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利用類轉錄活化因子核酸酶基因剃除小鼠模型探討微型核糖核酸 130a 與 B 型肝炎病毒之間的交互作用

In vivo Dissection of the Relationship between miR-130a and Hepatitis B virus using a TALEN knockout mouse model

莊景涵

Ching-Han Chuang

指導教授：施嘉和 教授

Advisor: Chiaho Shih, Ph.D

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本論文係莊景涵君（學號R04445126）在國立臺灣大學微生物學所完成之碩（博）士學位論文，於民國106年7月27日承下列考試委員審查通過及口試及格，特此證明

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碩士班 2 年的時間說長不長，說短但也並不短，實驗室就像一個大家庭，遇到不會的地方時，學長姐都很熱心地幫忙，一起想辦法解決，一路下來接受大家許多的幫忙與教導，我才能夠順利地完成此篇論文。首先，我很慶幸我能夠進入施嘉和老師的實驗室並且讓我研究這個計劃，施老師引導我的實驗方向與時常地鼓勵，讓我受益良多，他強調做科學要誠信，實事求是的精神影響了我對於科學的態度。

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中文摘要

B 型肝炎病毒(HBV)感染為全球主要的健康問題,B 型肝炎病毒感染可能會造成急性或慢性肝炎,長期的慢性肝炎可能會演變成肝硬化甚至是肝癌。雖然目前已經有疫苗能有效地預防 B 型肝炎病毒的感染,但仍然有許多人在施打疫苗後無法成功地產生對抗B型肝炎病毒的抗體以及疫苗對於慢性B型肝炎病人並無治療的效果。現今的第一線抗 B 型肝炎病毒藥物為第一型干擾素及核苷酸類似物,但兩者的作用效果皆有限且副作用強,容易產生抗藥性,目前的治療方法像是抑制病毒的進入或是抑制病毒顆粒的組裝等藥物皆無法根除病毒的共價閉合環狀去氧核醣核酸(cccDNA),病毒仍然潛藏在肝細胞中。慢性 B 型肝炎病毒感染仍影響全球 3.5 億人口且每年約有 78 萬 6 千人口死於慢性 B 型肝炎病毒導致的肝硬化及肝癌,目前仍急須找尋有效的治療方法來對抗 B 型肝炎病毒感染。

微型核醣核酸(micro-RNA)為小片段非編碼的核糖核酸,能透過影響轉錄後的標的信使核糖核酸(messenger RNA)的轉譯及降解來調控基因的表現,已經有許多研究指出微型核醣核酸影響病毒與宿主之間的關係以及病毒的生命週期,近年來有些微型核醣核酸被發現能夠調控細胞蛋白或 B 型肝炎病毒轉錄本(transcript),進而直接或間接地影響 B 型肝炎病毒的複製。

我們實驗室先前發現微型核醣核酸130a能夠調控兩個B型肝炎病毒轉錄因子/共同活化子: PGC1a 與 PPARg,進而抑制 B 型肝炎病毒的信使核糖核酸與蛋白質的表現及病毒的複製。

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本次實驗，我們利用類轉錄活化因子核酸酶(TALEN)方法製作微型核醣核酸130a剃除鼠，研究B型肝炎病毒與微型核醣核酸130a在活體內的關係，我們利用高壓流體注射法(hydrodynamic injection)從小鼠尾靜脈打入B型肝炎病毒質體，發現到帶有PGC1a與PPARg高度表現的微型核醣核酸130a剃除鼠會有較高的病毒複製，另外我們從剃除鼠的尾靜脈打入微型核醣核酸130a表現的腺病毒來回補微型核醣核酸130a，發現能夠回過頭來抑制B型肝炎病毒的複製。

總和來說，我們在小鼠模型中驗證了微型核醣核酸130a能夠抑制B型肝炎病毒的複製，微型核醣核酸130a或許能夠作為有效的治療對策來抑制B型肝炎病毒。

關鍵字:B型肝炎病毒、微型核醣核酸、微型核醣核酸130a、類轉錄活化因子核酸酶基因剃除鼠、過氧化物酶體增殖物啟動受體γ輔啟動因子1、過氧化物酶體增殖物啟動受體γ、高壓流體注射法、腺病毒

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Abstract

Hepatitis B virus (HBV) infection is a major public health problem throughout the world. HBV infection causes acute or chronic hepatitis which may cause liver cirrhosis or hepatocellular carcinoma (HCC). Although effective HBV vaccine are available to prevent HBV infection, some individual can't produce antibody against HBV and existing vaccine has no therapeutic effect on the chronically infected populations. The first-line antiviral HBV therapy reagents include type I interferon and nucleotide analogs but both have limited efficacy; severe side effects and drug resistance were found in many cases. Furthermore, current therapeutic methods such as viral entry inhibitors and capsid assembly inhibitors cannot eradicate HBV because viral cccDNAs still exist in hepatocytes. Chronic HBV infection still influences 350 million people worldwide and estimated 786,000 people die every year due to complications of HBV-related liver cirrhosis and HCC. Exploration of more effective therapeutic strategies for HBV infection is extremely urgent.

micro-RNAs (miRNAs) are small, non-coding RNAs that can regulate gene expression at post-transcriptional level through translational repression or degradation of targeted mRNAs. So far, several studies have implicated that miRNAs influence the virus and host interaction and affect the virus life cycle. Recently, some miRNAs have been found to directly or indirectly regulate HBV replication by targeting

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cellular protein or HBV transcript.

Previously, our laboratory found that miR-130a inhibited HBV replication by targeting two HBV transcriptional factor/coactivator: PGC1a and PPARg, thereby inhibits viral mRNA transcription and thus influence viral protein expression and virus replication.

In this study, we create the miR-130a knockout (KO) mice by TALEN approach to dissect the relationship between HBV and miR-130a in vivo. After HBV plasmids tail-vein hydrodynamic injection, these miR-130a KO mice can promote the HBV replication due to higher expression levels of PGC1a and PPARg. In addition, the I.V. injection of miR-130a-expressing adenovirus in miR-130a KO mice, which replenishes the miR-130a expression, would inhibit the HBV replication reversely. Taken together, we validate that miR-130a have an inhibitory effect on HBV replication in vivo and increase miR-130a might be an effective strategy to limit HBV replication.

Key words: Hepatitis B virus, micro-RNA, miR-130a, TALEN knockout mouse, PGC1a, PPARg, Hydrodynamic injection, Adenovirus

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Introduction

1. Hepatitis B virus

Hepatitis B virus (HBV) is a partially double strand DNA virus of hepadnaviradae family and one of most common human pathogens worldwide. It is an enveloped virus, and contains a partially double-stranded DNA genome (relaxed circular DNA; RC DNA) about 3.2 Kb within core (Seeger et al. 2000). A multiple transmembrane bile acid transporter in liver, sodium taurocholate cotransporting polypeptide (NTCP) was recently identified as a functional receptor for HBV (Huan et al. 2012). Virus entry is mediated by binding of PreS1 domain of L protein to NTCP and additional entry factors such as glypican 5 (Verrier et al. 2016).

After the virus enters the hepatocyte, the viral genome is delivered into the nucleus, and the RC-DNA-binding P protein (viral polymerase) is released by host DNA-repair factor, tyrosyl-DNA-phosphodiesterase 2 (TDP2). P-protein–free RC DNA is processed further by other factors of the host DNA repair machinery to form the covalently circular DNA (cccDNA) (Königer et al. 2014).

The cccDNA serves as the template for the RNA polymerase II-mediated transcription of viral transcripts. All transcripts are 5’ capped and 3’ polyadenylated and four separate promoters drive different viral transcripts but all use the same polyadenylation signal (Paran et al. 2000).

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The viral DNA contains four major open reading frames (ORFs: C, P, S and X): 1. The precore/core gene (C ORF), coding for the viral nucleocapsid protein (hepatitis B c antigen (HBcAg)) and the precore protein (hepatitis B e antigen (HBeAg)), which are translated by 3.5-kb transcripts but with different 5' ends. 2. The polymerase gene (pol) (P ORF), coding for the reverse transcriptase/HBV polymerase, which are translated by 3.5-kb pregenomic RNA. P ORF and is functionally divided into three domains: the reverse transcriptase (RT) domain, which catalyzes genome synthesis; the terminal protein domain, which is involved in encapsidation and initiation of minus-strand synthesis; and the ribonuclease H domain, which degrades pregenomic RNA and facilitates replication. 3. The S ORF (PreS1, PreS2, S genes), coding for the three envelope proteins, which are translated by 2.4, 2.1 and subgenomic transcripts to form the L protein (PreS1/PreS2/S), M protein (PreS2/S), and S protein (S). 4. The X ORF, coding for the hepatitis B virus X protein (HBx), translated by 0.7 kb transcripts (Urban et al. 2010).

The HBV replication cycle comprises translation of the hepatitis B proteins, including HBsAg, HBcAg, HBeAg, polymerase, HBx, and reverse transcription of pregenomic RNA to prime DNA synthesis. HBV DNA genome is replicated through the pregenomic RNA, which is produced by transcription of the entire viral DNA by cellular RNA polymerase. Pregenomic RNA is then transformed into the viral DNA

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genome by the HBV polymerase, multi-functional enzyme with reverse transcriptase, DNA-dependent DNA polymerase, and RNase H activities (Summers et al. 1982; Crouch et al. 2005). The nucleocapsid then interacts with the envelope proteins in the endoplasmic reticulum/golgi apparatus to constitute the mature virions, then secreted into the extra-cellular environment (Huovila et al. 1992) (Fig. 1).

2. HBV infection and treatment

Chronic hepatitis B virus infection influences more than 350 million people worldwide and estimated 786,000 people die every year due to complications of HBV-related liver cirrhosis and hepatocellular carcinoma (Trepo et al. 2014; Zuckerman et al. 2000; Lavanchy et al. 2005).

HBV is transmitted by contact with the blood or body fluids of an infected person and is commonly infected through perinatal, horizontal, sexual, and parenteral percutaneous transmission. The clinical pattern of HBV infection ranges from acute fulminant hepatitis to various forms of chronic infection, which may develop to liver cirrhosis and hepatocellular carcinoma (HCC) (Liaw et al. 2009). Serum HBsAg is a marker of HBV infection, and serum antibodies against HBsAg often means recovery from infection. Following infection, formation of stable cccDNA in the nucleus and HBV DNA integration into the host genome makes HBV difficult to eradicate

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(Dienstag et al. 2008).

Without effective vaccine, HBV remains a life-threatening pathogen to chronic HBV infected individuals, who are prone to develop liver cirrhosis, hepatocellular carcinoma (HCC) (Alter et al. 2012). Antiviral cytokine interferon alpha (IFN-α) (Type I interferon) and reverse transcriptase inhibitors (nucleotide analogs; NAs) are current treatment reagents for chronic HBV patients (Isorce et al. 2015; Gish et al. 2012). Either therapeutic agents may achieve control of infection but have limited efficacy to liver diseases/cancers and have numerous side effects (Chin et al. 2003). HBV patients can rarely be cured because cccDNA is not directly targeted and cccDNA is not eliminated completely even after recovery from acute self-limited hepatitis B (Rehermann et al. 1996; Seto et al. 2014). HBV patients still need lifelong therapy to suppress HBV until seroconversion.

Recently, there are some new strategies to control HBV such as viral entry inhibitors, destruction of cccDNA by CRISPR/Cas9, and breaking immune tolerance. However, there is still no to completely eliminate HBV virus. Exploration of more effective therapeutic strategies to eradicate HBV is extremely urgent (Shih et al. 2016).

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3. microRNA

The first microRNA (miRNA), lin-4, in C. elegans was identified by Victor Ambros's lab in 1993 (Lee et al., 1993). And the second miRNA, let-7, was discovered by Gary Ruvkun's lab in 2000 (Reinhart et al., 2000). They found that let-7 and lin-4 both took part in the regulation of later stages of C. elegans development. More and more miRNAs have been discovered in human and mouse, and all recorded in miRBase database which is a bioinformatic database of published miRNA sequences and annotation. According to the latest version of miRBase 21.0, there are 2588 and 1915 miRNAs have been found in Homo sapiens and Mus musculus (Kozomara et al. 2014).

miRNAs are endogenous non-coding RNA, approximately 21-23 nucleotides, regulate mRNAs expression through imperfect complementary pairing to the 3'untranslated region (3'UTR) of their target mRNAs (Bartel et al. 2009). Primary miRNAs (Pri-miRNAs) are transcribed by RNA Pol II then cleaved by Drosha in the nucleus to form the precursor miRNAs (Pre-miRNAs) and exported to the cytoplasm by Exportin 5 (Kim et al.2009; Lee et al. 2004; Denli et al. 2004). Pre-miRNAs are cleaved by an RNase III enzyme, Dicer, in cytoplasm to generate a miRNA duplex about 21-23 nucleotides in length (Lau et al. 2012; Lee et al.2002). Subsequently, guide strand of the duplex loads to the RNA-induced silencing complex (RISC) to

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produce mature miRNAs. Mature miRNAs can bind to the target mRNAs leading to translational repression or mRNA decay (Krol et al. 2010). miRNAs guide RISC to the mRNA target by complementary base-pairing to 2–8 nucleotides of the mature miRNA which is known as the seed sequence, and miRNAs can be grouped into families based on shared seed sequences (Lewis et al. 2003). It is reported that miRNAs could modulate genes that involved in many cellular processes such as development, differentiation, apoptosis, proliferation, inflammation (Bartel et al. 2004; Di Leva et al. 2014). Recently, miRNA and siRNA drugs have been studied in clinical trials by delivery of miRNA or siRNA targeting the specific proteins involved in the mechanism of disease. The key to develop miRNA and siRNA-based therapy need to achieve specific, efficient and safe systemic delivery of therapeutic miRNAs or siRNA. Chen Y et al. packaged the miRNAs and siRNAs into the LPH (liposome-polycation-hyaluronic acid) nanoparticle modified with tumor-targeting single-chain antibody fragment (scFv) for systemic delivery of siRNA and miRNA into experimental lung metastasis of murine B16F10 melanoma and demonstrated these miRNAs and siRNAs packaged nanoparticles had strong anticancer effects. (Chen et al. 2010).

Another siRNA-based drug ARC-520, a liver-tropic cholesterol-conjugated siRNA (chol-siRNA), transported by the Dynamic Polyconjugate delivery system is just

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reaching a Phase II clinical study. ARC-520 can directly target conserved sequences of different HBV strains and significantly suppress viral RNA, proteins and DNA in HBV-replicating human hepatocytes and HBV transgenic mice. Phase 2a clinical trial results showed that ARC-520 was well tolerated and resulted in significant, dose-dependent reduction in HBsAg for up to 57 days in CHB patients. RNAi-based therapies may play an important role in future therapeutic for HBV (Wooddell et al. 2013; Gish et al. 2015).

4. HBV and microRNA

Several miRNAs have been identified for regulating HBV infection or HBV related-disease by targeting cellular proteins or HBV proteins (Thirion et al. 2013). The first research for miRNAs which directly target HBV mRNA was done by Zhang GL et al (Zhang et al. 2010). They used a miRNA loss-of-function approach. A total of 328 human miRNAs were individually knockdown in HepG2.2.15 cells which are stably transfected with a complete HBV genome. They found that miR-199a-3p and miR-210 strongly reduced HBV replication and HBsAg expression. Bioinformatics analysis showed that miR-199a-3p and miR-210 inhibited HBV by directly targeting the HBsAg encoded region and the pre-S1 region of the HBV genome respectively (Zhang et al. 2010; Zhang et al. 2011).

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Furthermore, several studies reported that miR-122 can remarkably decrease HBV replication when overexpression in both cell lines and liver tissues. miR-122 is a liver-specific miRNA which expresses at high levels in normal hepatocytes (about 70% of the total miRNA population in the adult liver) (Lagos-Quintana et al. 2002). Many studies proposed several mechanisms underlying miR-122 can negatively regulate the viral gene expression and replication by direct binding to a highly conserved sequence of HBV. Chen et al. first demonstrated the inhibitory effect of miR-122 on HBV gene expression and replication. miR-122 bound to a highly conserved sequence on HBV pgRNA, coding the viral polymerase and core protein, resulting in a decrease in HBV core-associated DNA level (Chen et al. 2011). Qiu et al. demonstrated that miR-122 inhibited HBV replication through increasing heme oxygenase-1 (HO-1), which decreased HBV cccDNA levels both in vitro and in vivo (Qiu et al. 2010). Wang et al. revealed another regulatory mechanism of miR-122 which involved cyclin G1-modulated p53 activity. Cyclin G1 interacted with p53 and blocked the binding of p53 to HBV enhancers, which abolished p53-mediated inhibition of HBV transcription. Cyclin G1 was targeted by miR-122, thereby enhancing the anti-HBV activity of p53 (Wang et al. 2012).

HBV encoded miRNA called HBV-miR-3 was recently identified by Xi Yang et al. by deep sequencing and northern blot. HBV-miR-3 located at nts 373-393 of the HBV

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genome and was generated from 3.5kb, 2,4kb and 2.1kb HBV RNA in classic miRNA biogenesis (Drosha-Dicer dependent) manner. They found that HBV-miR-3 targets the region of HBV 3.5 kb mRNA encoding HBV core protein (HBc) and pregenomic RNA (pgRNA) to attenuate HBV replication, which serves as a negative feedback in virus replication so as to maintenance of viral persistent infection (Yang et al. 2016).

So far, many studies have implicated that miRNAs influence the virus and host interaction and affect the virus life cycle (Skalsky et al. 2010). And miRNAs affect HBV replication and HBV-related disease such as hepatocellular carcinoma (HCC) by translational targeting of virus factors or host factors (Thirion et al. 2013).

Several studies have demonstrated that microRNA expression is dysregulated in HBV infected patients and HBV-related HCC (Kitab et al. 2015; Naito et al. 2015; Lamontagne et al. 2015; Louten et al. 2015). Previously, our lab found that miR-204 and miR-1236 were downregulated in HBV-producing cells, and each could suppress HBV replication. The miR-1236 could reduce HBV replication and protein production by directly targeting at HBV specific mRNAs. The miR-204 could inhibit HBV pregenomic RNA encapsidation and capsid assembly. Both miR-204 and miR-1236 might be useful therapeutic target against HBV (Huang et al. 2016).

Recently, we found miR-130a was reduced in HBV-producing hepatoma cell lines by microRNA microarray approach. Furthermore, we discovered that miR-130a

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inhibited HBV replication by targeting two HBV transcriptional factor/coactivator: PGC1a and PPARg, which inhibited viral mRNA transcription and thus influence viral protein expression and virus replication. In contrast, HBV could downregulate miR-130a expression by enhancing the PGC1a and PPARg. Subsequently, PPARg inhibited the NF-kB/p65 which strongly activated the miR-130a promoter by 30-60 folds. We demonstrated two positive-feed-forward loops to explain the contrary relationship between miR-130a and HBV in cell culture (Fig. 2) (Huang et al. 2015). Recently, we successfully applied the United States Patent: Treating Hepatitis Virus Infection by Modulating MicroRNAs miR-130a, miR-130b, miR-204, and miR-1236 (6-06-17, approved (app. # 15/023,284) Inventor: Chiaho Shih).

Recently, analysis of miRNA knockout mice has been a well-developed tool for the research of the physiological importance of individual miRNAs in mammals. Additionally, studies of miRNAs as regulators of disease processes can help identify cellular factors and define molecular mechanisms of disease processes (Park et al. 2010). In this study, we created the miR-130a TALEN knockout mice to dissect the relationship between HBV and miR-130a in vivo.

The study here shows that the miR-130a expression is abolished in miR-130a KO mice, which contributes to these miR-130a KO mice expressing higher PGC1a and PPARg. Furthermore, these miR-130a KO mice promote the HBV replication due to

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higher expression of PGC1a and PPARg. In addition, the I.V. injection of miR-130a-expressing adenovirus in miR-130a KO mice, which replenishes the miR-130a expression, would inhibit the HBV replication reversely. Taken together, we validated that miR-130a have an inhibitory effect on HBV replication in vivo and miR-130a might be a useful target for developing new therapy against HBV.

Results

1. Establishment of miR-130a knockout mice by TALEN approach.

Knowing that miR-130a can inhibit HBV DNA and proteins expression, we created the miR-130a TALEN knock mice to verify the cell culture experiment (Huang et al. 2015). First, we designed the TALEN sequences which were complementary to mouse miR-130a 3p seed region and cleaved the DNA by its combined nuclease (Fig. 3A). We created five different TALEN sequences around the miR-130a 3p site and arranged two of them to be tested in Hep1-6 hepatoma cells (Fig. 3B). We chose the most effective TALEN pair (R2/L3), which reduced most of the miR-130a expression, and microinject the TALEN pair into mouse oocyte to create the miR-130a knockout mice (Fig. 3C). Next, we inbred heterogenous KO mice and cut small fragment of tail from the 4 weeks old offspring for genotyping. We then extracted the genomic DNA from the tail and amplify the mmu-miR-130a-3p region

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by PCR, then detect the DNA sequence of this region by DNA sequencing. In Fig. 3D, we demonstrated wild-type (WT), heterogenous (+/-) and homogenous (-/-) KO mouse sequencing data. The heterogenous (+/-) KO mouse had one mutant strand which had two base pair deletions in mmu-miR-130a-3p site, and the homogenous (-/-) KO mouse had two mutant strands (Fig. 3D). All the mice were confirmed the genotype by DNA sequencing for the sake of subsequent experiments accurately.

2. The expression of miR-130a are almost abolished in miR-130a-knockout mice.

Since the R2/L3 TALEN pair was the most effective pair to decrease the miR-130a expression, we used this pair to microinject into mouse oocyte to create miR-130a TALEN knockout mouse and verified mouse genotype by DNA sequencing. First, we made sure the miR-130a were almost abolish in the liver of KO mice by miRNA Northern blot (Fig. 4A) and stem-loop qPCR (Fig. 4B). Next, we checked not only the liver, but also others organ in wild-type, heterogeneous, homogeneous miR-130a KO mice such as white adipocyte, brown adipocyte, cerebellum, cerebrum, muscle and heart. They all abolished the miR-130a expression in miR-130a knockout mice (Fig. 4C). So, we confirmed the miR-130a expression were truly knockdown not only in the liver but also the whole body in miR-130a KO mice. Next, we wanted to

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make sure the relationship between miR-130a, PGC1a and PPARg in vivo. In Fig. 4D, we compared the liver PGC1a and PPARg proteins and mRNAs levels in the wild-type, heterogeneous, homogeneous miR-130a KO mice by western blot and q-RT-PCR. The miR-130a KO mice had higher PGC1a and PPARg proteins and mRNAs levels than wild-type mice (Fig. 4D). In these data, we confirmed that miR-130a had suppressing effect on PGC1a and PPARg in vivo.

3. miR-130a knockout mice have higher HBV DNA replication and protein expression than wild-type mice.

We bred the heterogeneous miR-130a KO mice to make the wild-type and KO mice, and all the genotype of mice were confirmed by the genotyping. 8~9 weeks old age-matched male mice were injected with HBV plasmid by tail vein hydrodynamic injection as well as miR-130a-expressing adenovirus 24 hours later (n=5 in each group). After 4 days post HBV plasmid hydrodynamic injection, we sacrificed the mice and collected liver and sera to analyze the HBV DNA replication (Fig. 5A), E- & S- antigens (Fig. 5B) and HBV core proteins expression (Fig. 5C&D). [Each lane means one individual mouse in each group: wild-type mice plus Ad-control: lane 1~5 in (A) & (C) ; miR-130a knockout mice plus Ad-control: lane 6~10 in (A) & (C) ; miR-130a knockout mice plus Ad-miR-130a: (lane 11~15 in (A) & (C)].

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Before we conducted the HBV replication experiment, we verified the miR-130a expression by stem-loop q-PCR to make sure that the miR-130a expression was abolish in miR-130a KO mice and increased after I.V. injection of miR-130a-expressing adenovirus (Fig. 5E). First, we extracted the HBV core-associated DNA in liver and analyzed the HBV DNA replication by southern blot. miR-130a KO mice (lane 6~10) had higher HBV DNA replication than wild-type mice (lane 1~5). After I.V. injection of miR-130a-expressing adenovirus in miR-130a KO mice (lane 11~15), the HBV DNA replication was downregulated than I.V. injection of adenovirus control (Fig. 5A). We also checked the HBV E-antigens and S-antigens in sera, which are secreted protein and surface protein of HBV, by ELISA. It had a similar pattern like the Fig. 5A, the miR-130a KO mice have higher E-&S-antigens than wild-type mice and these antigens were decreased after miR-130a-expressing adenovirus injection (Fig. 5B). Finally, we confirmed that the HBV core protein expression in liver was also upregulated in miR-130a KO mice (lane 6~10) compared to wild-type mice (lane 1~5), and the core proteins expression was declined after miR-130a-expressing adenovirus injection (lane 11~15) by western blot (Fig. 5C) and by immunohistochemistry (Fig. 5E). In summary, we testified that miR-130a KO mice had higher HBV replication than normal miR-130a-expressing wild-type mice, and the I.V. injection of miR-130a-expressing adenovirus in

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miR-130a KO mice, which replenished the miR-130a expression, would inhibit the HBV replication reversely. These data suggested that miR-130a had an inhibitory effect on HBV replication in vivo.

Materials and Methods

Ethics Statement

All animal experiments were conducted under protocols approved by Academia Sinica Institutional Animal Care & Utilizat1ion Committee (ASIACUC permit number 13-12-631). Research was conducted in compliance with the principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996.

Animals

C57BL/6J (B6/J) and C57BL/6J-miR-130a $^{-/-}$ (B6/J-miR-130a $^{-/-}$) were bred in the animal facility of the Institute of Biomedical Sciences, Academia Sinica. B6/J-miR-130a $^{-/-}$ mice were generated in our laboratory and backcrossed to B6/J mice for at least five generations. We used 6–8 weeks-old male mice. All experiments involving mice were approved by the Institutional Animal Care and Use Committee at Academia Sinica and conformed to relevant regulations. All animals were housed in a

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specific-pathogen-free environment in the animal facility of the Institute of Biomedical Sciences, Academia Sinica.

TALEN target site selection and TALEN construction

To target the seed region of the mouse miR-130a locus, various TALEN pairs were designed using an online tool, TAL Effector Nucleotide Targeter 2.0 (Doyle et al. 2012). To design efficient miRNA targeted TALENs, we followed these criteria: (1) TALEN binding sites were set to 20 bp, including the first T, to ensure high specificity of gene targeting; (2) spacer lengths of 13–20 bp were chosen to maximize cleavage efficiency; (3) the miRNA seed sequence was situated centrally within the spacer to direct cleavage to the seed region (Uhde-Stone et al. 2014). Following these criteria, the target sequences of TALENs for mmu-mir-130a are as follows; L1: 5'-TGTGCTACTGTCTAACGTG-3', L2: 5'-TGCTACTGTCTAACGTGTA-3' and L3: 5'-TACTGTCTAACGTGTACCG-3'; right arm: 5'-TGCCCTTTTAACAT-3' and 5'-TACAAGGCCGATGCCC-3'. We used custom TALEN service (Cold Spring biotech Co.Ltd) to construct plasmids coding for TALEN with an EF1α-driven expression cassette using the SIDANSAI TALEN Assembly Kit (Cat. No: 1801 -060, 1802-030, and 1803 -015; SIDANSAI Biotechnology Co. Inc). The final constructs were confirmed by DNA sequencing.

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Evaluation of TALENs activities in cultured cell line

Briefly, a total of 4×10^5 Hep1-6 hepatoma cells were transfected with 0.5 μg of each TALEN-encoding plasmid and cultured for 24 hrs. The puromycin was then added to the culture medium (final concentration 2 μg/mL), to kill untransfected cells. After 4 days of puromycin selection, puromycin-resistant clones were expanded for further DNA sequencing and RNA analysis.

Microinjection

TALEN plasmids were linearized by Not1and HindIII endonuclease digestion respectively. The procedures of linearized plasmid used as a template for in vitro transcription reaction and microinjection were manipulated by Institute of Molecular Biology Academia Sinica transgenic mouse core facility (Wefers et al. 2013).

Mouse genotyping

Tail genomic DNA of TALEN-transfected mice was isolated with REDxtract-N-Amp Tissue PCR Kit (Sigma Aldrich, St. Louis, MO, USA), done according to the manual. Genotyping miR-130a heterozygous and homozygous mutant mice were identified by performing PCR using following primers.

mmu-miR-130a KO forward: 5'-TCTTTCTCCTGCCTAAGCACCT-3';

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mmu-miR-130a KO reverse: 5'-GGGAGGGCTCCATATATCCAAAT-3'. The PCR program consists of a denaturation step at 95°C for 4 min, followed by 34 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension steps (72°C for 60 s). The program ends with a completion step at 72°C for 180 s. Each PCR tube contains 0.05 U of MyTaq™ HS DNA Polymerase in 14μL of reaction mix (react ion buffer, dNTPs and primers) and 100 ng of genomic DNA in final volume of 15 μL. The PCR product were purified by PCR clean-up kit for sequencing (Takada et al. 2013).

Adenovirus production

Human miR-130a (hsa-miR-130a) was cloned into pShuttle-CMV then recombinant with pAdEasy vector. Amplified this recombinant pAd-miR-130a in Ad-293 cells and extracted the adenovirus from supernatant by CsCl density-gradient centrifugation (AdEasy Adenoviral Vector System; Agilent Technologies®).

HBV ayw dimer plasmid tail vein hydrodynamic injection plus miR-130a-expressing adenovirus I.V. injection

The pSV2-neo-HBV ayw dimer plasmids (30 ug/mouse) (prepared in saline; 10% volume of body weight) were injected into 8~9 weeks old male mice through the

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tail-vein hydrodynamic injection (Yang et al. 2002). After 24 hours, these mice were injected with the miR-130a-expressing adenovirus (AV-CMV-miR-130a) or adenovirus control (AV-CMV) at a dose of 10^9 pfu (n=5 in each group) through the tail vein. Sacrifice the mice and collect the liver and sera after 4 days post HBV plasmid hydrodynamic injection.

Quantitative real-time RT-PCR

Briefly, Tissue was homogenized and extracted by TRIzol reagent (Invitrogen) and 2 μg of total RNA was reverse transcribed into cDNA using random primers and High Capacity cDNA Reverse Transcription kit (Applied Biosystem, Grand Island, NY) at 37℃ for 120 minutes. The cDNA product was then diluted 100 times for real-time PCR analysis using Power SYBR Green PCR master mix (Applied Biosystem, Grand Island, NY), and the default condition in a 20 μl reaction volume by Applied Biosystems 7500 Real-Time PCR System. Data were analyzed by relative quantification methods (ΔΔCt methods) using 7500 software V2.0.1.

Stem-loop qPCR for miRNA

For determination of miRNA expression levels, TaqMan RT and stem-loop real-time assay (Applied Biosystems) were used. Briefly, 100 ng RNAs were reverse

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transcribed by specific stem-loop primer, miR-130a (assayID: 000454), and further analyzed by Taq man real-time PCR assay using default setting. U6 snRNA (assayID: 001973) was used as an endogenous control. Data were analyzed by Applied Biosystems 7500 software V2.0.1.

Southern blot

To evaluate the HBV DNA replication levels, the HBV core particle-associated DNA was extracted and run 1% agarose gel. Probed with 3.2 Kb DIG probe then stain with CSPD to detect the RC, DL, SS form of HBV DNAs (Chua et al. 2010).

miRNA Northern blot

To check the miR-130a expression in miR-130a-/- mice, total cellular RNAs including miRNAs were extracted and run the denaturing acrylamide gel and transferred to nylon membrane. The membrane was then hybridized with the ^{32}P labeled DNA oligonucleotide probe which is complementary to the miR-130a-3p and detected with X-ray film.

Western blot and Antibodies

To detect the protein levels of the liver of mice, we homogenized the liver with

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magnesium beads in PBS. Extract the protein with RIPA on ice for 30 minutes. Run the 15% acrylamide gel to separate different sizes of protein then transfer the proteins on the PVDF membrane. Use primary antibodies (PPARg; Santa Cruze #sc-7196, PGC1a; Origene #TA309815, HBc; Dako #B0586) and secondary antibodies (anti-rabbit-HRP; GeneTex) to probe the proteins then stained with ECL to detect the protein levels of the livers.

Immunohistochemistry (IHC) of HBV core protein

Liver was perfused in neutral formalin then embed into wax to paste the section onto the slide. Probed with HBV core protein antibody (Dako #B0586) and secondary antibodies (anti-rabbit-HRP; Dako #K4002) to detect the HBV core proteins and then DAB staining. Finally, use the hematoxylin to stain the nucleus.

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Discussion

Current antiviral HBV therapy includes type I interferon or reverse transcriptase inhibitors (Liaw et al. 2008). Both therapies have been reported that have limited efficacy, numerous side effect and severe drug resistance in many cases (Rijckborst et al. 2010; Zoulim et al. 2009). Therefore, it is critical to continue the basic research on HBV replication and pathogenesis, in order to identify new therapeutic targets for treatment of chronic HBV infection.

MiRNA take part in several important biological processes by regulating mRNA translation or mRNA stability. Recently, numerous miRNAs are important regulators of several virus-host interactions, such as Herpesvirus, human papilloma virus, Adenovirus, polyomavirus, might be affected by various microRNAs (Umbach et al. 2009). In addition, some miRNAs increase viral replication, some others may even serve as potential anti-viral agents and HIV (Human Immunodeficiency virus), HCV (hepatitis C virus), and HBV (hepatitis B virus) have been studied enthusiastically in this respect.

And one of these mRNA, miR-122, has been developed to anti-HCV drug (Miravirsen) (Janssen et al. 2013). MiR-122 is a highly abundant in the liver and is crucial for the stability and propagation of HCV RNA (Jopling et al. 2005). miR-122 binds to two closely spaced target sites in the 5'-UTR of the HCV genome, which

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reside in the region involves in the regulation of both translation and replication. This binding can stabilize the HCV genome and protect the HCV genome from degradation, which results in the increased expression of the HCV RNA and accumulation of HCV RNA in vivo (Lanford et al. 2010; Henke et al. 2008; Machlin et al. 2011). Miravirsen is an LNA (locked nucleic acid)-antisense oligonucleotide complementary to mature miR-122, which can sequester and thus inhibit miR-122 to cause reduction of HCV RNA levels (Janssen et al. 2013).

The role of miRNAs in HBV biology is complicated and miRNA take part in complicated life cycle and host-virus response. HBV might modulate the expression of cellular miRNAs, which takes advantage of its own replication and allow evasion from innate immune responses in order to establish viral persistence and propagation in infected hepatocytes (Friedman et al. 2009). Furthermore, abnormal expression of miRNAs is associated with HBV-related pathogenesis through the modulation of signaling pathways such as proliferation, apoptosis, migration and immune responses. Recently, dysregulated circulating miRNAs have been discovered in the sera of chronic hepatitis B and HBV-associated acute-on-chronic liver failure (ACLF) patients. Therefore, circulating miRNAs might be a diagnosis maker in HBV patients (Ji et al. 2011).

Several studies have investigated the relationship between miR-130a and

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hepatocellular carcinoma (HCC) and all miRNA expression microarray profiles suggested that miR-130a was significantly reduced in HCC by comparison of the miRNA expression in tumorous and adjacent non-tumorous liver tissues (Thurnherr et al. 2016; Shen et al. 2016).

Li et al. detected miR-130a expression level in 102 paired HCC and adjacent non-tumor tissues and observed the 5-year overall survival rate of HCC patients. They found the expression of miR-130a in the HCC tissues were significantly lower than those in adjacent non-tumor tissues and HCC patients with low miR-130a expression level had lower overall survival rate than those with high miR-130a expression level. They suggested that miR-130a is downregulated in HCC and associates with poor prognosis. (Li et al. 2014). In other study, Zheng et al. compared the miRNA expression profiles in liver tissues from HBV-related ACLF (acute-on-chronic liver failure) patient and matched healthy control and detected the expression of selected miRNAs in sera. They found the serum expression of miRNA-130a was higher in recovered than nonrecovered ACLF patients. They suggested that reducing of serum miRNA-130a expression might be a useful prognosis biomarker in HBV-related ACLF patients. Yang et al. found that miR-130a-3p was downregulated in gemcitabine resistant (GR) HCC cells. miR-130a-3p targeted Smad4 which caused suppression the cell migration and invasion in GR HCC cells. Above studies all imply

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the competitive relationship exists between miR-130a and HCC (Liu et al.2016).

Few researches address about the relationship between miR-130a and HBV infection. Tang et al. found that HBV infection attenuated miR-130a expression and caused the upregulation of estrogen receptors alpha (ERα) which was targeted by miR-130a. The ERα play an important role in persistent HBV-infected hepatocarcinogenesis. ERα could lead to cell cycle progression or inhibition of apoptosis and HBV might affect ERα expression for promoting viral oncogenesis. The miR-130a level was difference between HepG2 and HepG2.2.15 cells which resulted in the difference of ERα expression. It indicated miR-130a mediated the host-virus interaction in viral pathogenesis (Tang et al. 2011). But the mechanism of how HBV inhibits miR-130a is still unknown.

In this study, we dissected the interaction between miR-130a and HBV by TALEN miR-130a knockout mice and found that miR-130a had a strong inhibitory effect on HBV replication in vivo. So far, lots of therapy against HBV have limited efficacy. We seek to find out new therapeutics toward HBV. In previous, we found that miR-130a expression was strongly decreased in HBV producing cell line by the approach of miRNA microarray. Furthermore, reduction of endogenous miR-130a would enhance HBV DNA replication and protein expression. In contrast, miR-130a overexpressing cell would attenuate HBV replication and gene expression. We found

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that miR-130a targeted two HBC transcriptional factor/coactivator: PGC1a and PPARg and thus inhibited HBV DNA replication and protein expression (Huang et al. 2015). Besides the cell culture system, we also wanted to validate the relationship between miR-130a and HBV in mouse model. We deplete the whole-body miR-130a expression including liver by TALEN approach and these miR-130a knockout mice have higher expression of PGC1a and PPARg in liver, which confirmed the inhibitory effect of miR-130a still existed on these two factors in mouse model (see in Fig. 4 & Fig. 5).

MiR-130a and miR-130b share the same seed sequences. To monitor the expression of miR-130b in miR-130a TALEN KO mice, we will set out to measure miR-130b expression, once the stem loop PCR primers for miR-130b is commercially available (still out of stock from the Vendor at present). In case of any off-target effect in the TALEN KO mice, we supplemented the level of miR-130a in miR-130a KO mice by injection of adenovirus expressing miR-130a.

We noted some inconsistent results between HBV DNA synthesis by Sothern blot assay and core protein expression by Western blot (compare lane 6 in Fig. 5A and Fig. 5C). We have no good explanation for this kind of experimental variation. Further studies using a larger number of mice could help reduce the noise of experimental variation. Above all, consistent with the cell culture model, miR-130a

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could inhibit HBV replication in vivo in this KO mouse model (Fig. 5). It is our hope that miR-130a might be a novel therapeutics against HBV in clinical medicine in the future.

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Figures

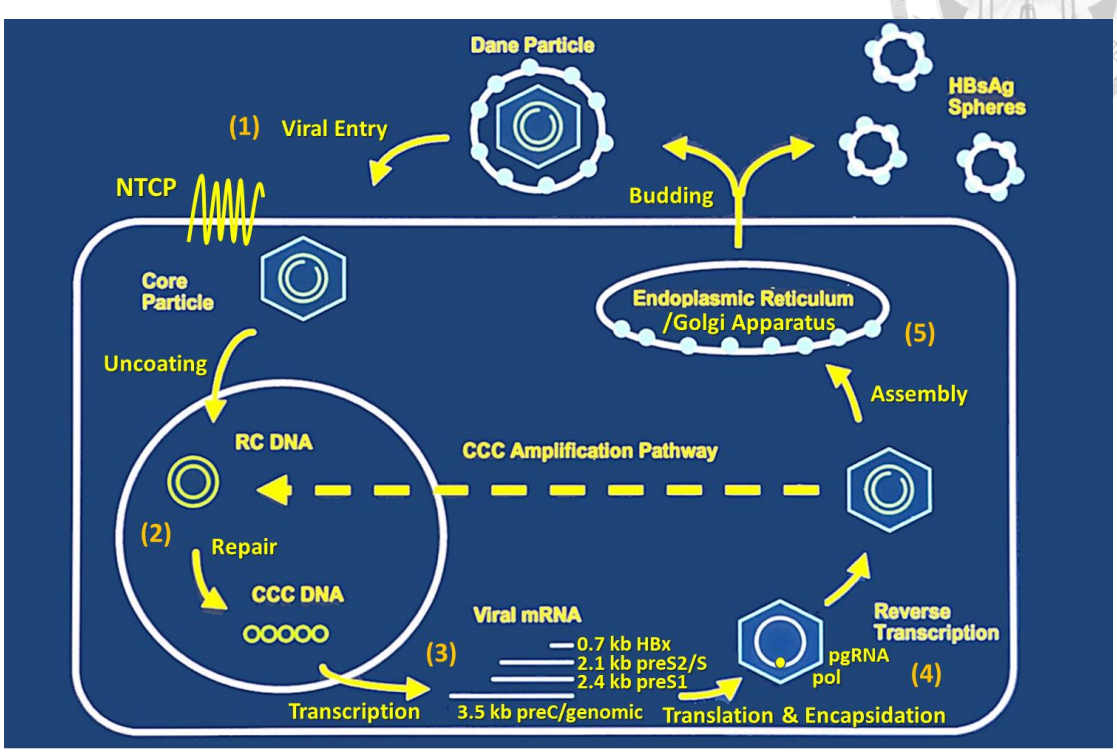

Fig. 1 Hepatitis B virus life cycle.

(1) HBV entries into the hepatocyte through NTCP and capsid is uncoated to release viral genome (RC DNA) into the nucleus.

(2) RC DNA is repaired by cellular DNA repair machinery to form the cccDNA.

(3) cccDNA serves as a template for viral transcription to form the 3.5 kb preC/pgRNA, 2.4 kb preS1 RNA, 2.1 kb preS2/S RNA and 0.7 kb HBx RNA.

(4) Viral proteins are translated in cytoplasm. pgRNA and polymerase are encapsidated into capsid particle then form the viral genome by reverse transcription.

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(5) Virus is assembled at ER/Golgi then secrets into the extra-cellular environment or returns into nucleus to amplify the cccDNA pool.

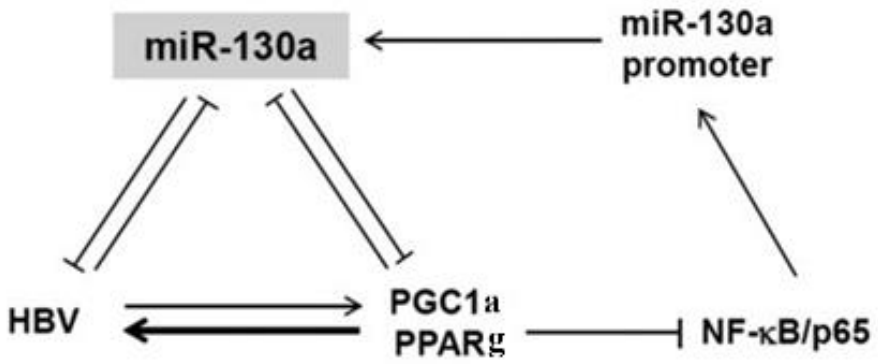

Fig. 2 Two positive-feed-forward loops between miR-130a and HBV.

miR-130a can inhibit HBV replication via targeting at two HBV transcriptional factor/coactivator PGC1a and PPARg. Reversely, HBV can downregulate the miR-130a expression by enhancing the PPARg, which makes NF-kB/p65 unable to activate the miR-130a promoter (Huang et al. 2015).

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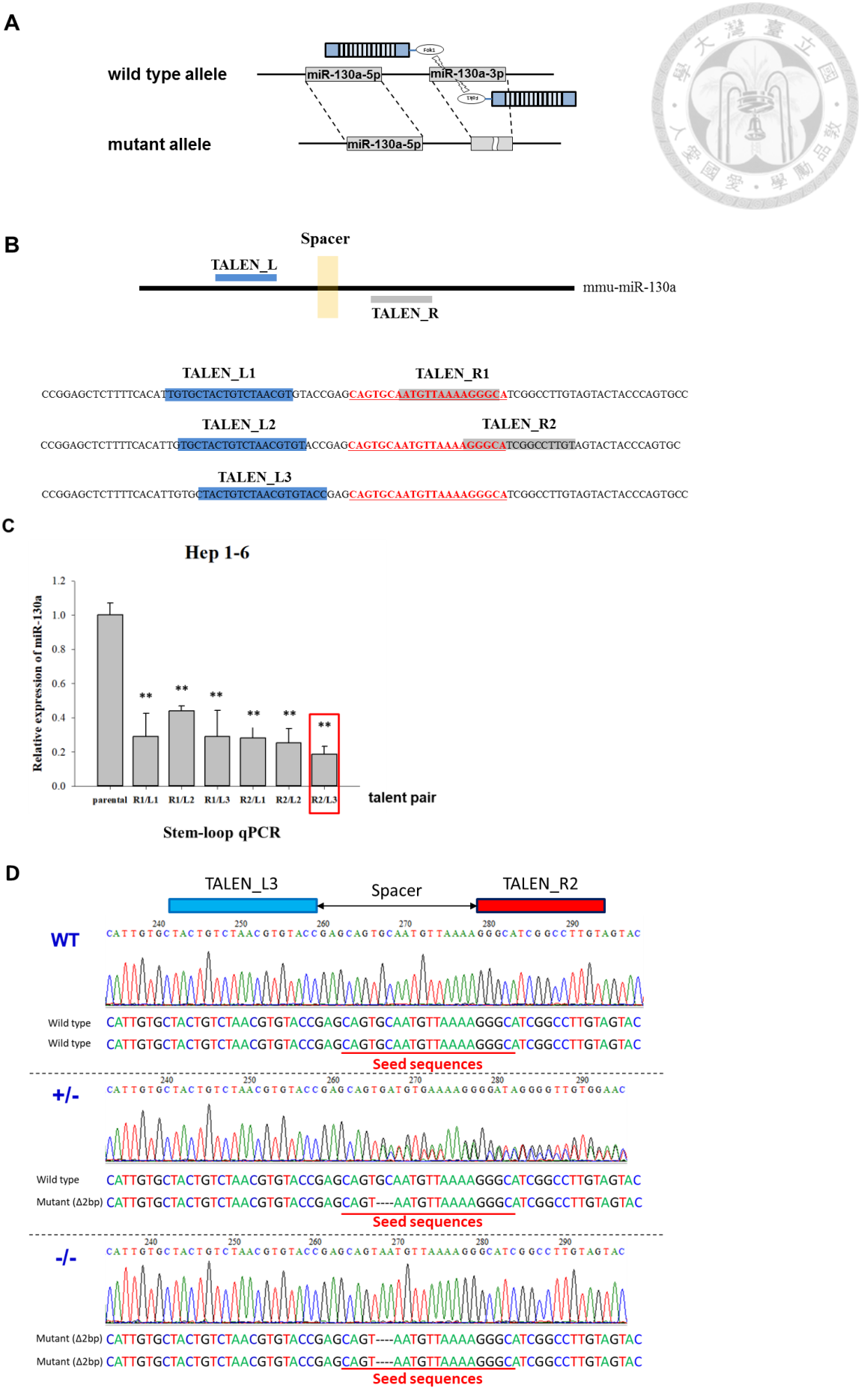

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Fig. 3 Establishment of miR-130a knockout mice by TALEN approach.

(A) The schematic view of the TALEN pair binding on miR-130a -3p site. (B) The illustration of the five different designed TALEN sequences on mouse miR-130a-3p site. (C) And test the six arrangement of TALEN pairs to select the most efficient TALEN pair to reduce the miR-130a expression in Hep 1-6 hepatoma cells. (D) PCR genotyping of wild-type (WT), heterogenous (+/-) and homogenous (-/-) miR-130a knockout mice.

(Δ2bp: two base pair deletions in mmu-miR-130a-3p seed sequences)

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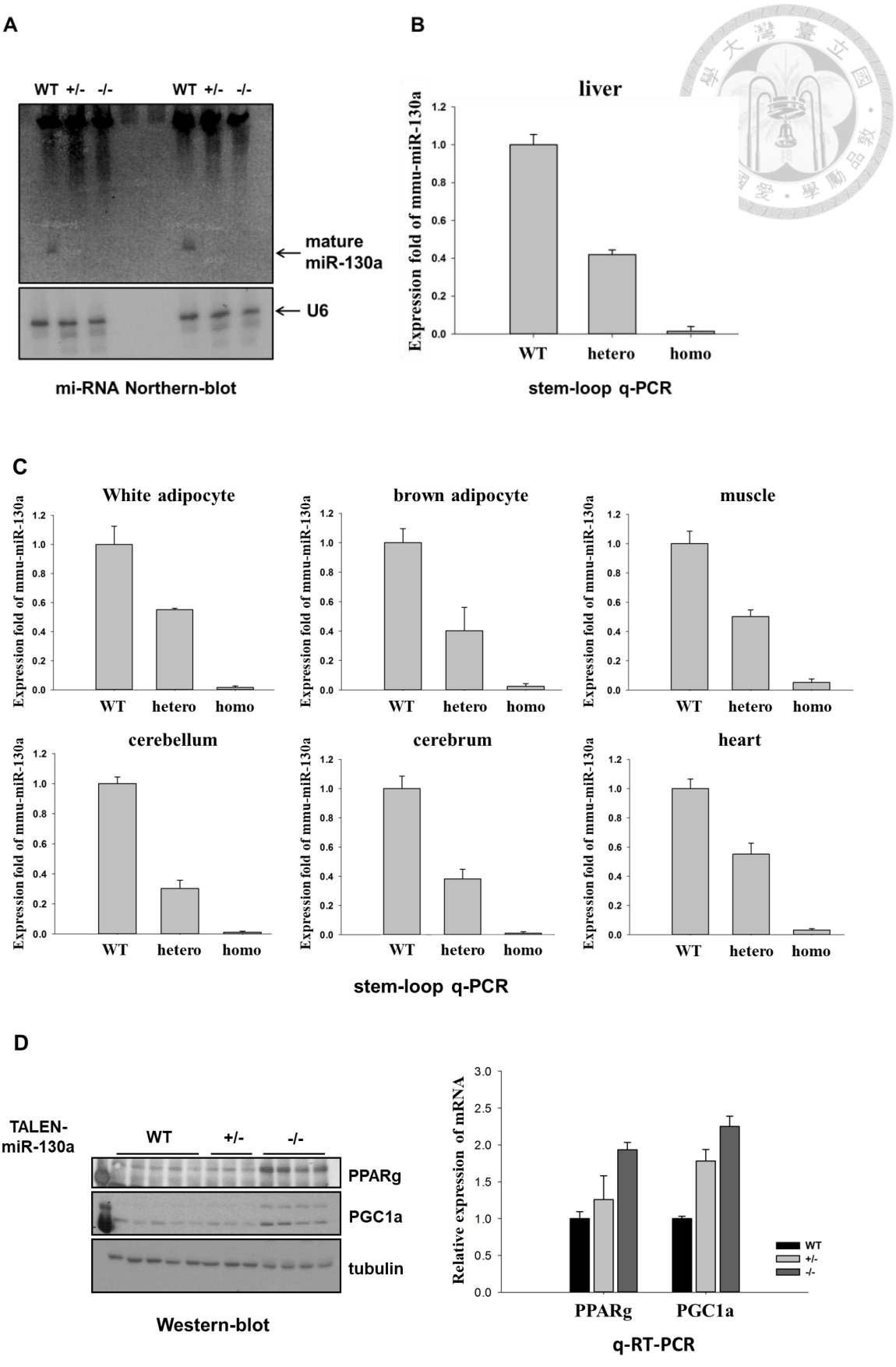

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Fig. 4 The expression of miR-130a are almost abolished in miR-130a-knockout mice.

Male wild-type mice (WT), heterogenous (+/-) and homogeneous (-/-) miR-130a knockout mice (6~8 weeks old) were sacrificed to analyze their feature as below.

The miRNA northern blot (A) and stem-loop qPCR (B) verify the deletion of miR-130a in the liver of miR-130a knockout mice. (C) Beyond the liver, the whole-body organs of in miR-130a knockout mice are almost knockdown (D) The PPARg and PGC1a protein and mRNA levels are upregulated in miR-130a knockout mice.

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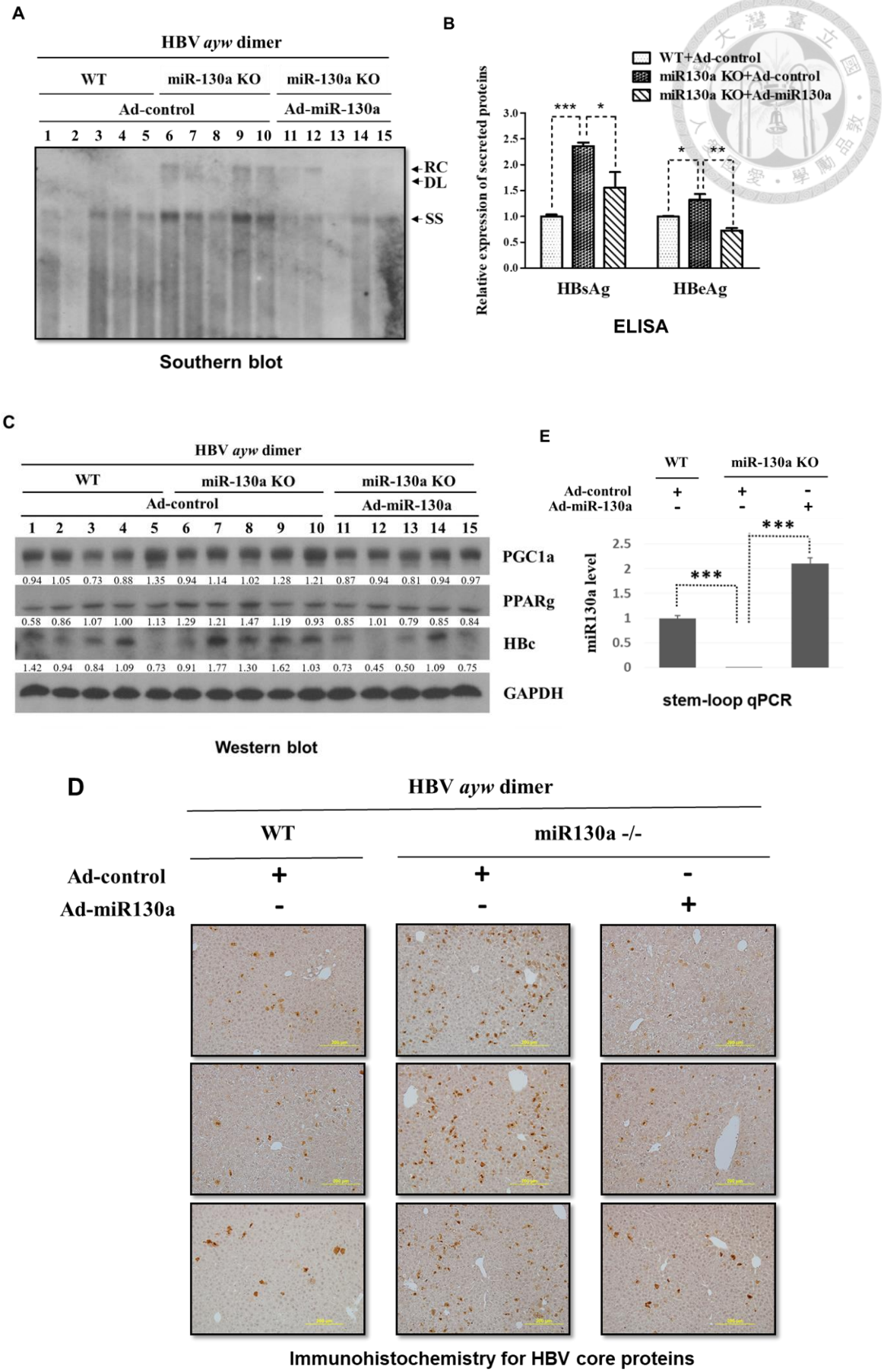

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Fig. 5 miR-130a knockout mice have higher HBV DNA replication and protein expression than wild-type mice.

Male wild-type mice (8~9 weeks old) were injected with HBV ayw dimer plasmid (30 μg/mouse) by tail-vein hydrodynamic injection. After 24 hours, these mice were injected with miR-130a-expressing adenovirus or adenovirus-control at a dose of 10^9 pfu (n=5 in each group) through the tail vein. Sacrifice and collect the liver and sera after 4 days post HBV plasmid hydrodynamic injection. [Each lane means one individual mouse in each group: wild-type mice plus Ad-control: lane 1~5 in (A) & (C) ; miR-130a knockout mice plus Ad-control: lane 6~10 in (A) & (C) ; miR-130a knockout mice plus Ad-miR-130a: (lane 11~15 in (A) & (C)].

(A) The HBV DNA replication is upregulated in miR-130a knockout mice (lane 6~10) compared to wild-type mice (lane 1~5) and supplemented miR-130a by miR-130a-expressing adenovirus (lane 11~15) decreases the HBV DNA replication in miR-130a knockout mice. (B) The HBV -E and -S antigens are increased in miR-130a knockout mice compared to wild-type mice and miR130a adenovirus I.V. injection causes the decrease in miR-130a knockout mice. (C) The protein expression of PPARg, PGC1a and HBV core proteins are increased in miR-130a knockout mice (lane 6~10) compared to wild-type mice (lane 1~5) and declined in miR-130a-expressing adenovirus supplied miR-130a knockout mice (lane 11~15). (D)

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IHC staining of HBV core proteins in livers of these three groups shows the similar core proteins expressing pattern like (C)'s WB data (wild-type mice plus Ad-control: lane 2, lane 3, lane 5 in Fig. 5C ; miR-130a knockout mice plus Ad-control: lane 2, lane 3, lane 4 in Fig. 5C ; miR-130a knockout mice plus Ad-miR-130a: lane 2, lane 3, lane 5 in Fig. 5C). (E) Use stem-loop qPCR to check miR-130a expression in wild-type, miR-130a knockout mice and miR-130a knockout mice replenished by miR-130a-expressing adenovirus I.V. injection.

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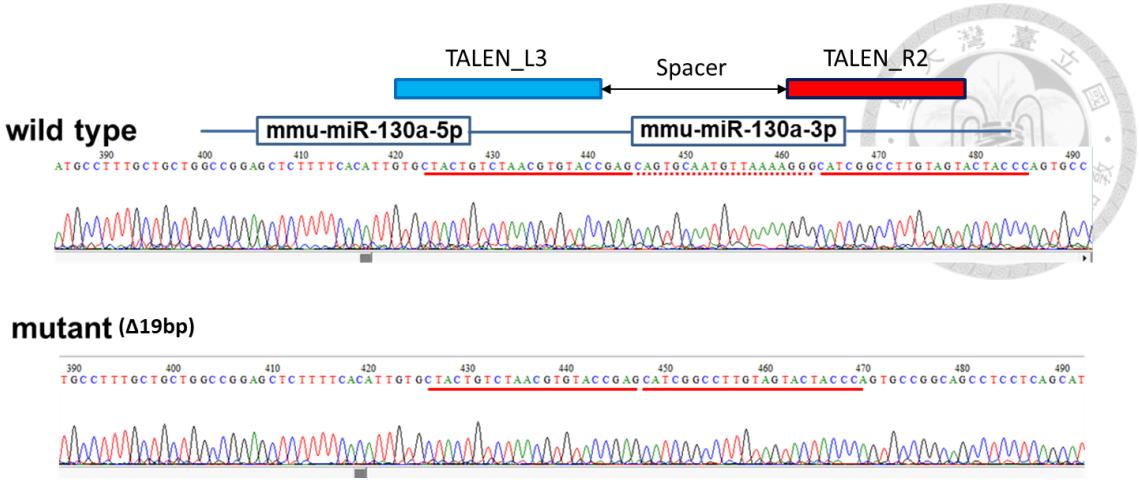

Fig. 6 miR130a-3p seed sequence is deleted in miR-130a defective mutant cell line.

The TALEN pair R2/L3 was transfected into Hep 1-6 cells for 24 hours then 4 days of puromycin selection. The puromycin-resistant clones were expanded for further DNA sequencing. (Δ19bp: nineteen base pair deletions in mmu-miR-130a-3p seed sequences)

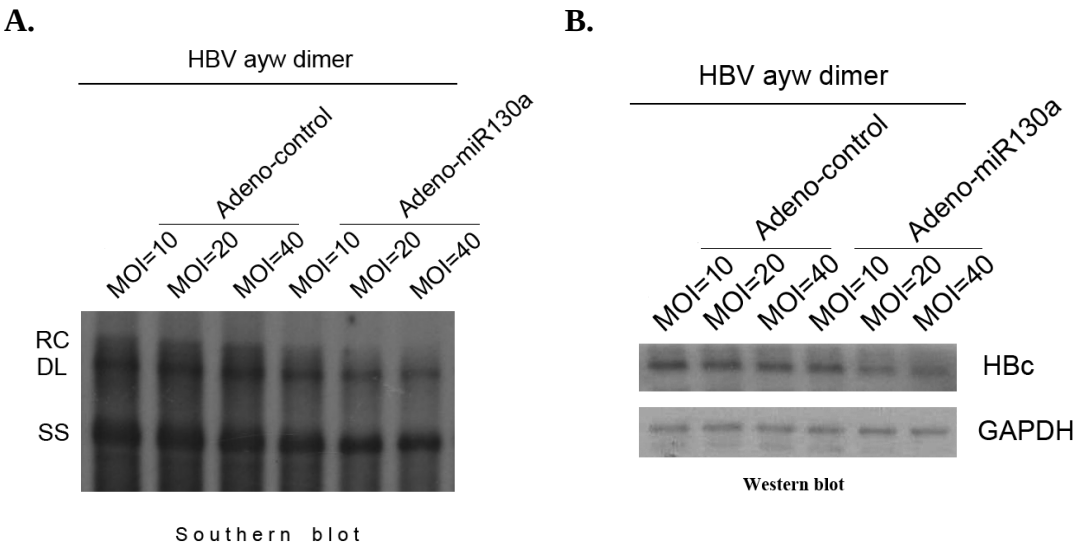

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Fig. 7 miR-130a-expressing adenovirus inhibited the HBV DNA replication and core protein expression in Huh-7 cells.

Huh-7 cells were transfected with HBV ayw dimer then infected with miR-130a expressing adenovirus or adenovirus control at a MOI of 10, 20, 40 after 24 hours post transfection. Collect cells after 5 days post transfection and extract the HBV core-associated DNA and total proteins to analyze with the southern blot (A) and the western blot (B).

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