay between HBV and cellular microRNAs

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**Abstract**

Chronic infection with the hepatitis B virus (HBV) is the leading risk factor for the development of hepatocellular carcinoma (HCC). With nearly 750000 deaths yearly, hepatocellular carcinoma is the second highest cause of cancer-related death in the world. Unfortunately, the molecular mechanisms that contribute to the development of HBV-associated HCC remain incompletely understood. Recently, microRNAs (miRNAs), a family of small non-coding RNAs that play a role primarily in post-transcriptional gene regulation, have been recognized as important regulators of cellular homeostasis, and altered regulation of miRNA expression has been suggested to play a significant role in virus-associated diseases and the development of many cancers. With this in mind, many groups have begun to investigate the relationship between miRNAs and HBV replication and HBV-associated disease. Multiple findings suggest that some miRNAs, such as miR-122 and miR-125 and miR-199 family members, are playing a role in HBV replication and HBV-associated disease, including the development of HBV-associated HCC. In this review, we discuss the current state of our understanding of the relationship between HBV and miRNAs, including how HBV affects cellular miRNAs, how these miRNAs impact HBV replication, and the relationship between HBV-mediated miRNA regulation and HCC development. We also address the impact of challenges in studying HBV, such as the lack of an effective model system for infectivity and a reliance on transformed cell lines, on our understanding of the relationship between HBV and miRNAs, and propose potential applications of miRNA-related techniques that could enhance our understanding of the role miRNAs play in HBV replication and HBV-associated disease, ultimately leading to new therapeutic options and improved patient outcomes.

**Key words**: Hepatitis B virus; MicroRNA; Hepatocellular carcinoma; Hepatitis B virus replication

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**Core tip:** In this review,we discuss the current state of our understanding of how hepatitis B virus (HBV) affects the expression profile of cellular microRNAs (miRNAs), how these miRNAs impact HBV replication, and the relationship between HBV-mediated miRNA regulation and hepatocellular carcinoma development. Importantly, we address challenges in studying the relationship between HBV and miRNAs, including the lack of an experimental system that effectively models HBV infection and a reliance on the use of transformed cell lines. Finally, we propose applications of techniques to address the functional impact of HBV-mediated regulation of miRNA expression, which could enhance our understanding of the role miRNAs play in HBV replication and disease.

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**INTRODUCTION**

The identification of microRNAs (miRNAs) as mediators of RNA-induced silencing[[1](#_ENREF_1)] has spawned an exciting new field of research into the role of miRNAs in cell physiology. As the central role of miRNAs in the regulation of cellular homeostasis has been established, it has become apparent that disruption of miRNA expression or function can contribute to the development of many diseases[[2](#_ENREF_2)] including neurodegenerative, cardiovascular, and kidney diseases, and many cancers[[3-6](#_ENREF_3)]. The results of numerous studies also support a role for miRNAs in many aspects of a viral infection and virus-associated diseases and suggest that some viruses have developed mechanisms to utilize miRNAs and the miRNA machinery to optimize cellular conditions for virus replication and survival[[7-10](#_ENREF_7)].

A number of groups have begun to examine the relationship between miRNAs and human hepatitis B virus (HBV) infection; these studies have produced compelling evidence that the interplay between HBV and cellular miRNAs is important to many facets of HBV replication and pathogenesis. The focus of this review will be to summarize these studies and our current understanding of the HBV-miRNA relationship as it relates to the overall liver miRNA profile, HBV replication and pathogenesis, and the development of HBV-associated hepatocellular carcinoma (HCC).

**MICRORNA OVERVIEW**

miRNAs are small, non-coding RNAs that are involved primarily in post-transcriptional regulation of gene expression through translational repression or degradation of targeted mRNAs[[2](#_ENREF_2),[11](#_ENREF_11),[12](#_ENREF_12)]. Regulation of miRNAs, their biogenesis, and target identification have been described in detail elsewhere (for biogenesis and regulation see[[11](#_ENREF_11),[12](#_ENREF_12)] and for targeting see[[2](#_ENREF_2)]) and are only briefly summarized here. Primary miRNAs (pri-miRNAs), the first product of the miRNA biogenesis pathway, are transcribed by RNA polymerase II and may contain a single miRNA or a cluster of multiple miRNAs. Once transcribed, the capped and polyadenylated pri-miRNA is processed by a microprocessor complex to remove the flanking sequence from a hairpin structure. This generates a ~70-90 nucleotide (nt) precursor miRNA (pre-miRNA) hairpin that is exported out of the nucleus and into the cytosol, where it is further processed to unwind and remove one strand of the dsRNA. Ultimately, a single-stranded 20-23 nt mature miRNA is assembled with specific proteins, including a member of the Argonaut family of RNA-binding proteins, to form the mature RNA-induced silencing complex (RISC)[[13-18](#_ENREF_13)]. Canonical RISC-mediated silencing is facilitated by complementary base pairing of a specific seed-sequence within nt ~2-7 at the 5’ end of the mature miRNA to a complementary target sequence, usually within the 3’ untranslated region (UTR) of the targeted mRNA[[2](#_ENREF_2)]. Recent evidence suggests that the majority of mRNAs that are targeted by miRNAs are regulated through mRNA destabilization and degradation, although the degree to which target mRNA sequestration and translational repression contribute to silencing is still under investigation[[19](#_ENREF_19)]. The recent recognition, through the application of transcriptome-wide target-identification techniques has expanded the rules of targeting beyond the seed-sequence regulated pathway to include non-seed sequence matching and target sites outside of the mRNA 3’ UTR[[20-23](#_ENREF_20)]. This highlights the importance of miRNA studies that use techniques such as high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) or photoactivatable ribonucleoside-enhanced (PAR)-CLIP, which assess miRNAs and target mRNA contained within functional RISC complexes[[22](#_ENREF_22),[24-26](#_ENREF_24)].

Since the discovery of the first miRNA, *lin-4*, the study of miRNA regulation and function has grown exponentially[[27](#_ENREF_27)]. Importantly, considerable conservation of the sequence of many miRNAs exists across distant species, implying an essential role in cellular function. In fact, recent studies estimate that as many as 60% of all mRNAs in a mammalian cell can be regulated in some way by miRNAs[[28](#_ENREF_28)], and this estimate can increase to over 90% when non-canonical target sites are considered[[29](#_ENREF_29)]. The same cellular signal transduction pathways that control the expression of protein-coding RNA transcripts can also regulate miRNA expression, and it is expected that disease-associated alterations to transcription-regulating pathways can also disrupt normal expression of miRNAs and affect cellular physiology.

**MICRORNAS IN THE LIVER**

The miRNA profile of the liver is unique in that 50%-70% of the total liver miRNA is a single miRNA, miR-122[[30](#_ENREF_30),[31](#_ENREF_31)]. Few studies have specifically examined the miRNA profile of the normal liver. Those studies that have been conducted indicate that nearly 90% of the total miRNAs in the liver may be comprised of only about 10 miRNAs, including miR-122[[30](#_ENREF_30),[32](#_ENREF_32)]. Multiple studies confirm abundant expression of members of the let-7 family as well as miR-125b, miR-92, miR-192, and miR-143. Other miRNAs, including miR-16, miR-126, and miR-199a/b, have also been shown to be highly expressed in the normal liver[[30](#_ENREF_30),[32](#_ENREF_32),[33](#_ENREF_33)]. Since hepatocytes account for 80% of the cell mass in the liver, miRNA profiling of liver tissue largely reflects miRNA expression in hepatocytes[[34](#_ENREF_34)]. The recognition that only a small number of miRNAs are significantly expressed in the liver suggests that studies attempting to assign physiological consequences to a change in expression of an miRNA in the liver should consider observed changes in miRNA expression levels in the context of the percentage of the overall expression of that miRNA in the liver.

**THE HUMAN HEPATITIS B VIRUS**

HBV is a hepatotropic virus that has a narrow host range and only naturally infects humans. An HBV infection can be an acute and self-limiting infection, a chronic, decades-long infection, or an occult infection in which the extremely low level of viremia is below the level of detection by standard diagnostic tools. HBV has a circular, partially double-stranded, 3.2 kb DNA genome with four overlapping open reading frames (ORF) that encode the viral capsid (core), envelope (surface antigens), polymerase/reverse transcriptase, and HBV X protein (HBx) proteins. The HBV genome is highly compact, with most nucleotides being utilized in more than one ORF (reviewed in [[35](#_ENREF_35),[36](#_ENREF_36)]). Interestingly, all viral transcripts share the same polyadenylation signal, and the sequence of each smaller transcript is contained within any larger transcript (Figure 1). For example, as the smallest transcript, the sequence of the HBx transcript makes up the 3’ end of all of the larger HBV RNA transcripts, which makes specifically targeting the HBx gene with small interfering RNAs (siRNAs) or miRNAs impossible without also targeting the other three transcripts. This is an important consideration when evaluating studies purporting to have specifically targeted the HBx transcript with siRNAs in cells expressing the entire HBV genome.

The HBx is a non-structural regulatory protein encoded by the smallest ORF of HBV. Various studies in cell lines, primary hepatocytes, and animal models have provided considerable evidence that HBx plays an important, likely essential, role during HBV replication[[37-41](#_ENREF_37)]. HBx is a multifunctional protein that can modulate hepatocyte calcium, apoptosis, and proliferation signals, among other pathways, and can activate multiple transcription factors, including activator proteins 1 and 2 (AP-1 and AP-2), nuclear factor of activated T cells (NFAT), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)[[39](#_ENREF_39),[40](#_ENREF_40),[42-51](#_ENREF_42)]. HBx can also regulate cellular signaling factors, such as Wnt/β-catenin, p53, and Akt, that have been implicated in HCC[[45](#_ENREF_45),[50-55](#_ENREF_50)]. Recently, modulation of miRNA expression has been included in the functions of HBx, as studies have begun to look at miRNA expression in response to HBV and HBx (reviewed below). It is possible that the many functions attributed to HBx could actually be the result of a few fundamental HBx functions that can affect multiple cellular signal-transduction pathways in a context-dependent manner.

Global epidemiological studies have identified chronic HBV infection of the liver as the leading risk factor for the development of HCC (reviewed in [[36](#_ENREF_36),[56](#_ENREF_56)]). Despite the availability of a vaccine, 350-500 million people worldwide are chronically infected with HBV, and as many as 25% of these individuals will go on to develop HBV-associated HCC (reviewed in [[56](#_ENREF_56),[57](#_ENREF_57)]). HCC is the second deadliest cancer worldwide with ~750,000 deaths per year and an incidence to mortality rate near 1[[58](#_ENREF_58)], underscoring the need for a better understanding of the mechanisms that lead to its development. In particular, defining HBV-mediated changes to the cellular miRNA profile, and how these changes affect cell physiology, could help identify molecular mechanisms that link a chronic HBV infection to the development of HCC.

**MODEL SYSTEMS THAT HAVE BEEN USED TO STUDY THE INTERPLAY OF HOST CELL MIRNAS AND HBV**

***Cell culture models of HBV infection***

The study of HBV has been hampered by the lack of model systems able to faithfully reproduce all aspects of a human HBV infection. Consequently, most HBV studies have relied on imperfect cell culture and animal models, each with its own advantages and disadvantages (see Table 1). For instance, the hepatoblastoma-derived cell line HepG2[[59](#_ENREF_59)] and the hepatoma-derived cell line Huh7[[60](#_ENREF_60)] are often used in studies of HBV biology. Similar to the majority of available human liver cell lines, HepG2 and Huh7 cells cannot be directly infected by HBV, in part due to lost expression of the HBV receptor[[61](#_ENREF_61)]. These cells do, however, support HBV replication when the HBV genome is introduced by transfection of a plasmid or infection with a virus-based delivery vehicle, such as adenovirus, that encodes a greater-than-unit-length copy of the HBV genome. Alternatively, HepG2.2.15 cells, derived from HepG2 cells and containing two integrated copies of a head-to-tail dimer of the HBV genome[[62](#_ENREF_62)], have been used in tandem with HepG2 cells as a means to study cells with and without replicating HBV. A disadvantage of all of these cell systems, however, is that these cell lines are derived from tumors and therefore are transformed. Because the cellular miRNA profile is significantly altered in cancer, the use of transformed cell lines means experiments are done in an already altered background of miRNA expression. Moreover, continuous passage over the decades since their development may have contributed to differences between HepG2.2.15 cells and the parental HepG2 cells, such as miRNA expression profiles, that are not solely due to the presence of replicating HBV.

Other cell systems that have been used to compare hepatocyte miRNA expression in the presence and absence of HBV replication or HBV gene products are described in Table 1. One cell line, HepAD38 cells, is a HepG2-derived cell line in which transcription of the HBV pgRNA, and therefore HBV replication, is regulated by an inducible, tetracycline-responsive element[[63](#_ENREF_63)]. Additional cell lines include Hep3B[[59](#_ENREF_59)] and PLC/PRF/5[[64](#_ENREF_64)] cells, which are derived from HBV-positive tumors and contain integrated HBV DNA. Both Hep3B and PLC/PRF/5 cells produce some HBV proteins, but neither produces infectious virus. Although HBV-infected primary human hepatocytes have been used in some miRNA-profiling studies, because cultured primary human hepatocytes quickly lose their ability to be infected by HBV after only a few days in culture, their use has been limited. Primary hepatocytes from other systems, namely mouse and rat, have also been used to study the impact of HBV replication on regulation of gene expression, although these cells cannot be directly infected by HBV. The recent discovery that NTCP serves as a receptor for HBV[[61](#_ENREF_61)] is an important step forward in the development of improved cell culture models for studies on the effects of natural HBV infection and the functional significance of any changes that might occur in miRNA levels in non-transformed hepatocytes.

***In vivo models***

Due to the limited host range of HBV, few suitable animal models exist for studying an *in vivo* HBV infection. The treeshrew, *Tupaia belangeri*, is one of the very few animals which can be experimentally infected with HBV, and *Tupaia belangeri* has been used as a model to study the immediate effects of HBV infection on gene expression in the liver[[65](#_ENREF_65)]. In fact, freshly isolated primary treeshrew hepatocytes were recently used in the identification of NTCP as the HBV receptor[[61](#_ENREF_61)] and have subsequently been used for assessing the miRNA profile of HBV-infected hepatocytes[[66](#_ENREF_66)].

HBV- and HBx-transgenic mice are another commonly used animal model for studying the impact of HBV on cellular miRNAs[[67-72](#_ENREF_67)]. Although mouse hepatocytes cannot be directly infected with HBV, the use of HBV-transgenic mice allows studies of the impact of HBV replication on the cellular miRNA profile. Similarly, HBx-transgenic mice aid in the study of HBx-mediated effects on the cellular miRNA profile. While these are valuable tools, they do have their drawbacks as well. For example, there is no inflammatory response against HBV in an HBV-transgenic mouse, which could be important in miRNA-related studies because of the important role miRNAs play in regulating the immune response. Additionally, because HBV-transgenic mice do not produce HBV cccDNA, there is some concern over whether this system accurately mimics all aspect of HBV replication in humans[[73](#_ENREF_73)].

HBV-induced changes in host miRNA expression have also been assessed by analyzing miRNA profiles in liver tissue, or in some cases serum samples, from individuals infected with HBV. Often, this approach has been used to compare miRNAs in patients at different stages of liver disease, progressing from chronic HBV infection through fibrosis, cirrhosis, or HCC, or with different underlying etiologies of liver disease, including both HBV and HCV infection[[74-76](#_ENREF_74)]. The goal in these studies has been to identify markers of disease etiology, progression, prognosis, or response to treatment, or to increase the understanding of these processes for the development of new therapies. Ideally, results from these studies would align with those of cell-based studies, but, as we describe below, that has often not been the case.

**METHODS USED TO ANALYZE MICRORNA EXPRESSION IN CELLS EXPRESSING REPLICATING HBV OR HBV PROTEINS**

Experimental protocols typically used in determining HBV-mediated changes to miRNA expression include targeted approaches such as quantitative reverse transcription polymerase chain reaction (qRT-PCR) and broader approaches such as microarray or deep sequencing of the cellular RNA transcriptome (Table 2). qRT-PCR is often used to analyze the expression level of a single miRNA, a small panel of miRNAs, or as a follow-up step for confirmation of results seen in broader studies such as miRNA microarray studies[[74](#_ENREF_74),[76-81](#_ENREF_76)]. Although microarray and qRT-PCR are reliable for assessing relative changes in miRNA expression, it is important to note that these techniques do not determine if the miRNAs are expressed at physiologically relevant levels.

An increasing number of studies have begun using broader techniques such as qRT-PCR arrays and microarrays as a means of identifying expression changes in the population of miRNAs in cells. This has resulted in multiple attempts to independently establish an “HBV-mediated miRNA profile” by identifying miRNAs with altered expression in the presence of HBV. Despite the widespread use of these techniques, whether it is due to technical limitations, heterogeneity of the samples, or still unknown factors, observations in these HBV-miRNA studies have often differed. For example, a comparison of the results of three different microarray analyses of HepG2.2.15 miRNAs, all done using the same microarray platform, shows that the studies respectively identified 18, 31, and 47 miRNAs with altered expression compared to the parental HepG2 cells. Of these, only four were in common among all of the studies (miR-146a, miR-181a, miR-181d, and miR-338-3p), and only ten were in common between any two of the studies[[82-84](#_ENREF_82)]. While this comparison highlights the potential variation between HepG2 and HepG2.2.15 cells, it also serves as an example of inconsistencies among HBV microarray studies and the need for follow-up studies to confirm altered miRNA expression.

One approach that could aid in establishing a consensus HBV-mediated miRNA profile is the application of techniques such as transcriptome-wide next-generation sequencing, which allows a determination of absolute miRNA levels instead of relative changes, as reported in microarray analyses. A determination of absolute miRNA levels is important because it distinguishes more abundantly expressed miRNAs from those present at only low levels. Abundant miRNAs have a higher potential to regulate cellular pathways in physiologically significant ways, whereas a several fold change in a low-abundance miRNA may have little effect. In one such study in primary treeshrew hepatocytes, the profile of all miRNAs expressed at a given time was established by deep-sequencing of primary treeshrew hepatocyte small RNAs. These studies identified 25 miRNAs that are both significantly expressed in the liver and show HBV-mediated expression changes. Interestingly, this included miR-146a, one of the four miRNAs consistently identified in the previously described microarray experiments, and miR-122 and let-7a, two highly abundant miRNAs in the liver. These results have particular relevance because of the use of infectious HBV instead of exogenous delivery of the viral genome by transfection or expression from an integrated transgene[[66](#_ENREF_66)]. As the use of transcriptome-wide techniques expands, it will be informative to apply a deep-sequencing approach to a larger population of HBV-infected individuals to discover and understand the functional significance of any alterations in miRNA expression profiles in HBV-infected cells. In addition, techniques that specifically investigate functional miRNAs (discussed in Future Directions)[[22](#_ENREF_22), [24-26](#_ENREF_24)] will help to determine the physiological impact of HBV-mediated alterations to the miRNA expression profile by identifying miRNAs that are actively involved in targeting, not simply the miRNAs with altered expression levels.

**EFFECTS OF HBV ON HOST CELL MIRNA EXPRESSION**

While there have been some inconsistencies in the results of studies that have sought to define HBV-induced changes in cellular miRNA expression profiles[[30](#_ENREF_30),[66](#_ENREF_66),[76](#_ENREF_76),[82-84](#_ENREF_82)], a small number of miRNAs have been identified more often than others as having expression changes that are associated with HBV infection. We focus our review first on these miRNAs, followed by a discussion on some additional miRNAs potentially altered by HBV (Figure 2, Table 3).

***miR-122***

The expression of miR-122 is highly specific to hepatocytes, where this single miRNA can account for 50 – 70% of the entire cellular miRNA population[[30](#_ENREF_30),[31](#_ENREF_31)]. miR-122 has a role in maintaining the differentiated phenotype of hepatocytes and is involved in the control of lipid metabolism, circadian rhythm, apoptosis, and iron homeostasis[[85-91](#_ENREF_85)]. The down-regulation of miR-122 levels in HCC has been observed in many studies[[30](#_ENREF_30),[76](#_ENREF_76),[92-94](#_ENREF_92)], consistent with the dedifferentiated status of transformed hepatocytes; the contribution of HBV infection to this down-regulation in the context of HBV-associated HCC is less well characterized.

The effect of HBV infection on the expression of miR-122 has often been studied by comparing its levels in uninfected cells with those in cells either stably or transiently transfected with the HBV genome. While many of these studies have found reduced levels of miR-122 in HBV producing cells[[69](#_ENREF_69),[79](#_ENREF_79),[95-98](#_ENREF_95)], others have not[[81](#_ENREF_81), [83](#_ENREF_83)]. Similarly, a down-regulation of miR-122 was observed in some analyses of liver tissue from chronically HBV-infected patients as compared to normal liver tissue[[98](#_ENREF_98)], but not in others[[30](#_ENREF_30),[76](#_ENREF_76),[99](#_ENREF_99)]. On the other hand, HBV infection of primary tree shrew hepatocytes was reported to result in an increase in miR-122 levels[[66](#_ENREF_66)]. It will be of interest to discover if this striking difference from reports of HBV down-regulation of miR-122 is due to the use of an authentic HBV infection system, as opposed to transfection of HBV genomic constructs into cell lines, or if it reflects an inherent difference between human and tree shrew hepatocytes.

A variety of hypotheses have been put forth to explain changes in miR-122 expression in HBV-producing cells. One report suggested that HBV regulates the transcription of miR-122 through interactions between HBx and peroxisome proliferator-activated receptor γ (PPARγ) that prevent ligand-induced activation of the miR-122 promoter by PPARγ[[97](#_ENREF_97)]. However, decreased miR-122 promoter activity is contrary to the established activation of multiple miR-122-activating transcription factors such as hepatocyte nuclear factor 4α (HNF4α) and CCAAT-enhancer-binding protein α (C/EBPα) in cells with replicating HBV[[69](#_ENREF_69),[100-102](#_ENREF_100)]. In fact, an increase in miR-122 promoter activity in the presence of HBV, although with decreased levels of mature miR-122, has been reported[[69](#_ENREF_69)]. It was proposed that this lower level of mature miR-122 was due to sequestration of miR-122 through binding to a conserved sequence detected at the 3’ end of all HBV transcripts (Figure 1). This miRNA sponge effect, in turn, may lead to a derepression of cellular mRNAs normally targeted by miR-122, such as pituitary tumor-transforming gene 1 binding factor (PBF), resulting in increased cell proliferation[[69](#_ENREF_69)]. However, it has been shown elsewhere that extremely high levels of competitive endogenous RNAs (ceRNAs), or endogenous RNAs that act to sequester miRNAs away from their target mRNAs, are required before a sponge effect can be demonstrated to derepress cellular targets of an miRNA, and miR-122 in particular[[103](#_ENREF_103)]. It remains to be tested whether a natural infection with HBV results in high enough amounts of HBV RNAs to sequester miR-122 at levels sufficient to alter the targeting pattern of endogenous mRNAs. Another report investigating HBV- or HBx-mediated effects on miR-122 promoter activity found no change to the miR-122 promoter activity, but suggested that HBx reduces the expression of Gld2, a poly(A) polymerase that can stabilize miRNAs through the addition of a single adenylate residue[[104](#_ENREF_104)]; reduced expression of Gld2 would ultimately contribute to destabilizing and reducing miR-122 levels[[96](#_ENREF_96)]. Clearly, a better understanding of the control of miR-122 expression will be necessary before conflicting reports of changes induced by HBV replication can be resolved, or the mechanism by which HBV gene products might affect miR-122 transcription, stability, and function can be understood.

***Let-7 family***

In humans, the let-7 family of miRNAs consists of 12 closely related miRNAs and represents some of the most highly expressed miRNAs in the liver. These miRNAs are classified as tumor-suppressor miRNAs, and the loss of members of the let-7 family is a common factor in the development of many forms of cancer, including HCC[[105](#_ENREF_105)]. In addition, miRNA microarray and qRT-PCR analyses have demonstrated that let-7 expression is altered in HepG2.2.15 cells, HBV-transfected cell lines, HBx-expressing cell lines, and HBV-infected human liver samples[[81](#_ENREF_81),[82](#_ENREF_82),[84](#_ENREF_84),[106](#_ENREF_106),[107](#_ENREF_107)]. In one study, multiple members of the let-7 family were identified by miRNA microarray as being down-regulated in HBx-expressing HepG2 cells, and a similar down-regulation of let-7a was shown in patients with HCC. Interestingly, this down-regulation of let-7a was correlated with an increase in cellular proliferation, in agreement with the established role of let-7 in the repression of cell proliferation factors such as Ras[[108](#_ENREF_108)] and Myc[[109](#_ENREF_109)]. These studies also demonstrated that both HBx expression and let-7 inhibition increased the levels of STAT3, a transcription factor that regulates cell proliferation, supporting the overall role of let-7 as an inhibitor of multiple proliferation pathways[[107](#_ENREF_107)].

Additional studies have shown that HBx can up-regulate the proteins Lin28A and Lin28B, known inhibitors of let-7 expression[[110-112](#_ENREF_110)]. In fact, Lin28 and let-7 form a double-negative feedback loop, with each inhibiting the expression of the other[[113](#_ENREF_113),[114](#_ENREF_114)]. Up-regulation of Lin28 expression has been observed in multiple HCC cell lines, HepG2.2.15 cells, and in HBx-transgenic mice, and Lin28 up-regulation was directly linked to increased tumor growth in HBx-transgenic mice[[71](#_ENREF_71)]. While levels of let-7 were not directly measured in these Lin28 studies, these observations suggest that HBx-mediated regulation of the Lin28/let-7 axis could play a role in the development of HBV-associated HCC. This suggestion is strengthened by the well-established oncogenic role of Lin28 and tumor suppressive role of let-7 that has been linked to both let-7-independent effects of Lin28 as well as to let-7 targeting of known oncogenes such as Ras and Myc, a let-7 effect that would be countered by up-regulation of Lin28 expression[[105](#_ENREF_105),[115](#_ENREF_115)].

***miR-125 family***

The miR-125 family is one of the most studied miRNA families because *lin-4*, its homolog in *C. elegans*, was the first described miRNA[[27](#_ENREF_27)]. Both miR-125a and miR-125b display altered regulation in various diseases including HCC[[30](#_ENREF_30),[116](#_ENREF_116)]. Targets of this family include regulators of apoptosis, such as p53[[117](#_ENREF_117)] and Bak1[[118](#_ENREF_118)], the stress response factor HuR[[119](#_ENREF_119)], and the Lin28 proteins[[120](#_ENREF_120)]. Interestingly, miR-125a has been identified in multiple profiling studies as being up-regulated in HepG2.2.15 cells as compared to HepG2 cells, and recent studies have also demonstrated that miR-125a expression is up-regulated in HBV-infected patient samples. A qPCR-based analysis of levels of miR-125a-5p in the liver directly correlated miR-125a-5p expression with liver HBV DNA levels and the severity of disease in chronically HBV-infected patients[[121](#_ENREF_121)]. A follow-up study attempted to determine the mechanism for HBV-mediated up-regulation of miR-125a by examining the role of HBx in this same cohort of chronically HBV-infected HBV patients. Of these patients, those with high HBV viral load had 3.6-fold higher levels of HBx compared to patients with low viral load, which correlated with a 3-fold increase in the level of liver miR-125a in the same patient groups. Transfection of HBx into HepG2 and Huh7 cells also increased the level of miR-125a expression[[122](#_ENREF_122)]. While these data suggest a correlation between the levels of HBx and the levels of miR-125a, additional research is needed to determine the mechanism by which HBV may be regulating expression of miR-125a. Recently, miRNA microarray analysis of HepG2.2.15 cells and HBV-expressing HepG2 cells identified a set of 35 miRNAs that exhibited similar regulation in both cell types when compared to HepG2 cells. Among these miRNAs, miR-125b was shown to be down-regulated in both HepG2.2.15 cells and HepG2 cells transfected with a HBV-expressing plasmid, and this regulation was subsequently confirmed by qRT-PCR[[123](#_ENREF_123)]. While these results suggest opposing HBV-mediated regulation of miR-125a and miR-125b, which have the same seed sequence and might target a similar set of mRNAs, together they indicate that HBV is likely playing a role in regulating the miR-125 family.

***miR-199 family***

Analysis of the total miRNA population in normal liver samples by next-generation sequencing of small RNAs identified members of the miR-199 family as being among the most abundant miRNAs in the liver, with miR-199a/b-3p representing an average of 4.9% of the total miRNAs in the liver[[30](#_ENREF_30)]. Because of the relative abundance of these miRNAs, changes in the levels of expression of miR-199 family members are likely to result in significant changes in the expression of mRNA targets. Although some studies suggest increased expression of miR-199a in HepG2.2.15 cells and HBV-positive liver samples[[30](#_ENREF_30),[124](#_ENREF_124)], many other studies report a down-regulation of miR-199 family members in HBV-expressing cells. This down-regulation has been shown in HepG2 cells transfected with a plasmid expressing the HBV genome, in HepG2.2.15 cells, and in HBV-infected patients[[82](#_ENREF_82),[84](#_ENREF_84),[99](#_ENREF_99),[125](#_ENREF_125)], and down-regulated miR-199b has been proposed as a biomarker for early detection of HBV-associated HCC[[74](#_ENREF_74)]. Additional studies are needed to determine the cellular impact of HBV-mediated altered miR-199 expression.

***miR-15 family***

The miR-15 family of miRNAs represents one of the earliest identified examples of a tumor-suppressor miRNA family[[3](#_ENREF_3)]. Decreased expression of many of the six miR-15 family members (miR-15a, miR-15b, miR-16-1, miR-16-2, miR-195, miR-497) is common in multiple forms of cancer, including HCC, and their targets include important cellular factors such as regulators of the cell cycle and apoptosis[[126](#_ENREF_126),[127](#_ENREF_127)]. Studies have shown decreased expression of members of this family in cell lines transfected with a plasmid expressing the HBV genome, in HepG2.2.15 cells, in HBV-infected liver samples, in HBV-associated HCC, and in cells expressing HBx[[83](#_ENREF_83),[84](#_ENREF_84),[106](#_ENREF_106),[128-131](#_ENREF_128)]. This alteration could be explained, in part, by the previously identified induction of c-Myc by HBx[[54](#_ENREF_54),[129](#_ENREF_129),[132](#_ENREF_132)] and c-Myc-mediated repression of the miR-15a/miR-16 cluster[[133](#_ENREF_133)]. Importantly, studies have confirmed the significance of this down-regulation of the miR-15a/16 cluster by demonstrating derepression of miR-15a/16 targets, including Cyclin D1, in HBx-expressing cells[[129](#_ENREF_129)].

Additional evidence of HBV-mediated regulation of the miR-15 family was obtained in two independent studies that suggested that HBV transcripts act as miRNA sponges to sequester miR-15 family members and decrease the interactions of miR-15 family members with endogenous targets. The first study used the TargetScan algorithm to identify four target sites for miR-15a and miR-16-1 within a sequence contained in all HBV mRNA transcripts. Interestingly, while transfection of HepG2 cells with HBV altered the expression of 8 miRNAs (miR-210, miR-602, miR-199a, miR-125a, and miR-29a up; miR-15a, miR-16-1, let-7a down), this regulation was retained when HepG2 cells were transfected with a plasmid encoding an HBV genome with a premature stop codon preventing production of HBx. Additionally, transfection of a plasmid encoding either HBx or a mutant that made HBx RNA but not HBx protein resulted in decreased expression of miR-15a and miR-16-1. This implied that while HBx over-expression was able to down-regulate miR-15a and miR-16-1, this regulation was not through the HBx protein. Pull-down assays conducted in this study also demonstrated that miR-15a can bind HBx RNA, and it was hypothesized that HBx RNA acts as an miRNA sponge to decrease the levels of miR-15a available in the cell. Support of this hypothesis was shown by up-regulation of miR-15a targets, such as the cell cycle regulator Cyclin D1 and anti-apoptotic Bcl-2, in the presence of HBx RNA[[106](#_ENREF_106)]. It should be noted, however, that because this target site is located within the HBx transcript, it is also within all HBV RNA transcripts (Figure 1). Therefore in cells with replicating HBV, each of the HBV transcripts would have the potential to have a similar sponge effect.

Additional studies have shown that the levels of miR-15a and miR-16 are significantly decreased in transformed cell lines expressing HBV, in HBV-transgenic mice, and in HBV-associated HCCs. Using TargetScan, another miR-15/16 target site was identified within the region of the HBV genome shared in all HBV transcripts and independent from the previously identified miR-15/16 target sites[[106](#_ENREF_106)] described above (Figure 1). Transfection of a plasmid expressing this target site or a plasmid expressing a greater-than-unit length copy of the HBV genome reduced levels of miR-15a and miR-16, while expression of an HBV genome in which the target site was mutated caused no change in the levels of miR-15a or miR-16. Together, these results suggest that HBV transcripts can bind to miR-15a and miR-16 and may act as an miRNA sponge. In HBV-associated HCC patient samples, a strong negative correlation was seen between expression of HBV transcripts and expression of miR-15/16, and a strong positive correlation was seen between HBV transcript levels and expression of the apoptosis inhibitor Bcl-2, a known target of the miR-15 family[[70](#_ENREF_70)]. These data support the hypothesis that HBV transcripts can divert miR-15 family members away from endogenous targets, as seen by increased expression of Bcl-2 and a significant decrease in apoptosis induction in cells expressing HBV. Together, these studies suggest a mechanism, potentially in addition to the HBx-mediated activation of c-Myc previously described[[129](#_ENREF_129)], by which HBV regulates the levels of the miR-15 family to optimize cellular homeostasis by decreasing apoptosis and modulating cell cycle regulators. As shown for miR-122[[103](#_ENREF_103)], it is important to point out that very high levels of the target transcript may be needed for an miRNA sponge to have a physiological effect, and it remains to be seen whether the levels of HBV transcripts in the cell reach this threshold[[103](#_ENREF_103)].

***miR-17-92 cluster***

The miR-17-92 cluster is composed of six miRNAs: miR-17, miR-18a, miR-19a, miR-19b-1, miR-20a, and miR-92a-1. This cluster is well characterized as having increased expression in multiple cancers and has been referred to as oncomir-1 due to its oncogenic potential[[134](#_ENREF_134)]. Analysis of expression of this miRNA cluster in the HepAD38 cell line, in which expression of the HBV pgRNA and therefore HBV replication, is under the control of a tetracycline-responsive promoter, showed that both the precursor and mature forms of multiple members of the miR-17-92 cluster were significantly up-regulated in the presence of HBV replication. It was further shown that c-Myc inhibition blocked HBV-mediated up-regulation of the miR-17-92 cluster, indicating that this regulation was through HBV-mediated activation of c-myc[[135](#_ENREF_135)]. Interestingly, next-generation sequencing of the miRNAs in HBV-infected liver samples showed significant up-regulation of miR-92a compared to normal tissue, and even higher expression in HBV-associated HCC[[30](#_ENREF_30)]. In contrast to observations in HepAD38 cells and patient samples, multiple studies have shown that members of the miR-17-92 cluster are down-regulated in HepG2.2.15 cells[[82-84](#_ENREF_82)]. These differences could reflect differences between the various cell types and assay methods used in these studies, and suggest that cellular context should be considered when interpreting these HBV-miRNA studies.

***Other miRNAs and miRNA-regulated pathways affected by HBV***

In addition to the miRNAs discussed above, other miRNAs and miRNA-regulated pathways that are altered in the context of HBV replication have been described. Because these studies of HBV-mediated miRNA regulation have been identified in fewer, sometimes only single, studies, they have been grouped together and are discussed here. In addition, some of these miRNAs are not highly expressed in the liver, and therefore their physiological role during an HBV infection may require careful interpretation. For example, miR-501 was shown by microarray and qRT-PCR to be up-regulated in HepG2.2.15 cells compared to HepG2 cells and in liver samples from HBV-associated HCC patients with a high viral load compared to patients with a lower viral load. Up-regulation of miR-501 led to increased targeting of hepatitis B X-interacting protein (HBXIP), a cofactor for the anti-apoptotic protein Survivin[[81](#_ENREF_81),[136](#_ENREF_136)]. Similarly, HBV-mediated up-regulated of miR-602 was shown in a study examining normal liver samples, HBV-infected liver samples, and HBV-associated HCC samples; Ras association domain family 1 isoform A (RASSF1A), a target of miR-602, important tumor suppressor, and regulator of apoptosis[[137](#_ENREF_137)], was down-regulated in the presence of HBV. Increased miR-602 expression was confirmed in both HepG2.2.15 cells and HepG2 cells stably expressing HBx, and targeting of RASSF1A was blocked in these cells with the addition of a miR-602 inhibitor[[99](#_ENREF_99)]. Another study demonstrated that HBV-associated HCCs and HepG2.2.15 cells express increased levels of the miR-371/372/373 miRNA cluster and that this up-regulation caused a decrease in the levels of nuclear factor I/B (NFIB)[[82](#_ENREF_82)]. A high-throughput sequencing analysis of the miRNAs expressed in the human liver, however, showed miR-501, miR-602, and the miR-371 cluster as having poor expression in the liver (< 10 transcripts per million reads)[[30](#_ENREF_30)]. Alternatively, miR-181a, an miRNA over-expressed in numerous cancers, and shown to have moderate liver expression (~150 transcripts per million reads in normal liver)[[30](#_ENREF_30)], was up-regulated in HepG2.2.15 cells and in HepG2 cells infected with an adenoviral vector containing a greater-than-unit length copy of the HBV genome[[82-84](#_ENREF_82),[138](#_ENREF_138)]. This up-regulation of miR-181a decreased expression of its predicted target E2F5, a transcription factor known to be involved in proliferation regulation, leading to increased cellular proliferation and tumor growth in nude mice[[138](#_ENREF_138)].

During an HBV infection, non-infectious HBsAg is secreted from the cell as empty viral particles, known as subviral particles, typically in a 1,000 to 100,000-fold excess compared to infectious particles[[139](#_ENREF_139)]. Next-generation sequencing of small RNAs from HepG2 cells over-expressing HBsAg showed that the miRNA profile is significantly altered in response to HBsAg expression. Some of the miRNAs up-regulated by HBsAg expression are known or predicted to target the major histocompatibility complex class I-related molecules A or B (MICA or MICB), two ligands involved in activation of natural killer (NK) cells. miR-373, miR-107, and miR-29a/b, three miRNAs shown to be up-regulated in HBsAg-expressing cells, were each shown to target MICA or MICB, resulting in decreased surface expression of these ligands and decreased NK cell-mediated cell death[[140](#_ENREF_140)]. Emerging evidence has supported a role for NK cells in the innate immune response to HBV[[141](#_ENREF_141)], and HBV down-regulation of NK cell-activating ligands such as MICA and MICB could contribute to the establishment of a chronic HBV infection and associated diseases. Further research is needed to determine the correlation between the amount of HBsAg expression required to induce expression of the MICA/MICB-regulating miRNAs and the amount of HBsAg that is present during a normal infection, but these studies suggest a potential role for MICA and MICB down-regulation in chronic HBV infection.

An interesting aspect of miRNA-related research has been the recognition of miRNA-mediated regulatory networks, in which multiple cellular factors and miRNAs combine to form a cascade of cellular signals. Together these regulatory networks, involving factors such as NF-κB /Lin28/let-7/IL-6 or miR-124/IL-6R/HNF4, are important for maintaining the normal function of the cell, and their disruption may be a cause of disease[[142](#_ENREF_142),[143](#_ENREF_143)]. Various studies have independently identified a number of HBx-mediated effects on cellular metabolism through disruption of cellular miRNAs (Figure 3). When considered together, these studies could imply a regulatory mechanism through which HBx modulates cellular metabolism by influencing the levels of specific miRNAs to regulate expression or activation of a group of important metabolism-related cellular factors. For example, one group demonstrated that HBx inhibits p53-mediated activation of miR-148a, resulting in increased expression of the miR-148a target, hematopoietic pre–B cell leukemia transcription factor–interacting protein (HPIP). This increase in HPIP activated Akt and extracellular signal-regulated kinase (ERK), and ultimately mechanistic target of rapamycin (mTOR)[[144](#_ENREF_144)]. HepG2.2.15 cells, HBx-transgenic mice, and cells over-expressing HBx each were also shown to have increased levels of miR-29a, which targets PTEN, a negative regulator of Akt activation, resulting in HBx-mediated Akt activation and increased cell migration[[67](#_ENREF_67)]. Studies in HBx-transfected HepG2 and Huh7 cells have also suggested that HBx up-regulates the oncogenic miR-21, resulting in decreased PTEN expression and increased Akt activation[[145](#_ENREF_145)]. In yet another finding, HBV-associated tumors and tumors from HBx-transgenic mice were shown to have increased levels of miR-224. This directly correlated with low levels of autophagy activation and low levels of the miR-224 target Smad4, which when inhibited results in increased tumorigenicity[[68](#_ENREF_68)]. Together these results support a role for HBx-mediated regulation of miRNA expression in the overall regulation of the metabolic processes of the cell. Because HBx modulates the levels of Akt activation through altered miRNA expression, HBx might also control the level of mTOR activation and autophagy to optimize the cellular environment for HBV replication.

HBx-mediated regulation of miRNAs has also been suggested to play a role in altered DNA methylation. Two different groups demonstrated HBx regulation of DNA methylation through HBx down-regulation of miR-101 and miR-152. In one study, miR-152 was down-regulated in HepG2.2.15 cells and in HepG2 cells expressing HBx, and this correlated with an increased expression of the miR-152 target DNA methyltransferase 1 (DNMT1). Increased DNA methylation by DNMT1, including at sites epigenetically silenced in HBV-associated HCC, was seen in cells treated with an inhibitor of miR-152[[146](#_ENREF_146)]. Similar results were observed with miR-101 and its target DNA methyltransferase 3A; HBx-mediated down-regulation of miR-101 increased expression of DNMT3A and increased DNA methylation by DNMT3A[[147](#_ENREF_147)]. Interestingly, additional evidence suggests that expression of both miR-122[[97](#_ENREF_97)] and miR-205[[148](#_ENREF_148)] may be regulated through HBx-mediated alteration of methylation of their transcription promoters. Taken together, these studies suggest that HBx can regulate expression of miRNAs to affect epigenetic modulation of gene expression.

**EFFECTS OF CELLULAR MIRNAS ON HBV REPLICATION**

The role of cellular miRNAs during viral infections of mammalian cells has been a matter of debate. In general, miRNAs could act as a cellular anti-viral defense, as siRNAs do in plants and lower eukaryotes, or miRNAs could be exploited by the virus to help establish a cellular environment that promotes virus replication or persistence. In the preceding discussion, we described the impact of HBV replication and HBV proteins on miRNA expression; in the following sections, we discuss evidence indicating that cellular miRNAs can directly affect the outcome of an HBV infection (Table 3).

***miR-122***

The high abundance and liver-specific expression of miR-122 has led many groups to investigate its effects on the replication of hepatotropic viruses. While miR-122 is a required factor for HCV replication (reviewed in[[149](#_ENREF_149)]), the effects of miR-122 on HBV replication remain less clear. Multiple studies, including those using libraries of miRNA mimics or miRNA inhibitors to examine the impact of miRNAs on HBV replication, failed to identify miR-122 as a candidate regulator of HBV replication[[80](#_ENREF_80),[124](#_ENREF_124),[150](#_ENREF_150)]. A growing body of evidence, however, suggests that there is a correlation between miR-122 levels and the presence of replicating HBV or the expression of HBV gene products. While many studies have examined alterations of miR-122 levels in the presence of replicating HBV (discussed above), others have found evidence for the down-regulation of HBV gene expression by miR-122. The various mechanisms that have been considered, including the direct targeting of HBV transcripts by miR-122, and indirect effects where miR-122 can suppress cellular targets that affect HBV replication, are discussed below.

One of the first studies to demonstrate miR-122-mediated effects on HBV gene regulation utilized Huh7 cells, which express moderate amounts of miR-122, and HepG2 cells, which express very low levels of miR-122. After co-transfection of Huh7 cells with a plasmid encoding the HBV genome and a miR-122 inhibitor, HBsAg and HBeAg were increased compared to cells transfected with HBV and a control miRNA inhibitor, indicating a negative regulatory effect of miR-122 on HBV gene expression. Similarly, when miR-122 was over-expressed in HBV-transfected HepG2 cells, HBsAg and HBeAg levels significantly decreased[[151](#_ENREF_151)]. In the same series of experiments, however, the over-expression of miR-122 was shown to decrease levels of heme oxygenase-1 (HO-1), and miR-122 inhibitors increased HO-1 levels. Consistent with the previously demonstrated role of HO-1 as a suppressor of HBV replication[[152](#_ENREF_152)], over-expression of HO-1 in HepG2 cells was sufficient to inhibit HBV protein production. Conversely, siRNA knockdown of HO-1 resulted in increased HBsAg and HBeAg, suggesting an indirect, but positive, regulatory effect of miR-122 on HBV replication. To explain these seemingly contradictory effects of miR-122 on HBV gene expression, a double regulatory mechanism was proposed in which miR-122 represses both HBV and HO-1, but the anti-HBV effects are dampened by the reduced ability of HO-1 to inhibit HBV replication[[151](#_ENREF_151)].

Another indirect mechanism of miR-122 regulation of HBV replication may be mediated through the activity of cyclin G1, a known target of miR-122[[91](#_ENREF_91)]. Cyclin G1 interacts with p53[[153](#_ENREF_153)], preventing p53 binding to HBV enhancer I and II elements, which normally suppresses HBV transcription, thus relieving repression of HBV transcription. A down-regulation of miR-122 in HBV-infected cells, then, would result in a feed-forward loop by allowing increased levels of cyclin G1 expression, increased sequestration of p53, and further increases in HBV transcription[[98](#_ENREF_98)].

Additional studies support the conclusion that miR-122 can suppress the expression of HBV proteins or HBV replication[[69](#_ENREF_69),[77](#_ENREF_77),[79](#_ENREF_79),[95](#_ENREF_95),[96](#_ENREF_96)]. For instance, it has been shown that HepG2.2.15 cells express lower levels of miR-122 than the already low levels found in HepG2 cells, and when miR-122 is over-expressed in HepG2.2.15 cells, there is a decrease in the production of HBV proteins[[95](#_ENREF_95)]. It should be noted, however, that while direct infection with HBV increased miR-122 levels in both HepG2 cells stably transfected to express the HBV receptor and in primary treeshrew hepatocytes, neither miR-122 over-expression nor inhibition had any impact on HBV replication[[66](#_ENREF_66)]. Although only a single study, the use of models of direct infection with HBV that more closely mimic an authentic HBV infection suggest that this study may more accurately reflect miR-122 effects on HBV replication and HBV protein expression during a natural HBV infection.

Direct targeting of HBV RNAs by miR-122 has been explored as a mechanism of post-transcriptional repression, and potential binding sites have been identified in conserved regions of the HBV genome (Figure 1)[[69](#_ENREF_69),[79](#_ENREF_79)]. One potential miR-122 binding site was identified in the HBV pgRNA, just downstream of the core protein ORF and within the polymerase ORF. miR-122 targeting of this site was investigated in studies with various HBV plasmids that included sequence variations at this site or in studies with miR-122 mimics carrying mutations designed either to prevent or restore miR-122 interactions with this site. HBsAg and HBeAg levels were reduced in HepG2 cells expressing HBV and co-transfected with a miR-122 mimic, but only when the HBV-expression plasmid and miR-122 mimic carried either wild type HBV sequence or compensating mutations to allow complementary interactions[[79](#_ENREF_79)]. These studies provide evidence that miR-122 regulates HBV replication through direct targeting of HBV transcripts. These studies also raise the interesting question, however, of why or how HBV, a very narrowly hepatotropic virus, has adapted to replicate in the same cells that express a potentially antiviral miRNA at unusually high levels. The authors propose that reduced viral replication mediated by miR-122 promotes HBV persistence by preventing excessive cell damage or a strong immune response. It is also possible, however, that the miR-122 binding site in HBV RNAs is not available for targeting by miR-122 in infected cells, either due to binding of other proteins at that site or to secondary structure in the RNAs. Similar masking of potential miRNA binding sites has been demonstrated in HIV-1 mRNAs[[154](#_ENREF_154),[155](#_ENREF_155)]. Additional experiments addressing these issues could help to explain how HBV tolerates the potentially anti-viral effects of miR-122.

***miR-125 family***

A study in the PLC/PRF/5 cell line, which expresses HBsAg from integrated HBV DNA, demonstrated that miR-125a down-regulates expression of HBsAg through direct targeting of HBV RNAs[[156](#_ENREF_156)]. Using a computational approach, seven potential miRNA target sites were identified within a conserved 995bp sequence of the HBV polymerase and HBsAg ORFs. Assays using luciferase reporter constructs, in which the target site is cloned into the luciferase 3' UTR, in a panel of human liver cancer cell lines confirmed targeting of one of these sites by miR-125a; this effect was lost when the predicted region complementary to the miR-125a seed sequence was mutated. Although the effects on HBV replication were not analyzed directly in this study, down-regulation of HBsAg expression was observed when a miR-125 mimic was transfected into PLC/PRF/5 cells, whereas transfection of a miR-125a inhibitor into these cells increased HBsAg expression[[156](#_ENREF_156)].

Another recent study correlated the regulation of miR-125a-5p expression to the opposing effects on HBV replication that are observed following treatment of HBV-expressing cells with either iron or TGFβ[[157](#_ENREF_157)]. HepG2.2.15 cells were treated with either iron, which significantly increased HBV replication, or TGFβ, which significantly decreased HBV replication, and the expression of 814 miRNAs was measured by high-throughput sequencing. Two miRNAs showed opposing regulation under these treatment conditions: TGFβ increased miR-125a-5p and miR-21 expression whereas iron decreased miR-125a-5p and miR-21 expression. In these studies, transfection of a miR-125a-5p mimic abrogated the iron-induced increase in HBV replication; however, inhibition of miR-125a-5p did not block the TGFβ-mediated decrease in HBV replication. When taken together with the results of the previously discussed study, these miR-125a-5p studies suggest a regulatory network in which miR-125a-5p decreases HBV replication through control of HBsAg translation. As with some other miRNAs that inhibit HBV replication, miR-125a-5p is paradoxically up-regulated in HBV-expressing cells and HBV-infected patients[[82](#_ENREF_82),[83](#_ENREF_83),[121](#_ENREF_121)]. In the studies that describe HBV-mediated up-regulation of antiviral miR-125a, this up-regulation was proposed to be a potential mechanism for HBV to regulate its own replication by controlling the levels of cellular miRNAs that inhibit the virus; additional studies are needed to validate this hypothesis.

In addition to studies examining miR-125a, a study identified miR-125b as down-regulated in HBV-expressing cells and showed that miR-125b negatively regulates HBV replication. In this study, HepG2.2.15 cells over-expressing miR-125b had decreased levels of HBV DNA and HBV proteins, and this effect was lost when the cells were transfected with a miR-125b inhibitor. This effect was proposed to be through regulation of the miR-125b target sodium channel, non-voltage-gated 1 alpha (SCNN1A)[[123](#_ENREF_123)]; however, further experiments will be needed to verify that this target is, in fact, playing a role in HBV regulation.

***miR-15 family***

The miR-15 family has also been shown to regulate HBV replication. In one study that described the ability of HBV transcripts to act as miR-15a and miR-16-1 sponges, over-expression of miR-15a or miR-16-1 decreased HBV replication in HepG2 cells transfected with a plasmid containing the HBV genome and decreased HBx expression in cells transfected with an HBx-expressing plasmid[[106](#_ENREF_106)]. While the mechanism(s) of miR-15a/16 regulation of replication was not directly examined, multiple miR-15a/16 targets were disrupted by the sponge effect of the HBV transcripts. Among these targets, Cyclin D1 has previously been shown to be up-regulated by HBx, and this up-regulation may be required for HBV replication in primary rat hepatocytes[[40](#_ENREF_40)]. Consequently, regulation of members of the miR-15 family could influence HBx-mediated alteration of the cell cycle and regulation of HBV replication.

Another study identified a complex regulatory cascade in which miR-15b may be playing a role in establishing HBV persistence. Expression of miR-15b was decreased in cell lines expressing HBx or stably expressing HBV, and ectopic expression of miR-15b in HepG2.2.15 cells increased HBV replication and increased production of viral antigens. In contrast, inhibition of miR-15b decreased HBV replication in HepG2.2.15 cells. Because HBV transcript levels were increased in the presence of over-expressed miR-15b, the authors reasoned that miR-15b was not likely directly targeting viral transcripts, unlike what had been shown for other members of this miRNA cluster[[70](#_ENREF_70),[106](#_ENREF_106)]. Instead, the authors hypothesized that miR-15b could be modulating activity at one of the viral transcription promoter or enhancer regions. After identifying the Enhancer I region as the regulatory region affected by miR-15b, the transcription factor HNF1α, which represses Enhancer I activity, was identified as a target of miR-15b. Targeting of HNF1α by miR-15b resulted in increased viral replication, which ultimately created a negative feedback loop that decreased the levels of miR-15b[[131](#_ENREF_131)]. While further studies are needed to determine how these findings relate to other studies that demonstrated that miR-15 family members inhibit HBV replication, together each of these studies supports the hypothesis that members of this miRNA family could be involved in regulation of HBV replication.

***miR-199a-3p***

In addition to studies describing HBV-mediated regulation of miR-199 family members, there is evidence that these miRNAs may play a role in regulating HBV replication. For example, in experiments where a pool of antisense miRNAs was used to screen for inhibitors of HBV replication, both miR-199a-3p and miR-210 were shown to inhibit HBV replication in HepG2.2.15 cells. This study used antisense oligonucleotides against 328 known human miRNAs to detect the loss of miRNA-dependent effects on HBV replication and surface antigen secretion. The predicted target sites of miR-199-3p and miR-210 within surface antigen and the PreS1 coding sequences were cloned into a luciferase reporter vector to confirm functional targeting of the sites[[124](#_ENREF_124)]. Interestingly, these sites are located in different transcripts across HBV genotypes, and the miR-210 target site is not completely conserved, which potentially alters the interpretation of these studies. The fact that miR-199a/b-3p is a highly abundant miRNA in the liver and is up-regulated in HBV-infected liver[[30](#_ENREF_30)] suggests that miR-199a-3p could be effective in targeting and regulating HBV during an infection. As with a number of other miRNAs reported to be inhibitory to HBV, the level of this anti-HBV miRNA was shown to be higher in HepG2.2.15 cells than HepG2 cells[[124](#_ENREF_124)], potentially as a mechanism to control the level of viral replication.

***miR-17/92 cluster***

Members of the miR-17-92 cluster of miRNAs have also been identified as regulators of HBV replication through direct targeting of viral transcripts. For example, inhibition of miR-20a and miR-92a-1, both members of the miR-17-92 miRNA cluster, increased the levels of HBV RNAs. Predicted target sites for these miRNAs within the HBx and polymerase ORFs were identified computationally, and confirmed using a luciferase reporter construct in which ~450 bp of the HBV genome that included the predicted target sites was cloned downstream of the luciferase ORF[[135](#_ENREF_135)]. Unfortunately, these studies were limited to this reporter assay and did not extend to analyses of mutated sites within the HBV genome nor did they introduce mutations into their reporter construct to attempt to inhibit targeting, so additional studies are needed to confirm this miRNA-mediated regulation of HBV.

***Other miRNAs***

While the most commonly studied miRNAs that have been proposed to regulate HBV replication are described above, additional miRNAs have been shown to regulate HBV replication. For example, the results of one study showed that HBV-mediated up-regulation of miR-501 led to increased targeting of HBXIP, an inhibitor of HBV replication, resulting in increased HBV replication[[81](#_ENREF_81)]. Increased levels of the miR-371/372/373 miRNA cluster, which was observed in HBV-associated HCCs and HepG2.2.15 cells, were also shown to increase HBV replication, and this occurred primarily through miR-372 and miR-373 targeting of nuclear factor I/B (NFIB), an inhibitor of HBV replication. Increased miR-372 and miR-373 levels increased HBV Enhancer I and core promoter activity, potentially through decreased NFIB-mediated transcriptional repression[[82](#_ENREF_82)].

Libraries of miRNA mimics have been transfected into HBV-expressing cells to identify miRNA-dependent changes in the level of HBV replication. In one study in HepG2 cells, miR-141, miR-125a, and miR-125b were identified as negative regulators, and miR-98 as a positive regulator, of HBV replication. PPARα, a transcription factor required for HBV replication, was identified as a target of miR-141[[80](#_ENREF_80)]. Using a similar library of miRNA mimics in HepG2.2.15 cells, another study identified miR-1, miR-146, and miR-214 as enhancing HBV replication and miR-210 as inhibiting HBV replication. miR-1 was further shown to enhance HBV replication by targeting histone deacetylase 4 (HDAC4), ultimately transactivating the HBV core promoter through enhanced farnesoid X receptor α(FXRA) expression[[150](#_ENREF_150)]. There did not appear to be overlap between the identified miRNAs in these two studies, although a similar study utilizing a pool of antagomirs transfected into HepG2.2.15 cells did identify miR-210 as an inhibitor of HBV replication[[124](#_ENREF_124)].

Finally, a purely computational approach was used to predict six potential miRNA target sites within the HBV genome by compiling results from four commonly used target prediction algorithms. Using a previously published next-generation sequencing dataset of miRNAs expressed in HBV-infected and normal liver samples, liver expression of the predicted miRNAs was examined. Four of the six predicted miRNAs were shown to be expressed in the liver in this sequencing dataset, but because no experimental data were presented to support the predicted target sites, it remains to be seen whether these predicted miRNA targets in the HBV genome are actually targeted[[158](#_ENREF_158)]. Moreover, the expression levels of each of these miRNAs, as determined in the next-generation sequencing dataset described above, was extremely low, and well below the threshold value set by the original study[[30](#_ENREF_30)]. This raises the question of whether these four miRNAs are expressed at high enough levels to have functional relevance for affecting HBV replication, and underscores the importance of considering the cellular context of miRNA expression when using computational approaches.

**HBV-MEDIATED REGULATION OF MICRORNA MACHINERY**

Recent studies have attempted to correlate HBV replication and protein expression with alterations in the cellular miRNA processing machinery. In HepG2 cells stably expressing HBV, the RISC protein Ago2 co-localized in multiple intracellular compartments with HBcAg and HBsAg, and this co-localization seemed to occur specifically, as it was not seen between Ago2 and HBx. Knockdown of Ago2 with siRNA decreased HBV DNA and HBsAg secretion, implying that Ago2 is important for HBV replication[[159](#_ENREF_159)]. As is the case with some other viruses, it was proposed that HBV might utilize some of the non-silencing related functions of Ago2, such as its role in P-body formation[[160](#_ENREF_160)]. This is an interesting hypothesis that warrants further study, as it could lead to a better understanding of cellular influences on HBV replication. These findings are, however, in direct contrast to studies that used siRNA knockdown of multiple components of the miRNA machinery, including Ago2, in HepG2 cells transfected with a plasmid expressing the HBV genome. In these studies, knockdown of the miRNA machinery increased HBV replication, implying an overall inhibitory role for miRNAs in HBV replication[[106](#_ENREF_106)]. Further studies will be required to determine if either effect is relevant to a natural HBV infection.

Two additional studies took a more direct approach and quantified the levels of miRNA machinery elements in HBV-expressing cells. In one study, levels of Drosha were significantly down-regulated in HepG2.2.15 cells compared to HepG2 cells, an effect that was proposed to be due to HBx-mediated regulation of SP1 and AP-2 transcription factors[[161](#_ENREF_161)]. In another study, it was shown that HepG2.2.15 cells and HepG2 cells expressing HBV have lower levels of DGCR8 than HepG2 cells, potentially through HBV-mediated up-regulation of the transcription factor YY1. It was shown that YY1 inhibits DGCR8 promoter activity, and that HepG2.2.15 cells express higher levels of YY1 than HepG2 cells. Although both of these studies showed a decrease in essential miRNA machinery components, no quantification of miRNA levels was conducted, and further studies are needed to determine the impact of this regulation on the cellular miRNA profile.

**MICRORNAS AND HBV-ASSOCIATED HCC**

Since HBV is the leading worldwide risk factor for developing HCC, many studies assessing the relationship between HBV and miRNAs have attempted to correlate the cellular impact of changes to the miRNA profile or specific miRNAs to tumorigenesis. There are also multiple studies describing the utility of serum or liver miRNA profiles as early detection markers for HCC. While we describe below the importance of miRNAs as they relate to HCC, particularly in the context of HBV-associated HCC, more extensive reviews of miRNAs in HCC are found[[162](#_ENREF_162),[163](#_ENREF_163)]

There are many reasons for the high mortality rate of HCC, but foremost among these are the lack of early detection methods and effective treatments (reviewed in[[164](#_ENREF_164)]). As with all cancers, HCC develops in an environment of cellular proliferation. In a normal liver only about 1 in 20000 hepatocytes is undergoing mitosis at a given time, so the uncontrolled proliferation associated with HCC requires a signal that induces and maintains hepatocyte proliferation[[165](#_ENREF_165)]. Many HBV-related miRNA studies have attempted to identify miRNA targets that are directly involved in increasing cellular proliferation. For example, in one study, decreased levels of miR-122 during HBV replication resulted in increased pituitary tumor-transforming gene 1 (PTTG1) binding factor (PBF) expression. This led to enhanced proliferation and invasiveness of HCC cells and tumorigenicity *in vivo* through PBF-mediated activation of the PTTG1 transcription factor. Importantly, decreased miR-122 and increased PBF were confirmed in liver samples from patients with chronic hepatitis or HBV-associated HCC[[69](#_ENREF_69)]. Similar relationships have been seen with miR-29c and its target TNFAIP3[[166](#_ENREF_166)], miR-22 and its target CDKN1A[[167](#_ENREF_167)], and miR-143 and its target FNDC3B[[168](#_ENREF_168)]. While the majority of these studies demonstrate altered regulation of their respective miRNAs in HBV-associated HCC samples, what is often lacking in these studies is direct confirmation of inverse expression of the identified mRNA target in the HCC samples. Because of the lack of an effective model system for studying HBV and HBV-associated HCC, for most studies the mRNA target identification and validation was performed in cell lines, without a definitive ability to confirm that the target is regulated by the miRNA in the original cell or tissue samples. Because of the heterogeneity of tumors, and the use of already transformed cell lines for confirmatory studies, it is difficult to determine which factors are involved in HCC development and which are consequences of the systems being used. This could be contributing to the extensive number of factors being identified as HCC-related miRNA-target pathways.

Profiling studies of miRNAs in HBV-associated HCC have mainly focused on identifying a distinct set of miRNAs that could be used for early detection or progression of disease. A study specifically designed to determine the effectiveness of using miRNAs as diagnostic markers utilized 454 sequencing and bioinformatics to show that using a panel of miRNAs was more effective at predicting HCC recurrence after resection than single miRNAs, validating this profile-based approach[[93](#_ENREF_93)]. Profiling studies have incorporated PCR-based arrays, microarrays, and next-generation sequencing to address the question of which miRNAs are consistently altered at various stages of HCC development. One example is the use of next-generation sequencing of small RNAs from normal liver samples, HBV-infected liver samples, HBV-associated HCC samples, HCV-infected liver samples, HCV-associated HCC samples, and non-viral HCC samples to characterize miRNA expression in the various etiologies of HCC. From these studies, it was concluded that there is not a distinct influence of HBV or HCV on the miRNA profile compared to normal tissue. In HCC, however, there was a significant reduction of multiple miRNAs, regardless of HCC etiology. Only one, miR-199a/b-3p, was consistently altered, and its down-regulation correlated with decreased patient survival. Confirmation of the sequencing results by qRT-PCR showed that 40 out of 40 HBV-associated HCC samples had a significant decrease in miR-199a/b-3p while only 45% had decreased miR-122. Additional studies showed that miR-199a/b-3p has tumor-suppressor capability; increased expression of miR-199a/b-3p in cell lines or with adeno-associated virus (AAV)-mediated delivery in human HCC-bearing nude mice inhibited tumor growth and increased tumor necrosis[[30](#_ENREF_30)].

In another study, qRT-PCR was used to analyze expression of 188 miRNAs in liver samples from normal liver, chronic HBV infected livers, chronic HCV-infected livers, HBV-associated HCC, and HCV-associated HCC. In contrast to the profiling study described above[[30](#_ENREF_30)], each of these groups could be segregated from its parent group based solely on a specific panel of miRNAs, indicating that miRNA expression profiles can differentiate between HBV and HCV, and between normal liver, chronically HBV- or HCV-infected livers, and HBV- or HCV-associated HCC. An investigation of the altered miRNAs and their putative targets suggested that pathways involved in apoptosis, DNA damage, recombination, and signal transduction were often affected in tumors from HBV-infected patients, while pathways involved in the immune response, antigen presentation, cell cycle control, proteasome activity, and lipid metabolism were more often affected in tumors from HCV-infected patients. Interestingly, a strong correlation between changes in miRNA expression in HBV- and HCV-infected liver samples and corresponding studies done in HBV or HCV cell line models was also seen, supporting the use of cell lines as models for miRNA studies with these viruses[[76](#_ENREF_76)].

Instead of directly comparing miRNA expression in HCC and normal tissue samples, another approach has been to examine miRNA expression through the multiple stages of HBV-associated HCC development. In one such study, qRT-PCR was used to examine a panel of seven miRNAs (miR-221, miR-21, miR-224, miR-10b, miR-145, miR-199b, and miR-122) in dysplastic nodules, small HCCs, and normal liver tissue from HBV-infected patients. While there was altered expression of these miRNAs at various stages of HCC development, altered expression of miR-145, miR-199b, and miR-224 occurred in pre-malignant dysplastic nodules and was maintained through the development of HBV-associated HCC. This study suggests that because of their consistently altered expression from the early stages of HCC, miR-145, miR-199b, and miR-224 might be useful as markers for the early detection of HBV-associated HCC[[74](#_ENREF_74)].

The current methods for screening and diagnosis of HCC involve a combination of imaging techniques, serum assays, and histology[[169](#_ENREF_169), [170](#_ENREF_170)]. Many of these tests are either invasive, in the case of liver biopsy, or unreliable, in the case of serum proteins such as AFP[[171](#_ENREF_171)]. Identification of an HCC-associated serum miRNA profile could relieve the need for invasive procedures and offer a reliable and specific method of detection. It has previously been shown that miRNAs in the serum are stable and reproducibly detected, supporting their use as serum biomarkers[[172](#_ENREF_172)]. A number of studies have characterized the serum miRNA profile in HBV-infected patients. A panel of 13 miRNAs that differentiated HBV-infected and HBV-associated HCC samples from control and HCV samples was identified in one study that used Solexa sequencing followed by confirmation with qRT-PCR to analyze serum miRNAs of 513 patients[[75](#_ENREF_75)]. Another study examined the expression of 723 miRNAs followed by confirmation of specific miRNAs with qRT-PCR in 137 plasma samples; these data were used to generate a panel of 7 miRNAs that was able to reliably diagnose HCC (sensitivity 83.2%, specificity 93.9%), chronic HBV infection (sensitivity 79.1%, specificity 76.4%), and cirrhosis cirrhosis(sensitivity 75.0%, specificity 91.1%) in an independent cohort of 390 patients[[78](#_ENREF_78)]. Although the development of HCC is a multi-stage process, and probably not caused by disruption of a single or a small handful of miRNAs, cumulatively, the results of these studies suggest that the use of an miRNA profile for early detection of HCC, especially in the serum, could prove to be an important diagnostic tool. Moreover, the significant contribution of HBV infection to the worldwide burden of HCC makes understanding the role of miRNAs in the development of HBV-associated HCC an important research goal.

**FUTURE DIRECTIONS**

***RNA silencing-based therapeutics***

Ideally, the identification of miRNAs that are important during an HBV infection and the progression of HBV-associated HCC could lead to the development of targeted therapeutics. Such an approach is currently in clinical trials for HCV with Miravirsen, a locked nucleic acid-modified antisense oligonucleotide that binds to and sequesters miR-122. Because efficient replication of HCV requires miR-122 binding in the HCV RNA 5’UTR, Miravirsen is a promising treatment for reducing HCV viral load and has proven successful in Phase 2a clinical trials[[173](#_ENREF_173)]. In contrast, no single miRNA or small group of miRNAs has emerged as being absolutely required for maintaining an HBV infection, and instead multiple miRNAs have been identified as playing a role in some facet of HBV replication and pathogenesis. Without a specific miRNA to modulate therapeutically, the inhibition of particular cellular miRNAs may not be a successful approach for treatment of HBV infections.

Another approach that currently holds more promise, and has been explored by multiple groups, is engineering small RNAs that target specific sequences within HBV RNAs. This approach has shown success in cell culture-based assays and *in vivo* studies using mouse models of HBV infection (reviewed in[[174-176](#_ENREF_174)]). Interfering RNAs have been tested as synthetic siRNAs, sometimes chemically modified to enhance their stability, and as RNAs that are expressed from plasmid or viral vectors as short hairpin (sh) RNAs or miRNA-formatted RNAs. In all cases, the interfering RNA can be designed with complete complementarity to target sequences in HBV transcipts, resulting in degradation of the targeted RNAs and highly effective down-regulation of those RNAs. Additionally, multiple interfering RNAs that target different regions of the HBV genome can be expressed from a single vector[[176](#_ENREF_176)] or even from a single transcript[[177](#_ENREF_177)] to promote efficacy against a range of viral serotypes and discourage the emergence of viral escape mutants. Together these studies have helped to lay the groundwork for the development of anti-HBV RNA silencing-based therapies. To date, the lack of a suitable animal model for early phase testing has meant that the majority of these studies have been done either in cell culture, or in small animal models with known deficiencies in their application to a true HBV infection. In addition, the effective *in vivo* delivery of these reagents to all or most cells of the liver has proven problematic. For a more extensive review on the development of RNAi based HBV therapeutics see Ivacik *et al*[[174](#_ENREF_174)]

***Studies of functional RISC complexes***

It is generally accepted that miRNA-mediated silencing acts as a “fine tuning” mechanism for regulating cellular protein expression[[178](#_ENREF_178),[179](#_ENREF_179)] and that *in vivo* changes in target protein levels in response to miRNAs often do not exceed two fold[[180](#_ENREF_180),[181](#_ENREF_181)]. In addition, results suggest that in most cell types, a small subset of miRNAs, maybe as few as 10-20, make up over 90% of the total miRNAs, and the pool of miRNAs functionally involved in miRNA-mediated silencing can be much smaller than that suggested by miRNA profiling studies[[30](#_ENREF_30),[182](#_ENREF_182)]. These findings support the idea that exogenous over-expression of single miRNAs or shifts in the expression of a few miRNAs in response to a stimulus, such as disease or viral infection, may have minimal physiological consequences, leading some to question the relevance of studies that claim that endogenous miRNAs mediate an antiviral response, directly or indirectly[[183](#_ENREF_183)]. What this means for the relevance of many of the studies described in this review remains to be seen. As only a handful of these studies dealt with miRNAs that are considered to have even moderate abundance in the liver, whether the described miRNA-mediated effects are actually occurring in the course of a natural HBV infection or the development of HBV-associated HCC cannot be determined until different approaches are taken for studying HBV effects on the profile of functional miRNAs in hepatocytes. Recent advances in technology have allowed researchers to attempt to answer these types of questions by specifically investigating miRNAs that are involved in miRNA-mediated silencing. By isolating and identifying miRNAs and their mRNA targets that can be found within cellular RISC complexes, a clearer picture of the physiological impact of changes in miRNA expression level and the mRNAs they are targeting can be determined. The application of these types of techniques that specifically investigate functional miRNAs, such as CLIP-based techniques[[25](#_ENREF_25),[26](#_ENREF_26)], CLASH[[22](#_ENREF_22)], or RISC-trap[[24](#_ENREF_24)], is an important next step in investigating the HBV-mediated profile of miRNAs that are actively involved in targeting instead of simply those miRNAs that exhibit HBV-mediated changes in expression level.

***Improved model systems***

The identification of NTCP as a functional receptor for HBV should help to establish a better understanding of the cellular functions and pathways involved in an HBV infection[[61](#_ENREF_61)]. Beyond that, however, is the potential use of systems expressing this receptor to generate more physiologically relevant data about the cellular impact of a natural HBV infection. The current standards of delivering the HBV genome through transfection or expression from viral vectors can themselves cause physiological changes within a cell that are independent of HBV-mediated effects. Eliminating this variable by directly infecting with HBV will generate a clearer picture of the miRNA response to HBV infection, although other issues will remain. Among these issues is the use of transformed cell lines, such as HepG2 cells, to make receptor-based HBV-infection model systems. While a direct HBV infection in these cells may remove some variables, it does not alleviate the concerns that the parental transformed cell line is far-removed physiologically from a normal primary hepatocyte. This is particularly important when investigating miRNAs, as an altered baseline of miRNA expression could introduce significant bias into experiments. The generation of receptor-complemented animal models will allow *in vivo* studies that could help to establish HBV-mediated changes to cell physiology over the course of a chronic HBV infection and the development of HBV-associated HCC, but recent studies showing that the human NTCP alone is not enough to render mouse hepatocytes susceptible to HBV infection[[184](#_ENREF_184)] indicates that more research is needed before these systems come to fruition.

**CONCLUSION**

Because of their role in regulating interactions and pathways that direct cell physiology, it is important to understand the consequences of altered expression of cellular miRNAs during the course of an HBV infection. Alterations to miRNA expression levels could simply be the byproduct of alterations to cell physiology that accompany an HBV infection, or could indicate that miRNAs are actively involved in how the cell interacts with and responds to HBV. As presented here, evidence exists to suggest that the later is, in fact, the case, and that miRNAs are likely playing an important role in the cellular response to HBV replication. Increasing evidence suggests that some miRNAs, such as miR-122, and miR-125 and miR-199 family members, have high expression in the liver and exhibit HBV-mediated expression changes. These changes in miRNA levels could ultimately prove to be having a significant cellular impact, both for the virus and the host. Just as important as studies identifying miRNAs involved in HBV replication are the studies designed to identify biomarkers of HBV-mediated disease, such as HBV-associated HCC. As technology, and our understanding of miRNA biology, improves, these studies will help to develop valuable tools that can help to diagnose both HBV and HBV-associated HCC sooner, leading to more effective patient care.

The application of newer techniques that account for miRNA function and expression level, combined with more relevant models of a genuine HBV infection, will help to solidify our understanding of the significance of miRNAs during an HBV infection. In addition, the potential clinical use of miRNA biomarker profiles, therapeutic targeting of an miRNA that positively regulates HBV replication, or expression of synthetic miRNAs that target HBV to inhibit viral replication add particular appeal to understanding the role that miRNAs could be playing during the course of an HBV infection and associated disease progression. As our understanding of the cellular roles of miRNAs continues to evolve along with advancing technologies for assessing miRNA levels and functions, the impact of HBV on cellular miRNAs and the role that these miRNAs play during an infection and the subsequent development of HBV-associated HCC will become clearer.

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**Figure 1 Genome structure of hepatitis B virus with proposed microRNA target sites.** A: The partially double-stranded DNA genome of hepatitis B virus (HBV) is depicted at the center, surrounded by the overlapping open-reading frames (ORFs) (thick colored arrows). The outer, black arrows represent the viral mRNA transcripts; B: Linear arrangement of overlapping ORFs and viral transcripts. Transcripts and ORF are aligned with each other according to genomic location and are to scale. Colored boxes on transcripts indicate proposed miRNA target sites discussed in the text.



**Figure 2 microRNAs altered by hepatitis B virus and -X protein or in hepatitis B virus-associated hepatocellular carcinoma.** Venn diagram depicting microRNAs discussed in the text that have altered expression levels in the context of hepatitis B virus (HBV) replication, HBV-X protein (HBx) expression, and/or HBV-associated hepatocellular carcinoma (HCC).



**Figure 3 Model of potential hepatitis B virus X protein-mediated metabolic regulatory network.** Potential regulatory network involving hepatitis B virus (HBV)-X protein (HBx)-regulated microRNAs (miRNAs) and their downstream targets. Together these HBx-regulated miRNAs may be playing a role in cellular homeostasis through the regulation of important metabolism-related factors such as Akt and mechanistic target of rapamycin (mTOR).

**Table 1 Cell-based model systems for the study of hepatitis B virus**

|  |  |  |
| --- | --- | --- |
| **Model System** | **Source** | **Characteristics** |
| **Cell Lines** |
| HepG2 | Human hepatoblastoma[[59](#_ENREF_59)] | Transformed cell line that is easy to grow and maintain. Serves as a model system for HBx-dependent HBV replication, although these cells cannot be directly infected by HBV. |
| HepG2.2.15 | HepG2 with two integrated head-to-tail dimers of HBV genome[[62](#_ENREF_62)] | Stably replicates HBV and produces infectious virus; however, continuous passage since development has created a large separation from the parental HepG2 cells so that differences between HepG2 and HepG2.2.15 cells may not be HBV-specific |
| HepAD38 | HepG2 stably expressing HBV pgRNA that is controlled by a tetracycline-responsive promoter[[63](#_ENREF_63)] | Presence or absence of tetracycline (tet) allows cell line to act as its own baseline. HBV replication easily controlled, but only pgRNA expression is under tet-control, so many viral proteins are still made in the presence of tet. |
| Huh7 | Human hepatoma[[60](#_ENREF_60)] | Transformed cell line that is easy to grow and maintain. Cells cannot be directly infected by HBV. For reasons that remain unknown, HBV replication in these cells is HBx-independent |
| PLC/PRF/5 | Human hepatoma[[185](#_ENREF_185)] | Transformed cell line that is easy to grow and maintain. These cells express HBsAg from integrated HBV DNA |
| HepaRG | Human hepatoma[[186](#_ENREF_186)] | Transformed cell line, but differentiation can be induced to promote primary hepatocyte-like characteristics. After differentiation, these cells can be directly infected with HBV, although the infection efficiency is low. Acquisition of a differentiated, hepatocyte-like phenotype requires two-week maintenance in 2% DMSO prior to induction of differentiation; this process generates a mixed culture of hepatocytes and cholangiocytes. |
| **Primary cells** |
| Cultured primary human hepatocytes | Hepatocyte isolation from liver tissue | Natural target of an HBV infection; however, quickly lose susceptibility to an HBV infection after isolation and culture. These cells can be difficult to acquire, difficult to maintain in culture, and begin to de-differentiate soon after isolation and plating. These cells can be difficult to transfect. |
| Cultured primary mouse/rat hepatocytes | Perfused liver and isolation of hepatocytes | Primary cells that can be cultured to maintain a "normal" phenotype and serve as a surrogate model for studies in primary human hepatocytes. . Support HBV replication although not direct HBV infection. Isolation requires access to animals and ability to reliably isolate high quality hepatocytes |
| Liver tissue samples | HCC and adjacent normal liver tissue; HBV and non-HBV liver tissue | Primary cells can give a more accurate profile of the liver RNA transcripts and expressed proteins than transformed cell lines. Disease versus normal tissue comparisons facilitate analysis of disease-mediated differences; however, it is often difficult to determine if differences are a cause or consequence of the disease. Requires access to patient samples that is often hampered by a finite supply and limited access such that these samples are often only used for primary screens or final confirmation |
| Peripheral blood mononuclear cells  | Circulation | Easier to acquire than primary hepatocytes, but only support low levels of HBV infection and replication |

HBV: Hepatitis B virus; HBx: Hepatitis B virus -X protein.

**Table 2 Methods for studying microRNA expression levels**

|  |  |  |
| --- | --- | --- |
| **Strategy** | **Advantages** | **Disadvantages** |
| qRT-PCR | Can determine absolute miRNA-expression level when compared to standard curve, requires minimal bioinformatics analysis | Analysis of single miRNA at a time; often used to determine expression of small numbers of miRNAs; requires prior identification of miRNAs of interest |
| miRNA microarray | Can be used to determine expression changes for a large group of miRNAs | Data are expressed as fold change relative to a baseline and not absolute expression values; can require prior identification of miRNAs of interest |
| Next-generation sequencing | Identifies the complete miRNome and determines absolute expression values in the context of all other cellular miRNAs | Expensive and requires extensive bioinformatics analysis |

miRNA: microRNA; qRT-PCR: Quantitative reverse transcription polymerase chain reaction.

**Table 3 Hepatitis B virus-mediated regulation of cellular microRNAs**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **miRNA** | **Regulation** | **Sample type** | **Proposed target** | **Proposed targeted pathway** | **Method of detection** | **Ref.** |
| **HBV-mediated regulation** |
| miR-122 | Down | HepG2.2.15 | HO-1 | Inflammation and apoptosis | qRT-PCR | [[151](#_ENREF_151)] |
| Down | HBV-transfected HepG2/Huh7/SK-Hep-01, HBV-transgenic mice, HBV-infected liver tissue | PBF | Cell proliferation | qRT-PCR | [[69](#_ENREF_69)] |
| Down | HepG2.2.15 | Cyclin G1 | Cell cycle regulation; p53-mediated repression of HBV transcription | *in-situ* hybridization, qRT-PCR | [[98](#_ENREF_98)] |
| Down | HepG2.2.15 | NDGR3 | Cell proliferation | qRT-PCR | [[95](#_ENREF_95)] |
| Up | HBV-infected primary treeshrew hepatocytes, HBV patient serum | - | - | Next-generation sequencing, qRT-PCR | [[66](#_ENREF_66)] |
| Up | HBV patient serum | - | - | microarray | [[77](#_ENREF_77), [159](#_ENREF_159)] |
| let-7a/g | Down | HBV-transfected HepG2 | - | - | microarray | [[84](#_ENREF_84)] |
| let-7a | Down | HBV-transfected HepG2 | - | - | qRT-PCR | [[106](#_ENREF_106)] |
| let-7 family | Up | HepG2.2.15 | - | - | microarray | [[84](#_ENREF_84)] |
| let-7a | Up | HepG2.2.15 | - | - | microarray | [[81](#_ENREF_81)] |
| miR-15/16 family | Down | HBV-transfected HepG2 | - | - | microarray | [[84](#_ENREF_84)] |
| Down | HBV-transfected HepG2, HepG2.2.15, HBV-transgenic mice, HBV-associated HCC | Bcl-2 | Apoptosis | qRT-PCR | [[70](#_ENREF_70)] |
| Down | HepG2.2.15 | - | - | microarray | [[83](#_ENREF_83)] |
| miR-22 | Down | HepG2.2.15 | CDKN1A | Cell proliferation | qRT-PCR | [[167](#_ENREF_167)] |
| miR-29c | Down | HepG2.2.15 | TNFAIP3 | Cell proliferation | qRT-PCR | [[166](#_ENREF_166)] |
| miR-125a-5p | Up | HBV-infected liver tissue | - | - | qRT-PCR | [[121](#_ENREF_121)] |
| Up | HepG2.2.15 | - | - | microarray | [[82](#_ENREF_82), [83](#_ENREF_83)] |
| Up | Transfected Cell Line | - | - | qRT-PCR | [[106](#_ENREF_106)] |
| miR-125b | Down | HepG2.2.15, HBV-transfected HepG2 | SCCND1 | - | microarray, qRT-PCR | [[123](#_ENREF_123)] |
| miR-181a | Up | HepG2.2.15, AdHBV-infected HepG2 | E2F5 | Cell proliferation | qRT-PCR | [[138](#_ENREF_138)] |
| mIR-199b | Down | HBsAg+ liver tissue | - | - | microarray | [[82](#_ENREF_82)] |
| miR-372/373 | Up | HepG2.2.15 | NFIB | HBV replication | microarray | [[82](#_ENREF_82)] |
| miR-501 | Up | HepG2.2.15 | HBXIP | HBV replication | microarray, qRT-PCR | [[81](#_ENREF_81)] |
| miR-602 | Up | HepG2.2.15 | RASSF1A | Apoptosis, tumor suppressor | microarray, qRT-PCR | [[99](#_ENREF_99)] |
| **HBx-mediated regulation** |
| miR-122 | Down | HBx-expressing HepG2 | PPARγ | miR-122 transcription | qRT-PCR | [[97](#_ENREF_97)] |
| let-7 family | Down | HBx over-expressing HepG2, HCC | Stat3 | Cell proliferation | microarray, qRT-PCR | [[107](#_ENREF_107)] |
| miR-15/16 family | Down | HBx-expressing HepG2/Huh7/SK-HEP-1 cells | CyclinD1 | Cell cycle | microarray, qRT-PCR | [[129](#_ENREF_129)] |
| miR-21 | Up | HBx-expressing HepG2/Huh7 | PDCD4, PTEN | Akt, cell proliferation | qRT-PCR | [[145](#_ENREF_145)] |
| miR-21 | Up | HBx-expressing MIHA | - | Cell migration | qRT-PCR | [[187](#_ENREF_187)] |
| miR-29a | Up | HepG2.2.15, HBx-expressing HepG2, HBx-transgenic mice | PTEN | Akt, cell migration | qRT-PCR | [[67](#_ENREF_67)] |
| miR-101 | Down | HepG2.2.15, HBx-expressing HepG2 | DNMT3A | DNA methylation | qRT-PCR | [[147](#_ENREF_147)] |
| miR-143 | Up | HBx-expressing HepG2, HBx-transgenic mice | FNDC3B | Cell proliferation | qRT-PCR | [[168](#_ENREF_168)] |
| miR-148a | Down | HBx-expressing LO2/HepG2 cells | HPIP | mTOR | qRT-PCR | [[144](#_ENREF_144)] |
| miR-152 | Down | HepG2.2.15, HBx-expressing HepG2, HBx-transgenic mice | DNMT`1 | DNA methylation | microarray, qRT-PCR | [[188](#_ENREF_188)] |
| miR-199a | Up | HBx-expressing HepG2, HepG2.2.15, HBV infected HCC | - | - | microarray, qRT-PCR | [[107](#_ENREF_107)] |
| miR-205 | Down | HBx-expressing HepG2, HBV-associated HCC |  | DNA methylation, cell proliferation | qRT-PCR | [[72](#_ENREF_72)] |
| miR-224 | Up | HBx-transgenic mice | Smad4 | Cell proliferation | *in-situ* hybridization, qRT-PCR | [[68](#_ENREF_68)] |
| **HBV-associated HCC** |
| miR-122 | Down | HBV-associated HCC | PBF | Cell proliferation | qRT-PCR | [[69](#_ENREF_69)] |
| Down | HBV-associated HCC | NDGR3 | Cell proliferation | qRT-PCR | [[95](#_ENREF_95)] |
| let-7 family | Down | HCC | Stat3 | Cell proliferation | microarray, qRT-PCR | [[107](#_ENREF_107)] |
| miR-199a/b-3p | Down | HCC liver samples (HBV and non-HBV) | PAK4 | ERK, cell proliferation | NGS, qRT-PCR | [[30](#_ENREF_30)] |
| miR-199b | Down | HBV-associated HCC | - | - | qRT-PCR | [[74](#_ENREF_74)] |
| miR-22 | Down | HBV-associated HCC | CDKN1A | Cell proliferation | qRT-PCR | [[167](#_ENREF_167)] |
| miR-29c | Down | HBV-associated HCC | TNFAIP3 | Cell proliferation | qRT-PCR | [[166](#_ENREF_166)] |
| miR-101 | Down | HBV-associated HCC | DNMT3A | DNA methylation | qRT-PCR | [[147](#_ENREF_147)] |
| miR-143 | Up | HBV-associated HCC | FNDC3B | Cell proliferation | qRT-PCR | [[168](#_ENREF_168)] |
| miR-145 | Down | HBV-associated HCC | - | - | qRT-PCR | [[74](#_ENREF_74)] |
| miR-224 | Up | HBV-associated HCC | - | - | qRT-PCR | [[74](#_ENREF_74)] |
| miR-224 | Up | HBV-associated HCC | Smad4 | cell proliferation | in-situ hybridization, qRT-PCR | [[68](#_ENREF_68)] |
| miR-501 | Up | HBV-associated HCC | HBXIP | HBV replication | microarray, qRT-PCR | [[81](#_ENREF_81)] |
| miR-602 | Up | HCC liver samples (HBV and non-HBV) | RASSF1A | Apoptosis, tumor suppressor | microarray, qRT-PCR | [[99](#_ENREF_99)] |

HBV: Hepatitis B virus; HBx: Hepatitis B virus -X protein; HCC: Hepatocellular carcinoma; miRNA: microRNA; qRT-PCR: Quantitative reverse transcription polymerase chain reaction.