MicroRNA-125b modulates cell growth and metabolism and HBV replication

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für

Biologie

an der

Universität Duisburg-Essen

Vorgelegt von

Wanyu Deng

Aus Nanchang, P.R.China

December 2013

Die der vorliegenden Arbeit zugrunde liegenden Experimente wurden am Institut für Virologie der Universität Duisburg-Essen oder durchgeführt.

1. Gutachter: Prof. Dr. Mengji Lu

2. Gutachter: Prof. Dr. Ralf Küppers

3. Gutachter:

Vorsitzender des Prüfungsausschusses: Prof. Dr.Peter Bayer

Tag der mündlichen Prüfung: 07.03.2014

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1 Introduction

1.1 Hepatitis B virus

1.1.1 Molecular structure

Hepatitis B virus (HBV) is a member of the hepadnavirus family and is one of the smallest enveloped animal viruses with a virion diameter of 42 nm (named Dane particle). The viral particle consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of protein. The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity similar to retroviruses (LOCARNINI 2004). The outer envelope contains embedded proteins which are involved in viral binding of, and entry into, susceptible cells. Besides the Dane particle, there are other two kinds of pleomorphic forms existing in patient serum, including filamentous and spherical bodies lacking a core (Figure 1.1). These two particles are not infectious and are composed of the lipid and protein that forms part of the surface of the virion, which is called the surface antigen (HBsAg), and is produced in excess during the life cycle of the virus (HOWARD 1986).

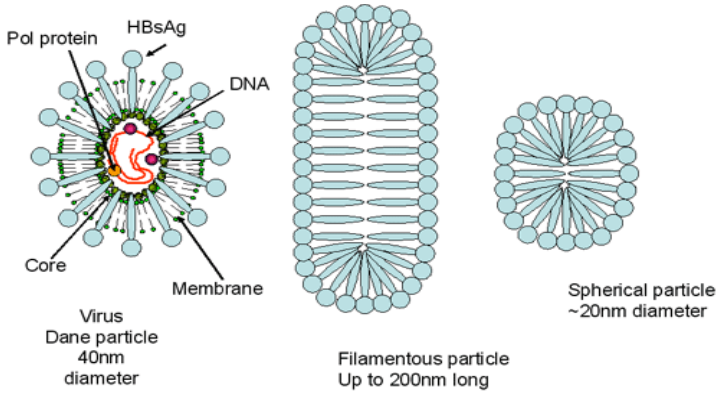

Figure 1.1 Schematic models of HBV three particle forms

From left to right: Dane particle, filamentous particle and spherical particle (HUNT 2011)

The HBV genome (3.2 kb) consists of a partially double stranded, relaxed circular DNA (rcDNA) and is extremely compactly organized with widely overlapping open reading frame encoding the four structural and two nonstructural viral proteins. In addition, regulatory elements like promoters, enhancers and the polyadenylation and encapsidation

signals overlap with coding sequences (QUASDORFF and PROTZER 2010) (Figure 1.2). HBV four known proteins encoded by the genome called C, X, P, and S. The core protein is coded by gene C (HBcAg), and its start codon is preceded by an upstream in-frame AUG start codon from which the pre-core protein is produced. HBeAg is produced by proteolytic processing of the pre-core protein. The DNA polymerase is encoded by gene P. Gene S is the gene that codes for the surface antigen (HBsAg). The HBsAg gene is one long open reading frame but contains three in frame of "start" (ATG) codons that divide the gene into three sections, pre-S1, pre-S2, and S. Because of the multiple start codons, polypeptides of three different sizes called large, middle, and small (pre-S1 + pre-S2 + S, pre-S2 + S, or S) are produced (BECK and NASSAL 2007).The function of the protein coded by gene X is not fully understood (BOUCHARD and SCHNEIDER 2004).

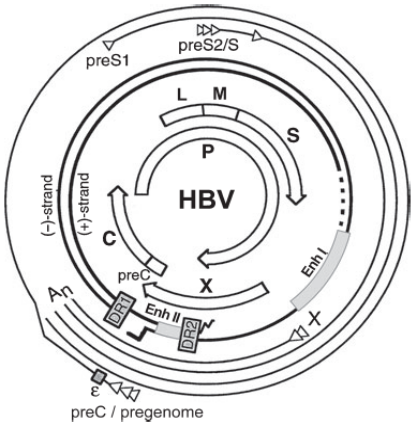

Figure 1.2 Genome organization of HBV

The HBV genome is a 3.2 kb, partially double stranded, relaxed circular DNA represented by the bold inner circles. The thin, outer lines represent the different classes of transcripts (3.5, 2.4, 2.1 and 0.7 kb) with arrowheads marking the start sites. Open arrows in the centre represent the four open reading frames encoding for the viral proteins (C, core protein; preC/C, hepatitis B e antigen; envelope proteins L, M and S; P, polymerase; X, X protein). Enhancer (Enh) I and II, direct repeat (DR) 1 and 2, the common polyadenylation site (An) and the encapsidation signal are indicated (QUASDORFF and PROTZER 2010).

1.1.2 Life cycle

HBV is one of a few known non-retroviral viruses which use reverse transcription as a part of its replication process so that its life cycle is complex (Figure 1.3). The early steps of how HBV virion infects hepatocytes are still unclear, but it is thought that first of all, HBV may be reversible and non-cell-type specific attachment to cell associated heparan

sulfate proteoglycans, then specific and probably irreversible binding to an unknown hepatocyte-specific preS1-receptor. This step presumably requires activation of the virus resulting in exposure of the myristoylated N-terminus of the L-protein (GLEBE *et al.* 2005).

There are two different entry pathways that have been proposed for viral entry: one is endocytosis followed by release of nucleocapsids from endocytic vesicles; the other is fusion of the viral envelope at the plasma membrane. The viral nucleocapsid in cytoplasm could then be transported along microtubules into nuclear and release rcDNA into the nucleoplasm (URBAN *et al.* 2010).

In the nucleus, rcDNA could be repaired and form circular covalently closed DNA (cccDNA) by viral polymerase, combined with host cellular enzymes (NASSAL 2008). The cccDNA serves as a transcriptional template in the nucleus and utilizes the cellular transcriptional machinery to produce all viral RNAs. The RNA transcripts are then transported to the cytoplasm and translate into associated proteins, while pgRNA is assembled with core protein and polymerase proteins to form the RNA-containing nucleocapsid in cytoplasm. Maturation of RNA-containing nucleocapsid, including synthesis of the (-) DNA strand, pgRNA degradation and synthesis of the (+) DNA strand by the different enzyme activities of viral polymerase (LUCIFORA 2011).

Newly formed DNA-containing nucleocapsid can be re-imported into the nucleus to form additional cccDNA molecule, this pathway is important for virus persistence in hepatocytes and also contributes to the relapse of viremia after stopping antiviral treatment in chronic HBV infected patients. On the other hand, nucleocapsids can also be directly bud into the endoplasmic reticulum (ER) or proximal Golgi membranes to acquire their glycoprotein envelope to trigger new virions secretion, or they are redirected towards to nucleus to amplify the cccDNA pool (GUIDOTTI and CHISARI 2006).

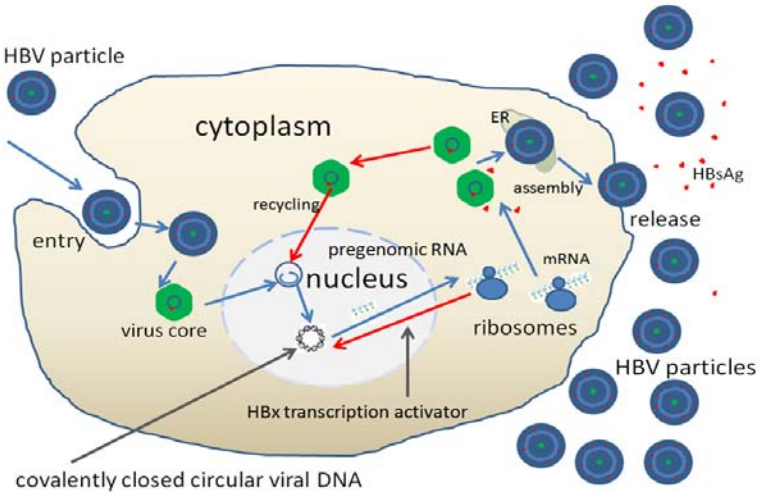

Figure 1.3 Schematic diagram of HBV life cycle

Attachment: The virus gains entry into the cell by binding to a receptor on the surface of the cell and enters it by endocytosis. **Penetration:** The virus membrane then fuses with the host cell membrane releasing the DNA and core proteins into the cytoplasm. **Uncoating:** Because the virus multiplies via RNA made by a host enzyme, the viral genomic DNA has to be transferred to the cell nucleus by host proteins called chaperones. The core proteins dissociate from the partially double stranded viral DNA which is then made fully double stranded and transformed into covalently closed circular DNA (cccDNA) that serves as a template for transcription of four viral mRNAs. **Replication:** The largest mRNA, named pgRNA, which is longer than the viral genome, is used to make the new copies of the genome and to make the capsid core protein and the viral DNA polymerase. **Assembly:** Four viral transcripts undergo additional processing and go on to form progeny virions which are released from the cell or returned to the nucleus and re-cycled to produce even more copies. **Release:** The long mRNA is then transported back to the cytoplasm where the virion P protein synthesizes DNA via its reverse transcriptase activity (BECK and NASSAL 2007; BRUSS 2007).

1.1.3 Host factors participate in HBV life cycle

Virus replication relies on host cells. HBV gene expression can be modulated both in transcriptional or posttranscriptional process and also depend on the nature of the antiviral stimulus applied. Host cellular factors participate in HBV life cycle in almost every step from cccDNA formation, transcription, core particle formation and progeny secretion.

The HBV cccDNA plays a key role in the life cycle of the virus and permits the persistence of infection. Nuclear cccDNA accumulates in hepatocyte nuclei as a stable minichromosome organized by histone and non-histone viral and cellular proteins and

then utilizes the cellular transcriptional machinery to produce all viral RNAs necessary for protein production and viral replication (LEVRERO *et al.* 2009; POLLICINO *et al.* 2006). Even though host factors that control cccDNA formation and cccDNA pool size are yet poorly defined, there is still a correlation between viremia levels and the acetylation status of cccDNA-bound histones (POLLICINO *et al.* 2006), indicating that epigenetic mechanisms can regulate the transcriptional activity of the cccDNA.

Viral gene expression is controlled mainly at the transcriptional level, via four promoters and two enhancers (BAR-YISHAY *et al.* 2011). The Enhancer I element is located upstream to the HBx open reading frame and is activated by ubiquitous transcription factors (TFs) such as CCAAT/enhancer-binding protein (C/EBP), cyclic-AMP (cAMP) response element-binding (CREB), Ap-1, cAbl and RFX1 as well as by hepatocyte nuclear factor (HNF) 3 (FoxA) and the liver-enriched nuclear receptor (NR) HNF4a (LOPEZ-CABRERA *et al.* 1991; QUASDORFF and PROTZER 2010). Enhancer II activation is liver-specific, owing to the large number of liver-enriched TFs and NRs recruited: C/EBP, forkhead Box O1 (FoxO1), HNF3/FoxA, HNF4a, oestrogen-related receptors, farnesoid X receptor (FXR) and peroxisome proliferator-activated receptors (PPARs) (LI *et al.* 1995; RAMIERE *et al.* 2008; SHLOMAI and SHAUL 2009).

The viral core protein is a critical regulatory factor of the HBV life cycle. It contains 183 amino acids in length which divides into an N-terminal assembly domain, a linker and a rich in serines and arginines C-terminal domain (CTD), as for CTD domain it is highly flexible and strongly binds to the encapsidated nucleic acids (MENG *et al.* 2011). Dynamic phosphorylation and dephosphorylation of the hepadnavirus core protein C-terminal domain are required for multiple steps of the viral life cycle, such as intracellular transport of HBV cores, core protein maturation, viral RNA packaging and DNA synthesis and also virion secretion (BASAGOUDANAVAR *et al.* 2007; KANN *et al.* 1999; LAN *et al.* 1999; LEWELLYN and LOEB 2011; MELEGARI *et al.* 2005; NASSAL 1992; PERLMAN *et al.* 2005; RABE *et al.* 2003). It is still unclear how the state of phosphorylation regulates core protein function in RNA packaging, DNA synthesis, nuclear import, and potentially additional steps in the viral life cycle. One possibility is that the core protein exerts its multiple roles by interacting dynamically with distinct viral or host factors at different stages of viral replication, in a CTD phosphorylation

state-dependent fashion (LUDGATE *et al.* 2011).

Moreover, HBV replication requires the host cells to be in G1 phase and may be associated with CDK2 activation, which is a kinase, incorporates into HBV capsids and could phosphorylate the HBV core protein CTD (LUDGATE *et al.* 2012). As for the mature nucleocapsid, since there is much dsDNA which is negatively charge, t it need cations to neutralize and stabilize (MENG *et al.* 2011). Cellular microRNAs, such as miR-125a-5p could up-regulate iron level so that they may regulate HBV replication (PARK *et al.* 2012).

Besides the above host regulation factors, there are other cell pathways that could also control HBV infection, such as host innate immunity. Type I IFNs, which are a factor for the innate immune response and are produced by all types of nucleated cells in response to virus infection, via activation of PRRs. IFNs elicit an antiviral response by binding to their cognate receptors, which trigger a signaling cascade (the JAK–STAT signaling pathway) leading to expression of IFN-stimulated genes (ISGs), whose products exhibit antiviral effects (CHANG *et al.* 2012). The effect of type I interferon on HBV replication is determined by viral load. They suppressed HBV replication when viral load is high and enhanced HBV replication when viral load is low. The suppression of HBV replication by IFN-I apparently involves both in transcriptional and post-transcriptional regulations whereas the enhancement of HBV replication by IFN- I is mediated by transcription factors HBF3r and STAT3 (TIAN *et al.* 2011). In addition to type I IFNs, other proinflammatory cytokines and chemokines also play essential roles in controlling HBV infection. At present, activation of innate immune response acts as therapeutic approaches for chronic hepatitis B infection.

1.1.4 HBV and hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the fifth most common cancer and currently ranks the third leading cause of cancer-related deaths worldwide (GIORDANO and COLUMBANO 2013). The liver tumor, which arises from hepatocytes, is often associated with liver cirrhosis resulting from chronic liver diseases. Among the environmental risk factors, the prevalence of chronic hepatitis B and C virus infections is linked directly to the incidence of HCC. It is well known that the HBV DNA genome is able to integrate into the cellular

chromosomal DNA, causing host genome rearrangements and enhancing the instability of the host chromosome, leading to large inverted duplications, deletions and chromosomal translocations (TAN 2011).

There is no evidence for persistence within the tumor cells of a low level HBV multiplication potential. HBV DNA replicative molecules and cccDNA are detectable by polymerase chain reaction (PCR). Moreover, the association between HCC and HBV recurrence after liver transplantation, and the detection of cccDNA in HCC cells points toward the possibility of HBV replication in tumor cells. The latter could act as potential reservoirs for HBV recurrence, especially in patients who present with a recurrence of HCC (FARIA *et al.* 2008). So far, chronic and persistent infection with hepatitis B virus is a major risk factor for the development of HCC. Worldwide an estimated 350 million individuals are chronically infected with HBV (PRANGE 2012). Approximately 25% of chronically HBV-infected individuals will develop HCC (GANEM and PRINCE 2004). Chronic carriers of HBV have up to a 30-fold increased risk of HCC (FRANCESCHI *et al.* 2006). In areas of high HBV endemicity, persons with cirrhosis have an approximately 16-fold higher risk of HCC than the inactive carriers, and a 3-fold higher risk for HCC than those with chronic hepatitis but without cirrhosis (FATTOVICH *et al.* 2004). Although the mechanisms of oncogenesis of HBV remain obscure, several factors have been identified to be associated with a high risk of developing HCC among chronic hepatitis B (CHB) patients. Such as HBx, which is not binding directly to DNA, but rather acts on cellular promoters by protein-protein interactions and by modulating cytoplasmic signaling pathways, appears to play a critical role in the development of HCC (KUO and CHAO 2010; NGUYEN *et al.* 2008; ZHANG *et al.* 2006; ZHU *et al.* 2010). HBV exerts its oncogenic potential through a multi-factorial process, which includes both indirect and direct mechanisms that likely act synergistically (HINO and KAJINO 1994).

1.2 MicroRNA

1.2.1 MicroRNA biogenesis

MicroRNAs (miRNAs) are a class of endogenous conserved short single-stranded RNA. The family of miRNA constitutes about 1–3% of the human genome. Most miRNA genes are situated within the intergenic regions and have their own transcription units. About a

quarter are located within exons or introns of other coding genes where their transcription is controlled by the host genes. MiRNAs can be transcribed as monocistronic transcripts or in polycistronic clusters, the latter involve several miRNAs situated on a single transcript being controlled by the same promoter (LAW and WONG 2011).

In the nucleus, miRNA genes are transcribed as primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II). Structurally, pri-miRNAs consist of a 5′-7-methyl guanylate (m7G) cap, a characteristic imperfect stem-loop secondary structure, and a 3′-poly (A) tail. Pri-miRNAs are cleaved to precursor miRNAs (pre-miRNAs) of about 50–150 nucleotides by Drosha, an endoribonuclease III (RNAse III), and its cofactor RNA-binding protein Pasha (DGCR8). The pre-miRNAs are then exported to the cytoplasm by exportin 5. This is further excised to double-stranded duplices of 20–23 nucleotides by Dicer, an RNAse III enzyme. The miRNA duplex later separates into single-stranded mature miRNA, and incorporates into the RNA-induced silencing complex (RISC), which is composed of Argonaute proteins. This complex binds to the 3′-untranslated region (3′-UTR) of its target transcript and negatively regulates protein translation by a mechanism that depends on the complementarity between the miRNA and target messenger RNA. Partial complementarity results in translational repression, while complete complementarity triggers mRNA degradation (Figure 1.4).

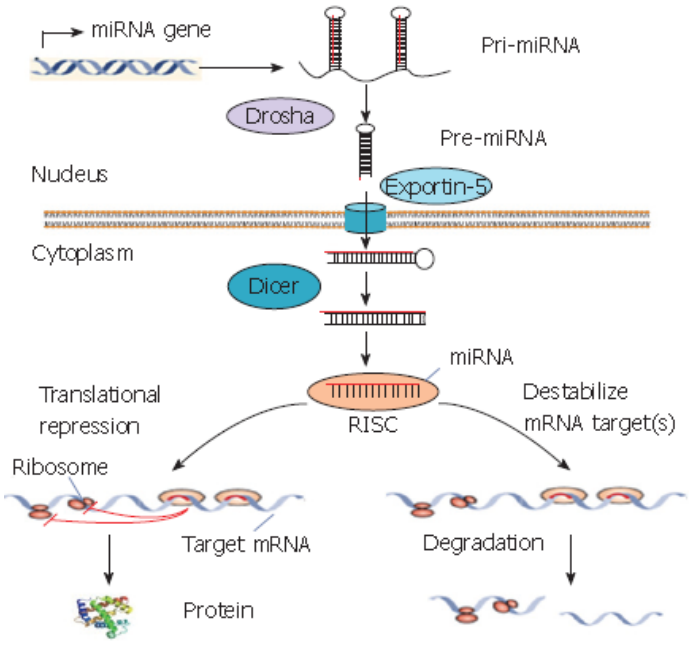

Figure 1.4 MiRNA biogenesis and functions in animal cells

In the nucleus, miRNAs are transcribed as either monocistronic or polycistronic pri-miRNAs by Pol II. Pri-miRNAs cleaved by Drosha and Pasha to pre-miRNAs are exported to the cytoplasm by exportin 5. In the cytoplasm, pre-miRNAs are excised to double-stranded miRNA: miRNA* duplex of 20–23 nucleotides by Dicer. The miRNA duplex unwinds to single-stranded mature miRNA, and incorporates into RNA-induced silencing complex (RISC), which is composed of Argonaute proteins. The miRNA/RISC complex binds to the ′3 -untranslated region (3′-UTR) of target cellular gene and negatively regulates gene expression with a mechanism depending on the complementarity between miRNA and its target mRNA. Perfect complementarity triggers mRNA degradation, while partial complementarity results in translational repression (LAW and WONG 2011) .

Although it is conventionally thought that miRNAs target the 3'UTR to down-regulate gene expression, there is evidence that miRNAs can also regulate mRNA expression in alternative. Some miRNAs target the coding regions or the 5'UTR to regulate gene expression whereas others can positively activate mRNA translation (KLOOSTERMAN *et al.* 2004; SAXENA *et al.* 2003).

1.2.2 MiRNA multiple functions

Although functional validation is frequently lacking, target prediction databases based primarily on Watson–Crick base-pairing (for example, TargetScan, miRanda and Pictar) have suggested that a single miRNA may simultaneously target more than 100 mRNAs (ROTTIERS and NAAR 2012). Similarly, a single mRNA could be regulated by many miRNA. Human miRNAs are predicted to control the activity of 30–60% of all protein-coding genes. Thus, miRNAs are integrated into vast regulatory networks that impinge upon a broad spectrum of biological events, including cell proliferation or differentiation, cancer development, viral replication and also immune response (HAGEN and LAI 2008). Moreover, miRNAs are stable in plasma, and differential plasma miRNA profiles have been described for many diseases, including fatty liver (CHEUNG *et al.* 2008), atherosclerosis (FICHTLSCHERER *et al.* 2011), and cancer (FRIEL *et al.* 2010; KOSAKA *et al.* 2010; RABINOWITS *et al.* 2009; TAYLOR and GERCEL-TAYLOR 2008). Circulating extracellular miRNAs have enormous potential as novel disease biomarkers. MiRNAs could, therefore, be considered like hormones as a possible form of intercellular communication.

1.2.3 MiRNA and HCC

More than fifty percent of genes that encode miRNAs are located at fragile sites or in cancer-associated regions of the genome and almost every type of human cancer analyzed has been associated with altered activities of miRNAs (WANG *et al.* 2012d). Thus, the potential of miRNAs to be robust biomarkers for cancer diagnosis, prognosis, and pathogenesis has been predicted.

Recently, there are more and more miRNAs reported as etopic expression in HCC compared with normal hepatic tissues, such as miR-34a, 99a, 101, 106, 124, 125b, 140-5p, 145, 195, 199a, 214, 216a/217, 224, 372, 375, 519d, 520b, 637 (GU *et al.* 2013; HE *et al.* 2012b; HOU *et al.* 2011a; HOU *et al.* 2011c; LI *et al.* 2011a; LI *et al.* 2009a; LIANG *et al.* 2010; NOH *et al.* 2013; SHEN *et al.* 2013; SU *et al.* 2009; WANG and LEE 2011; XIA *et al.* 2012; XIA *et al.* 2013; XU *et al.* 2009; YANG *et al.* 2013; ZHANG *et al.* 2011a; ZHANG *et al.* 2012b; ZHENG *et al.* 2012), which suggested the important roles of miRNAs in HCC development.

In HCC, miRNA dysregulation plays a key role in mediating the pathogenicity of several etiologic risk factors and, more importantly, they promote a number of cancer-inducing signaling pathways (LAW and WONG 2011). For example, DNA methylation occurs in the early stage of cancer development, including HCC. Genomic hypomethylation increases chromosome instability while localized hypermethylation decreases tumor suppressor gene expression, thus increasing the risk of HCC development (PARK *et al.* 2007). While miRNAs could modulate host methylase expression, such as miR-101, which could target DNA methyltransferase 3A (DNMT3A) to modulate hepatocarcinogenesis (WEI *et al.* 2013).

MiRNAs with tumor suppression functions are often less expressed in HCCs compared to paired normal tissues. Most of the HCC-related miRNAs discovered so far belong to this class. Two important members of *let-7* family, *let-7a* and *let-7g* inhibit HCC cell proliferation *via* down-regulating STAT3 and c-myc as well as up-regulating p16INK4A (LAN *et al.* 2011). The level of *let-7g* is significantly lower in metastatic HCCs compared to non-metastatic HCCs. High expression of miR-15b in HCCs predicts a low risk of HCC recurrence and is associated with antitumor effect *via* targeting Bcl-w, an

anti-apoptotic Bcl-2 family member (CHUNG *et al.* 2010). MiR-22 expression is down-regulated in HCCs and the low miR-22 expression is predictive of poor survival of HCC patients. In addition, ectopic expression of miR-22 significantly inhibits HCC cell proliferation and tumorigenicity (ZHANG *et al.* 2010b). miR-29c is significantly down-regulated in HBV-related HCCs. Over-expression of miR-29c in HepG2.2.15 cells effectively suppresses tumor necrosis factor alpha (TNFα)-induced protein 3 (TNFAIP3) and HBV DNA replication, it also inhibits cell proliferation and induces apoptosis (CHIANG *et al.* 2010; WANG *et al.* 2011a). MiR-99a, which is down-regulated in HCCs compared to the normal tissues, can induce cell cycle arrest in HCC cells, once miR-99a combines to its targets insulin-like growth factor 1 receptor (IGF1R) and mammalian target of rapamycin (mTOR), the cell cycle related proteins such as cyclin D1 are subsequently suppressed (LI *et al.* 2011b). MiR-101, which is also down-regulated in many human cancers including HCCs, targets the enhancer of zeste homolog 2 (EZH2), the key component of polycomb repressive complex 2 (PRC2) involving in the control of many cellular processes such as cell proliferation and differentiation. It also induces apoptosis of hepatoma cells *via* targeting Mcl-1, an anti-apoptotic member of Bcl-2 family (SU *et al.* 2009). Over-expression of miR-125b in HCC cells suppresses cell growth and phosphorylation of Akt, and high expression of miR-125b is correlated with optimal survival of HCC patients (LI *et al.* 2008). MiR-199a-3p is frequently down-regulated in HCCs and HCC cell lines. MiR-199a-3p can efficiently suppress HBV replication and suppress HCC growth *via* targeting tumor-promoting p21 protein-activated kinase 4 (PAK4), mTOR, and c-Met, while down-regulation of miR-199a-3p in HCCs is associated with a shorter time to recurrence after HCC resection (FORNARI *et al.* 2010; HOU *et al.* 2011b; SHEN *et al.* 2010). MiR-519d is down-regulated in HCCs and could suppress HCC growth *via* targeting MKi67, a 359-kDa nuclear protein reflecting cell proliferation rate (HOU *et al.* 2011c).

In contrast to the tumor suppressor-like miRNAs, oncogene-like miRNAs regulate targeted genes and/or pathways that facilitate HCC growth and invasiveness. These miRNAs are often undetectable or less expressed in normal livers, but their expression levels are significantly elevated in HCCs. MiR-17-5p is able to activate the p38 mitogen-activated protein kinase (MAPK) pathway and significantly increase the

phosphorylation of heat shock protein 27 (HSP27), and eventually promote the migration and proliferation of HCC cells (YANG *et al.* 2010a). MiR-30d is frequently up-regulated in HCCs and its expression is highly associated with HCC intrahepatic metastasis (YAO *et al.* 2010). MiR-143 is dramatically increased in metastatic HBV–HCC from both p21-HBx transgenic mice and HCC patients. It is partially transcribed by NF-κB and favors HCC invasiveness and metastasis *via* repressing fibronectin type III domain containing 3B (FNDC3B) expression (ZHANG *et al.* 2009). MiR-221/222, one of the most up-regulated miRNAs in HCCs, target the CDK inhibitor p27 and enhance hepatocarcinogenesis *via* targeting DNA damage-inducible transcript 4 (DDIT4), a modulator of mTOR pathway (PINEAU *et al.* 2010). MiR-221 can simultaneously affect multiple pro-oncogenic pathways *via* targeting Bmf and modulating p27 and p57 (FORNARI *et al.* 2008; GRAMANTIERI *et al.* 2009). Over-expression of miR-222 is common in HCCs and confers metastatic potentials in HCC cells, possibly *via* activating Akt signaling (WONG *et al.* 2010). MiR-423 behaves as an oncogene in HCCs by down-regulating p21 (LIN *et al.* 2011). MiR-602 expression is significantly higher in HBV-associated liver inflammation, liver cirrhosis and HCC than in normal livers. It plays a pro-carcinogenic role in HBV-related hepatocarcinogenesis by inhibiting the tumor suppressor gene RAS association domain family 1A (RASSF1A) (YANG *et al.* 2010b).

1.2.4 MiRNA and cell cycle

The cell cycle is the sequence of coordinated events resulting in reproduction of an individual cell into two daughter cells. A cell cycle can be divided into four distinct phases: S phase (synthesis phase), M phase (mitosis phase), and two gap phases G1 and G2. Two types of mechanisms are responsible for cell-cycle control: a protein phosphorylation cascade fine-tuned by cyclin/cyclin-dependent kinase (CDK) complexes and three supervisory restriction points, namely G1/S, G2/M, and metaphase checkpoints (CHEN *et al.* 2010). Cell-cycle control is a complicated process that is involved in the differential expression of a set of genes.

The mechanisms governing cell cycle control by miRNAs are increasingly well understood. Several miRNAs have been reported to play a role in the control of cell cycle,

in particular at the G1/S checkpoint (LAW and WONG 2011). MiR-26a targets G1/S cyclins (both cyclin D2 and E2) in murine liver cancer (KOTA *et al.* 2009). Whereas miR-195 targets multiple genes (including cyclin D1, CDK6, and E2F3) of the G1/S transition in primary HCC (XU *et al.* 2009). Both miR-26a and miR-195 have been found to be frequently down-regulated in HCC, where they have been shown to cooperate in overcoming the G1/S cell cycle blockade through repression of E2F transcription (KOTA *et al.* 2009; XU *et al.* 2009). In addition, miR-221 in HCC has also been reported to target CDKN1B/p27/Kip1 and CDKN1C/p57/Kip2, both of which are CDK inhibitors (FORNARI *et al.* 2008). Both miR-106b and miR-93 can also target E2F1 (LI *et al.* 2009b). MiR-223 could target stathmin1 (STMN1), which is a microtubule destabilizer that sequesters tubulin for depolymerization and affects microtubule assembly (WONG *et al.* 2008a). As microtubules have an important role in the segregation of metaphase chromosomes during mitosis, it is probable that miR-223 has a function in regulating the G2/M transition (LAW and WONG 2011) (Figure 1.5).

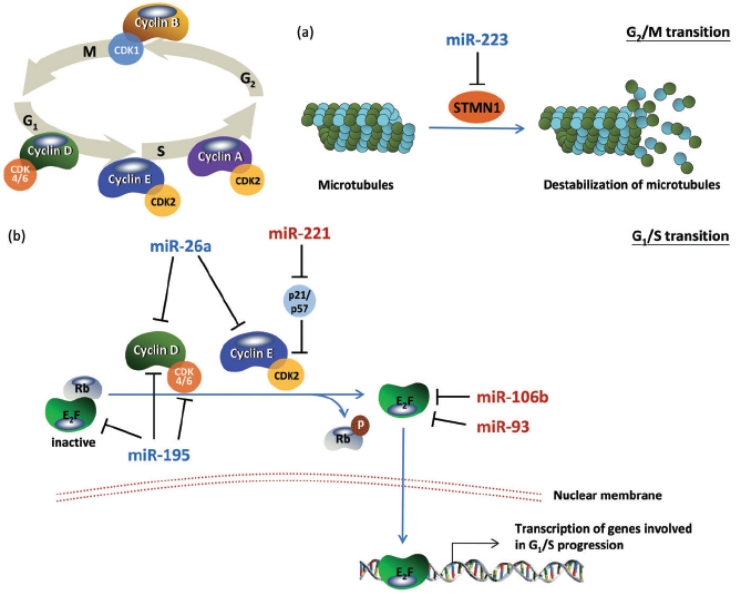

Figure 1.5 Regulation of cell cycle by miRNA

Several miRNAs take part in the control of cell cycle in HCC. (a) MiR-223 has been shown to target stathmin1 (STMN1), which is a regulatory protein involved in destabilizing the microtubules and plays a role in maintaining the dynamic nature of mitotic spindles at the G2/M transition. (b) MiR-26a can target both cyclin D2 and E2 while miR-195 has been shown to negatively regulate multiple genes, including cyclin D1, CDK6, and E2F3. In addition, miR-221

has been reported to target CDK inhibitors, p27 and p57. Members of miR-106b-25 cluster, miR-106b and miR-93, can repress E2F1 expression, whereby preventing excessive E2F1 accumulation that may paradoxically result in apoptosis (LAW and WONG 2011).

1.2.5 MiRNA and metabolism

Proper control of metabolic homeostasis is crucial to the maintenance of human physiology and health. Besides a number of key transcription factors, such as liver X receptors (LXRs), CCAAT-enhancer-binding protein (C/EBP) and forkhead box protein O1 (FOXO1), respond directly or indirectly to cholesterol, lipids, glucose and insulin to rapidly alter gene expression programmes governing metabolic homeostasis (HORTON *et al.* 2002; NAKAE *et al.* 2008; ZELCER and TONTONOZ 2006). MiRNAs have recently been found to represent another crucial regulatory layer overlaying and intersecting with transcriptional control mechanisms in guiding metabolic homeostasis (ROTTIERS and NAAR 2012).

Several miRNAs have recently been implicated in controlling both insulin signalling and glucose metabolism at multiple levels (Figure 1.6) (GUAY *et al.* 2011). For example, the pancreatic miRNA, miR-375, was shown to be required for the maintenance of pancreatic α-cell and β-cell mass in mice (POY *et al.* 2009). MiR-124a is also involved in pancreatic islet development, potentially through regulation of the FOXA2 transcription factor, which is involved in β-cell differentiation, and RAB27A, a GTPase required for insulin secretion (BAROUKH *et al.* 2007; LOVIS *et al.* 2008). MiR-9 may regulate insulin secretion through its inhibition of the transcription factor one cut homeobox 2 (OC2; also known as ONECUT2) and through SIRT1 (PLAISANCE *et al.* 2006; RAMACHANDRAN *et al.* 2011), whereas miR-29a and miR-29b, which are highly expressed in the pancreatic islets of diabetic mice, inhibit the expression of monocarboxylate transporter 1 (MCT1) and its function in insulin release (PULLEN *et al.* 2011). Besides the above miRNAs which have key roles in controlling insulin secretion, other miRNAs could act in target tissues to regulate responses to insulin and glucose homeostasis. For example, miR-223 was found to inhibit glucose uptake through targeting GLUT4 (LU *et al.* 2010). MiR-33a and miR-33b may also influence insulin signalling and glucose regulation by targeting IRS2, SIRT6 and AMPKα1 (DAVALOS *et al.* 2011; ROTTIERS *et al.* 2011). A number of miRNAs have also been implicated in metabolic disorders associated with aberrant

insulin response. For example, miR-103 and miR-107 were recently shown to be up-regulated in livers of leptin-deficient (ob/ob) and diet-induced obese (DIO) mice (TRAJKOVSKI *et al.* 2011).

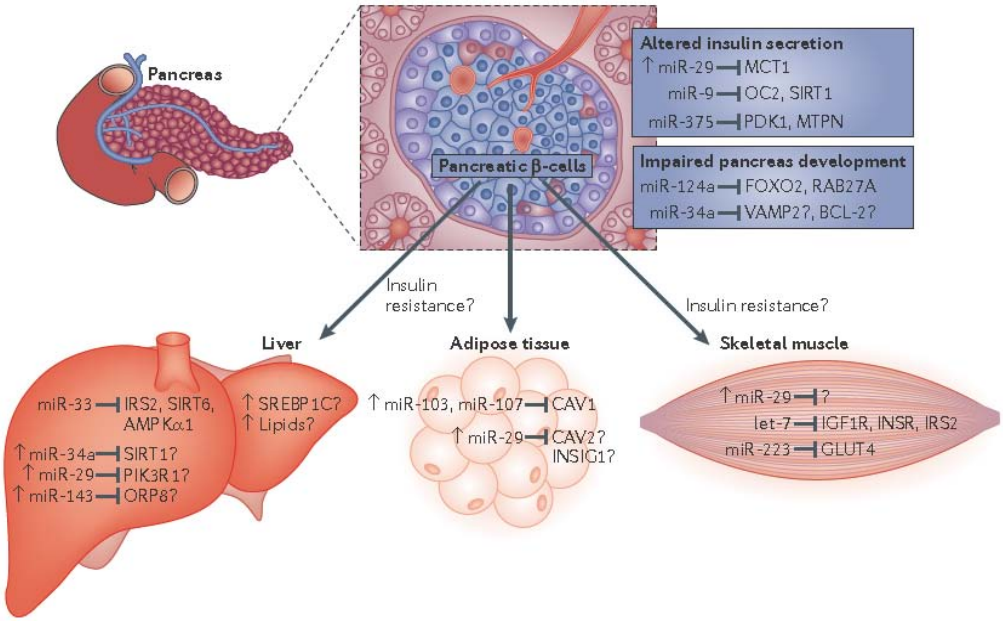

Figure 1.6 MiRNA regulation of insulin signalling and glucose homeostasis

Normally, following feeding, insulin is produced in pancreatic β cells and after release will reach target tissues such as the muscle, liver and adipose to cause uptake of glucose, reduce the production of glucose and activate fat production and storage. MiRNAs that affect diverse parts of insulin signalling in the pancreas, liver, muscle and adipose tissue have been identified. In disease conditions, such as impaired insulin secretion or insulin resistance, several miRNAs are up-regulated (marked with an arrow) (ROTTIERS and NAAR 2012).

Lipids are structural components of cell membranes (for example, cholesterol and phospholipid) and are important for energy storage (for example, triglycerides) but can also act as signalling molecules (for example, steroid hormones). Lipids such as cholesterol and fatty acids are taken up in the diet and are synthesized de novo, predominantly in the liver. Regulation of the biosynthesis of cholesterol, fatty acids and phospholipids is mediated by transcription factors such as SREBPs (ROTTIERS and NAAR 2012).

The role of miRNAs in the regulation of lipid metabolism is just beginning to be explored (ROTLLAN and FERNANDEZ-HERNANDO 2012). Several miRNAs have been described to regulate lipid metabolism, including miR-122 which is highly expressed in the liver, and

it is estimated to account for approximately seventy percent of all liver miRNA (KRUTZFELDT *et al.* 2005; LAGOS-QUINTANA *et al.* 2002). MiR-122 inhibition by antisense oligonucleotides (ASO) in mice resulted in increased hepatic fatty-acid oxidation and a reduced cholesterol synthesis. In addition, miR-122 inhibition reduced total plasma cholesterol by 25–35%, and this was reflected by changes in both the LDL and HDL fractions (ESAU *et al.* 2006) . Besides miR-122, miR-33, which located within the sterol regulatory element-binding protein genes (*Serbp)* and was co-transcribed with their host genes, regulated cholesterol and fatty acid metabolism (DAVALOS *et al.* 2011; NAJAFI-SHOUSHTARI *et al.* 2010; RAYNER *et al.* 2011). Other miRNAs such as miR-370, miR-758, miR-106b, miR-378/378*, miR-143, miR-27, miR-29a, miR-302a, and miR-335 have also been shown to regulate lipid homeostasis (CHEN *et al.* 2011b; ESAU *et al.* 2004; GERIN *et al.* 2010; HOEKSTRA *et al.* 2012; ILIOPOULOS *et al.* 2010; KIM *et al.* 2012; LIN *et al.* 2009; NAKANISHI *et al.* 2009; RAMIREZ *et al.* 2011).

1.2.6 MiRNA and viral replication

Viruses are a class of intracellular pathogens with well established roles in the development of many diseases. Many cellular mechanisms, such as innate immunity and cell signaling pathways may participate in controlling viral pathogenesis (LECELLIER *et al.* 2005). Alterations in miRNA expression profiles have emerged as important indicator of changes in gene expression that either favor or restrict virus replication (SCARIA *et al.* 2007; UMBACH *et al.* 2008).

Although HBV is a DNA virus, its transcripts might be targeted and regulated by cellular miRNAs, and accumulating evidence suggests that miRNAs modulate directly or indirectly on HBV transcription and replication (GUO *et al.* 2011a; LIU *et al.* 2011; POTENZA *et al.* 2011; WANG *et al.* 2012b; ZHANG *et al.* 2011b) (Figure 1.7). Thilde Nordmann Winther indicates the existence of a relationship between abundance of circulating miRNAs and immunological stages in the natural course of disease. Certain miRNAs may contribute to the establishment and maintenance of chronic hepatitis B infection in children (WINTHER *et al.* 2013).

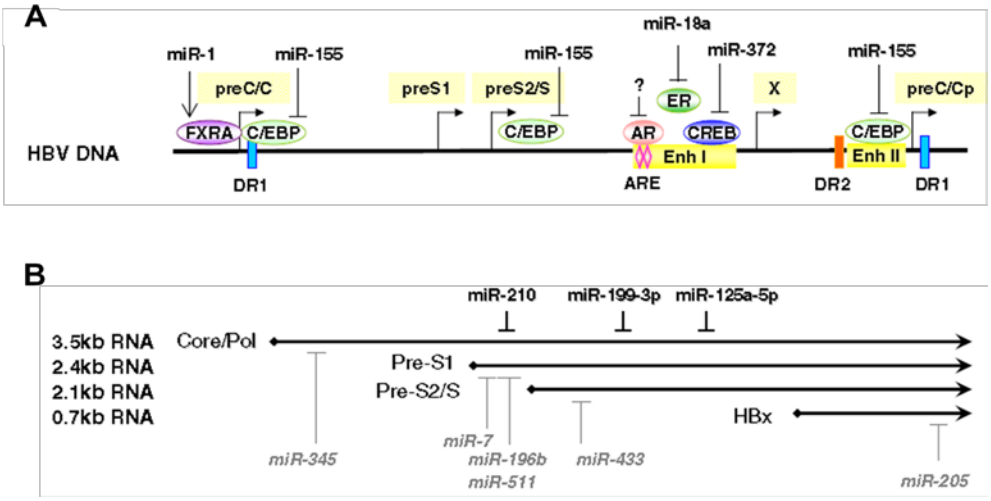

Figure 1.7 Summary of cellular miRNAs effect on HBV transcription

(A) Angled arrows indicate the HBV RNA start site for the major viral transcripts (Pre/core, PreS1, PreS, and X), and viral enhancer I and II are schematically depicted as boxes. DR I and DR II are two short repeats essential for viral replication. ARE represents two androgen response elements which were identified to locate at enhancer I region. Host cellular transcription factors binding position on HBV genome and the miRNAs targeting cellular factors known for regulating HBV transcription are shown. The arrow indicated an activated but the bars indicated an inhibited effect of the miRNAs on HBV transcription. (B) Positions of binding sequences in HBV transcripts proposed to be targeted by miRNAs are shown. Italic gray color represents miRNAs which were predicted to probably bind HBV transcripts by four well established target-prediction software (LIU *et al.* 2011).

Hepatitis C virus infection is a major risk factor for chronic hepatitis, liver cirrhosis and HCC worldwide (KUMAR 2011). MiR-122 was found to be required for the propagation of hepatitis C virus (HCV). MiR-122 binds two positions in the 5' UTR of the HCV genome, and this binding is essential to viral accumulation and propagation infected hepatocytes (JOPLING *et al.* 2005; RANDALL *et al.* 2007).

In contrast to activated $CD4^+$ T cells and differentiated macrophages, resting $CD4^+$ T cells and monocytes are non-permissive for HIV-1 replication. Recently, certain miRNAs which are highly expressed in resting cells have been implicated in the regulation of the resting or quiescent phenotype, inhibiting the expression of cellular proteins that are also viral co-factors and finally mediate restriction of HIV (CHIANG and RICE 2012; XIAO *et al.* 2007; ZHOU *et al.* 2007).

Besides host cellular miRNAs involving in mediation viral replication, virus could also encode miRNAs to benefit its survival. Such as Herpesviruses which have two distinct

phases in their lifecycle: latency and lytic replication (GREENE *et al.* 2007). Herpesviral miRNAs appear to promote viral latency by inhibiting viral lytic replication either through direct targeting of key viral replication genes or through manipulation of host pathways that regulate viral lifecycle (LEI *et al.* 2010; UMBACH *et al.* 2008).

On the other hand, virus infection can also lead to changes in miRNA expression. Winther et al. have investigated the plasma miRNA profile of children chronically infected with HBV, and found there was differential plasma miRNA profiles in HBeAg positive and HBeAg negative children with chronic hepatitis B (WINTHER *et al.* 2013). Moreover, HBx was found to significantly up-regulate the expression of seven miRNAs but down-regulate the expression of eleven cellular miRNA, respectively. An inverse correlation was noted between the expression of HBx and that of the highly-expressed members of the *let-7* family including *let-7a*, *let-7b* and *let-7c* in HCC patients (WANG *et al.* 2010b). HBV might also have evolved strategies to prevent infected cells from undergoing apoptosis and escape the innate or adaptive immune responses in host cells through regulating the expression of miRNAs (SARNOW *et al.* 2006). The well-defined miR-17-92 cluster and miR-29 are down-regulated following HIV-1 infection of Jurkat cells or PBMCs (HOUZET *et al.* 2008; SUN *et al.* 2012; TRIBOULET *et al.* 2007). *Herpesvirus saimiri* expresses a non-coding RNA, HSUR1 (*H. saimiri* U-rich RNA 1), which down-regulates miR-27 expression, resulting in low levels of miR-27 in herpesvirus-transformed marmoset T cells (CAZALLA *et al.* 2010). MiR-27 is also down-regulated following murine cytomegalovirus (MCMV) infection of cell lines and *in vivo* by a viral transcript which acts as a miR-27b target and mediates its degradation by the addition of post-transcriptional modifications (BUCK *et al.* 2010; LIBRI *et al.* 2012; MARCINOWSKI *et al.* 2012).

2 Aims of the study

HBV infection could induce liver tissue to develop into HCC, meanwhile, in HCC, HBV replicative ability is weaker compared with hepatocytes, which means host cells status has direct correlation with HBV replication. Recently, there are more and more groups focusing on the roles of miRNAs in tumor development. In HCC tissue, there are some miRNAs reported deregulated, which are defined as oncogenous miRNAs or anti-tumor miRNAs. All these mean that miRNAs have multiple functions in liver physiology and pathophysiology and also virus infection. But the exact functions or regulatory mechanisms for each miRNAs on HCC development and viral replication are far from being fully understood, so it is meaningful to study miRNAs which may participate in HBV life cycle or liver tumor progress and investigate their regulatory mechanisms.

In our previous study, Dr. Xiaoyong Zhang selected several miRNAs which were functionally related to cell differentiation, viral infection, innate immune response, hypoxia stress and cancer. He described miR-1 and 449a could enhance HBV replication and meanwhile block hepatoma cells staying at G1 phase. Thus, in the current study, we selected six miRNAs, including miR-99a, 101, 125b-5p, 199a-3p, 519d and 637, which are reported to be relatively highly expressed in normal liver, but decreased in HCC tissue so that they are considered to be as a kind of tumor suppression miRNAs, to verify their functions on hepatoma cells growth and test their influence on HBV replication, and also what mechanisms they may take. To elucidate these questions, we have investigated the following steps:

① Transfect or co-transfect these miRNA mimics with a HBV genome containing plasmid pSM2 into hepatoma cell lines to screen out miRNAs which regulate HBV replication.

② Transfect these miRNA mimics into hepatoma cells and treat cells with cell cycle inhibitors to analyse their influence on cell growth.

③ To further study which steps miRNAs may take in the HBV life cycle, we analyse HBV from transcription to core particle formation and also progeny secretion after transfection miRNA mimics into hepatoma cells.

④ Since different hepatoma cell lines have different background, we also analyse the different appearances and molecule mechanisms miRNAs take in different cell lines.

⑤ In order to figure out the molecule mechanisms these miRNAs may take in modulation of HBV replication, we use several molecular detection methods and also RNA sequence assay to analyse the possible target genes or pathways.

3 Materials and Methods

3.1 Materials

3.1.1 Cells

Human hepatoma cell lines HepG2, Huh7 and HepG2.2.15 which integrated with HBV dimers (GenBank accession number: number: U95551) were kept at the Institute of Virology, University Hospital Essen. Con-1 cells with a subgenomic HCV replicon were kindly provided by Prof. Dr. Ralf Bartenschlager from University of Heidelberg. Primary human hepatocytes were isolated from liver transplantation donors by perfusion.

3.1.2 Chemicals and reagents

Name	**Company**
3M Sodium Acetate pH 5.5	Ambion
Yeast RNA	Ambion
EDTA solution pH 8.0	AppliChem
20× Sodium dodecyl supphate (SDS)	AppliChem
Ethanol absolute p.A.	AppliChem
Tris buffer pH7.4 / 8.0 / 8.8	AppliChem
Fetal Calf Serum (FCS)	Biochrom AG
Tween 20	Biochemica
RNAstable	Biomatrica
30% Acrylamide Solution	BIO-RAD
William’s Medium E	Biotech GmbH
Nocodazole	CalbioChem
Red Loading Buffer	Cell Signaling
SmartLadder MW-1700-10	Eurogentec

Amersham™ Rapid-hyb Buffer	GE Healthcare
Rediprime Ⅱ DNA labeling system	GE Healthcare
Illustra™ MicroSpin™ S-200 HR columns	GE Healthcare
Amersham ECL Western Blotting Reagent	GE Healthcare
Alpha-P32 (dCTP)	Hartmann Analytic GmbH
TRIzol® Reagent	Invitrogen
10× TBE Buffer	Invitrogen
20× SSC Buffer	Invitrogen
D-PBS	Invitrogen
Lipofectamine® 2000 Reagent	Invitrogen
EcoR I and Buffer	New England BioLab
RPMI 1640 medium	PAA Laboratories
DMEM High Glucose medium	PAA Laboratories
L-Glutamine	PAA Laboratories
Trypsin-EDTA	PAA Laboratories
MEM Non Essential Amino Acids	PAA Laboratories
HEPES Buffer Solution	PAA Laboratories
Penicillin/Stretomycin	PAA Laboratories
PeqGOLD Protein-Marker Ⅳ	Peqlab
5× Green GoTaq™ Reaction Buffer	Promega
Proteinase K	QIAGEN
RNase A	QIAGEN
Cell proliferation Reagent WST-Ⅰ	Roche

2-propanol	Roche
Roti-phenol	Roche
2-propanol	Roche
DNAase 1	Roche
10×SDS-PAGE	Roche
Hydrochloric Acid	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
Chloroform	Sigma

3.1.3 Materials and instruments

Amersham Hybond^{TM}-N^{+}	GE Healthcare, USA
Blotting Paper Sheets	MUNKTELL & TILTRAK GmbH
BIO WIARD KOJAIR	BIO-FLOW Technik
Bio Imaging System	Syngene, UK
Bin DER	BIOTron Labortechnik GmbH
CAWOMAT 2000 IR	CAWO photochemisches Werk GmbH
Cell culture plates	Greiner Bio-One, Germany
Centrifuge Megafuge 1.0R	Heraeus, Germany
Centrifuge Avanti J-26Xpi	Beckman Coulter, Germany
Centrifuge 5415 R	Eppendorf, Germany
Centrifuge: Ultracentrifuge Optima L-70K	Beckman Coulter, Germany
CO_2 incubator	Thermo, Deuschland
Cyclone Storage Phosphor Screen	Packard, USA
Duomax 1030	Oehmen Laborechnik

FACS Calibur Flow Cytometer	Becton Dickinson, Germany
FilterMate Harvester	PerkinElmer, USA
Flat-bottom 96-well microplates	Falcon BD, Germany
Fridge/Freezer (-20℃)	AEG, Germany
Freezer (-80℃)	Thermo Forma, Germany
HYBIDIZATION OVEN/SHAKER	Amersham Pharmacia
Julabo U3	Oehmen Laborechnik
Light Cycler	Roche, Switzerland
Light Cycler Capillaries	Roche, Switzerland
Model 785 Vacuum Blotter	BIO-RAD, USA
Mini PROTEAN Tetra Cell	BIO-RAD, USA
Mini Trans-Blot Cell	BIO-RAD, USA
Nitrocellulose	GE Healthcare, USA
Power pack P25	Biometra, Germany
Pipette tips (10ul, 200ul, 1ml)	StarLab, Germany
Plastic sterile pipettes (5ml, 10ml, 25ml)	Greiner Bio-One, Germany
Shaker (Duomax 1030)	Heidolph, Germany
Single-, multichannel pipettes	Eppendorf, Germany
TopCount.NXT^{TM}	Packard, UK
T 3000 Thermocycler	Biometra, Germany
Vacuum Regulator	BIO-RAD, USA

3.1.4 Buffers

Name	Component
10× PBS (1 l)	80g NaCl 14.4g Na_2HPO_4 2.0g KCl 2.4g KH_2PO_4
12% Separation gel (10 ml)	3.3ml deionized water 4.0ml 30% acrylamide 2.5ml 1.5M Tris pH 8.8 100µl 10% SDS 100µl 10% AP 4µl TEMED
5% Concentration gel (5ml)	3.15ml deionized water 720µl 30% acrylamide 540µl 1.0M Tris pH6.8 45µl 10% SDS 25µl 10% AP 5µl TEMED
10× Transfer buffer (1 l)	142.6g Glycin 30g Tris-base
Lysis buffer for core particle extraction	10mM Tris pH7.5 1mM EDTA 50mM NaCl 8% sucrose

	0.25% Nonidet P-40
Lysis buffer for HBV RI extraction	50mM Tris pH 7.4 1mM EDTA 1 % Nonidet P-40
Denaturation buffer	1.5M NaCl 0.5M NaOH
Neutralization buffer	2.0M NaCl 1.0M Tris-base 2.5% hydrochloric acid

3.1.5 Commercial kits

Name	**company**
Dual-Glo Luciferase Assay Kit	Promega
QuantiFast SYBR Green RT-PCR Kit	QIAGEN
RNeasy Mini kit	QIAGEN
QIAamp DNA Blood Mini Kit (250)	QIAGEN
Gel Extraction Kit (50)	QIAGEN
MiScript II RT Kit (50)	QIAGEN
MiScript SYBR Green PCR kit	QIAGEN
Qiagen plasmid kits (Mini-,Midi-,Maxi-)	QIAGEN
Nuclear and Cytoplasmic Extraction Kit	Thermo Scientific

3.1.6 Plasmids

3.1.6.1 pSM2

pSM2 which contains a head-to-tail tandem dimer of the HBV genome (GenBank accession number: V01460) was provided by Dr. Hans Will (Heinrich-Pette-Institute,

Hamburg, Germany). The plasmid encodes HBV proteins under the control of HBV core promoter, and HBV genome could be digested from vector pUC19 by single restriction endonuclease EcoR I.

3.1.6.2 pGL3-HBV promoter report plasmids

The luciferase reporter plasmids containing HBV promoters were constructed by Dr. Xiaoyong Zhang (ZHANG *et al.* 2011b). The regions of HBV core promoter (nt1648-1853), HBV X promoter (nt1237-1375), SP1 promoter (nt2224-2784), SP2 promoter (nt2814-3123) were amplified from pSM2 plasmid and inserted into pGL3-basic vector between MluI and BglII restriction sites (Promega, Madison, WI), (Figure 3.1), resulting in the luciferase reporter vectors pSP1, pSP2, pCP and pXP respectively.

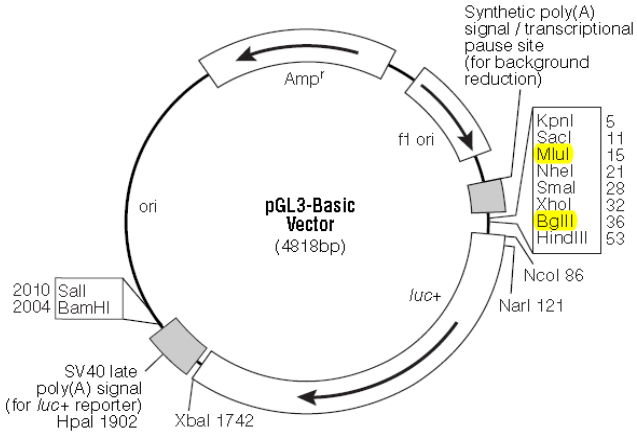

Figure 3.1 pGL3-basic vector circle map (adopted from Promega)

3.1.6.3 p$^{\text{MIR-REPORT}}$ system

Full length of HBV genome sequence was generated from pSM2 plasmid by restriction enzyme SpeI digestion and cloned into the 3'UTR of luciferase gene of p$^{\text{MIR-REPORT}}$ vector (Ambion, Invitrogen). Four partial fragments of the HBV sequence (nt2840-837, nt837-1840, and nt1830-2849) and HBV RNA 3'UTR sequence (nt1841-1964) were amplified from pSM2 plasmid, digested with restriction enzymes MluI and HindIII, and cloned into the 3'UTR of luciferase gene by Dr. Xiaoyong Zhang (ZHANG *et al.* 2011b) (Figure 3.2).

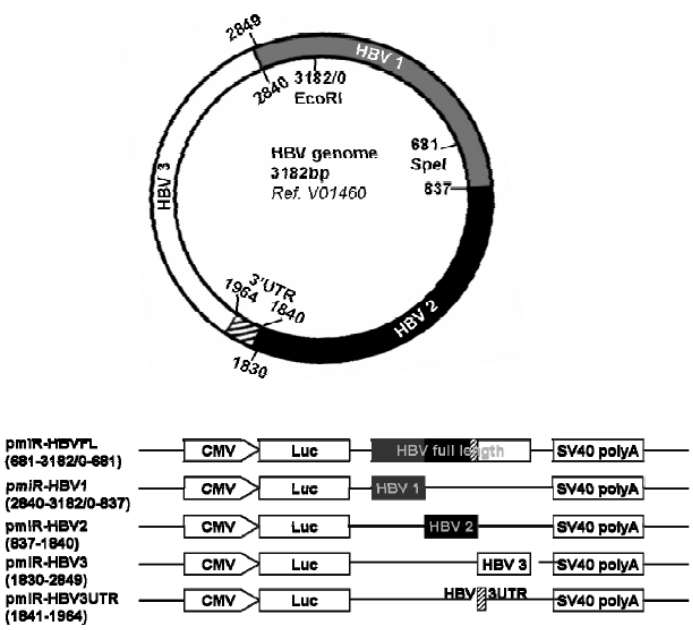

Figure 3.2 Luciferase reporter plasmids containing HBV fragments

(adopted from Dr. Xiaoyong Zhang) (ZHANG *et al.* 2011b)

3.1.6.4 Renilla luciferase report vector

The Renilla luciferase report plasmid was purchased from Clontech.

3.1.7 Antibodies

Name	**Source**	**Company**
Anti-HBcAg	Rabbit pAb	Abcam
Phospho-Rb (ser795)	Rabbit pAb	Cell Signaling
EZH2	Rabbit mAb	Cell Signaling
Cleaved PARP (Asp214) Antibody (Human Specific)	Rabbit pAb	Cell Signaling
Caspase-3 Antibody	Rabbit pAb	Cell Signaling
Anti-Albumin	Rabbit mAb	Cell Signaling
LIN28B	Rabbit pAb	Cell Signaling
Anti-human FXR/NR1H4	Mouse mAb	PPMX

Bcl-2 (12) Antibody	Mouse mAb	Santa Cruz Biotechnology
Anti-β-actin	Mouse mAb	Sigma

3.1.8 MiRNA mimics and siRNA sequences

Name	**Sequence (5'-3')**
Hsa-miR-1	UGGAAUGUAAAGAAGUAUGUAU
hsa-miR-99a	AACCCGUAGAUCCGAUCUUGUG
Hsa-miR-101	UACAGUACUGUGAUAACUGAA
Hsa-miR-122	UGGAGUGUGACAAUGGUGUUUG
Hsa-miR-125b-5p	UCCCUGAGACCCUAACUUGUGA
Hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA
Hsa-miR-125b-2-3p	UCACAAGUCAGGCUCUUGGGAC
Hsa-miR-199a-3p	ACAGUAGUCUGCACAUUGGUUA
Hsa-miR-519d	CAAAGUGCCUCCCUUUAGAGUG
Hsa-miR-637	ACUGGGGGCUUUCGGGCUCUGCGU
Hsa-let-7a-5p	UGAGGUAGUAGGUUGUAUAGUU
Hsa-miR-98-5p	UGAGGUAGUAAGUUGUAUUGUU
MiR-control	UCACAACCUCCUAGAAAGAGUAA
SiR-control (Cat NO. # D-001210-02)	Not provided by Thermo Scientic
SiEZH2 6#	CAGGATGGTACTTTCATTGAA
SiEZH2 7#	AACCATGTTTACAACTATCAA
SiLIN28B 1#	CTGGGATAACATAACTCCAGA
SiLIN28B 4#	AACATGATAAGCGTTGCTCAA

3.1.9 Prime sequences

Name	Sequence (5’-3’) / Cat. NO.
HBV progeny DNA	Forward: GTTGCCCGTTTGTCCTCTAATTC Reverse: GGAGGGATACATAGAGGTTCCTT
HBV xRNA	Forward: CCGTCTGTGCCTTCTCATCT Reverse: TAATCTCCTCCCCCAACTCC
HBV pgRNA	Forward: CTGGGTGGGTGTTAATTTGG Reverse: TAGGATAGGGGCATTTGGTG
HCV RNA	Forward: TCCCTGGAGAAGAGCTACGA Reverse: AGCACTGTGTTGGCGTACAG
Hs_NR0B2_1_SG	QT00061460 from QIAGEN
Hs_NR1H4_1_SG	QT00030870 from QIAGEN
Hs_NR1H3_1_SG	QT00065156 from QIAGEN
Hs_PPARA_1_SG	QT00017451 from QIAGEN
Hs_PPARG_1_SG	QT00029841 from QIAGEN
Hs_HNF4A_1_SG	QT00019411 from QIAGEN
Hs_HNF1A_1_SG	QT00085428 from QIAGEN

Hs_POU2F1_1_SG	QT00086856 from QIAGEN

All miScript primer assays and miRNA inhibitors are purchased from QIAGEN.

3.2 Methods

3.2.1 Plasmid extraction

The transformation of chemically competent *E.coli* strains (DH5α, Invitrogen) was performed according to manufacturer's instructions. Briefly, 50µl aliquots of bacteria were thawed on ice, mixed gently with 2-3µl of ligation mixture and incubated on ice for 30 min. To improve the DNA absorption by bacteria, a heat shock for 30s at 42℃ followed by a subsequent incubation on ice for 2 minutes. Afterwards 200µl of SOC medium (Invitrogen) was added to the cells and the mixture was incubated on a shaker for 2 hours at 37℃. Using a sterile spatula the complete mixture was spread over an LB-agar plate containing a selective antibiotic (100 µg/ml of ampicillin or 50 µg/ml kanamycin). The plates were incubated overnight at 37℃. One bacterial colony was picked up from the LB agar plate, using a sterile pipette tip and transferred into the flask with LB-medium containing selective antibiotic. The volume of 2ml of the culture was used for Mini, 50ml for Midi and 250ml for Maxi preparations. After overnight incubation on the shaker at 37℃ the plasmid DNA was extracted using Qiagen plasmids Kits according to the manufacturer protocol. And the DNA concentration was quantified by spectrophotometric OD 260nm measurement as follows:

$$\text{Concentration [µg/ml]} = \text{OD 260nm} \times \text{dilution factor} \times 50$$

The purified plasmid DNA was checked by restriction enzyme digestion.

3.2.2 Cell culture and transfection

HepG2 and Huh7 were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100μg/ml streptomycin, and maintained at 37℃ in a humidified 5% CO2 atmosphere. HepG2.2.15 and Con-1 cells were cultured with 500 μg/ml of G418 (Sigma-Aldrich). Primary human hepatocytes were cultured in William E medium with 250μl Insulin, 2% DMSO, and 125μl hydrocortisone hemisuccinate.

The transfection of nucleic acids by transfection reagents (lipofectamine 2000, Invitrogen) was performed according to manufacturer's instructions. Briefly, diluted lipofectamine 2000 transfection reagent and nucleic acids into Opti-MEM I reduced serum medium, mix gently and incubate for 5 minutes at room temperature, add nucleic acids diluents into transfection reagent diluents, mix gently by fingers 20 times and then incubate for 20 minutes at room temperature. Finally, add the mixtures into plates with hepatoma cells drop by drop. 4-6 hours after incubation, discard the mixture and add new medium into cells.

The volume of transfection reagents and Opti-MEM used in different plates

Reagents / well	**6-well**	**12-well**	**24-well**	**48-well**
Lipofectamine 2000	5-6μl	2-3μl	1.5μl	0.3μl
Opti-MEM for nucleic acids	250μl	100μl	50μl	50μl
Opti-MEM for reagent	250μl	100μl	50μl	50μl
Opti-MEM for plate	1ml	500μl	300μl	100μl

3.2.3 Dual-luciferase reporter assay

48 hours post transfection, collected cells for Dual-luciferase activity detection according to manufacturer's instructions. Briefly, add a volume of Dual-Glo® Luciferase Reagent equal to the culture medium volume to each well and mix. Wait at least for 10 minutes, then measure the firefly luminescence. Add a volume of

Dual-Glo® Stop & Glo® Reagent equal to the original culture medium volume to each well and mix. Wait at least for 10 minutes, and then measure luminescence. Calculate the ratio of luminescence from the experimental reporter to luminescence from the control reporter. Normalize this ratio to the ratio of a control well.

3.2.4 HBV progeny DNA detection

Purification HBV DNA from culture medium according to manufacturer's instructions (QIAamp DNA Blood Mini Kit, QIAGEN). Briefly, pipetting 20μl QIAGEN Protease (or Proteinase K) into the bottom of a 1.5ml microcentrifuge tube and add 200μl sample, then add 200μl Buffer AL to the sample. Mix by pulse-vortexing for 15s and incubate at 56℃ for 10min. Briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside of the lid and add 200μl of ethanol (96 – 100%) to the sample, mix again by pulse-vortexing for 15s. After mixing, briefly centrifuge the 1.5ml microcentrifuge tube to remove drops from the inside of the lid. Carefully apply the mixture from step 6 to the QIAamp spin column (in a 2ml collection tube) without wetting the rim, close the cap, and centrifuge at 6000 x g (8000rpm) for 1min. Place the QIAamp spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate. Carefully open the QIAamp spin column and add 500μl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1min. Place the QIAamp spin column in a clean 2ml collection tube (provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp spin column and add 500μl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000rpm) for 3min. Continue directly with step 10, or to eliminate any chance of possible Buffer AW2 carryover, perform step 9a, and then continue with step 10. Place the QIAamp spin column in a new 2ml collection tube (not provided) and discard the collection tube with the filtrate. Centrifuge at full speed for 1 min. Place the QIAamp spin column in a clean 1.5ml microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp spin column and add 100μl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1min. Use

as template for real time PCR or put the samples at -20℃ for long-term storage.

Reaction mixture for real-time PCR

Component	**Volume (total: 20µl)**
2×UDG mix	10µl
Hope-forward prime	0.4µl
Hope-reverse prime	0.4µl
$MgCl_2$	0.8µl
BSA	1.0µl
Template	2.0µl
Aqua	5.4µl

3.2.5 HBV core particle extraction and detection

Wash cells by ice-cold PBS for one time and collect cells with cell lysis buffer (200µl, 150µl, 100 µl/well for 6, 12, 24-well plate), mix with tip. Incubate on ice for 10min and centrifuge at 13000rpm for 10 min at 4℃. Transfer the supernatant to new tube, add 6uM $MgCl_2$ and 0.8 mg/µl DNase 1 and 1.5 mg/µl RNase, and incubate for 20min at 37℃. Centrifuge at 13000rpm for 10min at 4℃, transfer the supernatant to new tube. Add 5× green loading buffer (5µl buffer for 20µl sample) and mix. 1.6% agarose gel electrophoresis for 2.5 h (volt = 45 V) at 4℃.

Capsid protein detection by non-denaturing western blot as follows:

Nitrocellulose membrane is transferred by absorption in 20× SSC overnight at RT and then blocking the membrane with 5% milk for 1hour at RT. The following steps are as like normal western blot.

Capsid DNA detection by southern blot as follows:

Denature gel for 30min at RT by shaking gently. Neutralize gel for 30min at RT by

shaking gently. Transfer DNA from gel to Nylon membrane by absorption overnight at RT. Crosslink by UV and the following steps are the same with southern blot hybridization.

3.2.6 Hybridization probe preparation

Digest pSM2 which contain HBV dimer by restriction enzyme EcoRⅠ

Component	**Volume (total: 100μl)**
EcoRⅠ	1μl (20000 units/ml)
10× NEB 2	10μl
pSM2	10μg
Aqua	up to 100μl

After digested at 37℃ for 2 hours, add 25μl 5× green loading buffer and run 0.8% agarose gel at 130v for 2 hours to separate two bands (3.2kb for HBV fragment and 2.7kb for vector). Agarose gel extraction to quantify the HBV fragment concentration and diluted into 5μg/ul, the HBV fragment were put in -80℃ for long-term storage or used directly for southern blot hybridization.

3.2.7 HBV RI extraction and hybridization

Hepatoma cells were transfected and cultured for 3-4 days in 6-well plates. And then wash cells with PBS, add 800μl iced lysis buffer and incubate on ice for 10min. Collect the cell lysate, vortex vigorous for 15s and incubate on ice for 10min. Centrifuge at 13200rpm for 2min at 4℃ and transfer the supernatant to a new 2ml tubes which contain 8μl 1M $MgCl_2$ and 8μl 10 mg/ml DNase1, mix gently by up and down 3-4 times, briefly centrifuge and incubate for 30min at 37℃. Briefly centrifuge and add 40μl 0.5M EDTA pH8.0 to a final concentration of 25mM, 80μl 10% SDS, mix by vortex and briefly centrifuge. Add 20μl 20 mg/ml proteinase K and incubate for 2 hours at 55℃. Briefly centrifuge and add phenol/chloroform (450μl + 450μl), mix and centrifuge at 13000rpm for 8min at RT. Transfer the upper liquid to a new

2ml tube, add 0.7V isopropanol, 0.1V NaAc (3M, pH5.2) and 2μg yeast RNA, mix and incubate over night at -20℃. Centrifuge at 13200rpm for 15min at 4℃ to collect the pellet. Wash the pellet with 1ml 75% ethanol and centrifuge at 8000rpm 2-5min at RT. Discard the supernatant carefully and dry the pellet for 5-10min. The pellets could be stored in -80℃ for long-term storage or used directly as follows:

Dissolve the pellet in 15μl 1× TE buffer and add 5μl 5× green loading buffer. Load samples into 1% agarose gel and electrophoresis for 1.5-2 hours at 50v. Denaturation gel for 30min at RT with gentle agitation and wash gel with aqua. Neutralize for 30min at RT with gentle agitation, wash gel one time with aqua and soak gel in 20× SSC. Transfer the DNA from gel to the Nylon membranes at 13Hg for 2 hours. Crosslink DNA on membranes at 150 J/cm^2.

Hybridization

Dilute 5μl HBV DNA fragment (5μg/μl) in 41μl 1× TE in 1.5ml tube, mix and denature for 5min at 95℃, snap cool the DNA by placing on ice for 5min after denaturation. Prehybridize the membranes in 10ml AmershamTM Rapid-Hyb buffer at 68℃ for 10-20min, make sure there is no bubble between membrane and tube. Briefly centrifuge the denatured DNA and add into the reaction tube, add 2μl a-32P dCTP into the reaction tube and mix by pipette up and down about 12 times, moving the pipette tip around in the solution. Incubate at 37℃ for 10min and stop the reaction by adding 5μl of 0.2M EDTA. Snap off the bottom closure of the microspin columns and centrifuge for 1min at 3000rpm. Place the column in 1.5ml tube which contains salmon sperm and slowly apply the reaction sample to the resin, centrifuge at 3000rpm for 2min. Denature the labeled DNA by heating to 95-100℃ for 5min, then snap cool on ice for 5min and centrifuge the tube briefly. Add purified labeled DNA to the pre-hybridization solution by directly dropping into hybridization tube and hybridize at 68℃ overnight. Wash the membranes at RT with gentle rotation in 50ml wash buffer I for 30 min two times. Wash the membranes at 65℃ with gentle rotation in 50 ml wash buffer Ⅱ for 30min two times. Detect radioactivity.

3.2.8 Total protein collection

Three days after transfection, we collected cells according to manufacturer's instructions (Red Loading Buffer, Cell Signaling). Briefly, aspirate media from cultures and wash cells with 1× PBS one time. Lysis cells by adding moderate 1× loading buffer. Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube and keep on ice. Heat the samples to 95-100ºC for 5min, cool on ice and microcentrifuge for 2-5min. Load samples onto SDS-PAGE gel or put in -80℃ for long-term storage.

3.2.9 Cytoplasmic and nucleus protein purification

The separation of nuclear extract and cytoplasmic fractions from cultured cells were preformed according to manufacturer's instructions (Thermo Scientific). Perform all centrifugation steps at 4℃, keep all samples and extracts on ice. Briefly, harvest cells with trypsin-EDAT and then centrifuge at 500×g for 5min. Wash cells by suspending the pellet with PBS and centrifuge at 500×g for 2 min. Remove the supernatant and leave the cell pellet as dry as possible. Add 100μl ice-cold CER I to pellet and vortex the tube vigorously on the highest setting for 15s, incubate on ice for 10min. Add 5.5μl ice-cold CER Ⅱ and vortex the tube for 5s on the highest setting, incubate for 1min on ice. Vortexing 5s on the highest setting and centrifuge for 5min at maximum speed. Immediately transfer the supernatant (cytoplasmic extract) to a new pre-chilled tube and place on ice until use or storage. Suspend the insoluble (pellet nuclei) fraction in 50μl ice-cold NER, vortex on the highest setting for 15s, place the samples on ice and continue vortexing for 15s every 10min, for a total of 40min. Centrifuge the tube at maximum speed for 10min and immediately transfer the supernatant (nuclear extract) fraction to a new pre-chilled tube. Store at -80℃ until use.

3.2.10 Western blot

Load protein samples into 10%-15% separate SDS-PAGE gels, electrophoresis at 130v for 2 hours. Transfer protein to NC membrane at 100v for 1 hour on ice. Incubate the membrane in 5% blocking buffer by shaking gently for 1 hour at RT.

Incubate the membrane in primary antibody overnight at 4℃. Wash membrane by PBST for 10min by gentle shaking for three times. Incubate membrane in HRP-conjugated secondary antibody for 1 hour at RT with gentle shaking. Wash membrane by PBST for 10min by gentle shaking for three times. Detect enzyme activity by ECL reagents.

3.2.11 RNA extraction

Hepatoma cells were transfected and cultured in 12-well plates for three days. And then collect cells with 500μl Trizol reagent into 1.5ml tubes and incubate the homogenized sample for 5min at RT to permit complete dissociation of the nucleoprotein complex. Add 100μl chloroform and shake tubes vigorously by hand for 15s, incubate at RT for 2-3min, and then centrifuge at 12,000×g at 4℃ for 15min. Transfer the aqueous phase to a new tube and precipitate the RNA from the aqueous phase by mixing with 0.25ml 100% isopropyl alcohol. Incubate at RT for 10min and then centrifuge at 12,000×g at 4℃ for 10min. Remove the supernatant from the tubes, wash the RNA pellet with 1ml 75％ ethanol, and then centrifuge the tubes at 7500×g at 4℃ for 5min. Discard the wash and air dry the RNA pellet for 5-10min, dissolve RNA in RNase-free water.

3.2.12 Real-time RT-PCR

PCR for the detection of RNA was performed according to manufacturer's instructions (one-step SYBR Green RT-PCR, QIAGEN). Briefly, after purified (RNase-Free DNase Set and RNeasy Mini Kit, QIAGEN), the RNA samples are diluted into 100ng/ul, and then a reaction mix as follows:

Component	**Volume (total: 20μl)**
2× SYBR Green RT-PCR Master Mix	10μl
10× primers	2.0μl
QuantiFast RT mix	0.2μl

Template RNA	1.0μl
RNase-free water	6.8μl

Mix the reaction thoroughly and dispense appropriate volumes into PCR vessels. **Program real-time cycler according to the following program:**

Step	**Time**	**Temperature**
Reverse transcription	10min	50℃
PCR initial activation step	5min	95℃
Two-step cycling		
Denaturation	10s	95℃
Annealing/extension	30s	60℃
Number of cycles	35-40	

3.2.13 Northern blot

All procedures should be carried out carefully to avoid RNase contamination. **Preparation:** Wash gel electrophoresis chamber, tray and combs three times with clean reagent in VE-water, and then wash with DEPC-water for two times, 100% ethanol one time and finally dry in ventilate hood. **Prepare agarose gel:** Dissolve 1.2g agarose in 10ml NorthernMax 10× Gly prep running buffer diluted in 95ml DEPC water. **Prepare RNA samples:** Add 1V Glyoxal load Dye into 10μg RNA (final volume is less than 25μl), incubate at 50℃ for 30min and then cool in ice for at least 5min. **Electrophoresis:** Run agarose gel in 1× Gly runnig buffer at 50v for 2.5-3 hours at 4℃. **Transfer :** RNase-free DEPC-water wash gel for 5-15min, 10× SSC shake for 15min one or two times, and then transfer gel to positive-charged nylon membrane in 20× SSC at 13Hg for 2 hours. **Hybridization:** See previous methods as southern blot.

3.2.14 MiRNA quantification

First of all, extract total RNA from cells by Trizol. Then, 1μg total RNA is reverse-transcribed into cDNA according to manufacturer's instructions (miScript Ⅱ RT Kit, QIAGEN). Briefly, prepare the reverse-transcription master mix on ice as follows:

Component	**Volume (total: 20μl)**
5× miScript HiSpec Buffer	4.0μl
MiScript Reverse Transcriptase Mix	1.0μl
Template RNA	variable
RNase-free water	up to 20μl

Incubate for 60min at 37℃ and then incubate for 5min at 95℃ to inactivate miScript Reverse Transcriptase Mix. The reverse transcribed products used for mature miRNAs quantification by miScript SYBR Green PCR kit (QIAGEN).

Reaction mixture as follows:

Component	**Volume (total: 20μl)**
2× SYBR Green RT-PCR Master Mix	10μl
10× miScript Universal Primer	2.0μl
10× miScript Primer Assay	2.0μl
Template RNA	2.0μl
RNase-free water	4.0μl

Cycle parameters as followed: a single step 95℃ for 15min followed by 40 cycles at 94℃ (15s), annealing at 55℃ (30s) and extension at 70℃ (30s).

For positive control/standard curve: Dilute synthetic miRNA to get 10^{10} copies/μl.

Prepare 20µl reverse-transcription reaction just like above, using 5µl synthetic miRNA (10^{10} copies/µl) and 50ng carrier bacterial RNA (yeast mRNA, 10 µg/µl). Incubate for 60min at 37℃. Incubate for 5min at 95℃ to inactivate and place on ice. Add 480µl bacterial carrier RNA (1 ng/µl concentration) to the 20µl reaction. Mix gently by pipetting up and down and centrifuge briefly. Note: This dilution yields 10^8 copies cDNA/µl (assuming an efficiency of 100 %).

Using the diluted cDNA mix from step 5 and carrier RNA (1 ng/µl).

Dilution tube	Volume	Carrier RNA	Copies/µl	Use in PCR
1	5 µl diluted cDNA mix	45 µl	1×10^7	2 µl (2×10^7 copies)
2	5 µl from tube 1	45 µl	1×10^6	2 µl (2×10^6 copies)
3	5 µl from tube 2	45 µl	1×10^5	2 µl (2×10^5 copies)
4	5 µl from tube 3	45 µl	1×10^4	2 µl (2×10^4 copies)
5	5 µl from tube 4	45 µl	1×10^3	2 µl (2×10^3 copies)
6	5 µl from tube 5	45 µl	1×10^2	2 µl (2×10^2 copies)

3.2.15 Cell proliferation

Seed hepatoma cells into the 6-well plates and transfect with the miRNA. 24 hours post transfection, the cell were separated into 96-well plates at 10000 cells. After 24 hours, the cells were starved for additional 16 hours (12-16 hours) within 100µl serum-free 1640 medium. Serum-free medium was replaced with 100µl complete medium, and 30min later, 0.5 µl/well (diluted in 50µl complete medium) of H^3-thymidine was added. 6-8 hours later, cells were washed one time with PBS and then 100µl trypsin was added to digest cells for about 10min at 37℃. Suspend cells from 96-well plate and transfer the cells from plate into paper with scintillation counter. Detect radioactivity.

3.2.16 Cell cycle analysis

Wash cell with PBS, add 400µl Trypsin-EDTA and incubate for 5-8min at 37℃. Add 800µl complete medium to inactivate Trypsin and harvest cells in 2ml tube.

Centrifuge at 1200rpm for 5min at 4℃. Drop off the medium with pipette and resuspend cells with 1ml PBS. Centrifuge at 1200rpm for 5min at 4℃. Drop off PBS and resuspend cells with pipette with 300μl PBS to get single cell. Add 900μl 100% ethanol and mix by vortex gently. Fix cells for at least 1hour at 4℃.

PI staining

Centrifuge at 3000rpm for 10min at 4℃ to get cell pellet. Drop off supernatant, resuspend by 1ml PBS and centrifuge at 3000rpm for 10min at 4℃ to get cell pellet. Drop off supernatant and PI staining (PI solution: 500μl PBS + 200 μg/ml RNase A + 50 μg/ml PI + 0.1% Triton X-100), mix and incubate for 30min at 4℃. Add 1ml FACS and mix, then centrifuge at 3000rpm for 10min at 4℃. Drop off supernatant and wash cells once more, centrifuge at 3000rpm for 10min. Resuspend cells with 200-400μl FACS and transfer into FACS tube. Analyse by flow cytometry.

3.2.17 RNA sequence assay

The RNA quantification were detected and analyzed by BGI TECH SOLUTIONS (HONGKONG) CO., LIMITED SERVICE AGREEMENT.

3.2.18 Statistical analysis

Statistical analyses were performed using Graph Pad Prism software version 5.1. Analysis of variance with Student's t test was used to determine significant differences in multiple comparisons. P values < 0.05 were considered statistically significant. Data are presented as standard error of the mean (SEM). Gray-value analysis was processed by ImageJ software.

4 Results

4.1 MiRNAs modulate HBV replication

4.1.1 Liver tumor suppressor miRNAs enhance HBV replication specifically in different hepatoma cell lines

To identify miRNA candidates influencing HBV replication, six miRNA mimics (sequences information are in Materials and Methods), which are reported of being highly expressed in normal liver and usually down-regulated in hepatocellular carcinoma tissues, were selected and transfected into HepG2.215 cells at a concentration of 20nM. HBV replicative intermediate (HBV RI) were isolated at day 4 after transfection and analyzed by southern blot, culture media were collected for HBsAg and HBeAg analysis by CMIA test. As compared to control miR-C, HBV replication was enhanced slightly after transfected with miR-99a and strongly after transfected with miR-125b (Figure 4.1A, line 4 and line 6). We used miR-1 as positive control since our previous results confirmed that miR-1 could enhance HBV replication (ZHANG *et al.* 2011b). As like HBV RI, HBsAg and HBeAg from culture media were also increased after transfection miR-99a and miR-125b (Figure 4.1B). The other four selected miRNAs, including miR-101, 199a-3p, 519d and 637 could down-regulate HBV RI formation slightly compared with miR-C (Figure 4.1A, line 5 and 7-9), but they had no obvious effect on HBsAg and HBeAg secretion (Figure 4.1B).

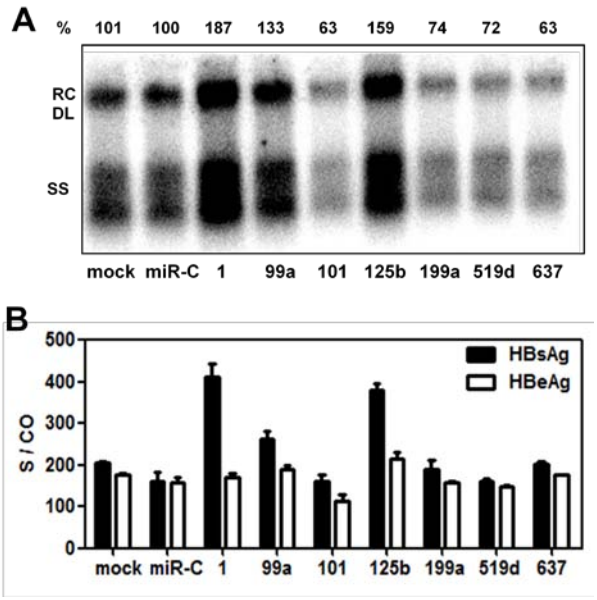

Figure 4.1 The ability of HBV replication in HepG2.215 cells

(A) HepG2.215 cells were transfected with different miRNA mimics at 20 nM. Cells were harvested at day 4 and the levels of HBV RI from intracellular core particles were determined by southern blot assay. The signals were analyzed and miR-C was set to 100. (B) Four days after transfection, we collected media for HBsAg and HBeAg detection by CMIA test.

To further evaluate these miRNAs effects on HBV replication, 20nM of miRNA mimics and a replication competent clone of HBV pSM2 were cotransfected into Huh7 and HepG2 cells. In Huh7, besides the positive control, miR-1 could enhance HBV replication remarkably, miR-125b also enhanced HBV replication remarkably, miR-101, miR-519d enhanced HBV replication slightly compared to miR-C or mock group (Figure 4.2A, upper panel, line 5, 6, 8). While different from HepG2.215, HBsAg and HBeAg in culture media from Huh7 were not increased for these miRNAs which could enhance HBV RI from intracellular core particle (Figure 4.2A, below panel, line 5, 6, 8). The other three miRNAs, including miR-99a, 199a-3p and 637 had no obvious effect on HBV replication. In HepG2, besides miR-1, only miR-125b could enhance HBV replication, other five miRNAs did not up-regulate HBV replication. Moreover, HBV proteins in culture media from HepG2 also have no difference compared with miR-C group for all groups (Figure B).

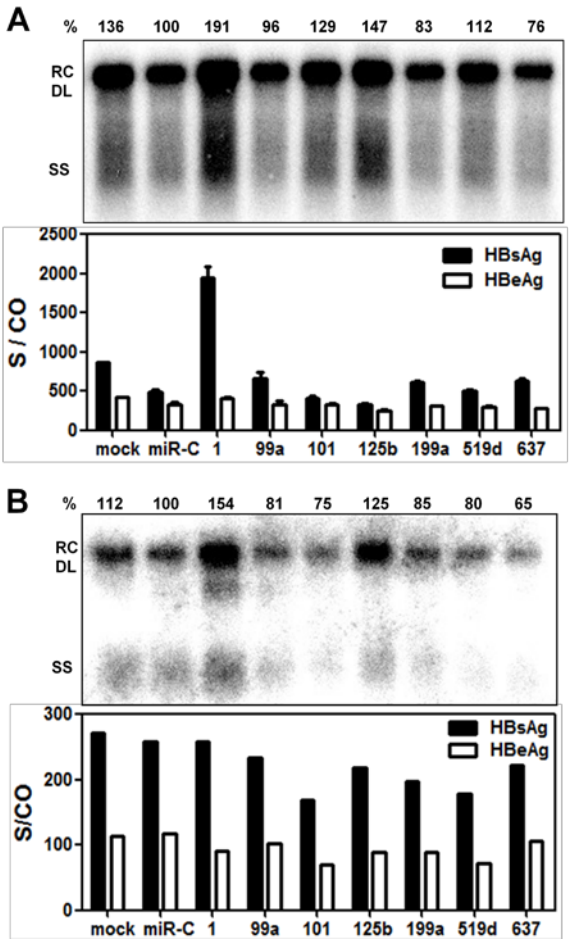

Figure 4.2 Influence of miRNAs on HBV replication in hepatoma cells

HBV RI, HBsAg and HBeAg from Huh7 cells (A) and HepG2 cells (B). We cotransfected an HBV genome expression plasmid and different miRNA mimics into hepatoma cells. Cells were harvested at day 3 and the levels of HBV RI were determined by southern blot (upper panel). HBsAg and HBeAg from culture media were tested by CMIA (below panel). The signals were analyzed and miR-C was set to 100.

In order to test if these miRNAs could also affect other hepadnavirus replication, such as hepatitis C virus, we transfected them into Con I cells which contain HCV subgenomic replicon, and we used miR-122 as positive control since miR-122 transfection results in an increase of HCV RNA copy number (JOPLING *et al.* 2005). 3 days after transfection, we extracted RNA for real time RT-PCR. Compared to miR-C or mock, positive miR-122 could up-regulate HCV replication significantly, while other six selected miRNAs did not increase HCV RNA copy number (Figure 4.3).

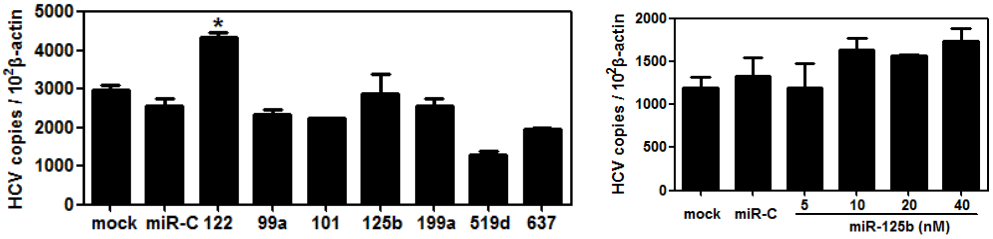

Figure 4.3 Influence of miRNAs on HCV replication

Con I cells harboring HCV subgenomic replicon were transfected with different miRNA mimics (left panel) or different concentrations of miR-125b mimic (right panel), a nonspecific control of miR-C was set at a concentration of 20nM and cultured for 3 days. Total RNA were extracted and HCV copies were determined by real time RT-PCR and normalized against 10^2 β-actin transcripts. * $P<0.05$.

4.1.2 The effect of miR-125b on HBV replication is seed sequence specific, but is not binding to HBV genome directly

Since miRNA acts functions by its 5 terminal seed sequence, we want to know if miR-125a-5p, which has consistent seed sequence with miR-125b-5p (Table 1, bold black characters), could also affect HBV replication.

Table 1 Sequence of hsa-miR-125b and hsa-miR-125a

miRNA	**Sequence (5'-3')**
has-miR-125b-5p	**UCCCUGAG**ACCCUAACUUGUGA
hsa-miR-125a-5p	**UCCCUGAG**ACCCUUUAACCUGUGA

We transfected 20nM of miRNAs into HepG2.215 or cotransfected pSM2 and miRNAs into Huh7 cells, then isolated HBV RI for southern blot analysis. As compared to miR-C, miR-125a-5p could also enhance HBV replication obviously both in HepG2.215 and Huh7 cell lines even though not as remarkably as miR-125b-5p (Figure 4.4).

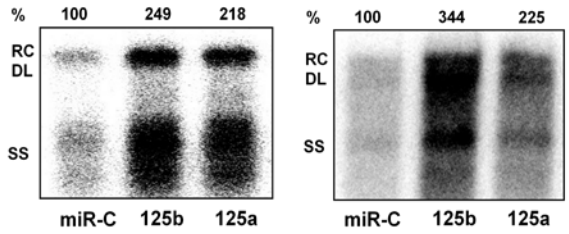

Figure 4.4 Influence of miR-125a and miR-125b on HBV replication

After transfection or cotransfection of pSM2 with 20nM miR-125b or miR-125a into HepG2.215 (left panel) and Huh7 (right panel), the HBV RI were extracted and determined by southern blot. The signals were analyzed and miR-C was set to 100.

Computational analysis showed that miR-125 seed sequence could target HBV genome at polymerase and surface genes region (Table 2) (POTENZA *et al.* 2011), this suggested miR-125b may regulate HBV genes expression by directly binding to HBV genome.

Table 2 Computational analysis of HBV genome targets for hsa-miR-125a/b-5p

Human miRNA	HBV genomic target (nt)	Target ORF	Mfe (kcal/ mol)	miRanda score	miRNA-mRNA pairing
125a-5p	3037-3065	P/S	-18.4	161	miRNA: 3′AGUGUCCAAUUUC-----CCA**GAGUCCC**U5′ |: ::|| |::|| | |||||| mRNA: 5′UUUUGGGGUGGAGCCCUCAGGCUCAGGGC3′
125b	3046-3065	P/S	-14	159	miRNA: 3′AGUGUUCAAUCCCA**GAGUCCC**U5′ | | ||| ||||||| mRNA: 5′GGAGCCCUCAGG--CUCAGGGC3′

The most common methods for miRNA target validation are based on reporter gene constructs transfected in hepatoma cells. The rationale for using this assay is that the binding of a given cellular miRNA to the target, transcribed together with the luciferase coding sequence, will repress reporter protein production thereby reducing luciferase activity compared to a control. The HBV full length genome or four genomic fragments containing firefly luciferase report plasmids were constructed (foe details, please see materials and methods) and cotransfected with miR-C or miR-125a, 125b and renilla luciferase report vector into Huh7 cells. Luciferase activities were measured 48 hours after transfection and the ratio of firefly to renilla luciferase activity was calculated, the luciferase activities were normalized to pMIR-REPORT vector not containing any HBV sequences. The results showed that miR-125b did not repress HBV genome containing luciferase report plasmids activity, and miR-125a

only weakened report plasmids which contained HBV fragment 1 activity slightly, but the difference between miR-C and miR-125a group was not significant (Figure 4.5).

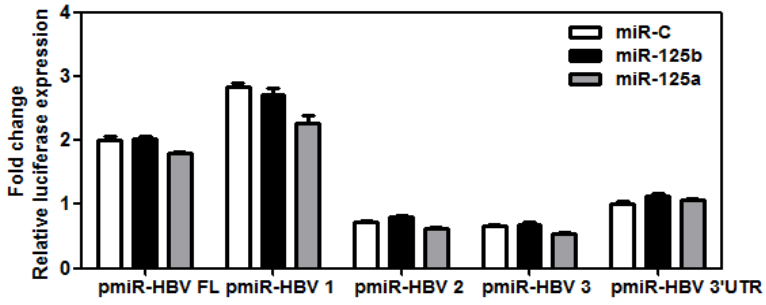

Figure 4.5 Luciferase activities from HBV genome containing plasmids

Renilla and firefly luciferase reporter plasmids which contain HBV fragments were cotransfected at a concentration of 50μg/ml into Huh7 with 20nM of miR-C, miR-125b, or miR-125a and assayed for luciferase activity at 48 hours. We calculated the ratio of firefly to renilla for each well and the luciferase activity was normalized to empty vector transfection. All detections were performed in triplicate.

4.1.3 MiR-125b enhances HBV RI and progeny production in dose dependence and its inhibitor could down-regulate HBV RI production

We then transfected different concentrations of miR-125b into HepG2.215 cells or cotransfected pSM2 and different concentrations of miR-125b into Huh7 cells, 4 days or 3 days post transfection, isolated HBV RI for southern blot analysis. Compared with miR-C, miR-125b could enhance HBV RI production in dose-dependent manner both in HepG2.215 and Huh7 cell lines (Figure 4.6).

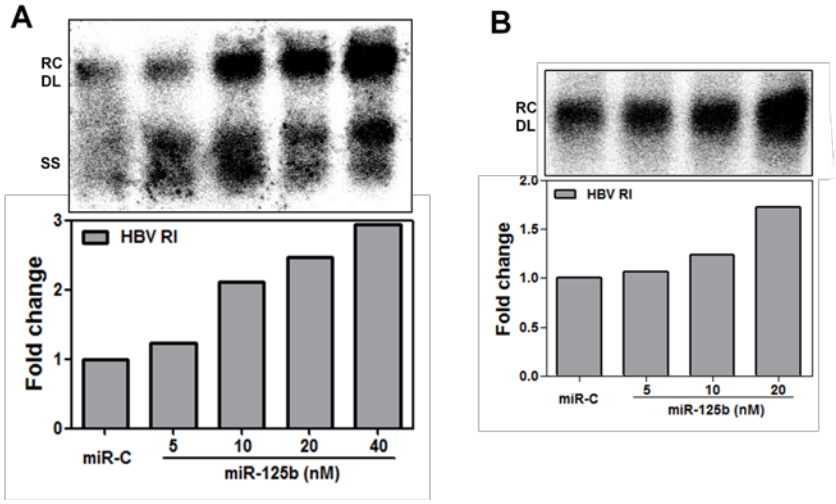

Figure 4.6 MiR-125b enhances HBV RI generation in dose dependent manner

HBV RI in HepG2.215 which were transfected with 0, 5, 10, 20 or 40nM miR-125b mimics for 4 days (A) and Huh7 which were cotransfected with an HBV genome expression plasmid pSM2 and 0, 5, 10 or 20nM miR-125b mimics for 3 days (B). The levels of HBV RI from intracellular core particles were determined by southern blot. The signals obtained from miR-C transfected samples were set to 1.

Besides HBV RI, we also tested HBV progeny secretion by real time PCR and CMIA assay. 4 days after transfection different concentrations of miR-125b into HepG2.215 cells, purified HBV DNA from culture media. Compared to miR-C or mock, miR-125b could enhance HBV progeny production in dose dependent manner remarkably (Figure 4.7).

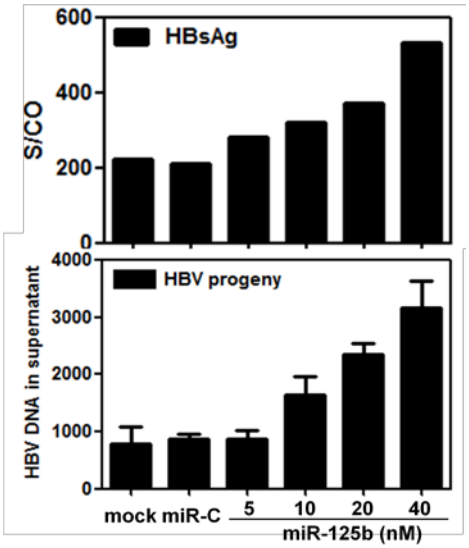

Figure 4.7 MiR-125b promotes HBV progeny secretion

Four days post transfection with 0, 5, 10, 20, 40nM of miR-125b or miR-C into HepG2.215, HBV DNA was purified for real time PCR analysis (below panel) and HBsAg from culture media for CMIA test (upper panel).

As previous results showed that exogenous transfected miR-125b could enhance HBV replication, this suggested that the inhibition of endogenous mature miR-125b may suppress HBV replication. We synthesized the hsa-miR-125b inhibitor named anti-miR-125b and transfected it into hepatoma cells to test its inhibition efficiency and influence on HBV replication. The real time PCR results showed that anti-miR-125b could suppress mature miR-125b generation remarkably both in HepG2.215 and Huh7 cell lines (Figure 4.8).

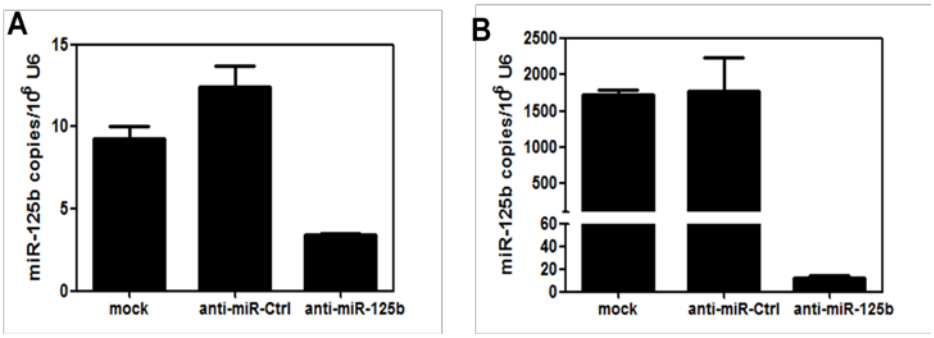

Figure 4.8 MiRNA inhibitor suppresses mature miR-125b generation

HepG2.215 (left) and Huh7 (right) were transfected with 20nM of miRNA inhibitors, and 3 days after transfection, we extracted total cellular RNA for reverse-transcription into cDNA and then real time PCR for mature miR-125b quantification. The relative mRNA copies were determined by a standard curve using serial dilutions of synthetic miR-125b mimic and normalized by U6 snRNA. All reactions were performed in triplicate.

Next we tested miRNA inhibitors effect on HBV RI formation. Inhibition of miR-125b in HepG2.215 did not only down-regulate HBV replication, but was slightly up-regulated (Figure 4.9A), the reason may be that in HepG2.215 cell line, mature miR-125b was already very low (Figure 4.8A), so exogenous anti-miR-125b may have non-specific effect on miRNAs generation. While in Huh7, after transfection of 20nM anti-miR-125b for 3 days, HBV RI was down-regulated, but there was no effect on HBV proteins secretion (Figure 4.9B). 3 days after cotransfection different concentrations of anti-miR-125b and pSM2 into Huh7, we isolated HBV RI from intracellular core particle for southern blot analysis. Compared to anti-miR-C or mock, even though transfected low concentration of anti-miR-125b, such as 5 and 10nM, seemed slightly up-regulated HBV RI. The trend for anti-miR-125b suppression HBV RI formation in dose dependent manner was obvious, so as to HBV proteins secretion (Figure 4.9C).

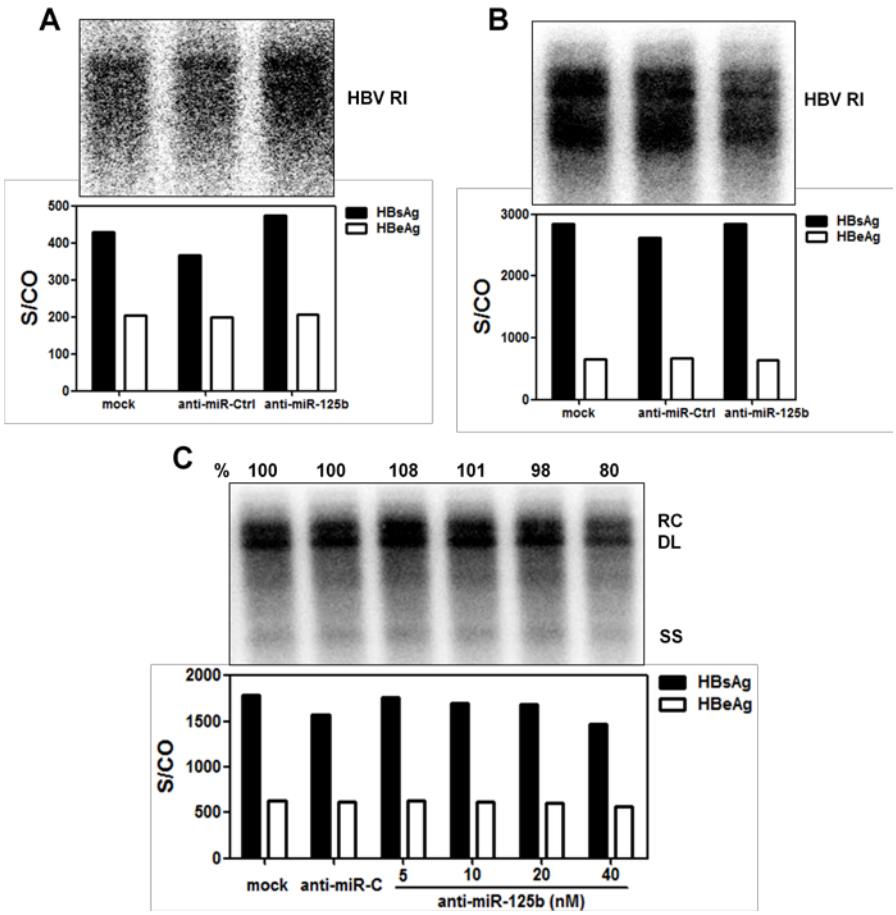

Figure 4.9 Influence of miRNA inhibitors on HBV RI formation

HBV RI, HBsAg and HBeAg in HepG2.215 cells (A) and Huh7 cells (B and C). 4 or 3 days post transfection miRNA inhibitors into hepatoma cells, HBV RI was isolated for southern blot analysis (upper panel) and culture media was collected for CMIA test (below panel). The signals were analyzed and miR-C was set to 100.

4.1.4 MiR-125b regulates HBV in the post transcription steps and enhances HBV replication with miR-1 synergistically

As HBV expresses proteins under the control of four promoters, so we supposed that miR-125b may regulate HBV promoter activities. We inserted the HBV four promoter regions into a luciferase report plasmid pGL3-basic to get pSP1, pSP2, pCP and pXP (Figure 4.10A and information in Materials and Methods), then cotransfected them with renilla luciferase report plasmid and miR-C or miR-125b into hepatoma cells. 48 hours after transfection, we collected cells for dual-luciferase activity detection. As compared to miR-C, miR-125b had no influence on HBV SP1 and SP2 promoter activities, for CP and XP promoters, the results showed there was a little suppression

on their activity, but the difference between control and CP, XP were not significant (Figure 4.10B).

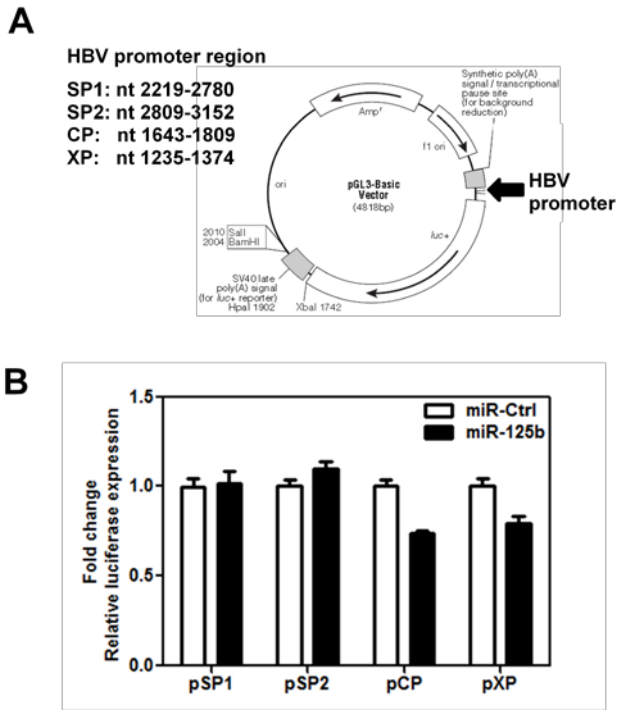

Figure 4.10 Influence of miR-125b on HBV promoter activities

(A) HBV promoter regions and the schematic for luciferase report plasmids. (B) The effect of miR-125b on HBV promoter activities. Renilla and luciferase reporters containing HBV promoter regions pSP1, pSP2, pCP, and pXP were cotransfected at a concentration of 50ng/ml with 20nM of miR-125b or miR-C into HepG2.2.15 cells and assayed for luciferase activity at 48 hours. We calculated the ratio of firefly to renilla for each well, and luciferase activity was normalized against pGL3-basic control transfection. The values obtained from miR-C transfected samples were set to 1. All detections were performed in triplicate.

In order to further verify that miR-125b has no effect on the four HBV promoters, we tested the RNA level of several host cellular transcription factors since they may participate in the regulation of HBV promoter activities. After transfection 20nM miR-C or miR-125b into HepG2.215 for 3 days, we purified RNA for real time RT PCR. And the results confirmed that miR-125b did not regulate these factors expression (Figure 4.11).

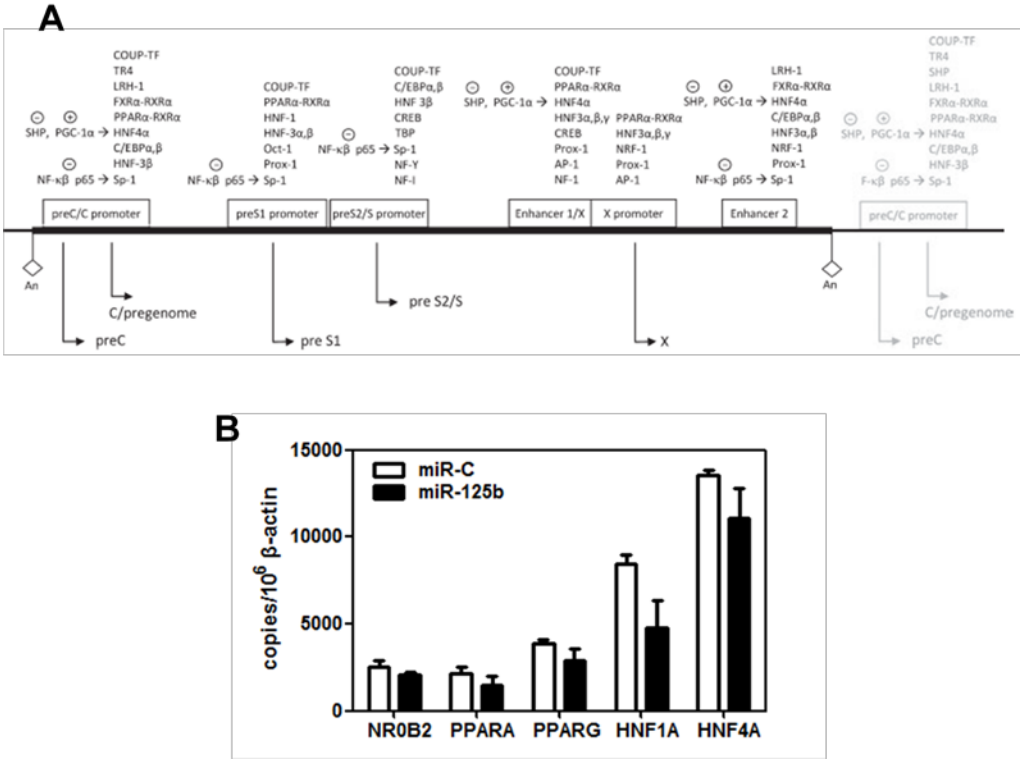

Figure 4.11 Influence of miR-125b on host cellular transcription factors

(A) Binding sites of hepatocyte enriched transcription factors within HBV promoter and enhancer regions (QUASDORFF and PROTZER 2010). (B) Several transcription factors RNA in HepG2.215 were selected and tested by real time RT PCR after transfection with miR-C or miR-125b for 3 days.

Besides the above experiments, we also tested HBV RNA level after transfection different concentrations of miR-125b into HepG2.215 or cotransfection pSM2 with different concentrations of miR-125b into Huh7 by real time RT PCR and northern blot, and all these results confirmed that miR-125b did not regulate HBV RNA transcription (Figure 4.12).

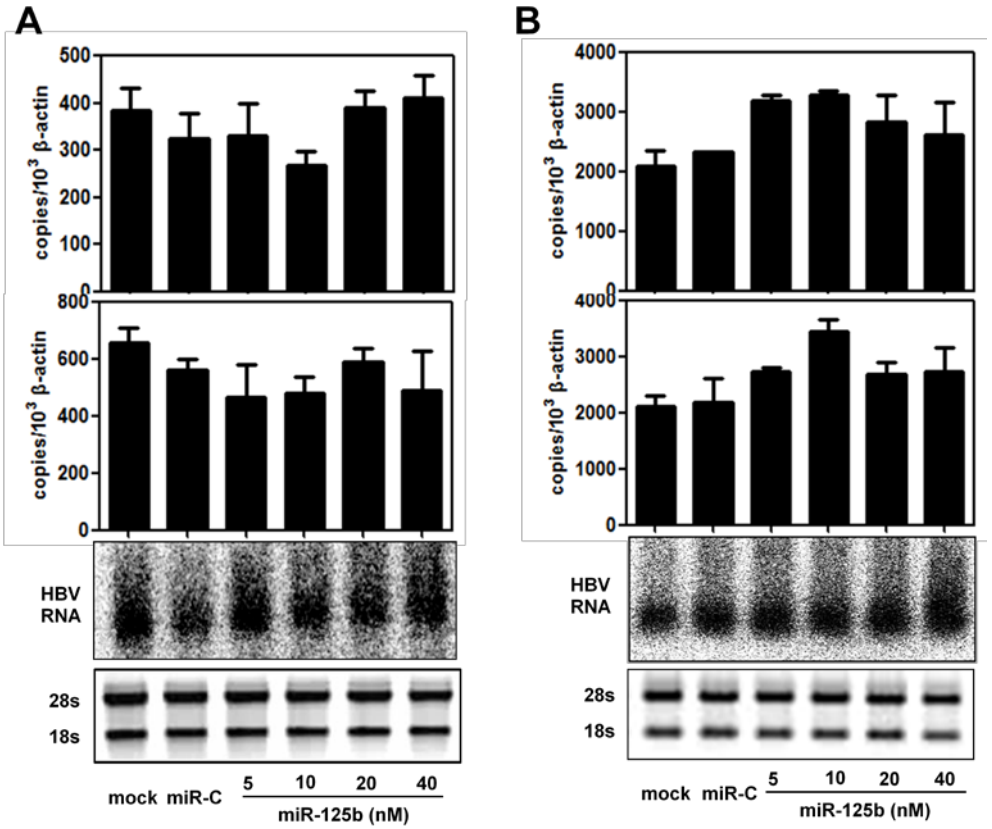

Figure 4.12 Influence of miR-125b on HBV RNA level

HBV RNA from HepG2.215 cells (A) and Huh7 cells (B). After transfection or cotransfection pSM2 with 0, 5, 10, 20 or 40nM miR-125b mimics into hepatoma cells, cellular RNA were extracted by Trizol. The HBV pgRNA (upper panel), xRNA (middle panel) levels were determined by real-time PCR, and HBV total RNA were also detected by northern blot , 28S and 18S RNA were used as loading control (below panel).

Then we wanted to figure out in which steps of the HBV life cycle miR-125b may play a role. Since the previous results showed that miR-125b could facilitate HBV RI from intracellular core particle accumulation in dose dependence, we supposed that miR-125b may be involved in HBV core protein expression and HBV nucleocapsid formation. We transfected different concentrations of miR-125b into HepG2.215 or cotransfected pSM2 and different concentrations of miR-125b into Huh7 cells, 3 days post transfection, collected cell lysate for HBcAg detection by western blot, isolated and tested HBV nucleocapsid both by western blot and southern blot. MiR-125b could up-regulate HBV core protein expression in dose-dependence slightly both in these two kinds of hepatoma cell lines (Figure 4.13A and B, upper panel). And compared with HBcAg accumulation, HBV nucleocapsid formation was up-regulated

by miR-125b in dose dependence more obviously in hepatoma cells (Figure 4.13A and B, below panel).

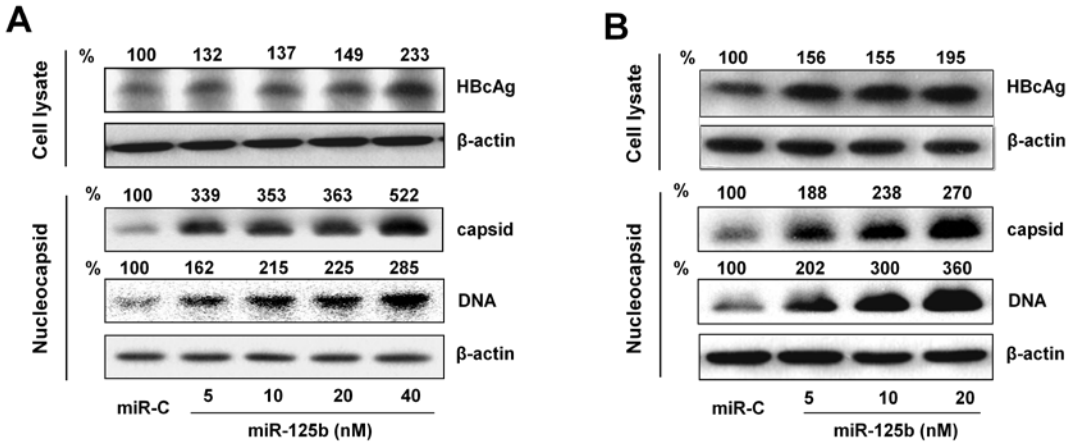

Figure 4.13 MiR-125b enhances HBcAg expression and nucleocapsid formation in dose dependence

HBcAg and nucleocapsid from HepG2.215 cells (A) and Huh7 cells (B). Hepatoma cells were transfected with 0, 5, 10, 20, 40nM miR-125b or cotransfected different concentrations of miR-125b with pSM2 for three days. Cell lysates were harvested at day 3 for total HBcAg detection (upper panel), nucleocapsid was extracted and detected both by western blot and southern blot (below panel). The signals were analyzed using β-actin as loading control and miR-C was set to 100.

Since Dr. Xiaoyong Zhang demonstrated that miR-1 enhanced HBV replication during transcription (ZHANG *et al.* 2011b), while miR-125b regulated downstream steps, we suggested that miR-1 and miR-125b may have a synergistic effect on the enhancement of HBV replication. We confirmed that miR-1 could up-regulate HBV core promoter activity while miR-125b did not up-regulate by dual luciferase activity assays (Figure 4.14A). By transfection and western blot, we confirmed that both miR-1 and miR-125b could up-regulate HBV core protein expression and capsid formation both in HepG2.215 and Huh7 cells. As for miR-125b, even though the enhancement on HBcAg was not as obvious as miR-1, its enhancement on capsid formation was more remarkable than miR-1 (Figure 4.14B and C). We also tested the difference for the effect on HBV RI between individually transfected miR-1/miR-125b and cotransfected miR-1 with miR-125b. Compared to transfection of miR-1 or miR-125b alone, cotransfection of miR-1 with miR-125b could

up-regulate HBV RI accumulation more effectively both in HepG2.215 and Huh7 cell lines (Figure 4.14C and D).

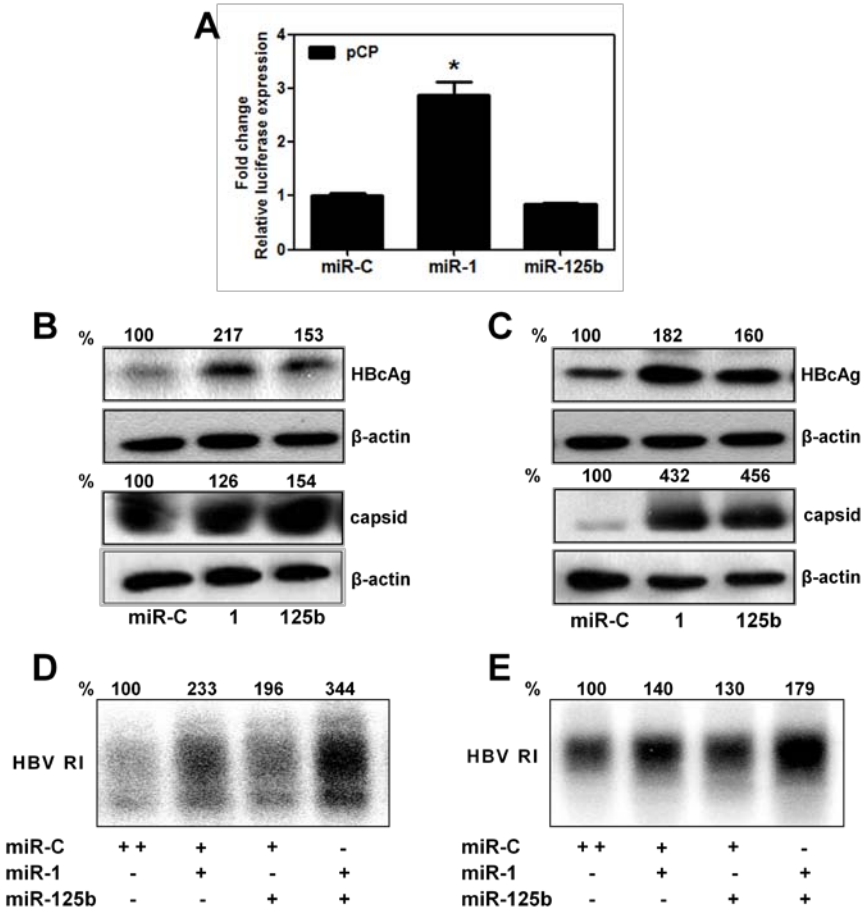

Figure 4.14 MiR-1 and miR-125b have synergistic effect on upregulation of HBV replication

(A) MiR-1 and miR-125b on HBV core promoter activity. The relative luciferase expression was determined and the values obtained from miR-C transfected samples were set to 1. * $P<0.05$. HBcAg and HBV capsid from HepG2.215 cells (B) and Huh7 cells (C). Three days after transfection or cotransfection of pSM2 and 20nM miR-C, miR-1 or miR-125b into hepatoma cells, the total cellular proteins and nucleocapsid was extracted for HBcAg detection. The signals were analyzed using β-actin as loading control and miR-C was set to 100. HBV RI from HepG2.215 cells (D) and Huh7 cells (E). Four or three days after transfection or cotransfection of pSM2 with 5nM miR-1, miR-125b or 5nM miR-1 plus 5nM miR-125b into cells, the HBV RI were extracted and detected by southern blot. We used miR-C to complement each transfection sample to 10 nM, and the signals of miR-C sample were set to 100.

As IFN-α could suppress HBV replication when viral load is high, and enhance HBV replication when viral load is low, the suppression of HBV replication by IFN-α

involves both transcriptional and post transcriptional regulations (TIAN *et al.* 2011). So we want to investigate if miR-125b has a synergistic or antagonistic effect on HBV replication with IFN-α. We transfected 20nM miR-C or miR-125b into HepG2.215 cells, and changed transfection mixture into new cell culture media containing 10000 UI/ml IFN-α 6 hours post transfection. Four days later, we isolated HBV RI from intracellular core particle for southern blot analysis. The results showed that even though miR-125b could enhance HBV replication significantly, IFN-α could weaken this effect, so as in Huh7 cell lines (Figure 4.15). This means that the suppression for the IFN-α, and the enhancement for the miR-125b on HBV replication do not interfere with each other.

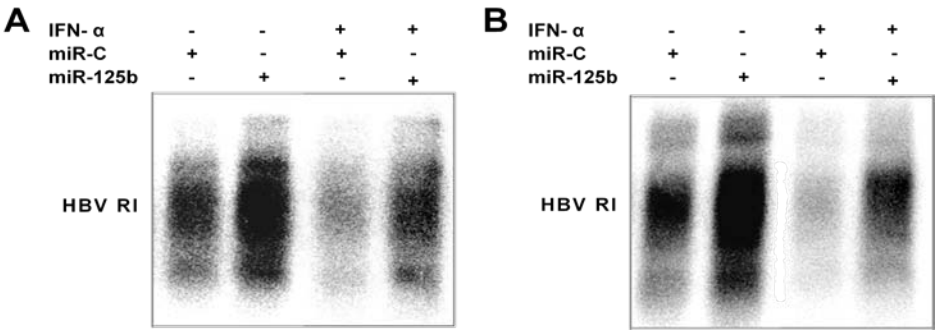

Figure 4.15 Influence of miRNA transfection and IFN treatment on HBV replication

(A) HBV RI from HepG2.215 transfected with 20nM miR-C or miR-125b, and meanwhile add 10000 UI/ml IFN-α was added for 4 days culturing. (B) HBV RI from Huh7 cotransfected with pSM2 and 20nM miR-C or miR-125b, and meanwhile 100 UI/ml IFN-α was added for 3 days culturing.

4.2 Tumor suppressor miRNAs are down-regulated in hepatoma cells

4.2.1 MiR-125b generation is weakened in hepatoma cells but is not regulated by HBx protein

We then selected and synthetised miR-99a, miR-101 and miR-125b primers to detect these mature miRNAs quantity in primary human hepatocytes (PHH) and hepatoma cells since they had different influence in HBV replication in different hepatoma cell lines. By miScript PCR, we confirmed that the expression of all these three miRNAs were significantly down-regulated in hepatoma cells compared with in PHH. For

miR-99a and miR-125b, their expression in HepG2.215 were more than 10^5 fold lower compared with in PHH. In HepG2 and Huh7, even though their differences were not so remarkable, there were still more than 2 fold and 40 fold differences for both miR-99a and miR-125b. As to miR-101, the expressions in three hepatoma cell lines were more than 10 fold lower compared with in PHH, especially in Huh7 cells (Figure 4.16).

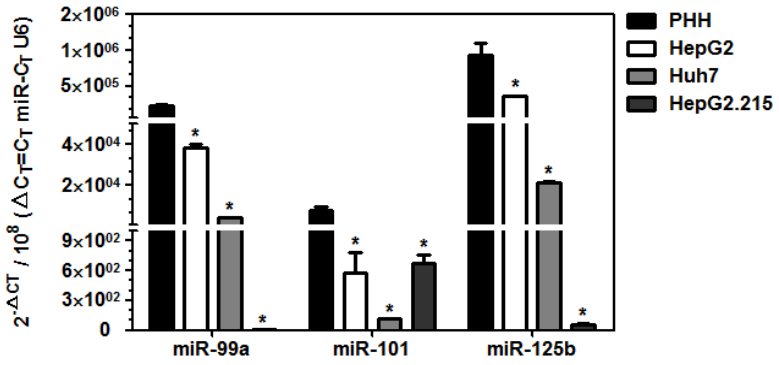

Figure 4.16 Quantification of mature miRNAs in PHHs and hepatoma cells

Total cellular RNA, including miRNA, was extracted from cells using Trizol. Total RNA (1μg) was reverse transcribed into cDNA (20μl) with miScript Reverse Transcription Kit Ⅱ from QIAGEN. The real time PCR for the quantification of miR-99a, 101, 125b was carried out with miScript Primer Assay and miScript SYBR Green PCR Kit from QIAGEN. Cycle parameters were as followed: a single step at 95℃ for 15min followed by 40 cycles at 94℃ (15s), annealing at 55℃ (30s) and extension at 60℃ (30s). An aliquot of cDNA (2μl) was used for each assay. The relative miRNA quantification was normalized by U6 snRNA. All reactions were performed in triplicate. MiRNA quantification in hepatoma cells was compared with in PHH. * $P<0.05$.

Song *et al*. found that HBx protein could inhibit the transcription of miR-122 by binding transcription factors PPARγ as they compared miR-122 in HepG2 to HepG2.215, which derived from HepG2 and is characterized by having stable HBV expression (SONG *et al.* 2013). And in our above results, we also found that mature miR-125b was much lower in HepG2.215 compared within Huh7 and HepG2, so we supposed that miR-125b generation could also be regulated by HBx. We knocked down HBx in HepG2.215 and then detected the generation of mature miR-125b, we used siR-ctrl and siHBs as control. While the real time RT PCR results showed that even though the knocking down efficience for both siHBs and siHBx in HepG2.215

cells was more than half percentage, they had no influence on mature miR-125b generation (Figure 4.17).

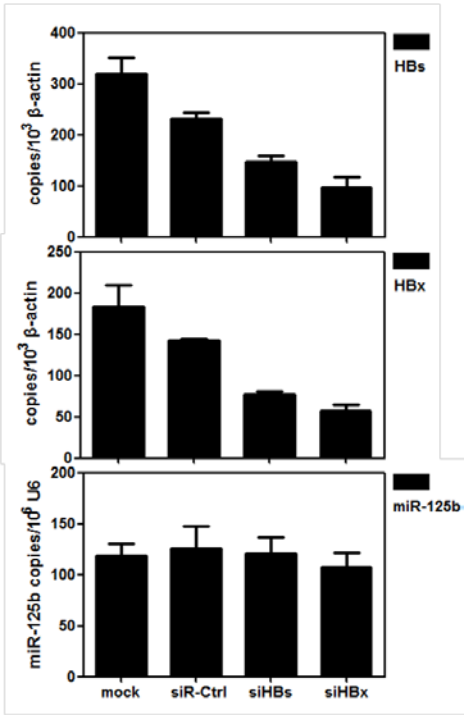

Figure 4.17 Knocking down HBs and HBx on mature miR-125b generation

Three days after transfection of 20nM siRNAs into HepG2.215 cells, we purified total cellular RNA, including miRNA using Trizol reagents. HBs, HBx RNA level (upper two panels) which were normalized against 10^3 β-actin transcripts and mature miR-125b which were normalized by U6 snRNA (below panel) were tested. All reactions were performed in triplicate.

4.2.2 Oncoprotein EZH2 has no influence on mature miR-125b generation while suppression of EZH2 protein could enhance HBV replication in Huh7 cell line

As mature miR-125b were down-regulated significantly in hepatoma cells compared to PHH, so we want to find out which molecules may participate in this phenomenon. MiR-125b gene promoter was enriched in CpG dinucleotides which are benefit for DNA methylation, so miR-125b transcription is modulated by DNA methylase (ALPINI *et al.* 2011; HE *et al.* 2012a; ZHANG *et al.* 2011c). Enhancer of zeste homolog 2 (EZH2) acts as a methyltransferase which is thought to be involved in targeting transcriptional regulators and is reported to epigenetically silence multiple tumor suppressor microRNAs in liver (AU *et al.* 2012; ONDER *et al.* 2012). So we supposed

that EZH2 may participate in the modulation of mature miR-125b generation in HCC. We synthesized two kinds of siRNAs against EZH2, including EZH2 6# and 7#, the knocking down efficiency for EZH2 6# in HepG2.215 was about 71%, EZH2 7# was about 79% (Figure 4.18A, upper panel). In Huh7 cells, the knocking down efficiency for EZH2 6# was about 51%, and EZH2 7# was about 71% (Figure 4.18B, upper panel). We tested mature miR-125b quantity 3 days post transfection of siEZH2 in hepatoma cells. Compared to siR-ctrl, knocking down of EZH2 had no obvious effect on mature miR-125b generation in hepatoma cells (Figure 4.18A and B, below panels).

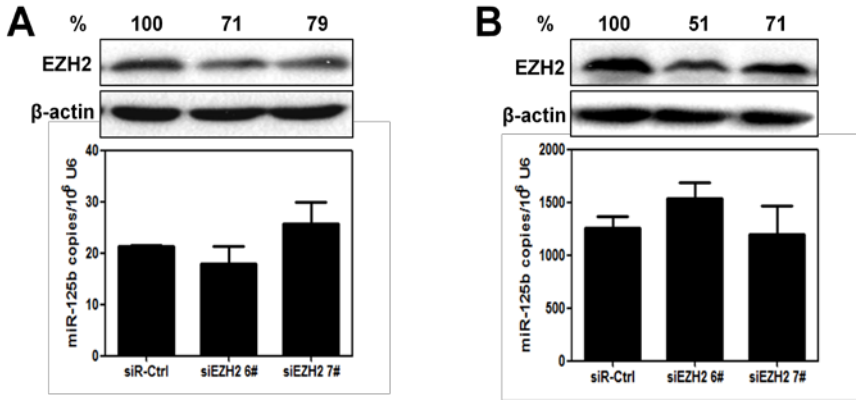

Figure 4.18 Influence of knocking down EZH2 on miR-125b generation

Protein EZH2 expression and mature miR-125b generation in HepG2.215 cells (A) and Huh7 cells (B). Three days after transfection with 20nM siR-C, siEZH2 6# or 7# into hepatoma cells, we collected total cellular proteins for EZH2 protein detection by western blot (upper panel) and extracted RNA for mature miR-125b quantification by miScript PCR (below panel). The signals were analyzed using β-actin as western blot loading control and miR-C was set to 100.

As oncoprotein EZH2 is up-regulated in HCC compared with in normal liver tissue, there are several miRNAs reported targeting EZH2 to regulate liver cancer metastasis (XIA *et al.* 2012; ZHENG *et al.* 2012). So we supposed that the tumor suppressor miR-125b may also modulate EZH2 expression and finally regulate HBV replication. We transfected different concentrations of miR-125b into HepG2.215 and Huh7 cells, 3 days post transfection, collected cell lysate to test EZH2 protein expression by western blot. Compared with miR-ctrl, miR-125b could down-regulate total EZH2 protein in hepatoma cells in dose dependent manner (Figure 4.19A). Next, we tested

HBV RI after knocking down of EZH2 in hepatoma cells. We selected siEZH2 6# for transfection since the knocking down efficiency for 6# was higher than 7# in both hepatoma cell lines. In HepG2.215, 4 days post transfection 20 nM siEZH2 6#, HBV RI not only increased, but decreased slightly (Figure 4.19B, left panel). While in Huh7 cell line, 3 days post cotransfection pSM2 and siEZH2 6#, HBV RI was increased obviously (Figure 4.19B, right panel). We then cotransfected pSM2 and different concentrations of siEZH2 6# into Huh7 for 3 days, and analyzed HBV core protein, HBV RI and nucleocapsid by western blot and southern blot assays. The results confirmed that knocking down of EZH2 in Huh7 could up-regulate HBV core protein expression, HBV RI and nucleocapsid formation in a dose dependent manner (Figure 4.19C).

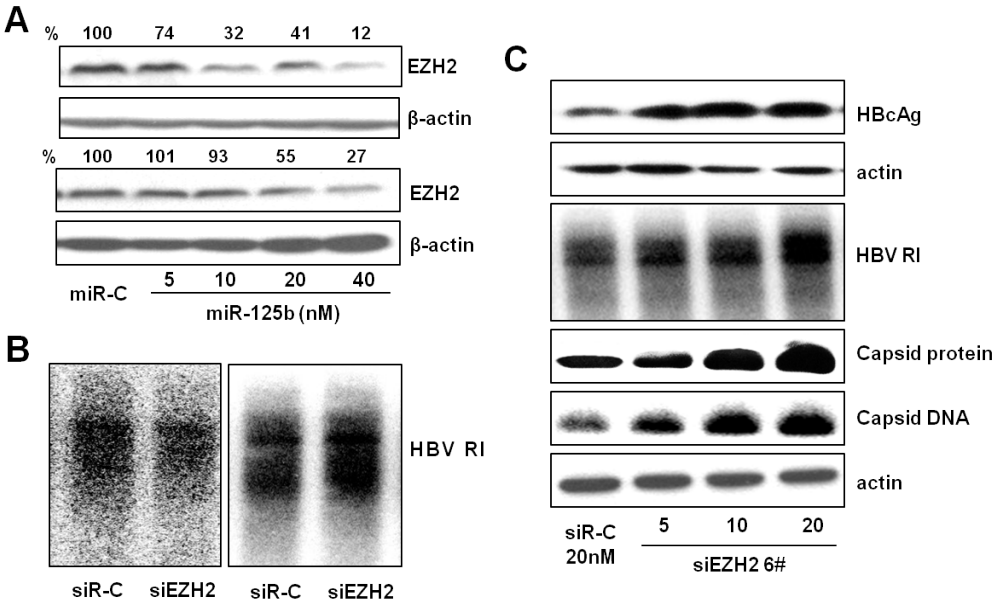

Figure 4.19 MiR-125b affects EZH2 expression and HBV replication in Huh7

(A) Three days after transfection with 0, 5, 10, 20 or 40nM miR-125b into HepG2.215 (upper panel) and Huh7 (below panel), collect total cellular proteins for EZH2 detection, using β-actin as loading control and miR-C was set to 100. (B) Four days after transfection 20 nM siRNAs into HepG2.215 (left panel) or three days post cotransfection 20 nM siRNAs and pSM2 into Huh7 (right panel), we isolated HBV RI for southern blot analysis. (C) Three days after cotransfection pSM2 and 0, 5, 10, 20nM siEZH2 into Huh7 cells, we collected HBcAg for western blot, isolated HBV RI and nucleocapsid for southern blot analysis. We used β-actin as loading control.

As knocking down EZH2 did not up-regulate HBV replication in HepG2.215 cells,

we wanted to know the different roles in different hepatoma cell lines for nuclear protein EZH2, whose total protein expression was suppressed by miR-125b both in HepG2.215 and Huh7 cell lines. We transfected different concentrations of miR-125b into HepG2.215 and Huh7 cells, 3 days post transfection, collected cells for separation of nuclear and cytoplasmic proteins individually. Western blot analysis showed that miR-125b could suppress cytoplasmic EZH2 protein but have no effect on nuclear EZH2 protein in HepG2.215 cell line (Figure 4.20A). Where as in Huh7 cells, miR-125b could suppress the EZH2 protein both in the cytoplasm and in the nucleus in dose dependent manner (Figure 4.20B).

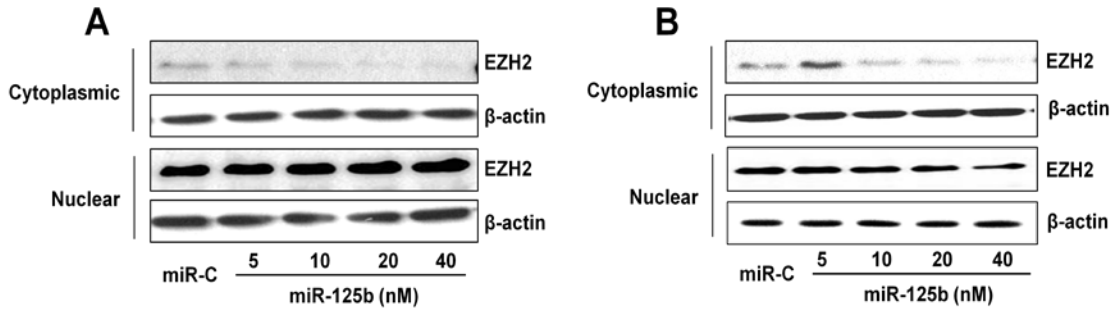

Figure 4.20 Distribution of EZH2 after miR-125b transfection

Three days after transfection with 0, 5, 10, 20 or 40nM miR-125b into HepG2.215 (A) or Huh7 (B), we collected cytoplasmic and nuclear proteins for EZH2 detection. We used β-actin as loading control.

In order to further verify EZH2-miR-125b roles in HepG2.215 cell line, we transfected siRNA or miRNA into cells for two times. We first transfected 20 nM miR-ctrl, miR-125b or siR-ctrl, siEZH2 6# into HepG2.215, and two days post transfection, split cells into new plates for an additional transfection. Three days after the second transfection, we separated cytoplasmic and nuclear proteins to test the EZH2 protein expression. And four days after second transfection, we isolated HBV RI from intracellular core particle for southern blot analysis. Compared to miR-ctrl, miR-125b enhanced HBV RI formation remarkably after two times transfection (Figure 4.21A, below panel). For the EZH2 protein, even though miR-125b could reduce its quantity in cytoplasm, there was still no effect in nuclear EZH2 protein accumulation (Figure 4.21A, upper and middle panels). On the other hand, after

transfection of siEZH2 into HepG2.215 for two times, EZH2 almost could not be detected in cytoplasm, and EZH2 in nucleus was also decreased to 28 percent compared with siR-ctrl (Figure 4.21B, upper and middle panels). While even though most EZH2 has already been knocked down after two times transfection with siEZH2, HBV RI was still not increased in HepG2.215 cell line (Figure 4.21B, below panel). This means that in HepG2.215 cell line, EZH2 is not a regulative protein for the modulation of HBV replication.

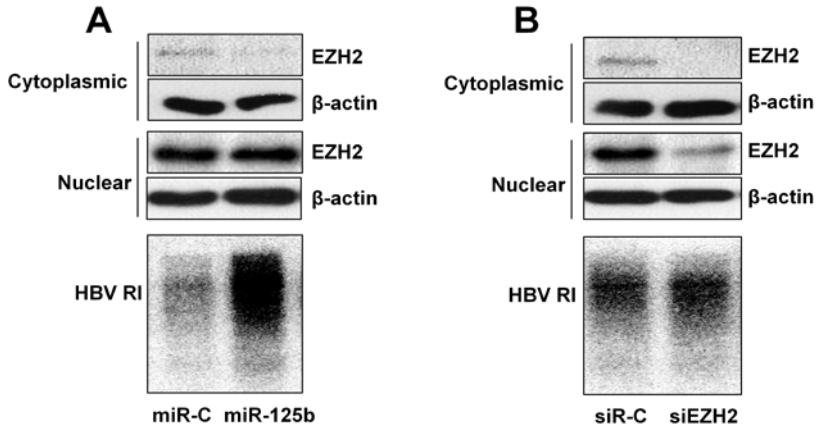

Figure 4.21 HBV replication and EZH2 expression in HepG2.215 cells after transfection with miRNA or siRNA for two times

Two days after transfection of 20nM miRNAs (A) or siRNAs (B) into HepG2.215, we split cells and then transfected miRNAs or siRNAs for additional time. After three or four days culturing, WE extracted cytoplasmic and nuclear protein to test EZH2 protein by western blot analysis (upper panel), extracted HBV RI for southern blot analysis (below panel). We used β-actin as loading control.

4.3 MiR-125b modulates hepatoma cells growth and metabolism

4.3.1 Tumor suppressor miRNAs do not regulate liver specific factors

HBV replication is dependent on host cell differentiation status and controlled by a variety of cellular transcription factors. Previously, Dr. Xiaoyong Zhang demonstrated that miR-1 could up-regulate the liver-specific protein albumin and host nuclear receptor NR1H4 so that it enhanced the HBV replication (ZHANG *et al.* 2011b). Therefore, we supposed that these selected miRNAs may also participate in regulating these factors expression. We transfected 20nM miRNA mimics into hepatoma cells, and three days post transfection, purified RNA for real time RT PCT and cell lysate

for western blot. As compared to miR-C or mock, miR-1 could up-regulate both RNA and protein level for albumin and NR1H4, while these six new selected miRNAs, except for miR-101, could enhance albumin protein slightly, the other five miRNAs had no obvious effect on these two protein expressions, especially for miR-125b (Figure 4.22).

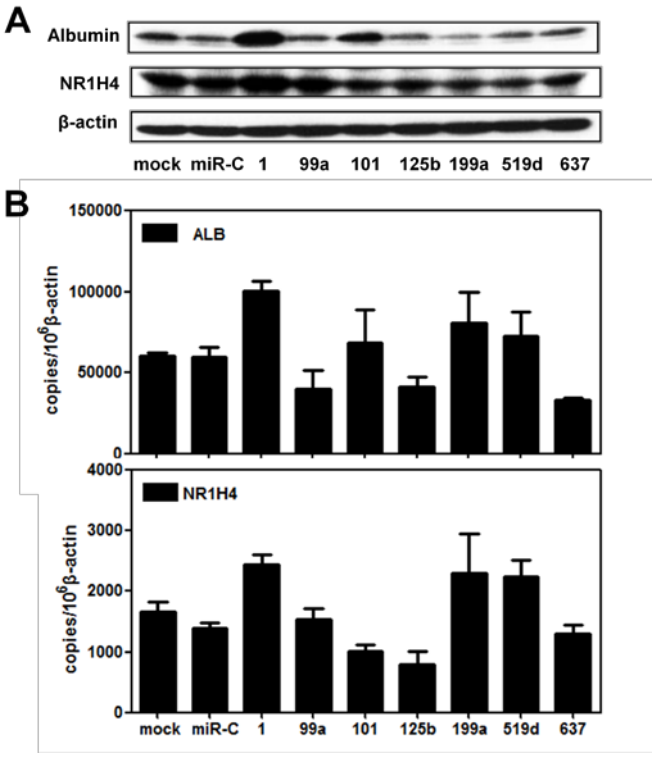

Figure 4.22 Influence of miRNAs on host cellular factors

(A) Western blot analysis. 3 days after transfection of 20nM miRNA mimics into HepG2.215 cells, we collected cell lysate to test albumin and NR1H4 protein expression by western blot, using β-actin as loading control. (B) Real time RT PCR analysis. 3 days after transfection of 20nM miRNA mimics into HepG2.215 cells, we purified total cellular RNA to test albumin and NR1H4 RNA and normalized against 10^6 β-actin transcripts.

4.3.2 MiR-125b arrests cell cycle at G1 phase and inhibits hepatoma cell proliferation

Next, we tested their effect on cell cycle distribution. We transfected 20 nM miRNA mimics into HepG2.215 and Huh7, and then treated cells with cell cycle inhibitors aphidicolin (APH), which could block cell cycle at G1 phase or nocodazole (NOC), which could block cell cycle at G2/M phase. As for HepG2.215, after treated with

APH for 24 hours, more than half percentage of cells stay at G1 phase, but after APH was removed for additional 6 hours, most cells could entry into S phase, and there were less than 10% cells stay G1 phase for all transfection groups except miR-125b group. Compared with miR-C group, there were much more cells in G1 phase in miR-99a, 199a-3p and 637, and for miR-125b group, there were about 4 fold cells still in G1 phase compared with miR-C group (Figure 4.23A). After treating HepG2.215 with NOC for 6 hours or 12 hours, even though there were about more than 35% and half percentage of cells at G2/M phase, there were still more than 15% of cells at G1 phase for miR-125b group. Compared with miR-C group, there were more than 2 fold and 4 fold cells at G1 phase for miR-125b group. The other groups had no such a remarkable effect except of miR-637, which had 2 fold cells at G1 phase compared with miR-C after treating cells with NOC for 12 hours (Figure 4.23B).

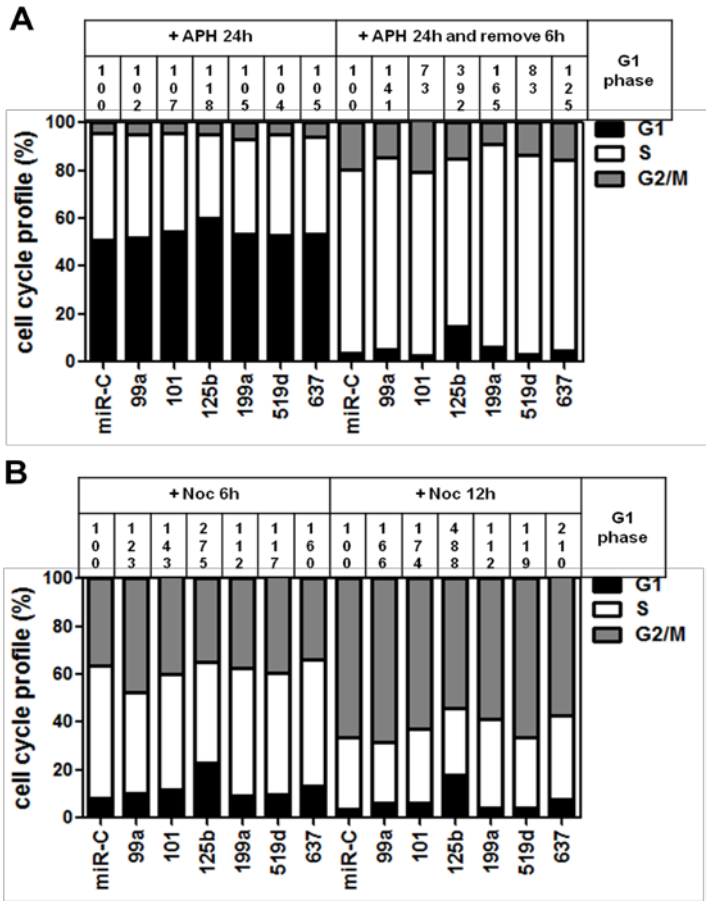

Figure 4.23 MiRNAs affect HepG2.215 cell cycle progress

(A) HepG2.215 cells were transfected with 20nM of different miRNA mimics for 24 hours, then they were split and treated with cell cycle inhibitor aphidicolin (4μg/ml) for 24 hours or

treated for 24 hours and removed for additional 6 hours. (B) After transfection miRNA mimics for 24 hours, the cells were split and treated with cell cycle inhibitor nocodazole (100nM) for 6 hours or 12 hours. We calculated G1 phase proportion and miR-C group was set to 100.

We also tested their effect on HepG2.215 cell proliferation by H^3-thymidine DNA synthesis assay. Compared to miR-ctrl, miR-125b could inhibit HepG2.215 cells proliferation significantly (Figure 4.24).

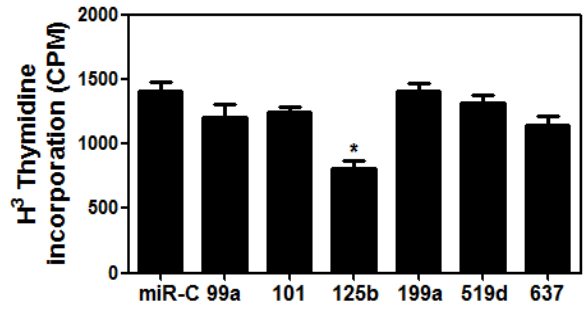

Figure 4.24 Influence of miRNAs on HepG2.215 cell proliferation

After transfection 20nM miRNA mimics for 24 hours, the HepG2.215 cells were split into 96-well plate for testing their cell proliferation ability by H^3 Thymidine labeled DNA synthesis assay.

Besides the HepG2.215 cell line, we also tested their effect on Huh7 cell cycle profile. In Huh7 cells, even though G1 phase for all groups was not so consistent as in HepG2.215 cells after being treated with APH for 24 hours, there were still about half percent cells at G1 phase. Then we compared two groups, the first group was treated with APH and removed 6 hours, and the second group was only APH treated for 24 hours without removed. The results showed that in miR-125b group there was still 1.5 fold cells at G1 phase compared with miR-C group, whereas, other miRNA groups had no obvious difference compared to miR-C (Figure 4.25A). So as to NOC treatment, after treated Huh7 cells with NOC for 6 hours or 12 hours, there were also much more cells at G1 phase for miR-125b group as compared to miR-C group, other miRNAs had no obvious difference compared to miR-C (Figure 4.25B).

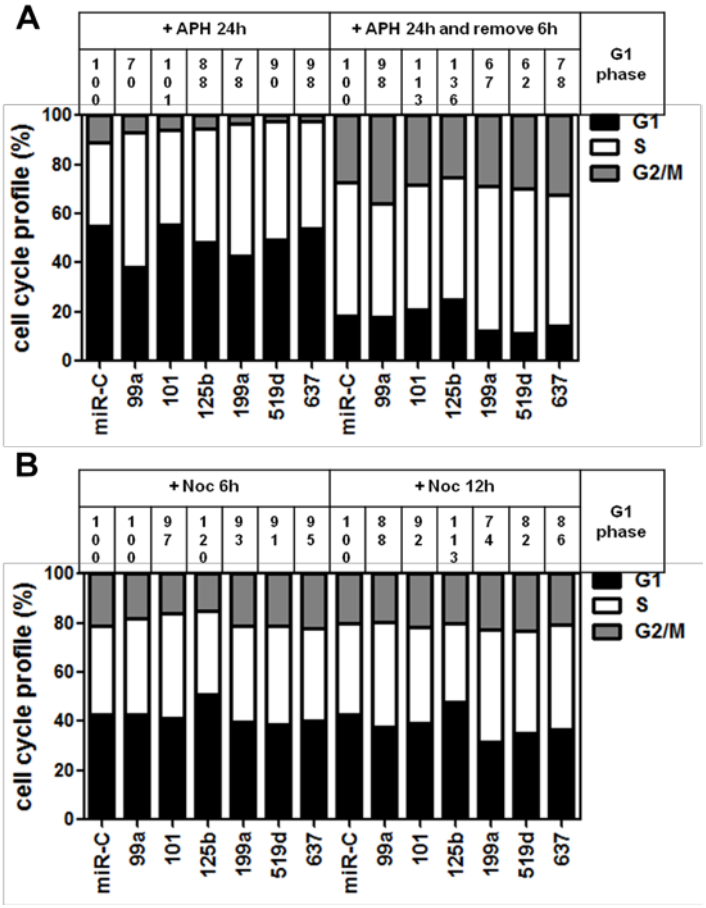

Figure 4.25 MiRNAs affect Huh7 cell cycle progress

(A) Huh7 cells were transfected with 20nM of different miRNA mimics for 24 hours, then split and treated with cell cycle inhibitor aphidicolin (4μg/ml) for 24 hours or treated for 24 hours and removed for additional 6 hours. (B) After transfection miRNA mimics for 24 hours, the cells were split and treated with cell cycle inhibitor nocodazole (100nM) for 6 hours or 12 hours. We calculated G1 phase proportion and miR-C group was set to 100.

The previous results demonstrated that miR-125b could arrest hepatoma cells at G1 phase, so we want to investigate the molecular mechanisms of miR-125b on cell cycle blockage. Retinoblastoma protein (RB) is a key factor that arrests cell cycle progress by repressing transcription genes such as E2Fs family which are required for the G1-to-S-phase transition. Meanwhile, phosphorylation for RB could inactivate RB and allow cell cycle progression from G1 to S phase (GIACINTI and GIORDANO 2006; HARBOUR and DEAN 2000; HENLEY and DICK 2012; SINGH *et al.* 2010). Thus, we supposed that miR-125b may regulate RB phosphorylation to disturb cell cycle progress. We transfected different concentrations of miR-125b into HepG2.215 and

Huh7, 3 days after transfection, we collected cell lysate for phosphorylated RB detection by western blot analysis. As compared to miR-C, miR-125b could down-regulate RB phosphorylation obviously in dose dependence both in HepG2.215 and Huh7 cell lines (Figure 4.26).

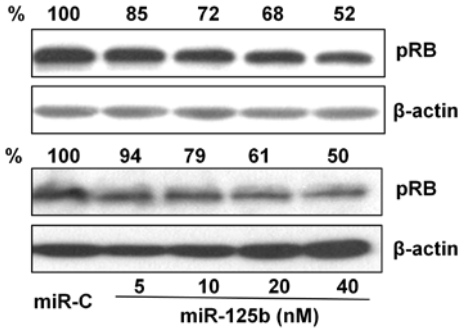

Figure 4.26 MiR-125b down-regulates RB phosphorylation

Three days after transfection with 0, 5, 10, 20 or 40nM miR-125b into HepG2.215 (upper panel) or Huh7 cells (below panel), we collected total cellular proteins for phosphorylated RB detection, The signals were analyzed by using β-actin as loading control and miR-C was set to 100.

Normally, cell proliferation and apoptosis are two opposite phenomenon. Our previous results showed that miR-125b could inhibit hepatoma cell proliferation, so we supposed that miR-125b may promote hepatoma cell apoptosis. The process of apoptosis is regulated by a series of signal cascades under certain circumstances. The caspase-cascade system plays vital roles in the induction, transduction and amplification of intracellular apoptotic signals (FAN *et al.* 2005). And the activation and function of caspases are regulated by various kinds of molecules, such as Bcl-2 family proteins. So we tested several apoptosis associated protein expressions after transfection of different concentrations of miR-125b in HepG2.215, including BCL-2, apoptosis marker caspase-3 and cleaved PARP since PARP is one of the main cleavage targets of caspase-3. Western blot analysis showed that after transfection of miR-125b into HepG2.215 cells, BCL-2 was up-regulated, and cleaved PARP was down-regulated in dose dependence, while caspase-3 had no obvious difference between miR-C and miR-125b transfected cells (Figure 4.27). The reason why miR-125b dose not suppress anti-apoptosis protein BCL-1 expression to up-regulate

capsase-3 protein and finally enhance the cleavage ability to PARP is that the caspase-cascade system is complicated in vivo, and there are several caspase-cascade pathways including, caspase-3, which means that miR-125b dose not simply modulate one or several molecules to participate in hepatoma cell apoptosis pathway.

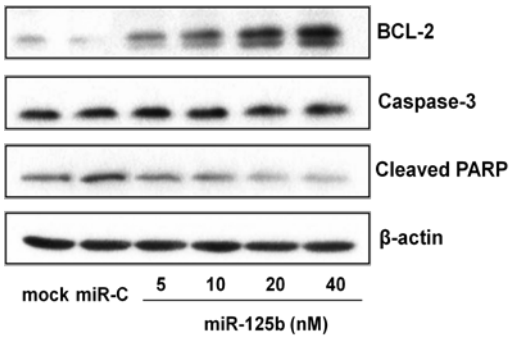

Figure 4.27 MiR-125b modulates apoptosis associated proteins

Three days after transfection with 0, 5, 10, 20, 40 nM of miR-125b into HepG2.215 cells, we collected cell lysates to test BCL-2, caspase-3, cleaved PARP protein expression by western blot analysis, and used β-actin as loading control.

4.3.3 MiR-125b regulates metabolism associated genes in hepatoma cells

Even though HBV gene expression is regulated mainly at the transcriptional level by recruitment of a whole set of cellular transcription factors and coactivators to support transcription, there are still many other indirect regulation mechanisms that miRNAs may take to participate in HBV life cycle, such as host cellular metabolism and cytoskeleton change. We used the RNA sequence assay to figure out pathways and genes miR-125b may regulate in hepatoma cells. We transfected 20 nM miR-ctrl or miR-125b into HepG2.215 and Huh7 cell lines, and then purified RNA for RNA abundance analysis. Our RNA sequence results showed that there were 180 pathways in HepG2.215 and 199 pathways in Huh7 which have been regulated by miR-125b, and there were about 20000 genes on the RNA level which have been changed by miR-125b in hepatoma cells. We sorted out twelve pathways which have more than five genes that have been changed after transfection miR-125b into hepatoma cells, and there were eight pathways related to cell growth and metabolism, including metabolic pathways, carbohydrate digestion and absorption, retinol metabolism,

purine metabolism, pancreatic secretion, protein digestion and absorption, bile secretion and MAPK signaling pathways. And in these pathways, there were several family genes that had both obviously changed in HepG2.215 and Huh7, such as Glucuronosyltransferase 2 family, aldo-keto reductase family and ATP-binding cassette family. Two pathways were related to the cell skeleton, including regulation of actin cytoskeleton and tight junction, such as myosin gene. And two pathways were related to immune response, including NF-kappa B signaling pathway and phagosome. In these pathways, IL8 and IL1R1 were up-regulated by miR-125b in both Hep2.215 and Huh7 cell lines significantly (Table 3).

Table 3 Pathways regulated by miR-125b in hepatoma cells

	Pathways	Genes changed in HepG2.215	Genes changed in Huh7	Examples
Cell growth and metabolism	Metabolic pathways	32	60	Glucuronosyltransferase 2 family Aldo-keto reductase family ATP-binding cassette family
	Carbohydrate digestion and absorption	5	9	
	Retinol metabolism	3	14	
	Purine metabolism	11	11	
	Pancreatic secretion	11	6	
	Protein digestion and absorption	7	10	
	Bile secretion	15	11	
	MAPK signaling pathway	9	8	
Cell skeleton	Regulation of actin cytoskeleton	8	8	Myosin
	Tight junction	10	6	
Immune response	NF-kappa B signaling pathway	8	7	IL8, IL1R1
	Phagosome	6	9	

4.3.4 MiR-125b regulates LIN28B/*let-7* axis to modulate HBV replication

It is reported that LIN28, which is a conserved RNA binding protein and highly expressed during embryogenesis and early larval development in the hypodermal, neural and muscle cells, is a primal regulator of growth and metabolism in stems cells. Loss of LIN28 function could accelerate the differentiation. Furthermore, LIN28 could inhibit the tumor suppressor miRNA *let-7* biogenesis, so that the LIN28/*let-7* pathway plays a specific and tightly regulated role in modulating glucose metabolism in mammals (SHYH-CHANG and DALEY 2013; ZHU *et al.* 2011). Besides, LIN28B is a

conserved target gene for miR-125b, so we supposed that miR-125b may regulate hepatoma cell metabolism through LIN28B/*let-7* axis and finally affect HBV replication.

Our RNA sequence data showed that LIN28B was down-regulated both in HepG2.215 and Huh7 cells significantly after transfection with miR-125b for three days (Table 4).

Table 4 Lin28b RNA level in hepatoma cells

	Cell lines	miR-ctrl-Expression	miR-125b-Expression	log2 Ratio (ctrl/125b)	Up-Down (ctrl/125b)	P-value
lin28b	HepG2.215	2241	1041	-1.199	Down	3.55E-117
	Huh7	6786	4117	-0.67381	Down	3.33E-127

And we also confirmed that miR-125b could down-regulate LIN28B protein expression in hepatoma cells in dose dependent manner by western blot analysis (Figure 28).

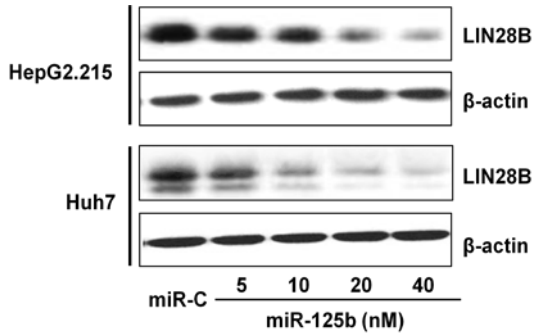

Figure 4.28 MiR-125b down-regulates LIN28B protein expression

Three days after transfection with 0, 5, 10, 20 or 40nM miR-125b into HepG2.215 (upper panel) or Huh7 (below panel), we collected total cellular proteins for LIN28B detection through western blot analysis by using β-actin as loading control.

We then selected two siRNAs to knock down LIN28B and detected their influence on HBV replication. Our western blot results showed that both siLIN28B 1# and 4# could down-regulate LIN28B protein expression efficiently, especially siLIN28B 4# (Figure 4.29A and B, upper panels), while only siLIN28B 4# could enhance HBV replication and HBsAg expression in HepG2.215 cells after transfection for four days

(Figure 29A, middle and below panels). However, in Huh7 cells, there was no difference for HBV RI between siR-ctrl and siLIN28B when we cotransfected siRNAs and pSM2 for three days (Figure 29B, middle panel).

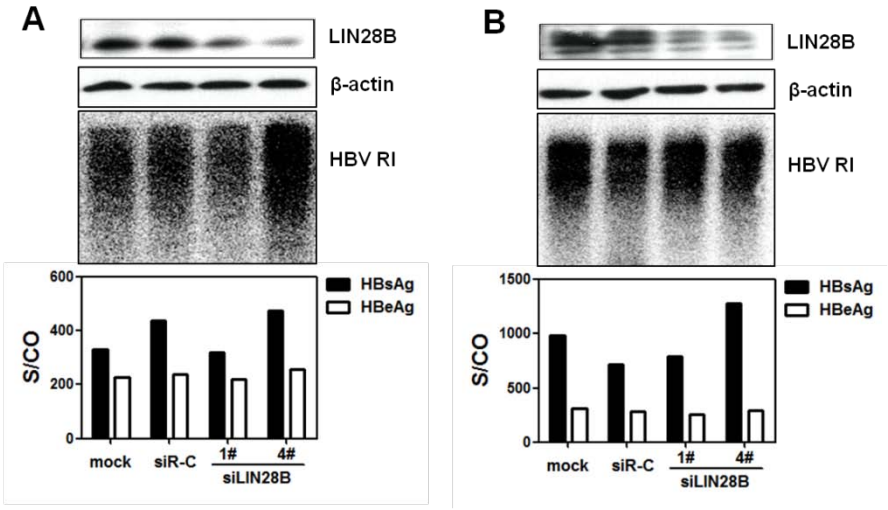

Figure 4.29 SiLIN28B knocking down efficience and influence on HBV

Three days after transfection of 20nM siRNAs into HepG2.215 (A) or Huh7 (B), we collected total cellular proteins for LIN28B detection, and extracted HBV RI for southern blot and supernatant for CMIA assay, and we used β-actin as loading control for western blot analysis.

As for HepG2.215 cells, after transfection with different concentrations of siLIN28B 4# for 3 days, the LIN28B protein expression could be down-regulated in a dose dependent manner. And we also tested HBV RI, nucleocapsid from intracellular and HBsAg and HBeAg from culture media after 4 days of transfection. And the results confirmed that in the HepG2.215 cell line, knocking down LIN28B could enhance HBV EcDNA, nucleocapsid formation and protein secretion in a dose dependent manner (Figure 4.30A). While in Huh7, when we transfected 20nM siLIN28B 4# for three days, and then split cells for cotransfection of pSM2 and siLIN28B 4# for additional three days, HBV RI could also be accumulated (Figure 30B).

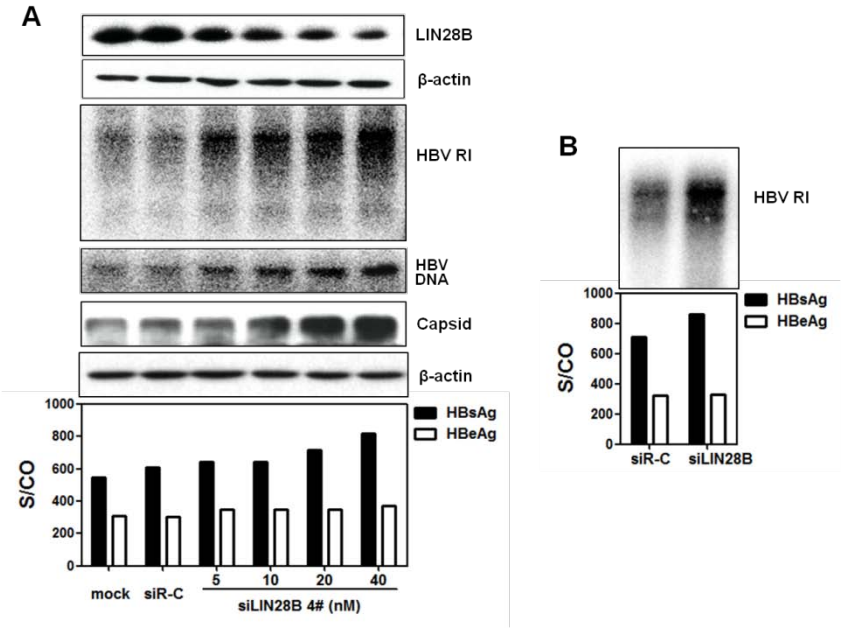

Figure 4.30 Knocking down LIN28B enhances HBV replication

(A) HepG2.215 cells were transfected with 0, 5, 10, 20 and 40nM of siLIN28B 4# for three days for the detection of LIN28B expression by western blot (upper panel), and four days for HBV RI, nucleocapsid and HBsAg and HBeAg detection by southern blot and CMIA assay (middle and below panels). We used β-actin as loading control for western blot analysis. (B) Huh7 cells were transfected with 20nM siR-ctrl or siLIN28B 4# for three days, then split cells and cotransfected of pSM2 with siRNAs for additional three days for HBV RI, HBsAg and HBeAg detection.

Up to now, nine members have been recognized in the *let-7* family, including *let-7a*, *let-7b*, *let-7c*, *let-7d*, *let-7e*, *let-7f*, *let-7g*, *let-7i*, and miR-98. And for some *let-7* family members, there are some sub-family members, such as for *let-7a*, there are *let-7a-1*, *let-7a-2* and *let-7a-3*. But all *let-7* family members are believed to exert similar functions since they share a common seed region (Table 5, thick black characters), which mediated miRNA interaction with target mRNAs.

Table 5 *Let-7* family members sequence

Members	Sequence
has-let-7a	3' -UUGAUAUGUUGGAU**GAUGGAG**U
has-let-7b	3'- UUGGUGUGUUGGAU**GAUGGAG**U
has-let-7c	3'- UUGGUAUGUUGGAU**GAUGGAG**U
has-let-7d	3'- UUGAUACGUUGGAU**GAUGGAG**A
has-let-7e	3' -UUGAUAUGUUGGAG**GAUGGAG**U
has-let-7f	3'- UUGAUAUGUUAGAU**GAUGGAG**U
has-let-7g	3'- -UUGACAUGUUUGAU**GAUGGAG**U
has-let-7i	3'- UUGUCGUGUUUGAU**GAUGGAG**U
has-miR-98	3' -UUGUUAUGUUGAAU**GAUGGAG**U

Our RNA sequence results showed that there were four *let-7* members that could be detected with a differential expression after transfected with miR-125b into hepatoma cells, including *let*-7a, 7c, 7d and miR-98 (Table 6).

Table 6 *Let-7* family members changed by miR-125b in hepatoma cells

Let-7 member	Cell lines	miR-ctrl-Expression	miR-125b-Expression	log2 Ratio (ctrl/125b)	Up-Down (ctrl/125b)	P-value
let-7a	HepG2.215	not detected				
	Huh7	18	34	0.964696	Up	0.964696
let-7c	HepG2.215	not detected				
	Huh7	1	0	-5.43426	Down	0.516476
let-7d	HepG2.215	1	1	-0.09283	Down	0.951778
	Huh7	2	2	0.047158	Up	0.969364
miR-98	HepG2.215	not detected				
	Huh7	0	1	4.978915	Up	0.483792

We also tested the above four *let-7* members by miScript real time PCR after knocking down LIN28B or the exogenous transfection of miR-125b into hepatoma

cells. The results confirmed that in Huh7 cell line, *let-7a, 7c, 7d* and miR-98 generation were enhanced significantly after knocking down LIN28B, and in HepG2.215 cell line, except *let-7c*, the other three *let-7* members were up-regulated by knocking down LIN28B. However, in HepG2.215 cells, the exogenous transfection of miR-125b could obviously enhance all four members generation, while in Huh7 cells, there was no obvious difference between miR-ctrl and miR-125b groups (Figure 4.31).

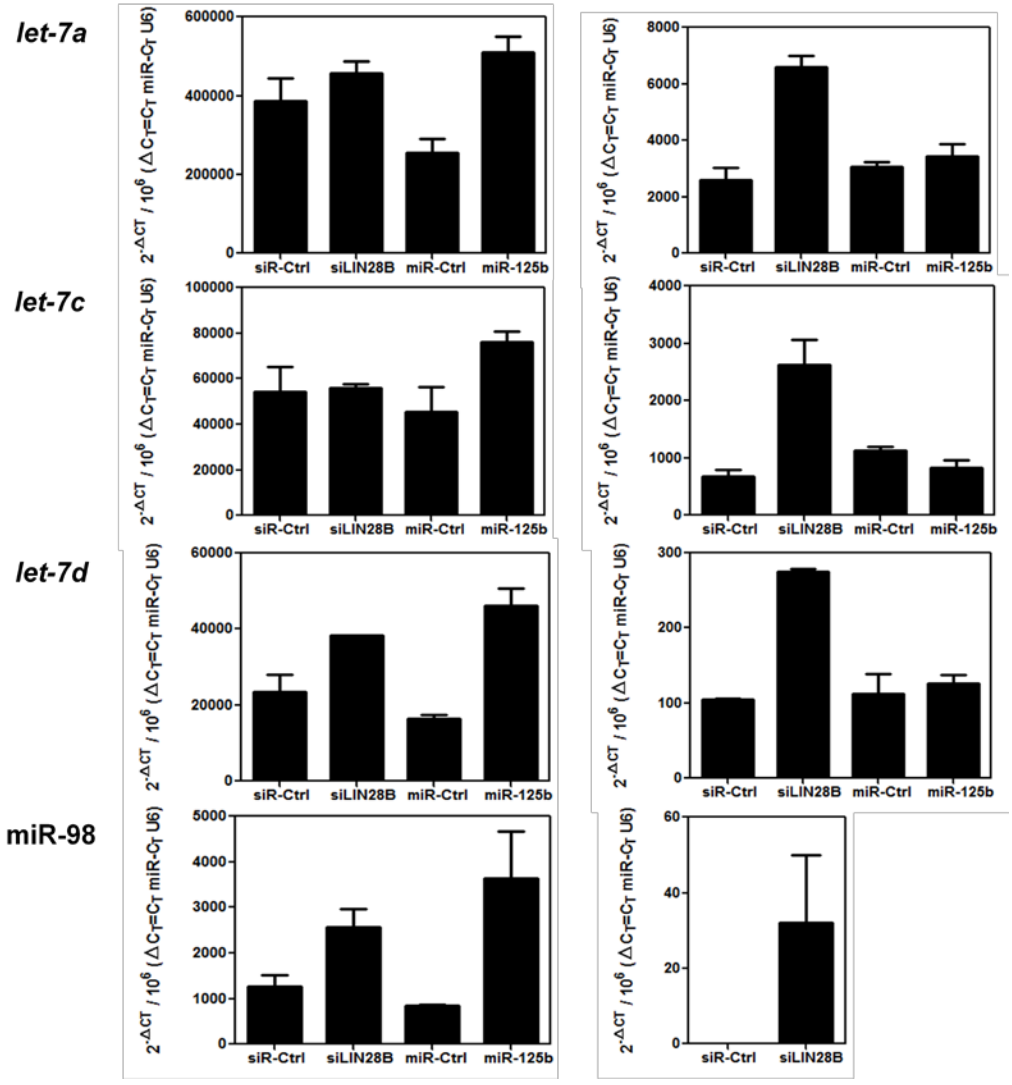

Figure 4.31 Influence of knocking down LIN28B or exogenous miR-125b on *let-7* generation in hepatoma cell lines

Three days after transfection with 20nM siR-ctrl, siLIN28B 4# or miR-ctrl, miR-125b into HepG2.215 (left panel) or Huh7 (right panel), we purified the total cellular RNA, including miRNA using Trizol. The quantification of *let-7a, 7c, 7d* and miR-98 were carried out with

miScript Primer Assay and miScript SYBR Green PCR Kit from QIAGEN. The relative miRNA quantification was normalized by U6 snRNA. All reactions were performed in triplicate.

Then we selected and synthesized two *let-7* member mimics, *let-7a* and miR-98 since the quantities for these two members were the highest and the lowest ones, and transfected them into hepatoma cells for detecting their influence on HBV replication. Four days after transfection of 20nM mimics into HepG2.215 or three days after cotransfection of 20nM mimics with pSM2 into Huh7, we extracted HBV RI from intracellular core particle for southern blot assay. The results showed that exogenous miR-98 could enhance HBV replication both in HepG2.215 and Huh7 cell lines significantly, while *let-7a* had no obvious influence (Figure 4.32). The reason for this phenomenon may be that the basic abundance for *let-7a* in hepatoma cells was already very high, so that the exogenous mimic could not enhance function. However, this did not appear for miR-98, since its abundance in hepatoma cells was really low so that exogenous miR-98 mimic could enhance HBV replication obviously (Figure 4.31).

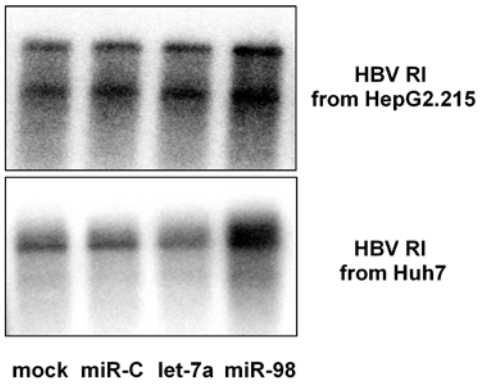

Figure 4.32 Exogenous *let-7* members on HBV replication

HepG2.215 (upper panel) or Huh7 (below panel) cells were transfected or cotransfected pSM2 plasmid with 20nM of miR-C or let-7 members for four or three days, and then we extracted HBV RI from intracellular core particle for southern blot assay.

5 Disscussion

Since initial observation, there are about 1733 mature human miRNAs, which have been registered in miRBase (WANG *et al.* 2012d). In liver, miRNAs are abundant, such as miR-122 which is described as a liver specific miRNA and has been reported in mouse, woodchuck and human livers, in human primary hepatocytes, and in cultured liver derived cells (CHANG *et al.* 2004; CHANG *et al.* 2003). Besides miR-122, many other miRNAs, such as miR-1, miR-16, miR-27b, miR-30d, miR-126, miR-133, miR-143, and the *let-7* family, are also abundantly expressed in adult liver tissue. While miR-122 appears as the most highly expressed miRNA in adult liver, miR-92a and miR-483 seem to be more specifically expressed in the fetal liver (GIRARD *et al.* 2008). Thus, the liver displays a differential miRNA expression profile during its development, so as to hepatocellular carcinoma development. Several studies have shown that specific miRNAs are aberrantly expressed in malignant HCC cells or tissues compared to non-malignant hepatocytes or tissue (JACOB *et al.* 2004; KUTAY *et al.* 2006; MENG *et al.* 2007; VARNHOLT *et al.* 2008; WONG *et al.* 2008b). Alteration of expression profile in HCC for some miRNAs may be the consequence of malignant transformation. Besides, drugs treatment could also change host cellular miRNAs. Pedersen for example, demonstrated the IFN-mediated modulation of the expression of numerous cellular miRNAs in the treatment of hepatocytes infected with HCV (PEDERSEN *et al.* 2007). For other miRNAs, they may play a role in the transformation process.

MiRNAs, which from the host cells also play a role in building up direct or indirect effect in regulating viral genes (BERKHOUT and JEANG 2007; GHOSH *et al.* 2009; LECELLIER *et al.* 2005). MiRNA-122 has been reported to facilitate the replication of HCV, targeting the viral 5'non-coding region (JOPLING *et al.* 2005). Besides host cellular miRNAs, genes encoding miRNAs have also been found in viruses, and viral miRNAs have a regulatory effect on their protein-coding genes (BERKHOUT and JEANG 2007). This regulatory effect may be beneficiary to the virus towards maintaining its replication, latency and evading the host immune system. As to HBV,

so far none of the HBV-encoded miRNAs have been identified. But cellular miRNAs have shown to be able to regulate HBV at the transcription level either by targeting to cellular transcriptions factors which are required for HBV gene expression, or by directly bind to HBV transcripts (LIU *et al.* 2011). And in our previous results, Dr. Xiaoyong Zhang found that miR-1 could increase HBV transcription under the control of the HBV core promoter in a farnesoid X receptor alpha dependent manner (ZHANG *et al.* 2011b). Other than miRNAs affecting viral translation, there are reports on HBV replication, such as miR-122, which is found to suppress HBV replication indirectly through HO-1 (QIU *et al.* 2010).

5.1 Cancer related miRNAs down-regulated in hepatoma cells could enhance HBV replication

The major risk factor for HCC is chronic HBV and/or HCV infection. HBV, HCV, and their functional proteins are likely to exhibit their pathogenic roles via regulating the expression of host-derived miRNAs in the affected liver. The deregulated miRNAs serve as mediators of virus–host interaction, playing an important role in persistent infection, carcinogenesis, and HCC treatment via regulating host gene expression. Host miRNAs cannot only be regulated by virus infection but also affect virus replication and viral protein expression via specifically binding to the viral sequences or targeting some related host genes, thus, playing an important role in the virus-induced hepatocarcinogenesis (ZHANG *et al.* 2012a). For example, Guo *et al*. demonstrated that miR-373 is up-regulated in HBV-infected livers. Furthermore, the enhanced expression of miRs-372/373 in HepG2 cells transfected with 1.3-fold HBV genome stimulates the production of HBV proteins and HBV core-associated DNA *via* targeting nuclear factor I/B (GUO *et al.* 2011b). Ladeiro *et al*. reported the overexpression of miR-96 in HBV-associated hepatocellular carcinoma (HCC) (LADEIRO *et al.* 2008), and Ura *et al*. proposed that differential miRNA expression contributed to the different progression between HCV and HBV infection to HCC (URA *et al.* 2009). Liu *et al.* observed that miR-181a was up-regulated in HBV-producing HepG2.2.15 cells, and that one of its putative targets, human

leukocyte antigen A (HLA-A), was repressed, possibly related to decreased viral resistance (LIU *et al.* 2009). All these reports suggest that there is a relationship between miRNAs and HBV in HCC. In our results, we tested the abundance of several cancer related miRNAs, including miR-99a, miR-101 and miR-125b, and found their quantities were remarkably reduced in hepatoma cells compared with in PHH. MiR-99a and miR-125b in HepG2.215, which comes from HepG2 cell line and contains HBV dimer, were much lower than in HepG2 (Figure 4.16). Moreover, after exogenously transfection miR-99a and miR-125b into HepG2.215 cell line, HBV replication was obviously up-regulated, and exogenous miR-101 could also enhance HBV replication in Huh7 cell line but not in HepG2.215 and HepG2. The reason for this phenomenon may be that the abundance for mature miR-101 in Huh7 cells was much lower than in the other two hepatoma cell lines. As for miR-125b, besides its enhancement of HBV replication in HepG2.215 cells, it also enhanced HBV replication in HepG2 and Huh7 cell lines (Figure 4.1 and 4.2). This phenomenon is consistent with Winther *et al.* who reported that the quantity for miR-125b in HBeAg positive CHB patients is higher than in HBeAg negative patients (WINTHER *et al.* 2013).

On the other hand, some miRNAs down-regulated in HCC are beneficial for HBV survival, such as miR-122. MiR-122 levels are significantly decreased in HBV-infected patients compared with healthy controls, which may facilitate viral replication and persistence (CHEN *et al.* 2011c; WANG *et al.* 2012c).

5.2 MiRNAs which could arrest hepatoma cell at G1 phase are beneficial for HBV replication

Viruses may transform host cell growth status to benefit its survival. Huang *et al.* demonstrated that HBV replication was active in quiescent hepatocytes but slowed when hepatocytes started to divide (HUANG *et al.* 2004). Wang *et al.* found that HBV x protein could inhibit the capability of proliferation of HepG2.2.15 cells by regulating cell cycle gene expression and inducing G1 arrest (WANG *et al.* 2011b). Gearhart *et al.* demonstrated that cell cycle progression affected HBV replication in

cultured primary rat hepatocytes, and that HBV replication requires primary rat hepatocytes in G1 phase (GEARHART and BOUCHARD 2010). All these studies reflect that HBV replication is affected by the hepatocytes' growth ability, and the G1 phase is more beneficial for HBV replication. In our results, we found one miR-125b could block cell cycle at G1 / S phase transition and inhibit hepatoma cells proliferation, and these results are consistent with several groups that miR-125b could interfere with hepatoma cell growth (BI *et al.* 2012; JIA *et al.* 2012; KIM *et al.* 2013; LIANG *et al.* 2010). Meanwhile, our results demonstrated that miR-125b could up-regulate HBV replication in different hepatoma cell lines (Figure 4.1, 4.2, 4.23, 4.24 and 4.25).

5.3 MiRNAs involved in multiple steps in HBV life cycle directly or indirectly

Host cellular miRNAs could affect both transcription and replication in HBV life cycle directly or indirectly. Although HBV is a DNA virus, its transcripts might be targeted and regulated by cellular miRNAs. Some cellular miRNAs were found to be capable of inhibiting or stimulating viral replication by directly targeting to viral RNAs (CULLEN 2009; LIN and FLEMINGTON 2011). For example, bioinformatics analysis of six miRNAs with ViTa database suggested a putative binding site for miR-199a-3p in the HBsAg coding region, and the other binding site for miR-210 in the HBV pre-S1 region. The direct effect of these two miRNAs on HBV RNA transcripts was also validated by GFP reporter assay. This study suggests up-regulation of miR-199a-3p and miR-210 in HepG2 2.2.15 cells resulting in a reduction of HBV replication by a direct binding to the viral RNAs (ZHANG *et al.* 2010a). In addition, Russo's group found miR-125a-5p is able to interact with HBV surface antigen and interfere with its expression, thus reducing the amount of secreted HBsAg (POTENZA *et al.* 2011).

HBV is controlled within the host hepatocyte by a number of cellular proteins at multiple steps of the HBV replication cycle in a complex way. Control of HBV at the step of transcription influences both HBV gene expression and replication. There are several studies on host cellular transcription factors modulated potentially by cellular miRNAs in host hepatocytes, and so affecting HBV transcription. CAAT

enhancer-binding protein (C/EBP) α and β, for example, are co-expressed in hepatocytes and are involved in the hepatocyte metabolism and proliferation. C/EBP can bind and activate the HBV Enhancer II in a dose-dependent manner (LOPEZ-CABRERA *et al.* 1991), and also binding to core promoter and S promoter to activate their transcription (BOCK *et al.* 1999; LOPEZ-CABRERA *et al.* 1990). Wang *et al.* showed that C/EBP-β was targeted and negatively regulated by miR-155 (WANG *et al.* 2009), which could down-regulate HBV transcription. A second example is miR-372. An inhibition of miR-372 results in reducing the expression of its target gene, PRKACB, which induces phosphorylation of CAMP- response element binding protein (CREB) and dissociated CREB from the promoter (WANG *et al.* 2010a). CREB was known to be required for the expression of all HBV transcription units for binding to viral enhancer I (KIM *et al.* 2008). And in our previous results, Dr. Xiaoyong Zhang found that miR-1 was able to enhance the HBV core promoter transcription activity by augmenting FXRA expression, a positive transcription factor (ZHANG *et al.* 2011b).

Other than miRNAs affecting viral translation, there are reports on HBV replication. In Qiu's study, silencing miR-122 in HBV transfected Huh-7 cells with antisense miR-122 significantly increases HBsAg and HBeAg secretion (QIU *et al.* 2010). Furthermore, miR-152 may be a factor in regulating the methylation of host and viral cccDNA by modulating DNMT1 expression (ZHANG *et al.* 2009). Collectively, host miRNAs can modulate HBV transcription and replication by regulating cellular factors that do not direct bind to viral genome.

In our results, we found that miR-125b dose not bind to the HBV genome directly even though it was predicted that the seed sequence of miR-125b could target HBV polymerase and s gene region by computational analysis (Figure 4.5). Besides, miR-125b neither regulated the HBV four promoters activity nor regulated the HBV RNA transcription process (Figure 4.10-12). But miR-125b could up-regulate HBV core protein expression, core particle formation, HBV RI accumulation as well as progeny section in a dose dependent manner in several hepatoma cell lines (Figure 4.6,

4.7 and 4.13). Moreover, miR-125b could enhance HBV replication with miR-1 in a synergistic manner (Figure 4.14). All these results demonstrated that, in contrast to miR-1, miR-125b participated in HBV life cycle in the post transcriptional process. Since phosphorylation of HBV core protein is important in post transcriptional steps in HBV life cycle, including viral RNA packaging, DNA synthesis, and subcellular localization (CHEN *et al.* 2011a; LUDGATE *et al.* 2011; LUDGATE *et al.* 2012; WANG *et al.* 2012a; WITTKOP *et al.* 2010), we supposed that miR-125b may strengthen HBcAg protein stability and nucleocapsid assembly ability through modulation core protein phosphorylation and dephosphorylation process, but this hypothesis should be further confirmed.

5.4 MiR-125b is involved in regulating multiple pathways in hepatoma cells which may affect HBV replication

One miRNA could regulate several target genes, this implies that in different tissues one miRNA may have different regulatory targets or pathways for one phenomenon, and on the other hand, one miRNA could target multiple signalling pathways for one phenomenon in one tissue (SURDZIEL *et al.* 2011). As to liver tissue, even though hepatoma cell lines used in different groups were all extracted from liver tumor tissue, there were still different physiological statuses, such as different differentiation degree, molecular components or activated pathways, which may all influence one miRNA modulation mechanism. Huang *et al.* for example, demonstrated that miR-125b could arrest bladder cancer cells at G1 phase through directly suppressing target protein E2F3 expression, which was a critical factor for G1/S transition and was over expressed in most of poorly differentiated bladder cancers (HUANG *et al.* 2011). Wu *et al*. reported that miR-125b regulated the proliferation of glioblastoma stem cells by targeting E2F2 (WU *et al.* 2012). However, in our results, we found that miR-125b did not down-regulate E2F3 protein expression in hepatoma cells, so as to other E2Fs family members (data not showed), but decreased RB phosphorylation which is a key factor that arrests cells during the G1 phase of the cell cycle by repressing transcription genes such as E2Fs family (Figure 4.26).

At the beginning, our results confirmed that the quantity for mature miR-125b in hepatoma cells was significantly decreased compared with PHH, so we want to find out the reasons. MiR-125b gene promoter is enriched in CpG dinucleotides, which is benefit for DNA methylation, so miR-125b transcription is modulated by DNA methylase (ALPINI *et al.* 2011; HE *et al.* 2012a; ZHANG *et al.* 2011c). Enhancer of zeste homolog 2 (EZH2) acts as a methyltransferase which is thought to be involved in targeting transcriptional regulators, and it is reported that EZH2 could epigenetically silence multiple tumor suppressor microRNAs in liver (AU *et al.* 2012; ONDER *et al.* 2012). Thus, we supposed that EZH2 may participate in modulating of mature miR-125b generation in HCC. However, our results showed that knocking down EZH2 in hepatoma cells had no influence on mature miR-125b generation (Figure 4.18). Unexpectedly, we found that exogenous miR-125b could suppress EZH2 protein expression both in HepG2.215 and Huh7 cell lines. After further study, we found that knocking down EZH2 in Huh7 could enhance HBV replication but not in HepG2.215 cell line (Figure 4.19, 4.21). This phenomenon showed that in different hepatoma cell lines, miR-125b have different regulatory mechanisms on HBV replication.

Moreover, Liang *et al*. elaborated that miR-125b suppressed human liver cancer cell proliferation and metastasis by directly targeting oncogene LIN28B while Alpini *et al*. showed that miR-125b inhibited hepatoma cells proliferation by targeting placenta growth factor but did not regulate LIN28B in HCC (ALPINI *et al.* 2011; LIANG *et al.* 2010). It is yet unclear which genes or pathways that miR-125b may take in regulating hepatoma cells growth and metabolism. And we want to sort out which genes or pathways miR-125b may take in hepatoma cells in modulating HBV replication. As HBV replication is related to host cell metabolism, for example, in 2006, Shlomai *et al.* reported that HBV was tightly regulated by changes in the body's nutritional state through the metabolic regulator PGC-1alpha (SHLOMAI *et al.* 2006). And recently, Shyh-Chang *et al.* reported that LIN28 is a primal regulator of growth and metabolism in stem cells, and that a loss of its function accelerates differentiation

(SHYH-CHANG and DALEY 2013). Moreover, LIN28 could inhibit *let-7* biogenesis which could control glucose homeostasis and insulin sensitivity. LIN28/*let-7* pathway plays a specific and tightly regulated role in modulating glucose metabolism in mammals (FROST and OLSON 2011; ZHU *et al.* 2011). And our results were consistent with these of Liang's group that miR-125b could down-regulate LIN28B protein expression in hepatoma cells (Figure 4.28), and that knocking down LIN28B could up-regulate *let-7* family members generation and also enhance HBV replication (Figure 4.30 and 4.31).

The molecular mechanisms for miR-125b on regulating HBV replication are complicated, there is still a lots of work needs to be done in the future to figure out more pathways and make clear what is the difference between the various hepatoma cell lines for miR-125b modulation.

6 Summary

In our present study, we tested the influence of six liver cancer related miRNAs on HBV replication and hepatoma cell growth, and found that miR-125b could up-regulate HBV replication and suppress hepatoma cell growth. Moreover, we found, different from previous investigated miR-1, that miR-125b neither regulates hepatoma cells differentiation nor participates in HBV transcription level. More detailed functions for miR-125b that we found in this study are summarized as follows:

① MiR-125b could specifically up-regulate HBV replication both in HBV stable expression or transient transfection system, while it has no influence on HCV replication.

② Mature miR-125b is down-regulated in HCC compared with PHH, while the reduction is neither attributed to methylase EZH2 nor to HBx. On the contrary, exogenous miR-125b could decrease EZH2 both in HepG2.215 and Huh7, but only the decrease in Huh7 causes the enhancement of HBV replication.

③ The action for miR-125b on HBV replication is seed sequence specific, anti-miR-125b inhibitor could suppress mature miR-125b generation and also HBV replication in hepatoma cells.

④ MiR-125b neither binds to HBV genome directly nor regulates the HBV transcription process, but it enhances HBV core particle formation and progeny secretion in a dose dependent manner.

⑤ Exogenous miR-125b could inhibit hepatoma cells proliferation and block cell at G1/S phase transition through a decrease of RB phosphorylation, but it does not regulate liver specific protein ALB or cellular transcription factor FXRA expression.

⑥ Exogenous miR-125b could regulate primal metabolism associated genes transcription in hepatoma cells, such as LIN28B, which is a target gene for miR-125b, and this may be benefit for the HBV replication.

7 Zusammenfassung

In der vorliegenden Arbeit wurde der Einfluss von sechs mit Lebertumoren in Beziehung stehenden miRNAs auf die Replikation von HBV und das Wachstum von Leberkrebszellen untersucht. Dabei zeigte sich, dass miR-125b die HBV-Replikation hochregulieren und das Wachstum von Leberkrebszellen unterdrücken konnte. Darüber hinaus hat sich gezeigt, dass, anders als im Falle der bereits untersuchten miR-1, miR-125b weder die Differenzierung von Leberkrebszellen reguliert noch an der Transkription von HVB beteiligt ist. Weitere Funktionen von miR-125b, die im Rahmen dieser Arbeit aufgedeckt wurden, werden im Folgenden im Detail zusammengefasst:

1. MiR-125b konnte besonders die HBV-Replikation sowohl im stabilen Expressions- als auch im transienten Transfektionssystem hochregulieren, wohingegen es keinerlei Einfluss auf die HCV-Replikation hatte.
2. Reife miR-125b wird, im Vergleich zu PHH, in HCC herunter reguliert, wobei die Reduktion weder mit Methylase EZH2 noch mit HBx in Zusammenhang steht. Im Gegenteil, exogene miR-125b konnte EZH2 sowohl in HepG2.215 als auch in Huh7-Zellen herabsetzen, wobei nur die Reduktion in Huh7 die HBV-Replikation erhöhen konnte.
3. Das Verhalten von miR-125b bei der HBV-Replikation ist abhängig von „seed sequence“; der anti- miR-125b-Inhibitor konnte sowohl die Generierung von reifen miR-125b als auch die HBV-Replikation in Leberkrebszellen unterdrücken.
4. MiR-125b bindet weder direkt an das HBV-Genom noch reguliert es den HBV-Transkriptionsprozess; allerdings erhöhte es dosisabhängig die Bildung und Sekretion von HBV Core Partikeln.
5. Exogene miR-125b konnte die Proliferation von Leberkrebszellen inhibieren und durch eine Verringerung von RB Phosphorylierung die Zellen in der G1/S-Phase blockieren, jedoch war es nicht in der Lage, das Leber-spezifische

Protein ALB oder die Expression des zellulären Transkriptionsfaktors FXRA zu regulieren.

6. Exogenes miR-125b konnte die Transkription von Primärmetabolismus assoziierten Genen in Leberkrebszellen regulieren, wie z.B. LIN28B, welches ein Zielgen für miR-125b darstellt, was für HBV-Replikation förderlich sein kann.

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9 Abbreviations

3'-UTR	3'-untranslated region
5'-UTR	5'-untranslated region
APH	aphidicolin
C/EBP	CCAAT/enhancer-binding protein
cccDNA	circular covalently closed DNA
CDK2	cyclin-dependent kinase 2
CHB	chronic hepatitis B
CREB	cyclic-AMP response element binding protein
CTD	C-terminal domain
DIO	diet-induced obese mice
DMEM	Dulbecco’s Modified Eagles’s Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3A
E.coli	Escherichia coli
EDTA	ethylenediaminetetraaceticacid
ER	endoplasmic reticulum
EZH2	enhancer of zeste homolog 2
FBS	fetal bovine serum
FNDC3B	fibronectin type Ⅲ domain containing 3B

FOXO1	forkhead box protein o 1
FXR	farnesoid X receptor
g	gram
HBcAg	hepatitis B core antigen
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HBV RI	hepatitis B virus replicative intermediate
HBx	hepatitis B x antigen
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HNF	hepatocyte nuclear factor
HSP27	heat shock protein 27
IFN	interferon
IGF1R	insulin-like growth factor 1 receptor
IU	international unit
kb	kilo base pair
l	liter
LXRs	liver X receptors
m	milli
MAPK	mitogen-activated protein kinase
MCMV	murine cytomegalovirus

MEM	minimum Essential Medium
min	minute
miRNAs	microRNAs
mTOR	mammalian target of rapamycin
NOC	nocodazole
ob/ob	leptin-deficient mice
PBS	phosphate buffered saline
pCP	plasmid contains HBV core promoter region
PCR	polymerase chain reaction
PCR	polymerase chain reaction
pgRNA	pregenomic RNA
pHBV1	plasmid contains HBV fragment 1
pHBV2	plasmid contains HBV fragment 2
pHBV3	plasmid contains HBV fragment 3
pHBV3'UTR	plasmid contains HBV 3'-termnal region
pHBVFL	plasmid contains HBV full length genome
PHH	primary human hepatocyte
pRB	phosphorylated retinoblastoma protein

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12 Acknowledgements

I am very grateful to my PhD project supervisor, Prof. Dr. rer. nat. Mengji Lu for giving me the opportunity to study and complete the present study in the Institute of Virology. I want to appreciate his patient guidance. Every time when I was depressed he could encourage me and let me having the courage to go on with my scientific work. And also thanks for his strict attitude which makes my experiments going on much well-off.

I am deeply appreciated Ms. Daniela Catrini for helping me to modify my dissertation and prepare the materials for PhD degree application.

I also would like to thank Prof. Dr. Ulf Dittmer and Prof. Dr. med. Michael Roggendorf. They gave me support and guidance, and also provided opportunity and welfare for our Chinese students in China office.

Many thanks to our technician Mrs. Thekla Kemper and former technician Mrs. Barbara Bleekmann. Their technical assistance and help are important for my work.

I also want to appreciate my colleagues Dr. Zhiyong Ma, Dr. Xiaoyong Zhang, Dr. Jia Liu, Dr. Jing Hu, Dr. Zhenhua Zhang, Mr. Jianbo Xia, Mr. Yong Lin, Mr. Weimin Wu, and all the Chinese friends in Essen. They gave me much help and care and make my life in Germany much joyous.

I would like to acknowledge Dr. Anna Kosinska, Dr. Susanne Ziegler, Ms. Maren Lipskoch, Mr. Hadi Karimzadeh and Ms. Christine Thöns for their help and support in my work and life. I would like to thank Mrs. Elisabeth Zimmerman, Ms. Ursula Schrammel, and Ms. Katrin Palupsky for giving me a lot of help during my stay in Germany.

I am deeply grateful to my parents, my sisters and brother for their sustained encouragement. And also my boyfriend for his understanding and supports.

I appreciate the financial support provided by PhD Graduate Course GRK1045.

13 Curriculum vitae

The biography is not included in the online version for reasons of data protection.

14 Erklärung

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „MicroRNA-125b modulates cell growth and metabolism and HBV replication“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von (Wanyu Deng) befürworte.

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