based sequence of miR-122 and the uses of locked nucleic acid-miR-122 to control the function of liver miR-122 (Janssen et al. 2013). Biomarkers and nucleic acid drugs will be applied clinically in the near future.

6 MicroRNAs in Hepatitis B Virus; Expression, Regulation and Function

6.1 Basic Knowledge of HBV Infection

The hepatitis B virus (HBV) is a small enveloped partially double-stranded DNA virus that belongs to the *Hepadnaviridae* family. This virus primarily infects hepatocytes and causes acute and chronic liver disease. Among the 2,000 million people worldwide infected with HBV, more than 350 million remain chronically infected and become carriers of the virus (Ganem and Prince 2004). Epidemiological studies have revealed that chronic HBV infection is the major etiological factor in the development of HCC. Indeed, more than a half of the HCC patients are HBV carriers (Parkin 2006). Despite the availability of an efficacious vaccine, persistent HBV infection remains a challenging global health issue that requires a better understanding of the virus biology and pathogenesis for improved control and treatment.

The life cycle of HBV is complex (Fig. 7.5a). The initial stages of the acute HBV infection, including virion attachment, uncoating and nucleocapsid transport to the cell nucleus, are still poorly understood (Seeger and Mason 2000; Yan et al. 2012). Once delivered into the nucleus, the 3.2 kb relaxed circular DNA genome is converted into a covalently closed circular DNA (cccDNA) from which all the viral RNAs are transcribed. These transcripts include the pregenomic RNA (pgRNA) that will serve as template for reverse transcription and the subgenomic mRNAs that derive from the four overlapping gene sequences composing the viral genome. These sequences comprise the pre-S and surface genes, the precore and core genes, the polymerase gene, and the X gene. The newly formed nucleocapsids can either assemble with envelope proteins in the endoplasmic reticulum and form mature virion that will be secreted, or return to the nucleus to maintain the cccDNA amplification. When the immune system fails to clear the virus, the HBV infection becomes chronic (Fig. 7.5b). Eventually, the viral genetic material or sequences can integrate into the host cellular DNA. The integration has been frequently observed and is associated with HCC (Brechot et al. 1980; Paterlini-Brechot et al. 2003).

MiRNAs play key roles in the regulation of almost every cellular process in all multicellular eukaryotes (Bartel 2009). As intracellular pathogens, viruses are affected by these post-transcriptional modulators and have found a way to subvert their effects. Several viruses, especially the herpesviruses, encode for miRNAs that increase their replication potential and/or allow the evasion from the innate immune system (Skalsky and Cullen 2010). This chapter will outline the implication of miRNAs in the HBV biology and the associated pathogenesis, including HCC development. We will also outline the present and future miRNA-based strategies for the diagnosis, prognosis and treatment of the HBV-related HCC.

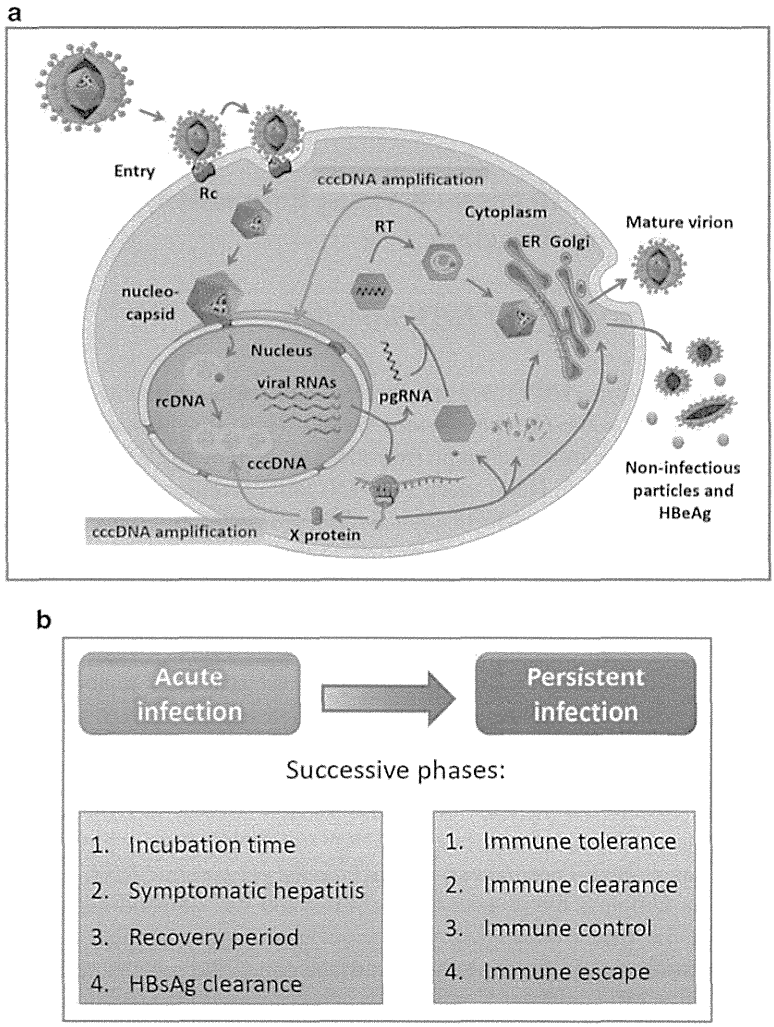

Fig. 7.5 The HBV infection (**a**) Schematic representation of the HBV life cycle. (**b**) HBV natural history of infection. Abbreviations: *cccDNA* covalently closed circular DNA, *ER* endoplasmic reticulum, *HBeAg* hepatitis B extracellular "e" antigen, *HBsAg* HBV surface antigen, *pgRNA* pregenomic RNA, *Rc* receptor, *rcDNA* relaxed circular DNA, *RT* reverse transcription

6.2 MiRNAs Involved in the Regulation of HBV Gene Expression, Replication and Effects on the Carcinogenesis

Viruses, nuclear DNA viruses in particular, need some time to complete their life cycle. During this period, the host cell can develop defense mechanisms such as cell cycle arrest and viral clearance. By taking advantage of the cellular miRNA machinery,

Table 7.2 Cellular miRNAs and their effects on HBV biology, pathogenesis or related-HCC HBV (↑): Promotes HBV replication, HBV (↓): Inhibits HBV replication, HCC (↑): Development and/or growth of HCC

Target genes	miRNAs	miRNA expressions	HBV or HCC status	Reference
Viral target genes				
HBsAg	miR-199-3p	Up	HBV(↓)	Zhang et al. (2010)
HBVpre-S1	miR-210	Up	HBV(↓)	Zhang et al. (2010)
Cellular target genes				
HDAC4	miR-1	Up	HBV (↑)	Zhang et al. (2011a)
c-myb	miR-15a	Down	HCC (↑)	Liu et al. (2009)
E2F1 (c-myc repressor)	miR-17-92 cluster	Up	HCC (↑)	Connolly et al. (2008)
PTEN (?)	miR-21	Up	HCC (↑)	Connolly et al. (2008)
cyclin G1 (p53 modulator)	miR-122	Down	HBV (↑), HCC (↑)	Wang et al. (2012)
DNMT1	miR-152	Down	HBV (↓)	Huang et al. (2010)
SOCS1 (STAT inhibitor)	miR-155	Up	HBV(↓)	Su et al. (2011)
HLA-A (miR-181)	miR-181a, -181b, 200b	Up	HBV (↑)	Liu et al. (2009)
NFIB	miR-372,-373	Up	HBV (↑)	Guo et al. (2011)
STAT3	let-7 family	Down	HBV (↑?), HCC (↑)	Wang et al. (2010)

these viruses can more easily and efficiently help to promote a favorable cellular environment for viral replication and achievement of the life cycle (Skalsky and Cullen 2010). The modulation of the machinery could be made by direct action on the cellular miRNAs (Backes et al. 2012; Jopling et al. 2005) (inhibition or up-regulation) or by expression of their own miRNAs that will mimic their cellular counterparts (Gottwein et al. 2007; Lu and Cullen 2004). Despite the fact that HBV is a nuclear DNA virus, none viral-encoded miRNA has been identified so far. Only one putative HBV-miRNA, with hypothetical regulation role on its own genome, was deduced by computational approach (Jin et al. 2007). However, several cellular miRNAs are involved in the HBV viral replication. They are presented here above and summarized in Table 7.2.

6.2.1 Cellular miRNAs That Promote HBV Replication

MiR-1 can enhance the HBV core promoter transcription and thus increase the viral replication by modulating the expression of several host genes such as transcription factors (Zhang et al. 2011a). The report has confirmed that the histone deacetylase 4 (HDAC4) expression is down-regulated by miR-1. Knowing that the cccDNA amplification is controlled by epigenetic regulation (Pollicino

et al. 2006), miR-1 could act in complementarity with the nuclear HBV X protein (HBx) in order to induce these modifications (Belloni et al. 2009). However, miR-1 can also inhibit the cell proliferation and even induce a reverse cancer cell phenotype (Zhang et al. 2011a). The roles of miR-1 in the cell proliferation and hepatocellular carcinogenesis (Datta et al. 2008) seem to be contradictory with the viral replication and with the characteristics of oncogenic virus but must represent benefit for HBV survival.

Another miRNA, miR-501, has also been suggested to work together with HBx for the benefit of viral replication (Jin et al. 2013). HBx itself has also the ability to deregulate the cellular miRNAs expression. This small protein is a key regulator of HBV infection. It is usually over-expressed in HCC and accumulated evidence indicates that HBx can promote hepatocarcinogenesis by disrupting the normal physiologic mechanisms of the host cell (Chirillo et al. 1997; Lee et al. 2005; Tian et al. 2013). The let-7 family of miRNAs has been demonstrated to be negatively regulated by HBx (Wang et al. 2010). This miRNA family is often observed down-regulated in many cancers including HCC (Guo et al. 2006; Johnson et al. 2005; Yu et al. 2007). The consequence of this down-regulation is the increase activity of that signal transducer and activator of transcription 3 (STAT3) that supports the cell proliferation, and potentially the hepatocarcinogenesis.

Finally, the miRNAs can promote the viral replication by the indirect stimulation the HBV enhancer element I or II. It is the case for the CCAAT/enhancer binding protein that binds and activates the HBV enhancer II in a dose-dependent manner (Lopez-Cabrera et al. 1991). miR-372, together with miR-373, targets the nuclear factor I/B, an important regulator of several viruses (Nagata et al. 1983), and so supports the HBV expression (Guo et al. 2011).

6.2.2 Cellular miRNAs That Prevent HBV Replication

One of the best studied miRNAs in liver-related diseases is miR-122. This liver-specific miRNA is expressed at high levels in normal hepatocytes (about 70 % of the total miRNA population in the adult liver) (Lagos-Quintana et al. 2002) and is pivotal in numerous aspects of the liver function such as lipid metabolism, liver development, differentiation, growth and neoplastic transformation (Girard et al. 2008). The essential role of mir-122 in the HCV replication reflects furthermore the importance of this miRNA in the infection process (Jopling et al. 2005). While the loss of miR-122 expression is impeding HCV replication, it is enhancing the replication in the circumstance of HBV infection (Wang et al. 2012). In fact, miR-122 can negatively regulate the viral gene expression and replication by direct binding to a highly conserved sequence of HBV (Chen et al. 2011). This repression effect can apparently be impeded by a negative feedback loop involving the Heme oxygenase-1 (Qiu et al. 2010). A recent study has reported the indirect implication of HBx in miR-122 deregulation (Song et al. 2013) that could, at least partially, explain the difference observed between the two viruses. Knowing that miR-122 expression is low in HBV and HCC tissues (Wang et al. 2012; Kutay et al. 2006)

and that HBV replication is usually low or absent in HCC cells (Wong et al. 2006), miR-122 is a highly potential linker between HBV infection and liver carcinogenesis (Wang et al. 2012; Fan et al. 2011) and therefore a predilected target for future clinical applications.

The miR-17-92 cluster is also important in the HBV-associated HCC. This polycistron includes six miRNAs (miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a-1) and its up-regulated expression is associated with malignancies (Hayashita et al. 2005). By using human HBV-positive human HCC tissues, hepatoma cell lines and woodchuck hepatitis virus -induced HCC animal model (Popper et al. 1987), Connolly and colleagues were able to demonstrate the elevated expression of miR-17-92 cluster and its implication in the malignant phenotype (Connolly et al. 2008). The expression could be amplified by c-myc activation (He et al. 2005), under HBx control (Terradillos et al. 1997), to contribute to HBV latency state (Jung et al. 2013). The consequence is the induction of liver oncogenesis. Since the RNA intermediates of HBV (pgRNA and transcripts) are good targets of miRNA action, it is not surprising to observe several cellular miRNAs with different binding sites. So, in addition to miR-122 that targets the polymerase region (Chen et al. 2011), the mir-199a-3p and mir-210 can repress the S and pre-S1 regions, respectively (Zhang et al. 2010).

All the examples illustrating cellular miRNAs as inhibitors of the viral replication are a bit difficult to comprehend initially because of their obvious negative effect on HBV infection. However, it can be understood by keeping in mind the survival of the virus into the host organism. The natural history of HBV infection shows often a transition from acute to chronic infection, especially in young children. This step corresponds to a failure of the immune system to eradicate the virus (Fig. 7.5b). One of the escape pathways is the successful adaptation to the immune-induced down-regulation of replication. The virus could evade the immune system by reaching a dormant state into the infected hepatocytes, under the cccDNA form, and survive until its eventual life cycle reactivation (Ganem and Prince 2004; Belloni et al. 2009, 2012; Huang et al. 2010). The study of Huang and colleagues reports the CpG islands methylation of the cccDNA by the DNA methyltransferase 1 (DNMT1) to prevent the viral gene expression and therefore the viral antigen presentation. DNMT1 over-expression is induced by a decrease of miR-152, under the effect of HBx (Huang et al. 2010).

6.3 MiRNAs in the Modulation of the Immune System and Effects on the Carcinogenesis

HBV must adapt to a very complex network in order to survive. It has to cope with the modification of homeostasis, the cell cycle arrest, the apoptosis and the destruction of the host cell by the immune cells. MiRNAs are also important in the development and function of immune system (Baltimore et al. 2008). Some miRNAs in particular are crucial for modulating innate and adaptive immune responses. MiR-155 has multi-roles during an innate immune response such as the regulation of the acute inflammatory response after recognition of pathogens by the toll-like

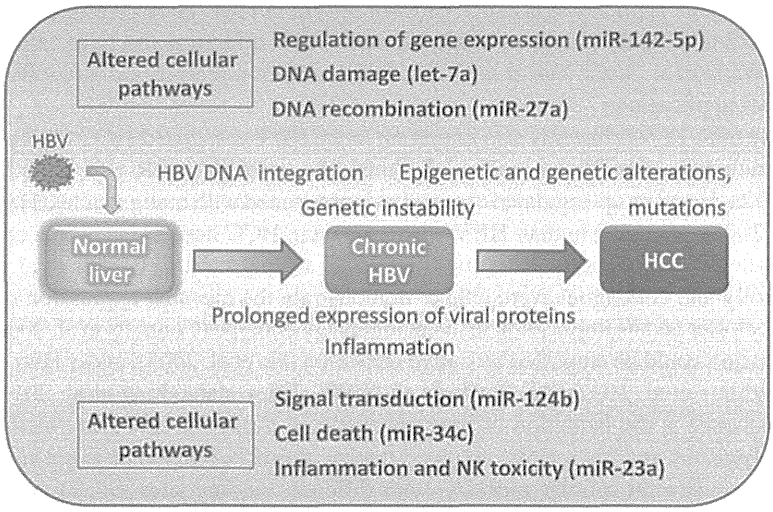

Fig. 7.6 Chronology of events from the HBV infection until HCC development. The indicated altered miRNAs and related pathways are based on the results from Ura et al. (2009)

receptors (O'Connell et al. 2007; Tili et al. 2007). The up-regulation of miR-155 can lead to prolonged exposure to inflammation, a well-known causal agent to cancers like HCC (Berasain et al. 2009). Two recent studies suggest a role of miR-155 in hepatocarcinogenesis and HBV infection (Table 7.2). Using HCC-induced mouse model, Wang and collaborators have demonstrated an oncogenic role of miR-155 at the early stages of the tumorigenesis (Wang et al. 2009a). On the other hand, the ectopic expression of miR-155 in human hepatoma cells enhances the innate immunity through promotion of the JAK/STAT pathway and down-regulates HBx expression (Su et al. 2011).

A study analyzing the modified expression profiles of miRNAs in a stable HBV-expressing cell line revealed the up-regulation of miR-181a (Liu et al. 2009) (Table 7.2). The deregulation of this miRNA in liver cell might participate to the establishment of HBV persistence through inhibition of the human leukocyte antigen A (HLA-A) -dependent HBV antigen presentation. To date, it is unclear if miRNAs altered in the host cell, like miR-181a and miR-146a also present in Liu's study, miRNAs involved in ubiquitous and cell-specific regulatory functions, could affect directly the immune cells. The presence of circulating miRNAs, as well as the existence of intercellular nanovesicle-mediated miRNA transfer and its impact on the environmental modulation, could potentially support that hypothesis (Arataki et al. 2013; Waidmann et al. 2012; Li et al. 2010, 2012; Zhou et al. 2011; Kogure et al. 2011). The current knowledge shows an altered miRNA profile expression between normal and HCC liver at the different stages, and between the HBV and HCV-induced HCC (Murakami et al. 2006; Li et al. 2008; Budhu et al. 2008; Ura et al. 2009). For the latest one, this reflects the variation in the cellular pathways that are modulated as a consequence of the viral infection (Fig. 7.6).

6.4 MiRNAs as Biomarkers and Treatment-Based Strategies for HBV Infection and HBV-Induced HCC

It is important to know the precise mechanisms, the cellular pathways that the viral infection or cancer cells alter in the different steps of the infection and/or tumor evolution. The knowledge will allow developing powerful targeted therapeutical strategies. The significance of miRNAs in antiviral immunity and liver carcinogenesis emphasizes their values as therapeutic targets for HBV infection and HBV-induced HCC. MiR-122 and miR-18a are of particular interest. They are both released in the blood and could be used as potential non-invasive biomarkers for HBV-related HCC screening (Liu et al. 2009; Waidmann et al. 2012; Li et al. 2012). Some other reports suggest using a miRNA panel in order to improve the specificity of the test (Li et al. 2010; Zhou et al. 2011). In addition with the current routinely used markers such as HBV surface antigen, HBV extracellular antigen and alanine aminotransferase, the circulating miRNAs represent a significant clinical value for better evaluation of the HBV-infection status, liver injury and early diagnosis of HCC.

In the therapeutic perspective, the work of Ura's group is valuable. They analyzed the livers of HBV and HCV positive patients with HCC to identify the miRNAs that are differentially expressed. Nineteen miRNAs were clearly differentiated between HBV and HCV groups, six specific for HBV and thirteen specific for HCV. Based on the miRNAs profile, they made a pathway analysis of candidate targeted genes and were also able to distinguish the cellular mechanisms altered in HBV or HCV-infected livers (Ura et al. 2009). The HBV infection alters mostly the pathways related to signal transduction, inflammation and natural killer toxicity, DNA damage, recombination, and cell death (Fig. 7.6), while HCV infection modifies those involved in immune response involving antigen presentation, cell cycle and cell adhesion (Ura et al. 2009).

Moreover, technological advances in the delivery of miRNA and RNA interference enable safe and efficient *in vivo* miRNA gene therapy, as exemplify by the recent study from Kota and collaborators on the liver cancer (Kota et al. 2009). They used an adeno-associated virus to deliver miR-26a in a mouse model of HCC. This resulted in the successful inhibition of the cancer cell proliferation, induction of the tumor-specific apoptosis, and protection from disease progression without toxicity.

7 Concluding Remarks

MiRNAs have emerged as novel key players in the control of gene expression in cells. Investigations of their profiling have unveiled specific miRNA deregulations in tumors and in condition of viral infection. On the viral point of view, the deregulated pathways mirror the strategies of the virus to allow its replication and evade the host defense mechanisms to survive. On the cellular point of view, they mirror the immune response that is trying to get rid of the intruder and that become

deregulated. In both cases, the viral infection leads to the alteration of miRNA expression by RSSs that can trigger tumorigenesis. Several oncogenic viruses, especially herpesviruses like EBV and KSHV, encode their own miRNAs to modify both cellular and viral gene expression (Pfeffer et al. 2004). This step is crucial for their latency phase. On the other hand, HPV, HBV and HCV do not express viral miRNAs but can affect the host miRNA pathway. The present and future knowledge about miRNA will broaden our understanding of the pathogenesis of oncogenic viruses and most certainly allow developing efficient oncogenic viral therapies.

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Successful Generation of Hepatitis B virus (HBV) Pseudotype Particle; a versatile tool for Identification of the HBV Receptor and Investigation of HBV infectivity

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Abstract

It is near a half century since hepatitis B virus (HBV) was identified. HBV receptor molecules and the entry mechanism of HBV into hepatocytes have not been elucidated completely, though there are some reports on infection systems and on the receptor molecules. Thus, we still have not reached finding a real HBV receptor and there have been no useful and convenient infection system in vitro and in vivo for HBV, which makes it impossible for us to understand a precise HBV life cycle and HBV involved related diseases. An HBV infection system is really needed to explore ways and means of treatment of HBV related diseases based on evidence as well. Here, we designed and tried to generate an HBV pseudotype, which has a viral particle containing a retrovirus capsid and a genome inside surrounded by HBV membrane proteins. We proved successful generation of this pseudotype by immunoprecipitation with anti-HBVs antibodies and by CsCl density gradient ultracentrifugation, followed by RT-PCR targeting a retroviral gene, an *EGFP* gene in this case, respectively. Though our established system is constructed on growth dependent integration of retroviral

34 genomes and thus was very hard to observe its infection in a primary human
35 hepatocytes culture system, successful generation of the HBV pseudotype will make it
36 possible for us to perform a biological assay to clone an HBV receptor based on
37 infectivity and will facilitate its separation and identification.
38
39
40 Key words; HBV, pseudotype, infection
41

Introduction

It has been near half a century, since hepatitis B virus (HBV) was identified by Blumberg [1]. Though treatment of chronic hepatitis B with interferons has been continued and nucleotide analogs, most of which were explored as anti-HIV agents, and prevention against HBV infection by vaccination has been developed, HBV infected patients yet reach three hundreds and fifty million people worldwide [2]. Thus, HBV infection has not been controlled and one of the biggest viral infections in the world.

There are several animal models; avian hepatitis B viruses such as duck hepatitis B virus (DHBV) and heron hepatitis B virus (HHBV), rodent hepatitis B viruses such as ground squirrel hepatitis virus (GSHV) and woodchuck hepatitis virus (WHV) [2; 3]. HBV shows highly species specific spectrum for its infection and can infect only primates and amplify mainly in parenchymal hepatocytes, but never infects the other animals including mice [2; 4]. Furthermore, even human hepatocyte-originated hepatocellular carcinoma cell lines such as HepG2, HuH7, HuH6, Hep3B and so on never permit HBV infection, or extremely limited even if possible,