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Effector pathway of the antiviral effect of interferons in Hepatitis B Virus infection

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List of abbreviations

2`-5`-OAS	2`-5`-oligoadenylate synthetase
3D-PCR	differential DNA denaturation PCR
A	Adenine
aa	Amino acid
ADV	Adefovir
AID	Activation-induced deaminase
ALT	Alanine aminotransferase
Ab	Antibody
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
APS	Ammonium persulphate
B2M	Beta-2 microglobulin
bp	Base pair
C	Cytosine
cccDNA	Covalently closed circular DNA form of HBV
CCL17	Chemokine (C-C motif) ligand 17
CCL20	Chemokine (C-C motif) ligand 20
cDNA	CopyDNA, to mRNA complementary DNA
CHB	Chronic hepatitis B
CPE	Cytopathic effect
C-terminal	Carboxy-terminal
Ctrl	Control

CXCL10	Chemokine (C-X-C motif) ligand 10
CXCL13	Chemokine (C-X-C motif) ligand 13
d	Day
ddH2O	Double distilled water
DMSO	Dimethylsulfoxid
DNA	Desoxyribonucleic acid
ds	Double-stranded
EDTA	Ethylenedinitrilotetraacedic acid
e.g.	exempli gratia
ELISA	Enzyme linked immunoabsorbent assay
Enh	Enhancer
EtBr	Ethidiumbromide
ETV	Entecavir
FCS	Fetal calf serum
G	Guanine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
h	Hour
HB	Hepatitis B
HBc	HBV core protein
HBeAg	HBV e-antigen
HBsAg	HBV s-antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma

HCV	Hepatitis C virus
HMBS	Hydroxymethylbilane synthase
HRP	Horse radish peroxidase
HIV	Human immunodeficiency virus
IC50	Inhibitory concentration 50
IFN	Interferon
IFNAR	Interferon α/β receptor
Ig	Immunoglobulin
IL-6	Interleucin-6
IP-10	Interferon-inducible protein 10
IRF9	Interferon regulatory factor 9
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
IU	International units
kb	Kilo base
kDa	Kilo Dalton
L	Liter
LAM	Lamivudine
L-protein	Large surface protein of HBV envelope
LPS	Lipopolysaccharide
mA	Milli-ampere
MDA-5	Melanoma differentiation associated gene-5
µg	Microgram

mg	Milligram
MHC	Major histocompatibility complex
Min	Minute
MYC	Myelocytomatosis Viral Oncogene
µl	Microliter
ml	Milliliter
µM	Micromolar
mM	Millimolar
MOI	Multiplicity of infection
M-protein	Middle surface protein of HBV envelope
mRNA	Messenger RNA
MxA	Myxoma resistance protein 1
n	Number
ng	Nanogram
NLS	Nuclear localization sequence
nM	Nanomolar
N-terminal	Amino-terminal
nt	Nucleotide
OD	Optical density
ori	Origin of replication
ORF	Open reading frame
P	P-value
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol
pgRNA	Pregenomic RNA template of HBV
PHH	Primary human hepatocytes
PKR	Protein kinase R
PML	Promyelocytic leukaemia
PolyA	Polyriboadenosine
Poly(I:C)	Polyinosinic-polycytidylic acid
preS1	C-terminal domain of HBV L-protein
preS2	C-terminal domain of HBV M-protein
p-STAT	phosphorylated Signal Transducer and Activator of Transcription
qPCR	Quantitative real-time PCR
rcDNA	Relaxed circular DNA: partial double-stranded HBV-genome
RIG-I	Retinoic acid inducible gene I
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RT-PCR	Reverse transcription PCR
SD	Standard deviation
S-protein	Small surface protein of HBV envelope
SRC	Sarcoma (Schmidt-Ruppin A-2) viral oncogene
ss	Single-stranded

STAT	Signal transducer and activator of transcription
SVP	Subviral particle
T	Thymine
TAE	Tris-acetate-EDTA buffer
TBP	TATA box binding protein
TBS	Tris buffered saline
TBV	Telbivudine
TDF	Tenofovir disoproxil fumarate
TEMED	N,N,N',N'-Tetramethylethylendiamin
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
TP	Terminal protein
U	Uracil
VP	Virus particle
VSV	Vesicular stomatitis virus
WHO	World Health Organization
WT	Wild type

Abstract

Having been used in hepatitis B therapy for more than 20 years, interferon-alpha (IFN-α) is still the only medication that may lead to virus clearance. Although the effect of IFN-α has been widely studied, its definite mode of action is still unclear.

In the first part of the thesis, I investigated antiviral effect of murine IFN-α on HBV. mIFN-α restricts HBV replication at the level of HBV-RNA transcription *in vitro* and *in vivo*. Different mIFN-α subtypes varied largely in their antiviral efficiency with mIFN-α4 and 11 showing the strongest anti-HBV effect. Analysis of protein alignment revealed several potential important residues in IFN-α receptor binding region.

The second part characterizes the IFN-α signaling pathway. Different HBV replicating hepatoma cell lines were used to examine anti-HBV activity of IFN-α and compared to PHH. IFN-α only elicited antiviral activity in HBV-infected HepaRG cells, but not in HBV-replicating Huh-7 or HepG2 cells. Detailed analysis of the IFN-α signaling pathway revealed that neither Huh-7 nor HepG2 cells responded properly to IFN-α, while HepaRG showed similar induction of IFN-stimulated genes as PHH did. Although the amount of IFN-α/β receptors and phosphorylated STAT (p-STAT) proteins were comparable in all cell lines tested, nuclear translocation of p-STATs was far more efficient in HepaRG cells. We therefore selected HepaRG cells as well as PHH to study IFN-α effects on HBV.

The third part describes a novel antiviral mechanism of IFN-α, which affects HBV binding to its host cell. In differentiated HepaRG cells, the supernatant of IFN-α treated cells limited HBV infection. The IFN-α induced factor(s) bound to heparin columns indicating that they interact with heparan sulfate to compete with HBV. Mass spectrometry analysis suggested that complement factor H or fibronectin 1 may be the HBV binding inhibitor.

The fourth part focused on the mechanism of noncytopathic viral clearance by IFN-α. In addition to its transcriptional and posttranscriptional effects, IFN-α significantly reduced HBV cccDNA, the template of HBV transcription, in HBV infected PHH and HepaRG cells. Specific 3D-PCR indicated HBV cccDNA sequence alterations. Sequence analysis showed C to U transition of the HBV cccDNA after IFN-α treatment. APOBEC3 family cytidine deaminases A3A and A3G were upregulated upon IFN-α treatment in a time and dose dependent manner. JAK-STAT signaling blockade or HIV-Vif expression proved that IFN-α induced cccDNA deamination by APOBEC3 leads to cccDNA degradation. Subcellular localization analysis and overexpression experiments demonstrated that A3A was the active effector. Treatment with a DNA repair enzyme cocktail corrected all mutations of cccDNA. AP endonuclease

incubation reduced cccDNA showing that the base excision pathway was involved. Lacking host genomic DNA deamination upon IFN-α treatment shown suggested that A3A is directed specifically to viral DNA. Since A3A co-localized with HBV core protein in confocal microscopy and interaction was confirmed by co-immunoprecipitation, we propose that A3A utilizes HBc to get access to cccDNA.

Taken together, this thesis leads to a better understanding of the antiviral function of IFN-α and opens new options for the development of novel and safe treatments to eradicate HBV and to cure chronic hepatitis B.

1. Introduction

1.1 Hepatitis B virus

1.1.1 Classification and epidemiology

The Hepatitis B virus (HBV) is a small and enveloped DNA virus belongs to the family of hepadnaviridae [1]. HBV is highly specific and replicates primarily in the hepatocyte of the liver. The replication of the DNA genome occurs via a ribonucleic acid (RNA) intermediate by reverse transcription within the viral capsid in the cytoplasm of host liver cells. Therefore the virus is classified as pararetroviruses. HBV is divided into four major serotypes (adr, adw, ayr, ayw) to distinguish between different variations in between subspecies of HBV [2], which is based on antigenic epitopes within the envelope proteins. Furthermore, HBV is divided into eight genotypes (A-H), according to diverse overall nucleotide sequence variations of the HBV genome [3]. The genotypes differ by at least 8% of their sequence, which has been associated with anthropological history and exhibit distinct geographical distributions [4]. For example, type A is prevalent in Europe, North America and Africa [5], genotype D has a worldwide distribution but predominates in Southern Europe, the Middle East, and India [6, 7]. The differences in between the genotypes A-H significantly affect the disease severity, the response to distinct treatment therapies and possible vaccination strategies against the virus.

Despite the availability of an HBV vaccine, infection rates of HBV increase constantly worldwide, especially in developing countries. Worldwide, an estimated two billion people have been infected with the hepatitis B virus and around 400 million have chronic (long-term) liver infections. About 600 000 people die every year due to the acute or chronic consequences of hepatitis B. (WHO, 2012). Infection with HBV occurs by exchange of body fluids through blood or sexual contacts with an infected person and proceeds in between adults by horizontal transmission or from an infected mother to a newborn by vertical transmission [8]. People at high risk of HBV infection therefore include parental drug users, people with multiple sex partners and infants born to HBV-infected mothers. After an acute phase of infection with HBV, some patients develop a chronic state with a lifelong infection.The two primary adverse outcomes of chronic HBV infection are liver cirrhosis and primary hepatocellular carcinoma (HCC), either of both can lead to a liver related death [9].

HBV infection, acute or chronic, holds variable manifestations. During the acute state, HBV infection can manifest as a subclinical or icteric hepatitis and rarely in 0.1% to 0.5% of patients, as acute fulminant hepatitis with acute liver failure.

Chronic HBV infection can be asymptomatic or can be manifested by symptoms and signs of cirrhosis or hepatocellular carcinoma or both. Subsequent course of disease after primary HBV infection is depends on age and general immune status of the patient. After primary infection with HBV the acute phase of infection starts with an incubation period without any symptoms (asymptotic) that ranges from four to six weeks. During the acute phase of infection approximately one third of all patients proceed asymptotic and develop neutralizing immunity. Thus, the majority of patients show after an asymptomatic incubation time symptoms like fatigue, malaise, anorexia or flu-like symptoms until jaundice may become apparent. Afterwards, the patients develop an acute liver inflammation (hepatitis). In 95% of infected adults the virus is cleared after an acute infection, only 5% of infected adults, but 20-50% of infants in early childhood (one to five years) and even 90% of infected perinatal develop a chronic state of infection [10]. Dependent on different phases of chronic infection, the annual rate of progression to cirrhosis in untreated patients has been estimated to be 2 – 9 % [11].

HBV is a non-cytopathic virus, but strongly induce liver cancer. Severe liver damage is due to a strong inflammatory response against the virus [12, 13]. The risk of hepatocellular carcinoma is 100 times higher in patients with HBV infection than in uninfected ones [14].

About half of the world's population has had contact with HBV, but HBV prevalence varies drastically worldwide (**Fig 1.1**). Hepatitis B is endemic in China and other parts of Asia. Most people in this region become infected with the hepatitis B virus during childhood and 8–10% of the adult population is chronically infected. Liver cancer caused by hepatitis B is among the first three causes of death from cancer in men, and a major cause of cancer in women in this region. High rates of chronic infections are also found in the Amazon and the southern parts of eastern and central Europe. In the Middle East and Indian subcontinent, an estimated 2–5% of the general population is chronically infected. Less than 1% of the population in Western Europe and North America are chronically infected (WHO, 2012).

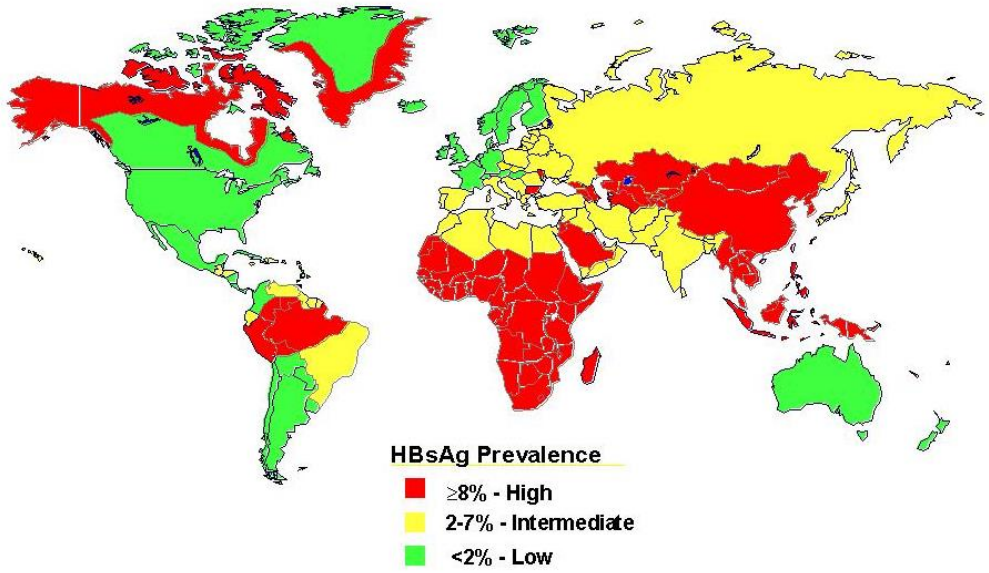


Fig. 1.1 Hepatitis B prevalence. For Multiple countries, estimates of prevalence of hepatitis B surface Ag (HBsAg), a marker of chronic infection are based on limited data and might not reflect current prevalence in countries that have implemented childhood hepatitis B vaccination. In addition, HBsAg prevalence might vary by subpopulation and locality. Picture from Worldwide distribution of chronic HBsAg carriers (WHO 2007).

1.1.2 HBV structure and proteins

Hepatitis Bviral particles, commonly termed Dane particles, are spherical lipid-containing structures with a diameter of approximately 42 nm **(Fig 1.2)**. The inner shell of the virus consists of an icosahedral nucleocapsid, which is assembled from 120 dimers of the core protein. The nucleocapsid is covered with a membrane made up of three forms of the viral envelope protein: large (L), middle (M) and small (S), which are acquired together with the host's lipids during budding into the endoplasmic reticulum (ER). The three surface proteins are commonly defined as hepatitis B surface antigens. They are translated from their own start codons but share the same C-terminal amino acids, called the S domain. As a consequence, the M protein contains an extra domain, terned the preS2 domain, compared with the S protein, and the L protein contains two extra domains compared with the S protein: the preS2 and preS1 domains. Nucleocapsids contain a single copy of the HBV genome consisting of a 3.2 kb partially double-stranded relaxed circular (rc) DNA molecule. This rcDNA is covalently linked to the VP at the 5′ end of the complete strand, also called viral (-) strand DNA. Besides the Dane particles, HBV infection also leads to the secretion of subviral particles, which consist of empty viral envelopes with filamentous or spherical shapes. Subviral particles are the most abundant HBV structures released into the blood, and are

thought to facilitate virus spread and persistence in the host by adsorbing virus neutralizing antibodies.

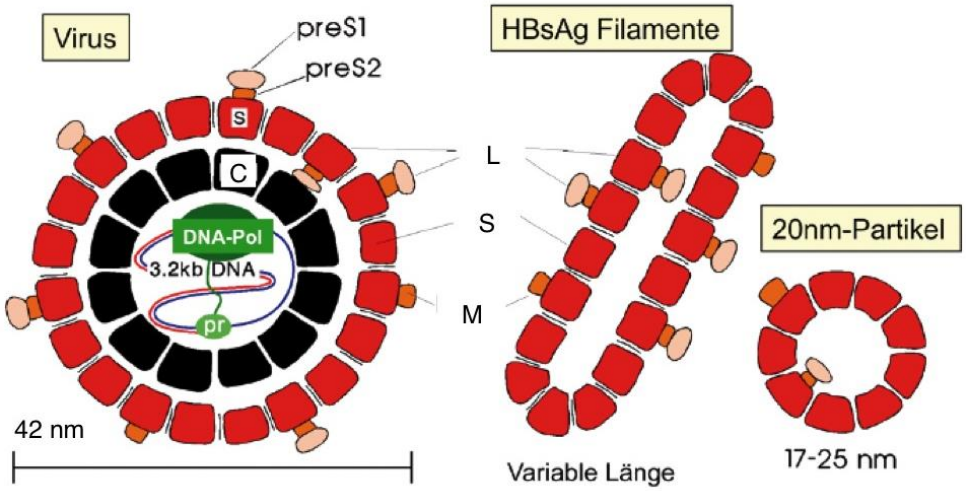


Fig. 1.2 Structures of hepatitis B virus particles. The infectious Dane particles with a diameter of ~42 nm are composed of a host derived lipid bilayer with integrated HBV surface proteins (L-, M- and S-protein). This envelope covers the nucleocapsid, composed of viral core proteins. The nucleocapsid harbors the 3.2kb HBV DNA genome, covalently linked via the terminal protein to the viral polymerase. The non-infectious subviral particles (SVP), filaments and spheres, differ in structure, size and HBV surface-protein composition. SVPs contain neither viral capsids nor viral DNA. Modified from [15].

In addition to polymerase and the structural proteins, the HBV genome also encodes two nonstructural proteins, which, currently, have less-well defined functions. Secreted HBeAg may have immunoregulatory functions [16-19], whereas the X protein (HBx) seems to have multiple functions. HBx interacts with various cellular partners and modifies several cellular processes, including transcription, cellcycle progression, cancer signaling, DNA-damage repair and apoptosis [20-26]. Using *in vitro* HBV infection models, it has been demonstrated that HBx is essential to initiating and maintaining HBV cccDNA transcription [27].

1.1.3 HBV genome

The HBV genome present in virions is a 3.2 kb partially double-stranded relaxed, circular DNA (rcDNA) molecule and the smallest known full replicative mammalian viral genome. The genome has a very complex organization with multiple overlapping open reading frames (ORFs). Every nucleotide in the HBV genome encodes for one of the HBV-proteins.

The viral polymerase is covalently attached via the terminal protein (TP) to the 5`-end of the full-length antisense minus-strand, which is complementary to the viral mRNA. The 3`-end of the sense plus-strand is of variable length, hence a part of the viral genome is single stranded (ss). After entry of the host cell the viral DNA translocates into the nucleus, where plus-strand of the rcDNA molecule is repaired, resulting in circularized cccDNA. The cccDNA serves as a template for the viral pre- (pg-) andsubgenomic (sg) messenger RNAs (mRNAs). The small and compactly organized HBV genome consists of four overlapping ORF on the (-)-antisense DNA-strand[28]. The ORF preC/Core encodes for the precore protein (HBeAg) and the core protein (C), ORF preS1/preS2/S encodes for the surface proteins (L, M and S), ORF polymerase encodes for the viral polymerase (Pol) and the ORF X encodes for the viral X protein (X). The transcription of viral mRNAs is regulated by four promotors, the preC/C-, preS1 (L)-, preS2 (S)- and the X-promotor.

Starting at these different promotor sites, transcription in all cases ends at one common polyadenylation (polyA) signal, resulting in one pregenomic and three subgenomic viral mRNAs [29]. Two internal enhancer elements (Enh1/Enh2) differently influence the promotors. Enh1 increases transcription of all four promotors, whereas the liver-specific Enh2 [30] only upregulates the transcription-rate of preS2/S.

Once formed, the cccDNA in the nucleus serves as a template for the transcription of four groups of viral RNA. First these are the 3.5 kb pre-core mRNA (pre-C) and pregenomic (pg) RNAs. PreC mRNA is translated to produce a precore protein that is further proteolytically cleaved into HBeAg.

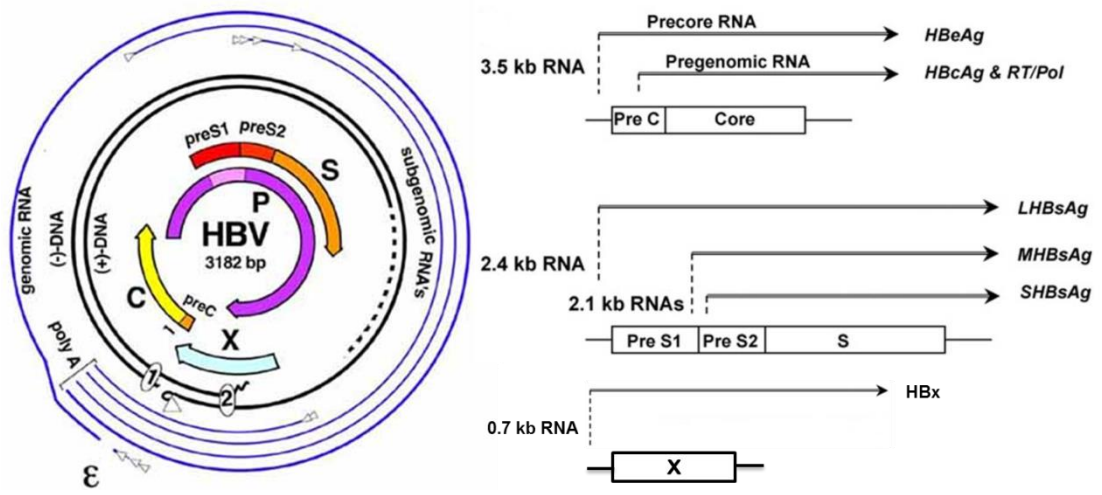


Fig. 1.3 The HBV genome. The genomic organization, RNA transcripts and gene products are shown with several key regulatory elements. The transcription start sites of various HBV transcripts and the proteins they encode (see text for details). Modified from [31].

The 3.5 kb pgRNA transcript is bi-functional. On one hand it serves as mRNA for the translation of viral capsid protein and the DNA polymerase, initiated at different start codons. On the other hand pgRNA serves as a template for reverse transcriptional synthesis of the viral genome and is afterwards packaged together with the complex of viral polymerase and terminal protein into the nucleocapsid. The core protein has the intrinsic property to self-assemble into capsid structure [32] and it contains a cluster of aminoacids at the C-terminus which bind to the pgRNA [33].

1.1.4 HBV life cycle

Hepatitis B virus is thought to be internalized into cells through receptor-mediated endocytosis [34]. Proteolytic cleavage of the surface protein occurs within the endosomal compartment, resulting in a conformational change that exposes some translocation motifs at the surface of the viral particle. The high density of translocation motifs allows endosomal escape of the nucleocapsid into the cytosol [35]. The naked nucleocapsid is then directed towards the nucleus, and the HBV genome is translocated to the nucleus [36]. At this stage, the rcDNA genome is converted into a cccDNA, the template for viral transcription. The 3.5 pgRNA serves as mRNA for the synthesis of polymerase and core proteins. Additionally, it is used as a template for reverse transcription. The 2.4/2.1 kb subgenomic RNAs encode for three viral envelope proteins. The pgRNA is reverse transcribed within the nucleocapsid in

the cytoplasm into new rcDNA. Mature nucleocapsids are then either directed to the secretory pathway for envelopment or are redirected towards the nucleus to establish a cccDNA pool. An overview of the HBV life cycle is depicted in **Fig 1.4.**

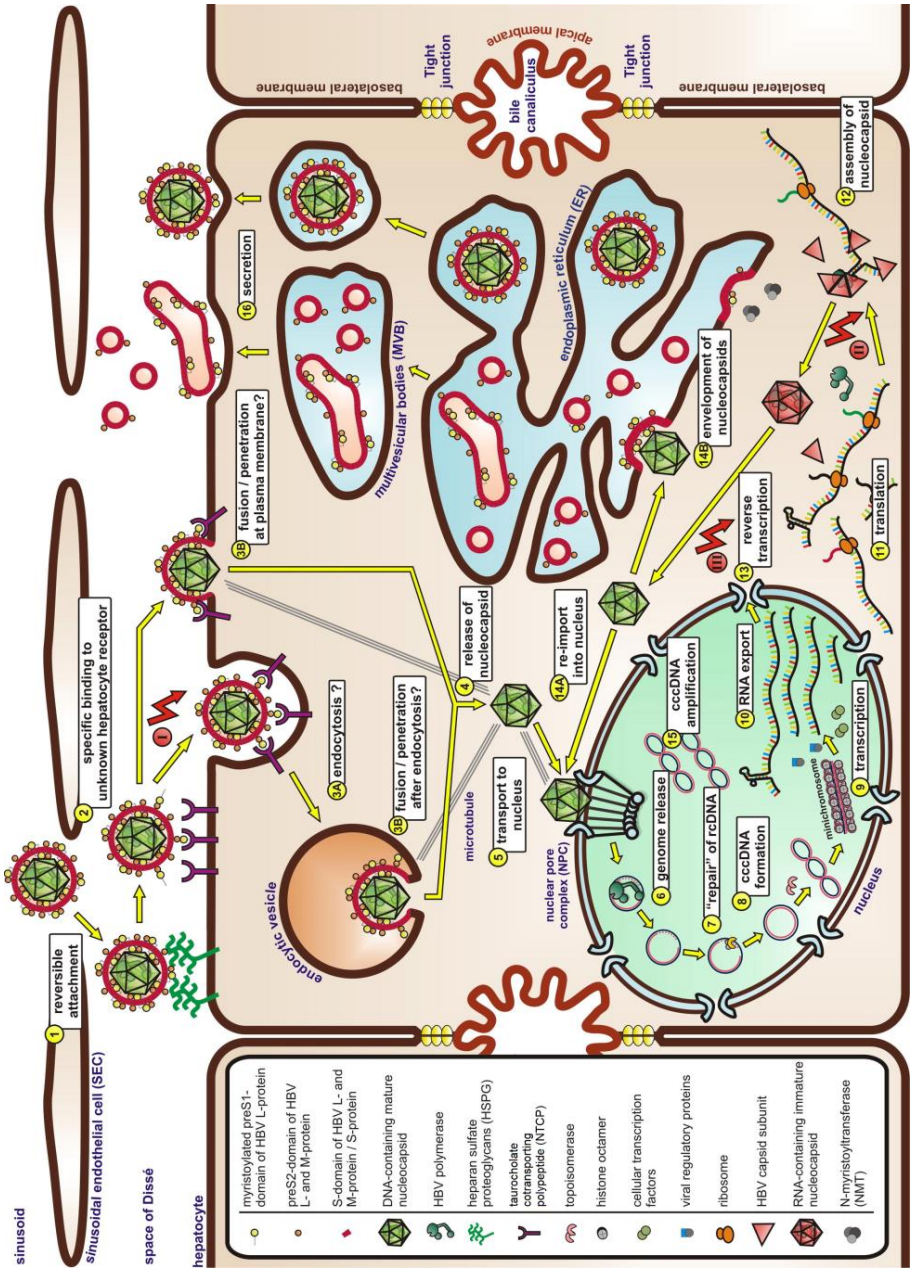


Fig. 1.4 The replication cycle of HBV. HBV virions attach to the cell surface of the hepatocyte via heparin sulfate proteoglycan (HSGP) and sodium taurocholate cotransporting polypeptide (NTCP) or other unknown receptor. During the entry step the nucleocapsid gets uncoated and is released into cytoplasm of the cell. The partially double-stranded DNA genome is imported into the nucleus, where cellular enzymes repair the plus-strand gap, leading to the formation of cccDNA. The episomal viral cccDNA genome is transcribed into viral pre- and subgenomic RNAs. After the export of the RNAs into the cytoplasm, translation into viral proteins takes place. The pregenomic RNA gets encapsidated and reverse transcribed into the HBV DNA genome. The viral genome of mature nucleocapsids is either re-imported into the nucleus or the capsid buds into the ER, where it receives its envelope containing the HBV surface proteins. Virions and subviral particles are transported through the ER/Golgi network to the cell surface and get secreted. Modified from [37].

1.1.4.1 HBV entry

Hepatitis B virus infection is restricted to hepatocytes, but the early steps of the HBV life cycle, including virus attachment to the cell surface, are poorly understood. However, recent developments from cell culture (HepaRG cells [38], primary tupaia hepatocytes [39]) as well as primary human hepatocyte and animal (severe combined immunodeficiency–uPA mice [40]) models have significantly improved our understanding of mechanisms leading to HBV entry into hepatocytes. The S domain of the surface protein crosses the lipid bilayer several times. The pre-S domain is located in the cytoplasmic side during the initial steps of assembly, and is located both outside and inside the virus after budding of the viral particle [41]. The L protein plays a major role in the HBV infection process. The first clues to its involvement came from studies that used synthetic peptides spanning the pre-S1 domain and from antibodies against this domain that strongly inhibited HBV binding to HepG2 cells [42]. Later mapping experiments showed that a domain that includes amino acids 2–48 of the L protein mediates attachment of the virus to hepatocytes, whereas the S protein seems to be involved in other steps [31].

Hepatitis B virus entry into hepatocytes is now thought to be a multistep process. Dane particles are the first to be trapped at the surface of the cell by heparan sulfate proteoglycans [43], and can then bind to a high-affinity receptor that confers uptake of the virus into the cells [44]. Until recently, sodium taurocholate cotransporting polypeptide (NTCP), a multiple transmembrane transporter predominantly expressed in the liver, was identified as a functional receptor for HBV [45, 46].

1.1.4.2 HBV capsid transport towards the nucleus and genome release

After entry, HBV uses the cellular machinery located within the nucleus for transcription of its RNA. As a consequence, the HBV genome within the capsid has to be transported across the cytoplasm to reach and enter the nucleus.

As *in vitro* infection models are rather inefficient, most likely because of a defect(s) in the entry process, lipofection assays have been performed to study the behavior of nucleocapsids after their release from the endosomal compartment into the cytoplasm. Capsid lipofection into Huh-7 cells, consisting of the replacement of the HBV envelope by a lipid shell, has led to highly productive HBV replication and has demonstrated capsid accumulation at the nuclear envelope within 15 min [36]. Further experiments using nocodazole – a depolymerizing microtubule drug – together with binding and co-immunoprecipitation assays, have revealed active microtubule-dependent capsid transfer

towards the nucleus [36]. Upon phosphorylation within the cytoplasm, capsids undergo structural changes that lead to exposure of increasing numbers of the C-terminal part of the core proteins, which contain a nuclear localization signal [47, 48]. Exposure of the nuclear localization signal increases the probability of an interaction with the importin a/b proteins and binding to the nuclear-pore complexes [47]. Using permeabilized cells, it has been further demonstrated that nucleocapsids are imported in an intact form through the nuclear-pore complexes into the nuclear basket where they interact with nucleoporin. Once inside the nuclear-pore complex, mature capsids (containing dsDNA and, thus, less stable than immature capsids containing RNA) disassemble and release HBV genomes within the karyoplasm [41, 48].

Viral capsids released from the endosomal pathway and newly synthesized progeny capsids have the same structure and, thus, can both be directed to the nucleus via the same mechanisms. It was shown that immature capsids (from newly synthesized progeny capsids) are also transported through the nuclear-pore complexes, but remain arrested within the nuclear basket and do not show any release of the immature genome [48, 49], thus preventing disruption of the viral life cycle. Immature capsids may complete genome maturation while they are arrested in the nuclear basket and then lead to HBV genome release within the karyoplasm to increase the number of cccDNA molecules.

1.1.4.3 cccDNA formation

Conversion of rcDNA into cccDNA requires several steps: removal of the VP linked to the 5′ end of the (-) strand DNA as well as one of the redundant sequences (either in the 3′ or in 5′ end); removal of the RNA primer linked to the 5′ end of the (+) strand; completion of the viral (+) strand DNA; and ligations of DNA extremities for both (+) and (-) strands.

Using transient transfection of the enveloped deficient HBV genome or stable cell lines supporting replication of the enveloped deficient HBV genome in an inducible manner, it was demonstrated that surface proteins negatively regulate HBV cccDNA formation before the removal of the VP linked to the 5′ end of the rcDNA (-) strand [50, 51]. Furthermore, this rcDNA deproteinization appears to occur within the capsid before translocation to the nucleus, by cleaving the phosphodiester bound between the tyrosine of the polymerase and the 5′ phosphoryl group of the (-) strand DNA [51]. It was also observed that deproteinized rcDNA contains only full-length (+) strand DNA, and completion of this strand has to occur earlier [50, 51]. These results and the latter finding that *in vitro* deproteinization is inhibited by viral DNA polymerase inhibitors [52] suggest that completion of (+) strand DNA and removal of polymerase linked to the (-) strand DNA are probably catalyzed by the VP itself and/or by

cellular proteins packaged into the nucleocapsid. A recent study that precisely analyzed rcDNA and cccDNA sequences showed that, once in the nucleus, the redundant sequence at the 5′ end of the (-) strand is removed and that both DNA strands then undergo DNA-repair reactions, which are probably carried out by cellular DNA-repair machinery [53]. The hypothesis that the initial cccDNA formation may at least partially depend on cellular enzymes is consistent with data showing that the initial formation of cccDNA from incoming virions cannot be prevented using potent NUCs that inhibit VP or (+) strand DNA synthesis [54-56]. However, a recent study reported that generation of cccDNA via intracellular recycling is also probably regulated in a virus-specific manner. Indeed, it was shown that, whereas HBV and duck HBV (DHBV) cccDNA formation were not cell-type dependent, DHBV cccDNA conversion was much more efficient than that of HBV [57].

1.1.4.4 HBV RNA transcription

Once formed, the cccDNA serves as a transcriptional template for viral RNA synthesis by cellular RNA polymerase II (the enzyme also responsible for cellular mRNA synthesis). Histones and nonhistone proteins are bound to cccDNA, resulting in a chromatin-like structure named viral minichromosome [58, 59]. Epigenetic modification of the cccDNA molecule, which leads to the control of HBV RNA transcription, has been recently reported. For instance, it was shown that HBV transcription is regulated by the acetylation status of the cccDNA-bound H3/H4 histones [60]. Recent studies have highlighted HBx as a key regulator of transcription from cccDNA, either by favoring histone acetylation or by preventing histone deacetylation [27, 61]. As HBx plays a central role in HBV infection, it would be a very interesting target for new therapies that prevent HBV viral replication. By contrast, methylation of the cccDNA CpG island was shown to inhibit HBV transcription *in vitro* [62], and the ratios of rcDNA to cccDNA molecules in HbeAg positive individuals have revealed that cccDNA methylation correlates with impaired virion productivity [63].

1.1.4.5 Capsid formation and maturation

Once synthesized, pgRNA is encapsidated together with VP. The HBV capsid spontaneously self-assembles from many copies of core dimers present in the cytoplasm. It has been shown that the formation of a trimeric nucleus and the subsequent elongation reactions occur by adding one dimeric subunit at a time until it is complete [64]. Three components are involved

in specific packaging of pgRNA into the capsid: VP, the nucleic acid-binding domain of the core protein and the e stem–loop in the 5′ region of pgRNA [65-67]. A recent study has

reported that core protein dimers can bind and encapsidate both pgRNA and heterologous RNA molecules with a high level of cooperation, irrespective of the phosphorylation status [68]. This strongly suggests that VP is probably the factor required for specific packaging of pgRNA.

1.1.4.6 Virus release

Hepatitis B virus budding has been shown to be strictly dependent on the L protein [69], and when the ratio between L proteins and nucleocapsids is not optimal, the latter are preferentially recycled to the nucleus to amplify the cccDNA molecule pool [70]. Moreover, the interaction of L protein with capsids is critical for HBV assembly as it can be disrupted by peptides interacting with the core [71]. Based on data obtained recently in different studies, it has been proposed that HBV virions can bud into late endosomes or multivesicular bodies, and exit the cell via the exosome pathway.

1.1.5 Experimental models for HBV

Due to the narrow host-range and the organ-specificity of HBV, experimental models are still limited. However, over the last forty years a multiplicity of models has been developed that allow the study of different steps in the HBV life cycle, or even virushost immune reactions.

1.1.5.1 Cell culture models

Different hepatoma cell lines have been established like Huh-7 or HepG2 that support the HBV life-cycle upon transfection with plasmids carrying an HBV-genome [72, 73]. In addition, stably HBV-transformed cell lines like HepG2.2.15 and HepG2-H1.3 have been generated by molecular cloning that produce high amounts of HBV particles [74, 75]. HBV-Met cells, based on immortalized, highly differentiated hepatocytes prepared from mice transgenic for both c-Met and HBV, support HBV gene expression, HBV replication, and virus production [76]. Importantly, HBV replication in this in vitro system is inhibited by the intracellular antiviral mechanism(s) induced by cytokines. Nevertheless, the cell lines are not permissive for natural infection. Only primary human hepatocytes (PHH) and surprisingly hepatocytes from the Tupaia belangeri, a squirrel-like animal, are susceptible to HBV particles [39, 77, 78]. Since 2002, a cell line (HepaRG) has been established that also becomes susceptible to HBV after differentiation under special cell culture conditions [38]. Furthermore, the availability of PHH from surgical liver resections and the isolation procedure by collagenase digestion and perfusion are extremely sumptuous [78]. This is combined with an unpredictable and permanently altered quality of cells because the donors are mostly HCC

patients. Moreover, the infection of PHH and HepRG cells is polyethyleneglycol (PEG)-dependent and the cultivation of these cells needs high concentrations of dimethylsulfoxide (DMSO).

1.1.5.2. Animal models

To analyze the early steps of HBV infection and especially virus-host interactions in combination with immune responses, an animal model is necessary.

Besides humans only chimpanzees are naturally susceptible to HBV but the access to chimpanzees is extremely limited due to ethical aspects, availability, and high costs. The woodchuck and Peking duck are alternative natural animal models that are based on HBV-related hepadnaviruses. Both models brought fundamental knowledge into the biology of hepatitis B [79, 80]. However, these animals are not convenient to keep and furthermore they lack genetic characterization.

HBV transgenic mouse lineages expressing partial or complete HBV genomes have been generated as an inbreed animal model with well-defined immune system [81, 82]. These transgenic mouse models provided important insights into viral pathobiology hepatocellular injury, especially upon lymphocyte transfer [83-87], and most importantly in control of HBV replication.

However, it is important to note that hepatocytes of mice are not permissive to HBV infection, although they support HBV-life cycle and secrete decent amounts of virions upon transduction with viral genomes [81]. The reason why viral entry is hampered remains still an unknown. Moreover, mouse hepatocytes do not establish cccDNA, the natural template for HBV transcription. Thus, viral RNA is synthesized from a linearized transgene.

In the last few years, two different mouse models of acute-self limiting HBV infection by either adenoviral HBV genome transfer or by hydrodymamic injection of plasmids carrying an HBV genome have been independently developed [88-90]. These models allow detailed studies of virus-host interactions in an immune competent environment. Thus, they are suitable to analyze the onset, the establishment and the dynamic of an acute HBV-infection in a well-defined immune system. A further advantage of the transient genome transfer is the fact that HBV genomes, used for viral transcription, stayed in an extra-chromosomal organization like cccDNA.

A chimeric mouse with humanized hepatocytes represents an additional small-animal system. The advantage of this system is that the humanized liver can be infected by HBV, cccDNA

will be formed and the virus can spread. However, this mouse model is very complex: it is based on a liver-toxic phenotype in urokinase-type plasminogen activator (uPA) immunodeficient transgenic mice. In this model, the mouse-hepatocytes expressing the uPA transgene become depleted and transferred PHH repopulate in a functional manner [40, 91]. The major disadvantage of this system is the use of immune deficient mice (RAG-I, SCID).Therefore, this model system cannot be used to address immunological questions.

1.1.6 Therapeutic approaches

1.1.6.1 Interferon-α

Standard IFN-α was the first therapy used for treatment of chronic HBV infection and has been used for over the last two decades.

The main advantage of IFN-α is the possibility of immune mediated viral clearance and the absence of viral resistance. A meta-analysis of 15 randomized controlled trials revealed that IFN-α was superior to placebo in HBeAg-positive patients [92].

The conjugation of the IFN-α molecule with polyethyleneglycol, which decreases the renal excretion of IFN-α, thus prolonging its half-life and allowing for once weekly dosing intervals has considerably improved patient compliance and have replaced thrice weekly standard IFN-α for the treatment of CHB. In HBeAg-positive patients, treatment with peg-IFN was found superior to conventional IFN-α [92, 93]. Three large randomized controlled trials have confirmed the improved efficacy of peg-IFN over lamivudine in HBeAg-positive and negative patients [93-95].

HBV genotype appears to predict response to PEG-IFN-α, with a higher HBeAg seroconversion rate in patients with genotypes A > B > C > D [92, 95, 96]. In HBeAg-positive patients with CHB, the rate of HBsAg loss after 1 year of treatment with Peg-IFN α-2b stratified by genotype is 14% for genotype A, 9% for genotype B, 3% for genotype C, and 2% for genotype D [97]. About a third of patients achieve HBeAg loss after 52 weeks of Peg-IFN α-2b therapy. Long term follow-up over a period of 3 years after stopping treatment shows that durability of HBeAg and HBsAg loss is also higher in genotype A. Sustained HBeAg and HBsAg loss in genotype A vs. non-A genotype after Peg-IFN-α-2b is 96% vs. 76% and 58% vs. 11%, respectively. The exact role of genotype before embarking therapy is currently in a state of evolution and is an area on intense research.

1.1.6.2 Nucleoside and nucleotide analogues

As described earlier, hepadnavirus polymerase plays an important role in genome replication. Similar to the HIV virus, viral reverse transcriptase (or HBV polymerase) is a good target for inhibiting viral replication. Nucleos(t)ide analogues are chemically synthesized selective competitive inhibitors of HBV polymerase. These agents are incorporated into the viral DNA strand, resulting in chain termination. Nucleos(t)ide analogues may interfere with the synthesis of the negative DNA strand by reverse transcription, synthesis of the positive DNA strand, and possibly cccDNA formation in newly infected cells [98]. These agents are orally administered and therefore, adherence to the prescribed treatment regimen is better than IFN-based therapy. However, the drawback with these agents as compared to finite therapy with IFN-based regimen is that therapy is usually required for several years. Prolonged use of these agents is challenging due to the development of drug resistance.

Lamivudine was the first oral agent approved for the management of HBV. Lamivudine (LAM) is rapidly absorbed after ingestion and it is phosphorylated in hepatocytes acquiring its antiviral properties [99]. It acts by terminating viral DNA synthesis and competitively inhibiting the viral polymerase. A randomized placebo-controlled trial involving patients who were treated with lamivudine or placebo daily for 52 weeks revealed that lamivudine led to virological, biochemical, and histological response [100]. However, longterm follow-up of patients treated for 2 to 4 years noted limitations of lamivudine monotherapy as demonstrated by the emergence of viral resistance after 8 months of therapy and eventually involving 76% of HBeAg-positive patients [101]. The development of resistance is lower for HBeAg negative disease. Lamivudine has been safely used in patients with cirrhosis and has been shown to decrease the risk of hepatic decompensation and hepatocellular carcinoma [102].Resistance develops when mutations occur at M204I/V within the YMDD motif of the HBV gene encoding for polymerase. Resistance is more likely to occur in patients with high baseline levels of HBV DNA. Clinically, the emergence of drug resistance is usually associated with rise in HBV DNA levels that is followed by a flare in serum. Therefore, it is critical that patients be followed at regular intervals despite an initial favorable response to lamivudine therapy.

Adefovir (ADV) was the second nucleos(t)ide analogue licensed for use in the United States. It is an oral adenosine nucleotide analogue that acts by selectively inhibiting HBV polymerase. Efficacy with ADV therapy at 10mg per day was first shown in HBeAg-positive patients where 48 weeks of therapy showed significant normalization of ALT, suppression of HBV DNA, and rates of HBeAg seroconversion compared to placebo [95]. The rates of

clearance of HBeAg and HBV DNA are lower with ADV compared to lamivudine, but biochemical and histological responses are similar. A study with HBeAg-negative patients treated for 144 weeks showed continued long term therapy revealed a durable response but discontinuation of treatment led to a rebound in HBV DNA levels [103]. The ADV-resistance mutations rtN236T and rtA181V were identified in 5.9% of patients after 144 weeks and 29% of patients after 5 years of therapy, although a more recent long-term follow-up study showed 5 year mutation rates of 20% in HBeAg-positive patients [97, 104, 105]. This high rate of resistance limits ADV use as stand-alone therapy in HBeAg-positive patients. However, it may be used as first-line therapy in individuals with HBeAg-negative CHB with HBV DNA levels below 10^7 copies/ml. Higher doses of ADV have greater potency, but also have higher rates of nephrotoxicity, the major limiting side effect of this therapy [106].

Entecavir (ETV) is a cyclopentyl guanosine nucleoside analogue that blocks both the priming and elongation steps of viral replication, which results in potent inhibition of HBV DNA polymerase [107]. It was approved for use in the United States in 2005. ETV (0.5 mg/day) was shown to be more potent than lamivudine (100 mg/day) in a 48-week randomized-controlled trial in 648 nuleoside-naïve patients with HBeAg-negative CHB. The rate of normalization of ALT, undetectable serum HBV DNA levels, and histologic response in ETV vs. lamivudine group in this study were 78% vs. 71%, 90% vs. 72%, and 70% vs. 61%, respectively. In HBeAg-positive patients, HBeAg seroconversion rates between lamivudine vs. ETV were not statistically significant but ETV treated patients had higher rates of normalization of serum ALT, reduction in HBV DNA levels, and histologic improvement as compared to lamivudine treated patients. In patients with lamivudine resistant mutations, 1mg daily ETV is the preferred dose as it is more potent than 0.5 mg daily ETV. The rate of development of ETV resistance after 2 years of therapy is higher in patients with lamivudine resistance vs. lamivudine naïve patients (9% vs. 0%) [108-110]. In nucleoside-naïve patients, continued treatment through 96 weeks has yet to reveal any evidence of ETV resistance and preliminary data from 5 years of treatment shows that emergence of resistance is only 1.2% [110]. In addition, ETV use in lamivudine-refractory HBeAg positive patients has shown a continued clinical benefit through 96 weeks with a safety profile comparable to lamivudine [111].

Telbivudine (TBV) is a β-L-nucleoside analogue of thymidine with specificity for hepadnaviruses. It is highly specific and selective inhibitor of both HBV first and second-strand DNA synthesis, targeting the viral DNA polymerase [112]. TBV was approved for use in the United States in 2006. A double-blind, randomized-controlled, phase III trial including CHB patients randomized to either TBV 600 mg daily or lamivudine 100 mg daily showed

that TBV was superior to lamivudine in terms of mean reduction in serum HBV DNA levels, complete virologic response, and reduced rates of HBV drug resistance [113]. TBV is a potent and safe antiviral agent for HBV but is associated with a high rate of resistance and TBV-resistant mutations are cross-resistant with lamivudine.

Tenofovir disoproxil fumarate, an acyclic nucleoside analogue, is the prodrug of tenofovir (TDF). It has been available in the United States for treatment of HIV, and later was approved for use in chronic HBV infection in 2008. Its molecular structure is closely related to ADV and it is directly incorporated into viral DNA causing inhibition of DNA polymerase. Despite its chemical similarities to ADV, it has been shown to have greater efficacy at HBV DNA suppression, histological improvement, and higher rates of HBsAg loss than ADV in HBeAg-positive patients treated for 48 weeks [113]. 72 week data showed that 89% of HBeAg-positive patients continued on TDF had HBV DNA viral suppression and 78% of patients who did not achieve complete viral suppression with ADV did so 24 weeks after switching to TDF [114]. TDF was well tolerated in all of the above studies without evidence of significant renal toxicity and no resistance to TDF has been detected to date.

1.2 Interferon

1.2.1 Interferon genes and proteins

IFNs are a multigene family of inducible cytokines [115, 116]. They possess antiviral activity [117]. Indeed, the biological activity of IFN is most commonly assayed by determining the antiviral activity in cell culture, although radioimmunoassays and enzyme-based immunoassays are also available for IFNs [117]. IFNs are commonly grouped into three types. Type I IFNs are also known as viral IFNs and include IFN-α, IFN-β, and IFN-ω. Type II IFN is also known as immune IFN (IFN-γ). Type III interferon group consists of four IFN-λ (lambda) molecules called IFN-λ1, IFN-λ2, IFN-λ3 (also called IL29, IL28A and IL28B respectively) [118] and the recently classified IFN-λ4 [119]. The viral IFNs are induced by virus infection, whereas type II IFN is induced by mitogenic or antigenic stimuli. Most types of virally infected cells are capable of synthesizing IFN-α/β in cell culture. By contrast, IFN-γ is synthesized only by certain cells of the immune system including natural killer (NK) cells, CD4 Th1 cells, and CD8 cytotoxic suppressor cells [120, 121]. The natural IFN-α-producing cells appear to be precursor dendritic cells [122, 123]. Purified CD4+CD11c− type 2 dendritic cell precursors (pDC2s) from human blood produce up to 10^3 times more IFN in cell culture than do other blood cells following microbial or viral challenge. IFN-α genes can be divided into two groups: an immediate-early response gene (IFN-α4), which is induced rapidly and

without the need for ongoing protein synthesis, and, a set of IFN-α genes, consisting of IFN-α2, IFN-α5, IFN-α6, and IFN-α8, that display delayed induction and are synthesized more slowly and require protein synthesis [124].

The large numbers of viral IFN genes in the human include 13 IFN-α genes, 1 IFN-β gene, and 1 IFN-ω gene [125]. They all lack introns and are clustered on the short arm of chromosome 9. The single IFN-γ gene possesses three introns and maps to the long arm of chromosome 12. Although some IFNs are modified posttranslationally by N- and O-glycosylation, the major human IFN-α subspecies are not glycosylated. The IFN-α gene products appear to function as monomers, whereas IFN-β and IFN-γ appear to function as homodimers [126].

A total of 14 IFN-α genes were detected in the mouse genome, in addition to three IFN-α pseudogenes [127, 128]. Mouse interferons are coded by an intronless multigene family clustered on murine chromosome 4. mIFN-α, as well as mIFN-β, mIFN-κ, mIFN-ε, and limitin, are thought to bind the same receptor [128, 129].

1.2.2 Induction of interferon

Type I IFN can be produced by nearly all cell type, including leukocytes, fibroblasts and endothelial cells. The signaling pathways that lead to the induction of type I IFN differ depending on the stimulus and the responding cell types, but they ultimately lead to the activation of some common signaling molecules, including TNF receptor-associated factor 3 (TRAF3) [130] and the transcription factors IFN regulatory factor 3 (IRF3) and IRF7. Dimerized IRF3 and IRF7 translocate to the nucleus and, concomitantly with the transcription factor nuclear factor-κB (NF-κB), bind to both the IFNA and IFNB promoters [131] to initiate the transcription of these IFN genes. Although the most important function of type I IFNs is typically considered to be in the induction of an antiviral immune response, these cytokines are also induced in response to many bacterial pathogens or their products, mainly through Toll-like receptor (TLR)-dependent pathways [132, 133].

TLRs are the key sensors of microbial invasion in mammals [134], and they activate an innate defence programme that is crucial for host survival. Each TLR senses a particular subset of microbial signature molecules. Most TLRs that recognize bacterial products are linked to the induction of type I IFNs10; these TLRs include TLR3, TLR4, TLR7 and TLR9. Signalling through TLR3 and TLR4 induces type I IFN production in a broad range of cell types in a manner dependent on TIR-domain-containing adaptor protein inducing IFNβ (TRIF). By contrast, TLR7, TLR8 and TLR9 induce type I IFN production in dendritic cells

(DCs) — mainly plasmacytoid DCs (pDCs) — via a pathway dependent on myeloid differentiation primary-response protein 88 (MYD88).

However, it became evident that TLR-deficient animals can still produce type I IFNs in response to RNA and DNA ligands [135, 136]. These TLR-independent pathways include the cytoplasmic sensors retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5). In addition, stimulator of IFN genes (STING) and DNA dependent activator of IRFs have been reported to induce type I IFNs in response to cytosolic DNA [137, 138]. STING is an endoplasmic reticulum-associated protein that has been shown to respond to DNA from various pathogens — including Listeria monocytogenes and the DNA virus herpes simplex virus 1 (HSV-1) — in macrophages,DCs and epithelial cells [137]. DAI, which was the first cytosolic DNA receptor to be described, recognizes viral, bacterial and mammalian double-stranded DNA and induces type I IFN production through the activation of TANK-binding kinase 1 (TBK1), which subsequently phosphorylates IRF3 [138]. In addition, DAI stimulates the production of pro-inflammatory cytokines — such as IL-6 and tumour necrosis factor (TNF) — through the activation of the kinase receptor-interacting protein 1 (RIP1), which leads to the phosphorylation of NF-κB inhibitor-α (IκBα) and the subsequent activation of NF-κB [139]. However, DAI-deficient cells can still induce type I IFN production in response to foreign DNA [140], suggesting the existence of additional mechanisms that contribute to type I IFN production in response to cytosolic DNA. Indeed, a recent study showed that DNA dependent RNA polymerase III can use cytosolic DNA as a template to synthesize RNA containing a 5’-triphosphate group, and that this RNA activates the RIG-I–IPS1 (IFNB promoter stimulator 1; also known as MAVS) signalling pathway[141].

1.2.3 Type I interferon signaling pathway

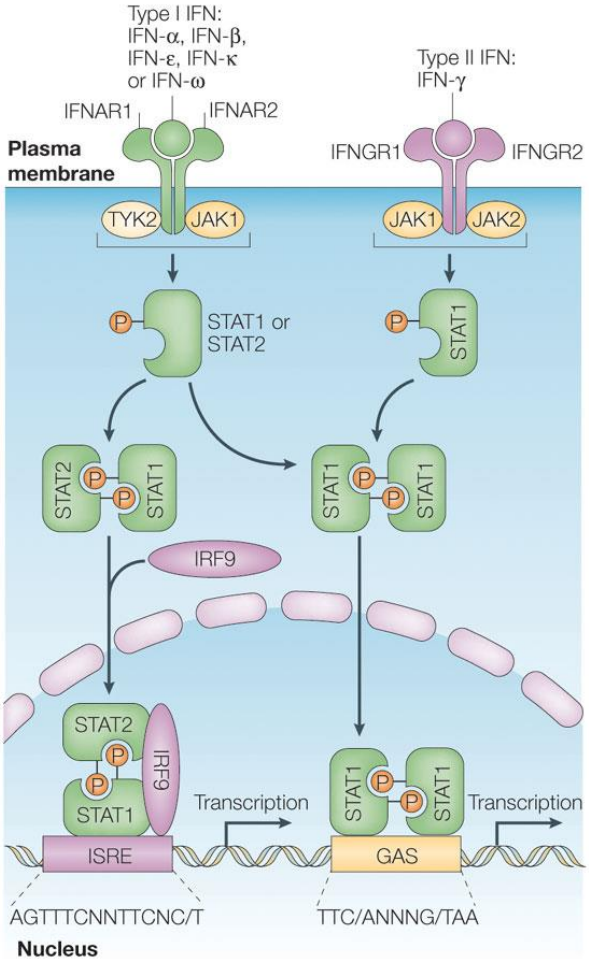


Fig. 1.5 Interferon receptors and activation of classical JAK–STAT pathways by type I and type II interferons.

All type I interferons (IFNs) bind a common receptor at the surface of human cells, which is known as the type I IFN receptor. The type I IFN receptor is composed of two subunits, IFNAR1 and IFNAR2, which are associated with the Janus activated kinases (JAKs) tyrosine kinase 2 (TYK2) and JAK1, respectively. The only type II IFN, IFN-, binds a distinct cell-surface receptor, which is known as the type II IFN receptor. This receptor is also composed of two subunits, IFNGR1 and IFNGR2, which are associated with JAK1 and JAK2, respectively. Activation of the JAKs that are associated with the type I IFN receptor results in tyrosine phosphorylation of STAT2 (signal transducer and activator of transcription 2) and STAT1; this leads to the formation of STAT1–STAT2–IRF9 (IFN-regulatory factor 9)

complexes, which are known as ISGF3 (IFN-stimulated gene (ISG) factor 3) complexes. These complexes translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA to initiate gene transcription. Both type I and type II IFNs also induce the formation of STAT1–STAT1 homodimers that translocate to the nucleus and bind GAS (IFN--activated site) elements that are present in the promoter of certain ISGs, thereby initiating the transcription of these genes. Type III IFNs signal through a receptor complex consisting of IL10R2 (also called CRF2-4) and IFNLR1 (also called CRF2-12).

1.2.4 Interferon stimulate genes and antiviral effect

1.2.4.1 Protein kinase R (PKR)

PKR is synthesized in an inactive form and, in response to the cofactor dsRNA, produced during viral replication, undergoes dimerization and activation. In addition to activation by dsRNA, PKR is activated by a stress-activated protein called protein kinase R (PKR)-activating protein (PACT) [142, 143]. The best-characterized substrate for PKR is the α subunit of the eukaryotic translational initiation factor 2 (eIF2α). Phosphorylation by PKR prevents recycling of eIF2α such that initiation is halted. Additionally, eIF2α phosphorylation can activate autophagy, by which the contents of a cell can be degraded [144] and, for HSV-1, this limits viral replication [145]. As discussed below, the involvement of PKR has been invoked in a number of other antiviral mechanisms, including the induction of apoptosis and cell-cycle arrest.

1.2.4.2 2´,5´-oligoadenylate synthetase (2´,5´ OAS)/RNaseL

One of the principal IFN antiviral pathways involves activation of the ubiquitous cellular endoribonuclease RNase L (formerly 2-5A-dependent RNase) [146]. Recently, there has been progress in understanding how RNase L affects a range of different types of viral infections and how viruses counteract RNase L. The 2´,5´-oligoadenylate synthetase (OAS)/RNase L system is an innate immunity pathway that responds to a pathogen-associated molecular pattern to induce degradation of viral and cellular RNAs and thereby block viral infections. The pathogen-associated molecular pattern is double-stranded RNA (dsRNA), a type of nonself-RNA produced during infections by both RNA and DNA viruses. Viral dsRNAs include replicative intermediates of single-stranded RNA (ssRNA) viruses, viral dsRNA genomes, annealed viral RNAs of opposite polarities, and stem structures in otherwise single-stranded viral RNAs.

1.2.4.3 Myxoma resistance protein 1 (MxA)

Mx1 gene encodes large GTPases related to dynamin MxA. The precise functions of MxA and the superfamily of GTPases are unknown, but they show antiviral activity against a wide range of RNA viruses. The MxA proteins act by recognizing nucleocapsid-like structures and restricting their localization within the cell, thereby restricting virus replication. For example, human MxA recognizes the viral nucleoprotein of Thogoto virus (THOV) and prevents transport of the incoming viral nucleocapsids into the nucleus [147, 148]. Recently, MxA has also been demonstrated to have activity against HBV, it displays antiviral activity against HBV involving a mechanism of MxA HBcAg interaction that may interfere with core particle formation. [149].

1.2.4.4 Ubiquitin-like protein ISG15 (ISG15)

ISG15 is related to ubiquitin and, like ubiquitin, it becomes joined covalently to many cellular proteins [150, 151]. Interestingly, ISG15 is also released from IFN-treated cells and acts as a cytokine. Evidence for the importance of ISG15 in the antiviral response comes from studies of knockout mice that are deficient in Ubp43 (also called Usp18), a deconjugating enzyme that removes ISG15 from substrates. These mice are hypersensitive to IFN and polyI:C [152] and resistant to LCMV and VSV [153], and MEFs derived from these mice show restricted replication of VSV and Sindbis virus (SINV) [154]. Similarly, siRNA directed against Ubp43 enhances the ability of IFN to inhibit hepatitis C virus (HCV) replication and infectious particle production [155]. Mice lacking the ISG15 gene are more susceptible to infection by influenza A and B viruses, SINV and several herpesviruses although, curiously, these mice show no alteration in sensitivity to VSV infection [156]. It seems likely that ISG15 acts to upregulate the efficiency of the IFN response globally; thus, macrophages from Ubp430/0 mice show an increase in the strength and duration of the IFN-inducible transcription response [157]. The importance of ISG15 modification is underscored by the fact that the influenza B virus non-structural protein 1 (NS1) protein interacts with ISG15 and prevents its activation and conjugation to its substrates [158].

1.2.4.5 Promyelocytic leukaemia nuclear bodies (PML)

The role of PML nuclear bodies in the antiviral response has been of considerable interest recently [159]. These bodies are heterogeneous in size and composition, and so are functionally poorly defined, but contain, amongst other proteins, the IFN-inducible protein PML and other IFN-inducible proteins, such as Sp100. They play roles in transcriptional responses to stress and may regulate chromatin structure and promoter accessibility [160].

Overexpression of certain isoforms of PML impairs the replication of both RNA and DNA viruses, and depletion of key PML nuclear-body components enhances the growth of herpesviruses, indicating that the PML nuclear bodies do indeed play a role in restricting viral replication, although the details of their involvement remain to be determined. The best evidence that these functions are antiviral is that some viruses encode factors to disrupt these bodies.

1.2.4.6 Apolipoprotein B mRNA-editing enzyme–catalytic polypeptide-like (APOBEC)

Extensive studies on cellular genes that restrict the replication of retroviruses have revealed the existence of 'restriction factors'. These factors are members of protein families that control normal cellular functions, and the antiviral family members are evolving rapidly, suggesting that they are under positive selection pressure. The best characterized of these restriction factors are the apolipoprotein B mRNA-editing enzyme–catalytic polypeptide-like (APOBEC) 3F and 3G genes (APOBEC3F and 3G). Although expressed constitutively (as are many of the 'classical' IFN-response genes, such as PKR), these proteins are upregulated strongly by IFN-α/β in certain cell types [161-165] indicative of a role in the innate immune response. APOBEC3F and 3G show antiviral activity against a range of and against hepatitis B virus (HBV) [164-169]. The mechanism of action of these enzymes involves both cytidine deamination and subsequent mutation of template, and inhibition of reverse transcription, but nothing published about their effect on cccDNA. Both APOBEC3F and 3G can be incorporated into retroviral virions; the HIV Vif protein prevents this, and also targets these two proteins for degradation by cellular proteasomes [170-175].

1.2.4.7 Viperin

Viperin is a cellular protein which could inhibit many DNA and RNA viruses such as CHIKV, HCMV, HCV, DENV, WNV, SINV, influenza, HIV LAI strain [176].Initially identified as an IFN-γ induced antiviral protein in human cytomegalovirus (HCMV) infected macrophages, viperin is reported that it could be induced by HCMV glycoprotein B in fibroblasts but inhibits HCMV viral infection and down-regulates viral structural proteins, which is essential for viral assembling and maturation. The mechanism of how the virus protein induces viperin against itself is still not clear. However, the viral induced redistribution of viperin is also found in HCMV infected cells, which may reflect the mechanism of virus evading antiviral activities of viperin [177]. Interestingly, viperin could also be induced, and then interact with HCMV viral proteins and relocate to mitochondria in HCMV viral infected cells, and finally enhance viral infectivity by the disrupted cellular metabolism [178]. In the inhibition of influenza virus budding and release, viperin could disrupt the lipid rafts on cell plasma membrane by

decreasing the enzyme activities of farnesyl diphosphate synthase (FPPS), which is an essential enzyme in isoprenoid biosynthesis pathway [179]. Besides, viperin can also inhibit the viral replication of HCV via the interaction with host protein hVAP-33 and NS5A and disruption of the formation of the replication complex [180].

1.2.4.8 IFN-inducible microRNAs (miRNAs)

Recently, it has been reported that IFN induces the expression of several cellular miRNAs and that some of these have sequence-predicted targets within HCV genomic RNA [181]. Neutralization of these miRNAs with anti-miRNAs reduced the antiviral effects of IFN-β against HCV, whilst the introduction of synthetic miRNA mimics that corresponded to these miRNAs reduced HCV replication, suggesting that IFN-inducible miRNAs may have activity against HCV, and thus potentially other viruses. However, given the high mutation rates of RNA viruses, it might be expected that viruses such HCV would rapidly evolve resistance to such miRNAs. Furthermore, as it is not clear that HCV has played a significant role in human evolution, it is debatable as to how anti-HCV IFN-inducible miRNAs might have evolved. Nevertheless, this is a potentially important area of research in which progress into the possible roles, and importance, of IFN-induced miRNAs is likely over the coming years.

1.3. Aims of the study

The aims of the study are to investigate the mechanisms involved in the antiviral effect of IFNs in HBV infection and to provide reasonable approaches to improve current treatments.

The use of IFN-α in treating chronic hepatitis B patients has generated inconsistent results, with less than 20% of the patients responding to the treatment. There are well established murine *in vitro* and *in vivo* models like HBV-Met cells and HBVtg-mice. So the first question we wanted to answer was: whether IFN-α can affect HBV replication in these experimental models?

IFN-α2 is the most common subtypes in clinic usage, but the effect is not satisfying. Whether different IFN-α subtypes affect differently in HBV replication is not clear. Recent studies have shown that the various IFN-α subtypes can interact with interferon receptor in different ways thus may elict different antiviral effect. So the question we wanted to answer is: are there any differential antiviral activities between IFN-α subtypes? Which subtype is most superior in HBV inhibition?

The precise mechanism(s) of IFN-α involved in HBV infection is still unknown, which is mainly due to the lack of suitable experimental models. Since mouse based models do not support real HBV infection (HBV entry and cccDNA formation steps are missing), and murine ISGs are different from human ISGs, it is important to find suitable human models for the IFN mechanism research.

With suitable tools in hands, the questions should be addressed are: how IFN-α inhibits HBV and what ISGs and pathways are involved in this process?

Persistence of cccDNA is a major problem of hepatitis B treatment, and cccDNA clearance is the only way to cure chronic HBV infection. It has been shown that innate immunity strongly suppresses viral replication through noncytopathic mechanisms in chimpanzees, which may also contribute to clearance viral infection as well as cccDNA. In this study, we wanted to figure out whether IFN-α can lead to cccDNA degradation, and if it´s true, what´s the mechanism involved.

2. Results

2.1 Murine IFN-α inhibits HBV replication

2.1.1 Interferon bioassay

Since we have murine cell line HBV-Met and *in vivo* model HBV transgenic mice which support HBV replication from HBV 1.3 genome integration, we first wanted to know how efficiently recombinant murine IFN-α (mIFN-α) can inhibit HBV replication in these models.

To determine the activity of mIFN-α expressed and purified by our collaborative partners, IFN bioassay was performed. It is a well-established cell/virus system which is highly sensitive for antiviral test.

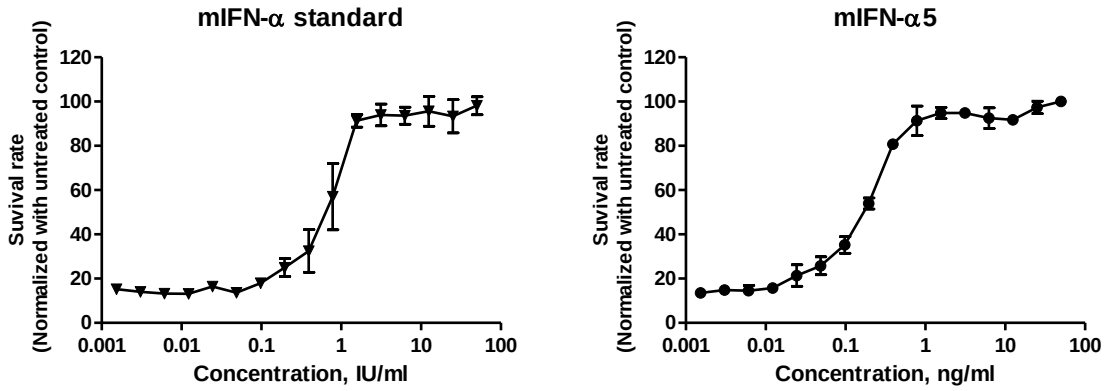


Fig.2.1.1 Interferon bioassay of mIFN-α. The biological activity of mIFN-α5 was determined in an assay in which L929 cells were treated with a dilution series of the mIFN-α5 for 24 hours, subsequently infected with VSV and finally the viability of the cells was measured using the XTT cell viability assay.

As expected, IC50 of commercial mIFN-α standard was around 1IU/ml, and for mIFN-α5 it was 0.2 ng/ml (**Fig.2.1.1**). So we calculated the activity of mIFN-α5 as 1IU=0.2ng.

The result indicated that recombinant mIFN-α5 is able to inhibit VSV propagation. Thus we performed experiments to check whether it also affects HBV.

2.1.2 mIFN-α inhibits HBV in HBV-Met cells

Type I interferon elicits its antiviral effect via interferon stimulated genes (ISG). The next question we wanted to know was whether mIFN-α can induce ISG in a HBV replication

model. HBV-Met cells are murine hepatocyte cell line which supports HBV gene expression, HBV replication, and virus production [76]. So we first differentiated the cells to get a high HBV replication level, and then treated the cells with mIFN-α. mRNA of Mx1 was evaluated by qPCR.

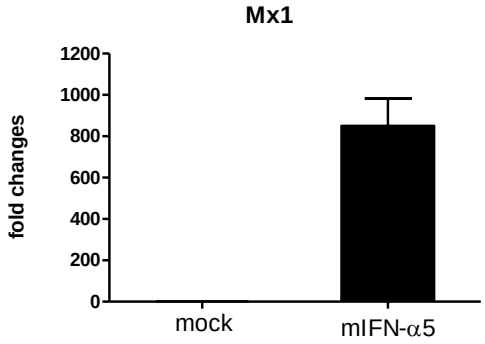


Fig.2.1.2 mIFN-α stimulates ISG in HBV-Met cells. HBV-Met cells were differentiated and treated with 1000 IU/ml of mIFN-α5 for 6 hours. All samples were followed by RNA extraction and reverse transcription. Real-time PCR was used to quantify the mRNA level of different Mx1 gene.

After 6 hours stimulation, more than 800 fold induction of Mx1 mRNA level was observed (**Fig.2.1.2**).The result suggested that ISG can be upregulated in HBV-Met cells by mIFN-α treatment.

Then, its antiviral effect on HBV was investigated. Differentiated HBV-Met cells were treated with different dose of mIFN-α5, and HBV replication markers from cell culture supernatant were measured.

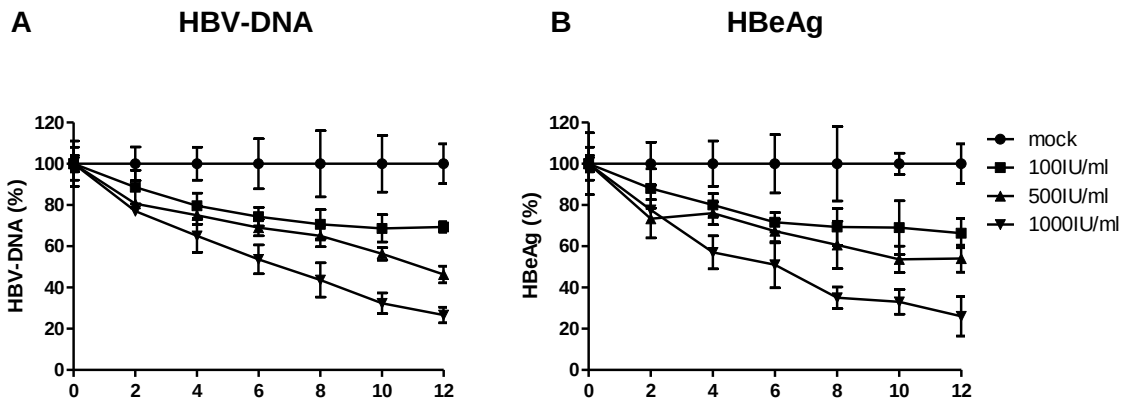


Fig.2.1.3 mIFN-α inhibits HBV replication in HBV-Met cells. HBV-Met cells were differentiated and treated with indicated dose of mIFN-α5 every two days.HBV-DNA (**A**) and HBeAg (**B**) from cell culture supernatant were evaluated by qPCR and Elisa respectively.

Both HBV-DNA and HBeAg were inhibited upon mIFN-α5 treatment in a dose dependent manner (**Fig.2.1.3**). The HBV replication markers went down two days after treatment. The higher the dose was applied, the more reduction was obtained. HBV replication derived from HBV1.3 genome integration in this model. Since both viral antigen secretion and viral production were reduced upon mIFN-α treatment, the result indicated mIFN-α affect HBV-RNA.

2.1.3 mIFN-α inhibits HBV in the HBVtg-mouse

HBV transgenic (HBVtg) mice created the opportunity to examine the influence of viral and host factors on HBV pathogenesis and replication and to assess the antiviral potential of pharmacological agents and physiological processes, including the immune response [81].

In our study, 12 female HBVtg-mice with comparable HBeAg were grouped and injected with increasing doses of mIFN-α5 as shown in **Table 2.1.1**.

Group No.	I (PBS)			II (10^4 IU/injection)			III ($3x10^4$IU/injection)			IV (10^5IU/injection)		
Mouse No.	F139	F141	F190	F157	F166	F167	F174	F180	F201	F151	F171	F172
HBeAg (S/CO)	27,02	23,36	15,33	11,96	25,67	27,03	17,27	21,17	14,13	20,98	18,41	14,76

Table.2.1.1 HBVtg-mice used in the study.

Four groups of mice got 4 injections within 8 days, and were then sacrificed. Serum ALT level and HBV replication markers as well as HBV core protein were evaluated (**Fig.2.1.4A**).

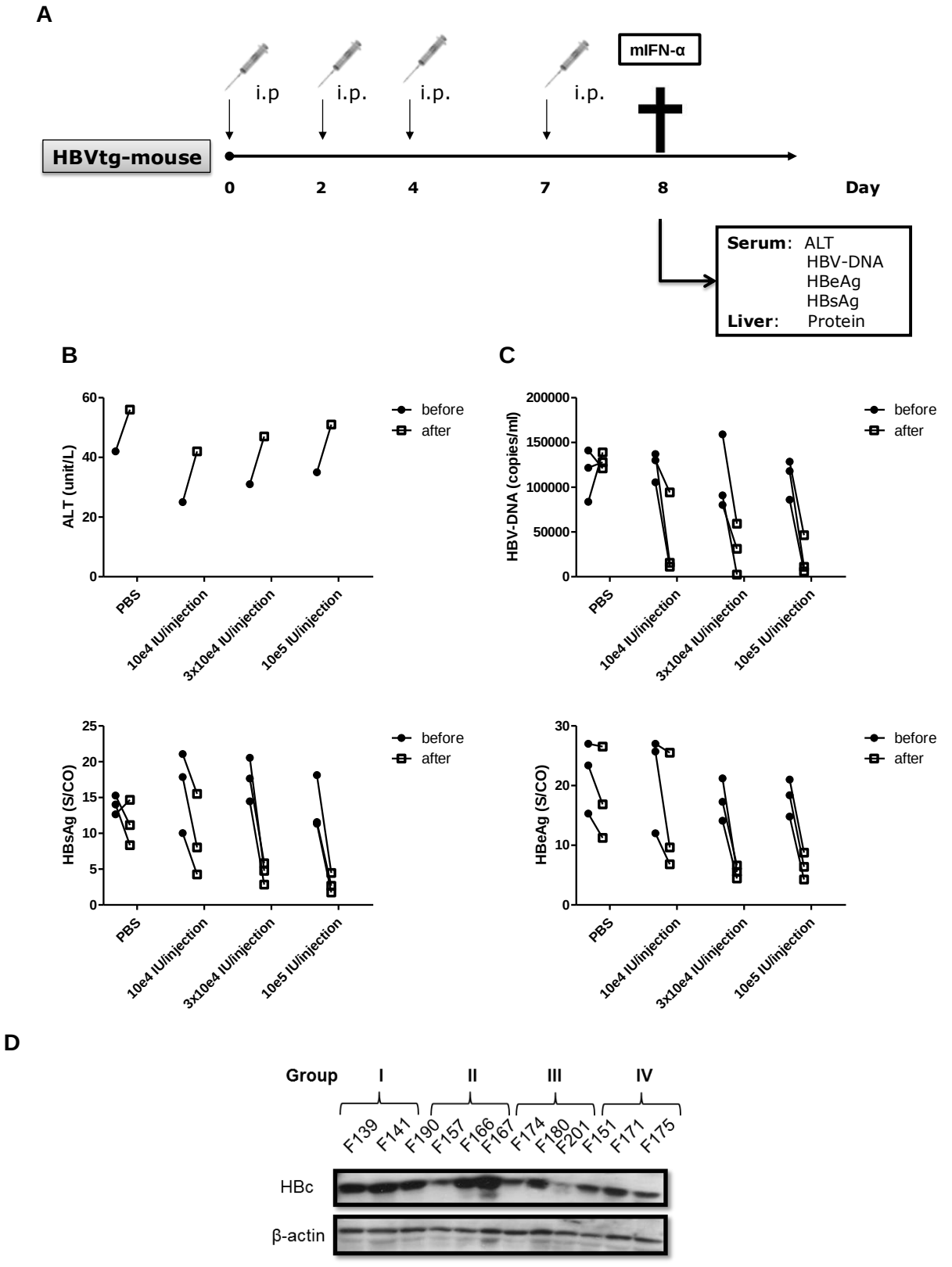


Fig.2.1.4 mIFN-α inhibits HBV replication in HBVtg-mice. HBVtg-mice were injected with different dose of mIFN-α5 as indicated (**A**). Serum ALT level (**B**) and HBV replication markers (**C**) were measured at day 8. Liver HBV core protein was determined by Western blot (**D**).

Compared to PBS, three doses of mIFN-α5 injections did not induce significant elevation of ALT level (**Fig.2.1.4B**), but strongly reduced HBV-DNA, HBsAg, HBeAg and HBc (**Fig.2.1.4C&D**). So the inhibition was not mediated by liver damage. $3x10^4$ IU/ injection showed similar antiviral effect as 10^5 IU/injection. In accordance with the results in HBV-Met cells, mIFN-α reduced HBV viral production and protein synthesise, indicated an inhibition at the level of HBV RNA transcription.

2.1.4 Anti-viral effect of different mIFN-α suptypes

Several studies compared the antiviral effects of different murine and human IFN-α subtypes in cell cultures, and they found that antiviral activities vary greatly [182-185]. But the effects of different IFN-α suptypes on HBV was still unclear. We tested mIFN-α1, mIFN-α 4, mIFN-α 9 and mIFN-α11, which were reported have better antiviral effect then the others subtypes [186].

We co-transfected four mIFN-α suptypes expression plasmids with secreted alkaline phosphatase (seap) plasmid into HBV-Met cells and checked the induction of ISG. mRNA values of ISG were normalized with secreted seap amount.

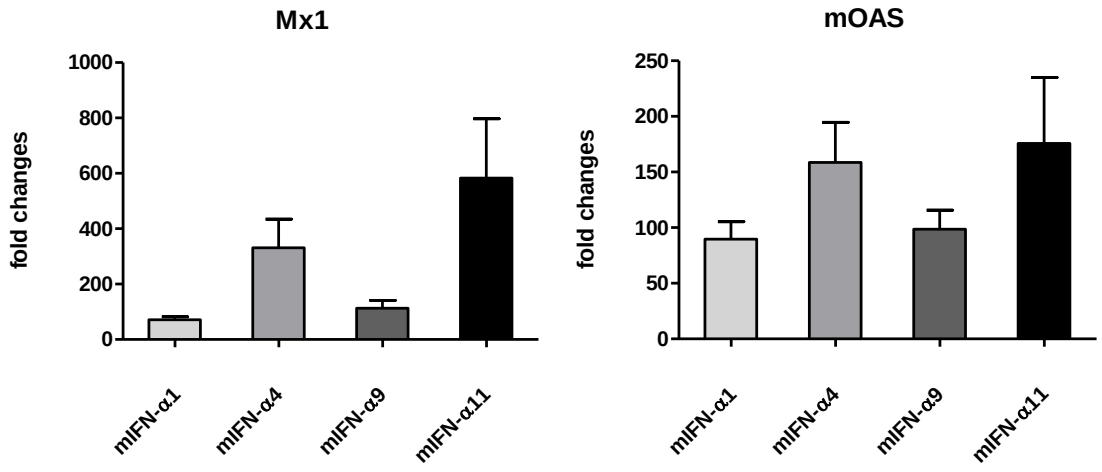
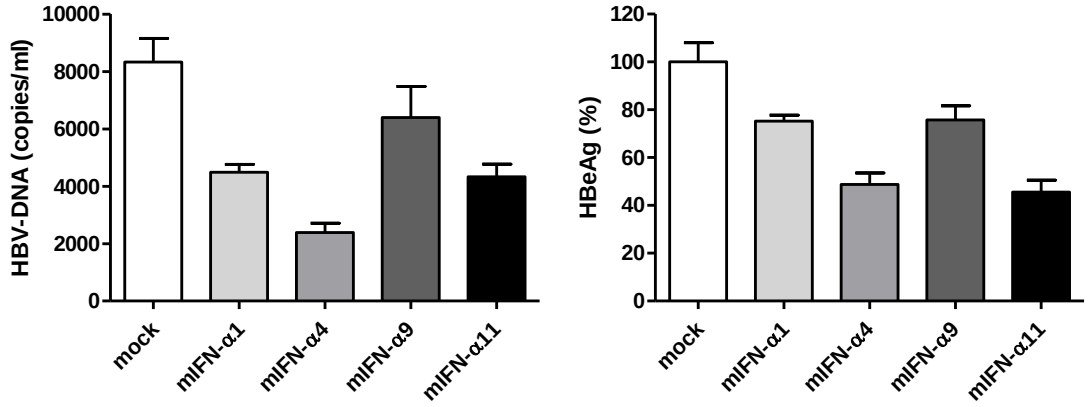


Fig.2.1.5 Induction of ISG by different mIFN-α suptypes. HBV-Met cells were co-transfeced with indicated different mIFN-α suptypes expression plasmid and seap plasmid. 48 hours after transfection, samples were followed by RNA extraction and reverse transcription. Real-time PCR was used to quantify the mRNA level of different Mx1 and mOAS genes.

We observed that after transfection, all four mIFN-α suptypes were able to induce Mx1 and mOAS (**Fig.2.1.5**). ISG mRNA levels induced by mIFN-α4 and mIFN-α11 were higher than mIFN-α1 and mIFN-α9.

The next question was whether the different ISG induction leads to a different antiviral effect on HBV. To investigate that, two kinds of treatment were used on HBV-Met cells.

A Plasmid transfection



B Recombinant mIFN-α

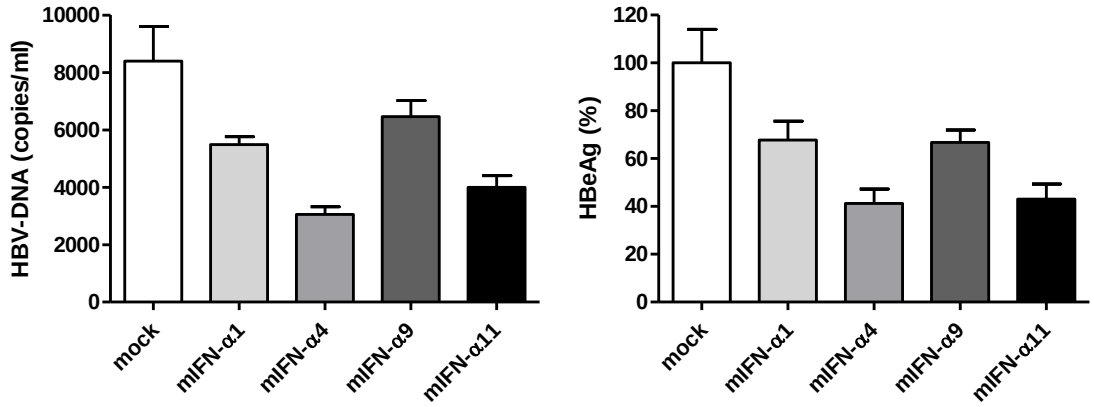


Fig.2.1.6 Anti-viral effect of different mIFN-α suptypes. (**A**) HBV-Met cells were co-transfeced with indicated different mIFN-α suptypes expression plasmid and seap plasmid. Seven days after transfection, HBV-DNA and HBeAg from cell culture supernatant were evaluated. (**B)** HBV-Met cells were differentiated and treated with 1000 IU/ml of indicated mIFN-α suptypes for 4 days. HBV-DNA and HBeAg from cell culture supernatant were evaluated.

We transfected plasmids expressing different mIFN-α suptypes into HBV-Met cells and quantified HBV-DNA and HBeAg amount in the cell culture supernatant. We observed mIFN-α4 showed highest inhibition while mIFN-α9 showed lowest (**Fig.2.1.6A**).

Then we used purified and titrated recombinant mIFN-α protein expressed in *E.coli* to treat HBV-Met cells. Same as transfection experiments, we observed mIFN-α4 and mIFN-α11 showed better reduction than mIFN-α1 and mIFN-α9 (**Fig.2.1.6B**).

To figure out the differences at molecular level, the sequences of mIFN-α suptypes 1,4,9,11 were aligned.

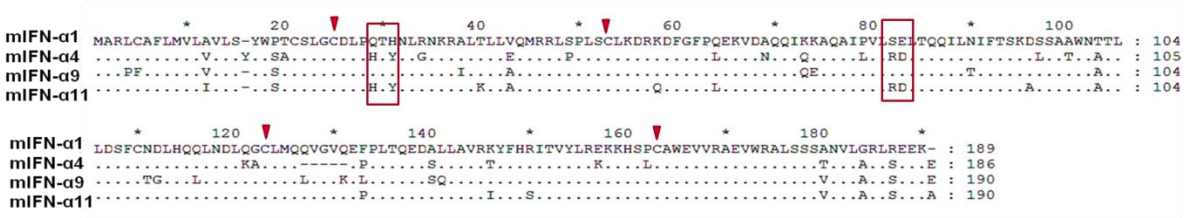


Fig.2.1.7 Multiple aligment of mIFN-α suptypes. Sequences of mIFN-α suptypes 1,4,9,11 were aligned. Four cysteines that are involved in disulfide bond formation are indicated with triangle. Unique residues of mIFN-α4 and mIFN-α11 proteins suspected to influence their activities are boxed.

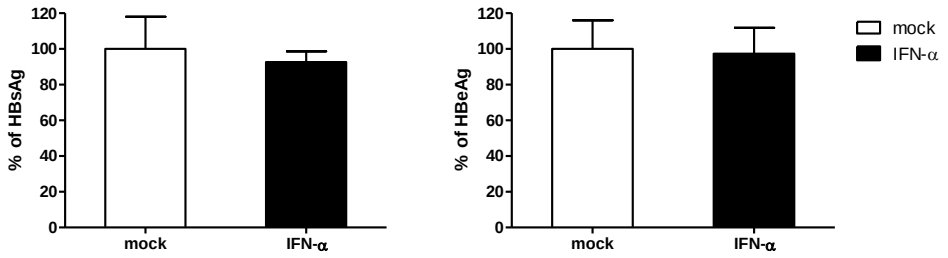
Alignment of protein sequences showed an overall conservation among various mIFN-α suptypes (**Fig.2.1.7**). In particular, the four cysteines that are involved in disulfide bond formation are conserved in all four subtypes. Unique residues within IFNAR1 binding region of mIFN-α4 and mIFN-α11 which showed higher antiviral potencies were identified.

2.2 Characterization of IFN-α signaling pathway in hepatocyte cell lines

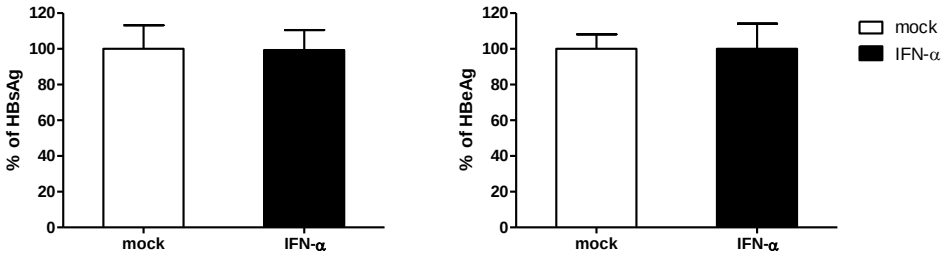
2.2.1 Anti-HBV effect of IFN-α in different cell culture models

There are differences between human and murine interferon signaling, and mouse models do not support real HBV life cycle. To find out suitable human *in vitro* models for studying the antiviral mechanism of IFN-α on HBV replication, we first compared anti-viral response of three cell culture lines bearing HBV replication upon IFN-α treatment. Huh-7 cells were transfected with pEntry-HBV1.3 plasmid, which contains HBV1.3 fold genome and express all HBV transcripts and proteins as well as viral particle after transfection. HepG2-H1.3 cells, which have HBV1.3 fold genome integration, were differentiated for 10 days to obtain high level of HBV replication. HepaRG cells were differentiated and infected with HBV (M.O.I= 200) for 10 days. All cells were then treated with two times 1000 IU/ml IFN-α for one week, and HBsAg and HBeAg were measured by ELISA.

A.Huh-7 cells transfected with HBV expressing plasmid



B. Stable HepG2-H1.3 cell line



C. HepaRG cells infected with HBV

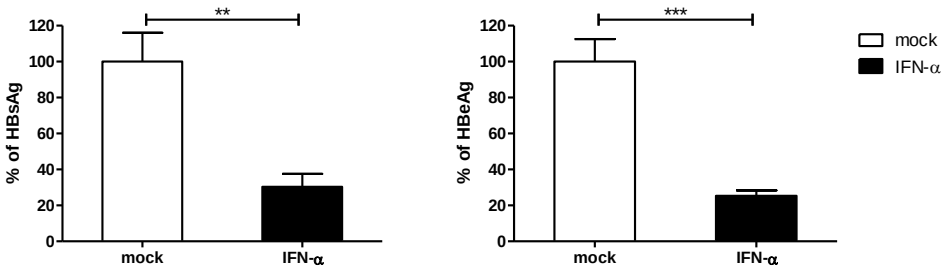


Fig.2.2.1 Inhibition of HBV replication markers by IFN-α in different hepatocytes. (**A**) Huh-7 cells were transfected with pEntry-HBV1.3 plasmid, (**B**) differentiated HepG2-H1.3, (**C**) differentiated HepaRG cells were infected with HBV, all cells were treated with two times 1000 IU/ml of IFN-α for one week, HBsAg and HBeAg were evaluated. Data are means ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student´s unpaired two-tailed *t* test.

In all three models, we could detect the expression of HBeAg and HBsAg **(Fig.2.2.1)**. IFN-α treatment significantly reduced viral antigen secretion from HBV infected HepaRG cells but had no effect on Huh-7 and HepG2-H1.3 cells.

2.2.2 Interferon response of different hepatocyte cells

To investigate whether different cells respond differently to IFN-α treatment, we performed interferon bioassay. Undifferentiated HepaRG cells and Huh-7 cells were seeded in 96-well plate, and incubated with serial diluted IFN-α. 24 hours later, cells were infected with VSV (M.O.I= 0.1). XTT test was performed to evaluate the viability of the cells two days after infection.

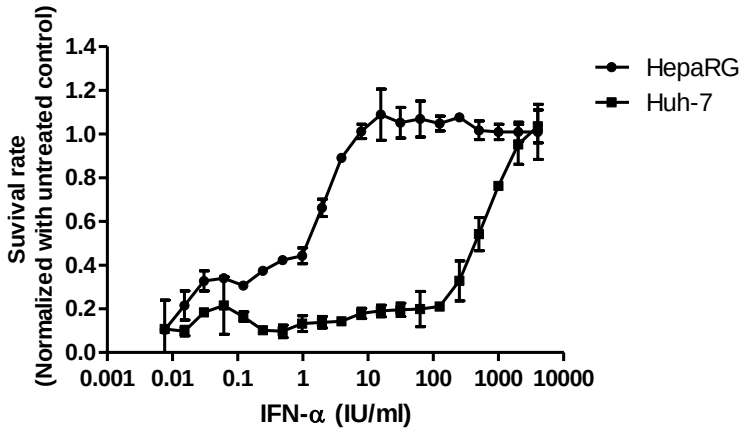


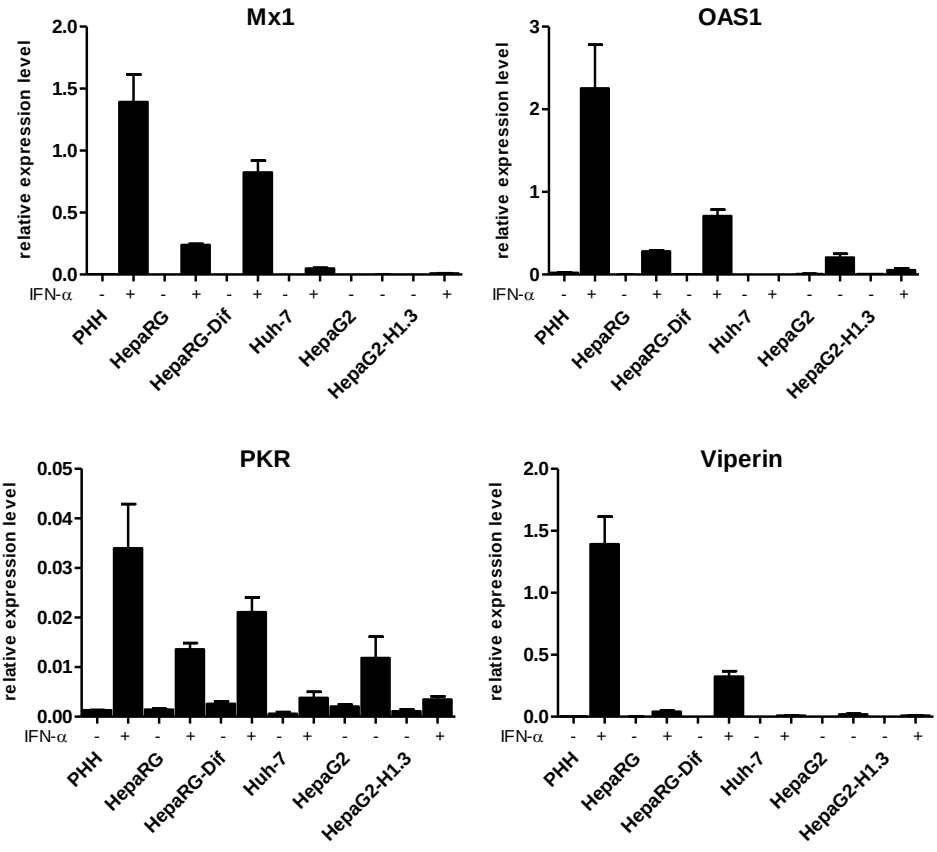
Fig.2.2.2 Interferon bioassay on HepaRG and Huh-7 cells. Differentiated HepaRG and Huh-7 cells were incubated with serial diluted IFN-α for 24 hours, and infected with VSV (M.O.I= 0.1). Two days after infection, XTT test was performed to measure the survival of the cells.

We could see that only high concentration of IFN-α (4000 IU/ml) protected Huh-7 cells from VSV challenging, where10 IU/ml were sufficient in HepaRG cells. Low concentration (0.01 IU/ml) showed nearly no protection in both cell lines (**Fig 2.2.2**). When we focus on the IC50 of IFN-α in both cell lines, we found it was 1 IU/ml in HepaRG cells and 500 IU/ml in Huh-7 cells. It means IFN-α is 500 times more efficient in HepaRG than in Huh-7 cells.These data indicated HepaRG have much higher response to IFN-α treatment than Huh-7 cells.

2.2.3 Expression of interferon induced genes in different hepatocytes

Since different ISGs have direct antiviral abilities, we hypothesized that the expressions of ISGs in HepaRG are higher. In order to ascertain that, different cells were treated with 1000IU/ml IFN-α.Total RNA were extracted 6 hours after treatment, followed by reverse transcription. Real-time PCR was used to quantify the mRNA level of Mx1, OAS1, PKR and Viperin. Two days after treatment, cells were lysed for Western blot to detect the expression of MxA protein.

A



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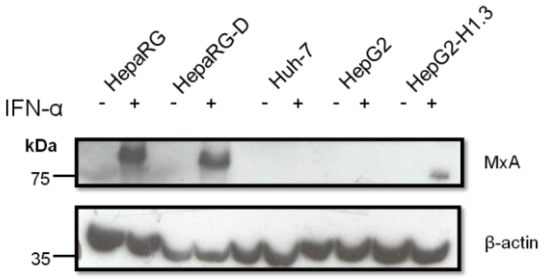


Fig.2.2.3 Expression of ISGs in different hepatocytes. (**A**) Different cells were treated with 1000 IU/ml IFN-α for 6 hours followed by RNA extraction and reverse transcription. Real-time PCR was used to quantify the mRNA level of different ISGs. (**B**) Different cells were treated with 1000 IU/ml IFN-α for 48 hours. The expression of MxA was analyzed by Western blot.

The results showed that, IFN-α stimulated these ISGs expression in all hepatocyte cell lines (**Fig 2.2.3A**). PHH expressed highest level of all four ISGs after stimulation. After differentiation, HepaRG responded better to IFN-α. The level of ISG induction in differentiated HepaRG cells was closer to PHH compared to the other cell lines. In Western blot, HepaRG and differentiated HepaRG expressed higher amounts of MxA protein than Huh-7, HepG2 and HepG2-H1.3 (**Fig 2.2.3B**).

2.2.4 Levels of IFN-α/β receptors in different hepatoma cells

The expressions of ISGs are mediated by IFN-α induced JAK-STAT signaling. IFN-α first binds to IFNAR1 and IFNAR2, and trigger the phosphorylation of STAT1 and STAT2. p-STAT1 and p-STAT2 then form dimer and translocate into nucleus together with IRF9 [187].Since different cells reacted differently to IFN-α stimulation, we wanted to know whether there are some difference among the essential steps.
We first checked IFNAR1 and IFNAR2 expression by three methods.

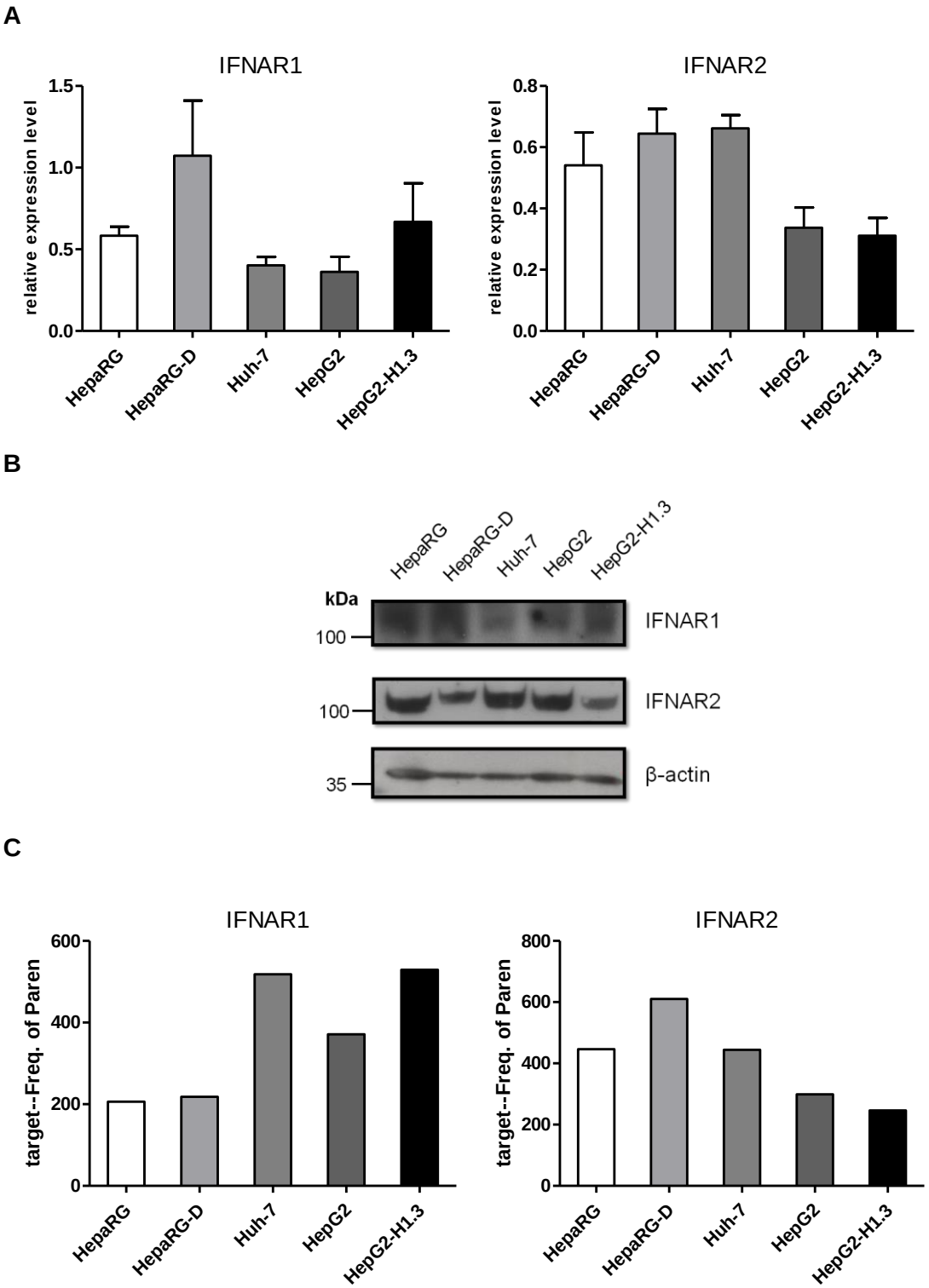


Fig.2.2.4 Inhibition of HBV replication markers by IFN-α in different hepatocytes. (**A**) mRNA level of IFNAR1 and IFNAR2 were analyzed by qPCR. (**B**) Protein level of IFNAR1 and IFNAR2 were analyzed by Western blot. (**C**) Surface protein level of of IFNAR1 and IFNAR2 were evaluated by FACS.

Total RNA from different cells were extracted followed by reverse transcription. mRNA level of IFNAR1 and IFNAR2 were evaluated by qPCR(**Fig 2.2.4A**). The whole cell lysates were

run on a SDS-PAGE gel and transferred onto PVDF membrane. IFNAR1 and IFNAR2 specific antibodies were used for protein detection (**Fig 2.2.4B**). FACS was used to determine the amount of receptors on the cell membrane (**Fig 2.2.4C**). From the above results, we could conclude that IFN-α/β receptor expression levels vary between the cell lines but that cannot explain the lack of sensitivity observed before.

2.2.5 Phosphorylated STATs in different cells

Then we asked ourselves if the differences are caused by the STAT phosphorylation. Cells were treated with 2000 IU/ml of IFN-α for 20 min. The whole cell lysates or cell nuclear extracts were run on a SDS-PAGE gel and transferred onto PVDF membrane. p-STAT1 and p-STAT2 specific antibodies were used for protein detection.

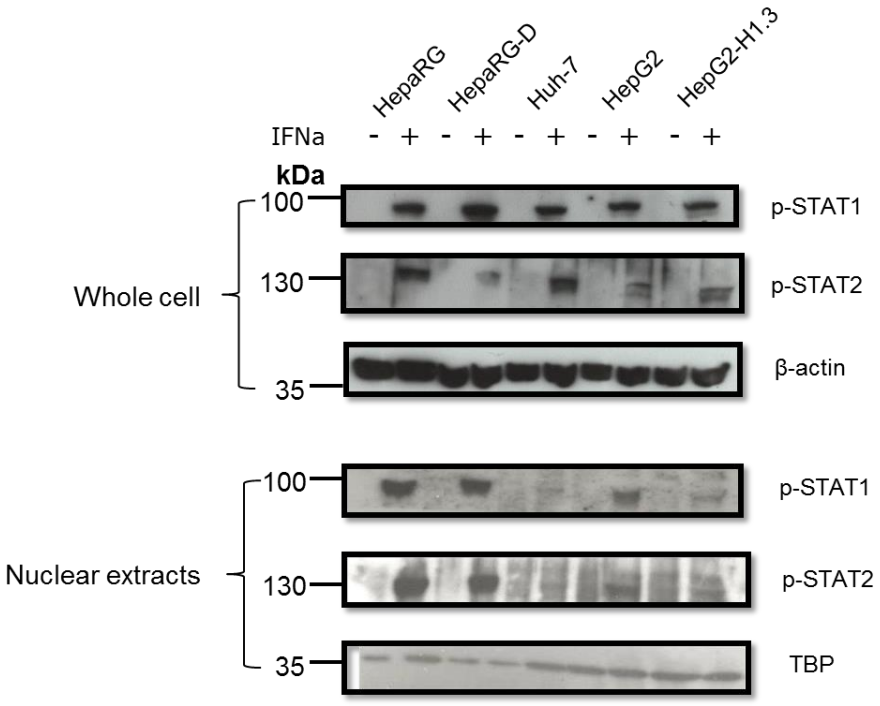


Fig.2.2.5 Activation of STAT1 and STAT2 by IFN-α in different hepatocytes. Different cells were treated with 2000 IU/ml IFN-α for 20 minutes. p-STAT1 and p-STAT2 were analyzed from whole cell lysate or nuclear extracts by Western blot.

In whole cell lysate, p-STAT1 and p-STAT2 level were comparable among the cells (**Fig 2.2.5**). When we looked into nuclear extracts, HepaRG and differentiated HepaRG showed much stronger signals than other cells. The results indicated that nuclear translocations of p-STATs were more efficient in HepaRG cells than in the other cell lines. This means the transcription and activation of ISGs in HepaRG cells was stronger than in the others.

Altogether, our results suggested PHH and differentiated HepaRG are the suitable cells to study effect of IFN-α on HBV.

2.3 Interferon inducible secreted factors restrict HBV binding

2.3.1 Pretreatment of IFN-α inhibits HBV replication

It has been generally accepted that in cells pretreated with interferon, many virus replications are blocked at the level of gene expression [188]. In addition, there are evidences that IFN may block the maturation and release of viruses like VSV and MLV [189-192]. Because several studies showed that IFN pretreatment can inhibit replication of viral genome but does not affect virus entering into host cells, for a long time it has been assumed that IFN has no effect on virus entry [193-198]. Later studies well described that pre-treatment of cells with IFN elicits a potent antiviral activity in the presence of IFN during infection [199-201].

To investigate whether IFN-α pre-treatment would result in inhibition of HBV replication, differentiated HepaRG cells were pre-treated with IFN-α for 24 hours and then infected with HBV without addition of IFN-α. HBV replication markers were measured at 7 days or 10 days post infection.

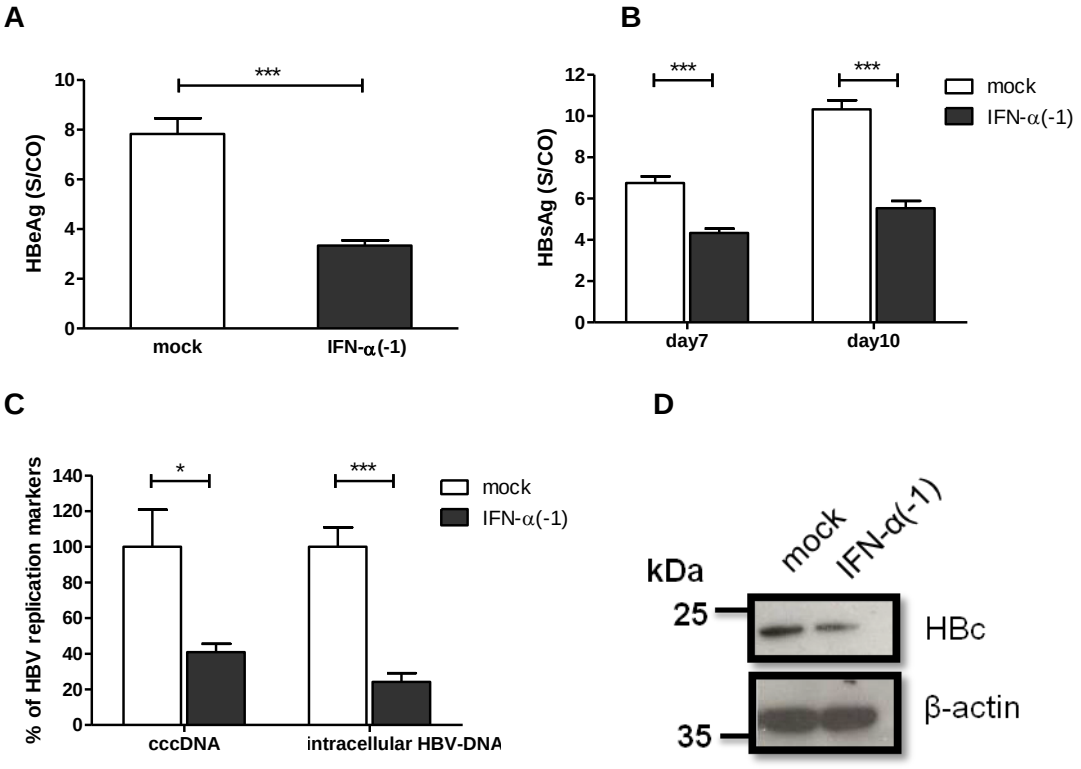


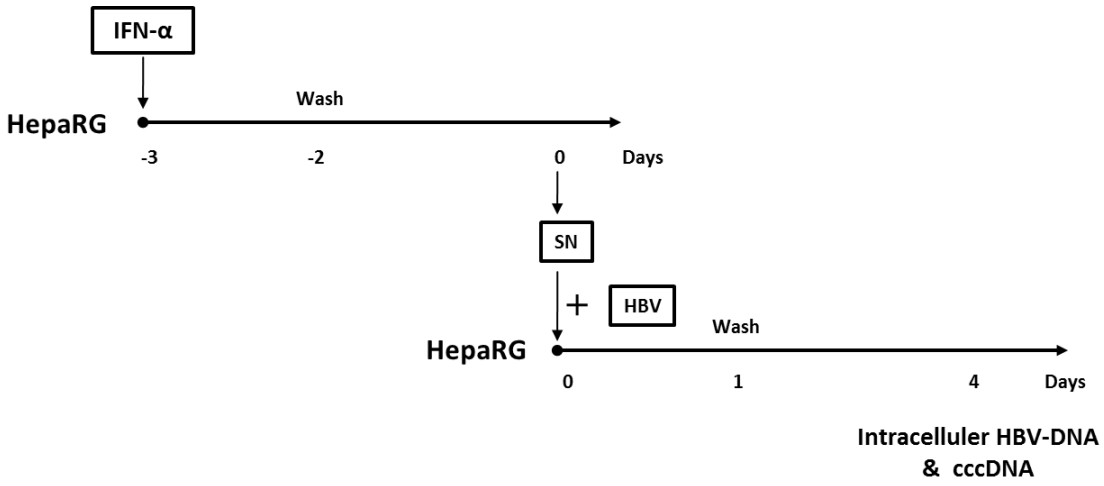
Fig.2.3.1 IFN-α pretreatment inhibits HBV infection. Differentiated HepaRG cells were treated with 1000 IU/ml of IFN-α for 1 day (IFN-a(-1)) and then infected with HBV (M.O.I= 200). HBeAg from cell culture supernatant was measured by ELISA at day 10 (**A**). HBsAg was measured at day 7 and day 10 (**B**). HBV cccDNA and intracellular DNA were evaluated by qPCR (**C**). HBV core was detected by Western blot (**D**). Data are means ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student´s unpaired two-tailed *t* test.

In HepaRG cells only pretreated with IFN-α for 24 hours there was still a significant decline of HBeAg at day 10 (**Fig.2.3.1A**). HBsAg Elisa revealed inhibition at both day 7 and day 10 (**Fig.2.3.1B**). Concomitantly, we analyzed intracellular HBV replication markers at day10. More than 50% of cccDNA and intracellular HBV-DNA were reduced upon pretreatment (**Fig.2.3.1C**). Western blot analysis revealed the decline of intracellular HBV core protein production (**Fig.2.3.1D**). These results suggested that pre-treatment of cells with IFN-α for 24 hours was sufficient to induce an antiviral effect and the IFN-induced antiviral factors sustained this activity during infection. These results also implied that IFN-α induces an antiviral strategy in HBV early infection resulting in the production of antiviral factors such as soluble secreted factors, which are known to be produced rapidly following stimulation of cells [202-205]. Therefore, we investigated whether IFN induces such soluble secreted factors to restrict HBV infection.

2.3.2 Interferon inducible secreted factors restrict HBV early infection steps

In order to assess the possibility that IFN-induced soluble, secreted antiviral factors are produced following IFN treatment, conditioned medium was prepared from IFN-α treated cell (**Fig.2.3.2A**). Differentiated HepaRG cells were treated with 1000 IU/ml IFN-α for 24 hours, followed by intensive washing to remove exogenous IFN-α. Fresh medium was added to washed cells, and after 48 hours the medium (ISG+) was collected, and inoculated to fresh differentiated HepaRG cells together with HBV.

A



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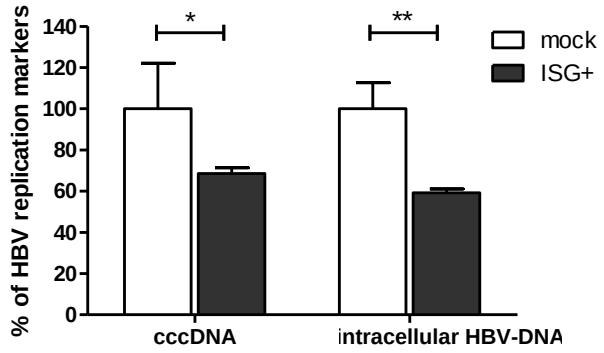


Fig.2.3.2 Secreted ISGs restrict HBV early steps. (**A**)Differentiated HepaRG cells were treated with 1000 IU/ml of IFN-α. One day later, the medium was removed. Cells were washed three times with PBS and refilled with new medium. The resulting medium containing interferon induced factors (ISG+) was collected 48 hours later, then mixed with HBV and PEG. The mixture then transferred onto fresh differentiated HepaRG cell for HBV infection. (**B**)HBV cccDNA and intracellular DNA were evaluated by qPCR 4 days after infection. Data are means ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student´s unpaired two-tailed *t* test.

Four days after infection, cells were lysed for DNA extraction. HBV cccDNA and intracellular DNA were evaluated by qPCR (**Fig.2.3.2B**). Compared with untreated cells, ISG+ medium treated cells showed decreased cccDNA and intracellular HBV-DNA which demonstrate inhibition effect of ISG+ medium.

Although ISG+ medium was prepared from IFN-α treated cells, the antiviral activity of ISG+ medium was unlikely due to residual amount of IFN-α present in the conditioned medium nor

de novo synthesized IFN-α/β from ISG+ medium treated cells. First, the remaining IFN-α, if there was any, would be fast endocytosed and rapidly degraded by ISG producing cells [206]. Second, IFN-α treatment does not induce IFN-α/β production [207].

To further confirm the antiviral action elicited by ISG+ medium is not due to leftover of IFN-α, we did following assays. Differentiated HepaRG cells were infected with HBV. Seven days later after inoculation, cells were treated with ISG+ medium with or without IFN-α specific neutralizing antibody (IFN-αAb). Neutralizing effect of IFN-αAb was controlled by cocomitently application of IFN-α + IFN-αAb (**Fig.2.3.3A**). Alternatively, cells received different treatment first and then infected with HBV (**Fig.2.3.3B**). Intracellular HBV-DNA was evaluated by qPCR.

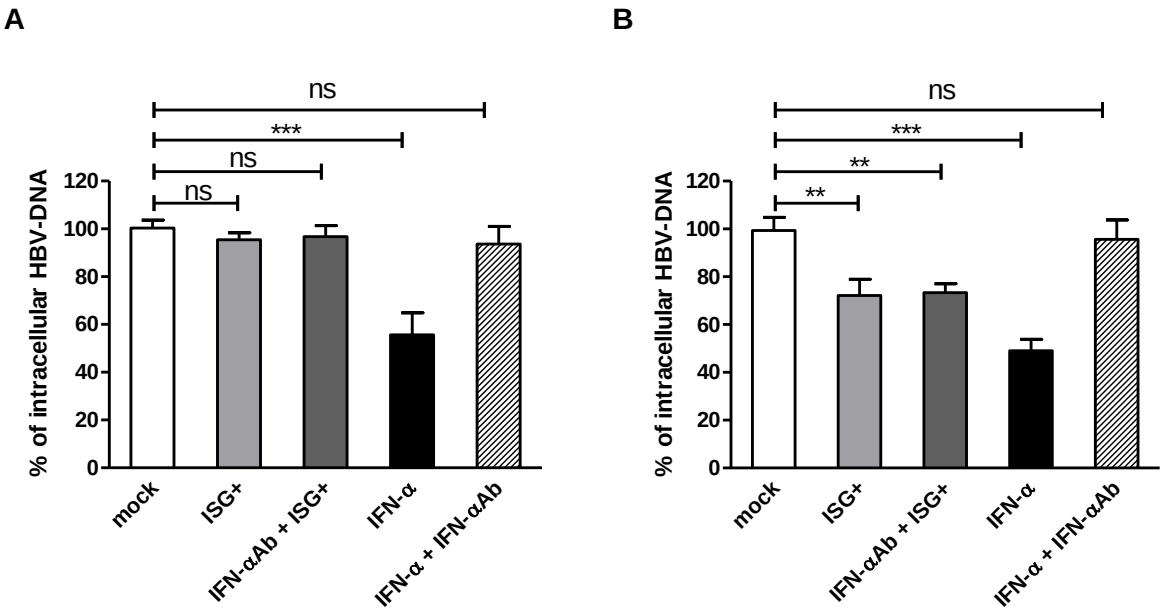


Fig.2.3.3 Antiviral effect is not mediated by remaining IFN-α. (**A**)Differentiated HepaRG cells were infected with HBV (M.O.I= 200) and 7 days later different treatments were applied as indicated. Intracellular HBV-DNA was measured 4 days after treatment. (**B**) Differentiated HepaRG cells were incubated with indicated treatment for 24 hours and then infected with HBV (M.O.I= 200). Intracellular HBV-DNA was measured 4 days after infection. Data are means ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student´s unpaired two-tailed *t* test.

As shown, if ISG+ treatment was applied after establishment of HBV infection, ISG+ medium showed no inhibition of HBV replication while IFN-α reduced HBV-DNA significantly (**Fig.2.3.3A**). When ISG+ medium was added concomitantly with HBV inoculum, HBV infection was inhibited (**Fig.2.3.3B**).

Taken together, our results clearly showed that the antiviral activity of ISG+ medium was not elicited by remained IFN-α. Not like IFN-α, ISG+ medium only decreased HBV infection in cells treated prior to HBV infection, whereas no effect was observed if cells were treated following HBV infection. IFN-α treatment could induce hepatocytes to secret soluble factors, and those soluble factors inhibit HBV infection by targeting early entry steps. We thus concluded that interferon induced soluble factors inhibiting HBV early steps and have no influence on further steps of HBV life cycle.

2.3.3 Interferon induced factors interrupt HBV binding

To further characterize the effect of Interferon induced factors, we incubated ISG+ medium, heat inactivated ISG+ medium (ISG+/-) or synthetic anti-lipopolysaccharide peptides (SALPs) Pep19-2.5 [208] with differentiated HepaRG cells for 24 hours. After that, cells were transferred to 4°C and incubated with virus particles for 4 hours. After extensive washing, HBV rcDNA qPCR was performed (**Fig.2.3.4A**). Since low temperature (4°C) prohibit HBV entry into host cell but does not affect cell binding, the amplified HBV-DNA could only come from the cell membrane bounded viron.

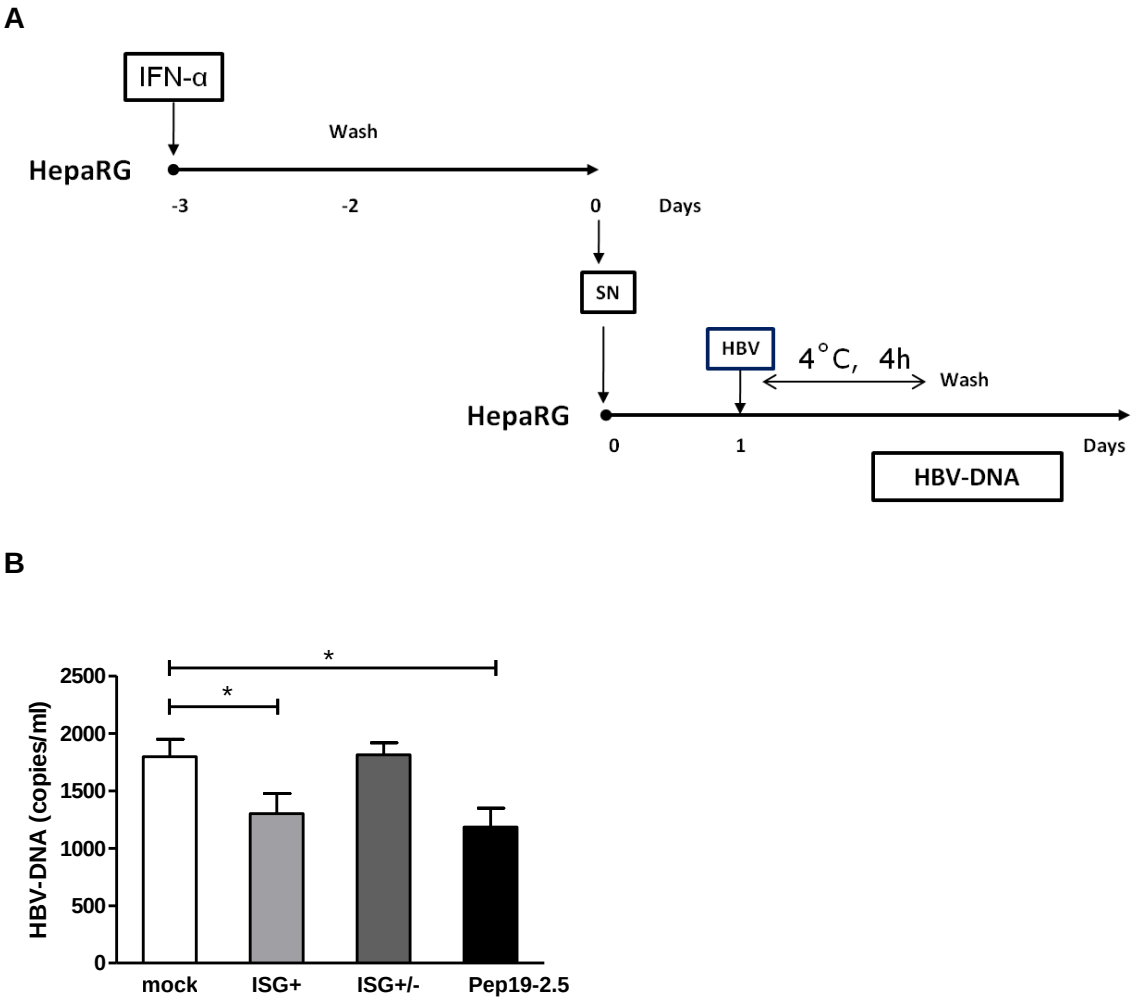


Fig.2.3.4 Secreted ISGs interrupt HBV binding. (**A**) Differentiated HepaRG cells were treated with 1000 IU/ml of IFN-α. One day later, the medium was removed. Cells were washed three times with PBS and refilled with new medium. The resulting medium containing interferon induced factors (ISG+) was collected 48 hours later. Part of the medium was cooked at 99°C for 10 minutes to inactivate proteins (ISG+/-). The medium was incubated with fresh differentiated HepaRG cells for 24 hours followed by 4 hours HBV incubation at 4°C. Peptide 19-2.5 was used as an positive control. (**B**) Cells were lysed and HBV-DNA were evaluated by qPCR. Data are means ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student´s unpaired two-tailed *t* test.

Pep19-2.5 has been showed to inhibit the binding step of many enveloped viruses including HBV [208]. Here, we observed that interferon induced soluble factors could inhibit HBV binding as efficient as Pep19-2.5, and heat inactivation led to extinguishment of the anti-viral ability (**Fig.2.3.4B**).

Together, these results demonstrated that, in differentiated HepaRG cells, interferon induced soluble factors treatment lead to interruption of HBV binding. After heat inactivation, IFN-

induced soluble secreted factors lost their anti-viral activity, which indicated the factors are heat-sensitive proteins [209]. And we speculated that the mechanism(s) involved may be induced factors associated with virus or with viral entry receptors which lead to reduced binding.

2.3.4 Interferon induced factors interrupt HBV binding to heparin sulfate

Experimental evidence has been presented that HBV initiates infection of hepatocytes by binding to heparan sulfate proteoglycans [43]. We therefore analyzed whether interferon induced factors can interact with heparan sulfate and thus block HBV binding.

It has been shown that HBV from chronically infected patients bind to heparin, the closest homologue of liver heparan sulfate, and that heparin chromatography is an efficient and gentle method to purify these viruses from human serum [210]. HiTrap Heparin HP columns which prepacked with heparin sepharose for high-resolution purification of proteins with affinity for heparin were used in our study. We applied normal differentiated HepaRG cell culture supernatant, ISG+ medium, or Pep19-2.5 on heparin columns, and followed with HBV containing PBS. After washing, bound HBV were eluted and quantified by qPCR.

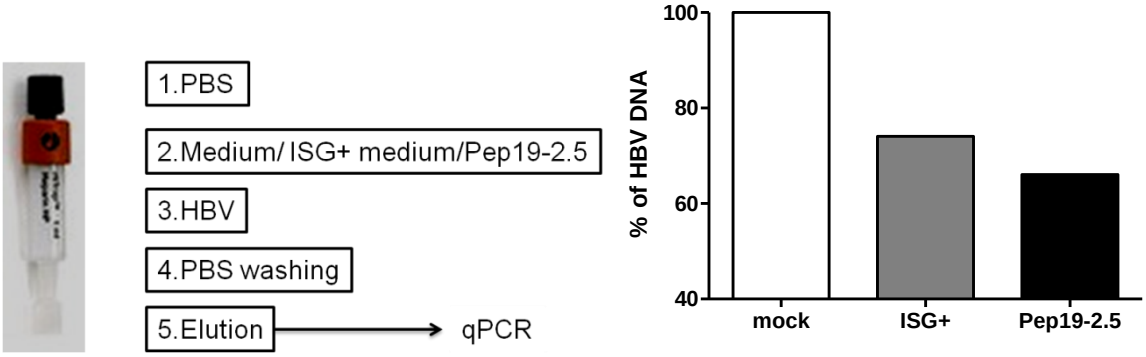
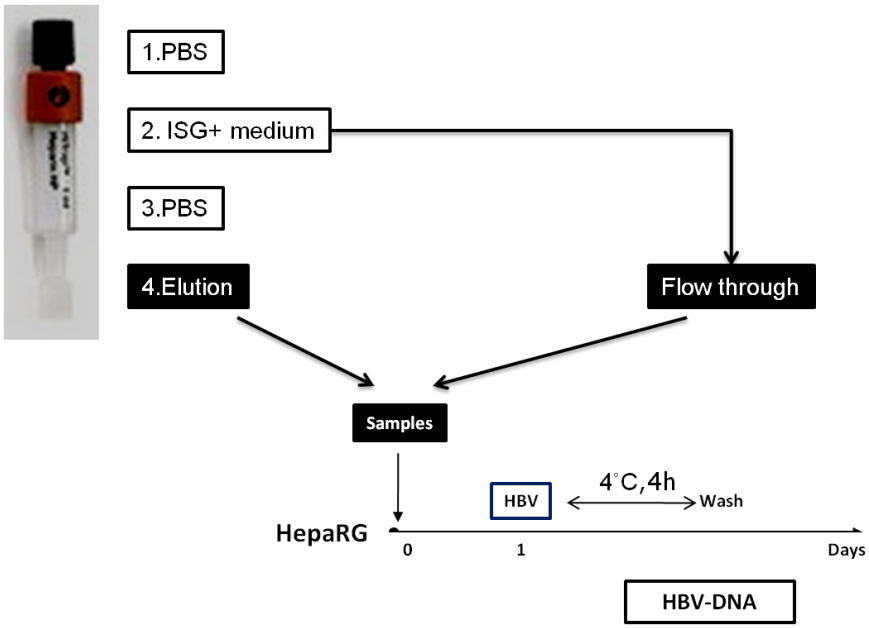


Fig.2.3.5 IFN-α induced products compete with HBV for binding to heparin column. (**A**) Heparin columns were washed with PBS and then applied with Differentiated HepaRG cell culture medium, or IFN-α treated differentiated HepaRG cell culture medium or Pep19-2.5. Then columns were applied with PBS containing HBV. After washing, HBV was eluted by elution buffer. (**B**) HBV amount from different elutions were analyzed by HBV-DNA qPCR.

Results showed that most HBV virions bound to heparin-sepharose under physiological salt conditions and could be eluted with a high salt concentration (**Fig.2.3.5**). HBV particle binding to heparin was reduced by 30% when particles were loaded after ISG+ medium. As a positive control, Pep19-2.5 inhibited HBV binds to heparin column as described before [208]. The result indicated that ISG+ medium competes with HBV for binding to heparin.

To confirm that, ISG+ medium was applied on heparin column, and separated into flow through (FT) and elution. Differentiated HepaRG cells then incubated with both samples for 24 hours and followed by 4 hours HBV incubation at 4°C. HBV particles bound to cell surface were evaluated by qPCR.

A



B

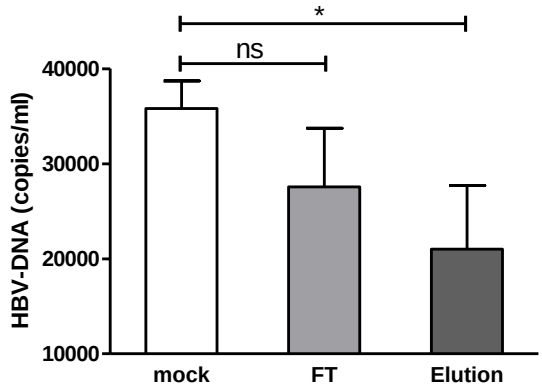


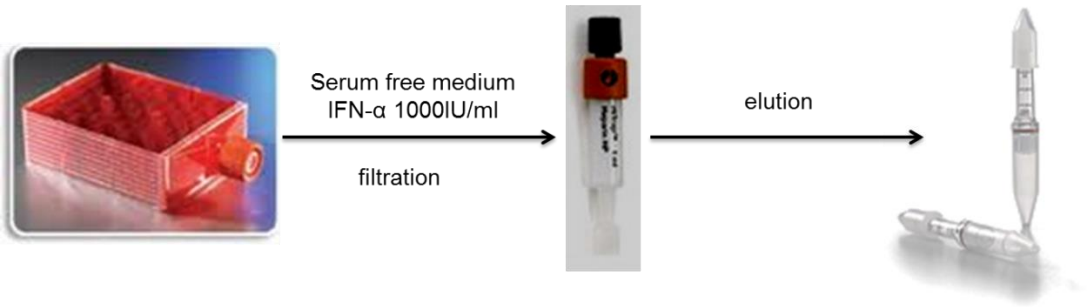
Fig.2.3.6 IFN-α induced products inhibit HBV binding to the cells. (**A**) Heparin columns were washed with PBS and then applied with IFN-α treated differentiated HepaRG cell culture medium. Then columns were washed with PBS. Fresh differentiated HepaRG cells were incubated with elution or flow through for 24 hours followed by 4 hours HBV incubation at 4°C. (**B**) Cells were lysed and cellular HBV-DNA were evaluated by qPCR. Data are means ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student´s unpaired two-tailed *t* test.

After heparin column purification, ISG+ medium was divided into high heparin affinity part (elution) and low heparin affinity part (Flow through) (**Fig.2.3.6A**). The elution from heparin column showed reduction of HBV binding to differentiated HepaRG cells while flow through showed no significant inhibition (**Fig.2.3.6B**). Therefore, our results showed that interferon induced factors inhibit initial binding of HBV to the cell surface by direct interaction to heparan sulfate.

2.3.5 Identification of IFN-α induced binding inhibitors

In order to identify the active factors in ISG+ medium, we performed with size exclusion experiment. HepaRG cells were cultivated and differentiated in hyperflask which offers higher cell yields to increases productivity of interferon induced soluble factors. To reduce the protein background of our sample, serum free medium was used after IFN-α stimulation in this case. Heparin binding fraction was purified from the column, and protein concentration columns with different cutoffs were used afterwards to further separate the fractions by protein size (**Fig.2.3.7A**). Three kinds of protein concentration columns were applied, with 10 kDa, 30 kDa and 100 kDa cutoff respectively. We collected both flow through and concentrate from each column, and thus we obtained six different fractions containing different size of proteins (**Fig.2.3.7B**).

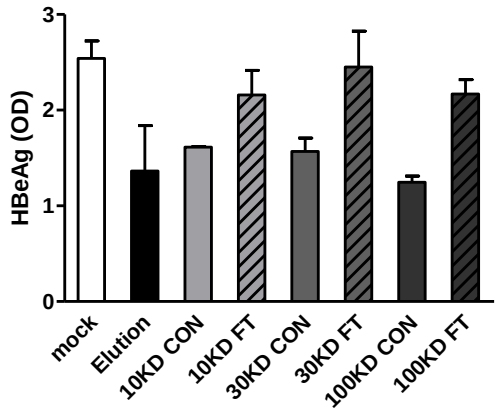
A



B

Column	Flow-through	Concentrate
10kDa	<10kDa	≥10kDa
30kDa	<30kDa	≥30kDa
100kDa	<100kDa	≥100kDa

C



D

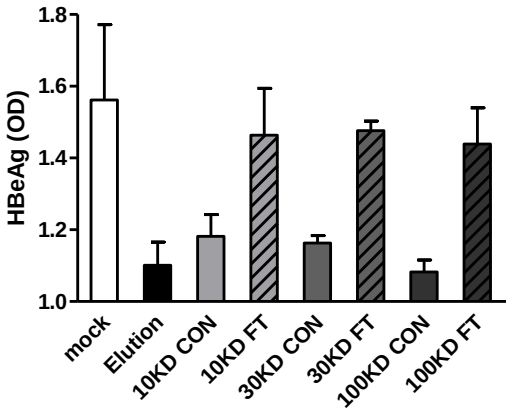


Fig.2.3.7 Size exclusion of IFN-α induced binding inhibitors. (**A**) HepaRG cells were cultivated in hyperflask. After differentiation, cells were stimulated with 1000IU/ml IFN-α for one day and then fed fresh serum free medium. Heparin binding fraction was purified from heparin column, and after that protein concentration columns with different cutoffs were used to further separate the fractions by protein size. (**B**) Protein size of each fraction was summarized. (**C**) Differentiated HepaRG cells were incubated with indicated samples for 24 hours and then infected with HBV. HBeAg was evaluated by ELISA 4 days after infection. (**D**) Highly purified HBV SVPs from chronic HBV carriers were added to heparin-coated (25 µg/ ml) 96-well-plate. Plates were incubated for 2 h at 37°C, and heparin-bound SVPs were detected using an HBsAg ELISA kit.

Differentiated HepaRG cells were incubated with heparin column elution or different fractions for 24 hours and then infected with HBV. HBeAg was evaluated by ELISA 4 days after infection (**Fig.2.3.7C**). Elution from heparin column inhibited HBV replication as we expected. With different fractions, all three concentrates showed anti-viral effect while all three flow-through didn´t reduce HBeAg.

To determine binding specificity of our samples to heparin, we coated 96-well-plate with heparin and determined binding of purified HBV particles. Using elution or different fractions as competitor, we determined the amount of HBV bound to plates by HBsAg ELISA (**Fig.2.3.7D**). Similar to the result from cell culture system, complete inhibition of HBV binding to heparin could either be achieved with elution, or concentrates from size exclusion columns. All three flow-through showed no inhibition effect. This suggested that the interferon induced secreted factors which inhibit binding of HBV are larger than 100 kDa. To further identify the factors, we used mass spectrometry which is the primary method for protein identification from complex mixtures of biological origin.

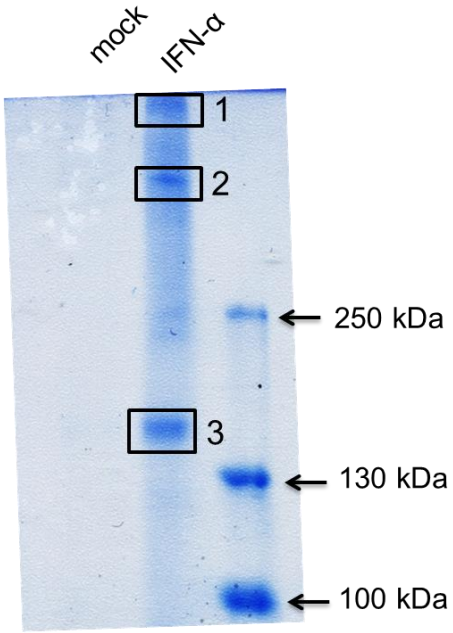


Fig.2.3.8 SDS-PAGE of heparin column elution. Differentiated HepaRG cells were treated with or without 1000IU/ml IFN-α. Two days later, cell culture supernatants were collected and applied on heparin columns. Elution then concentrated with 10 kDa cutoff column. 7.5% SDS-PAGE was loaded with concentrates and stained with coomassie bright blue.

We collected supernatant from mock or IFN-α treated differentiated HepaRG cells, purified with heparin column, and then concentrated with 10 kDa cutoff column. With SDS-PAGE, we observed three distinguish bands from IFN-α treated sample (**Fig.2.3.8**). Band 1 and band 2 presented very big molecule weight, with more than 250 kDa. Band 3 was between 130 kDa and 250 kDa. The result in accordance with our earlier conclusion that interferon induced secreted factors are larger than 100 kDa (**Fig.2.3.7**).

Three bands were cut and digested by trypsin, and then analyzed by Orbitrap mass spectrometry. Identified proteins were ordered by numbers of unique peptides as shown in **Table.2.3.1.**

No	Identified proteins	Molecular weight (kDa)	Number of unique peptides		
			Bnad1	Band2	Band3
1	fibronectin 1	259	29	45	67
2	complement factor H	139	1	63	30
3	calsyntenin 1	109	33	12	
4	complement component 3	187	1	16	16
5	apolipoprotein B	516	1	14	17
6	collagen, type XII, alpha 1	333	4	9	18
7	complement C4-B-like preproprotein	193		7	14
8	inter-alpha-trypsin inhibitor heavy chain 2	106	10		
9	nidogen 1	136	15		
10	neogenin 1	160	11		
11	inter-alpha-trypsin inhibitor heavy chain 1	101	5	1	
12	tenascin C	241	9		
13	albumin	69		5	2
14	gelsolin	81	5		
15	amyloid beta (A4) precursor-like protein 2	87	5		
16	serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 5	46		4	3
17	calsyntenin 3	106	7		
18	collagen, type VIII, alpha 1	73		2	2
19	complement factor H-related 3	37		2	1
20	ceruloplasmin (ferroxidase)	122	5		

21	coagulation factor II (thrombin)	70	4		
22	nidogen 2 (osteonidogen)	151	3		
23	protein tyrosine phosphatase, receptor type, F	213	1	4	
24	coagulation factor XI	64	1		3
25	activating transcription factor 6 beta	77	4		
26	protein tyrosine phosphatase, receptor type, D	215	4		
27	collagen, type XIV, alpha 1	194	4		
28	thrombospondin 1	129		2	2
29	amyloid beta (A4) precursor protein	87	3		
30	coagulation factor V (proaccelerin, labile factor)	252	1	2	
31	Beta-lactoglobulin	20		1	2
32	protein tyrosine phosphatase, receptor type, S	215	3		
33	alpha-2-macroglobulin	163	3		
34	vitronectin	54	3		
35	hemoglobin, alpha 2	15	2		
36	valyl-tRNA synthetase	140	2		
37	proteoglycan 4	147	2		
38	alpha-2-HS-glycoprotein	39	2		
39	actin, gamma 1	42	2		

Table.2.3.1 Result of mass spectrometry.

Thirty nine proteins were identified from mass spectrometry, some of them are traditionally not considered as secreted proteins or interferon induced proteins. The exist of cell surface proteins (such as nidogen and neogenin) and intracellular proteins (such as gelsolin and activating transcription factor 6 beta) indicated that the cell culture supernatant also contained some proteins released from cell debris.

According to the coverage of unique peptides and characteristics, we speculated that fibronectin1 and complement factor H may involve in the binding inhibition of HBV. Further investigation should be performed to prove that.

2.4 An IFN-α induced base excision repair pathway leads to HBV cccDNA degradation

2.4.1 IFN-α induces degradation of HBV cccDNA

Current treatments for chronic hepatitis B are not satisfying because of the persistence of HBV cccDNA in infected hepatocytes. Viral cccDNA forms minichromosomes and serves as a matrix for viral mRNA transcription and replication [27]. It has been shown that innate immunity strongly suppresses viral replication through noncytopathic mechanisms in chimpanzees, which may also contribute to clearance viral infection as well as cccDNA[211]. For chronic hepatitis B patients, PEG-IFN-α treatment leads to intrahepatic HBV cccDNA decline [212, 213]. The aim of the following study was to determine the mechanism involved.

In order to study the effect of IFN-α on HBV, differentiated HepaRG cells were infected with HBV and treated with LAM or IFN-α.

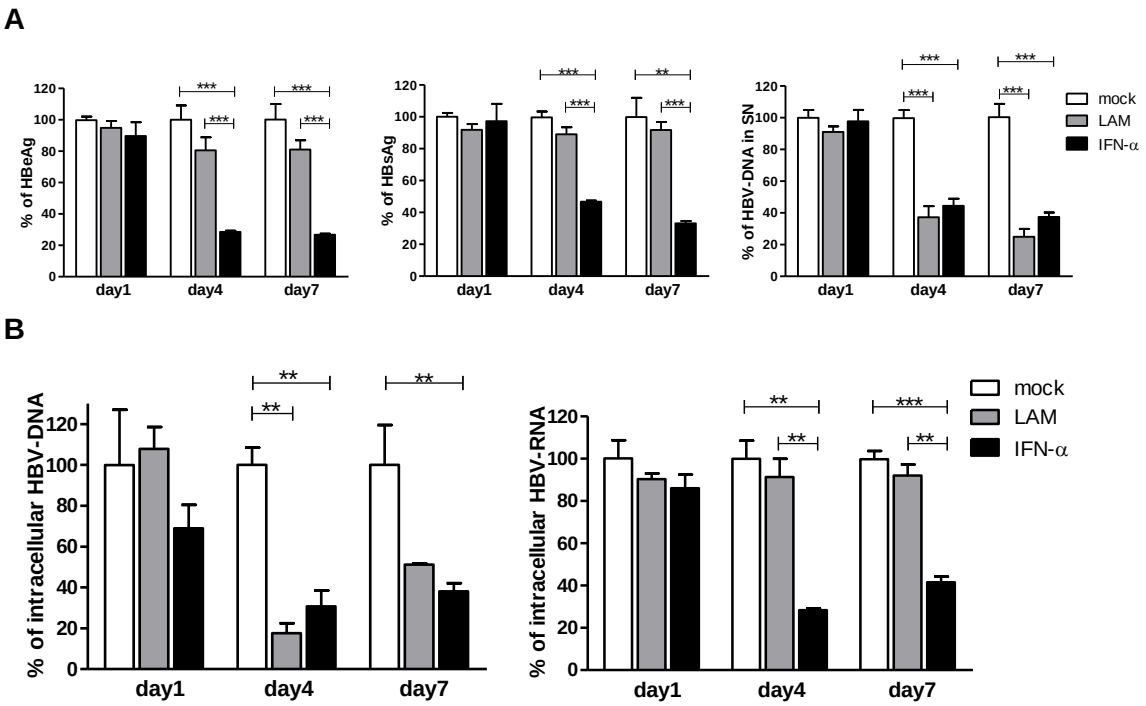


Fig.2.4.1 IFN-α inhibits HBV replication in HepaRG cells. Differentiated HepaRG cells were infected with HBV (M.O.I= 200). Ten days post infection, cells were treated with 1000 IU/ml of IFN-α or 1 μM of Lamivudine (LAM) at day 0. Cell culture supernatant (**A**) and intracellular (**B**) HBV replication markers were measured on day 1, 4 and 7 after treatment. Data are means ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student´s unpaired two-tailed *t* test.

Looking at HBV replication markers in the cell culture supernatant, we observed that LAM inhibited only HBV-DNA while IFN-α also reduced HBeAg and HBsAg (**Fig.2.4.1A**). We also observed that LAM reduced HBV-DNA and IFN-α inhibited both HBV-DNA and RNA

(**Fig.2.4.1B**) as described before [214-216].It has been shown that LAM inhibits reveres-transcription [217, 218], so it only reduces DNA synthesis as expected. Since IFN-α hindered viral DNA replication, transcription and antigen secretion, these results indicated that IFN-α influences viral RNA synthesis and/or directly targets on the template of HBV replication.

To investigate whether this template can be reduced upon IFN-α treatment, we performed a rebound experiment (**Fig.2.4.2)**. For different groups of HBV infected HepaRG cells, we started IFN-α treatment at the same time point, and stopped the treatment at different time point.

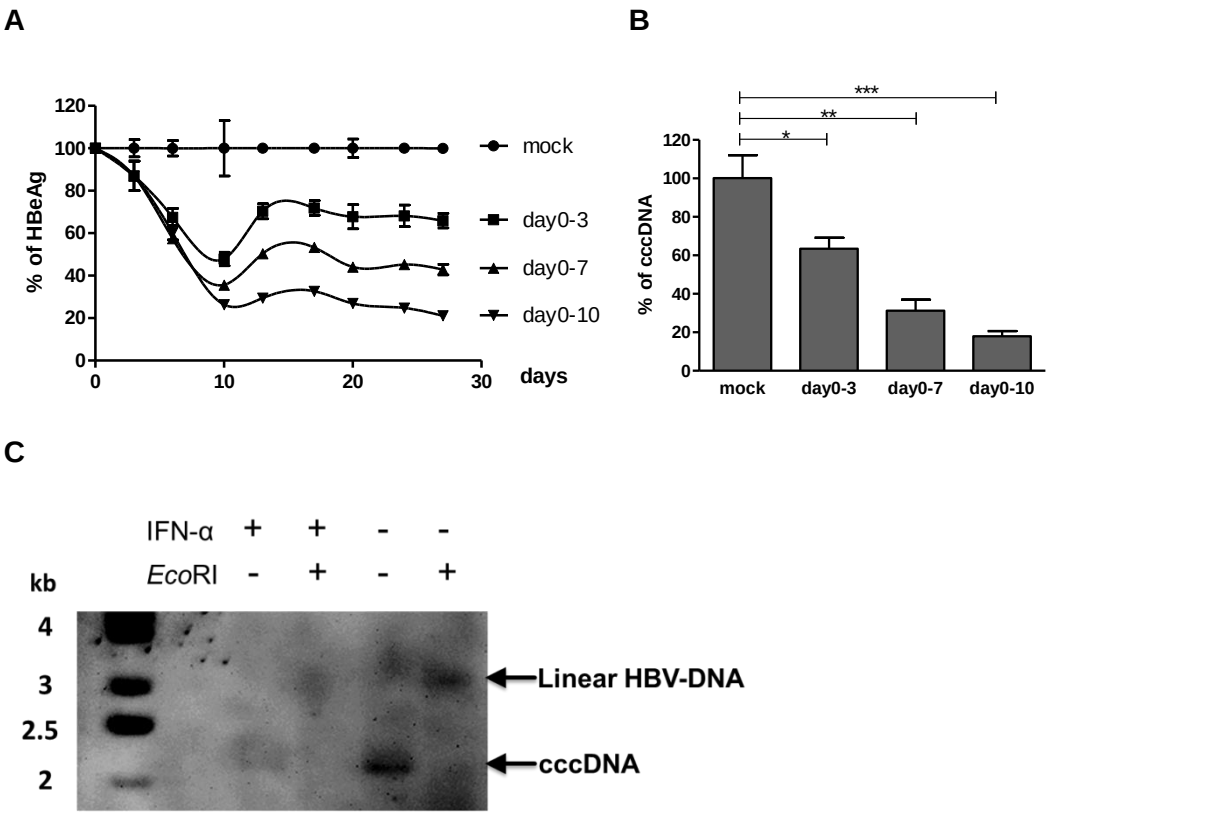
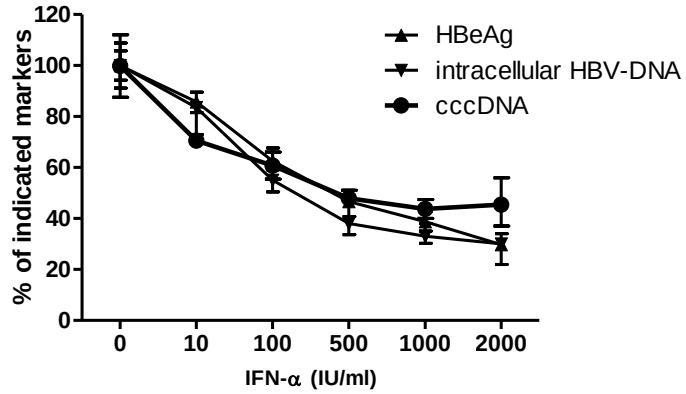


Fig.2.4.2 IFN-α reduces HBV cccDNA in HepaRG cells. (**A**) Differentiated HepaRG cells were infected with HBV (M.O.I= 200). Treatment was started ten days post infection. Cells were treated with 1000 IU/ml of IFN-α once (day0-3), twice (day0-7) or three times (day0-10), HBeAg was measured twice a week. (**B**) HBV cccDNA was evaluated by the end of the experiment. (**C**) Protein-free viral DNA from day 10 was analyzed by Southern blot. Data are means ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student´s unpaired two-tailed *t* test.

We observed a partial rebound of HBeAg, but the antigen levels didn't rebound to the level of untreated group. The longer the treatment, the less rebound of HBeAg we could observe (**Fig.2.4.2A**). These results suggested that the matrix of HBV replication was reduced, which is HBV cccDNA. Then HBVcccDNA specific q-PCR was used for quantification. We could see a significant reduction of cccDNA under IFN-α treatment, in a time dependent manner (**Fig.2.4.2B**). The decrease of cccDNA was confirmed by Southern blot (**Fig.2.4.2C**).

A



B

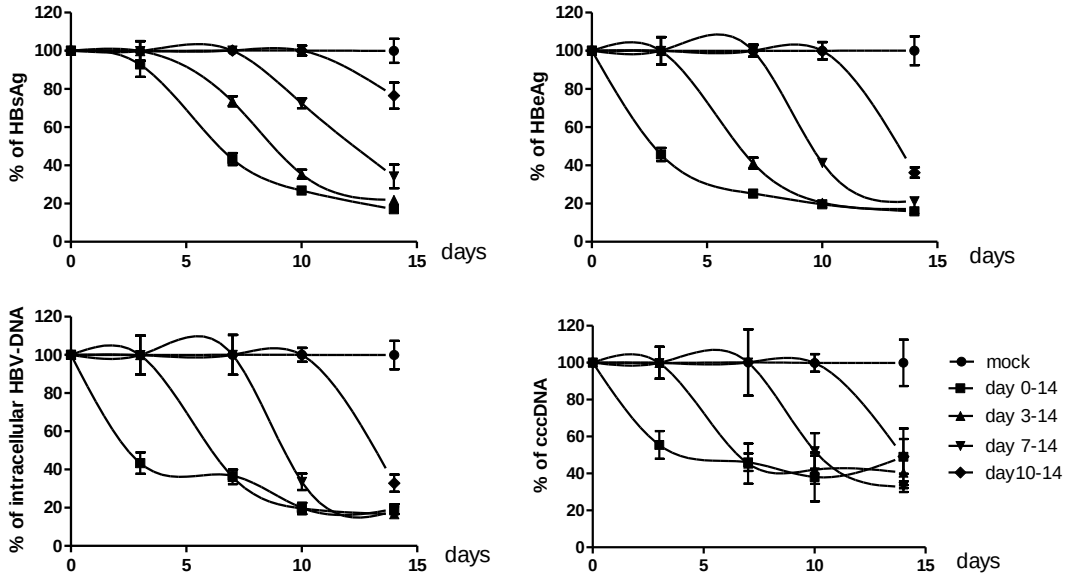


Fig.2.4.3 IFN-α reduces HBV replication in a dose and time dependent manner. (A) HBV infected HepaRG cells were treated with indicated doses, different HBV replication markers were measured 7 days after treatment. **(B)**HBV infected HepaRG cells were treated with 1000 IU/ml of IFN-α four times (day0-14), three times (day3-14), twice (day7-14) and once (day 10-14), HBV cccDNA were evaluated.

The reduction of HBV cccDNA showed a dose dependent effect upon IFN-α treatment (**Fig.2.4.3A**). We also checked the kinetic of HBV replication markers during IFN-α treatment. In this case, we initiated the treatment at different time point, and stopped the treatment at the same time point. All HBV replication markers were suppressed by IFN-α include cccDNA no matter when we started the treatment (**Fig.2.4.3B**).

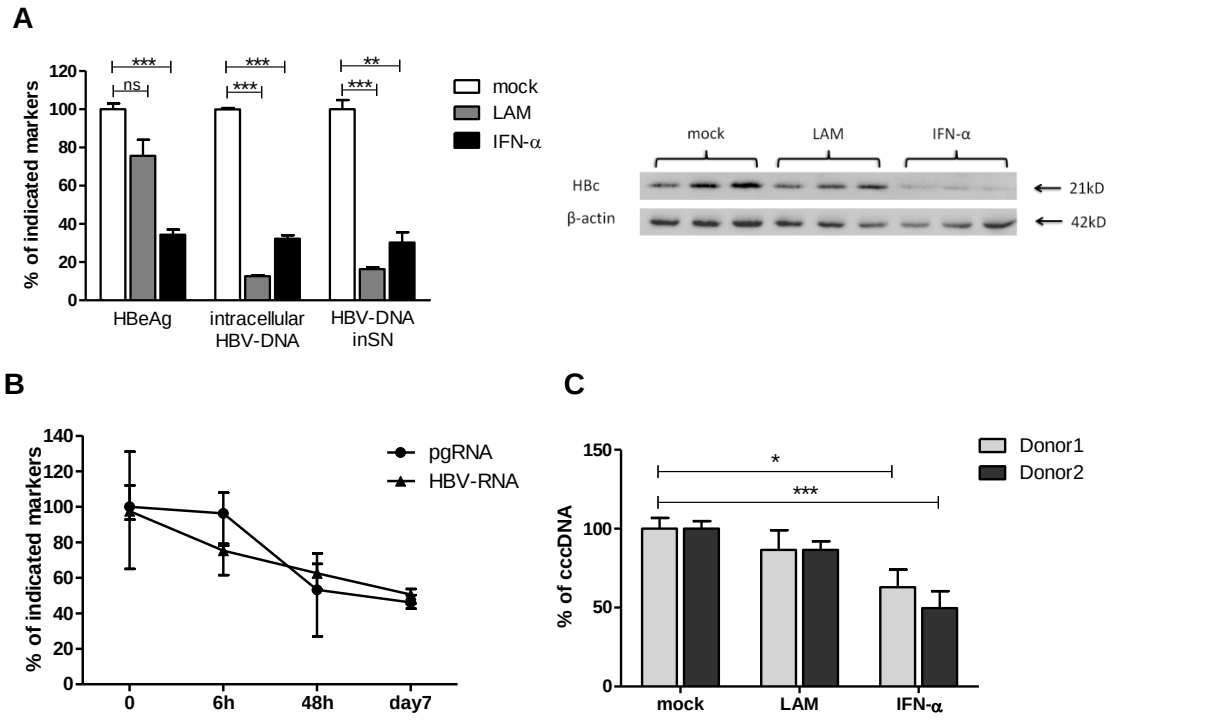


Fig.2.4.4 IFN-α inhibits HBV replication and reduces cccDNA in PHH cells. (A) Primary human hepatocytes (donor2) were infected with HBV (M.O.I= 200), three days after infection, cells were treated with 500 IU/ml of IFN-α or 1 μM of Lamivudine (LAM). Cell culture supernatant and intracellular HBV replication markers were measured 13 days after treatment. HBV core was detected by Western blot. **(B)**HBV infected PHH (donor3) were treated with 500 IU/ml of IFN-α, cells were lysised for RNA extraction at indicated time point, HBV-RNA and pgRNA were measured by qPCR. **(C)**HBV cccDNA from two donors were evaluated by qPCR. Data are means ± s.d. **P*<0.05, ***P*<0.01, ****P*<0.001 by Student´s unpaired two-tailed *t* test.

The inhibition of HBV replication and decrease of HBV cccDNA can also be observed on HBV infected and subsequently IFN-α treatment primary human hepatocyte (**Fig.2.4.4**). LAM only inhibited HBV-DNA but IFN-α affected HBV DNA and protein synthesis (**Fig.2.4.4A**). IFN-α reduced HBV RNA in a time dependent manner (**Fig.2.4.4B**). IFN-α showed the ability to affect cccDNA in PHH isolated from different donors (**Fig.2.4.4C**).

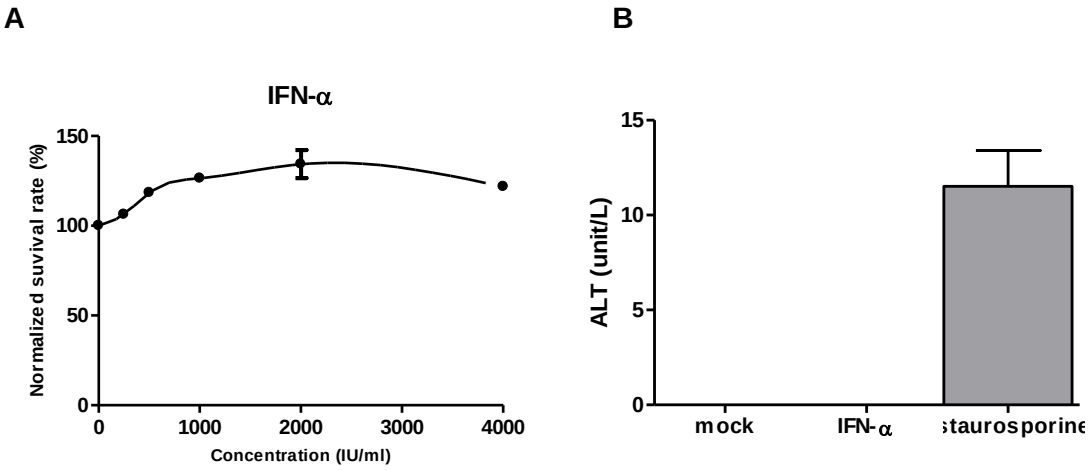


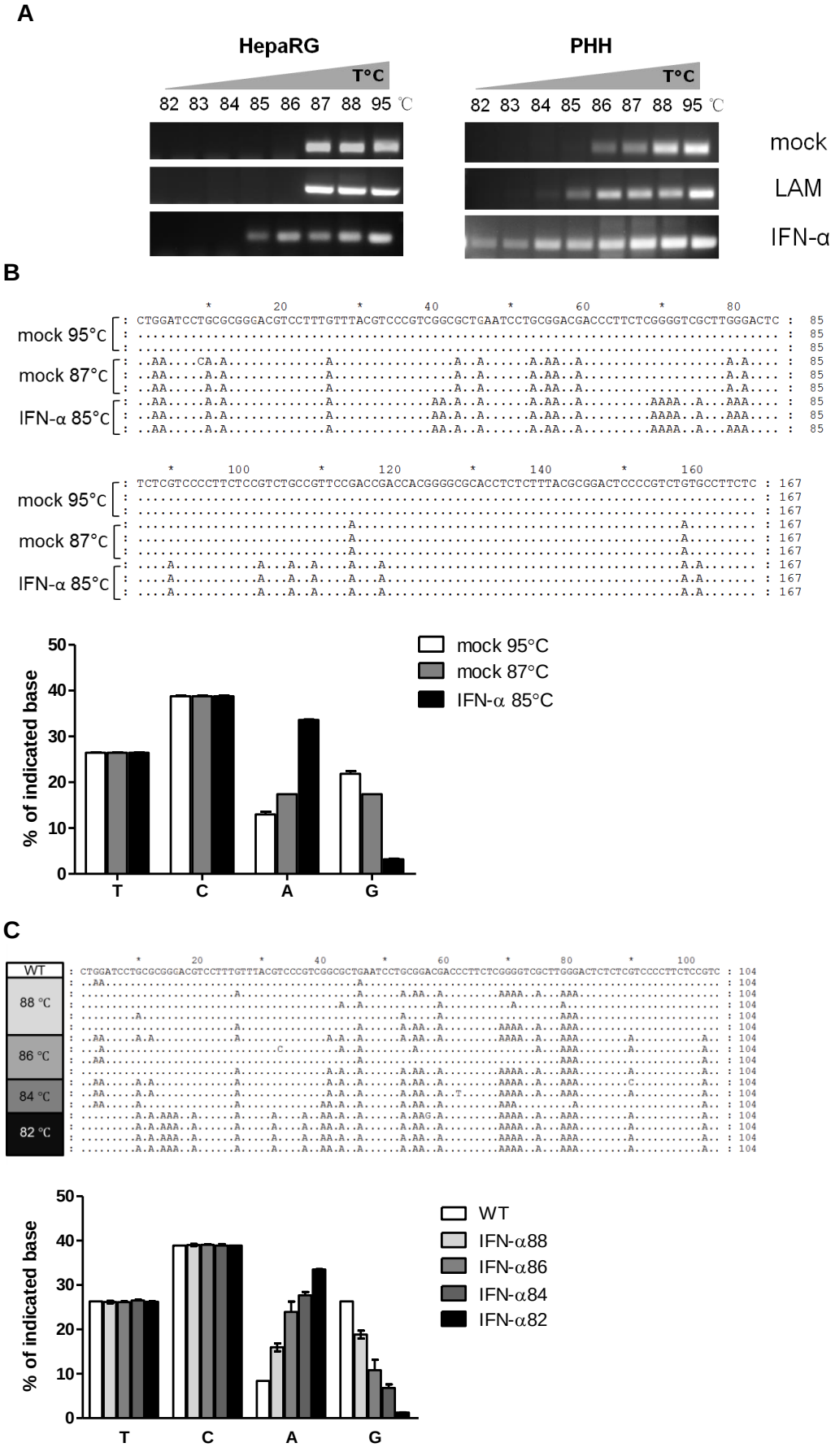
Fig.2.4.5 Cytotoxicity test of IFN-α on HepaRG and PHH. (A) HBV infected HepaRG cells were treated with indicated doses of IFN-α for 7 days, cell viability was determined by XTT assay. **(B)** HBV infected PHH were treated with1000 IU/ml IFN-α or staurosporine for 10 days, ALT levels from cell culture supernatant were measured.

Standard IFN-α or PEG- IFN-α treatment for chronic hepatitis B patients is normally associated with side effects. To make sure the antiviral effect we observed was not due to cytotoxicity, cell viability and hepatocyte damage were measured by XTT test and ALT test respectively. Results showed that the antiviral effect induced by IFN-α is not due to cytotoxicity **(Fig.2.4.5)**. This suggested that IFN-α elicits an antiviral mechanism to leads to HBV cccDNA degradation and clear viral infection.

2.4.2 IFN-α induces HBV cccDNA sequence alterations

The decrease of HBV cccDNA upon IFN-α treatment indicated DNA modification. To determine whether the reduction of HBV cccDNA refers to DNA damage, we performed differential DNA denaturation PCR (3D-PCR) to detect potential DNA damage in DNA sequence [169].

3D-PCR is a method which can be used to discover mutants with GC to AT transitions. AT-rich DNA melts at lower denaturation temperatures than GC-rich DNA due to the two hydrogen bonds between A and T versus the three between G and C. Therefore, doing PCR with a lower denaturing temperature allows differential amplification of AT-rich sequences.


A
HepaRG
PHH
T°C
82 83 84 85 86 87 88 95 ℃
mock
LAM
IFN-α
B
mock 95°C
mock 87°C
IFN-α 85°C
% of indicated base
mock 95°C
mock 87°C
IFN-α 85°C
T
C
A
G
C
WT
88 ℃
86 ℃
84 ℃
82 ℃
% of indicated base
WT
IFN-α88
IFN-α86
IFN-α84
IFN-α82
T
C
A
G

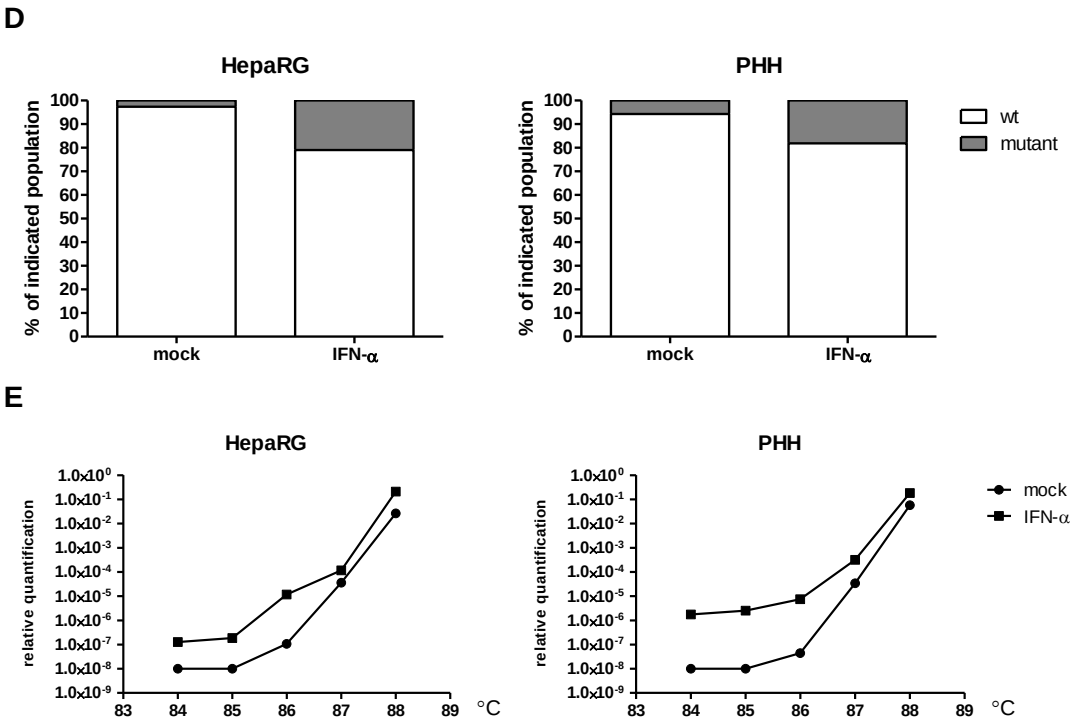


Fig.2.4.6 IFN-α induces HBV cccDNA sequence alterations. HBV infected HepaRG cells were treated with 1000 IU/ml of IFN-α or 1 µM of Lamivudine (LAM). 3D-PCR was performed to detect HBV cccDNA damage 4 days after treatment **(A)**. PCR products with low denature temperature were cloned and sequenced. Different content of nucleotides between IFN-α treated and untreated sample were summed up **(B)**. **(C)**Primary human hepatocytes (donor2) were infected with HBV (M.O.I= 200), three days after infection, cells were treated with 500 IU/ml of IFN-α or 1 µM of Lamivudine (LAM), 3D-PCR was performed to detect HBV cccDNA damage 10 days after treatment. Different content of nucleotides between IFN-α treated and untreated sample (WT) were summed up. **(D)** Quantitative 3D-PCR was performed to determine the proportion of wild type cccDNA and mutants in cell culture and relative amount of mutants at different temperature **(E)** were evaluated

At higher denaturation temperatures, we observed PCR products for all mock, LAM and IFN-α treated samples. However, at lower denaturation temperatures, we only obtained PCR products from IFN-α treated samples, which indicated DNA damage **(Fig.2.4.5A)**. To confirm that, lower denaturing PCR products were cloned. Subsequent sequencing revealed G to A transition in HBV cccDNA PCR products. Although there was moderate background level also in mock samples at 87°C, IFN-α treatment strongly induced G to A transitions **(Fig.2.4.5B)**. Likewise, form PHH we could observe the same. IFN-α treatment induced a lot of G to A transition. The lower denature temperature we achieved, the more G to A transition we got **(Fig.2.4.5C)**.These data inferred that IFN-α treatment correlated with the level of G to A transition, however, whether it also related to the quantity of mutants was still unknown. Quantitative 3D-PCR was performed to determine the proportion of wild type cccDNA and

mutants. We detected very low background levels of mutants without treatment, but we could observe an increased population of mutants in both HepaRG and PHH models **(Fig.2.4.5D)**. Furthermore, we looked into the details of mutants selected by different temperature, and we discovered the increased amount of mutants at each point **(Fig.2.4.5E)**. These results suggested that IFN-α increases both level and quantity of G to A transition. Interestingly, G to A transition in cccDNA from patient liver-needle biopsies can be found after 48 weeks of IFN-α treatment (personal communication with Yong Li, Tongji hospital, Wuhan, China). G to A transition is a typical consequence of hypermutation [219]. From different models we could find G to A transition, which implied IFN-α induced G to A transition causes cccDNA sequence alterations.

2.4.3 IFN-α upregulates human cytidinedeaminases

A lot of studies revealed hypermutation as an innate immunity againsts several viruses [219-223], it may cause by ROS[224] or deamination[225]. We first checked whether IFN-α induces ROS in the HBV infected hepatocyte.

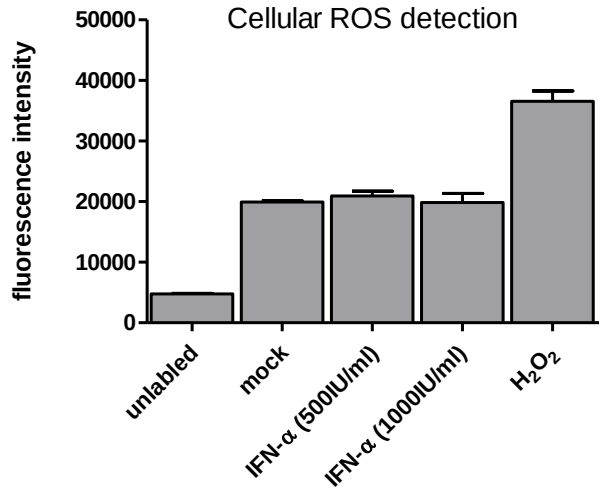


Fig.2.4.7 IFN-α does not induce reactive oxygen species. HBV infected HepaRG cells were treated with 1000IU/ml IFN-α for 7 days or 20μM H_2O_2 for 3 hours. Cellular reactive oxygen species were measured by DCFDA cellular ROS detection assay kit (abcam) followed by instruction.

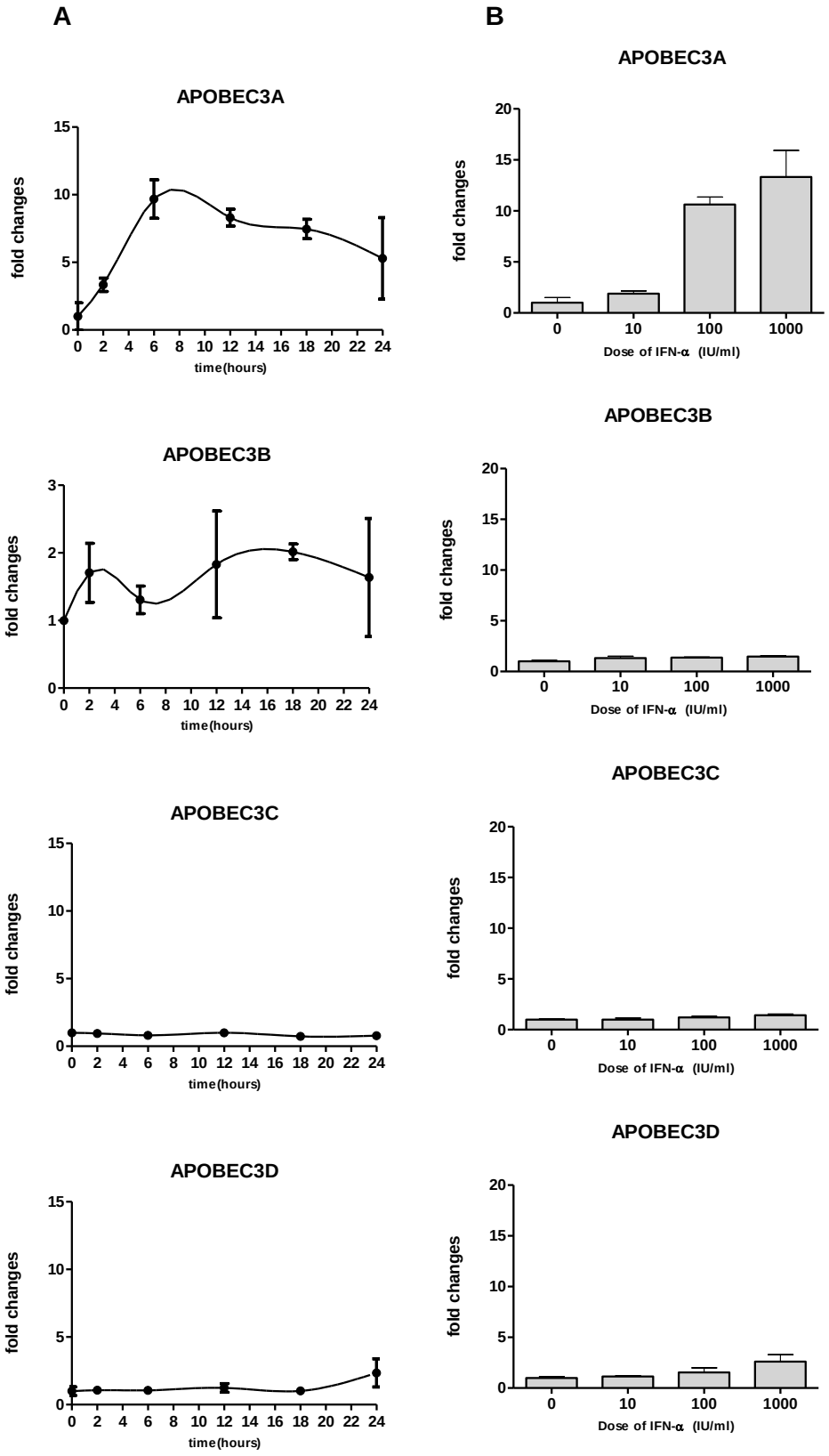
IFN-α treatment with two different concentrations displayed no difference compared to mock control, but H_2O_2 treatment strongly increased ROS signal (**Fig.2.4.7**). This suggested that other mechanisms might be involved in cccDNA hypermutation.

We then performed qPCR to detect the induction of different genes which play a role in DNA damage pathway, and we found several deaminases were upregulated after IFN-α stimulation which all belong to APOBEC3 family (**Table 2.4.1**).

Symbol	Gene Name	Fold induction
UNG1	uracil-DNA glycosylase 1	1
UNG2	uracil-DNA glycosylase 2	1
TDG	thymine-DNA glycosylase	1
MBD4	methyl-CpG binding domain protein 4	1
APEX1	APEX nuclease (multifunctional DNA repair enzyme) 1	1
SMUG1	single-strand-selective monofunctional uracil-DNA glycosylase 1	1
APOBEC1	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	1
APOBEC2	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 2	1
APOBEC3A	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3A	**9**
APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	2
APOBEC3C	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3C	1
APOBEC3D	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3D	2
APOBEC3F	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3F	**7**
APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	**9**
APOBEC3H	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3H	1
APOBEC4	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 4	1
AICDA	activation-induced cytidine deaminase	1
CTSE	cathepsin E	1
UBD	ubiquitin D	9
BIRC3	baculoviral IAP repeat containing 3	5
PLK1	polo-like kinase 1	1
TYMS	thymidylate synthetase	1
NEIL3	nei endonuclease VIII-like 3 (E. coli)	1
SOD2	superoxide dismutase 2, mitochondrial	1
TOP2A	topoisomerase (DNA) II alpha 170kDa	1
RRM2	ribonucleotide reductase M2	1

Table 2.4.1 HBV infected HepaRG cells were treated with 1000 IU/ml IFN-α for 6 hours followed by RNA extraction and reverse transcription. Real-time PCR was used to quantify the mRNA level of different genes related to DNA modification.

To confirm the result, we then checked the upregulation of different APOBEC3 in HepaRG and PHH by qPCR.


A
APOBEC3A
fold changes
time(hours)
APOBEC3B
fold changes
time(hours)
APOBEC3C
fold changes
time(hours)
APOBEC3D
fold changes
time(hours)
B
APOBEC3A
fold changes
Dose of IFN-α (IU/ml)
APOBEC3B
fold changes
Dose of IFN-α (IU/ml)
APOBEC3C
fold changes
Dose of IFN-α (IU/ml)
APOBEC3D
fold changes
Dose of IFN-α (IU/ml)

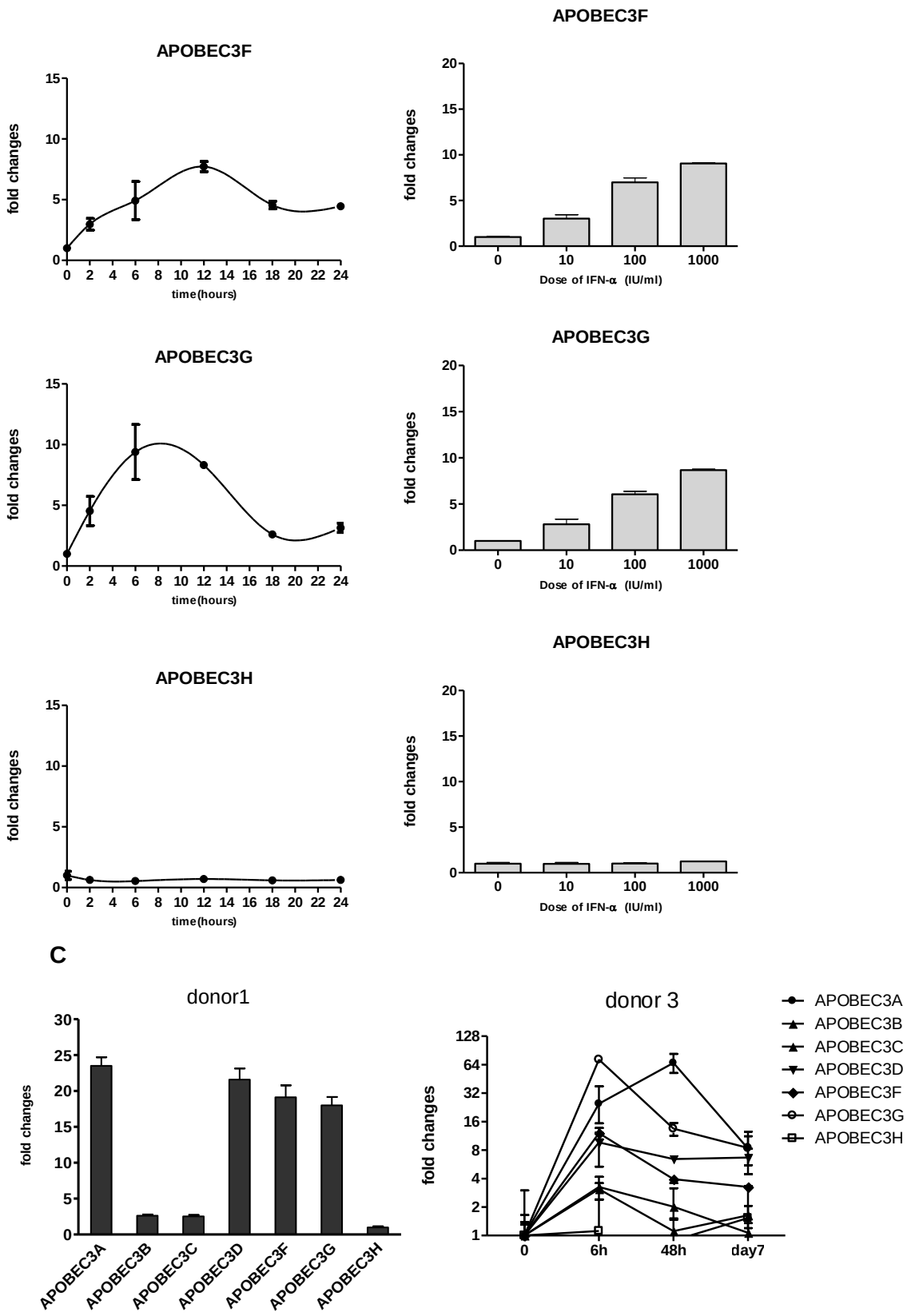


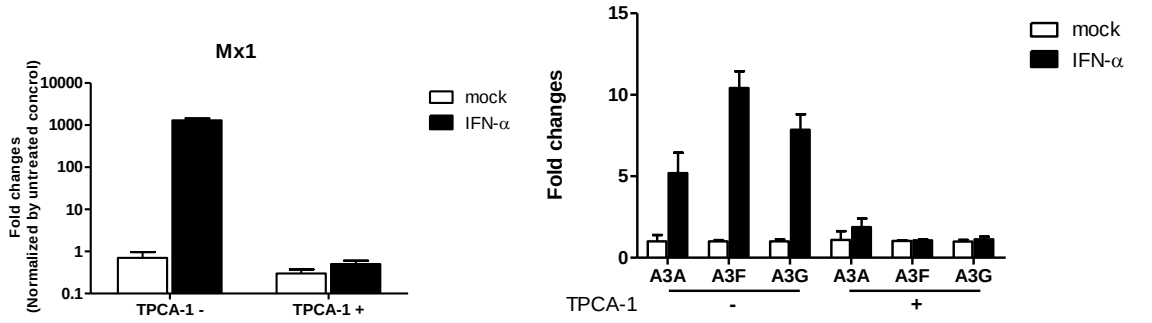
Fig.2.4.8 IFN-α upregulates human cytidine deaminases. HBV infected HepaRG were treated with 1000 IU/ml of IFN-α for 24 hours **(A)** or indicated dose for 6 hours **(B)**. PHH from donor 1 were infected with HBV and treated with 1000 IU/ml of IFN-α for 6 hours, PHH from donor 3 were infected with HBV and treated with 1000 IU/ml of IFN-α for 7 days **(C)**.All the samples were followed by RNA

extraction and reverse transcription. Real-time PCR was used to quantify the mRNA level of different APOBEC3 genes.

We treated cells with different does of IFN-α. APOBEC3A, APOBEC3F and APOBEC3G were induced dose dependently upon IFN-α stimulation (**Fig.2.4.8A**). At the same time, the transcription kinetics revealed upregulation of the same gene (**Fig.2.4.8B**). The induction of APOBEC3 RNAs in PHH was quite similar to HepaRG cells. Upregulation of APOBEC3A, APOBEC3D, APOBEC3F and APOBEC3G upon IFN-α stimulation was observed in PHH from two different donors (**Fig.2.4.8C**). In chronic hepatitis B patients with or without IFN-α treatment, the upregulation of APOBEC3A and APOBEC3G were significantly higher after one year of PEG-IFN-α treatment (personal communication with Yong Li, Tongji hospital, Wuhan, China).

Then, we wanted to know whether IFN-α induced JAK-STAT signal pathway was essential for the activation of APOBEC3. TPCA-1 was first described as an IKK inhibitor which block NF-κb signaling [226], and a later study shows it also inhibits type I IFN mediated antiviral action [227]. We applied TPCA-1 on HBV infected HepaRG cells and treated them with or without IFN-α.

A



B

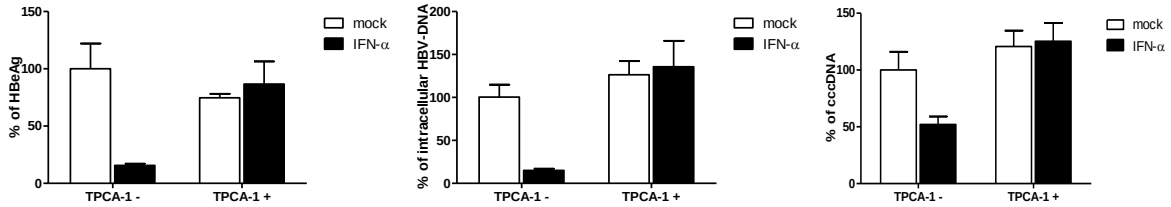


Fig.2.4.9 Blockade of IFN signal pathway. HBV infected HepaRG were treated with 1000 IU/ml of IFN-α together with or without 5μM TPCA-1.Induction of different interferon stimulated genes was measured 6 hours after treatment(**A**).HBV replication markers were measured 10 days after treatment(**B**).

We observed that TPCA-1 can block the induction of APOBEC3 on transcriptional level **(Fig.2.4.9A)**. When we looked into HBV replication markers, the antiviral effect of IFN-α was

reversed **(Fig.2.4.9B)**.These results indicated that activation of APOBEC3 and degradation of cccDNA via IFN-α signaling.

2.4.4 APOBEC3A is essential for HBV cccDNA deamination and degradation

HIV-Vif interacts with several APOBEC3 proteins and triggers the ubiquitination and degradation of them via the proteasomal pathway [170, 228-231]. To investigate the role of APOBEC3 in this antiviral activity, we generated a HIV-Vif inducible cell line based on HepaRG cells to specifically diminish APOBEC proteins.

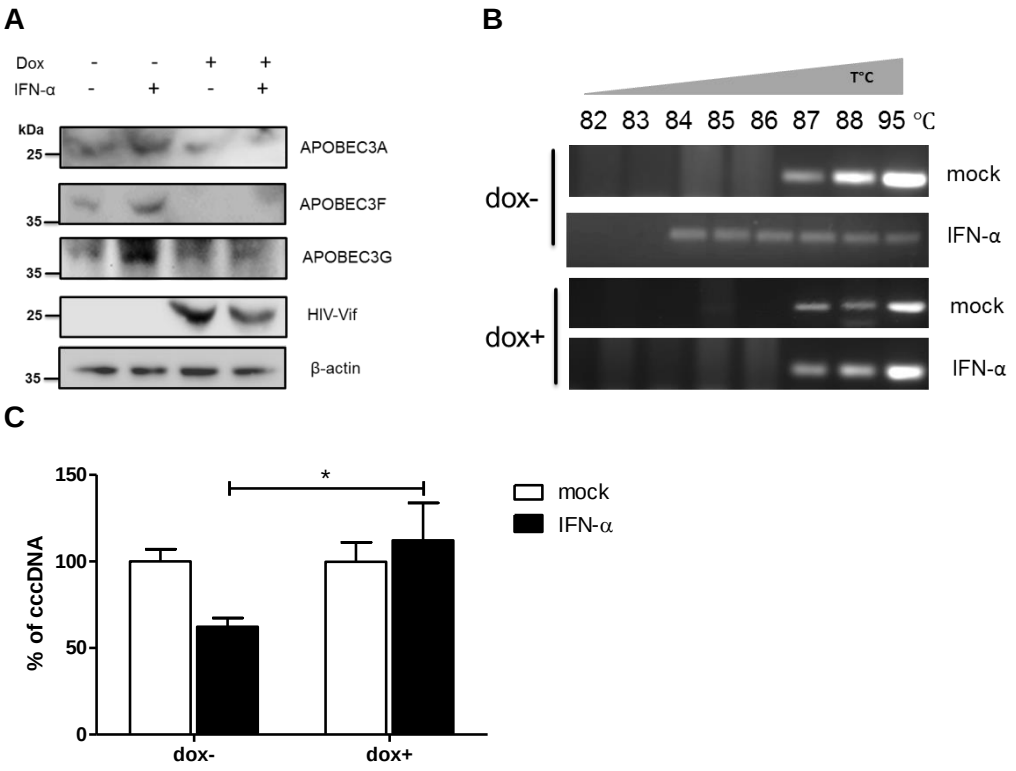


Fig.2.4.10 HIV-Vif counteracts IFN-α induced HBV cccDNA deamination and degradation. HBV infected HepaRG-tA-Vif cells were treated with 1000 IU/ml of IFN-α, doxycycline (dox) was used to induce the expression of HIV-Vif. **(A)** The expression of different proteins was analyzed by Western blot. **(B)** 3D-PCR was performed to detect HBV cccDNA deamination. **(C)** HBV cccDNA were measured 10 days after treatment. Data are means ± s.d. **P*<0.05 by Student´s unpaired two-tailed *t* test.

We confirmed that IFN-α stimulated APOBEC3A, APOBEC3F and APOBEC3G expression in the cells. But when HIV-Vif expression was switch on by adding doxycycline, all three APOBEC3 proteins were degraded (**Fig.2.4.10A**). 3D-PCR was performed to detect the deamination state of cccDNA, and the results showed cccDNA deamination induced by IFN-α were counteracted by HIV-Vif expression (**Fig.2.4.10B**). By quantifying cccDNA amount

from qPCR, we observed a rescue of cccDNA (**Fig.2.4.10C**), further supporting an important role of APOBEC3 in cccDNA deamination and degradation.

We next wanted to know which one of these three are real active effector. We used the following methods to answer the question.

Different APOBEC3 proteins showed a different preference of dinucleotide context [169]. APOBEC3G strongly prefer CpC, which means the mutated C residues were generally flanked at the −1 position by a C. APOBEC3F prefers TpC and GpC, whereas APOBEC3A shows no significant bias but slightly prefer TpC and CpC than GpC and ApC [232-236].

A. HepaRG

	-1	0	1
A	27	0	31
T	21	0	0
G	24	0	41
C	28	100	26

B. PHH

	-1	0	1
A	24	0	19
T	17	0	0
G	27	0	50
C	32	100	31

Table 2.4.2 Dinucleotide context preference of deamination. Dinucleotide context from Fig.2.2.6 B and C were summed up.

We couldn't find any clear pattern in terms of dinucleotide context of low denaturing PCR products from both PHH and HepaRG (**Table 2.4.2**) indicating that APOBEC3F and APOBEC3G are not pivotal in cccDNA deamination.

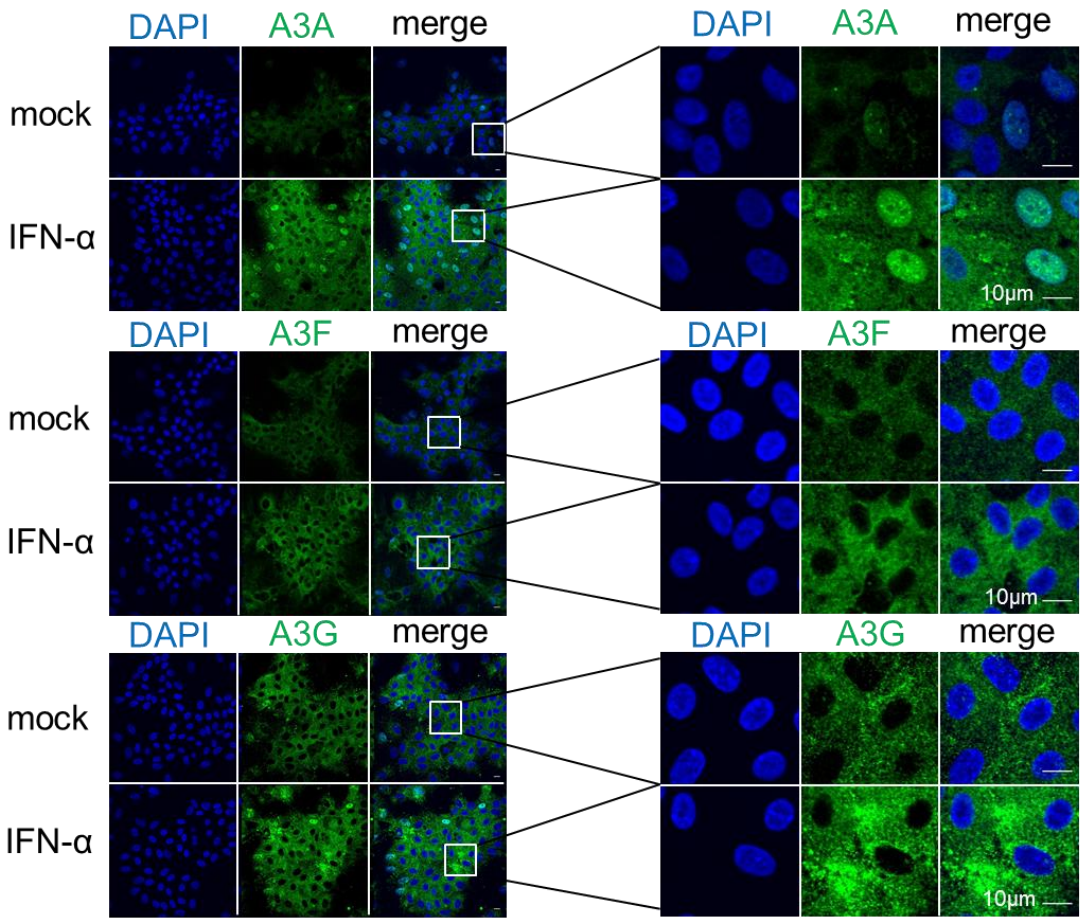


Fig.2.4.11 Immunostanning of APOBEC3 in HepaRG cells. Differentiated HepaRG cells were treated with or without 1000 IU/ml of IFN-α, cells were fixed three days after treatment and different APOBEC3 proteins were determined by immunofluorescence staining. Scale bar = 10μm.

Immunostaining result of HepaRG cells clearly showed the induction of APOBEC3A, APOBEC3F and APOBEC3G by IFN-α treatment. APOEC3F and APOEC3G localized in cytoplasm while APOBEC3A existed in both cytoplasm and nucleus (**Fig.2.4.11**).Our results are accordance with previous studies demonstrating the subcellular localization of these proteins[237, 238]. Thus, APOBEC3A is the only one that can access HBV cccDNA which is located in the nuclei.

To confirm this, we performed overexpression experiments. We cloned APOBEC3A and APOBEC3G, and transfected them into HepG2-H1.3 cells.

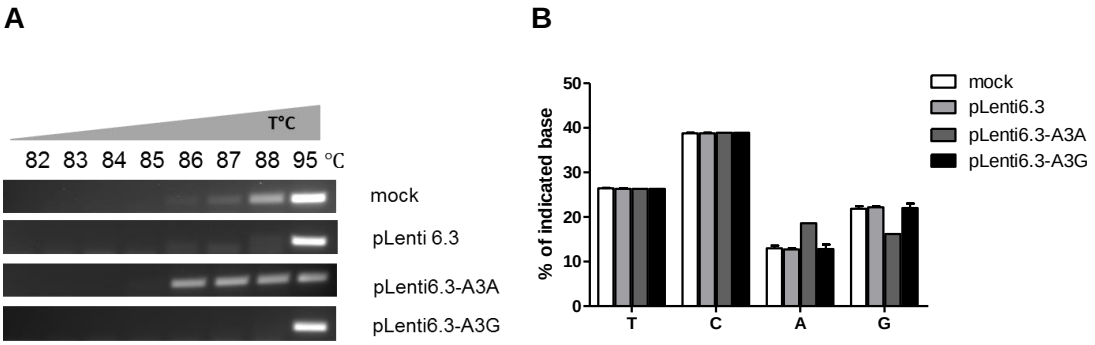


Fig.2.4.12 Overexpression of A3A and A3G in HepG2-H1.3 cells. HepG2-H1.3 cells were transfected with indicated plasmids, HBV cccDNA deamination were detected by 3D-PCR **(A)**. PCR products with low denature temperature were cloned and sequenced. Different content of nucleotides between IFN-α treated and untreated sample were summed up **(B)**.

After 3D-PCR, we observed that only cells transfected with APOBEC3A plasmid but not control (pLenti6.3 empty vector) or APOBEC3G plasmid showed deamination (**Fig.2.4.12,** repetition of Fig 19, Daniela Stadler Master thesis**)**.

Taken together, although IFN-α induced different APOBEC3 proteins in our model, APOBEC3A is the only one can access to HBV cccDNA and causes deamination.

2.4.5 IFN-α induced base excision repair pathway leads to HBV cccDNA degradation

APOBEC3A can induce deamination in cccDNA, but it not sufficient to lead to its degradation. Some other enzyme(s) or pathway(s) have to be involved. DNA deamination normally results in DNA repair or degradation, which is usually mediated by base excision repair pathway[239].

We used an enzyme cocktail called PreCR® Repair Mix, which is formulated to repair damaged DNA, to treat DNA samples isolated from HBV infected afterwards IFN-α treated HepaRG and PHH cells.

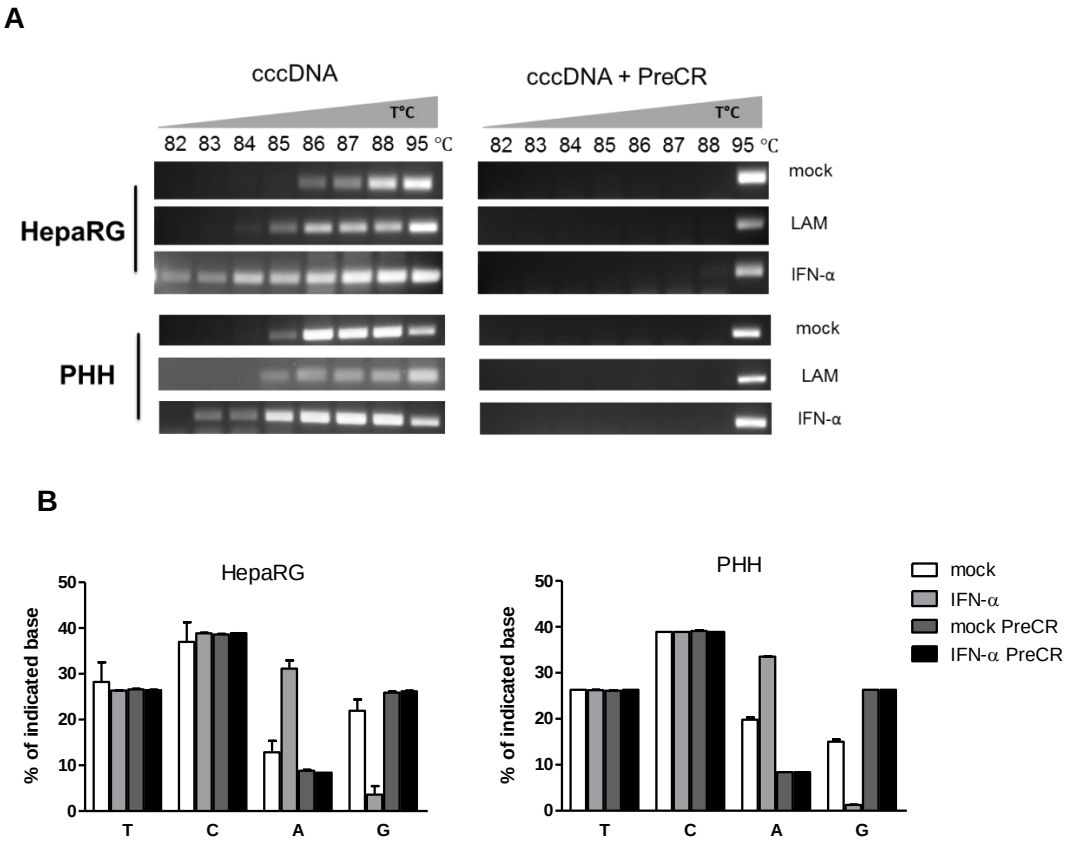


Fig.2.4.13 IFN-α induced uracil in cccDNA can be cleaved by enzyme mix. (A)DNA extracts from HBV infected HepaRG or PHH cells were incubated with PreCR mix. 3D-PCR was used to detect cccDNA deamination. **(B)**PCR products with low denature temperature were cloned and sequenced. Different content of nucleotides were summed up.

We observed that after PreCR® treatment, deamination of cccDNA was abolished (**Fig.2.4.13A**), which confirmed by subclonal sequencing (**Fig.2.4.13B**). The disappearance of bands from mock samples demonstrated even the background level of deamination which we observed before (**Fig.2.4.6B & D)** was repaired. Since there is one uracil-DNA glycosylase (UDG) in the mix, the results indicated that there are uracil residues in the cccDNA but even more after IFN-α treatment.

The consequence of uracil-DNA glycosylase treatment is the formation of apurinic/apyrimidinic site (AP site). The AP site can then be cleaved by an AP endonuclease during base excision repair [240]. To investigate whether cccDNA can be also digested via the same pathway, we extracted DNA from treated cells, and incubated DNA with APE1, a common endonuclease that exists in the cells, and then performed qPCR to quantify remaining cccDNA.

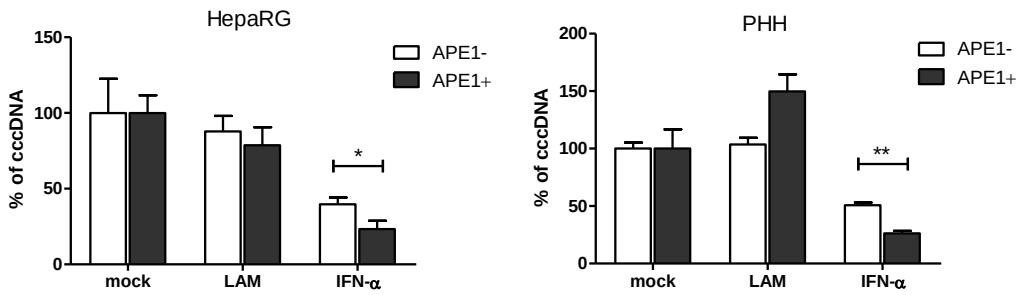


Fig.2.4.14 IFN-α induced AP sites in HBV cccDNA. DNA extracts from HBV infected HepaRG or PHH cells were incubated with APE1. HBV cccDNA was quantified by qPCR.

We observed that IFN-α degraded HBV cccDNA while LAM showed no effect as we demonstrated before (**Fig.2.4.14**). After incubation with APE1, LAM treated samples maintained cccDNA at the same level but in IFN-α treated samples we obtained further digestion of cccDNA in both HepaRG and PHH models. Thees results suggested that IFN-α treatment induces endonuclease-sensitive AP sites in cccDNA.

Taken together, these data implied that IFN-α reduces HBV cccDNA via base excision repair pathway. But, does this effective antiviral mechanism also affect genomic DNA?

2.4.6 IFN-α shows no effect on host genomic DNA

By using a commercial quantification kit, we compared AP site formation between HBV infected and uninfected HepaRG, and also evaluated the difference among mock, LAM treated and IFN-α treated HepaRG or PHH.

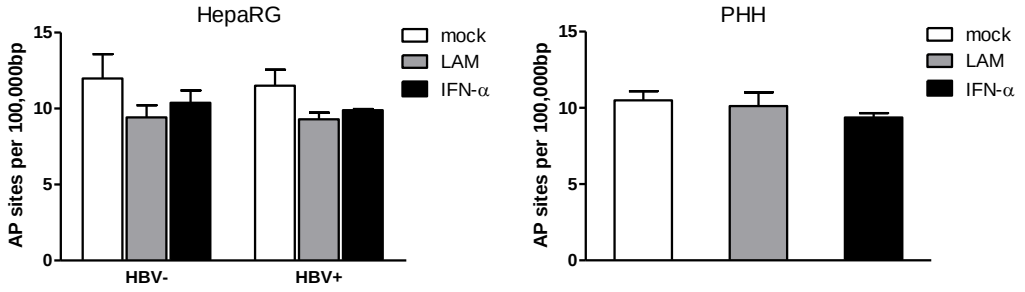


Fig.2.4.15 Genomic DNA total AP sites quantification. Cell total DNA was extract from HepaRG or HBV infected PHH cells, and AP sites were measured by quantification kit.

Total AP site quantification from genomic DNA shows no difference between HBV infected or non-infected HepaRG cells. And IFN-α treated or untreated samples from both HepaRG and PHH models have equal amount of AP sites in their genome **(Fig.2.4.15)**. The data indicated

that neither HBV infection nor IFN-α treatment stimulated AP site formation in the cellular genome.

Furthermore, we focused on the deamination process of genomic DNA. Prn-p gene, which used as a reference in our cccDNA qPCR, was chosen for 3D-PCR.

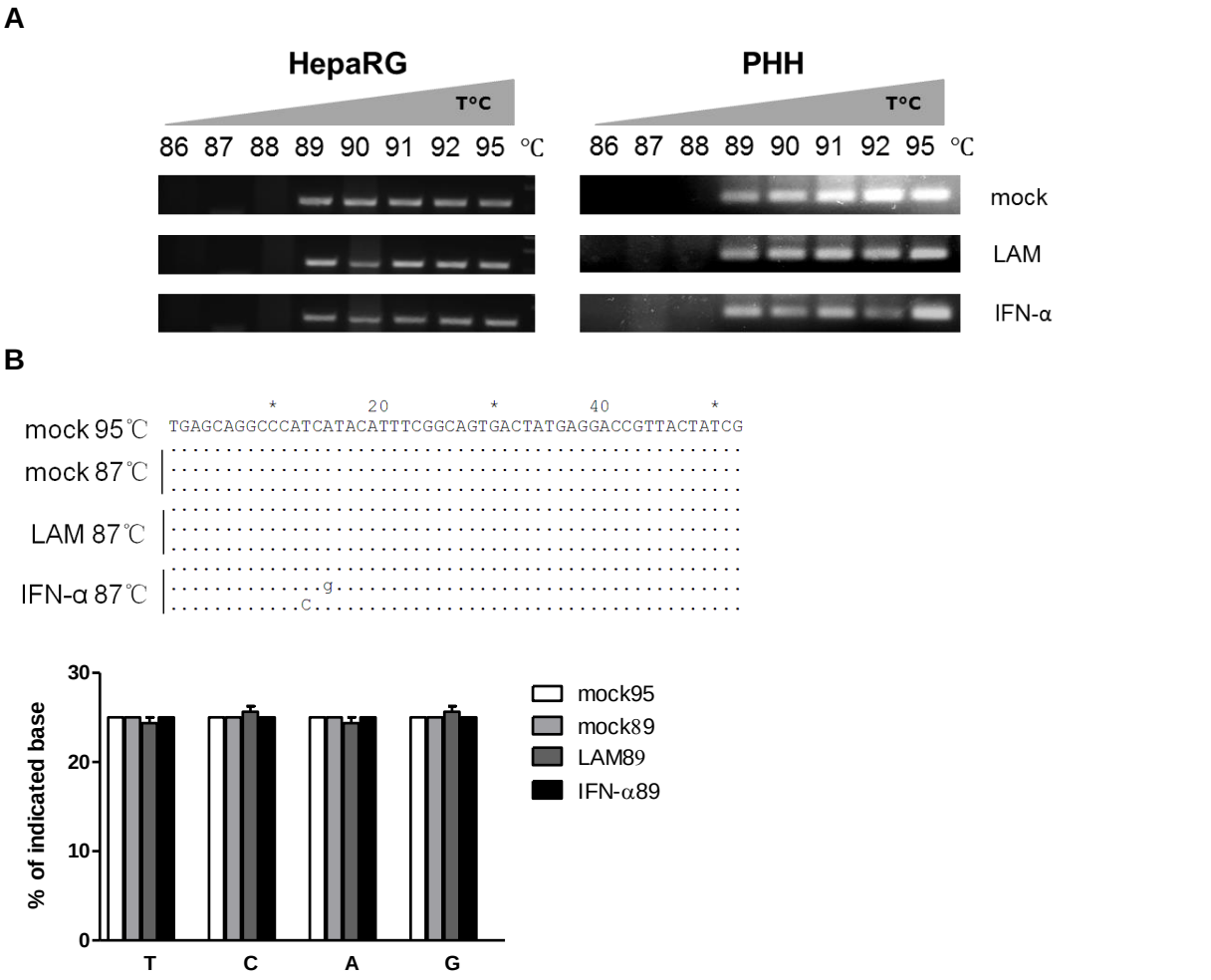
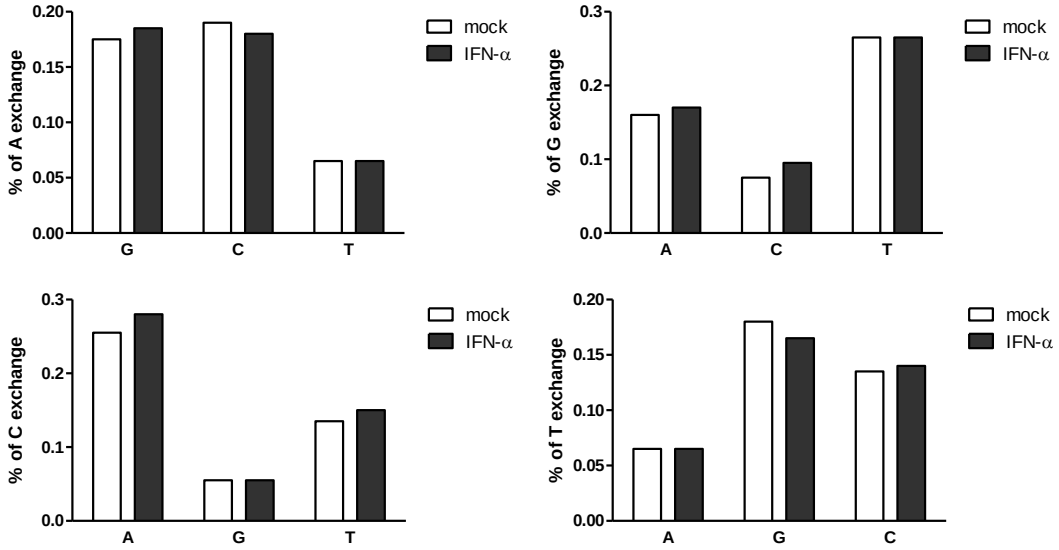


Fig.2.4.16 3D-PCR for Prn-p gene. (A)DNA extracts from HBV infected HepaRG or PHH with different treatment. 3D-PCR was used to detect Prn-p deamination. **(B)**PCR products with low denature temperature were cloned and sequenced. Different content of nucleotides were summed up.

According to the result, in both HepaRG and PHH models, the pattern of PCR products were identical among mock, LAM treated and IFN-α treated samples **(Fig.2.4.16A)**. We also cloned and sequenced low denaturing temprature PCR products, and no G to A transition was found in all samples **(Fig.2.4.16B)**.

To confirm that, we performed deep sequencing analyses for genomic DNA. Thirteen different gene regions were chosen: three housekeeping genes B2M, TBP, HMBS; two oncogenes MYC, SRC; three IFN stimulate genes MxA, PKR, PML; five other signal pathway related genes CXCL10, CXCL13, CCL17, CCL20, IL6.

A



B

Gene Name	Amplicon Length	Genomic Position	% GC	Average coverage	Overall Mutation Rate % (mock/IFN-α)	G-A Mutation Rate (mock/IFN-α)	C-T Mutation Rate (mock/IFN-α)
B2M	2157	Chr. 15: 15798407 - 15800563	40,10	85.543	0,31/0,31	0,050/0,045	0,055/0,050
TBP	2249	Chr. 6: 75031948 - 75034196	47,13	134.116	0,32/0,32	0,055/0,055	0,055/0,055
HMBS	2372	Chr. 11: 22521107 - 22523478	52,40	124.475	0,45/0,44	0,18/0,18	0,23/0,23
MYC	1794	Chr. 8: 42024572 - 42026365	49,39	3.191	5,5/5,3	6,6/6,4	3,5/3,2
SRC	1863	Chr. 20: 6168000 - 6169862	52,76	120.392	0,41/0,40	0,14/0,14	0,055/0,050
MxA	1882	Chr. 21: 28473387 - 28475268	49,15	4.631	3,4/3,2	1,1/1,1	2,6/2,4
PKR	1713	Chr. 2: 16170524 - 16172236	42,97	115.858	0,69/0,65	0,25/0,22	0,32/0,29
PML	2084	Chr. 15: 45116397 - 45118480	53,50	50.881	0,48/0,58	0,16/0,20	0,080/0,10
CXCL10	1577	Chr. 4: 1490756 - 1492332	37,67	103.739	0,44/0,44	0,16/0,16	0,055/0,055
CXCL13	1884	Chr. 4: 3074754 - 3076637	37,58	114.082	0,31/0,30	0,055/0,055	0,055/0,055
CCL17	2124	Chr. 16: 11062012 - 11064135	56,36	87.806	0,71/0,74	0,27/0,30	0,20/0,25

CCL20	1767	Chr. 2: 78889580 - 78891346	34,24	121.839	0,32/0,31	0,055/0,050	0,060/0,055
IL6	2070	Chr. 7: 22759130 - 22761199	45,17	118.519	0,41/0,45	0,13/0,13	0,11/0,15

Fig.2.4.17 Deep sequencing of genomic DNA. Thirteen different amplicons from different genomic DNA regions were submitted to deep sequencing. The overall % of base exchanged for each base **(A)** has been analyzed as well as the mutations rate for each amplicons. **(B)**

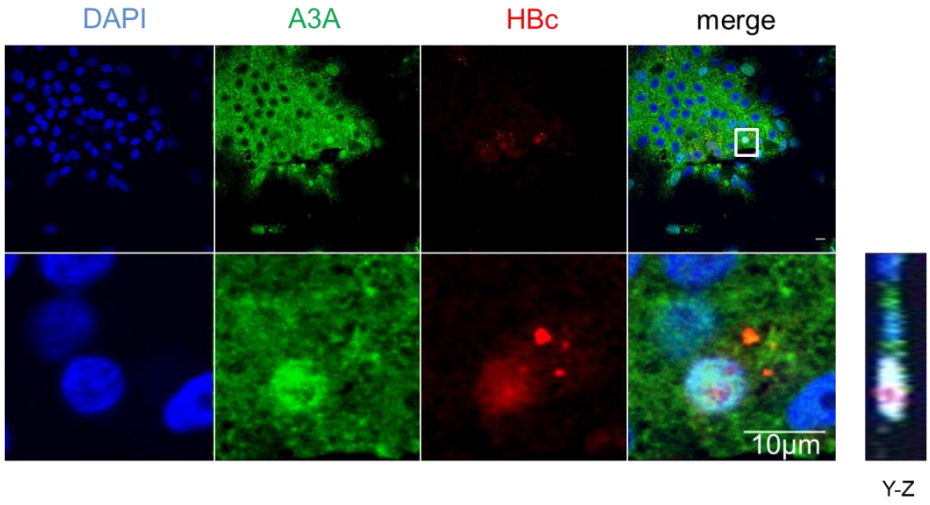
No mutation was induced by IFN-α treatment from overall mutation pattern (**Fig.2.4.17A**). When we focused on mutation detail of every single gene, we couldn't find that G to A or C to T mutation levels rised after IFN-α treatment (**Fig.2.4.17B**). Accordingly, MYC showed relatively high G to A mutation rate compared to other genes, which confirmed that MYC is a target of Activation-induced Cytidine Deaminase (AID) [241, 242]. The results also implied that our deep sequencing experiment was sensitive enough to detect genomic deamination.

Together these data indicated that IFN-α induced base excision pathway is specific for HBV cccDNA.

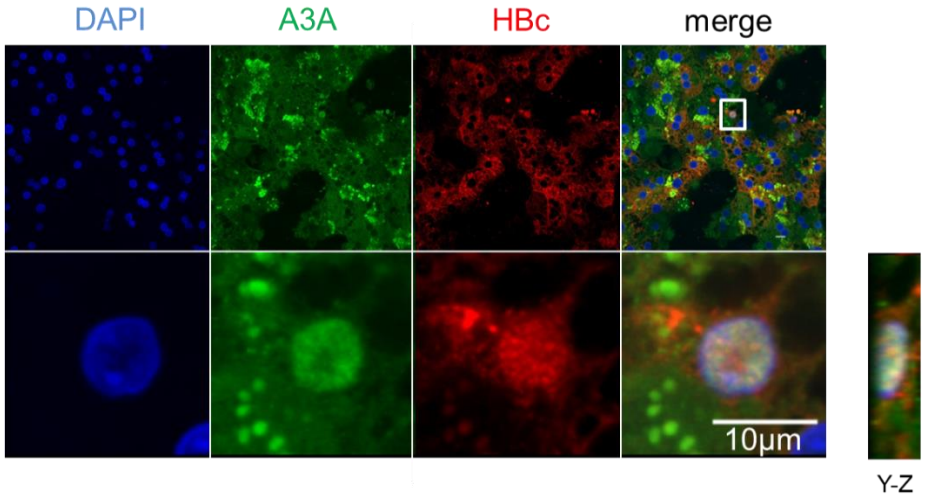
2.4.7 APOBEC3A utilizes HBV core to specifically targets cccDNA minus strand

To determine the mechanism of cccDNA specifity, we proposed APOBEC3A utilizes HBV core protein (HBc) to targets cccDNA. HBc is known to interact with APOBEC3G [243, 244] as well as HBV cccDNA [245, 246] and could thus physically link the nuclear APOBEC3 proteins to cccDNA.

A. HepaRG



B. PHH



C

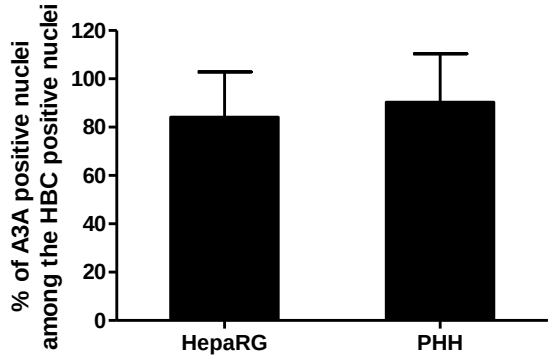


Fig.2.4.18 Immunostanning of APOBEC3A and HBc in HepaRG and PHH cells. Differentiated HepaRG **(A)** and PHH (donor3) **(B)** were infected with HBV for 7 days and then treated with 1000 IU/ml of IFN-α for three days. APOBEC3A and HBV core were determined by immunofluorescence staining. **(C)** Quantification of nuclear A3A is presented as the mean +/- standard deviation of different field. Scale bar = 10µm.

Confocal microscopy showed a co-localization of HBc and APOBEC3A in the nucleus of HBV-infected HepaRG cells **(Fig.2.4.18A)** and PHH **(Fig.2.4.18B)**. From statistics, more than 80% of APOBEC3A positive nuclei showed co-localization of HBc in both models **(Fig.2.4.18C)**.

To proof the association of APOBEC3A and HBc, co-Immunoprecipitation experiments were performed.

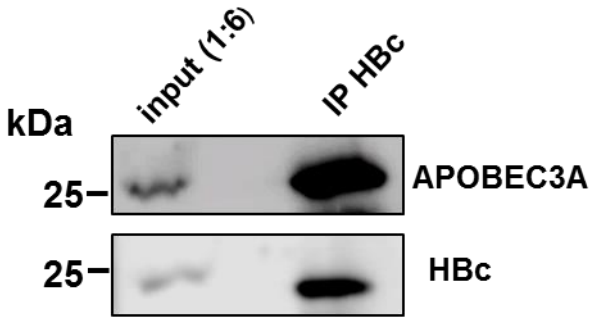


Fig.2.4.19 Co-Immunoprecipitation of APOBEC3A and HBc. HepG2-H1.3 cells transiently transfected with APOBEC3A expressing plasmid were lysed and protein immunoprecipitation was performed with an anti-HBc antibody followed by Western blot analyses to detect both APOBEC3A and HBc.

HBc co-immunoprecipitated APOBEC3A in HepG2H1.3 cells, which expressed high amounts of viral proteins, indicating physical interaction **(Fig.2.4.19)**.

To investigate if APOBEC3A can binds to cccDNA, Chromatin Immunoprecipitation experiments (ChIP) were performed.

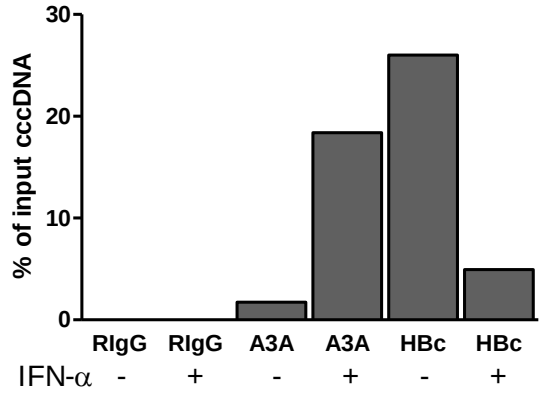


Fig.2.4.20 Chromatin Immunoprecipitation of APOBEC3A and HBc. HBV infected HepaRG after IFN-α treatment were lysed. Chromatin immunoprecipitation assays were performed with indicated antibodies. Immunoprecipitated chromatins were processed and analyzed by real-time PCR using cccDNA selective primers.

HepaRG cells were infected with HBV and treated with IFN-α. ChIP experiments confirmed that HBc and APOBEC3A both bind to cccDNA minichromosome but not rabbit IgG (RIgG) control **(Fig.2.4.20)**. Binding between cccDNA and APOBEC3A increased after IFN-α treatment, while in contrast, binding of HBc is decreased. These results indicated that IFN-α activates the induction of APOBEC3A and reduces total HBc.

Members of the APOBEC3 family are considered to be single-stranded DNA cytidine-deaminases [247]. For double-stranded DNA, exposing single-stranded DNA coupled with transcription is necessary for deamination [248-250]. G to A transition observed indicated a strand specific deamination.

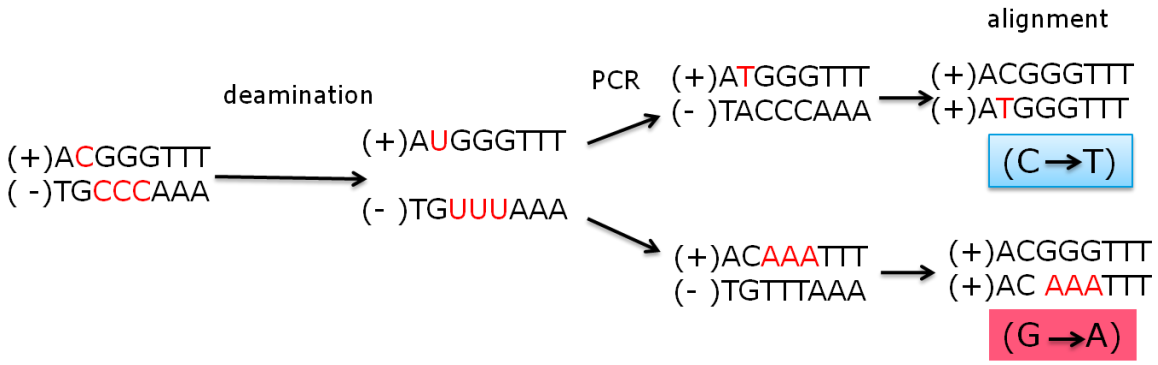
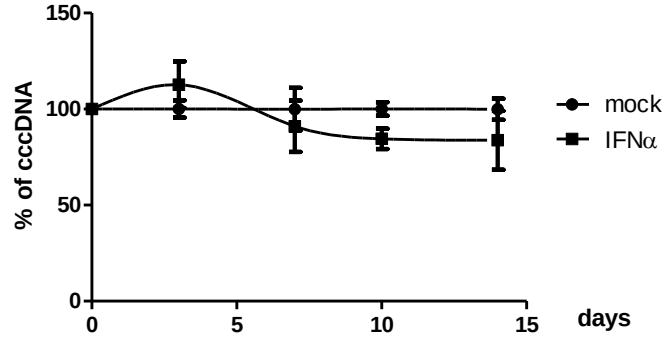


Fig.2.4.21 G to A transition and minus strand specificity.

Deamination is the biological process that removes an amine group from a molecule. For DNA, the consequence is that a cytosine loses its amine group and converts to uridine. If deamination occurs in both DNA strands, we will get C to U mutation in both strands as well. Since these two mutated strands are not paring to each other, during PCR amplification, there will be two different PCR products. When we perform alignment, we compare the plus strand sequence of PCR product with wild type. If the original deamination happened in plus strand, we will get C to T transition, otherwise, G to A transition. Since we only observed G to A transition, deamination only occured in cccDNA minus strand (**Fig.2.4.21**).

Because HBx is essential for cccDNA transcription [27], we then used HBV(X-) infection to investigate the role of transcription in cccDNA deamination.

A. HepaRG+ HBV(X-)



B. HepaRG-TR-X + HBV(x-)

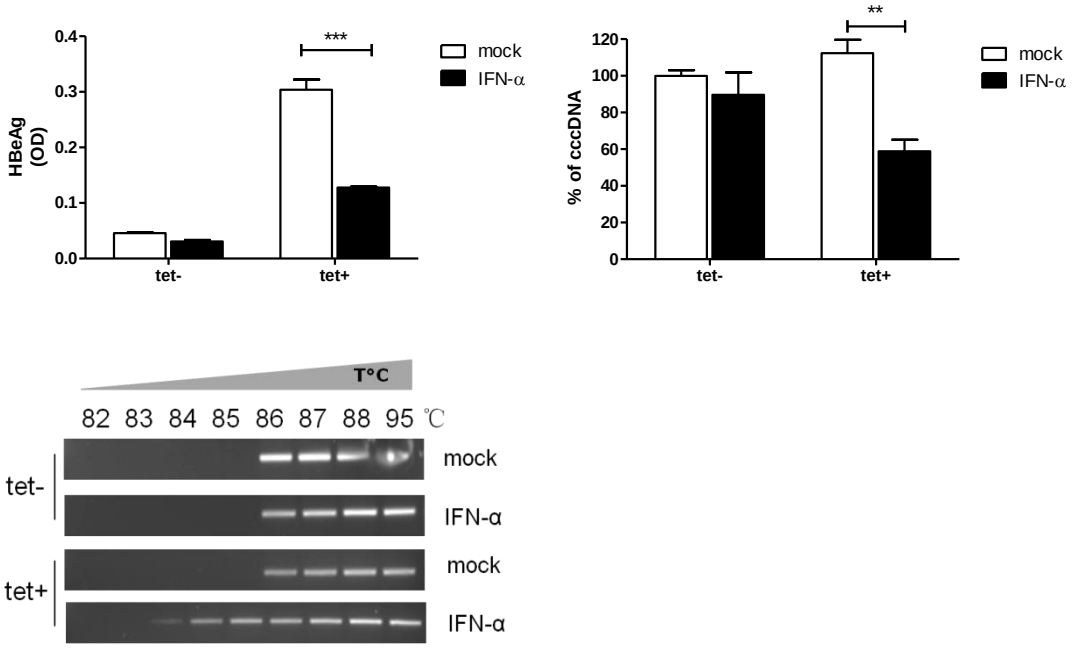


Fig.2.4.22 Transcription dependent deamination and degradation of cccDNA. (A) Differentiated HepaRG cells were infected with HBx deficient HBV (M.O.I=200).Cells were treated with 1000 IU/ml of IFN-α 10 days after infection (day0),and then cells were lysed at different time point for total DNA extraction. HBV cccDNA was measured by real-time PCR. 3D-PCR was performed to detect HBV cccDNA deamination. **(B)** Differentiated HepaRG-TR-X cells were infected with HBx deficient HBV (M.O.I=200).Cells were induced with or without 5μg/ml tetracycline (tet) and treated with or without 1000 IU/ml of IFN-α 10 days after infection(day0), HBV cccDNA were measured at day 7. 3D-PCR was performed to detect HBV cccDNA deamination.

When we use HBV(X-) infected HepaRG, cccDNA can be established without transcription activity. IFN-α treatment showed no effect on HBV(X-) cccDNA **(Fig.2.4.22A)**.When we use HBV(X-) infected and HBx inducible HepaRG, IFN-α was only active when we switched on cccDNA transcription by adding tetracyclin **(Fig.2.4.22B)**.

Taken together, these data suggested that HBc may help to target APOBEC3A to minus strand of cccDNA during transcription.

Noncytopathic HBV clearance has been described for more than 10 years [211], but the mechanism involved is still unclear. In our study, we described innate immunity such as IFN-α can exert degradation of HBV cccDNA, the matrix of HBV replication, via base excision repair pathway.

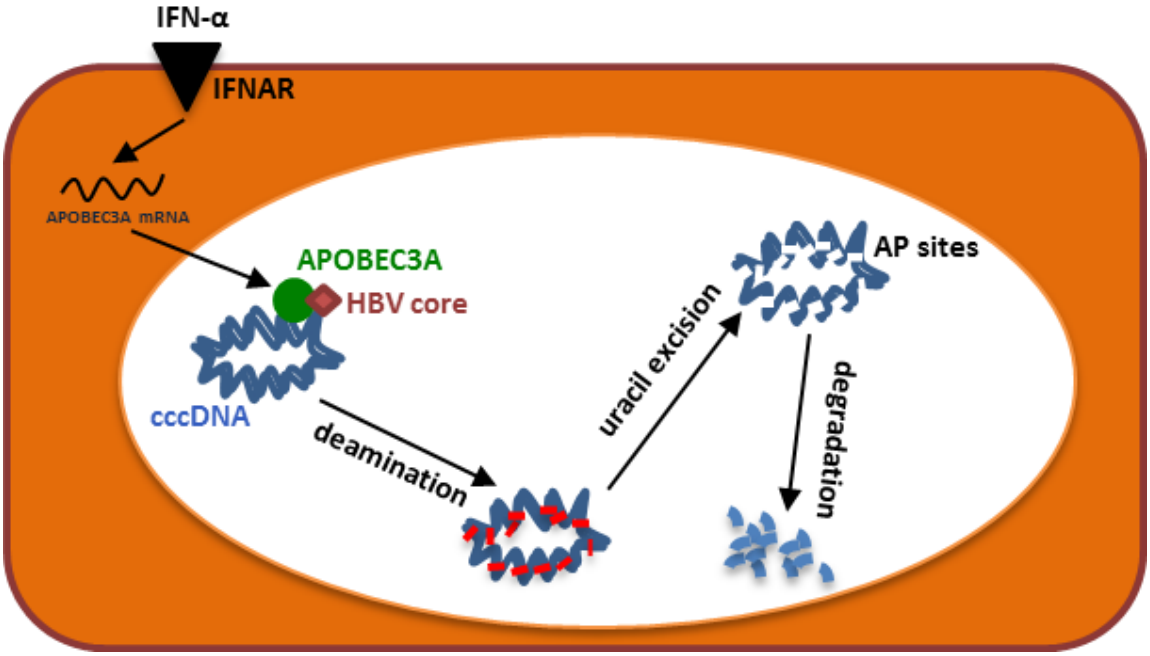


Fig.2.4.23 Proposed mechanism.IFN-α upregulates APOBEC3A in HBV infected hepatocytes. APOBEC3A utilize HBV core to target on HBV cccDNA and causes C to U conversion in minus strand of cccDNA. This mutation could be excised by uracil-DNA glycosylase. The resulting nuclease-sensitive abasic site could be further cleaved by endonuclease and lead to cccDNA degradation.

IFN-α upregulates APOBEC3A in HBV infected hepatocytes. APOBEC3A utilizes HBV core to target HBV cccDNA and causes C to U conversion in minus strand of cccDNA in a transcription dependent fashion. This uracil is then excised by uracil-DNA glycosylase.The resulting nuclease-sensitive abasic site can be further cleaved by endonucleases like APE1and lead to cccDNA degradation **(Fig.2.4.23)**.

3. Discussion

3.1 Murine IFN-α inhibits HBV replication

Bioassays are the method of choice for titrating IFNs because of their high sensitivity and because the results obtained in such assays indicate the proportion of the biologically active molecules present in the sample [251]. IFN-mediated protection of cultured cells against a challenge virus is determined either directly on the basis of a reduced cytopathic effect induced by the virus or indirectly in terms of reduced yields of the challenge virus. A challenge of IFN-pretreated cells with VSV is frequently used to quantify this cytokine because, on the one hand, the replication of VSV is highly sensitive to IFNs and, on the other hand, in unprotected cells this virus induces a rapid cytopathic effect that can readily be quantified. In this study, we used XTT test in substitution for traditional crystal violet staining, which highly increased the sensitivity of the test (**Fig 2.1.1**).

HBV was reported to interfere with IFN-α signaling [252-254]. To investigate whether IFN-α signaling is blocked in our model, we analyzed the induction of ISG, and we observed strong activation (**Fig 2.1.2**). These data don´t exclude the possibility of IFN-α signaling inhibition mediated by HBV, because we didn´t want to compare the situation with and without HBV replication, but wanted to show if we can study the effect on HBV. IFN-α response was not blocked in our experiments, and our model allowed us to investigate the antiviral effect of IFN-α.

In HBV-Met cell culture and HBVtg-mice, HBV replication is derived from the integration of an HBV1.3 fold genome [76, 81]. The integration is transcribed into different viral pre- and subgenomic RNAs. After the export of the RNAs into the cytoplasm, translation into viral proteins (such as HBsAg and HBeAg) takes place. The pregenomic RNA gets encapsidated and reverse transcribed into the HBV DNA genome. Since in both models, we observed the decrease of viral protein and HBV-DNA (**Fig 2.1.3 & Fig 2.1.4**), we concluded that IFN-α affect HBV-RNA transcription.

Throughout a long evolutionary history vertebrates have maintained numerous Type I interferons, while Type II IFNs are represented by a single member. Both human and mouse genome encodes several IFN-α subtype proteins, all of which apparently signal through the same receptor complex. The reasons for the high level of redundancy of Type I IFNs are unclear but they may include differences in tissue specific expression, temporal differences in expression and/or differences in the spectrum of activities by the different Type I IFNs

[255-257]. In our study, by transfection of different mIFN-α subtypes plasmids, we observed higher induction of ISG induced by mIFN-α4 and mIFN-α11 (**Fig 2.1.5**), which correlates with stronger HBV inhibition (**Fig 2.1.6A**). Purified mIFN-α directly treatment confirm a superior antiviral effect of mIFN-α4 and mIFN-α11 on HBV (**Fig 2.1.6B**).

The antiviral activity of IFN-α mainly depends on the interaction of IFN-α with its receptors [258, 259]. A recent study provided a baseline for the interaction of the different IFN-α subtypes with the receptor components [258]. The concordance of the binding with the activity for most of the subtypes suggests that receptor binding events play the major role in the activity profiles of these molecules. By analysis of protein sequence, we found several unique residues within IFNAR1 binding region of mIFN-α4 and mIFN-α11(**Fig 2.1.7**). We speculate these unique residues are related to antiviral potencies of mIFN-α subtypes. The differing activity-binding relationships highlight the limits of the currently available IFN therapeutics. Further investigation should focus on the important amino residues, and new approach to improve interferon such as protein design.

The discovery of the IFN-lambda (IFN-λ) family opened an exciting new chapter in the field of IFN research [260]. There are 4 IFN-λ genes that encode 4 distinct but highly related proteins denoted IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4. Collectively, they comprise the type III subset of IFNs. First three proteins are also known as interleukin-29 (IL-29), IL-28A, and IL-28B, respectively. IFN-λ4 is a newly identified gene that interferes with the clearance of hepatitis C virus infection [119], but the remains unknown. Further studies will explore molecular function of this novel protein in normal and disease conditions. Although type I IFNs and type III IFNs signal via distinct receptor complexes, they activate the similar intracellular signaling pathway and many of the same biological activities, including antiviral activity, in a wide variety of target cells. Consistent with their antiviral activity, expression of the IFN-λ genes and their corresponding proteins is inducible by infection with many types of viruses. The potential clinical importance of IFN-lambda as a novel antiviral therapeutic agent is already apparent [261], its effect on HBV should be investigated.

3.2 Characterization of IFN-α signaling pathway in hepatocyte cell lines

In vitro models are essential tools to study the interaction of HBV and its host cell during virus infection. In this study, we used HBV replication models based on different human hepatocyte cell lines to examine anti-HBV activity of IFN-α and compared them to primary human hepatocytes. The results show that IFN-α only elicits antiviral activity in HBV infected HepaRG cells, but not in HBV replicating Huh-7 or HepG2 cells. Detailed analysis of the IFN-

α signaling pathway in the different cell lines revealed that Huh-7 and HepG2 cells do not respond properly to IFN-α, but undifferentiated as well as differentiated HepaRG show the induction of IFN-stimulated genes similar to primary human hepatocytes. Although the amount of IFN-α/β receptors and p-STATs were comparable in all cell lines tested, nuclear translocation of p-STATs was far more efficient in HepaRG cells. Therefore only HepaRG cells and PHH are the suitable models for the study of IFN-α effect on HBV *in vitro.*

The major obstacle of HBV research on the development of drug and other therapies has been the lack of an efficient cell culture system or a readily available small-animal model, permissive for viral infection and replication. However, over the last forty years a multiplicity of models has been developed that allow the study of different steps in the HBV life cycle, and virus-host immune reactions. Different hepatoma cell lines have been established. For example Huh-7 or HepG2 can support the HBV life-cycle upon transfection with plasmids carrying an HBV-genome [72, 73]. Stably HBV-transformed cell lines like HepG2.2.15, HepG2-H1.3 have been generated by molecular cloning that produce high amounts of HBV particles [74, 75]. These were confirmed by our results (**Fig 2.2.1A,B**). Although HBV can be generated from transfected plasmid or HBV genomes integrated into host cell chromosomes, in these models the mode of viral replication is different from that in natural infection[262]. *In vitro* infection models are important for the experimental analysis of the early steps of the viral life cycle, including attachment, entry and uncoating, and in particular for studies of the underlying molecular interactions between viral particles and host cells.

Primary human hepatocytes have been used for several years as an *in vitro* model for a variety of investigations. They are not only dispensable in drug toxicity test and enzyme induction [263, 264], but also serve as an infection system for human pathogenic HBV due to the lack of infectable cell lines [265, 266]. In 1969, Berry and Friend for the first time used two step collagenase perfusion and successfully obtained primary hepatocytes from rat liver [267]. The method they introduced has become a standard protocol for PHH nowadays [268-272]. PHH show low mitotic activity *in vitro* [273, 274]. Culture of PHH for more than 3 months is possible [275] and PHH can retain phenotypic characteristics for a long culture period [276]. The use of PHH for in vitro experiment is limited because of lack of sources and difficulties in isolation and cultivation. Another disadvantage is the high variability of human cell preparations, which is mainly caused by individual hepatocyte donors. Thus, standardization of infection protocols and quantitative analysis of viral replication markers are the basis for the direct comparison of results from different experiment. Together with substantial improvement of in vitro infection and increased access to primary hepatocytes, PHH is still a valuable tool for basic investigation of HBV.

In 2002, HepaRG cell line was established. These cells become susceptible to HBV after differentiation under special cell culture conditions [38]. HepaRG is a human progenitor cell line capable to differentiate toward two different cell phenotypes, biliary-like and hepatocyte-like cells. It has been established from a liver tumor associated with chronic hepatitis C. This cell line represents a valuable alternative to *ex vivo* cultivated PHH, as HepaRG cells share some features and properties with adult hepatocytes. The progenitor nature of HepaRG cells and their ability to undergo differentiation toward biliary and hepatocyte phenotypes make them a very interesting tool for studying differentiation, liver metabolism, drug effect/metabolism/toxicity, hepatotropic viruses, and some aspect of carcinogenesis[277]. Currently, beside PHH, HepaRG is the only human cell line that can be efficiently infected and supports a complete viral life cycle, as shown in **Fig 2.2.1C**. Susceptibility of HepaRG cells to HBV infection is strongly dependent on the differentiation status induced by DMSO treatment as described [38], and well-defined culture conditions are required to achieve successful infection [218]: a long incubation phase of HBV virions with differentiated HepaRG cells is requested for a productive infection. Antiviral innate response during HBV infection remains an open question. In the case of HBV infection of HepaRG, it is difficult to quantify a potential interferon response, as this response is likely unregularly [278], meaning that the expression of endogenous interferon may be weak and restricted to few cells, and that the overall response does not need to be intense to be efficient. Due to the island-like structure of hepatocyte-like cells together with the fact that only few cells are infected by the virus, it explains why this response in the case of HBV, which is not a strong inducer of innate response and is very efficient to inhibit IFN signaling [252, 279], has not yet been evidenced. When the replication of HBV is artificially enhanced by using recombinant baculoviruses carrying HBV genome to establish infection [280], IFN-β response specific of HBV can be induced in HepaRG cells, which can lead to a non-cytopathic clearance of HBV [281].

IFN-α is used to treat viral infections as well as various cancers [187, 282]. However, the development of resistance to IFN-α has been a significant drawback. For our study, we need *in vitro* models susceptible to HBV infection as well as activation of IFN-α signaling pathway. Since we found Huh-7, HepG2 and HepG2 derived cell line were resistance to IFN-α but not PHH or HepaRG (**Fig 2.2.1, Fig 2.2.3**), which may due to the same reason why many individuals don´t react to IFN-α treatment during clinic therapy. It has been shown that cells express variant form of IFN-α/β receptors [283], and that´s maybe important for the anti-viral effects of IFN-α. Another study demonstrated that deficiency in ISGF3 components causes interferon-resistance of human melanoma cells [284]. In our case, we observed the translocation of ISGF3 was blocked in Huh-7, HepG2 and HepG2-H1.3 (**Fig 2.2.5**), these may due to different reasons. Many factors are involved in this process. The N-terminal

domain of STAT1 is essential for dimerisation [285], and mutation may cause the failure of dimerisation and nucleus translocation. Mutation or dysfunction of nuclear localization signal is another potential reason. Interferon regulatory factor 9 (IRF9) is an important component for ISGF3, so the difference of IRF9 can lead to difference consequence of nucleus translocation. Interferon stimulation triggers tyrosine phosphorylation of STAT1 at position 701, which is associated with switching from carrier-independent nucleocytoplasmic shuttling to carrier-mediated nuclear import. Unlike most substrates that carry a classical nuclear localization signal and bind to importin alpha1, STAT1 possesses a nonclassical NLS recognized by the isoform importin alpha5 [286]. So the variance of different importins may contribute to IFN-α response. Further study may focus on these possible reasons.

3.3 Interferon inducible secreted factors restrict HBV binding

In the present study, we found a novel antiviral mechanism of IFN-α targeting the HBV binding step. IFN-α treated cell culture supernatant restrict HBV infection. The inhibition is led by one or more secreted interferon induced proteins. The eluate from heparin column showed the same effect. This result indicates that the proteins in the IFN-α treated cell culture supernatant can bind to heparan glycosaminoglycan -the unspecific attachment receptor of HBV, leading to binding of HBV. Mass spectrometry analysis of the sample revealed complement factor H and fibronectin 1 maybe the induced factors. Future studies aimed at characterizing the IFN-induced soluble secreted factors using different method such as SILAC labeling and comparative proteomics.

Over the past 20 years, the development of novel therapies for the treatment of human pathogenic viruses provided an entire array of new antiviral drugs [287]. So far, antiviral development focused mainly on small-molecule drugs. However, this is gradually changing, as biologicals, including nucleic acids (eg, microRNA), antibodies, and peptides, are being tested in viral disease [288]. First, biologics are more specific than traditional low molecule drugs, which also lead to fewer side effects. For example in HBV T cell therapy, killing cells could recognize HBV infected hepatocyte which express HBV antigen but ignore healthy cells [289]. Second, administering a biologic often uses the body’s own physiological system. A chemical drug can inhibit a reaction, but a biologic can do that and also recruit the immune system to help out. Third, biologics have longer half-life in the human body. A traditional chemical drug has a half-life of a few to several hours, so a patient might need to take a new pill several times a day. An antibody can stay active for several days or even weeks, so one injection can remain effective for weeks [290]. While in HBV life cycle, quite some steps are potential targets for therapeutic intervention. But with no doubt, virus binding and entry are

most essential steps in initiating an infection and those steps are conserved among different genotypes, which makes them even more attractive therapeutic target.

Initial attachment of HBV to susceptible hepatocytes occurs via heparan sulfate proteoglycans that are presumably used to concentrate the virions on the cellular surface [43, 44]. That´s the reason we use heparin column to study HBV binding (**Fig 2.3.5**). Such primary attachment is relatively unspecific and occurs with low-affinity [15, 291]. Thereafter, HBV binds via its preS1 domain to sodium taurocholate cotransporting polypeptide (NTCP) [45] or any other unknown high-affinity receptor to actively promote further entry steps [15, 44, 292, 293]. In addition to the receptor-binding site in the preS1-domain of the LHBs, a determinant of infectivity resides within the S-domain of LHBs possibly needed for fusion [294].

Heparan sulfate is ubiquitously expressed on the surfaces or in the extracellular matrix of virtually all cell types. It normally binds to a wide variety of growth factors, chemokines, enzymes and matrix components [295], and has been reported to play an important role in the attachment of a number of viruses [296]. The first viral strain reported to use heparan sulfate as a receptor is HSV type 1. Heparan sulfate carrying a specific sulfation pattern interact with virus and functionally equals a tranditional protein receptor [297]. Although the overall picture is still far from complete, it has become clear during the past few years that cellular heparan sulfate is used as a receptor by a growing number of viruses, including DNA virus and RNA virus, some of which can cause severe disease epidemics in humans, such as HIV, HBV, HCV, Dengue Virus and HPV [298-302]. Thus it has been proposed that the binding of the viral surface to heparan sulfate may play an important role in viral pathogenicity. That further underscores the importance of IFN induced factors which may not only inhibit HBV but also effect on other viruses.

Specific inhibition of virus entry is an attractive therapeutic target not only for acute but also for chronic viral infections. In the case of chronic infection entry inhibition prohibit infection from spreading to naive cells, which together with antiviral therapy eliminate infected cells provide higher chance of cure. For example, in HIV infection this has been accomplished with the interference of virus entry using a gp41 protein derived peptide, enfuvirtide, which prevents fusion of the virus with the host cellular membrane [303]. The discovery of interferon induced binding inhibitors immediately posed the question of its property in therapeutic development of HBV infection might be useful for the treatment of HBV infections. Previous study demonstrated that acylated HBV preS-derived lipopeptides targeting viral envelope protein component can prevent interaction of HBV with its cellular receptor thus prevent *de novo* HBV infection in humanized mice [304]. Although there is no evidence

indicating that HBV can propagate by using cell to cell transmission, numerous enveloped viruses have shown to employ modes of spreading involving both direct cell-cell transmission or the release of progeny viruses into the extracellular space [305]. Further study showed the myristoylated preS-derived peptide Myrcludex-B could block HBV cell to cell dissemination among human hepatocytes in the liver of humanized mice [306]. All these studies together with our data indicate that HBV entry or binding inhibitors are highly interesting in application like preventing vertical transmission during birth, prevention of re-infection after liver transplantation, or possibly in chronic HBV infection in combination with established therapies.

Although the IFN-α induced antiviral response is known to be multifaceted for a long time, it has been thought that IFN-α had no effect on the virus life cycle at the step of binding or entry. Until recently, the interferon-inducible transmembrane (IFITM) protein family has been shown to block early stages of viral infection [307, 308]. IFITM was originally identified through RNAi genetic screening and was shown to inhibit infections of VSV, influenza A virus, West Nile virus and Dengue virus. Later IFITM proteins were found to potently restrict entry and infections by a number of highly pathogenic viruses, including HIV-1, filovirus, HCV and SARS coronavirus [309-314]. Recently, researchers identified cholesterol-25-hydroxylase (CH25H) as a broad antiviral ISG [315]. CH25H converts cholesterol to a soluble antiviral factor, 25-hydroxycholesterol (25HC). 25HC treatment in cultured cells inhibited growth a broad group of enveloped viruses including VSV, HSV, HIV, and MHV68 and acutely pathogenic EBOV, RVFV, RSSEV, and Nipah viruses. It suppressed viral growth by blocking membrane fusion between virus and cell. The effects of IFITM and 25HC on HBV are still unknown, further investigation should be performed.

Compared to other antiviral effects of IFN-α, the inhibition of virus binding is somewhat less dramatic and probably only serves as a first line of defense. In our case, the inhibition is only 20% to 40% (**Fig 2.3.2, Fig 2.3.5**). Although viral uptake is only partially blocked by IFN-α treatment, at low M.O.I this level of inhibition could protect a significant proportion of a cell population from infection.

Mass spectrometry is already a well-established protein identification tool. We analyzed three distinguished bands from SDS-PAGE, but there may be still a low abundant but active factor which invisible on the PAGE gel. So if we want to explore all possibilities, other methods should be applied. Recent methodological and technological developments have made quantification of protein abundance in the extraction possible in studies. Several strategies have been developed which differs in specific measurement and later on analysis wokflows have been developed. For example, SILAC - stable isotope labeling by amino acids in cell

culture and ICPL- Isotope coded protein label. Currently, we are analyzing whole elution from heparin column by SILAC.

From mass spectrometry, around 40 proteins were identified (**Table 2.3.1**). We screened the result based on cellular localization, protein size, heparin binding ability and interferon inducibility, we speculate complement factor H and fibronectin could be the interferon induced secreted viral binding inhibitor we were looking for.

Complement factor H, alternatively factor H, is one of the regulators of complement activation family and is a complement control protein. It is a large soluble glycoprotein that circulates in human plasma (at typical concentrations of 200–300 μg/ml [316]). The major source of factor H is the liver, and IFN-γ stimulation can up-regulates factor H expression [317-319]. Its physiological function is regulating the alternative pathway of the complement system, ensuring that the complement system is directed towards pathogens or other dangerous material and does not damage host tissue. Factor H exerts its protective action on cells via binding to glycosaminoglycan, which prevents the immune system from attacking a person's own cells, but allows it attack against bacteria and viruses which have no glycosaminoglycan on their surface [320, 321]. Because factor H is a key regulator of complement activation, some bacteria have manipulated its function to evade complement system detection, as first reported for the M6 protein of Streptococcus pyogenes [322]. In the currently favored model, factor H is recruited to M protein to protect the bacteria from complement attack and rapid killing, in particular through phagocytosis [323, 324]. Immune evasion mechanisms by viruses utilizing factor H have not been described. But what has been shown is that hepatitis B infection is associated with factor H dysfunction [325], and some factor H gene variants were associated with advanced fibrosis stages in HCV infection [326].

Fibronectin is a high-molecular weight (~440kDa) glycoprotein of the extracellular matrix that binds to membrane-spanning receptor proteins integrins [327]. Similar to integrins, fibronectin binds extracellular matrix components such as collagen, fibrin, and heparan sulfate proteoglycans. Fibronectin exists as a protein dimer, consisting of two nearly identical monomers linked by a pair of disulfide bonds. The fibronectin protein is produced from a single gene, but alternative splicing of its pre-mRNA leads to the creation of several isoforms. Two types of fibronectin are present in vertebrates.Soluble plasma fibronectin, a major protein component of blood plasma (300 μg/ml), is produced in the liver by hepatocytes. Fibronectin plays a major role in cell adhesion, growth, migration and differentiation. It is important for processes such as wound healing and embryonic development. Serum fibronectin levels are generally decreased in pathological blood coagulation and inflammation [328]. Decreased level of serum fibronectin in patients with chronic hepatitis B before

interferon treatment is related to hepatic injury and inflammation [329]. Increased levels of serum fibronectin in patients having interferon therapy are important and are related to the effects of interferon including antiviral property in patients with chronic HBV infection. One study showed that human liver fibronectin binds HBV in vivo by the pre-S2 region-encoded epitopes in a species-restricted manner [330]. And binding of the circulating virus could inhibit subsequent viral uptake by hepatocytes. A recent study showed a correlation between development of hepatic fibrosis and decrease of plasma fibronectin concentration in adult patients with chronic liver disease [331]. Therefore, the serum level of fibronectin may be a useful marker of hepatic fibrosis in chronic liver disease and interferon may be an important drug for prevention of liver fibrosis. Fibronectin may be also a useful marker in predicting IFN response.

3.4 An IFN-α induced base excision repair pathway leads to HBV cccDNA degradation

We achieved HBV cccDNA degradation by using high dose of IFN-α on cell cultures. As a treatment for chronic hepatitis B patient, the dose of IFN-α applies is relatively low. That may explain why only less than 20% of patient get viral clearance upon IFN-α treatment [332]. Clinical studies demonstrated that only IFN-α but not nucleos(t)ides analogues may lead to cccDNA decline[213, 333], which is in the same line with our observation **(Fig.2.4.4)**. The cccDNA deamination we observed from patient liver biopsy (personal communication with Yong Li, Tongji hospital, Wuhan, China) together with former study on serum HBV-DNA deamination [334] further support the idea that IFN-α can induce deamination of HBV genome as well as cccDNA *in vivo*. The background level of APOBEC3 as well as cccDNA deamination suggests one possible reason for viral clearance during acute phase.

APOBEC3 family members are considered as an innate defensive network against several viral infections. Their expression patterns varied in different human tissues: lower in skeletal muscle, heart brain and relatively higher in spleen, thymus and lymph node [163]. The mRNA level of APOBEC3F and APOBEC3G are higher than other APOBEC3s in the liver. With APOBEC3A, it was reported to express at a higher level in monocytes comparing to other family members [335]. Different APOBEC3 members can be induced by IFN-α, IFN-γ, poly I:C,TNF-α or LPS through different signaling pathways [168, 336, 337]. In this study, we described IFN-α stimulates APOBEC3A via JAK-STAT signaling in hepatocytes **(Fig.2.4.8, Fig.2.4.9)**, which in accordance with the observation on other cell types or studies [163, 168, 337, 338]. Subcellular localization of these APOBEC3 proteins affect their functions. Our results indicate APOBEC3A is a nucleus deaminase while APOBEC3F and APOBEC3G mainly exist in cytoplasm **(Fig.2.4.11)**, which in accordance with published studies [168, 228,

339, 340] . Among all APOBEC3 family members, APOBEC3A is the only one showing ability to lead to deamination and degradation of foreign DNA [236, 341]. Not like other nucleus deaminases, APOBEC3A is always exist in the nuclear during mitosis[340]. This property provides an opportunity to clear viral infection and prevent *de novo* HBV infection during liver regeneration.

The normal outcome of base excision is repairment of DNA by cellular DNA repair machinery. Using a mixture of various enzymes, we were able to repair *cccDNA in tubo* **(Fig.2.4.13)**. We currently don´t know why cccDNA is degraded instead of being repaired, but there are several possibilities: First, the number of AP sites introduced after IFN-α treatment induced deamination is too high thus DNA repair machinery was overloaded. Second, type I and II interferon were reported to down-regulate DNA repair capacity [342, 343], these could shifts the equilibrium towards cccDNA degradation rather than repairment by modulating components of the repair machinery. Third, HBV interferes with cellular DNA repair protein, which could also influence the recovery of cccDNA. Some studies revealed HBx interacts with several DNA repair proteins such as XAP-1, XPB and XPD [344-346], which support our results that there was no cccDNA degradation without HBx **(Fig.2.4.22)**.

Interplay between HIV-Vif and APOBEC proteins **(Fig.2.4.10)** raise an interesting question about HIV-HBV co-infection. The course of acute HBV infection may be modified in the presence of HIV infection, with lower rates of spontaneous clearance of HBV [347]. Persons with HIV and chronic HBV co-infection have higher levels of HBV DNA and lower rates of HBeAg clearance [348-350]. Studies of IFN-α therapy in HIV-HBV co-infected patients suggest a decreased response compared to patients without HIV infection [351, 352]. All these studies, together with our results propose that HIV may attenuate innate response against HBV by interact with APOBEC3 proteins. It also reminds us to be more cautious to choose proper treatment for HIV-HBV co-infected individuals.

Genomic damage may cause carcinogenesis, as demonstrated by AID in B cell lymphomagenesis [353] and, recently, APOBEC3B in breast cancers [339] . Although IFN-α as a well-practiced treatment brings no safety issue, it still raise a concern about the potential problematic effect of IFN-α induced APOBEC3A on genomic DNA. In this study, we used three different methods to check genomic DNA damage after IFN-α treatment. We couldn't find genomic AP site formation **(Fig.2.4.15)**. No genomic deamination was detected by 3D-PCR **(Fig.2.4.16)** and no mutation found by deep sequencing **(Fig.2.4.17)**. We propose that the specificity of APOBEC3A targeting HBV cccDNA is due to HBc. It has been shown that APOBEC3G can binds to the capsid protein of HIV [354]and HBV [243, 244]. APOBEC3A has significant homology to the C-terminus of APOBEC3G, and reported binds to HIV

nucleoprotein complex [228]. Here, we reported that APOBEC3A interact with HBV core protein as well by using two different methods **(Fig.2.4.18, Fig.2.4.19)**. HBc preferentially binds to HBV-DNA and arranges HBV cccDNA mini chromosome [245, 246]. After the association of HBc with APOBEC3A, APOBEC3A might bring to close contact with cccDNA. Our ChIP data confirmed this hypothesis **(Fig.2.4.20)**. From cccDNA kinetic, we observed a fast decline in the beginning and then getting slower during IFN-α treatment **(Fig.2.4.3B)**. That may due to the absence of sufficient HBc. According to former studies, APOBEC3 proteins only target single stranded DNA. Since we could only observe G to A transition, which confirms that APOBEC3A selectively deaminates minus strand. With the results of HBV(X-) infection experiment **(Fig.2.4.22)**, we conclude that transcriptional activation of cccDNA is necessary for APOBEC3A to access single strand when transcription bubble formed. Since HBc was reported to involves in viral transcription [245], the transcription dependent activation of APOBEC3A can be explained by its interaction with HBc. Although it was described that HBc may also bind to cellular DNA [246], we could not detect any binding between APOBEC3A and genomic DNA (Daniela Stadler, master thesis). One recent publication demonstrates that human tribbles 3 protein, serves as a guardian for the genomic DNA, interacts with APOBEC3A and keep it away from the genome [355]. These data imply that there are multiple mechanisms exist to regulate APOBEC3A and protect host genome.

Current treatments for chronic hepatitis B are not satisfying. Although IFN-α treatment achieved viral clearance in some cases, but majority of the patients don´t response well. Due to the side effects, it´s impossible to use comparable high dose of IFN-α as we used for cell culture to patients. Novel antivirals directly targeting cccDNA is of highly importance to curing HBV infection, and several approaches can be considered. First is to investigate new interferon treatments. There are several novel interferons in development or under clinic trail, such as Albinterferon-α-2b, Omega IFN, IFN-alpha-2b-XL and Pegylated Interferon Lambda [356]. The effect of these new treatments should be carefully evaluated. Second, since different APOBEC3 protein can be induced via different signaling pathways [168, 336, 337], the ligands of these pathways should be tested as potential treatments. Third, a focus could be put on developing small molecules directly activating APOBEC3A promoters to increase expression of APOBEC3A concomitantly or separately in an IFN independent manner. In contrast to treatments with nucleos(t)ides analogues, up-regulation of certain deaminases could potentially lead to a complete cure of HBV infection within a shorter time frame since they directly target cccDNA. Our study opens new possibilities for the development of novel and safe treatments for complete eradication of chronic HBV infection.

4. Summary

Hepatitis B virus chronically infects about 400 million people worldwide. IFN-α, the only licensed immunomodulatory drug for hepatitis B therapy, has been used for more than 20 years and is the only drug that may induce virus clearance, but its definite mode of action is still unclear.

By using mouse based *in vitro* and *in vivo* models (HBV-Met and HBVtg-mouse), we investigated the antiviral effect of murine IFN-α on HBV replication. mIFN-α restricts HBV-DNA and antigen secretion in both models, which means the inhibition is on HBV-RNA.

To answer the question whether there are differential antiviral activities between mIFN-α suptypes, we used either overexpression of mIFN-α suptypes by plasmid transfection or direct mIFN-α treatment on HBV-Met cells. We observed various induction level of ISG, which correlated antiviral activity. To figure out the difference on molecular level, protein sequences were alignment. Result revealed several potential important residues in mIFN-α receptor binding region.

There are differences between human and murine interferon signaling, and mouse models do not support real HBV life cycle. It is necessary to find out human cell culture system for the IFN-α mechanism study. We used HBV replication models based on different human hepatocyte cell lines to examine anti-HBV activity of IFN-α and compared them to primary human hepatocytes. The results showed that IFN-α only elicits antiviral activity in HBV infected HepaRG cells, but not in HBV replicating Huh-7 or HepG2 cells. Detailed analysis of the IFN-α signaling pathway in the different cell lines revealed that Huh-7 and HepG2 cells do not respond properly to IFN-α, but undifferentiated as well as differentiated HepaRG show a similar strong induction of IFN-stimulated genes as primary human hepatocytes. Although the amount of IFN-α/β receptors and phosphorylated STAT proteins were comparable in all cell lines tested, nuclear translocation of p-STATs was far more efficient in HepaRG cells. Therefore HepaRG cells as well as PHH are the suitable models that allow studying the mechanism of IFN-α affects HBV. Using these models to further study the effects of IFN-α on HBV, we found that IFN-α inhibits HBV replication on different levels.

In differentiated HepaRG cell culture, IFN-α treated cell culture supernatant restrict HBV replication. The inhibition is led by one or more secreted interferon induced proteins. When we applied the cell culture supernatant to heparin column and the elution from the column showed same effect. This result indicates that the proteins in the IFN-α treated cell culture

supernatant can bind to heparan sulfate, the unspecific receptor of HBV, to interrupt the binding step of HBV. Mass spectrometry analysis of the sample revealed complement factor H and fibronectin 1 maybe the viral binding inhibitors. Future studies aimed at characterizing the IFN-induced soluble secreted factors use different method such as SILAC.

Persistent of cccDNA in HBV infected hepatocyte is the major problem of chronic hepatitis B treatment. We investigated if IFN-α has an effect on the stability of HBV cccDNA, the template of HBV transcription and replication. In HBV infected primary human hepatocytes and HepaRG cells, IFN-α significantly reduced HBV cccDNA. HBV cccDNA specific 3D-PCR indicated sequence alterations. Sequence analysis showed C to U transition of the HBV cccDNA minus strand after IFN-α treatment. A detailed analysis of the underlying mechanism after IFN-α treatment revealed upregulation of the APOBEC3 family cytidine deaminases A3A and A3G in both cell types in a time and dose dependent manner. JAK-STAT signaling blockade or HIV-Vif expression proved that IFN-α induced cccDNA deamination by A3 lead to cccDNA degradation. Subcellular localization analysis and overexpression experiments demonstrated that A3A, which locates to the nucleus, was the active effector. Treatment of cccDNA with a DNA repair enzyme cocktail corrected all mutations indicating that uracil generated by deamination could be removed by uracil-DNA glycosylase inducing apurinic/apyrimidinic sites. AP endonuclease reduced cccDNA levels in IFN-α treated cells showing that the cccDNA can be further digested by this endonuclease. We did not observe any deamination of host genomic DNA upon IFN-α treatment by 3D-PCR or deep sequencing. This suggested that A3A is directed specifically to viral DNA. Since A3A co-localized with HBV core protein in confocal microscopy and interaction was confirmed by co-immunoprecipitation, we propose that A3A utilizes HBc to get access to cccDNA. Chromatin immunoprecipitation confirmed that both HBc and A3A were bound to the cccDNA minichromosome. In HBV(X-) infection, reduction of cccDNA by IFN-α depended on trans-complementation with HBx, which is required to activate cccDNA transcription and HBc expression. This as well as preferential mutation of the HBV minus-strand indicated transcription dependence. The results allowed us to make a conclusion that IFN-α is able to trigger HBV cccDNA deamination and subsequent degradation by endonucleases via the base excision pathway. C to U transition is due to APOBEC3A activation and depends on active transcription of HBV cccDNA. APOBEC3A utilizes HBc to specifically targets cccDNA minus strand during transcription.

Put all together, IFN-α is a multifunctional cytokine able to inhibits several steps of HBV life cycle. We confirmed the HBV-RNA degradation caused by IFN-α treatment which has been

published before. More importantly, we described two novel antiviral mechanisms. IFN-α inhibits HBV binding and in addition leads to HBV cccDNA degradation.

The whole study established experiment models for IFN-α and HBV study, leads to a better understanding of the innate immune antiviral function of IFN-α and opens new options for the development of novel and safe treatments for eradication of chronic hepatitis B infection.

5. Materials and methods

5.1 Materials

5.1.1 Cell cultures

HepaRG were cultured in Williams E medium (Gibco) supplemented with 10% fetal calf serum Fetalclone II (Hyclone), 20mM L-glutamin(Gibco), 50 U/ml penicillin/streptomycin (Gibco), 80µg/ml gentamicin (Ratiopharm), 0.023IE/ml human insulin (Sanofi-Aventis) and 4.7µg/ml hydrocortisone (Pfizer).Same medium containing extra 10µg/ml Blasticidin(InvivoGen) were used for HepaRG-TR-X cells as described [27]. The cell cultures were maintained in a 5% CO2 atmosphere at 37 °C. For differentiation and infection, cells were maintained for 2 weeks in standard medium and then for 2 more weeks in medium supplemented with 1.8% DMSO (Sigma).

HepaRG-TA-VifAU1 cell line was obtained after transduction with a lentivirus carrying doxycycline inducible HIV-Vif gene. Briefly, HIV-Vif gene with AU1 tag was cut out from pCG-NL43VifAU1-IRES-GFP plasmid, and cloned into Plvx-Tight-Puro lentiviral vector (Clontech). Then the construct was transfected into HEK-293T cells together with Lenti-X HTX Packaging Mix. Lentivirus from the cell culture medium was concentrated by Amicon Ultra-15 (Millipore).HepaRG standard medium with 0.25 µg/ml Puromycin was used to maintain the cell line after transduction.

Primary human hepatocytes (PHH) were isolated from surgical liver specimens obtained during partial hepatectomy and seeded onto plastic dishes coated with collagen type IV plates in HepaRG differentiation medium. Informed consent was obtained from each patient, and the procedure was approved by the local Ethics Committee.

HepG2, HepG2-H1.3 and HepG2-H1.3-A3A were seeded on collagen coated culture dishes. Therefore, dishes were incubated with a collagen solution (Serva, Heidelberg) diluted 1:10 in sterile ddH2O water for at least 30 min at 37°C. Cells are maintained in complete DMEM medium at 37°C and 5% CO2 atmosphere. Cells were passaged/splitted at a ratio of 1:5 when they were 90-100% confluent.

Huh-7 cells were maintained in complete DMEM medium at 37°C and 5% CO2 atmosphere. Cells were passaged/splitted at a ratio of 1:5 when they were 90-100% confluent.

The HBV-Met cell line used in this study is an immortalized hepatocyte cell line derived from

HBV-transgenic mice. Cells were maintained in RPMI medium 1640 containing 10% heat-inactivated FCS, 2 mM l-glutamine, 100 µg of penicillin per ml, 100 units of streptomycin per ml (Invitrogen), 10 µg of insulin per ml (Sigma), 100 ng of epidermal growth factor per ml (BD Biosciences, Bedford MA), and 16 ng of insulin-like growth factor 2 per ml (Calbiochem) .

5.1.2 Plasmids

pkCMV-mIFN-α1, pkCMV-mIFN-α4, pkCMV-mIFN-α9,pkCMV-mIFN-α11 plasmids, gifts from Prof. Ulf Dittmer

pCG-NL43VifAU1-IRES-GFP plasmid, gift from Dr.Micheal Shindler

pEntry-HBV1.3 plasmid, generated by Martin Sprinzl

pLenti6.3 plasmid, Invitrogen, USA

pLenti6.3-A3A plasmid, generated by Daniela Stadler

pLenti6.3-A3G plasmid, generated by Daniela Stadler

5.1.3 Oligonucleotides for PCR

Name	Sequence 5′-3′
rcDNA1745 fw	GGAGGGATACATAGAGGTTCCTTGA
rcDNA1844 rev	GTTGCCCGTTTGTCCTCTAATTC
pgRNA 383 fw	CTCCTCCAGCTTATAGACC
pgRNA 705 rev	GTGAGTGGGCCTACAAA
cccDNA 92 fw	GCCTATTGATTGGAAAGTATGT
cccDNA 2251 rev	AGCTGAGGCGGTATCTA
Prnp fw	GACCAATTTATGCCTACAGC
Prnp rev	TTTATGCCTACAGCCTCCTA
GAPDH fw	ACCAACTGCTTAGCCC
GAPDH rev	CCACGACGGACACATT
HBxin fw	ATGGCTGCTARGCTGTGCTGCCAA
HBxin rev	AAGTGCACACGGTYYGGCAGAT
OAS1 fw	CAGTTAAATCGCCGGG
OAS1rev	AGGTTATAGCCGCCAG
Mx1 fw	TTCAAGGATCACTCATACTTCAGC
Mx1 rev	GGGAGGTGAGCTCCTCAGT
APOBEC1 fw	AGGGACCTTGTTAACAGTGGAG
APOBEC1 rev	CCAGGTGGGTAGTTGACAAAA
APOBEC2 fw	GGAGAAGTTGGCAGACATCC
APOBEC2 rev	TGGCTGTACATGTCATTGCTG

APOBEC3A fw	AAGGGACAAGCACATGGAAG
APOBEC3A rev	TGTGTGGATCCATCAAGTGTC
APOBEC3B fw	CGCCAGACCTACTTGTGCTA
APOBEC3B rev	GCCACAGAGAAGATTCTTAGCC
APOBEC3C fw	TCAACTGCAAGGACGCTGT
APOBEC3C rev	ATTGCCTTCATCGGGTTTCT
APOBEC3DE fw	ACCCAAACGTCAGTCGAATC
APOBEC3DE rev	CACATTTCTGCGTGGTTCTC
APOBEC3F fw	GCCTATGGTCGGAACGAAA
APOBEC3F rev	TGGGTCTCAGGATCCACCT
APOBEC3G fw	CCGAGGACCCGAAGGTTAC
APOBEC3G rev	TCCAACAGTGCTGAAATTCG
APOBEC3H fw	AGCTGTGGCCAGAAGCAC
APOBEC3H rev	CGGAATGTTTCGGCTGTT
APOBEC4 fw	TTCTAACACCTGGAATGTGATCC
APOBEC4 rev	TTTACTGTCTTCTAGCTGCAAACC
AICDA fw	GACTTTGGTTATCTTCGCAATAAGA
AICDA rev	AGGTCCCAGTCCGAGATGTA
SMUG1 fw	CTGCAGTGCCTGTCATGTG
SMUG1 rev	GCAGGCTCATGGATGGAC
TDG fw	GAACCTTGTGGCTTCTCTTCA
TDG rev	GTCATCCACTGCCCATTAGG
MBD4 fw	GGCAACGACTCTTACCGAAT
MBD4 rev	CCCAAAGCCAGTCATGATATTT
C5MTF fw	GGAAATTAGAATCAAGGAAATACGA
C5MTF rev	AATTTGTCTTGAGGCGCTTG
UBD fw	CCGTTCCGAGGAATGGGATTT
UBD rev	GCCATAAGATGAGAGGCTTCTCC
BIRC3 fw	TTTCCGTGGCTCTTATTCAAACT
BIRC3 rev	GCACAGTGGTAGGAACTTCTCAT
PLK 1 fw	CGAGGACAACGACTTCGTGTT
PLK1 rev	ACAATTTGCCGTAGGTAGTATCG
TYMS fw	CTGCTGACAACCAAACGTGTG
TYMS rev	GCATCCCAGATTTTCACTCCCTT
NEIL3 fw	TCTCCTGTTTTGGAAGTGCA
NEIL3 rev	CATTAGCACATCACCTAGCATCC
SOD2 fw	GGAAGCCATCAAACGTGACTT
SOD2 rev	CCCGTTCCTTATTGAAACCAAGC
RRM2 fw	ATTGGGCCTTGCGATGGATAG
RRM2 rev	GAGTCCTGGCATAAGACCTCT

5.1.4 Antibodies

IFN alpha (G16) neutralizing antibody, GeneTex, USA

anti-HBV core rabbit serum (H800) kindly provided by H. Schaller

anti-HBV core polyclonal antibody, B0586, Dako, Denmark

anti–phpspho-STAT1 (Tyr701) antibody, #9171, Cell Signaling, USA

anti–phpspho-STAT2 (Tyr690) antibody, #4441, Cell Signaling, USA

anti–APOBEC3A polyclonal antibody,AP31973PU-N, Acris, Germany

anti–APOBEC3F polyclonal antibody, H00200316-A01, Abnova, Taiwan, China

anti–APOBEC3G polyclonal antibody, AP23049PU-N, Acris, Germany

anti-AU1 polyclonal antibody, A190-125A, Abnova, Taiwan, China

anti–IFNAR1 polyclonal antibody, 21375-1, PBL Biomedical Laboratories, USA

anti–IFNAR2 polyclonal antibody, 31385-1, PBL Biomedical Laboratories, USA

anti–MX1 polyClonal antibody,13750-1-AP, ProteinTech Group, USA

anti–Beta-actin, A5441,Sigma, USA

anti–TBP polyclonal antibody, H00006908-A01, Bethyl, USA

Goat anti rabbit, HRP-conjugated Sigma, Deisenhofen, Germany

Goat anti mouse, HRP-conjugated Sigma, Deisenhofen, Germany

Donkey anti goat, HRP-conjugated Sigma, Deisenhofen, Germany

5.1.5 Enzyems

DNase I, Fermentas, Germany

Hind III restriction enzyme (hc. 10 U/µl), Roche, Germany

Other restriction endonucleases, Fermentas, Germany

RNaseA 10 mg/ml Roche Diagnostics, Germany

RNAse free DNase, Qiagen, Germany

Proteinase K, Roth, Germany

Proteinase K inhibitor “Complete”, Roche, Germany

T7 RNA polymerase, Fermentas, Germany

APE1, New England BioLabs, USA

PreCR Mix New England BioLabs, USA

5.1.6 Kits

Super Script III First-Strand Synthesis Super Mix Kit, Invitrogen, USA

DNeasy Blood & Tissue Kit, Qiagen, Germany

DNA Tissue Kit, Macherey-Nagel, Germany

AllPrep DNA/RNA/Protein Mini Kit, Qiagen, Germany

peqGOLD Total RNA Kits, Peqlab, Germany

TA cloning Kit, Invitrogen, USA

GeneJET Plasmid Miniprep Kit, Thermo, USA

XTT Cell Proliferation Kit II, Roche, Germany

NovaBright™ Chemiluminescent SEAP Reporter Gene Assays, Invitrogen, USA

Co-Immunoprecipitation Kit, Pierce, USA

Chromatin Immunoprecipitation Kit, Pierce, USA

DCFDA-Cellular Reactive Oxygen Species Detection Assay Kit, Abcam, UK

Murex HBsAg Version 3, ABBOTT, Germany

Hitrap Heparin HP, 17-0406-01, GE Healthcare, Sweden

5.1.7 Reagents

RPMI 1640 medium Cambrex, Taufkirchen, Germany

Williams medium E, Gibco, Invitrogen, USA

Dulbecco's Modified Eagle Medium, Gibco, Invitrogen, USA

FCS, heat-inactivated Biochrom AG, Berlin, Germany

Trypan blue Gibco, Invitrogen, USA

PBS, Gibco, Invitrogen, USA

Collagenase Type IV Worthington, Lakewood, USA

Human interferon alpha standard, PBL Biomedical Laboratories, USA

IFN-α (Referon-A), Roche, Germany

mIFN-α standard, PBL, USA

mIFN-α1, mIFN-α4, mIFN-α5, mIFN-α9, mIFN-α11, kindly provided by Prof Arne Skerra

SYBR Green I Master Mix Roche Diagnostics, Mannheim, Germany

TRIzol reagent Invitrogen, Carlsbad, USA

Bradford Reagent, SIGMA, USA

M-PER Mammalian Protein Extraction Reagent, Thermo, USA

NE-PER Nuclear and Cytoplasmic Extraction Reagents, Thermo, USA

LDS Sample Buffer, Thermo, USA

SmartLadder, 0.2-10 kb Eurogentec, Liege, Belgium

SmartLadder, 0.1-2 kb Eurogentec, Liege, Belgium

Pageruler plus Protein ladder Fermentas, Karlsruhe, Germany

Amersham ECL Prime Western Bloting Detection Reagent, GE Healthcare, Sweden

Pep19-2.5 kindly provided by M. Krepstakies

5.1.8 Laboratory equipments

Equipment	**Manufacturer**

Centrifuge Rotanta 400R Micro 200R	Heraeus, Hanau, Germany
Centrifuge 5417C / 5417R	Eppendorf, Hamburg, Germany
Ultracentrifuge Beckmann SW40 rotor	Beckmann
Flow Cytometer FACSCantoII	Beckton Dickinson
Light Cycler 480 II	Roche Diagnostics
Light Cycler 96	Roche Diagnostics
Freezer -20°C	Liebherr, Biberach, Germany
Freezer -80°C	Sanyo, Pfaffenhofen, Germany
Fridge 4°C	Liebherr, Biberach, Germany
CO2 Incubator	Tuttlingen, Germany
Microscope Telaval 31	Oberkochen, Germany
Fluoview FV101	Olympus, Hamburg, Germany
Vortexer	Lab Dancer IKA , Staufen, Germany
Laminar Flow Hera Safe ClassII, Type A7B3	Heraeus,Hanau,Germany
Pipettes Pipetman, P10-1000	Gilson, Middleton, USA
Multi-channel pipette, Pipetman Ultra	Gilson, Middleton, USA
Pipettus, accu-jet pro Brand	Wertheim, Germany
Gel chambers	BIO-RAD Laboratories, Hercules, USA
Heating block	Thermomixer comfort Eppendorf, Hamburg, Germany
UV-Oven	GS Gene LinkerTM BIO-RAD Laboratories, Hercules, USA
ALT Reader	Reflotron® Roche Diagnostics, Mannheim, Germany
Photometer Nanodrop	Implen, München, Germany

5.1.9 Software

FlowJo v8.5.3 Treestar, Ashland, USA

LightCycler96, Roche Diagnostics

Quantity One, 4.2.1, BIO-RAD Laboratories, Hercules, USA

MEGA 5.1, Arizona State University, USA

GeneDoc,Pittsburgh Supercomputing Center,USA

Scaffold3, Proteome Software, USA

ImageJ, NIH, USA

CLC Genomics Workbench 6.01,CLC bio, Aarhus, Denmark

Graphpad Prism 5 Graph Pad Software, San Diego, USA

MS Office Microsoft, Redmond, USA

5.2 Methods

5.2.1 HBV virus production

HBVwt and HBV(X-) were produced from HepG2.2.15 cell and HepG2-H1.3-K6 cell lines, respectively. The cells were cultivated until confluent in complete DMEM medium. Then the medium was exchanged to 50% PHH medium and 50% complete Williams E medium. Every 2 to 3 days the virus-containing medium was collected and cell debris was removed by centrifugation at 1000 rpm for 10 min. The supernatant was transferred to centrifugal filter devices (Centricon Plus-70, Biomax 100, Millipore Corp.) The first centrifugation was performed at 3500 x g for 1 h. In this step, the virus particles are captured in a filter. Because of the exclusion limit of 100 kDa, serum proteins flow through, while proteins larger than 100 kDa are kept. In a second, invert centrifugation step, performed at 1000 x g for 3 min, the filter system was turned upside down, to elute the virus. The virus containing concentrate was filled to 1 ml with PBS and a final concentration of 10% glycerol. The virus concentrate was stored in 100 µl aliquots at -80°C until further use. The titer of the produced wtHBV was measured as enveloped, DNA-containing viral particles. A CsCl density-gradient, followed by dot blot analysis, was performed.

5.2.2 HBV infection

Inoculation of differentiated HepaRG or PHH was performed with multiplicity of infection 200 in differentiation medium containing 5% PEG 8000 (Sigma) for 16 h at 37 °C. At the end of the incubation period, cells were briefly washed three times with PBS and cultured in differentiation medium.

5.2.3 Analysis of HBV replication

HBsAg was measured using AXSYM system (Abbott), and HBeAg was measured by BEP III system (Siemens). Total cellular DNA or DNA from cell culture supernatant were extracted from infected cells using NucleoSpin Tissue Kit (Macherey-Nagel). Total RNA was extracted from infected cells by Trizol reagent (Invitrogen) and transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen). Real-time PCRs (qPCR) were performed using the LightCycler™ system (Roche), HBV-DNA, pgRNA, and cccDNA were detected using specific PCR primers (**4.1.3**). HBV-DNA from cell culture supernatant was quantified relative to an external plasmid standard. Intracellular HBV-DNA and cccDNA are expressed as normalized ratio to a genomic gene Prnp, pgRNA as normalized ratio to GAPDH as previously described [357].

5.2.4 DNA extraction

Intracellular DNA has been extracted using the “NucleoSpin® Tissue”-kit (Macherey-Nagel, Düren, Germany). The standard protocol for cultured cells was used but the silica membrane was dried for two minutes and incubation time before elution was increased to approximately five minutes.

DNA from cell culture supernatant has been extracted via the “Biosprint 96 One-For-All Vet”-kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions adjusted to an initial sample volume of 100 µl. To this volume, 20 µl proteinase K were added. 100 µl lysis buffer AL, 100 µl isopropanol (100 %), 1.3 µl carrier RNA and 12.5 µl magnetic beads were used per reaction, elution volume was 200 µl.

5.2.5 RNA extraction

Cell layers were washed with 1xPBS and lysed by adding 400 µl RNA lysis buffer T from “peqGOLD Total RNA Kit” (Peqlab, Erlangen, Germany). Samples were either stored at -80 °C before incubation at 37° C for five minutes or after. Further steps were carried out following the instruction manual of the kit for cell suspensions, beginning with loading of the

lysate onto the DNA removing column. In step 2 only 400 µl of ethanol were added, step 4 was skipped, washing at step 5 was done with 20 seconds of centrifugation but without repetition, 100 µl water were used at step 7 and samples have been incubated at room temperature for three minutes before elution.

5.2.6 RT-PCR

For the synthesis of the first strand of cDNA, “SuperScript® III First-Strand Synthesis SuperMix for qRT-PCR” (Invitrogen, Karlsruhe, Germany) was used. 5 µl of 2x RT reaction mix were combined with 1 µl of RT enzyme mix and 4 µl of extracted RNA. cDNA was transcribed in thermocycler with following temperature profile: 25° C for 5 min, 50° C for 30 min, 85° C for 5 min, 4° C. Then 0.5 µl of RNaseH (5 000 U/ml) were added to each well. After centrifugation, samples were incubated at 37° C for 20 min.

5.2.7 qPCR

qPCR was carried out. 4 µl of cDNA sample were mixed with 0.5 µl of reverse primer (20 µM), 0.5 µl of forward primer (20 µM) and 5 µl SYBR® Green Mix (Invitrogen, Karlsruhe, Germany). qPCR runs were performed using TBP (TATA binding protein),Prn-p, or GAPDH as reference gene.

5.2.8 Southern blot

Before separation, the genomic DNA was digested with high concentrated (10 U / µl) restriction enzyme Hind III over night at 37°C, which affected the HBV-DNA. Isolated HepG2 2.15 DNA as size control was additionally restricted with EcoR I, an HBV genome single-cutter restriction enzyme, for 3 h at 37°C to perform linearization of the HBV-genome. DNA was separated in a vertical agarose-gel (0.8% w/v agarose in 1x TAE buffer) at 35 V for 16 h. After separation, the gel was washed with ddH_2O, incubated for 15 min with 0.4 M NaOH to denature the DNA and then incubated with 0.2 M HCl for 5 min for depurination of the DNA. Transfer onto a positively charged nylon-membrane was performed with 0.4 M NaOH via capillary forces during 12 – 16 h. for neutralization, membrane was washed for 3 min with 2 x SSC and then dried at RT for 10 min. DNA was cross-linked to the membrane under UV light at 125 mJ /cm2. Membrane was stored at RT until hybridization with radioactive labeled HBV genome specific probe.

5.2.9 3D-PCR

Differential DNA denaturation PCR (3D-PCR) is a method which can be used to discover

mutants with GC→AT transitions. AT-rich DNA melts at lower denaturation temperatures than GC-rich DNA due to the two hydrogen bonds between A and T versus the three between G and C. Therefore, doing PCR with a lower denaturing temperature allows differential amplification of AT-rich sequences. Here, amplificates from qPCR of cccDNA were used at templates for 3D-PCR, diluted 1:50 with PCR grade water. 0.5 µl of 5'HBxin (20 µM), 0.5 µl of 3'HBxin primer (20 µM), 1 µl template DNA and 23 µl water were added to "PuReTaq Ready-To-Go PCR Beads" (GE Healthcare, Munich, Germany). Amplifications were carried out in LightCycler™ 96 system (Roche Diagnostics, Mannheim, Germany) by using a gradient in denaturing temperature: (92-82° C) for 5 min; then (92-82° C for 1 min; 60° C for 30 sec; 72° C for 30 sec) x35, 72° C for 10 min. Amplification at denaturing temperature of 95 °C was used as positive control. Amplificates were detected in a 2 %-agarose gel electrophoresis, selected ones were recovered for DNA extraction from gel.

5.2.10 Cloning and sequencing

DNA was purified from gel following the protocol from "GeneJET Gel Extraction Kit" (Fermentas, St. Leon-Rot, Germany). Purified DNA was ligated into pCR®2.1 vector from "TA Cloning® Kit" (Invitrogen, Karlsruhe, Germany) following the cloning protocol by using only 1 µl of pCR®2.1 vector (25 ng/µl) but 2 µl of purified PCR product.

Thereafter, transformation of competent E. coli Stbl3 cells, was performed as described in the transformation protocol from "TA Cloning® Kit" (Invitrogen, Karlsruhe, Germany). Deviating from protocol, 2.5 µl of ligation product were added, mixing was done without stirring with the pipette tip, vials were shaken about 30 minutes, LB plates with ampicillin (100 µg/ml) were used and X gal (4 %) and IPTG (1 M) were added. The next day, white colonies were picked and grown into LB medium with ampicillin (50 µg/ml) over night at 37° C and 225 rpm.

Bacteria cells were pelletised by centrifugation for ten minutes at 5 000 g and supernatant was discarded. Then, plasmids were extracted using the "GeneJET Plasmid Miniprep Kit" (Thermo Scientific, Schwerte, Germany). Deviating from protocol, centrifugation in step 9 was carried out for two minutes, incubation before elution lasted more than two minutes and samples were eluted at 5 000 g. After measurement of DNA concentration, samples were brought to a con-centration between 30 and 100 ng/µl and send for sequencing. Sequencing was carried out by GATC Biotech, Konstanz, Germany.

Sequences have been analysed in a multiple sequence alignment (http://www.ebi.ac.uk/Tools/msa/muscle/) adding the HBV wild type (WT) sequence for the

appropriate fragment from the databank of the Institute of Virology, Munich. The software “GeneDoc” was used to process the alignment, nucleotide compositions were calculated with “MEGA 5” .

5.2.11 Quantitative 3D-PCR

Amplificates from qPCR of cccDNA were used at templates for 3D-PCR, diluted 1:50 with PCR grade water. 0.5 µl of 5'HBxin (20 µM), 0.5 µl of 3'HBxin primer (20 µM), 4 µl template DNA and and 5 µl SYBR® Green Mix. Amplifications were carried out in LightCycler™ 96 system by using a gradient in denaturing temperature: (95-84° C) for 5 min; then (95-84° C for 1 min; 60° C for 30 sec; 72° C for 30 sec) x35, 72° C for 10 min. Amplification at denaturing temperature of 95 °C was used as 100% for the evaluation.

5.2.12 Western blot

Lysates from HepaRG cells were achieved by adding 200 µl “M-PER Mammalian Protein Extraction Reagent” (Thermo Scientific, Schwerte, Germany) onto cells per well and incubation at 37 °C for five minutes. 50 µl “LDS sample buffer Non-Reducing” were added and samples shaken for five minutes at 800 rpm, 99 °C. Proteins wre separated on 7.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, proteins were blotted onto a PVDF membrane. Membrane was blocked with 5 % milk powder in 1xPBS for one hour at room temperature (RT), incubations with primary and secondary antibody followed (in 1xPBS, 0.5 %milk powder) at 4 °C, overnight or at RT for two hours, respectively. Antibodies and used dilutions are listed

5.2.13 Immunofluorescence staining

Cells grown on 4-well-glass slide (Lab-Tek II, Fisher Scientific - Germany, Schwerte, Germany) were fixed with 4% paraformaldehyde in PBS pH 7.4 for 10 min at room temperature and permeabilized with 0.5% saponin. 10% serum from the species that the secondary antibody was raised in PBS with 0.5% saponin was used for blocking. Blocking was performed at room temperature for two hours. Primary antibodies were diluted in PBS with 0.5% saponin and 2% blocking serum, and incubated with cells overnight at 4°C. After three times washing, the cells were incubated with the secondary antibody in PBS with 0.5% saponin and 2% blocking serum for 2 hours at room temperature in dark. Then the slide was mounted with Dapi Fluoromount-G (SouthernBiotech, Birmingham, Alabama, USA).

5.2.14 Co-immunoprecipitation

Co-Immunoprecipitations were performed using Pierce Co-Immunoprecipitation Kit (Fisher Scientific - Germany, Schwerte, Germany) according to the manufacturer's instructions with polyclonal anti–APOBEC3A and anti-HBV-core antibodies (Dako). Chromatin immunoprecipitation was performed using Pierce Agarose ChIP Kit (26156, Fisher Scientific - Germany, Schwerte, Germany) and polyclonal anti–APOBEC3A, anti-HBV-core antibody. Non-sheared DNA were analysed by cccDNA specific qPCR.

5.2.15 Transfection

Transfection in suspension followed by seeding (seeding-transfection) showed highest efficiency. "Lipofectamine® 2000 Reagent" (Invitrogen, Karlsruhe, Germany) was used for transfection according to protocol and advises of the manufacturer. During transfection, cells were kept in antibiotic free media and changed to standard.

5.2.16 AP site quantification

AP site quantification was performed with OxiSelect™ Oxidative DNA Damage Quantitation Kit (AP sites) (STA-324, Cell Biolabs). Genomic DNA were isolated by tissue kit (Macherey Nagel, Germany) and dissolve with TE buffer to 100 µg/mL. 5 µL of purified genomic DNA (100 µg/mL) was mixed with 5 µL of ARP solution in a microcentrifuge tube and incubated at 37ºC for 1 hour. 90 µL of TE buffer and 1 µL of Glycogen Solution were added and mixed to each tube. 10 µL of Sodium Acetate Solution and 300 µL of absolute ethanol were added to each tube and incubated at -20ºC for 30 minutes. Mixtures were centrifuged for 10-20 minutes at 14,000 g and the pellets were washed three times with 70% ethanol. DNA pellet then dissolved in TE buffer to 1 µg/mL. 50 µL of ARP-derived DNA sample or each dilution of the prepared ARP-DNA standards were added to the DNA High-binding plate together with 50 µL of DNA Binding Solution and incubate at room temperature for 2 hours.Then microwell strips were wash 3 times with 250 µL 1X Wash Buffer and 100 µL of diluted Streptavidin-Enzyme Conjugate was added to each well and incubate at 37ºC for 1 hour. After three times washing, 100 µL of Substrate Solution was added to each well and incubated at room temperature for 10 minutes. Enzymatic reaction was stoped by adding 100 µL of Stop Solution into each well, absorbance of each microwell on a spectrophotometer using 450 nm as the primary wave length was read.

5.2.17 APE1 digestion

1µg of total cellular DNA was mixed with NEBuffer 4, 10unit APE1(M0282L, New England

Biolabs) and H_2O upto 20µl. The reaction was incubated at 37°C for 2 hour and inactivated at 70°C for 5 minutes.

5.2.18 PreCR mix treatment

1µg of total cellular DNA was mixed with ThermoPol Buffer, 100 µM dNTPs, NAD+, 1 µl of PreCR Repair Mix (M0309L, New England Biolabs) and H_2O up to 20 µl. The reaction was incubated at 37°C for 20 minutes.

5.2.19 Deep sequencing

Genomic DNA was extracted uing the “tissue kit” (Macherey Nagel, Düren, Germany) and target genes were amplified by PCR (for primers see table below) designed to span around 2000bp of genomic sequence containing at least one exon and one intron. PCR reaction was performed with the Advantage®-HF2 PCR-kit (Clontech, Saint-Germain-en-Laye, France). Subsequently, PCR-reactions were loaded onto a 0.8% agarose gel and amplicons were purified for deep sequencing using the S.N.A.P.TM UV-free gel purification kit (Life Technologies Darmstadt, Germany). Purified amplicons were submitted for deep sequencing by a commercial provider (GATC Biotech, Konstanz, Germany) using Hiseq2000 sequencing system (Illumina, San Diego, CA, USA). Final sequencing data were analyzed with CLC Genomics Workbench 6.01 (CLC bio, Aarhus, Denmark).

Nr.	Oligoname	Sequence (5'->3')
1	B2M fwd	CTGCCGTGTGAACCATGTGA
2	B2M rev	GCTACCTGTGGAGCAACCTG
3	CCL17 fwd	ACACAGAGACTCCCTCCTGG
4	CCL17 rev	GCTCCAGTTCAGACAAGGGG
5	CXCL10 fwd	AGCAGAGGAACCTCCAGTCT
6	CXCL10 rev	TGTGGTCCATCCTTGGAAGC
7	CXCL13 fwd	CGACATCTCTGCTTCTCATGC
8	CXCL13 rev	AGACTGAGCTCTCTTGGACAC
9	HMBS fwd	GGGCTCAGTGTCCTGGTTAC
10	HMBS rev	CCCAGAGCCCTCTAGACCTT
11	IL-6 fwd	GAGGAGACTTGCCTGGTGAAA

12	IL-6 rev	GCCCATGCTACATTTGCCG
13	MxA fwd	TCCATGACTCGCAGAGAGGA
14	MxA rev	TCCACTGTAGAACACGCCAC
15	MYC fwd	CCACCTCCAGCTTGTACCTG
16	MYC rev	GCTGCGTAGTTGTGCTGATG
17	PKR fwd	CTTGCCGTCACGGGGATAAT
18	PKR rev	CTTGGCATTAAGTCGCTGCC
19	PML fwd	GAAAGCCCAAAGCCAACAGG
20	PML rev	ATTGGCAGGATGGTTGAGGG
21	SRC fwd	TGGAACTTGCCAGGGTTGTT
22	SRC rev	ACCAGGGCAGGAAATGTAGC
23	TBP fwd	TTGGTAGTTGAATCCCGCCC
24	TBP rev	TCACTGTGAAGAGAGCGCAG

5.2.20 Virus Heparin Attachment Assay

96-well plates are coated with Heparin by adding 100 µl of a 25 µg/ml Heparin solution to each well and incubation at 4 °C overnight. Afterwards the plates can be stored for several days if the heparin solution is kept in the wells. For performing the assay, the Heparin solution is discarded and wells are washed 3 times with PBS. Then 100 µl of the solution containing the inhibitory factor are added and the plate is incubated over night at 4 °C. The inhibitor solution is aspirated and the plate washed 3 times with PBS. 300 µl of blocking buffer (1% BSA solution in PBS-T) are added to each well and incubated for 2 h at 37 °C to saturate free valences of the plastic surface. After that, wash 3 times with PBS-T.

Prepare virus premix: for each well use 100 µl of blocking buffer, 1 µl of virus stock solution and 50 µl Murex Conjugate solution (from Murex HBsAg Version 3 Kit). Prepare this solution in a reaction tube of sufficient size and distribute 150 µl of it to the wells. Shake plate for 10 seconds. Incubate at 37 °C for 1 h. Wash 4 times with PBS-T. Add 100 µl of Murex HBsAg Version 3 Substrate Solution. Incubate for 1 h at 37 °C. Add 50 µl Stop Solution (1 M H_2SO_4 in ddH2O). Controls were performed by substituting the protein extract with PBS. Light absorption at 450 nm, with 670 nm reference wavelength, was measured in a plate reader

(Infinite 200 series, Tecan).

5.2.21 Cytotoxicity assays XTT

Viability of the cells was tested with XTT - Cell Proliferation Kit II (Roche), according to manufacturer's instructions at indicated time points. 25µl XTT labeling mixture was added to each well and incubated for 2 h. The cell viability assay determining cleavage of tetrazolium salts (XTT) to formazan by the "succinate-tetrazolium reductase" system. This system belongs to the respiratory chain of mitochondria, and is only active in metabolically active cells. The assay is based on the cleavage of the tetrazolium salt XTT in the presence of an electron-coupling reagent, producing a soluble formazan salt. This conversion only occurs in viable cells. Cells grown in a 96-well tissue culture plate are incubated with the XTT labeling mixture for approximately 2 - 20 hours. After this incubation period, the formazan dye formed is quantitated using an ELISA reader. The absorbance revealed directly correlates to the cell number.

5.2.22 ROS detection

Reactive oxygen species was quantified by DCFDA cellular ROS detection assay kit (ab113851, abcam). 1. Grow HepG2 cells in standard media so that 3x106 to 4x106 cells are obtained the day before the experiment. Harvest cells the day before the experiment and seed a dark 96-well microplate with 25,000 cells per well. On the day of the experiment, make a 1X buffer solution as follows: 90 mL of sterile deionized water + 10 mL of 10X buffer. On the day of the experiment, make a 1X supplemented buffer solution as follows: 18 mL of 1X buffer solution + 2 mL of FBS. On the day of the experiment, prepare DCFDA mix as follows: 10mL of 1X buffer solution + 12.5 µL of 20mM DCFDA (final concentration 25µM). Wash the HepG2 cells seeded on the 96-well plate with 100 µL/well of PBS once. Add 100 µL/well of DCFDA mix and incubate for 45 minutes at 37°C in the dark. Include blank wells (with non-stained cells). Wash the plate once with 1X buffer solution. If performing cytotoxicity assays, add compounds of interest and treat for desired period of time. Set excitation wavelength at 485nm and emission wavelength at 535nm.

5.2.23 Flow cytometry

Cells were collected and resuspend cells briefly in 0.5–1 ml PBS. Add formaldehyde to a final concentration of 2–4% formaldehyde. Fix for 10 min at 37°C. Chill tubes on ice for 1 min. Step 1 or store cells in PBS with 0.1% sodium azide at 4°C The supernatant was discarded

and the pellets resuspended in 100 µL FACS buffer each + ethidium monoazide bromide (EMA, 1:2000) as a marker for discrimination of live/dead cells. The plate was then exposed to light for 20 min on ice to allow EMA staining reaction. The wells were then filled up to 180 µL volume with FACS buffer, followed by a centrifugation as described above, the supernatant was discarded and the pellets washed two times with 180 µL FACS buffer (2.5x wash). To avoid undesired antibody-binding by FC-receptors (e.g. on macrophages), cells were suspended in 50 µL FC blocking solution (FC-antibody diluted 1:100 in FACS buffer) and incubated for 20 min in the dark on ice. Afterwards, the pellets were washed 2.5x and resuspended in 50 µL/well antibody solution (surface staining antibodies diluted in FACS buffer). The single colour samples were resuspended in 50 µL FACS buffer and 0.1 µL of the respective staining antibody added. The plate was incubated for 25 min in the dark on ice. After that the plate was washed 2.5x with 180 µL FACS buffer. Then the pellets were resuspended in 100 µL FACS buffer each, while the single colours were transferred directly into FACS-tubes prepared with 150 µL FACS buffer. The plate with the remaining wells was incubated in the dark at 4°C for 15 min. Following this incubation, the cells were washed 1.5x with 1x Perm and resuspended in 50 µL/well antibody solution. Incubation for 30 min on ice in the dark followed, then 2x wash with 1x FACS buffer. At last, the pellets were taken up in 150 µL 2% PFA and transferred to FACS tubes prepared with 150 µL FACS buffer. This staining procedure was followed by flow cytometrical analysis. Flow cytometry was performed on a FACSCantoII TM.

5.2.24 Interferon bioassay

L929, HepaRG or Huh-7 cells were cultured in flat bottom 96-well plates containing 100 µl cell culture medium. For the assay, IFN-α were added in triplicates to the culture wells and a 1:1 dilution series from 50 to 0.0004 ng/ml was performed. After 24 h of incubation with the mIFNa the cells were infected with 104 Pfu/ml VSV which corresponds to 103 Pfu/well. Therefore the MOI (multiplicity of infection) in this experiment was 0.1 (1 Pfu per 10 cells). At the beginning of the experiment not all cells are infected simultaneously and the total infection requires the virions produced during the experiment. The titer of the uses VSV-stock was 1.36x108 Pfu/ml from which a dilution of 2.2 µl in 30 ml of culture media was prepared which was then used to infect the cells. After an incubation of 48 h the cell viability was measured using the cell proliferation kit II (XTT) (Roche). For this purpose the supernatant of the cell culture wells was discarded and the media exchanged for a cell culture media that does not contain phenol red which would interfere with the photometric XTT assay. Before using the XTT assay to quantify the viability of the cells the effect of the VSV infection was controlled microscopically by determining the relation of cells showing a cytopathic effect

(CPE) to cells which retained a healthy appearance. The XTT assay can directly be performed in cell culture by adding the dye to the media and after 4 h of incubation the viability of the cells was determined by measuring the absorbance at 450 nm which was corrected by an absorbance measurement at 650 nm. Before the absorbance was quantified, the multi-well plates were applied to a centrifugation step to remove air bubbles that would corrupt the photometric measurement. The corrected absorption values were exported from the plate reader. The evaluation of the data was done by subtraction of the value for cells that showed the maximal CPE and dividing the values through the value for uninfected cells that were defined to have 100 % viability.

5.2.25 Statistical analysis

For statistical analysis, Student's unpaired two-tailed t-test with or without Welch's correction and statistical significance were determined setting confidence intervals of 95% using Prism 5 software (Graph Pad Software, La Jolla, CA).

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2013年6月11日
德国 慕尼黑

Declaration

I solemnly declare that I have written the dissertation entitled

Effector pathway of the antiviral effect of interferons in Hepatitis B Virus infection

and submitted it to the Faculty of Medicine of the Technical University Munich for doctoral examination at the
Institute of virology

under the guidance and supervision of
Prof. Dr. Ulrike Protzer

without other help and, while writing it, only used aids in accordance with the academic and examination regulations of the PhD program in Medical Life Science and Technology.

[X] I have not involved any organization that offers doctoral advisors for a fee or partially or entirely fulfills examination obligations required of me on my behalf.

[ ] I have submitted the dissertation in this or a similar form in no other examination procedure as an examination achievement.

[ ] The complete dissertation was published in
_____.
The Faculty of Medicine has approved the advance publication.

[ ] I have not yet acquired the Doctor of Philosophy and I have not definitely failed in a previous doctoral procedure for the Doctor of Philosophy.

[ ] I have already submitted a dissertation with the subject
_____
at the Faculty of _____ of the university _____ on _____ to apply for admission to the doctoral examination with the result:
_____

I am familiar with the academic and examination regulations of the PhD program in Medical Life Science and Technology of the Technical University Munich.

Munich, _____

_____
Signature

Curriculum vitae

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Sep 2001 – Jul 2005 Bachelor study
College of Life Science, Wuhan University
Major: Biology Technology

Membership:

EASL (The European association for the study of the liver) member

Publications and meetings

Papers

"Specific and non-hepatotoxic degradation of nuclear hepatitis B virus cccDNA"
Xia *et al.* Submitted to Science

"Interferon-γ and Tumor Necrosis Factor α treatment induce covalently closed circular DNA degradation in hepatitis B virus infected hepatocytes"
Xia *et al.* Manuscript in preparation

Congresses and Meetings

November 29-30, 2013, Lyon, France
EASL Monothematic Conference - Translational Research in Chronic Viral Hepatitis - Bridging Basic Science and Clinical Research
- Oral presentation: Specific and non-hepatotoxic degradation of nuclear Hepatitis B Virus cccDNA by interferons
- Young Investigator award

November 1-5, 2013, Washington, DC, USA
The Liver Meeting 2013
- Oral presentation: Interferons induce degradation of HBV cccDNA

April 24-28, 2013, Amsterdam, Netherlands
The International Liver Congress™ 2013
- Oral presentation: Interferon-alpha eliminates HBV cccDNA via base excision repair pathway
- Publication: Journal of Hepatology, Volume 58, Supplement 1, Page S57,
- Young Investigator award

September 22-25, 2012, Oxford, England
2012 International Meeting on the Molecular Biology of Hepatitis B Viruses
- Oral presentation: An interferon-alpha induced base excision repair pathway eliminates HBV cccDNA

June 22-25, 2012, Shanghai, China
14th International Symposium on Viral Hepatitis and Liver Disease
- Oral presentation: Interferon-alpha induced base excision repair pathway eliminates HBV cccDNA

October 9-12, 2011, Florida, USA
2011 International Meeting on Molecular Biology of Hepatitis B Viruses
- Poster presentation: Activation of interferon alpha signalling pathway in human hepatocyte cell lines