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Review

Roles of microRNAs in the Hepatitis B Virus Infection and Related Diseases

Muriel Thirion and Takahiro Ochiya *

Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan; E-Mail: mthirion@ncc.go.jp

* Author to whom correspondence should be addressed; E-Mail: tochiya@ncc.go.jp; Tel.: +81-3-3542-2511; Fax: +81-3-3545-3567.

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Abstract: The hepatitis B virus (HBV) is a small enveloped DNA virus that belongs to the *Hepadnaviridae* family. HBV can cause acute and persistent infection which can lead to hepatocellular carcinoma (HCC). MicroRNAs (miRNAs) play a crucial role in the main cellular events. The dysregulation of their expression has been linked to the development of the cancer as well as to viral interference. This chapter will describe the involvement of miRNAs in the case of HBV infection and their implication in the development of the HBV-related diseases.

Keywords: hepatitis B virus; microRNA; hepatocellular carcinoma

1. Introduction

The microRNAs (miRNAs or miRs) are small non-coding RNAs of 19–23 nucleotides that play key roles in the regulation of almost every cellular process in all multicellular eukaryotes [1]. As intracellular pathogens, viruses are affected by these post-transcriptional modulators and have evolved to subvert them. Several viruses, especially the herpesviruses, encode for their own miRNAs that increase their replication potential and/or allow the evasion from the innate immune system [2]. Other viruses, such as the hepatitis B virus (HBV), modulate the cellular miRNAs in order to achieve the same effects.

HBV is a small enveloped DNA virus that belongs to the *Hepadnaviridae* family. It 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 [3]. Epidemiological studies have uncovered chronic HBV infection as the major etiological factor in the development of hepatocellular carcinoma (HCC) [4]. Despite the availability of an efficient vaccine, persistent HBV infection remains a challenging global health issue. The recent discovery of miRNAs involvement in HBV infection provides new insights into the virus biology and pathogenesis [5,6].

This chapter will outline the roles of miRNAs in the HBV biology and associated pathogenesis. We will also outline present and future miRNA-based strategies for the diagnosis, prognosis and treatment of the HBV-related diseases.

2. Biogenesis and Functions of miRNAs

miRNAs are most commonly transcribed in the nucleus by the RNA polymerase II (Pol II), as monocistronic or polycistronic pri-miRNAs that are further processed in pre-miRNAs (Figure 1). These pre-miRNAs are exported to the cytoplasm where they undergo cleavage by the RNAse III enzyme called Dicer that produces a miRNA duplex. This duplex splits to generate the single-stranded mature miRNA that incorporates the RNA-induced silencing complex (RISC). Based on the complementarity with its target gene sequence, the mature miRNA induce either translational repression (partial complementarity) or mRNA degradation (perfect complementarity) [7]. Besides, the mature miRNA can increase the expression of the target gene under growth arrest condition [8]. Finally, it has been recently reported that miRNA can also act in a RISC-independent manner on the transcriptional level by interaction with ribonucleoprotein or direct binding to DNA [9–11].

One single miRNA has the ability to regulate multiple targets and thereby to affect a broad network of genes (up to 100 genes) [12]. This specific characteristic makes the miRNAs key mediators of most of the cellular events. In animal, miRNAs mainly regulate mRNAs by interacting with their 5' end (5p) to the 3'-untranslated region (3'-UTR) of their target [13]. However, recent studies have revealed miRNAs target sites in the 5'-UTR, which interacts with the 3' end (3p) of miRNAs, and even simultaneous 5'-UTR and 3'-UTR interaction sites [14,15].

The abnormal expression levels of miRNAs have been revealed in various diseases such as cancer [16,17], inflammation [18,19], Alzheimer [20], cardiovascular disease [21] and viral infection including HBV [2,22].

3. Role of miRNAs in HBV Infection

Despite the fact that HBV is a nuclear DNA virus, none viral-encoded miRNA has been so far identified. Only one putative HBV miRNA, with hypothetical regulation role on its own genome, was deduced by computational approach [23]. However, HBV can modulate the expression of several cellular miRNAs in order to promote a favorable environment for its replication and survival. They are presented in this section and summarized in Table 1.

3.1. Brief Description of HBV Infection

The HBV infection is characterized by two phases; the acute and the chronic infection [24]. The initial stages of the acute infection include virion attachment [25], uncoating and nucleocapsid transport to the cell nucleus (Figure 2, steps 1 and 2). The 3.2 kb relaxed circular DNA genome is released into the nucleus and converted into a covalently closed circular DNA (cccDNA) from which all the viral RNAs are transcribed (Figure 2, steps 3 to 5). The pregenomic RNA (pgRNA) serves as template for reverse transcription (Figure 2, steps 8 and 9). The subgenomic mRNAs comprise the pre-surface (S) and S genes, the pre-core (C) and C 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 virions that will be secreted (Figure 2, steps 10 and 11), or return to the nucleus to maintain the cccDNA amplification. When the immune system fails to clear the virus, the HBV infection becomes chronic and it remains under a dormant state into the cell. 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 [26,27].

Figure 1. Schematic representation of miRNA biogenesis. The mature miRNAs originate from successive different steps. An initial DNA transcription generates pri-miRNAs that are cleaved in pre-miRNAs before their exportation to the cytoplasm. There, an RNAse III enzyme, Dicer, cleaves it to generate a miRNA duplex that subsequently joins an RNA-induced silencing complex (RISC) to produce the mature miRNA. The sequence complementarity to the target will decide its fate. Some miRNAs can act on the transcriptional level independently from the RISC.

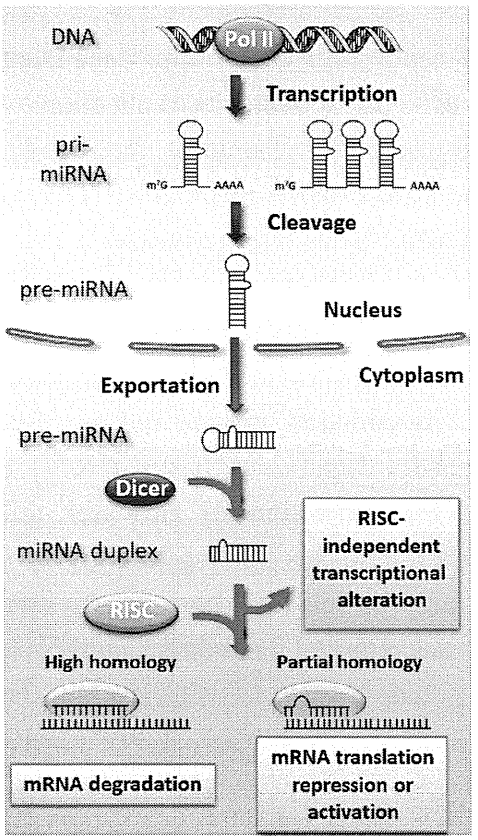


Table 1. Cellular miRNAs and their effects on HBV infection or HBV related-diseases. HBV (↑): Promotes HBV replication; HBV (↓): Inhibits HBV replication; HCC (↑): Development and/or growth of HCC; Fibrosis (↑): Promotes liver fibrosis.

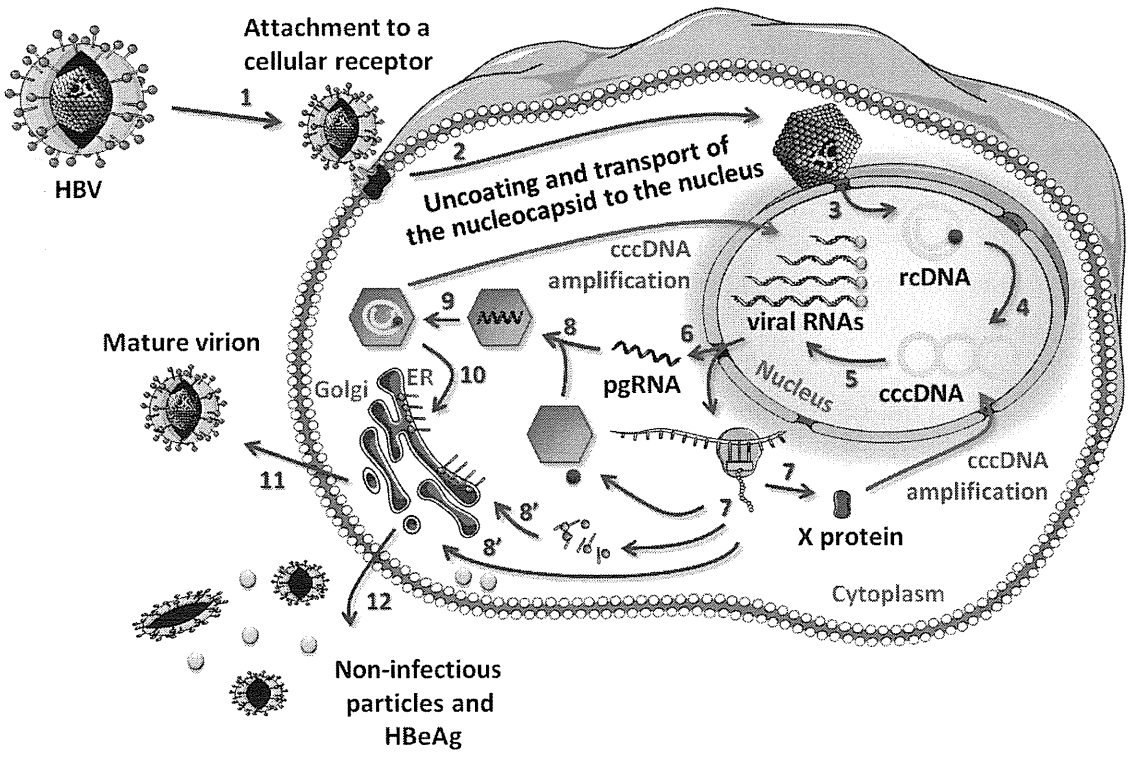
miRNAs	miRNA expression	Target genes	HBV or disease status	Ref.
		Cellular targets		
miR-1	up	HDAC4 (histone deacetylase 4)	HBV (↑)	[28]
miR-17-92 cluster	up	E2F1 (c-myc repressor)	HBV (↑?), HCC (↑)	[29]
miR-155	up	C/EBPβ (CCAAT/enhancer binding protein)	HCC (↑)	[30]
		SOCS1 (JAK/STAT signaling)	HBV (↓)	[31]
miR-181a	up	HLA-A? (MHC class I)	HBV (↑)	[5]
miR-372	up	NFIB (nuclear factor I/B)	HBV (↑)	[32]
miR-373	up	NFIB (nuclear factor I/B)	HBV (↑)	[32]
miR-501	up	HBIP (HBx inhibitor)	HBV (↑)	[33]
mir-29 family	down	collagen	Fibrosis (↑)	[22,34]
miR-122	down	cyclin G1 (p53 modulator)	HBV (↑), HCC (↑)	[6]
miR-152	down	DNMT1 (DNA methyltransferase 1)	HBV (↓)	[35]
let-7 family	down	STAT3 (transcription factor)	HBV (↑?), HCC (↑)	[36]
		Viral targets		
miR-122	up	HBV DNA polymerase	HBV (↓)	[37]
miR-125a-5p	up	HBsAg (HBV surface antigen)	HBV (↓)	[38]
miR-199-3p	up	HBsAg	HBV (↓)	[39]
miR-210	up	HBV pre-S1 (pre-surface 1)	HBV (↓)	[39]

3.2. Role of miRNAs in the HBV Replication

The role of miRNAs in HBV replication is therefore dependent on the phase of HBV infection. During the acute phase, the virus must activate its replication while avoiding destruction by the immune system. During the chronic phase, the virus reaches a "dormant" state where the viral replication must be restricted and viral evasion maintained. This leads to a time-dependent intricate interaction network in which miRNAs play an important role.

One of the best studied miRNAs in HBV infection and other 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) [40] and is pivotal in numerous aspects of the liver function such as lipid metabolism, liver development, differentiation, growth and neoplastic transformation [41]. While the loss of miR-122 expression impedes hepatitis C virus (HCV) replication [42], it enhances the replication in the circumstance of HBV infection [6]. However, miR-122 can negatively regulate the viral gene expression and replication by direct binding to a highly conserved sequence of HBV [37]. This repression effect can apparently be impeded by a negative feedback loop involving the Heme oxygenase-1 [43]. A recent study has reported the indirect implication of the HBV X protein (HBx) in miR-122 dysregulation [44] that could, at least partially, explain the difference observed between the two viruses.

Figure 2. Schematic representation of HBV life cycle. The virus infects a cell by an initial attachment to a cellular receptor that allows its internalization (step 1). In the cytoplasm, the virus is uncoated and the nucleocapsid is transported to the nuclear membrane (step 2). The viral genome is released into the nucleus under its relaxed circular form (rcDNA) and converted into a covalently closed circular DNA (cccDNA) from which all the viral RNAs are produced (steps 3 to 5). The viral RNAs transfer to the cytoplasm for traduction of the different viral proteins (steps 6 and 7) or for subsequent reverse transcription of the pregenomic RNA (pgRNA, steps 6, 8 and 9). All the viral components move to the proper place and assemble together to form new mature virions (steps 8, 10 and 11). The virus also produces non-infectious particles and extracellular antigen (HBeAg) as a decoy for the immune system of the host (step 12). The nucleocapsid containing the rcDNA and the HBV X protein (HBx) can go back to the nucleus in order to amplify the cccDNA and maintain the viral production.



On the other hand, miR-1 can enhance the HBV core promoter transcription by down-regulating the expression of the histone deacetylase 4 (HDAC4) [45]. This miRNA might act complementary to the nuclear HBx in order to induce epigenetic modifications on the cccDNA and amplify the viral genome [45,46].

miR-372, together with miR-373, also supports HBV gene expression by targeting the nuclear factor I/B [47]. This cellular protein is known to be an important regulator of several viruses [48].

The let-7 family of miRNAs has been demonstrated to be negatively regulated by HBx [36]. The consequence of this down-regulation is the increase activity of the signal transducer and activator of transcription 3 (STAT3) that supports cell proliferation, and potentially viral replication and hepatocarcinogenesis.

Finally, miR-501 has been suggested to work with HBx for the benefit of viral replication [33]. HBx itself has also the ability to dysregulate the cellular miRNAs expression. This small protein is a key regulator of HBV infection. It is usually overexpressed in HCC and is involved in hepatocarcinogenesis [48].

3.3. Role of miRNAs in the Immune Evasion of HBV

miRNAs are important in the development and function of immune system [49]. In particular, miR-155 has multi-roles during innate immune response such as regulation of the acute inflammatory response after recognition of pathogens by the toll-like receptors [50,51]. Su and collaborators demonstrated that ectopic expression of miR-155 in human hepatoma cells could enhance the innate immunity through promotion of the janus kinase (JAK)/STAT pathway and down-regulate HBx expression [31].

On the other hand, a study analyzing the modified expression profiles of miRNAs in a stable HBV-expressing cell line revealed the upregulation of miR-181a [5]. The dysregulation of this miRNA in liver cell might participate to HBV replication through inhibition of the human leukocyte antigen A (HLA-A)-dependent HBV antigen presentation.

It is now unclear if the miRNAs altered in the infected hepatocytes, such as miR-181a and miR-146 [5], that have specific regulatory functions in the immune cells as well [49], could affect directly these cells to support viral evasion. The presence of circulating miRNAs and the existence of intercellular nanovesicle-mediated miRNA transfer that modulates the environment, could potentially support that hypothesis [52–57].

3.4. Role of miRNAs in the Establishment of HBV Chronic Infection

The natural history of HBV infection shows often a transition from acute to chronic infection, especially in young children. The virus reaches a "dormant" state into the infected hepatocytes, under the cccDNA form, and survive until its eventual life cycle reactivation [3,35,45,58]. One study reported the CpG islands methylation of the cccDNA by DNA methyltransferase 1 (DNMT1) to prevent the viral gene expression and therefore the viral antigen presentation. The DNMT1 overexpression is induced by a decrease of miR-152, under the effect of HBx [35].

miR-1 illustrates the duality of actions that can be observed in the course of HBV infection. As said previously, this miRNA can promote viral replication but it can also inhibit the cell proliferation and even induce a reverse cancer cell phenotype [28]. The effect on HCC was confirmed in another study [59].

Also, miR-122 can bind directly to the polymerase region in order to repress its expression [37]. Similar observations were made for miR-125a-5p, miR-199a-3p that can affect the S region and miR-210 that can affect the pre-S1 region [38,39]. Since the RNA intermediates of HBV (pgRNA and transcripts) are good targets of miRNA action, it is not surprising to observe several cellular miRNAs targeting them. However, it remains to be determined whether the targeting of HBV transcripts represents an active anti-viral mechanism of the host or if the virus has evolved to hijack these cellular miRNAs in order to reach its "dormant" state.

4. Role of miRNAs in HBV-Related Diseases

The modifications induced as a result of HBV infection profoundly alter the cellular and overall organism homeostasis. They are usually associated with diseases, including liver cirrhosis with fibrosis and HCC. The liver cirrhosis turns most of the time into HCC.

4.1. Role of miRNAs in HBV-Related Cirrhosis

Numerous studies have tried to identify and characterize the miRNAs involved in liver cirrhosis and therefore differently expressed during this intermediate phase. Roderburg et al. investigated the role of miRNAs in liver fibrosis on a carbon tetrachloride-induced hepatic fibrogenesis and bile-duct ligation mouse models [34]. They observed a significant down-regulation of all the members of the miR-29 family in the two models. The decreased expression was induced by the transforming growth factor beta (TGF-β), inflammatory signals and the nuclear factor kappa B (NFκB) pathways. miR-29c was also identified in another report focusing on the miRNA expression profile in patients with HCC-positive or HCC-negative chronic hepatitis B and hepatitis C virus [22] (Table 1).

Nevertheless, the global miRNA expression profile analysis of human liver tissues from different inflammation, infection and cancer states are not always consistent. It sometimes revealed a particular profile due to the association of both viral hepatitis and cirrhosis [60] or regarding to the type of viral hepatitis [22] and sometimes showed no difference [61]. Further experiments are therefore required to identify the exact molecular mechanisms implicating miRNAs and viral components in the development of cirrhosis and in the transition from cirrhosis to HCC in the patients with chronic viral hepatitis.

4.2. Role of miRNAs in HBV-Related HCC

When the cellular modifications and inflammation are too high and maintained for too long, the liver cirrhosis usually evolves into HCC.

The miR-17-92 cluster is important in the HBV infection and associated HCC. This polycistron includes six miRNAs (mir-17-5p, miR-18a, miR-19a, miR-19b, miR-20a and miR-92a-1) and its upregulated expression is associated with malignancies [62]. By using human HBV-positive human HCC tissues, hepatoma cell lines and woodchuck hepatitis virus-induced HCC animal model [63], Connolly and colleagues were able to demonstrate the elevated expression of miR-17-92 cluster and its implication in the malignant phenotype [29] (Table 1). The expression could be amplified by c-myc activation [64], under HBx control [65], to contribute to HBV latency state [66]. The consequence is the induction of liver oncogenesis.

Because of its role in immune response, miR-155 is also implicated in hepatocarcinogenesis. Indeed, its upregulation can lead to prolonged exposure to inflammation, a well-known causal agent to cancers like HCC [67]. Using HCC-induced mouse model, Wang and collaborators have demonstrated the oncogenic role of miR-155 at the early stages of the tumorigenesis [30] (Table 1).

To conclude, the liver-specific miR-122 has been extensively studied in the liver-associated diseases. Its expression is low in HCC tissues, including those with viral chronic hepatitis [6,68] (Table 1). As described in point 3.2, the regulation of miR-122 is very complex and helps either promotion or

inhibition of the HBV replication. In HCC cells, the "dormant" state of HBV implicates a replication rate very low or inexistent [69]. The recent data accumulate evidence of miR-122 as a highly potential linker between HBV infection and liver carcinogenesis [6,70]. Because of its characteristics, miR-122 is therefore a target of choice for future clinical applications.

5. miRNAs as Molecular Tools Against HBV Infection and HBV-Related Diseases

The significance of miRNAs in viral replication, antiviral immunity and liver carcinogenesis emphasizes their values as diagnostic, prognostic and therapeutic targets for HBV infection and HBV-induced diseases.

miR-122 and miR-18a are of particular interest for diagnostic and/or prognostic applications. They are both released in the blood and could be used as potential non-invasive biomarkers for HBV-related HCC screening [5,53,54]. Some other reports suggest the use of a miRNA panel in order to improve the specificity of the test [55,56]. In addition with the current routinely used markers such as HBV surface antigen (HBsAg), HBV extracellular antigen (HbeAg) and alanine aminotransferase (ALT), 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 liver cirrhosis is an event prior to HCC development and being able to interfere with this process would prevent carcinogenesis. For example, a strategy based on administration of miR-29 mimic might prevent liver fibrosis (Section 4.1) [34]. However, the disease is often discovered when hepatocarcinogenesis has already developed and HCC does not always show underlying cirrhosis [71]. Finding therapeutic targets involved in HCC is thus a major issue.

For this purpose, the work of Ura's group is valuable [22]. 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. The HBV infection alters mostly the pathways related to signal transduction, inflammation and natural killer toxicity, DNA damage, recombination, and cell death, while HCV infection modifies those involved in immune response involving antigen presentation, cell cycle and cell adhesion. Although very interesting, their results are not consistent with those presented in other reports [60,61] and confirmation of the targets needs to be done before considering their clinical application.

Finally, 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 colleagues on the liver cancer [72]. 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.

6. Conclusions

miRNAs have emerged as new key players in the control of gene expression in cells. Investigations of their profiling have unveiled specific miRNA dysregulations in tumors and during viral infection.

The HBV is a widespread pathogen that is implicated in HCC development. Numerous cellular miRNAs interacting with HBV have been identified. They reflect the cellular pathways that are altered as a result of the viral infection, viral infection that triggers the liver cirrhosis and carcinogenesis as side effects. On the viral point of view, the dysregulated 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 tries to get rid of the intruder and that becomes dysregulated. The present and future knowledge about the interaction between miRNA, HBV infection and HCC development and progress will probably allow developing strategies and tools to cope, efficiently and at various steps, with the liver carcinogenesis induced by HBV infection.

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Conflicts of Interest

The authors declare no conflict of interest.

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LIVER BIOLOGY/PATHOBIOLOGY

miR-148a Plays a Pivotal Role in the Liver by Promoting the Hepatospecific Phenotype and Suppressing the Invasiveness of Transformed Cells

Luc Gailhouste,[1] Laura Gomez-Santos,[1,2] Keitaro Hagiwara,[1] Izuho Hatada,[3] Noriyuki Kitagawa,[4] Kazushi Kawaharada,[5] Muriel Thirion,[1] Nobuyoshi Kosaka,[1] Ryou-u Takahashi,[1] Tatsuhiro Shibata,[4] Atsushi Miyajima,[6] and Takahiro Ochiya[1]

MicroRNAs (miRNAs) are evolutionary conserved small RNAs that post-transcriptionally regulate the expression of target genes. To date, the role of miRNAs in liver development is not fully understood. By using an experimental model that allows the induced and controlled differentiation of mouse fetal hepatoblasts (MFHs) into mature hepatocytes, we identified miR-148a as a hepatospecific miRNA highly expressed in adult liver. The main finding of this study revealed that miR-148a was critical for hepatic differentiation through the direct targeting of DNA methyltransferase (DNMT) 1, a major enzyme responsible for epigenetic silencing, thereby allowing the promotion of the "adult liver" phenotype. It was also confirmed that the reduction of DNMT1 by RNA interference significantly promoted the expression of the major hepatic biomarkers. In addition to the essential role of miR-148a in hepatocyte maturation, we identified its beneficial effect through the repression of hepatocellular carcinoma (HCC) cell malignancy. miR-148a expression was frequently down-regulated in biopsies of HCC patients as well as in mouse and human HCC cell lines. Overexpressing miR-148a led to an enhancement of albumin production and a drastic inhibition of the invasive properties of HCC cells, whereas miR-148a silencing had the opposite consequences. Finally, we showed that miR-148a exerted its tumor-suppressive effect by regulating the c-Met oncogene, regardless of the DNMT1 expression level. *Conclusion*: miR-148a is essential for the physiology of the liver because it promotes the hepatospecific phenotype and acts as a tumor suppressor. Most important, this report is the first to demonstrate a functional role for a specific miRNA in liver development through regulation of the DNMT1 enzyme. (HEPATOLOGY 2013;58:1153-1165)

MicroRNAs (miRNAs) constitute a group of evolutionary conserved small noncoding RNA molecules that finely regulate gene expression by complementary base pairing with the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs). The past decades have seen an increasing recognition of the overall significance of miRNAs in regulating a wide variety of fundamental biological phenomena and diseases,[1,2] including cancer.[3-5] The functional significance of miRNAs in cell specification and vertebrate development has been recently tackled.[6] For instance, miR-124 and miR-9,[7,8]

Abbreviations: 5-Aza, 5-Aza-2'-deoxycytidine; Afp, alpha-fetoprotein; Alb, albumin; Ab, antibody; Ck19, cytokeratin 19; CLD, chronic liver disease; c-Met, hepatocyte growth factor receptor; COBRA, combined bisulfite restriction analysis; Cyp, cytochrome P450; Dnmt, DNA methyltransferase; E-cadherin, epithelial cadherin; FBS, fetal bovine serum; G6pc, glucose-6-phosphatase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HGF, hepatic growth factor; MFH, mouse fetal hepatoblast; mRNAs, messenger RNAs; miRNA, microRNA; PAS, periodic acid-Schiff; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; siRNA, small interfering RNA; Tat, tyrosine aminotransferase; 3'-UTR, 3'-untranslated region.

From the [1]Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo, Japan; [2]Metabolomics Unit, CIC bioGUNE, Bizkaia, Spain; [3]Laboratory of Genome Science, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi, Japan; [4]Division of Cancer Genomics, National Cancer Center Research Institute, Tokyo, Japan; [5]DS Pharma Biomedical Co. Ltd., Research and Development Division, Osaka, Japan; and [6]Institute of Molecular and Cellular Biosciences, The University of Tokyo, Tokyo, Japan.

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two brain-enriched miRNAs, appear to be essential in neurogenesis, whereas miR-27b is relevant for myogenesis.[9] To date, little is known regarding the role and function of miRNAs in liver development. Hand et al. provided the first link between miRNAs and hepatobiliary development by emphasizing the functional role of miR-30a during biliary morphogenesis in zebrafish.[10] In humans, miR-122 might be of prime interest because it represents more than 70% of the total amount of miRNAs expressed in the adult liver, where it acts in metabolism regulation and hepatic homeostasis.[11]

During development, epigenetic modifications are essential for the modulations of tissue-specific gene expression that promote cell differentiation.[12] Epigenetic silencing includes reversible DNA methylation, which is primarily orchestrated by DNA methyltransferases (DNMTs). DNMT1 represents the major enzyme responsible for the maintenance of DNA methylation patterns during replication.[13] In contrast, DNMT3a and DNMT3b have been identified as *de novo* methyltransferases, which methylate DNA during early development and gametogenesis,[14] although DNMT1 also possesses *de novo* methylation activity. Inactivation of the DNMT1 enzyme in mice results in loss of genomic imprinting and leads to early embryonic lethality.[15] In addition, studies using methylation-deficient mouse embryos (*Dnmt1*$^{-/-}$, *Dnmt3a*$^{-/-}$, and *Dnmt3b*$^{-/-}$) have demonstrated that restoring DNA methylation is essential for development.[14,16] More recently, Sen et al. observed the enrichment of DNMT1 protein in epidermal progenitors, where it is required to maintain proliferative strength and suppress differentiation.[17] Their study also showed that DNMT1 depletion was associated with the altered proliferation and transition from progenitors to premature epidermal cells. In the liver, DNMT1 expression is frequently increased in tissues affected by chronic hepatitis and cirrhosis and, more dramatically, in hepatocellular carcinoma (HCC), in which DNMT1 augmentation correlates with poor prognosis.[18,19]

This study aimed to investigate the potential role of miRNAs in hepatic development. By taking advantage of an experimental primary cell-culture model that can trigger hepatic differentiation, we performed mouse miRNA microarray analyses and identified 10 miRNAs, which were selected for their predicted aptitude to target DNMT1. Among those miRNAs, miR-148a showed a strong induction in differentiating liver progenitors. Conversely, DNMT1 expression presented a rapid decline after stem cell entry into the differentiation process. We reported a correlation between the elevation of miR-148a and the promotion of the hepatospecific phenotype through the silencing of DNMT1. Because a significant down-regulation of miR-148a was observed in HCC, the role of miR-148a in liver cancer was also considered. We demonstrated the ability of miR-148a to suppress the invasive properties of transformed hepatic cells by inhibiting c-Met expression. In line with these findings, miR-148a was shown to play an essential role in the fate of the liver by inducing hepatospecific gene expression and suppressing tumor cell invasion.

Materials and Methods

Mouse Fetal Hepatoblast Model. Mouse fetal hepatoblasts (MFHs) were isolated and triggered to differentiate into mature hepatocytes as previously described.[20] Briefly, the method was based on the selective harvesting of hepatic parenchymal stem cells from mouse fetuses (E14.5). After their isolation, fetal liver tissues were dissociated physically and enzymatically in the presence of liberase (Liberase TM Research Grade; Roche Diagnostics, Mannheim, Germany). The sorting of epithelial cadherin (E-cadherin)-positive progenitors was performed using the biotin anti-CD324 (E-cadherin) antibody (Ab) (eBioscience, Inc., San

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Address reprint requests to: Takahiro Ochiya, Ph.D., Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. E-mail: tochiya@ncc.go.jp; fax: +81-3-5565-0727.

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Diego, CA) and the EasySep Mouse Biotin Positive Selection Kit (STEMCELL Technologies Inc., Vancouver, British Columbia, Canada). From seeding, MFHs were maintained in a medium composed of the following mixture: William's E Medium, L-glutamine (2 mM), penicillin (50 IU/mL), and streptomycin (50 μg/mL), all from Gibco (Grand Island, NY), insulin (5 μg/mL; Sigma-Aldrich, St. Louis, MO), epidermal growth factor (25 ng/mL; Sigma-Aldrich), and 10% fetal bovine serum (FBS; HyClone; Thermo Fisher Scientific, Waltham, MA) supplemented with essential hepatocyte phenotype-promoting factors, including hepatic growth factor (HGF; 25 ng/mL; PeproTech Inc., Rocky Hill, NJ), oncostatin M (12.5 ng/mL; Sigma-Aldrich), hydrocortisone 21-hemisuccinate (5×10^{-7} M; Sigma-Aldrich), and dexamethasone (10^{-7} M; Sigma-Aldrich). The medium was replaced daily.

HCC Cell Lines and Human Samples. Mouse Hepa 1-6 and human HepG2 and Hep3B cells were purchased from the American Type Culture Collection (Manassas, VA). Huh-7 cells were from Riken BioResource Center (RIKEN BRC, Ibaraki, Japan). Cells were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented with penicillin (100 IU/mL), streptomycin (100 μg/mL), and 10% FBS. Human samples included 39 pairs of primary HCCs and their corresponding nontumor tissues. All patients exhibited chronic liver disease (CLD) related to hepatitis B (HBV) or C virus (HCV) infection (n = 18 and 21, respectively). Normal liver samples were collected from patients who had surgical resection of metastasis in the liver. Human fetal livers were obtained from spontaneously aborted fetuses (see Supporting Table 2 for clinical data).

Additional Methods. miRNA and small interfering RNA (siRNA) transfection procedures for primary cultures and cell lines, DNA extraction, methylation assay, immunoblotting, total RNA extraction, miRNA microarray, miRNA, and mRNA expression analysis by reverse-transcription quantitative polymerase chain reaction (RT-qPCR), miRNA assessment in the serum of HCC patients, periodic acid-Schiff (PAS) staining, luciferase reporter assays, apoptotic activity, cell growth, wound healing, transwell invasion assays, and statistical tools are described in the Supporting Materials.

Results

MFH Is an Adequate Model for the Study of Hepatic Differentiation. To clarify the function of miRNAs in liver development, we used an *in vitro* model previously developed by our group based on the sorting of E-cadherin-positive fetal liver cells, called MFHs, and their induced differentiation into hepatocytes (Fig. 1A). MFHs underwent remarkable changes in morphology during the maturation-induced process that resulted in the formation of pronounced cell aggregates with cuboidal shape, polarity, and frequent binucleation (Fig. 1B). Importantly, mature-induced hepatocytes exhibited prominent glycogen storage ability. The molecular data were consistent with those observations and revealed a hepatospecific phenotype and progressive maturation of MFHs, as evidenced by the expression of the early (alpha-fetoprotein; Afp), mid- (albumin; Alb), and late (glucose-6-phosphatase [G6pc] and tyrosine aminotransferase [Tat]) hepatic markers (Fig. 1C). In addition, the major cytochrome P450s (CYPs) were similarly induced (Supporting Fig. 1). Conversely, the mRNA level of cytokeratin 19 (Ck19), which is commonly associated with liver stem cells and epithelial cells of the biliary tract, decreased rapidly after the initiation of the maturation process. Our data also indicate the rapid decline of Dnmt1 expression in association with MFH differentiation, whereas Dnmt3a and Dnmt3b increased progressively (Fig. 1D).

miR-148a Induction Is Observed in Hepatic Cells During Mouse Liver Development. To analyze the expression profile of miRNAs during hepatic differentiation, we performed an miRNA microarray by using the MFH model at different stages of maturation (Fig. 2A; all the miRNA microarray data are displayed in Supporting Table 1). Then, taking advantage of the combination of the publicly available search engines, miRNA (miRanda), TargetScan, and PicTar, we obtained a list of 12 miRNAs that could putatively target Dnmt1 (Table 1). Among those miRNAs, 10 were significantly expressed in differentiating MFHs. A family of three conserved miRNAs (miR-148a, miR-148b, and miR-152) was highlighted as a result of its remarkable expression pattern during the maturation process of MFHs (Fig. 2B). More explicitly, both microarray and RT-qPCR analyses revealed that miR-148a and miR-152 were gradually up-regulated from the MFH to the mature-induced hepatocyte stage (Fig. 2C). In contrast, miR-148b stayed unchanged. Obviously, miR-148a exhibited the most significant induction and highest expression level in mature hepatocytes. Similar profiles of expression for these miRNAs were obtained from fetal liver tissues during mouse development (Supporting Fig. 2). In addition, Dnmt1 expression was inversely correlated with the level of miR-148a

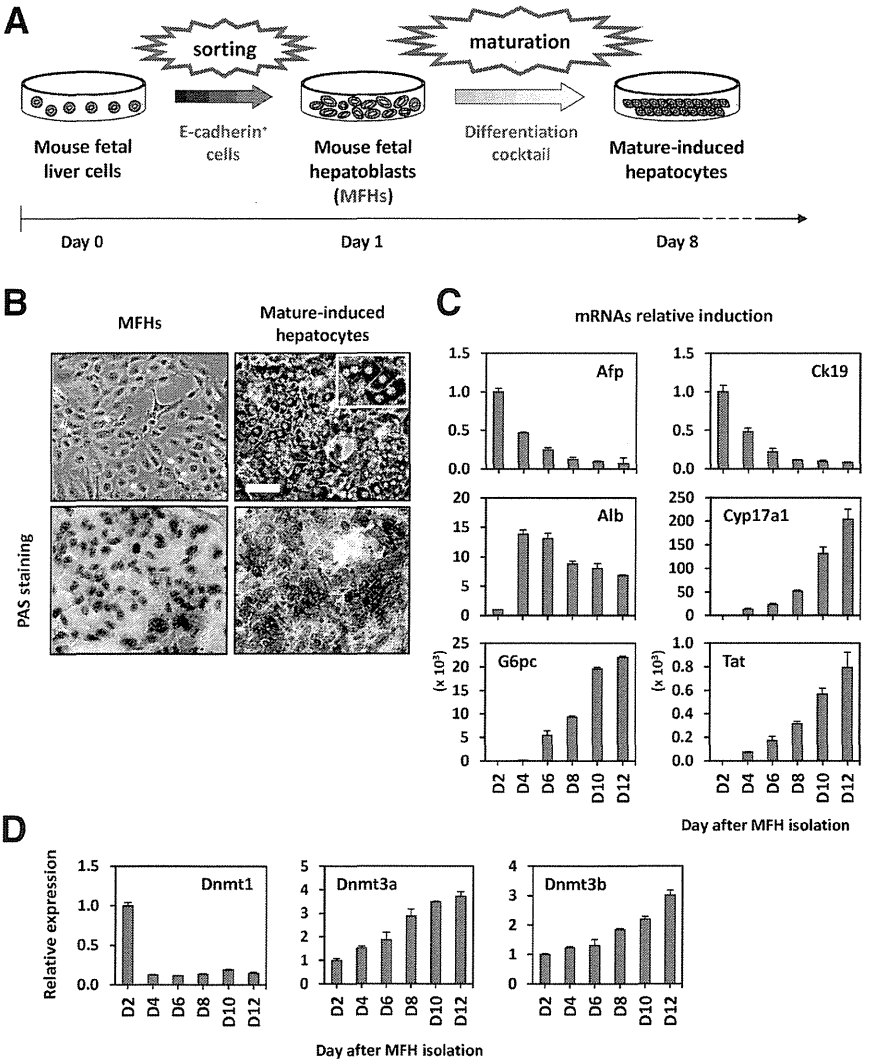

Fig. 1. Characterization of the MFH model. (A) Schematic representation of MFH purification and induced differentiation into mature hepatocytes after hepatotrophic factor stimulation. (B) Primary cultures of MFHs showing the radical changes undergone by undifferentiated hepatic progenitors to adopt the characteristic morphology of mature hepatocytes with polarity and frequent binucleation (white square). PAS staining revealed extensive glycogen storage in mature-induced hepatocytes, whereas MFHs were devoid of glycogen. Scale bar, 50 μm. Time course showing mRNA relative expression determined by RT-qPCR of (C) major hepatic markers and (D) Dnmt family members in the MFH model. The housekeeping gene, Gapdh, was used as an internal control to normalize the amount of complementary DNA.

in both *in vitro* and *in vivo* models, whereas Dnmt3a and Dnmt3b did not correlate. Consequently, it was hypothesized that miR-148a could play a critical role in liver development by regulating Dnmt1 expression.

miR-148a Is Down-Regulated in Human and Rodent HCC Cells. To explore the significance of miR-148a in the liver, we first compared expression profiles of miR-148a among mature-induced hepatocytes (MFH D8), undifferentiated hepatic stem cells (MFH D2), and the mouse HCC cell line, Hepa 1-6. Human Huh-7, HepG2, and Hep3B cells were also characterized in regard to normal adult and fetal hepatic tissues. As a result, a dramatic diminution of miR-148a was observed in both rodent (Fig. 3A) and human cell lines as well as in fetal livers (Fig. 3B). Moreover, the reduced expression of miR-148a was consistent with Dnmt1 augmentation in both species, arguing for a probable connection between miR-148a and Dnmt1. Thus, Spearman's rank correlation analysis showed that expression levels of DNMT1 and miR-148a in human samples were inversely correlated (rho: -0.609; $P = 0.0034$; Fig. 3C). To test the functional relevance of miR-148a down-regulation caused by DNA methylation, HCC cells were exposed to 5-Aza-2'-deoxycytidine (5-Aza). We found that demethylation treatment dramatically restored miR-148a expression in a dose-response manner in both Hepa 1-

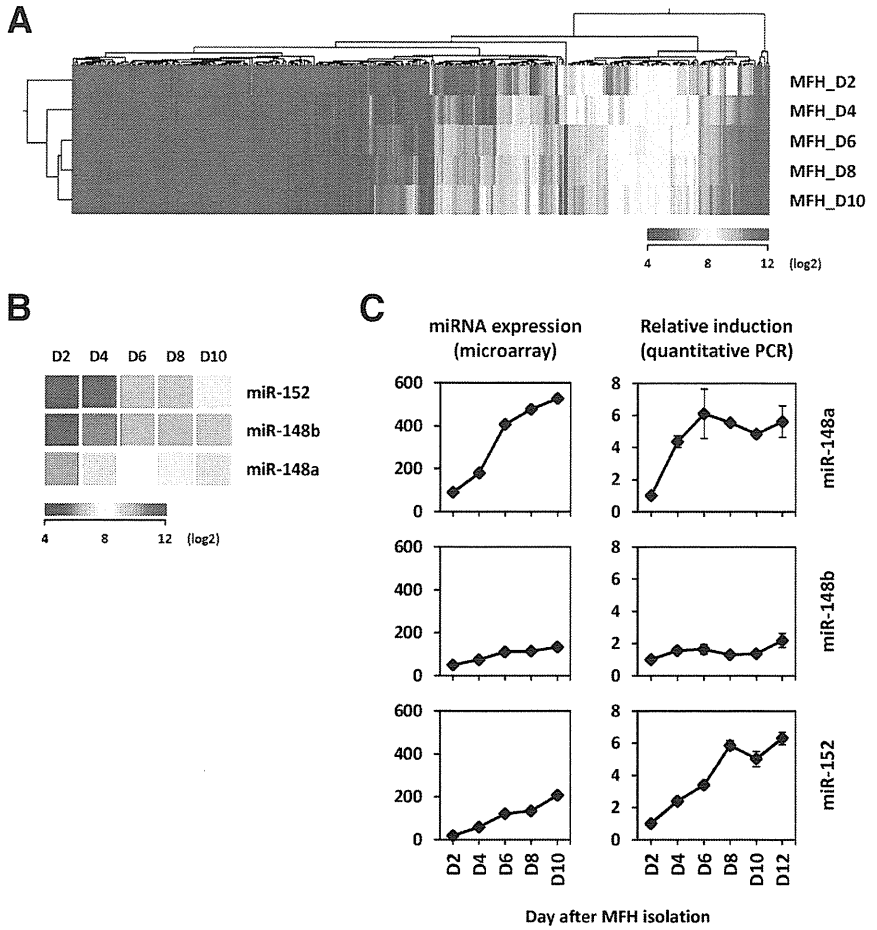

Fig. 2. Identification of miR-148a as a preponderant miRNA during hepatic differentiation. (A) miRNA global expression pattern during the process of MFH differentiation into mature hepatocytes. The scale bar encodes the logarithm of relative miRNA expression level. The 2-fold threshold was set to identify the miRNAs with significant differential expression. Microarray data are shown in Supporting Table 1. (B) Representative expression of miR-148a, miR-148b, and miR-152 selected for their significant induction during MFH differentiation and their predicted ability to target Dnmt1. (C) Differential expression of the miR-148a/148b/152 family evaluated by microarray and RT-qPCR. Relative expression levels determined by RT-qPCR were normalized against the endogenous control, RNU6B.

6 and HepG2 cell lines (Fig. 3D), indicating that a hypermethylation phenomenon is most likely responsible for the silencing of miR-148a in liver cancer cells. To verify this hypothesis, we first analyzed the genomic DNA sequence spanning of miR-148a and found that this gene had many CpG-rich regions (CpG islands) in its promoter. Subsequently, combined bisulfite restriction analysis (COBRA) was performed to examine the methylation status of the miR-148a promoter, which revealed hypermethylation of CpG islands in the miR-148a promoter in HepG2 cells, compared to normal human hepatocytes (Fig. 3E). We also observed that demethylation treatment by 5-Aza dramatically decreased the methylation status of the miR-148a promoter in both human and rodent HCC cell lines. Although the COBRA method did not reveal demethylation of the analyzed miR-148a CpG sites during the maturation process of MFHs (Supporting Fig. 3), bisulfite sequencing showed that the average methylation level of miR-148a was higher in undifferentiated MFHs (17.6% in MFH_D2), compared to differentiating cells (5.7% in MFH_D4), suggesting that a hypermethylation mechanism may participate in the regulation of miR-148a expression during development.

miR-148a Directly Modulates Dnmt1 Expression. We postulated that Dnmt1 inhibition during MFH maturation could be the result of its direct targeting by miR-148a. To explore this possibility, we first analyzed the consequences of miR-148a silencing or overexpression in HCC cell lines. The use of miR-148a mimics clearly affected Dnmt1 expression (Fig. 4A). Conversely, we observed a significant enhancement of Dnmt1 level after transfection with miR-148a antagonists up to 72 hours post-transfection. Dnmt1 contains a 3'-UTR element that is partially complementary to