Dottorato di Ricerca in Tecnologie Biomediche in Medicina Clinica

Ciclo XXIX

"Regulation of viral expression by the HBV core protein and the characterization HBc as a potential therapeutic target for HBV cure"

Candidato:

Leonardo Lupacchini

Matricola: 692740

Relatore:

Prof. Massimo Levrero

Anno Accademico 2016/2017

SUMMARY

INTRODUCTION

Hepatitis B virus (HBV) is one of the most common chronic viral infections in the world a potentially life – threatening liver infection (McMahon, 2009b). It is a major global health problem as it can cause chronic liver disease and chronic infection and puts people at high risk of death from cirrhosis of the liver and liver cancer (McMahon, 2009a). Over 240 million people have chronic (long – term) liver infections (Ott *et al.*, 2012). More than 780.000 people die every year due to the acute or chronic consequences of hepatitis B (El-Serag, 2012). A vaccine against hepatitis B has been available since 1982. Hepatitis B vaccine is 95% effective in preventing infection and its chronic consequences, and was the first vaccine against a major human cancer.

HBV GENOTYPES, SUBGENOTYPES AND GLOBAL DISTRIBUTION

HBV is the prototypic member of a small group of enveloped DNA viruses termed hepadnaviridae (hepatitis DNA viruses) which are defined by a distinct liver tropism and a very narrow host range. The hepadnaviridae can be divided into two genera, the orthohepadnaviridae infecting mammals and avihepadnaviridae which exclusively infect birds. The HBV of human, woodchucks (WHV), ground squirrels (GSHV) and woolly monkeys (WMHV) are members of the orthohepadnaviridae (Dane *et al.*, 1970); (Lanford *et al.*, 1998); (Marion *et al.*, 1980); (Summers *et al.*, 1978). The genus of avihepadnaviridae includes the hepatitis B virus of the Peking ducks (DHBV) and herons (HHBV) (Mason *et al.*, 1980); (Sprengel *et al.*, 1988). Similar to the family of retroviridae, the replication of hepadnaviridae occurs via a RNA – intermediate using the viral polymerase. To emphasize this relation, hepadnaviridae are often termed as pararetroviruses.

Eight genotypes of HBV worldwide have been identified. When sequencing the entire viral genome, these genotypes differ from each other by more than 8%;

(Fung and Lok, 2004); (Kramvis and Kew, 2005); (Kramvis *et al.*, 2005); (Miyakawa and Mizokami, 2003); (Schaefer, 2005). In addition, multiple subgenotypes have been and are continued to be identified, which differ from each other by 4 – 8%. The HBV genotypes identified to date are A, B, C, D, E, F, G, and H, of the HBV genome have been defined. Moreover, two new genotypes, I and J, have also been identified. Over 30 related subgenotypes belonging to HBV genotypes have been determined to date, but the mechanisms of different pathogenic characteristics of HBV genotypes are not known for certain. Many studies have reported that different genotypes and subgenotypes show different geographical distribution, and are related to disease progression, clinical progression, response to antiviral treatment, and prognosis. Subgenotypes for each genotype have been labeled by using the nomenclature 1, 2, 3, etc. Genotype A is divided into A1, found in sub – Saharan Africa, A2, in Northern Europe, and A3 in Western Africa. The subgenotypes of HBV genotype B have been divided into two major groups: those found to be ‘‘pure’’ genotype B including B1 (formerly called Bj or B Japan) and B6, and those subgenotypes that have a recombination of part of the core region of HBV genotype C onto the genotype B core area including B2, B3, B4, and B5 (formerly called Ba or B Asia). Genotype B1 is found in Japan, B2 – 5 are found in East Asia, and B6, the newest identified B genotype, is found in indigenous populations living in the Arctic. Genotype C is divided into C1, C2, and C3 and is found in China, Korea, Southeast Asia, and in several South Pacific Island countries. Genotype D is widely spread across Eastern Europe, the Mediterranean regions, including North Africa, Russia, the Middle East, the Indian subcontinent, and across the Arctic. Genotype E is found in West Africa. HBV genotype G has been found only in small areas of the world, in the United States, Vietnam, and Southern Europe, and appears primarily to be present as a coinfection with another HBV genotype, most commonly genotype A. Genotypes F and H are the ‘‘New World’’ genotypes found in indigenous populations in Alaska and Central and South America. Genotype F is divided into 4 subgenotypes: F1 – F4. Genotype H is very closely related to genotype F and was initially thought to be a clade of genotype F. In the 48 contiguous US states, genotypes A2, B, C, and D are more commonly found, with immigrants born in

endemic areas reflecting the HBV genotype of their country of origin (Chu *et al.*, 2003). Genotype I has recently been reported in Vietnam and Laos. The newest HBV genotype, genotype J, has been identified in the Ryukyu Islands in Japan. Geographic distribution of HBV genotypes may be related to route of exposure. For example, genotypes B and C are more common in high-endemic regions of perinatal or vertical exposure, which plays an important role in viral transmission. Other genotypes are primarily observed in regions of horizontal exposure (Schaefer, 2007). Therefore, genotyping provides an epidemiological clue in the investigation of acquisition, because this lies in the geographical distribution of HBV (Sunbul, 2014). Figure 1 shows genotype distribution across the world.

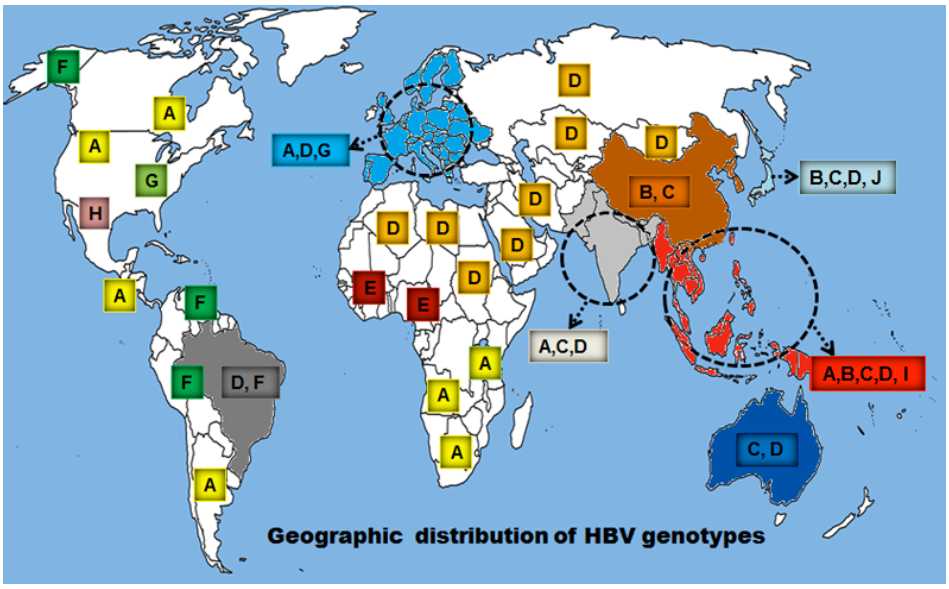

Figure 1 shows genotype distribution across the world.

MODE OF TRANSMISSION

HBV is transmitted by percutaneous or mucosal exposure to infected blood or other body fluids. HBV transmission has been observed with numerous forms of human contact: perinatal/mother – to – child; household (nonsexual); sexual; needle – sharing; and occupational/health – care – related. The highest concentrations of infectious HBV are found in blood and serum. However, other serum – derived body fluids, such as semen and saliva, are also infectious (Bond *et al.*, 1977). Persons with chronic HBV infection are the major reservoir for transmission. Because HBV can remain stable and infectious on environmental surfaces for at least 7 days, transmission may occur indirectly viacontaminated surfaces and other objects. Transmission from a chronically infected woman to her infant during delivery is efficient and is one of the most common routes of HBV infection worldwide. Perinatal transmission of HBV most often occurs during the birth process; in – utero transmission can occur but is rare and accounts for less than 2 percent of perinatal transmissions (Xu *et al.*, 1985). Transmission of HBV can also occur in situations where there is frequent and prolonged close personal contact with an infected person (Davis *et al.*, 1989). Although the exact mode of transmission is unknown, transmission is hypothesized to occur from inapparent blood or body fluid exposures from parents, siblings, or playmates that inoculate HBV into cutaneous scratches, abrasions, or other lesions or onto mucosal surfaces (Francis *et al.,* 1981). HBV is efficiently transmitted by sexual contact. Sexual contacts of chronically infected persons have been shown to have a higher seroprevalence of HBV infection than control populations, including household (nonsexual) contacts of infected persons (Alter and Margolis, 1990). Men who have sex with men (MSM) have long been known to have high rates of disease, and they have persistently higher HBV seroprevalence rates than the general population (MacKellar *et al.*, 2001). Injection drug users are athigh risk for HBV infection because of behaviors suchas sharing of needles, syringes, and other drug paraphernalia. However, the risk among injection drug users can vary

depending on the prevalence of chronic HBV infection in the community, as well as drug – sharing and preparation practices. Outbreaks linked to other percutaneous exposures besides injection drug use, such as tattooing and acupuncture, have been reported (Limentani *et al.*, 1979); (Kent *et al.*, 1988). Health – care – related transmission has long been recognized as an important source of new HBV infections worldwide. Provider – to – patient, patient – to – provider, and patient – to – patient transmission have all been observed, although the frequencies with which these types of transmission occur are widely divergent. Patient – to – provider transmission was common before widespread hepatitis B vaccination of health care workers. A health care worker's risk of infection has been shown to correlate with his orher level of blood and needle exposure (Hadler *et al.*, 1985). Patient – to – patient HBV transmission is a major source of new HBV infections in the developing world. Patient – to – patient HBV transmission can result from percutaneous exposure to contaminated equipment used for injections or other procedures, or from blood or mucosal exposure to contaminated medication. In developing countries, exposures to contaminated therapeutic injection equipment are common in many settings because of lack of awareness of infection control practices, lack of resources for sterilization and the purchase of new disposable equipment, and economic incentives and cultural preferences favoring overuse of injections. In the developed world, outbreaks involving this type of transmission remain a persistent problem as well, and they usually stem from lapses ininfection control practice by health care workers. Implicated vehicles for transmission include multidose vials, finger – stick devices, acupuncture needles, and jet injection guns. Contaminated environmental surfaces in health care settings have also served as a reservoir for HBV transmission, particularly in dialysis units (Williams *et al.*, 2004). Provider – to – patient HBV transmission is rarely reported. Most events have been associated with health care workers' performing invasive procedures, and most occurred before the widespread use of hepatitis B vaccine and the implementation of universal precautions in standard infection control practice (Gunson *et al.*, 2003). Transmission of HBV via transfusion of blood products has been largely eliminated in most parts of the world by screening blood donors and

implementing techniques that ensure viral inactivation of products made from blood, such as factor concentrates (Busch *et al.*, 2003).

GLOBAL PATTERNS OF TRANSMISSION

The global epidemiology of HBV infection has traditionally been described according to three categories of endemicity – high, intermediate, and low – depending on the proportion of the population that is seropositive for HBsAg Figure 2. Countries with high endemicity are those where HBsAg seroprevalence is greater than or equal to 8 percent; countries with intermediate endemicity are those where seroprevalence is 2 – 7 percent; and those with low endemicity are those where seroprevalence is less than 2 percent. HBsAg seroprevalence has marked geographic variations, and the degree of HBV endemicity often correlates with the predominant mode of transmission. In highly endemic settings, perinatal and horizontal (exposure to chronically infected household members) routes are responsible for most disease transmission, and 70 – 90 percent of the adult population has serologic evidence of prior infection. Because hepatocellular carcinoma is a potential sequela of chronic HBV infection, highly endemic countries have markedly higher rates of liver cancer than countries with lower endemicity, and hepatocellular carcinoma is a major cause of mortality in these areas. Countries with intermediate endemicity have a mix of perinatal, horizontal, health – care – related, sexual, and other forms of transmission. In countries with low endemicity, most new infections occur among young adults and are acquired sexually or through injecting drug use. Highly endemic population subgroups may be present within low – endemicity countries, however, depending upon seroprevalence rates of immigrant groups and native/indigenous populations.

Approximately 60 percent of the world's population lives in areas where HBV infection is highly endemic, including China (total population, 1.3 billion), Indonesia (222 million), Nigeria (132 million), and much of the rest of Asia and Africa. Some notable examples of high pre – vaccine – era burdens of disease include Taiwan, where 15 – 20 percent of the general population had chronic

HBV infection and 30 percent of those chronically infected were HBeAg – positive, and the Gambia, where the prevalence of chronic infection among children was 36 percent.

Southern Europe, the Middle East, and South Asia have an intermediate level of HBV endemicity. HBsAg seroprevalence in India is approximately 5 percent, and the major modes of HBV transmission are perinatal, child – related/horizontal, and health – care – related, particularly unsafe injections. In Italy, Russia, and Turkey, the prevalence of chronic HBV infection ranges from 3 percent to 10 percent, and unsafe injections have been implicated as a major route of HBV transmission.

Most of Central and South America is considered a region of low HBV endemicity. However, the western Amazon basin, including Brazil and Peru, is a highly endemic area, with observed HBsAg seroprevalence rates greater than 10 percent.

Many developed nations, including the United States, fall into the low endemicity category. Just prior to the era of widespread hepatitis B vaccine use (1988–1994), 0.42 percent of the US population was HBsAg – seropositive, and 4.9 percent had serologic markers of previous or current HBV infection. In the prevaccine era, some indigenous populations and immigrant groups within the United States had seroprevalence rates similar to those of highly endemic countries, and members of these populations comprised a disproportionate share of new HBV infections nationwide (Shepard *et al.*, 2006).

Figure 2: Geographic distribution of the prevalence of chronic hepatits B virus infection (Shepard *et al.*, 2006)

MORPHOLOGY AND GENETIC ORGANIZATION

MORPHOLOGY

In electron microscopy, the infectious hepatitis B particle, called Dane particle, appears as a spherical double – shelled structure with a diameter of 42 – 47 nm as depicted in Figure3 (Dane *et al.*, 1970). The virion consists of a nucleocapsid which is surrounded by a lipid membrane. Beside host cell endoplasmic reticulum (ER) – derived lipids, the three viral surface proteins, the large – (L), the middle – (M) and the small – (S) protein are embedded in the viral outer membrane. The surface proteins differ in the length of their N – terminal domain and share a common hydrophobic part, the S – domain of 226 aminoacids aa. The M – protein contains the 55 aa N – terminal extension termed preS2 and the L – protein consists of an additional extension of 108 to 119 aa depending on the genotype, termed pre S1 (Patient *et al.*, 2009). All three proteins are modified post translationally by glycosylation and the L – protein is additionally modified by myristoylation at its N – terminus (Heermann *et al.*, 1984); (Persing *et al.*, 1987). The preS1 – domain of the L – protein displays a dual orientation and is directed to the lumen or exposed to the outside of the viral particle. Thereby the L –

protein is involved both in the envelopment of the nucleocapsids (inside) or receptor binding (outside) (Glebe and Urban, 2007). The icosahedral nucleocapsid is formed by HBcore – protein dimers and displays a T=3 (90 HBcore – protein dimers) or T=4 symmetry (120 HBcore – protein dimers) depending on the number of HBcore – proteindimers (Bottcher *et al.*, 1997). Both nucleocapsid types are present in infectious particles (Roseman *et al.*, 2005). As a characteristic feature of hepadnaviridae, two types of subviral particles – spheres and filaments – can be detected in the serum of infected patients Figure3. In contrast to Dane particles, subviral particles only consist of a lipid envelope containing the viral surface proteins and therefore they are non – infectious (Heermann *et al.*, 1984). They are secreted with an excess of 1000 – 100000 fold relative to the infectious particles and their function is still poorly defined, but it is suggested that they contributes to immune tolerance by neutralizing antibodies.

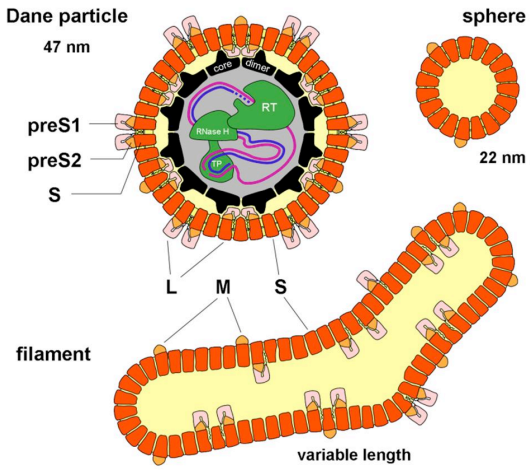

Figure 3: HBV morphology. Infectious particles (Dane particles) are composed of a nucleocapsid (black) formed by HBcore – protein dimers which is surrounded by lipid layer which consists of the three viral surface proteins, the Large – (L), the Middle – (M) and the Small – (S) protein. In the nucleocapsid, the viral genome (red and blue line) is located which is covalently linked to the viral polymerase (green). Subviral particles-spheres and filaments – are only composed of a lipid layer.

VIRAL GENOME ORGANIZATION

The HBV genome is a partially double – stranded circular DNA molecule called relaxed circular DNA (rcDNA). It represents one of the smallest genomes among

the DNA viruses and is formed by two linear strands of different length Figure 4 (Summers *et al.*, 1975*)*. The coding minus strand comprises the complete coding capacity of approximately 3.2 kilo bases Kb, depending on the genotype, while the non – coding plus strand contains a variable 3´end and thus comprises only 50 – 80% of the genome length (Hruska *et al.*, 1977). The ends of the minus strand are not connected but the 5´end of the minus strand is covalently linked to the polymerase (Gerlich and Robinson, 1980). The 5´end of the plus strand consists of a short RNA – cap structure of about 17 – 19 nucleotides. The circularization of both strands is achieved by complementary regions at the 5´end of both strands containing the directed repeated sequences, DR1 and DR2 (Seeger *et al.*, 1986) (Sattler and Robinson, 1979). A packaging signal, termed epsilon signal, is encoded by the minus strand. The HBV genome displays a highly compact structure and contains four partially overlapping open reading frames ORF which cover the entire genome and encode seven different HBV proteins. The three viral surface proteins (protein L, M and S) are encoded by the preS/S – ORF which consists of three 5´ in phase start codons for the initiation of the translation (Glebe and Urban, 2007). The HBcore – protein as well as soluble and secreted 17 kDa HB e antigen (HBeAg) is encoded by the preC/C – ORF. The polymerase with its typical domain structure is encoded by the P – ORF and the X – ORF encodes for the regulatory Hepatitis B virus proteinX (HBx).

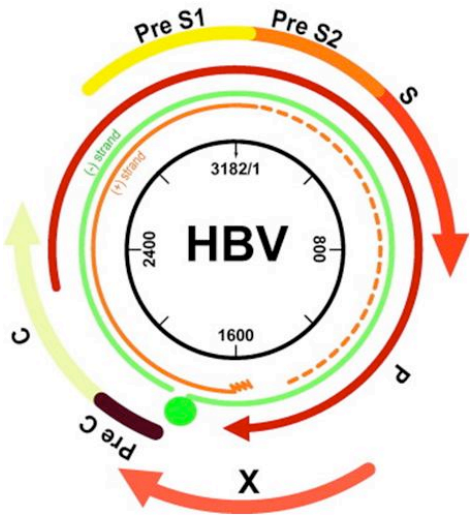

Figure 4: HBV genetic organization

REGULATORY ELEMENTS OF HEPATITIS B VIRUS TRANSCRIPTION

HBV gene expression is tightly regulated by four different promoters and two enhancers (Enh I and Enh II) which contain binding sites for different transcription factors Figure5. The preC/C promoter is a strong promoter; it allows the transcription of two RNAs: PreCore RNA and pgRNA of 3,5 kb each. The first transcript, longer for few nucleotides compared to the second, starts upstream of the protein PreCore ATG and brings to the sysnthesis of HBeAg protein. The second RNA starts downstream of PreCore ATG and it is called pgRNA. The pgRNA is the template for the translation of Core and Pol proteins. The preC/C promoter contains the region BCP (Basal Core Promoter) which brings non-canonic TATA boxes, binding sites for ubiquitaries factors (SP1, COUP-TF1, PPAR, RXR and TR2) and hepatic nuclear factor (HNF3 and HNF4). It has been also shown that HNF4, COUP-TF1 and TR2 negatively regulates the synthesis of PreCore RNA, whilst PPAR, SP1 and RXR can activate PreCore and pgRNA expression (Moolla *et al.*, 2002). The sequence CURS (Core Upstream Regulatory Sequence) is upstream of BCP and positively regulates the promoter (Yu and Mertz, 1996). Contrarily the NRE domain upstream of BCP inhibits the activity of PreCore promoter (Chen and Ou, 1995).

The PreS1 promoter allows the initiation of transcription of 2,4 Kb mRNA for PreS1 protein. This promoter has a canonic TATA box interacting with TBP and TFIID transcriptional factor and with HNF1. PreS1 promoter activity can bee it her positively regulate by a positive regulation sequence binding NF1, SP1 and HNF3 (Raney *et al.*, 1994) and negatively regulate by a CCAAT sequence in the PreS2 promoter in order to maintain the optimal stoichiometry of envelop proteins (Lu *et al.*, 1995).

The PreS2 promoter initiate the transcription for two 2,1 kb RNAs: PreS2 RNA and sRNA. This promoter presents a CCAAT sequence binding NFY and CBF and two positive regulatory elements also able to repress the transcriptional activity of PreS1 promoter (Zhou and Yen, 1991).

The X promoter starts the transcription of 0,9 kb RNA coding for X – protein (Zheng *et al.*, 1994). It localizes 140 bp upstream of the transcription start site and it over laps the enhancer I in its 3' region. It has LSR binding sites for hepatic factors (HNF3 and HNF4) and for ubiquitous transcription factor such as NF1, CREB/ATF, NRF1, RXR, COUP0TF, C/EBP, AP and c-Jun stimulating the mRNA expression (Guo *et al.*, 1991); (Fukai *et al.*, 1997).

The enhancer I is composed of about 270 bp and it is located between the ORF S and X and it partially overlaps the X promoter. The enhancer I strongly stimulates the transcription of pgRNA, PreCore/CRNA and XRNA but it has amodest effect on the transcription of RNAs for enveloppe proteins. It is made of distinct domains: regulatory domain at 5', a core central domain and a regulatory domainat 3' (Fukai *et al.*, 1997). The central domain presents binding sites for HNF3, RFX1, EFC, NF1, HNF1, HNF4 PPAR, COUP-TF and STAT3 (Bock *et al.*, 2000); (Waris and Siddiqui, 2002). The regulatory domains stimulates the enhancer I activity via its interaction with HNF1 and C/EBP. The enhancer I also has a role in the sequential activation of transcription of the viral RNA in the early maturing transactivating genes and allowing full functionality of the enhancer II rather responsible for the transcription of the late genes (Doitsh and Shaul, 2004). Also a ISRE/IRE motif has also been discovered at the domain of the enhancer I overlapping with ORF X. This pattern thus interacts with transcription factors of the IRF family and STAT allowing better transactivating activity of enhancer I (Belloni *et al.*, 2012).

The 105 bp enhancer II is localized upstream of BCP and it overlaps the sequence CURS in the PreCore/C promoter. It is composed of two regions (IIA and IIB) and stimulates the transcription of RNAs for enveloppe protein sand for X – protein. The region II A and II B don't have trans activator activity *perse* and they need to sinergistically cooperate (Yuh and Ting, 1990). The enhancer II presents many binding sites for ubiquitous and hepato – specific transcription factors such

SP1, HNF3, HNF4, PPAR, C/EBP, FTF, RXR and HFL but canal so be negatively regulated by NRE domain (Li and Ou, 2001); (Li *et al.*, 1995); (Ishida *et al.*, 2000).

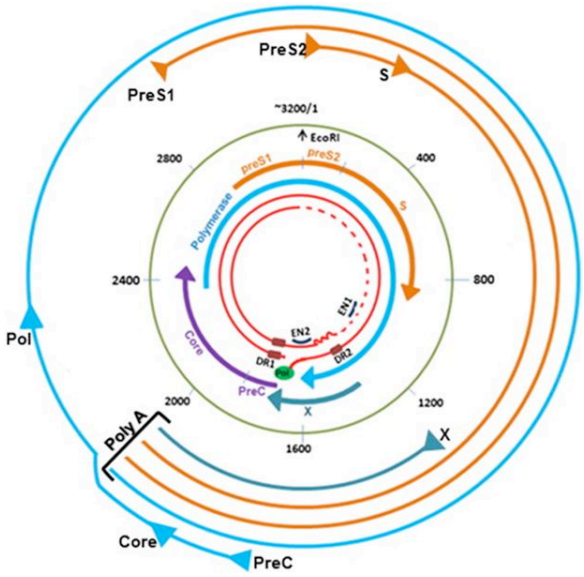

Figure 5: viral transcripts

PROTEINS OF THE HEPATITIS B VIRUS

The viral polymerase carries out several enzymatic functions that in other viruses are executed by indipendent enzymes, firstly the generation of minus starnd DNA from an RNA template (reverse transcriptase activity), secondly the degradation of the RNA template by its RNaseH activity and thirdly the generation of plus starnd DNA from a DNA template (DNA polymerase activity). The polymerase is attached covalently to viral minus strand DNA due to an unique mechanism of priming reverse transcription (Beck and Nassal, 1997); (Nassal *et al.*, 1990). The viral polymerase is composed of around 850 aminoacids organized into four functional domains. The terminal protein (TP) domain is covalently attached through a tyrosine residue at position 63 to the 5' end of the negative strand of the viral DNA. The TP domain is followed by a spacer domain containing a hinge in

the protein backbone that allows the catalytic domain to be brought into the proximity with the DNA sequence attached to the TP domain. Since the function of the spacer domain is structural rather than enzymatic, it can accept a significant degree of sequence variability and numerous variants have been described in this region. The active site has two activities: a revesre transcriptase function (RT) and a DNA – dependent DNA polymerase activity. The RT functions is responsible of the reverse transcription of the pgRNA from the minus strand. The active site contains a tyrosine – methionine – aspartate – aspartate (YMDD) motif that is identical to that identified in retrovirus reverse transcriptase. The DNA – dependent DNA polymerase activity allows the synthesis of the positive strand using the minus strand as template and thus the formation of the relaxed circular DNA. Finally the C – terminal domain of the protein presents an RNaseH activity that allows degradation of the RNA template once reverse transcription has taken place (Radziwill *et al.*, 1990).

The viral capsid is formed by the HBc antigen, a small protein of 183-185 aminoacids. Two protein domains with specific functions can be distinguished: the N – terminal domain (NTD) and the C – terminal domain (CTD) (Chain and Myers, 2005). The NTD is involved in the formation of core dimers and in their self – assembly into capsids. The core protein homodimers assembles in ichosaedrical capsids with a T=3 to T=4 symmetry via oligomerization of 180 or 240 monomers respectively (Crowther *et al.*, 1994). Each monomer contains a conserved cysteine at position 61 that can form an intradimer disulfide. *In vitro* HBV assembly is nucleated by a trimer of dimers and proceeds by the addition of individual dimeric subunits. Empty capsids assemble at a rate that is a function of protein concentration and ionic strength (Zlotnick *et al.*, 1999). The CTD domain is involved in pgRNA incapsidation and DNA replication (Gallina *et al.*, 1989). A basic sequence mostly composed of arginines and serines undergoes phosphorylation and lead to the interaction between capsid and pgRNA or viral DNA (Nassal *et al.*, 1990). Particularly three serines in position 155, 162 and 170 are important for pgRNA encapsidation and viral replication (Liao and Ou, 1995). The phosphorylation state of the core protein is associated with nucleocapsid mauration: immature nucleocapsids, highly phosphorilated, are gradually

dephosphorilated during rcDNA synthesis (Basagoudanavar *et al.*, 2007). Only mature capsids can interact with envelop proteins at the ERGIC (post ER, pre Golgi compartment) (Dryden *et al.*, 2006). Newly synthetized nucleocapsids interplay with the NPC as the CTD domain act as a NLS sequence (Kann *et al.*, 2007).

The protein PreCore is synthesized as a precursor polypeptide of 25 kDa. The N – terminal part, coded by precore, is very hydrophobic and plays the role of signal peptide to address the protein in the ER, where it undergoes different stages of maturation. The first clivage of 19 aminoacids in the PreCore domain gives the P22 only 10 aminoacids different from the core protein. Then, at the Golgi, the basic C – terminal tail of the precursor P22 can be cleaved to form the 17 kDa HBe antigen. The intracellular form of PreCore protein is able to assemble to form empty capsids (Kimura *et al.*, 2005). This pseudo – capsids can be envelopped and secreted (Watts *et al.*, 2011).

The HBs antigen, composed of 226 aminoacids, is the principle envelope protein of the virus. The HBs protein can self – assembly into empty viral envelopes even in the absence of other viral proteins or nucleic acid. Together with two longer proteins, the M (middle) and the L (large) proteins, it is inserted in the lipid bilayer surroundig the viral capsid. They are encoded in one open reading frame and share the C – terminal S – domain which provides four trans – membrane helices 4 and is probably involved in fusion. In the M and L proteins, the amino acids sequence of HBs is extended at the N – terminal by 55 (PreS2 domain) and 100 – 120 (PreS1 domain) additional aminoacids (Glebe and Urban, 2007). These extended sequence contain glycosylation sites that are processed in the Golgi apparatus. This myristoylation of the preS1 site is essential to render the viral particle infectious, in particular the myristoylation of a short sequence of the 2048 aa in the preS1 has been identified as responsable for the binding to the virus receptor, the Na – taurocholate transporter (NTCP) (Meier *et al.*, 2013); (Ni *et al.*, 2014).

The hepatitis B virus X – protein (HBx) with a molecular mass of 17kDa is the smallest protein expressed by HBV. HBx is an unstructured protein with a folded C – terminal and a disordered N – terminal region. HBx exhibits a dual

localization. In transfected hepatoma cells and in liver biopsies of HBV – infected patients, HBx could be detected in the nucleus and in the cytoplasm (Haruna *et al.*, 1991); (Su *et al.*, 1998); (Nomura *et al.*, 1999). Moreover, a portion of the cytoplasm fraction is localized at the outer membrane of the mitochondria (Rahmani *et al.*, 2000); (Huh and Siddiqui, 2002); (Kim *et al.*, 2007); (Clippinger and Bouchard, 2008). The dual localization displays the multiple functions of HBx in different cellular processes in the cytoplasm and nucleus including cell cycle regulation, apoptosis, activation of signaling pathways, autophagy, proteasomal protein degradation, DNA repair and transcription (Benhenda *et al.*, 2009). Since HBx cannot bind directly to DNA, it is suggested that the pleiotropic transcriptional activity is caused by two mechanisms, first the interaction with transcriptional regulators in the nucleus, such as TFIIB, TFIIH, TBP, RBP5, CBP/p300 (Lee *et al.*, 2001); (Cougot *et al.*, 2007) and secondly by activating different signal cascades in the cytoplasm including MAPK, SAP/JNK, p38, JAK/STAT pathway. Activation of the signaling cascades in turn results in the activation of different transcription factors including NF-kB, hypoxia – inducible factor 1a (HIF-1) or activator protein 1 (AP-1) (Benn and Schneider, 1994); (Lee and Yun, 1998); (Kim *et al.*, 2001). HBx can modulate many different pathways by direct protein – protein interaction with HBXIP, p53, DDB1, HDAC1 and CREM1 (Fujii *et al.*, 2006); (Feitelson *et al.*, 1993); (Lee *et al.*, 1995); (Forgues *et al.*, 2001). Several studies showed the importance of HBx in the viral replication by demonstrating that HBx expression in tissue cultures or animal models could restore the low replication from HBx – deficient HBV plasmids (Zhang *et al.*, 2004); (Keasler *et al.*, 2009); (Tang *et al.*, 2005). Recently, two studies have proved that HBx is indispensable for the complete authentic HBV infection *in vitro* and *in vivo*. In authentic tissue culture systems (HepaRG cells and PHHs) and in human hepatocytes chimeric mice, the replication of HBV deficient for HBx was strongly impaired and could be restored by WT HBx expression (Tsuge *et al.*, 2010). It is suggested that HBx either regulates the HBV DNA transcription, DNA replication, pgRNA endcapsidation, phosphorylation of HBcore – protein or polymerase activity (Zhang *et al.*, 2004); (Tang *et al.*, 2005); (Melegari *et al.*, 2005); (Cha *et al.*, 2009). Recently, it was shown that HBx is

essential for the transcriptional regulation of the cccDNA: HBx is recruited onto the cccDNA, binds to CBP/p300 and prolongs the activity of CREB and thereby regulates the acetylation status of cccDNA bound histones 3 and 4 resulting in the stimulation of cccDNA transcriptional activity (Cougot *et al.*, 2007); (Belloni *et al.*, 2012); (Lucifora *et al.*, 2011). In the absence of HBx, cccDNA – bound histones are hypo – acetylated, and the cccDNA transcribes significantly less pgRNA (Belloni *et al.*, 2009). HBx also binds and blocks the inhibitory activity on HBV transcription exerted by the PRMT1 methyltransferase (Benhenda *et al.*, 2013), the Tudor – domain protein Spindlin-1 (Ducroux *et al.*, 2014) and the SETDB1 histone methyltransferase (Riviere *et al.*, 2015). Additional mechanisms by which HBx can potentiate HBV replication include: a) the downregulation of DNMT3A expression through the induction of miR-101 (Wei *et al.*, 2013); b) a direct transcriptional activation of genes and miRNAs that potentiate endocytosis (RAB family) and autophagy (Guerrieri, 2015) both required for viral replication (Huang *et al.*, 2012); (Macovei *et al.*, 2013); (Sir *et al.*, 2010); c) binding to the UV-DDB1 protein (Hodgson *et al*., 2012) and inactivation of the Smc5/Smc6 – mediated restriction of cccDNA transcription (Strubin et al. presented at the HBV Molecular Biology Meeting 2015); d) the elevation of cytosolic calcium levels (Yang and Bouchard, 2012) and e) the direct transcriptional repression of miRNAs (miR-138, miR-224, miR-596) that inhibit HBV replication by directly targeting the HBV pgRNA (Guerrieri, 2015).

The genome – wide analysis of HBx chromatin recruitment in HBV replicating cells (Guerrieri, 2015) revealed a specific binding of HBx to a large number target sequences, including protein – coding genes and non – coding RNAs (16 lncRNA promoters and 32 lncRNA intragenic regions, 44 snoRNA, 3 snRNA and 75 miRNA promoter regions) (Guerrieri, 2015). Pathway analysis showed an enrichment in genes/non – coding RNAs involved in cell metabolism, chromatin dynamics and cancer as well as genes/non – coding RNAs known to modulate HBV replication (RAS, calcium transport, endocytosis, MAPK/WNT pathways, SRC and the EGF/ HGF family) (Guerrieri, 2015).

Mechanistically, the activity of HBx on transcription of both cellular genes and the viral genome rely on the modulation of epigenetic modifications and the

interaction with multiple transcription factors (ATF/CREB, ATF3, c/EBP, NF-IL6, ETS, EGR, SMAD4, OCT1, RXR receptor and p53), chromatin modifying enzymes (CBP, p300 and PCAF) and component of the basal transcriptional machinery (RPB5, TFIIB, TBP and TFIIH).

VIRAL REPLICATION CYCLE

Replication of the hepadnaviral genome can broadly be divided into three phases: infectious virions contain in their inner icosahedral core the genome as a partially double – stranded, circular but not covalently closed DNA of about 3.2 kb in length (relaxed circular, or rcDNA); upon infection, the rcDNA is converted, inside the host cell nucleus, into a plasmid – like covalently closed circular DNA (cccDNA); from the cccDNA, several genomic and subgenomic RNAs are transcribed by cellular RNA polymerase II; of these, the pregenomic RNA (pgRNA) is selectively packaged into progeny capsids and is reverse transcribed by the co – packaged P protein into new rcDNA genomes. Figure 6 pictures the HBV replication cycle.

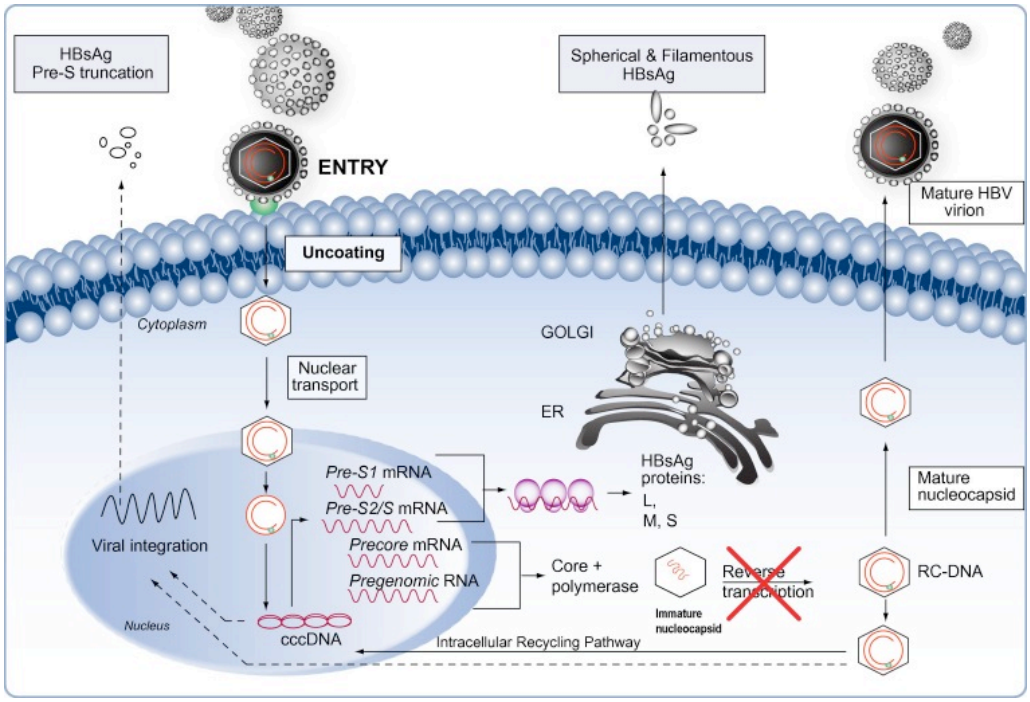

Figure 6: HBV replycation cycle (Chan *et al.*, 2011)

ENTRY AND RELASE INTO THE CYTOPLASM

Although there has been much research into the pathogenesis and treatment of hepatitis B virus (HBV) and hepatitis D virus (HDV) infections, we still do not completely understand how these pathogens enter hepatocytes. This is because *in vitro* infection studies have only been performed in primary human hepatocytes. Development of a polarizable, HBV – susceptible human hepatoma cell line and studies of primary hepatocytes from Tupaia belangeri have provided important insights into the viral and cellular factors involved in virus binding and infection (Urban, 2008). According to a general concept of viral infection, the first step is an energy – independent attachment of the viral particle to a structure at the host cell surface. After the primary attachment, which is characterized by low affinity and reversibility, the virus particle is transferred to a more specific receptor. HBV infection is thought to follow a multistep process. There are reports about the relevance of the initial attachment to the carbohydrate side chains of hepatocyte – associated heparan sulphate proteoglycans as attachment receptors for HBV

infection (Schulze *et al.*, 2007). This interaction is suggested to initiate the multistep entry process of HBV and is followed by a high-affinity step mediating HBV uptake. Using PHHs, 2 groups showed that N – terminal myristoylation of the HBV L protein is essential for virus infectivity (Gripon *et al.*, 1995); (Bruss *et al.*, 1996). Because myristoylation also occurs in the L proteins of woodchuck HBV and duck HBV (Macrae *et al.*, 1991), this modification has a general role in hepadnaviral infection, presumably by increasing receptor binding (De Falco *et al.*, 2001). In a systematic approach, Le Seyec et al. mapped functionally important sites in the preS1 and preS2 domains of the L protein. Consecutive deletions of 5 aa within the N – terminal 77 aa of the L protein blocked infectivity (Le Seyec *et al.*, 1998). A second determinant of infectivity is located in theantigenic loop AGL of the S domain. Mutation analysis revealed that Gly-119, Pro-120, Cys-121, Arg-122, and Cys-124 are required for infectivity (Jaoude and Sureau, 2005). Besides these envelope protein determinants, the lipid composition of the viral membrane is crucial for infectivity. Although depletion of cholesterol from cellular membranes with methyl – b – cyclodextrin has little effect on infection, depletion from the virus membrane strongly reduces infectivity in a reversible manner. This indicates a raft – independent localization of the virus receptor and suggests that a defined membrane association of HBV envelope proteins is required for productive entry (Bremer *et al.*, 2009). Evidence that the preS1 domain of the L protein is involved in recognition of a hepatocyte – specific receptor came from the observation that a myristoylated peptide, thepreS1 infectivity determinant, comprising 2–78 aa (Le Seyec *et al.*, 1999), inhibited HBV infection of HepaRG cells and PHH. Consistent with this observation, L protein – containing SVP (small viral particle) bound specifically to PTH. Mutational sequence analyses revealed the requirement for 9-NPLGF aa (F/L) P-15 motif, located at slightly variable positions within the N – terminus of the preS1 domain (Glebe *et al.*, 2003). Studies of HepaRG cells, PHH, and PTH found that heparin and highly sulfated polysaccharides interfere with HBV infection (Leistner *et al.*, 2008). HBV infection is sensitive to heparinases or sodium chlorate, indicating that the viruses use HSPGs as attachment sites (Sureau and Salisse, 2013). For many years groups worldwide had struggled to

identify, and confirm the functionality of host molecules needed for HBV and HDV entry. Many candidates were identified but none were shown to be sufficient for virus entry and initiation of replication (Urban, 2008). This situation was changed dramatically in late 2012 by a report from Yan et al. They used a synthetic peptide corresponding to the myristoylated N – terminus of the HBV preS1 protein to affinity select a candidate virus receptor from hepatocyte cultures. They identified the protein as the sodium taurocholate cotransporting polypeptide, NTCP, also known as SLC1-A1 (Yan *et al.*, 2012). NTCP transports bile acids from the blood into the liver. Their subsequent findings included evidence that the cDNA clone of human NTCP, when transfected into human hepatocellular carcinoma cell lines, specifically HepG2 and Huh7, conferred susceptibility to both HBV and HDV. Susceptibility could be inhibited by the synthetic preS1 peptide. In a different approach, Ni et al. took advantage of the observation that DMSO induces HepaRG cell differentiation and expression of the HBV receptor (Ni *et al.*, 2014). Comparing the expression profiles of undifferentiated and differentiated HepaRG cells and using stringent selection criteria, they found hNTCP to be the most strongly induced membrane protein. They confirmed the findings of Yan et al, showing that hNTCP-expressing HepG2 and HuH7 cells support high – level infection by HBV and HDV when cultured in the presence of DMSO. Once bound the NTCP the virus enter the cell. HBV does not possess a classic fusion peptide sequence (Berting *et al.*, 2000); (Marsh and Helenius, 2006). In a recent report, a fusogenic function was ascribed to the PreS1 domain of HBV *(*Nunez *et al.*, 2009). Based on sequence analysis, it was suggested by Rodriguez-Crespo et al. that the N – terminus of the S – domain (1–23 aa) might act as a fusogenic sequence (Rodriguez-Crespo *et al.*, 1999), but so far there is no direct experimental evidence for fusion to host cell membranes during HBV entry. In the case of genotype D, the PreS2 domain of HBV harbors between 41–52 aa a membrane – permeable peptide designated TLM (translocation motif). The presence of this TLM is conserved in all hepadnaviridae (Oess and Hildt, 2000). The TLM belongs to the family of membrane – permeable peptides. Fusion of the TLM to other peptides or proteins enables their energy and receptor – independent translocation across cellular membranes into the

cytoplasm. Fusion of the TLM to HBcAg revealed that fully assembled nucleocapsids that are decorated on their surface with TLM – peptides are able to translocate across cellular membranes and deliver the packaged nucleic acid to the nucleus (Brandenburg *et al.*, 2005) experiments revealed that in the endosomal compartment a proteolytic processing of the internalized viral particle occurs, resulting in an unmasking of the TLM peptide. It was concluded that, due to the endosomal proteolytic processing, unmasked TLM enabled the translocation across the endosomal membrane into the cytoplasm, where the proteolytically processed envelope dissociates from the nucleocapsid (Stoeckl *et al.*, 2006). Recently it has been described in a immortalized PHH line that HBV can use a clathrine – dependend endocytosis mechanism to enter the hepatocyte.

rcDNA TO cccDNA CONVERSION

At the end of the viral entry process, the viral genome is delivered into the nucleus. The viral genome exists at this stage as rcDNA. rcDNA consists of a complete (-)-DNA strand covalently linked to the viral polymerase P at its 5' end, and an incomplete (+)-DNA strand with an RNA oligonucleotide at its 5' end, which serves as primer for the (+)-strand synthesis. To establish a viral infection, the viral genome has to be present in a stable form within the infected cell. In the case of HBV, the viral rcDNA is converted into a nuclear, episomal cccDNA, which represents the central intracellular intermediate in viral replication (Jun-Bin *et al.*, 2003); (Sun and Nassal, 2006). In addition to incoming virion DNA, cccDNA can also be produced from newly synthetized cytoplasmic core DNA through an intracellular amplification pathway during the early phase of infection (Wu *et al*., 1990). This two pathways culminate in the formation of a regulate steady – state population of 5 to 50 cccDNA molecules per infected hepatocyte. The longevity of cccDNA is still in debate (Newbold *et al.*, 1995). At least four steps are involved in the conversion of gnomic rcDNA to cccDNA: repair of the single – stranded gap region; removal of the 5' – terminal structures, including the

capped oligoribonucleotide on the plus strand and HBV Pol protein on the minus strand; removal of the short terminal redundancy of the minus strand; and covalently ligation of both strand, thus forming cccDNA. Considering the structural feature of core – associated rcDNA, removal of viral DNA polymerase from the 5r terminus of minus strand DNA ought to be an essential step in cccDNA biosynthesis. Indeed, it has been demonstrated that the hypothetic deproteinized rcDNA (DP-rcDNA) species existed in the virally infected hepatocytes *in vivo* and transfected hepatoma cells in cultures. Detailed characterization of DP-rcDNA led to propose a working model of cccDNA biosynthesis pathway (Guo *et al.*, 2010). Further synthesis of plus strand DNA toward completion triggers the removal of genome – bound polymerase protein and nucleocapsid structure change, which leads to the exposure of a nuclear localization signal (NLS) at the carboxyl – terminus of capsid protein. The NLS in turn mediates the importation of the DP-rcDNA containing capsid into the nucleus (Kann *et al.*, 2007). It was demonstrated that unlike rcDNA, which formed cccDNA through faithful repair of the nicks in both plus and minus strand DNA, the double – stranded linear DNA (dslDNA) was converted into either cccDNA with deletions or insertions around the junction site, or oligomeric forms in which monomers were joined near the ends in random orientation, apparently via intra- or inter- molecular recombination (Yang and Summers, 1995). Similar with deproteinization of rcDNA, Guo et al. observed that deproteinized dslDNA (DP-dslDNA) appeared 24h earlier in the cytoplasm than cccDNA in the nucleus, suggesting that deproteinization of dslDNA also primarily takes place in the cytoplasm. These observations further supported the hypothesis that the removal of covalently attached viral polymerase from hepadnaviral mature genome DNA takes place in the cytoplasm and the resulting DP -rc and -dslDNA are subsequently imported into the nuclei, where they are converted into cccDNA. As illustrated in Figure 7, based on their unique structural features, DP -rcDNA and -dslDNA have been speculated to be converted into cccDNA by distinct cellular DNA repair machinery. While it is generally believed that multiple DNA repair components/pathways might participate in repair of the two gaps in rcDNA during cccDNA formation (Sohn *et al.*, 2009), it is also postulated that rcDNA may at

first be converted into a double – stranded linear DNA containing terminal repeats (TR-dslDNA) through extension of both plus and minus strand DNA over the cohesive – end region by viral and/or host DNA polymerases, and cccDNA is subsequently formed via intra - molecular homologous recombination of TR-dsl DNA. Although the TR-dsl DNA is undetectable in virally infected hepatocytes by conventional hybridization methods, previous sequence analysis of cccDNA recombinant joints in the livers of DHBV – infected ducks and WHV – infected woodchucks provided evidence supporting that cccDNA could be formed from two types of linear DNA, the dslDNA derived from in situ priming and the putative TR-dsl DNA, through nonhomologous recombination (Yang *et al.*, 1995). Guo and coworkers vigorously confirmed that NHEJ pathway is indeed required for cccDNA formation from the dslDNA, but not rcDNA precursor.

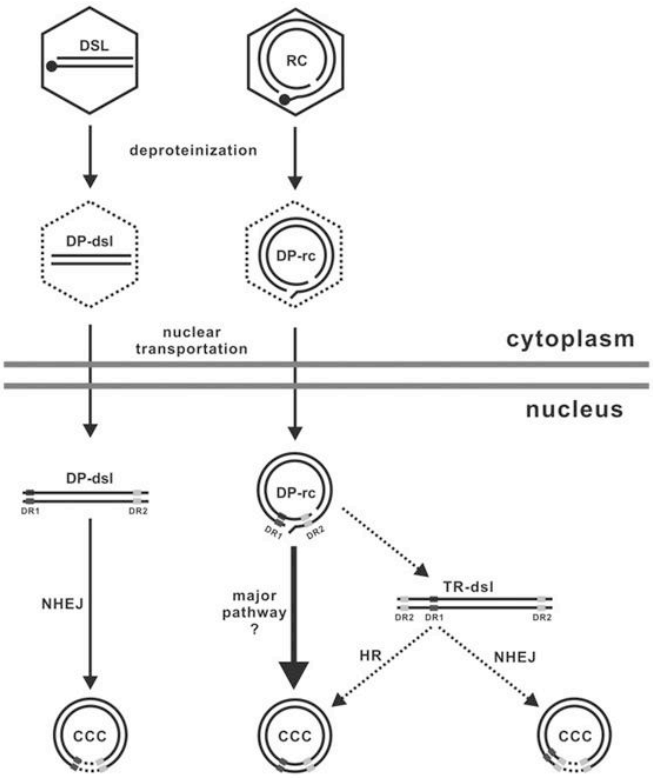

Figure 7: Schematic representation of cccDNA biosymthesis pathways from rc and dslDNA

The HBV genome gain access to the nucleus via the importin pathway using nuclear transport receptors Imp-β/Imp-α. The cccDNA is organized into minichromosomes in the nucleus of the infected cell by histone and non-histone proteins Figure 8. Histone proteins H3 and H2B are the most prominent species but lower levels of histone proteins H4, H2A and H1 are also detectable. In contrast to cellular chromatin the nucleosomal spacing of the HBV minichromosome has been shown to be unusually reduced by approximately 10%. *In vivo* and *in vitro* experiments indicated that HBV core protein is a component of the HBV minichromosome, binds preferentially to HBV double – stranded DNA, and its binding results in a reduction of the nucleosomal spacing of the HBV nucleoprotein complexes by 10% (Bock *et al.*, 2001).

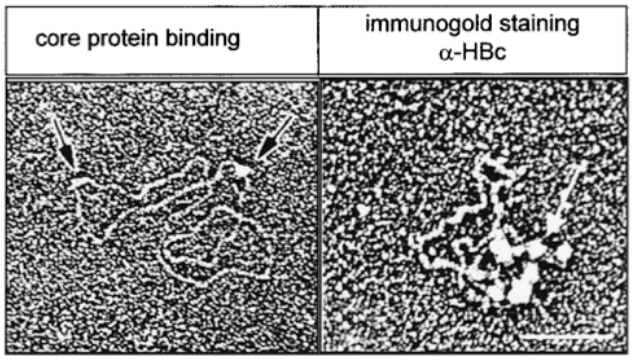

Figure 8: electron microscopy of *in vitro* reconstituted HBV nucleoprotein complexis

cccDNA TRANSCRIPTION

All known hepadnaviral RNAs, i.e. the subgenomic RNAs as well as the greater – than – genome length pgRNA and preCORE RNA, are transcribed by cellular RNA polymerase II (the enzyme responsible for cellular mRNA synthesis) using cccDNA as the template. All contain 5′ cap structures, all are 3′terminally poly – adenylated at a common site, and all serve as mRNAs for viral gene products. Spliced transcripts do exist, and even can be packaged into progeny virions, yet their functional role is still obscure (Sommer *et al.*, 2000). The transcript relevant

for virus replication is the pregenomic RNA (pgRNA), encompassing the entire genome length plus a terminal reduncancy of, in HBV, about 120 nt that contains a second copy each of the direct repeat 1 (DR1) and the ε signal, plus the poly – A tail Figure 9. The pgRNA starts immediately after the preCORE initiator codon. Its first essential role is that as mRNA for the core protein and the reverse transcriptase; unlike retroviral Gag-Pol proteins, P is expressed as a separate polypeptide by an unconventional mechanism (Sen *et al.*, 2004). Secondly, pgRNA is the template for generation of new DNA genomes by reverse transcription. The 5′ terminally extended preCORE RNA contains the initiator codon of the preC region and gives rise to the 25 kDa preCORE precursor protein of secreted 17 kDa HBeAg. It is unsuited as a pregenome, and is excluded from participating in replication on the level of encapsidation (Nassal *et al.*, 1990). Efficient transcription of HBV genes requires a number of ubiquitous transcription factors. NF-1 and Sp1 probably contribute to differential regulation of HBV gene expression. NF-1 has three different binding sites within HBV genome: the site 190 bp upstream S – ptomoter is essential for its optimal activity (Shaul *et al.*, 1986); the binding site between the Enh I and core promoter is responsible for the suppression of enhancer function (Spandau and Lee, 1992); the site within the Enh I has a central role in HBV gene transcription *(*Ori *et al.*, 1994). Sp1 binding site in the Enh II positively regulates transcription of all HBV genes; the upstream Sp1 binding site in contrast was described to negatively regulate transcription of S and X genes (Li and Ou, 2001). TBP binds to the putative TATA-like sequence and the initiator sequence in the preS2/S promoter region that is necessary for the promoter activity (Bogomolski-Yahalom *et al.*, 1997). Qin et al showed that Enh II/Core, preS1 and Enh I/ X promoter are targets for Prox1 – mediated repression (Qin *et al.*, 2009). Lin et al showed the requirement of CREB for expression of all HB antigens, HBV pgRNA and virus replication. The NF-KB indirectly inhibits synthesis of all HBV RNAs (Lin *et al.*, 2009). Although ubiquitous transcription factors do not confer hepatocyte tropism to HBV, they are essential for basal activity of HBV promoters and enhancers, for differential expression of HBV genes as well as for adaptation of HBV gene transcription to change the intra- and extra- cellular milieu. A number

of liver – enriched transcription factors and nuclear receptors have been shown to bind HBV promoter/enhancers elements and to be critical in activating and regulating HBV transcription. Chen et al found that HNF3α, HNF3β, HNF3γ, bind to Enh I and increase its activity 15 fold. Li et al moreover showed that HNF3α and HNF3β bind to Enh II and activate it in a dose dependent manner (Li *et al.*, 1995). As HNF3 was shown to be able to bind its sites in compacted chromatin to open local nucleosomal domain (Cirillo *et al.*, 2002), it is reasonable to speculate that the key role of HNF3 may be to bind the cccDNA and thus to provide acces for other transcriptional regulators. C/EBP can bind and activate the Enh II in a dose – dependent manner (Lopez-Cabrera *et al.*, 1991); another binding site of C/EBP was found in the S promoter and shown to be necessary for full S promoter activity (Bock *et al.*, 1999). Hepatocyte nuclear factor1 is expressed in two forms: HNF1α and HNF1β. HNF1α increases transcription from preS1 promoter (Raney *et al.*, 1991), interacting with Oct1 (Zhou and Yen, 1991). Raney et al. overexpressed HNF4a in HepG2.1 cells and found that it increased the level of transcription from preS1, preS2/S and core promoters; they identified two binding sites in the core promoter and in the Enh I/X promoter region. HNF4 inhibited transcription from the preC promoter and activated transcription from the pregenomic promoter. Overexpression of HNF4 increased synthesis of HBV pregenomic RNA (Yu and Mertz, 2003). HNF4a appears to be a major regulator of pgRNA transcription and thus HBV core and polymerase protein production and HBV replication. Raney et al. found that PPARa-RXRa heterodimers increase transcription from the pres1, the core and the Enh I/X promoters. Overexpression of PPAR-RXR resulted in stimulation of pregenomic RNA transcription (Yu and Mertz, 1997). FXRa-RXRa heterodimers can bind to HBV Enh II and core promoter (Ramiere *et al.*, 2008). TR4 represses the HBV core promoter via inhibition oh HNFa – mediated transactivation by protein – protein interaction (Lin *et al.*, 2003).

REVERSE TRANSCRIPTION: FROM THE pgRNA TO THE rcDNA

The next crucial step in hepadnaviral replication is the specific packaging of pgRNA, plus the reverse transcriptase, into newly forming capsids. Key actors are cis-elements on the pgRNA, most notably the encapsidation signal ε and P protein which binds to ε. This interaction, in a still poorly understood fashion, mediates recruitment of core protein dimers and thus leads to packaging of the pgRNA-P complex. Remarkably, the preCORE RNA is not packaged although it contains all of the sequence comprising the pgRNA. Likely, active translation from the upstream precore ATG through the ε sequence prevents the P-ε interaction (Nassal *et al.*, 1990). This implies, in turn, that P binding to ε on the pgRNA interferes with translation of the core ORF, and evidence supporting this view has been forwarded for DHBV (Cao and Tavis, 2006). Once pgRNA and P protein are being encapsidated a second key function of the P-ε interaction is brought to bear, namely the initiation of reverse transcription. At this stage the first DNA nucleotide (nt) is covalently linked to P protein, extended into a complete (-) strand DNA, and (+) strand DNA synthesis ensues, giving rise to a new molecule of rcDNA; the various immature DNA forms in *status nascendi* are termed replicative intermediates. The absolute requirements for replication are a template nucleic acid, plus an enzyme that is able to read the template information and use it for synthesis of a complementary nucleic acid. Clearly, these basic components are the pgRNA (and later the (-)-strand DNA) and P protein. However, generation of a functional genome also depends critically on precise start and end points, provided by cis – elements on the template Figure 9; a further specialty of P protein is its strict dependence, for activity, on cellular factors, namely heat shock proteins (Hsp´s) or chaperones. The best understood cis – element on the hepdnaviral pgRNA is ε, a stem – loop structure initially defined as the sequence from the 5´ end of HBV pgRNA that P – dependently mediated encapsidation of pgRNA, and also of heterologous transcripts to which it was fused (Junker-Niepmann *et al.*, 1990); later, the P-ε interaction was found to constitute the first step in initiation of reverse transcription (Wang and Seeger, 1993); (Tavis *et al.*, 1994); hence ε also acts as the replication origin. HBV ε, however, does not act as a completely autonomous encapsidation element. The 3´ copy of ε cannot

substitute for 5´ ε in the context of otherwise authentic pgRNA (Rieger and Nassal, 1996). Furthermore, 5´ ε mediates encapsidation of heterologous RNAs only up to a limited distance from the 5´ end (about 65 nt), and seems to require the 5´ cap structure (Jeong *et al.*, 2000). Hence the 5´ cap and factors bound to it appear to have a role in the process, possibly in concert with the 3´ poly – A tail and its associated cellular proteins. in all P proteins two conserved domains have been found, namely the polymerase/reverse transcriptase (RT) domain, and the C – terminal RNase H [RH] domain. Both are necessary as structural components for pgRNA encapsidation (Bartenschlager *et al.*, 1990). An absolutely hepadnavirus – specific feature is, however, the Terminal Protein (TP) domain at the N terminus, separated from the RT domain by a highly variable, and dispensable, spacer (Radziwill *et al.*, 1990). TP was first identified as the (-)-DNA linked protein (Bartenschlager and Schaller, 1988) and later was shown to provide a specific Y residue to which the first nt of the (-)-DNA becomes covalently linked. The P protein activity dependes on cellular chaperones. It was initially proposed that Hsp70 and Hsp90 and their respective co – chaperones, are essential for P activation (Hu *et al.*, 1997). It has been shown that the chaperones convert the inactive P protein transientely into an active form, P*, which is able to bind ε RNA (Tavis *et al.*, 1998). P activation appears to follow a double – hit, then run mechanism (Stahl *et al.*, 2007): the first hit is the transient, energy consuming conformational change in P that exposes the RNA binding site, creating P*; this remains without consequences unless ε RNA is bound. However, after ε RNA binding and/or priming, P may gain autonomous, chaperone – indipendent DNA synthesis activity (P'), perfectly suited to continue DNA synthesis after e has been replaced as template by the 3' proximal DR1*. A first – though not the only – requirement for a productive P-ε interaction is specific binding. In many RNA – protein interactions, structural diversity and hence specificity is achieved by deviations from a fully base – paired doublehelical structure, e.g. by interspersed single – stranded bulges and loops. Indeed, the ε bulge structure (but not its actual sequence, unless it affects structure) is absolutely necessary for P binding. Mutants in the upper stem which favor stable non – bulged structures do not bind to P (Beck and Nassal, 1997). Similarly critical is the sequence and structure at

the junction between the lower stem and the bulge. The existence of Dε RNA variants which bind P but have a much reduced or no template activity indicated that a productive P-ε interaction requires more than binding *(*Pollack and Ganem, 1994). It is likely that the RNA, after an initial binding step, must experience an induced – fit alteration into a new structure, and that only this is usable as a template. Also P protein undergoes structural alterations in this process. Proteolysis of *in vitro* translated DHBV P protein yielded a distinct proteolytic fragment only in the presence of priming – competent but not priming – inactive RNA variants (Tavis *et al.*, 1998). Hence RNA and P protein mutually alter each others conformation, likely to properly arrange the ε template region and the priming Y residue of TP in the active site of the RT domain. Notably, of the various P binding RNAs only those that are priming – active also support pgRNA encapsidation. Hence the abilities to initiate reverse transcription, to package pgRNA, and to adopt a distinct RNA-P protein complex conformation appear strictly coupled. In effect, this represents a quality control mechanism ensuring that only RNAs suitable as templates for reverse transcription are packaged.

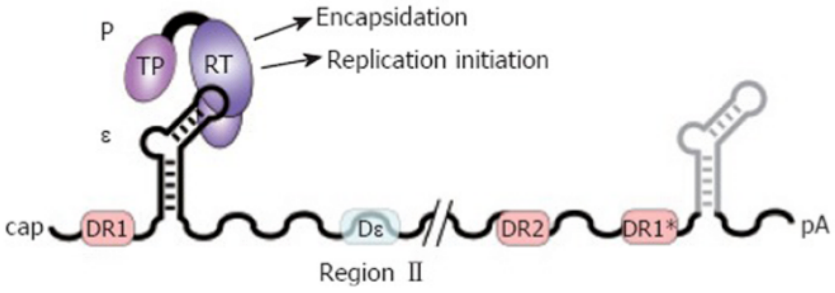

Figure 9: pgRNA organization. The pgRNA is shown with some major cis – elements, i.e. ε (hairpin structure), DR1, DR2, and DR1*

The initial model of hepadnaviral (-)-DNA formation assumed that synthesis would start inside the 3´ copy of DR1 (DR1*), for HBV at the motif 5´ UUCA. The complementary sequence 3´ AAGT at the 5´ end of (-)-DNA is instead copied from the UUCA motif in the ε bulge. Hence the oligonucleotide bound to TP must

specifically be translocated to the 3´ DR1*, nearly 3 kb apart from 5´ ε. One model is that DR1* and 5´ ε are brought into close proximity. A general mechanism would be closed-loop formation of the pgRNA via cellular proteins such as elongation initiation factor 4G (eIF-4G) which links 5´ cap and 3´ poly – A binding proteins. The end product of (-)-DNA synthesis is a unit length DNA copy of the pgRNA from its 5´ end to the UUCA motif in the 3´ DR1* Figure 10A; hence it contains a small, about 10 nt, terminal redundancy ("r"). Most of the pgRNA template is degraded concomitantly to (-)-DNA synthesis by the RNase H domain of P. The fate of the non – copied 3´ end of the pgRNA from DR1* to the poly – A tail is not exactly known. The 5´ terminal about 15 to 18 nt of the pgRNA including the 5´ DR1 sequence are spared from degradation. This capped 5´RNA oligo is essential as primer for (+)-DNA synthesis. Extension of the RNA from its original position ("in situ priming") gives rise to a double – stranded linear (dsL) DNA Figure 10E which occurs to a small percentage in all hepadnaviruses (Staprans *et al.*, 1991). For rcDNA formation, the RNA primer must be transferred to the 3´ proximal DR2 Figure 10B. Why the RNA primer predominantly jumps to DR2 although its complementarity to the initial site is larger is not obvious. From its new location on DR2 the RNA primer is extended towards the P bound 5´ end of the (-)-DNA, including the 5´r redundancy. Further elongation requires a third template switch, i.e. circularization Figure 10C. In effect, the growing (+)-DNA end is transferred from 5´ r to 3´ r on the (-)-DNA template from where it can further be extended to yield rcDNA Figure 10D. Though sequence identity between 5´ r and 3´ r is important, additional cis – elements are again required to ensure efficient RNA primer translocation and circularization. The Loeb laboratory has defined several such cis – elements on the (-)-DNA (Habig and Loeb, 2003); (Liu *et al.*, 2004); (Mueller-Hill and Loeb, 2002), e.g. 3E, M, and 5E, which are located at both termini and in the middle of pgRNA.

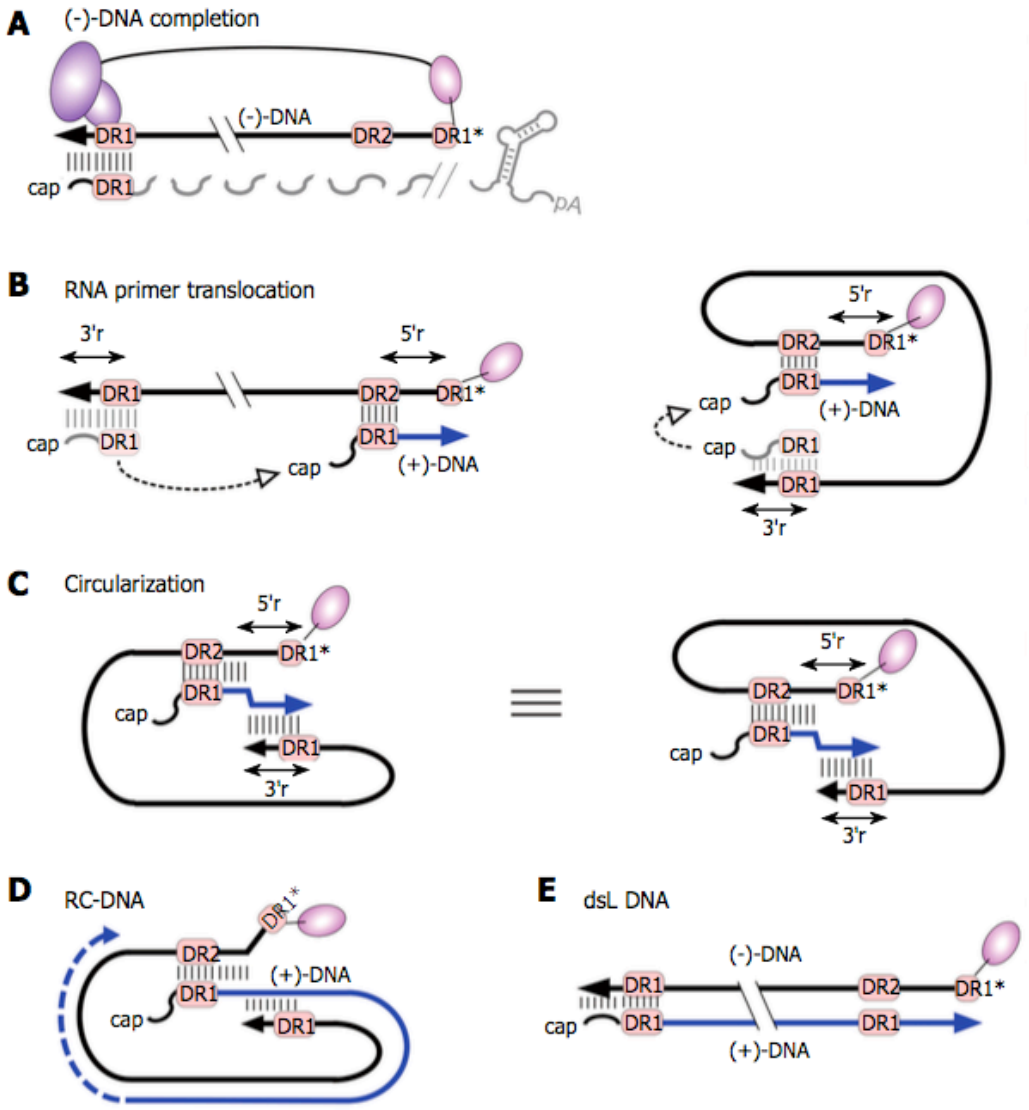

Figure 10 RCDNA formation. A: (-)-DNA completion. The DNA primer, still linked to TP, is extended from DR1* to the 5′ end of pgRNA. The RNA is simultaneously degraded by the RH domain, except for its capped 5′ terminal region including 5′ DR1; the fate of the poly – adenylated 3′ end is unclear; B: RNA primer translocation (second template switch). The RNA primer translocates to DR2, and is extended to the 5′ end of (-)-DNA. 3′ r and 5′ r denote an about 10 nt redundancy on the (-)-DNA. As above, several cis – elements appear to promote close proximity of the DR1 donor and the DR2 acceptor, as schematically indicated in the right hand Figure; C: Circularization (third template switch). Having copied 5′ r, the growing 3′ end of the (+)-DNA switches to 3′ r on the (-)-DNA, enabling further elongation. This reaction must involve juxtaposition of 5′ r and 3′ r. For easier comprehension, the switch is also depicted on the basis of the representation shown on the right of Figure 10B; both are topologically equivalent; D: RCDNA. Extension on the (-)-DNA template creates a set of (+)-DNA strands of various length; E: Double – stranded linear (dsL) DNA. This minor DNA form originates when the RNA primer, having failed to translocate to DR2, is extended from its original position ("in situ priming").

CAPSID MATURATION

HBV nucleocapsid formation starts when the complex of the RNA pregenome, HBV polymerase and HBcAg dimers has formed (Bartenschlager and Schaller, 1992); (Junker-Niepmann *et al.*, 1990). When nucleoapsid assembly is completed, the conversion of the RNA into single – stranded and then into partially double – stranded DNA takes place. In contrast to the nucleocapsids isolated from secreted virus, that contain only mature partially double – stranded DNA, intracellular nucleocapsids show all these different stages of the viral DNA synthesis. Based on these observations, it was concluded that the early RNA – containing capsids (the immature nucleocapsid) are not incorporated in to viral particles (Gerelsaikhan *et al.*, 1996). Detailed analyses revealed that capsid maturation is associated with a dephosphorylation of the nucleocapsid. Phosphorylation is required for efficient RNA packaging. *In vitro* experiments in HepG2 cells revealed that Ser-162 in the HBV core protein is necessary and sufficient for the encapsidation of HBV RNA. However, both Ser-162 and Ser-170 are required for the production of HBV DNA replicative intermediates. The core Ser-155 is essential for the formation of relaxed circular DNA intermediates (Liao and Ou, 1995). Based on the data from the HBV (Roseman *et al.*, 2005) and DHBV (Basagoudanavar *et al.*, 2007) systems, it has been concluded that nucleocapsid maturation can be described by a sequential phosphorylation (immature nucleocapsid) and dephosphorylation (mature nucleocapsid) (Roseman *et al.*, 2005). This dephosphorylation during capsid maturation is associated with significant differences in the structure between the RNA- and the DNA-containing cores.

ENVELOPMENT AND BUDDING

The envelopment of the mature nucleocapsid strictly depends on the presence of the viral surface proteins. Formation of LHBs and SHBs are strictly required. Moreover, virion formation requires that in a fraction of LHBs the PreS1 PreS2 domain faces the cytoplasm (Bruss and Ganem, 1991). To identify nucleocapsid residues that are crucial for envelopment, a variety of natural and engineered

mutants were analyzed. Based on these experiments, it was concluded that the spike tip seems to have no impact on the capsid envelopment (Ponsel and Bruss, 2003). Cryo – electron microscopy of HBV particles supports the observation that the spike tip interacts via electrostatic interactions with HBsAg (Seitz *et al.*, 2007). Mature hepadnaviral nucleocapsids form in the cytoplasm. For DHBV, it has been shown that mature nucleocapsids attach to intracellular membranes. This attachment does not require the presence of envelope proteins. Immature nucleocapsids do not bind (Mabit and Schaller, 2000). The exact mechanism that mediates the delivery of mature nucleocapsids to the post – ER, pre – Golgi – compartment (Huovila *et al.*, 1992), where envelopment occurs, is presently not understood. Inhibition of different MVB (multivesicular bodies) proteins. revealed that MVB functions are required for efficient budding and release of enveloped HBV virions. Moreover, HBV virions and subviral particles are all released by distinct pathways with separate host factor requirements (Watanabe *et al.*, 2007).

NATURAL HISTORY OF HBV INFECTION

The natural history of chronic HBV infection Figure 11 in individuals is complex, and infected persons can pass through several phases. Patients can move from a state of high viral load and no liver disease to one of active liver disease, followed by inactive disease, and then revert back to active liver disease years later. Progression to advanced fibrosis can be rapid, slow, or sporadic. During the inactive periods, hepatic inflammation, fibrosis, and even early cirrhosis can be reversed over time only to reappear again if the disease reactivates. Thus, chronic hepatitis B is a dynamic condition and it is difficult to predict what will happen over time to an individual with this chronic infection (McMahon, 2009b).

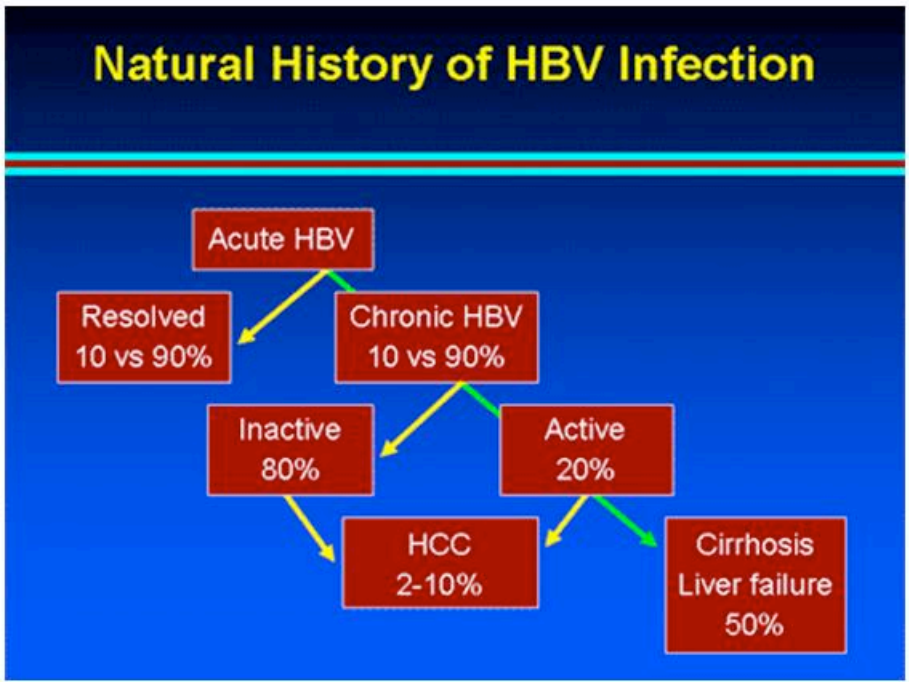

Figure 11: Phases of HBV infection

ACUTE INFECTION

In acute HBV infection, hepatitis B surface antigen (HBsAg) becomes detectable in the serum after an incubation period of 4 to 10 weeks, followed shortly by the appearance of antibody against the hepatitis B core antigen, which is predominantly of the IgM isotope in the early phase (Hoofnagle *et al.*, 1981). Levels of HBV DNA are generally very high, frequently in the range of 200 million IU/mL to 200 billion IU/mL (10 9010 12 copies/mL) (Ribeiro *et al.*, 2002). Circulating HBeAg can be detected in most patients with acute HBV infection, and these patients can readily transmit the infection (Koff *et al.*, 1977) Figure 12. Aminotransferase levels do not increase until after viral infection is well established because time is required for specific cytotoxic T lymphocyte responses to develop against virally infected hepatocytes. Approximately 30% to 50% of infected adults present with an icteric illness after an incubation period of 6 weeks to 6 months (McMahon *et al.*, 1985). The outcome of acute HBV

infection depends on age and immune competence at the time of infection.). For example, chronic HBV infection will develop in as many as 90% of infected neonates and infants but only in 1% to 5% of immunocompetent adults (excluding those with acute exacerbations of chronic HBV infection). Children aged 1 to 5 years have an intermediate risk (approximately 30%) (Tassopoulos *et al.*, 1987); (Beasley *et al.*, 1982). Most persons with acute HBV infection are adults. As a rule, acute HBV infection resolves without the need for intervention or antiviral treatment. Fulminant hepatitis occurs in 0.1% to 0.5% of those with acute HBV infection and often demonstrates no evidence of HBV replication because of the massive immune – mediated lysis of infected hepatocytes (Wright *et al.*, 1992). In endemic areas, exposure to HBV at birth or in early childhood results in higher rates of chronic HBV infection. Persons infected as children may present in adulthood with clinical manifestations similar to those of acute hepatitis if they have acute exacerbation of chronic HBV infection. These exacerbations frequently may be associated with elevated levels of IgM antibody to hepatitis B core antigen, which may lead to misdiagnosis of acute HBV infection (Liaw *et al.*, 1985) and an increase in the serum α-fetoprotein concentration, which may raise concerns for the presence of hepatocellular carcinoma (HCC) (Lok and Lai, 1989). Thus, it is important to define and understand the phases of acute and chronic HBV infection. Hepatitis B virus was thought to be cleared completely in those who recover from acute HBV infection. However, with the development of sensitive assays for HBV DNA detection, traces of the HBV genome have been frequently identified in the liver or serum up to 10 years after clinical recovery from acute HBV infection, despite the disappearance of viral antigens and the appearance of antiviral antibodies and specific cytotoxic T lymphocytes (Rehermann *et al.*, 1996). These observations suggest that HBV is rarely completely eradicated after recovery from acute infection, which may account for several reports of reactivation of HBV replication in persons with serologic markers of recovery from HBV who receive chemotherapy or immunosuppression after organ transplantation (Blanpain *et al.*, 1998).

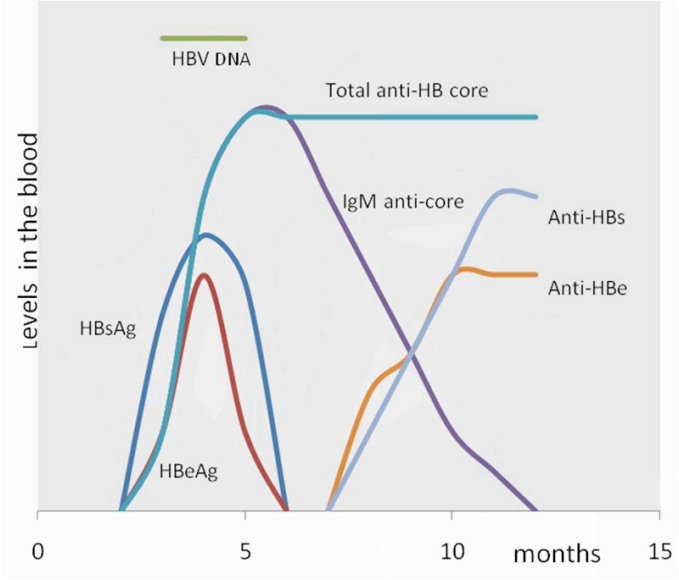

Figure 12: HBV serum markers during acute infection

PHASES OF CHRONIC HBV INFECTION

Those with chronic HBV infection may present: 1. in a state of immune tolerance, 2. with HBeAg- positive chronic hepatitis, 3. as an inactive HBsAg carrier, or 4. with HBeAg-negative chronic hepatitis Figure13.

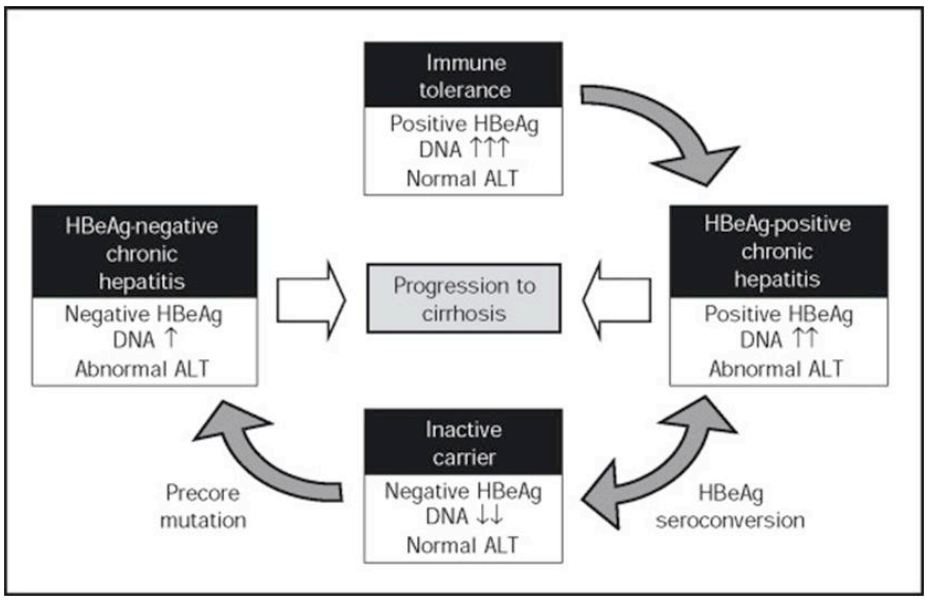

Figure 13: Phases of chronic hepatitis B virus infection. White arrows represent changes of histopathology, whereas gray arrows represent the changes in serologic markers between phases. Up- and down-facing arrows represent an increase or decrease of DNA level (↑= low increase; ↑↑= moderate increase; ↓↓= moderate decrease; ↑↑↑= high increase). ALT= alanine aminotransferase; HBeAg= hepatitis B e antigen.

Phase 1: Immune Tolerance

HBV – infected persons in the immune tolerant phase are HBeAg – positive, have normal ALT levels, and elevated levels of HBV DNA that are >20,000 IU/mL and commonly well above 1 million IU/mL. The immune tolerant phase is thought to occur most frequently in persons who are infected via perinatal transmission from HBeAg – positive mothers (Livingston *et al*., 2007). HBeAg may act as an immune tolerant protein that aids the virus in avoiding detection by the immune system. In immune competent persons, HBV is not cytopathic and hepatocellular damage is induced by the host immune system's efforts to eliminate HBV. The immune tolerant phase can last for a few years to more than 30 years (Hui *et al.*, 2007). During this phase, there is either no or minimal liver inflammation or fibrosis. However, because the HBV polymerase gene has reverse transcriptase properties, HBV integrates randomly into the host's hepatocyte DNA and during the immune tolerant phase, persistently high levels of HBV DNA over many years would likely mean an accumulation of integration sites, increasing the risk of HCC over time even in the absence of active liver inflammation and fibrosis.

Phase 2: HBeAg – Positive Chronic Hepatitis

As the host immune system matures and begins to recognize HBV – related epitopes on hepatocytes, immune – mediated hepatocellular injury ensues. Although HBV replication continues in the liver and viremia is continual, the viral level in the serum becomes lower than during theimmune tolerance phase when viral replication is completely unopposed (Tedder *et al.*, 2002). The immune active phase, also sometimes referred to as the "chronic hepatitis B phase" or the "immune clearance phase", is characterized by elevated ALT levels and an elevated HBV DNA level above at least 2000 IU/mL and histological findings of active inflammation and often fibrosis in the liver (Liaw *et al.*, 1988). Most patients with HBeAg – positive chronic hepatitis remain asymptomatic. However, some patients present with a symptomatic flare of hepatitis that mimics acute hepatitis or even with fulminant hepatic failure (Davis *et al.*, 1984). Spontaneous HBeAg seroconversion, which occurs annually in as many as 10% to 20% of those with HBeAg – positive hepatitis, is an important landmark in the natural history of chronic HBV infection (Hoofnagle, 1981). Factors associated with a higher rate of spontaneous HBeAg seroconversion include older age, higher aminotransferase levels and certain HBV genotypes (Yuen *et al.*, 2003); (Chu *et al.*, 2002). In contrast, spontaneous HBeAg clearance or seroconversion occurs in fewer than 5% of patients with normal or mildly elevated levels of alanine aminotransferase (ALT) (Ribeiro *et al.*, 2002). Many HBeAg – positive persons undergo seroconversion over time. However, those who remain HBeAg positive continue to be at risk for progressive liver disease. Approximately 12% to 20% of them will develop serious liver injury that results in cirrhosis and complications within 5 years (Liaw *et al.*, 1988); (Fattovich *et al.*, 1991) depending on the duration of the chronic hepatitis and the frequency and severity of flares (McMahon *et al.*, 2001). In a small proportion of patients with HBeAg – positive chronic hepatitis B, HCC may develop without cirrhosis. Although this phenomenon is widely recognized among clinicians, the rate at which it occurs is low (Liaw *et al.*, 1986).

Phase 3: Inactive HBsAg Carriers

After seroconversion, most patients remain negative for HBeAg and positive for anti – HBe antibody. Seroconversion is usually accompanied by stabilization of hepatitis, characterized by normalization of ALT levels and decreases in HBV DNA to low (<1000 copies/mL) or undetectable levels, depending on the assays used. This condition is commonly referred to as the "inactive carrier state" (Lok *et al.*, 2001). Histologically, minimal to mild hepatitis may be observed, although the degree of fibrosis may be variable. For example, inactive cirrhosis may be identified in patients who had severe liver injury before seroconversion (Yim and Lok, 2006). Most patients remain in this phase for many years, if not indefinitely (de Franchis *et al.*, 1993). Their prognosis is generally favorable, particularly if this phase is reached early in the disease course. Unlike patients with continued active viral replication, most inactive carriers do not have progressive liver disease. Hepatitis B e antigen reversion occurs in a minority of patients who have seroconversion (McMahon, 2004). Spontaneous clearance of HBsAg was delayed in a small number of inactive HBV carriers, at the estimated annual rate of 0.5% to 2% in Western countries and at a much lower rate of 0.1% to 0.8% in Asian countries. Patients with delayed spontaneous clearance of HBsAg are thought to have a favorable prognosis (i.e., lack of progression to cirrhosis) (Liaw *et al*., 1991); (Alward *et al.*, 1985); (Chen *et al.*, 2002).

Phase 4: HBeAg – Negative Chronic Hepatitis

Chronic hepatitis may recur in up to one third of inactive HBV carriers without reversion of HBeAg in their serum (Sung *et al.*, 2002). Most patients progress to this phase after a variable length of time in the inactive HBV carrier state, whereas some progress to HBeAg – negative chronic hepatitis directly from HBeAg – positive chronic hepatitis (Hsu *et al.*, 2002). This phase is characterized by the absence of HBeAg, the presence of anti – HBe antibody, detectable levels of HBV DNA, elevated levels of serum ALT, and histological findings of continued necroinflammation of the liver (Hadziyannis and Vassilopoulos, 2001). Compared to those with HBeAg – positive chronic hepatitis, patients with HBeAg – negative chronic hepatitis are generally older, have more advanced disease as

evidenced by liver histology, and have lower serum HBV DNA levels (Yim and Lok, 2006). The natural course of HBeAg – negative chronic hepatitis B is incompletely understood. In some patients, disease may progress silently for years, escaping clinical recognition. In such patients, serum HBV DNA levels may increase only transiently before serum ALT levels increase (Hadziyannis and Vassilopoulos, 2001). In general, HBeAg-negative chronic hepatitis represents a potentially severe and progressive form of chronic liver disease (Lai *et al.*, 1994). Because most, if not all, of these patients have gone through the HBeAg – positive chronic hepatitis phase, varying degrees of hepatic fibrosis are already present (Di Marco *et al.*, 1999). Moreover, continued hepatitis activity (persistent or intermittent) in the absence of spontaneous, sustained remission further increases the risk of progressive fibrosis. Spontaneous clearance of HBsAg is rare (Papatheodoridis *et al*., 2001).

OCCULT HBV INFECTION

Occult HBV infection is defined as the existence of HBV DNA in serum, although it is not considered as a phase of CHB (Torbenson and Thomas, 2002). A 2008 international workshop on occult hepatitis B virus (Yang et al.) infection (OBI), endorsed by the European Association for the Study of the Liver EASL (Raimondo et al., 2008b), as well as The Taormina Consensus Conference in 2008, defined "OBI" as the "presence of HBV DNA in the liver of individuals testing HBsAg – negative with currently available assays" (Raimondo et al., 2008a) and introduced a cutoff value for serum HBV DNA (< 200 IU/mL). In addition to a symptomatic and serologically evident infection, occult persistent HBV carriage has been identified since nucleic acid amplification assay enhances its sensitivity to hepadnaviral genomes and their replicative intermediates. There is evidence that occult HBV infection is a common and long – term consequence of acute hepatitis B resolution. This form of residual infection is termed as secondary occult infection (SOI) (Mulrooney-Cousins and Michalak, 2007). Several possible mechanisms have been hypothesized for the pathogenesis of OBI and the condition is probably multifactorial. Both host and viral factors are

important in suppressing viral replication and keeping the infection under control (Hollinger and Sood, 2010). It was suggested that long – term maintenance of an active anti – viral T cell response several years after clinical recovery from acute hepatitis B could be important, not only for protection against reinfection, but also for keeping the persisting virus under tight control where detection of minute amounts of virus in some recovered subjects was confirmed (Penna et al., 1996). Humoral and cellular immune pressure on the HBV envelope proteins are major mechanisms generating OBI. Masking of HbsAg by HbsAg – anti – HBs immune complexes is another postulated mechanism for the development of OBI (Hu, 2002). The severe consequences of occult HBV infection have not been fully recognized. There is evidence that occult HBV can be a source of virus contamination in blood and organ donations, as well as a reservoir from which full blown hepatitis can arise (Schreiber et al., 1996). Case reports also indicate that immunosuppression caused by chemotherapy or immunomodulatory agents or immunodeficiency due to HIV infection or hematological malignancies can induce reactive occult infection (Hui et al., 2006); (Chamorro et al., 2005).

cccDNA DURING THE NATURAL HISTORY OF CHRONIC HEPATITIS B

Chronic infection is believed to be maintained by a replicative form of HBV DNA termed cccDNA. During infection, HBV cccDNA accumulates in cell nuclei where it persists as a stable episome and acts as a template for the transcription of viral genes (Tuttleman *et al.*, 1986); (Newbold *et al.*, 1995). Considering the long half – life of hepatocytes, the limiting factor in eliminating infection is thought to be the clearance of cccDNA reservoirs from infected cells (Moraleda *et al.*, 1997); (Mason *et al.*, 1980). Three immune mechanisms have been proposed to mediate cccDNA clearance. In the first scenario, cccDNA is primarly eliminated by cytokines and cell death or cell proliferation do not contribute significantly (the cure model). In the second, cytokines suppress cytoplasmic viral replication and new cccDNA formation but do not act on pre – existing cccDNA that is eliminated by both cell death and mitotic loss and would need a turnover of approximately 0,7 of the total liver mass (The "death and compensatory

proliferation" model). In the third, cccDNA survives hepatocytes mitosis, is distribuited in a binomial fashion to the progeny hepatocytes and it is eliminated only by the elimination of infected cells, requiring an estimated turnover exceeding 2,5 times the total liver mass (the "cell death" model) (Mason *et al.*, 1980). There is evidence from animal models supporting each mechanism, and while these mechanisms are not mutually exclusive, it is currently unclear how infection is resolved in patients. To gain insight on the persistence of cccDNA and clearance mechanisms, Werle-Lapostolle et al. studied patients in different phases of the natural history of CHB and a group of patients participating in a placebo – controlled trial of the nucleotide analog adefovir dipivoxil. Intrahepatic cccDNA levels in patients with hepatitis B e antigen positive (HBeAg+) CHB (1.4 copies/cell) were significantly higher than in HBeAg – patients (0.01 copies/cell; $P<0.001$), or inactive carriers (0.02 copies/cell; $P<0.001$) (Werle-Lapostolle *et al.*, 2004). Patients with evidence of hepatitis B surface antigen (HBsAg) clearance had extremely low levels of cccDNA (0.002 copies/cell; $P<0.001$, compared to HBeAg+ patients). Similarly, median total intracellular HBV DNA levels in HBeAg+ patients (155 copies/cell) were significantly higher compared to HBeAg-patients (0.6 copies/cell; $P<0.001$), inactive carriers (2 copies/cell) ($P<0.001$), and HBsAg-patients (0.19 copies/cell; $P<0.001$). This result is consistent with observations of greater levels of serum HBV DNA in HBeAg+ patients (compared to the later groups). The observation that cccDNA remained detectable in all HBeAg – patients explains why viral reactivation has been observed, to varying frequencies, in all these patient groups.

SEQUELAE OF CHRONIC HBV INFECTION

Cirrhosis

Individuals with chronic HBV infection are at an increased risk of developing end – stage liver diseases including cirrhosis, hepatic failure, and HCC. The annual incidence of cirrhosis in patients with HBeAg – negative chronic hepatitis may be as high as 8% to 10%, compared with 2% to 5% in those with HBeAg – positive chronic hepatitis (Liaw *et al.*, 1988) The higher rate of cirrhosis in patients

presenting with HBeAg – negative chronic hepatitis, a late phase in the natural history of chronic HBV infection, is not surprising because these patients tend to be older and have more advanced liver disease (Fattovich, 2003). In addition to HBeAg status, HBV genotype and high levels of HBV replication have been found to affect the natural history of HBV infection (Iloeje *et al.*, 2006); (Sumi *et al.*, 2003). Hepatitis B virus DNA levels were the strongest predictor of progression to cirrhosis in a multivariable regression model. In addition, HBeAg positivity (HR, 1.7; 95% CI, 1.2-2.3), abnormal ALT levels (ALT>45; HR, 1.5; 95% CI, 1.1-2.1), male sex, and increasing age were associated with increased risk of cirrhosis (Iloeje *et al.*, 2006). Additional risk factors associated with progression to cirrhosis include habitual alcohol intake102 and concurrent infection with hepatitis C or D virus (HCV, HDV) or human immunodeficiency virus (Liaw *et al.*, 2004); (Thio *et al.*, 2002).

Hepatocellular carcinoma

It has recently been estimated that about 53% of HCC cases in the world are related to HBV infection. The lifetime risk of developing HCC is increased even in patients with cleared HBsAg or occult HBV infection. Further risk factors include chronic HCV infection, exposure to aflatoxin B1, alcohol abuse, obesity and diabetes (Lupberger and Hildt, 2007). Thus, it is important to identify HBV-infected patients at a higher risk of progressing to HCC. The reason why some CHB patients progress to HCC remains unknown. Host factors, such as immune response to HBV, genetic predisposition to HCC, high HBV replication rate, mutations within the HBV genome, are related with HCC. Many observations revealed that the major factor for the development of HBV-associated HCC is the immune system (Ganem and Prince, 2004). The same T – cell response has different effects. If T – cell response is strong enough, HBV can be eliminated from the liver. If not, a pro – carcinogenic effect can be induced by triggering necrotic inflammatory disease without final eradication of HBV from the liver. It can, thus, be concluded that the immune system – mediated chronic inflammation of the liver, continuous cell death and subsequent cell proliferation may increase the frequency of genetic alteration and the risk of developing cancer. However,

the molecular basis of inflammatory liver carcinogenesis caused by HBV remains largely unsolved. Cytokines modulate inflammation and the presence of inflammatory cells with the production of inflammatory cytokines activates cellular oxidant-generating pathways. Reactive oxygen species that are generated in inflammatory conditions induce oxidative DNA damage and increased oxidative stress caused by chronic inflammation can produce genetic mutations and chromosomal alterations (Coussens and Werb, 2002). HBV genotype C infection is associated with a higher risk of developing HCC than HBV genotype B infection (Kakimi *et al.*, 2002). The BCP A1762T/G1764A mutant is associated with an increased risk of developing HCC compared with the double wild type variant, whereas the precore G1896A mutation is associated with a decreased risk of developing HCC compared with the wild – type variant. Several mechanisms of liver carcinogen are related to the BCP A1762T/G1764A mutation which may enhance HBV virulence by increasing host immune response and viral replication, or by altering the coding region of the X antigen. Mutant BCP may augment the host immune response to HBV – infected hepatocytes by diminishing circulating HBeAg and increasing hepatocyte apoptosis and regeneration, thus leading to liver injury (Tong *et al.*, 2007). The BCP mutation appears to enhance the efficacy of viral replication either by modulating the relative levels of precore and core RNAs or by creating a transcription factor binding site for hepatocyte nuclear factor 1. Mutations in the BCP region over lapping the coding sequence of the X antigen of HBV may result in changes of amino acids, K130M and V131I, in the X gene. These amino acid changes may interfere with cell growth control and DNA repair, thus leading to HCC (Yang *et al.*, 2008). There is experimental evidence that HBx, a multifunctional protein with oncogenic potentials, can interact with a large number of cellular factors and modulate their normal function, thus leading to deregulation of normal cell activities and HCC (Hagen *et al.*, 1994). Despite its importance in HCC development, the clinical significance of genetic variability in the x genetic region still remains poorly understood (Bouchard and Schneider, 2004).

DEFINITION OF HBV – CURE

The term “cure” has been used recently in HBV – infected patients in a somewhat confusing way and needs clarification. Loss of HBsAg with or without seroconversion to anti – HBs antibodies is believed to be an effective end point of resolved acute HBV or of antiviral treatment in chronic HBV where antiviral treatment can be stopped ("functional cure"). It represents in general the end of detectable viral replication in the serum with sustained immunological control of persistent cccDNA in the liver of infected individuals. This is clearly different from "sterilizing cure" which, for HBV, would imply the eradication of all HBV – DNAs, including the viral template in the nucleus of infected hepatocytes, cccDNA. "Clinical" or "functional cure" implies a remaining risk that transcriptionally inactive cccDNA may become active again under certain clinical conditions, such as immunosuppression. True eradication of cccDNA remains an elusive therapeutic goal, but will be pursued intensively with new drugs in the coming years (Dandri and Petersen, 2016). It is important to underline that even if a "sterilizing cure" with complete elimination of cccDNA will be achieved, HBsAg might be still detectable, due to its cccDNA-independent production from integrated HBV sequences. Notably, functional cure of HBV with HBsAg loss might be sufficient to clear HDV infection, because HDV cannot replicate in the absence of HBsAg (Petersen *et al.*, 2016).

ACHIEVEMENTS AND LIMITS OF CORRENT HBV THERAPIES

Currently approved regimens include two classes of antivirals: 1) Nucleos(t)ide analogs (NAs), and 2) Interferon alpha2a, usually in its pegylated form (PEG – IFN). NAs do not affect the transcriptional activity of the cccDNA (Dandri and Locarnini, 2012); (Nguyen and Locarnini, 2009) numerous clinical studies have reported a significant decrease in cccDNA levels (Werle-Lapostolle *et al.*, 2004); (Sung *et al.*, 2005); (Wong *et al.*, 2006); (Lutgehetmann *et al.*, 2008); (Wursthorn *et al.*, 2006), but long – term antiviral therapy is needed to achieve significant reduction of the cccDNA pool (Dandri and Locarnini, 2012). Moreover, entecavir (ETV) and tenofovir (TDF), the two current first line NAs, only modestly affect

the levels of serum HBsAg (Brunetto *et al.*, 2009) and immunological control is rarely achieved, making such treatments a life time commitment, especially in HBeAg-negative patients. Interestingly, transitory hepatic flares after stopping long – term nucleoside therapy facilitate recognition and destruction of HBV – infected cells (Hadziyannis *et al.*, 2012) and cell killing occurring during hepatic flare would further contribute to reduce cccDNA and viral antigen levels, both of which may be instrumental to gain immunological control. Nevertheless, stopping long – term NA therapy in the presence of HBsAg is currently not an approved therapy – only in areas of the world with budget restrictions it is being considered to stop NA treatment in the presence of HBsAg after more than 12 months of undetectable HBV – DNA. Moreover, recent studies indicated that NA – treatments are not able to eliminate the risk of developing hepatocellular carcinoma (Arends *et al.*, 2015). Thus, there is an urgent need to develop therapeutic concepts leading to loss of HBsAg and, ideally, seroconversion to anti – HBs antibodies in a much larger proportion of individuals.

COMBINING PEG – IFM AND NAs – SIMULTANEOUSLY

Emerging data support a small but definite clinical benefit associated with combination therapy using both NA plus PEG – IFN. The intention is to harness both direct antiviral and immunomodulatory mechanisms. Clinical trials have evaluated de novo combination, as well as add-on or switch strategies (Dandri and Petersen, 2016). In one of the first studies to evaluate combination therapy, patients were treated for 1 year simultaneously with adefovir plus PEG – IFN. Stronger HBV suppression with approximately 2-log cccDNA reduction and 99% inhibition of intrahepatic virion productivity was observed, suggesting a synergistic function (Lutgehetmann *et al*., 2008); (Wursthorn *et al*., 2006). Unfortunately, in this study a PEG – IFN monotherapy control arm was missing. Significantly greater decreases in HBV DNA were achieved with ETV add-on on-treatment, but were not sustained posttreatment. Moreover, a recent study reported unexpected severe peripheral neuropathy in some patients who received combination therapy of PEG – IFN and telbivudine, compared to patients who

received telbivudine alone (Marcellin *et al.*, 2015). The study was terminated early because of safety concerns, indicating that in depth safety studies must be performed in ongoing combination trials. Switching treatment from NAs to PEG – IFN might be another treatment option to induce higher rates of HBsAg loss (Ning *et al.*, 2014). For Hepatitis B and Delta co – infection or superinfection, the results of combining PEG – IFN with NAs were disappointing. There was neither an extra benefit in combination therapy nor in extension of treatment duration to two years, compared to one year of PEG – IFN monotherapy (Rizzetto, 2016). Combining drugs with the same virological target, such as different NAs, may not have additive therapeutic effects. Therefore, EASL HBV CPG is considering combination therapy of potent NAs only as rescue therapy under highly selected circumstances such as occurrence of multi – resistant virus strains, and the number of published studies involving this segment of patients is very limited. To date, combination treatment strategies cannot be recommended, neither for HBV monoinfection, nor for HBV/HDV coinfection (Petersen *et al.*, 2016). EASL considers combination therapy in CHB to be an area requiring further research and supports further assessment of the safety and efficacy of the combination of PEG – IFN with potent NAs to increase anti – HBe or anti – HBs seroconversion rates.

NOVEL ANTIVIRAL STRATEGIES

Several strategies targeting HBV entry, HBV cccDNA production and processing, viral replication and viral protein expression are currently being explored Figure 14; Table 1. The discovery of the sodium taurocholate co – transporting polypeptide (NTCP) as the main receptor for HBV entry has been a major breakthrough for the HBV field. The identification of NTCP has allowed the establishment of cell lines susceptible to HBV infection in which the complete viral life – cycle, including the cccDNA mini – chromosome, can be studied thus enabling new research possibilities and drug discovery efforts (Petersen *et al*., 2016).

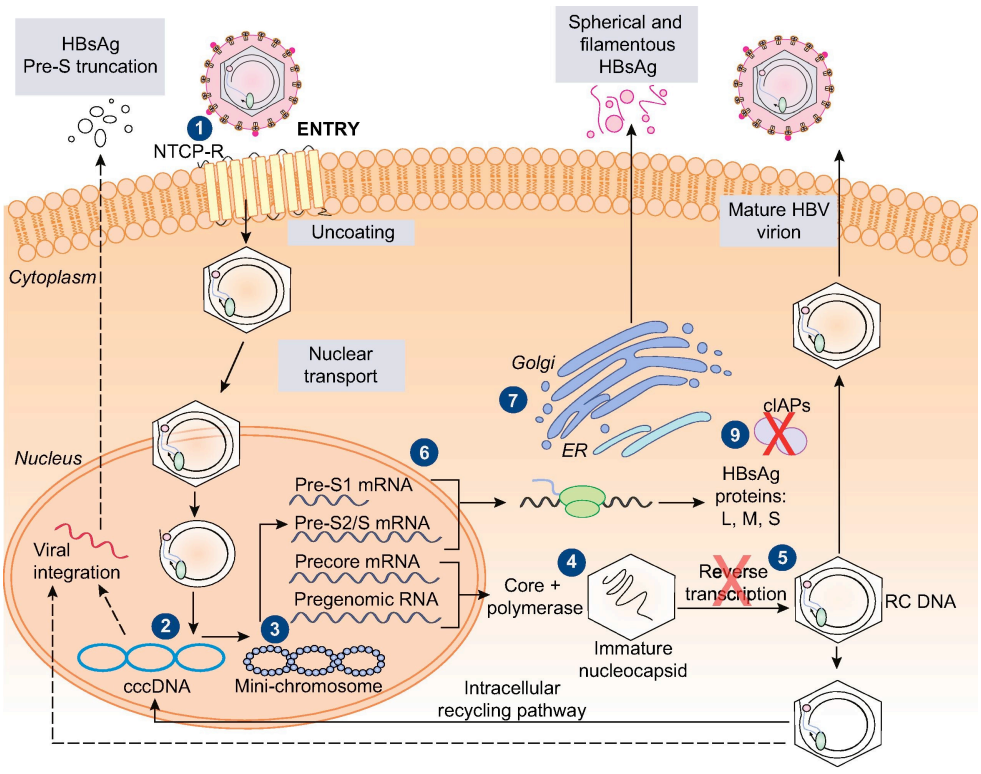

Figure 14: New anti – HBV approaches. A graphical representation of the drug target points within the HBV life – cycle. Adapted from (Chan *et al.*, 2011).

Strategy		Candidate	Clinical development phase
Entry inhibitors			
1	Entry inhibitors	Myrcludex-B	Phase 2
Direct antiviral agents			
2	cccDNA inhibitors		
	Elimination	LT-βR agonist	Preclinical
		Zinc finger nucleases	Preclinical
		TALENs	Preclinical
		CRISPR-Cas9	Preclinical
3	Silencing	Epi-drugs	Preclinical
4	Capsid inhibitors	NVR 3-778	Phase 1/2
		HAPs	Phase 1
		Phenylpropenamide	Preclinical
5	HBV polymerase inhibitors	Tenofovir alafenamide	Phase 3
6	HBV RNA interference	ARC-520	Phase 2
		TKM-HBV	Phase 1
		ALN-HBV	Preclinical
7	HBsAg release inhibitors	REP-9AC'	Phase 2
		REP-2139-Ca	
Host targeting agents			
8	TLR agonist	GS-9620 (TLR7)	Phase 2
	Anti-PD-1	Nivolumab	Preclinical
		Pembrolizumab	Preclinical
	Therapeutic vaccine	ABX302	Phase 2b/3
		GS-4774 (tarmogen)	Phase 2
		INO-1800	Phase 1
		TS1050	Phase 1
9	cIAP inhibitor	Birinapant	Phase 1

Table 1: Drug candidates targeting specific points in the HBV life – cycle. Strategy numbers refer to numerical points shown graphically in Figure 14.

Entry Inhibitors

In the setting of CHB, the rationale of HBV and HDV entry inhibitors is to impair viral propagation and, in the presence of ongoing liver damage and regeneration, to allow the expansion of uninfected hepatocytes and cure of HBV and HDV infection (Volz *et al.*, 2013); (Urban *et al.*, 2014). Cyclosporine A, that irreversibly binds NTCP, and the cholesterol absorption inhibitor ezetimibe, that act as a transport substrate for NTCP, both inhibit HBV/HDV entry in cell culture models but, although licensed for other clinical settings, have not so far been tested *in vivo* against HBV (Urban *et al.*, 2014). A number of small molecule compounds have been identified in functional screenings for HBV-entry inhibition (Watashi, 2015); (Tsukuda *et al.*, 2015) and are currently in early pre-clinical evaluation. Myrcludex B, a myristylated PreS1 peptide that competes with HBV/HDV for binding to NTCP has been shown to prevent HBV/HDV entry *in vitro* and *in vivo* and to impair viral dissemination when administered post-inoculation in human liver- chimeric uPA/SCID mice (Petersen *et al.*, 2008); (Lutgehetmann *et al.*, 2012). The future role for Myrcludex B remains unclear due to the limited efficacy as monotherapy for HBV and the impact on bile acids

metabolism advice for caution in planning long – term treatments with Myrcludex B (Xinfeng, 2015). Myrcludex B must be explored in several further combination regimens to improve efficacy against HBV. More interesting are the results in HBV/HDV co – infected patients, who have very limited therapeutic options and might benefit from further exploration of Myrcludex B in combination therapies provided that long – term safety issues are clarified. NTCP co – receptors have been identified, including Heparan Sulfate Proteoglycans (HSPGs) and, more recently, Glypican 5 (Verrier *et al.*, 2016) but their potential value as new targets for HBV entry inhibition strategies remains to be established.

Targeting cccDNA

Strategies targeting the cccDNA for HBV cure aim at preventing cccDNA formation, eliminating existing cccDNA or silencing cccDNA transcription. Mechanistically, control of cccDNA can be obtained, in principle, by capsid disassembly, inhibition of rcDNA entry into nucleus, inhibition of conversion of rcDNA to cccDNA, physical elimination of cccDNA, inhibition of cccDNA transcription (epigenetic control), or inhibition of viral/cellular factors contributing to cccDNA stability/formation. These objectives can be achieved by direct – acting antivirals (DAAs), or host-targeting agents (HTAs), that inhibit key host factors required for the viral life cycle, and immune – modulatory agents, that elicit signals converging on the cccDNA mini – chromosome. All these approaches are currently at the preclinical stage. An important limitation is represented by the lack of standardized assays for specific cccDNA quantification in cells and tissues. Moreover, direct methods to discriminate between active and inactive cccDNA pools and suitable surrogate markers for cccDNA activity to treatment efficacy assessment in trials and clinical practice are needed.

a) cccDNA formation. The precise mechanisms and the viral/cellular players involved in the conversion of rcDNA into cccDNA, the critical step for both the establishment of HBV infection after viral entry and the replenishment of the cccDNA pool from mature capsids re – cycling to the nucleus, are still unknown. TDP2 mediates the first step of cccDNA formation from incoming rcDNA (Koniger *et al.*, 2014) but its role in the formation of active cccDNA precursors

has been challenged (Cui *et al.*, 2015) and its value as a target for drug development remains to be established. Unbiased screenings led to identify di-substituted sulfonamide (DSS) compounds as inhibitors of de novo cccDNA formation (Cai *et al.*, 2012) but their molecular target is still unknown. New ongoing screening efforts in HBV – infected NTCP – HepG2 cells might identify new classes of cccDNA formation inhibitors.

b) cccDNA silencing. cccDNA molecules in the nucleus are assembled with cellular histone proteins into chromatinized minichromosome and associate with the viral proteins HBx and HBc (Levrero *et al.*, 2009). cccDNA transcription and HBV gene expression are controlled by the regulation of HBV chromatin and cccDNA-bound histone post – translational modifications (PMTs) (Levrero *et al.*, 2009); (Pollicino *et al.*, 2006). HBx is critical for cccDNA transcription but not for cccDNA chromatinization or maintenance (Belloni *et al.*, 2009); (Lucifora *et al.*, 2011); (Riviere *et al.*, 2015). Members of different classes of histone modifying enzymes have been shown to bind to and impact on cccDNA transcription. PEG – IFN, in addition to induce the expression of genes encoding intracellular or secreted proteins with direct or indirect anti – HBV properties, to activate natural killer (NK) and NKT cells and to destabilize viral nucleocapsids, has been shown to inhibit cccDNA transcription by decreasing cccDNA – bound histones acetylation (Belloni *et al.*, 2012); (Liu *et al*., 2013). Modulation of the same enzymatic activities with small compounds has been shown to induce the expected PMTs on cccDNA bound histones, to reduce cccDNA transcription and to inhibit viral replication in cell systems, providing the proof of concept for an epigenetic silencing of cccDNA (Tropberger *et al.*, 2015). However, its efficacy must be confirmed *in vivo* and the issues of functional redundancy between the members of the different classes of chromatin modifiers, the possible off-target effects on the host genome should be carefully considered. In this respect more selective approaches to affect cccDNA transcription and/or stability by targeting HBx, HBc and their host – interacting proteins (Belloni *et al*., 2012); (Liu *et al*., 2013); (Benhenda *et al.*, 2013); (Decorsiere *et al.*, 2016); (Zlotnick *et al.*, 2015) are actively pursued.

c) cccDNA destruction. Targeting cccDNA to destruction includes two different approaches. The first is based upon the ability, apparently shared by members of the IFN and TNF family of cytokines, to decrease cccDNA stability via the induction of deamination and apurinic/apyrimidinic site formation in the cccDNA and the up – regulation of nuclear APOBEC3 deaminases (Lucifora *et al.*, 2014); (Xia *et al.*, 2016). These results await confirmation and TNF – α and IFN – γ have little chances to be used as therapeutic agents. IFN – α activity on cccDNA stability at physiological or even pharmacological concentrations remains to be established *in vivo*. The safety and therapeutic index of antibodies activating the lymphotoxin – β receptor (LTβR) in patients with chronic HBV infection and liver disease remains to be explored. The second and more direct approach makes use of DNA cleavage enzymes delivered within viral vectors to target hepatocytes. These include homing endonucleases or meganucleases, zinc – finger nucleases (ZFNs) (Weber *et al.*, 2014), transcription activator – like effector nucleases (TALENs) (Bloom *et al.*, 2013) and, more recently, the clustered regularly interspaced short palindromic repeats (CRISPR)/CAS based – strategies (Kennedy *et al*., 2015a); (Kennedy *et al*., 2015b); (Ramanan *et al*., 2015); (Seeger and Sohn, 2014). Most of the available results have been generated in cell models or after hydrodynamic injection of mice with both HBV DNA and CRISPR/CAS expression plasmids, resulting in an *in vivo* co – transfection of the mouse liver that mimicked an acute HBV infection rather then an established chronic HBV infection. Moreover, not all the cell models allowed a correct evaluation of the impact on cccDNA levels. Despite their great potential, a number of critical issues need to addressed before DNA cleavage approaches will be amenable to enter clinical development: a) proof of efficacy in relevant animal models of chronic HBV infection (i.e. immune – deficient humanized mice); b) evaluation of off – target effects; c) careful assessment of resistant viruses selection *in vivo*; d) potential targeting of integrated viral sequences and its consequences on viral pathogenesis and host genome stability.

Targeting HBV Capsids

The HBV capsid is essential for HBV genome packaging, reverse transcription, intracellular trafficking and the re – import of encapsidated HBV genomes into the nucleus. The elucidation of the HBc crystal structure and the clarification of core dimers assembly process led to the development of several compounds that target HBc and capsid assembly and display anti – HBV therapeutic potential (Zlotnick *et al.*, 2015). Since HBV capsid proteins can also traffic to the nucleus of infected cells, those compounds that are capable to target HBc nuclear functions, i.e. regulation of ISGs expression and/or regulation of cccDNA transcriptional activity, will have a great potential for enhanced antiviral activity (Petersen *et al.*, 2016).

The phenyl – propenamide derivatives AT – 61 and AT – 130 increase capsid assembly reaction rate (Delaney *et al.*, 2002); (Feld *et al.*, 2007), interfere with HBV RNA packaging and with the formation of apparently normal empty capsids (Feld *et al.*, 2007); (Katen *et al.*, 2010). It causes tertiary and quaternary structural changes without disrupting the capsid structure (Petersen *et al.*, 2016); Figure 15A.

Despite their favorable toxicity profile this class of compounds has not been moved into clinical trials. Heteroaryldihydropyrimidines (HAPs) act as allosteric effectors to increase the kinetics of assembly, strengthen dimer – dimer association and prevent the proper formation of viral capsids (Deres *et al.*, 2003) with the formation of aberrant core particles at high concentrations (Stray *et al.*, 2005); Figure 15B. In a short – term study in HBV – infected uPA/SCID human liver – chimeric mice Bay 41-4109 reduced HBV DNA by 1 log but HBV replication showed rebound soon after treatment cessation (Brezillon *et al.*, 2011). Crystallographic studies indicate that HAP – 1 binds to a hydrophobic pocket at the dimer – dimer interface (Bourne *et al.*, 2006), an observation that is supported by the generation of a HAP – resistant mutation of core protein, V124W, where the incorporated tryptophan partially fills the binding pocket and results in a protein that confers HAP – like activities (Tan *et al.*, 2013). More recent studies in the optimization of the HAP series have focused on increasing potency (Wang *et al.*, 2012); (Guan *et al.*, 2015), aqueous solubility and the application of structural

constraints (Zhu *et al.*, 2010). Structural elaboration of the peripheral functionality surrounding the HAP core identified GLS-4 (Wang *et al.*, 2012). GLS-4 is reported to be more potent than BAY-41-4109 *in vitro*, demonstrating an EC50 of 12 nM in stably transfected HepG2.2.15 cells (Wang *et al.*, 2012), and in comparative studies measuring cell viability in primary human hepatocytes following treatment with increasing compound concentrations, GLS-4 demonstrated no toxicity up to 25 mM, whereas BAY-41-4109 showed 60% toxicity at the same concentration (Wu *et al.*, 2013). *In vivo* testing of GLS-4 in nude mice inoculated with HepAD38 cells showed a strong and sustained suppression of viral DNA (similar to that of BAY-41-4109) (Wu *et al.*, 2013), and the compound is reported to be the subject of initial clinical evaluation in China (Zhou *et al.*, 2013). Metabolite identification studies on BAY-41-4109 with mouse and human liver microsomes indicate that aromatization of the HAP core to the corresponding pyrimidine is the major metabolic pathway, and is consistent with the suboptimal pharmacokinetic profile that has been observed for HAPs. A recent study has focused on the chemical optimization of the series and the inclusion of a methyl group at the 4 - position of the dihydropyrimidine core to block aromatization, resulting in analogs with a significantly improved pharmacokinetic profile (Qiu *et al.*, 2016). Morphothiadine mesilate (GLS4) is the first member of the HAP family of compounds that entered early clinical development. Phase I and II clinical trials have been conducted in China (Wang *et al.*, 2012); (Zhou *et al.*, 2013), but no clinical results have been reported to date. Studies have proded that for HAP-1 the strongest HAP density was located in the pocket of the C – subunit (Bourne *et al.*, 2006), whereas for AT – 130, the strongest density was in the B – subunit pocket. HAPs and phenyl – propenamides both synergize with NRTIs and are active against NRTI resistant strains *in vitro* (Delaney *et al.*, 2002); (Billioud *et al.*, 2011), highlighting the potential for combining capsid inhibitors with other antivirals.

NVR 3-778, a member of the Sulfamoyl Carboxamide class of core – inhibitors, induces the misassembly of HBV core proteins *in vitro* and blocks the secretion of both infectious HBV DNA – containing virions and HBV RNA – containing particles by inhibiting pgRNA encapsidation in HBV replicating cells. NVR 3-

778 antiviral activity is additive with NRTIs and other core – inhibitors in cell models and is conserved against all HBV genotypes from A to H and many NRTI – resistant strains (Petersen *et al.*, 2016). In the humanized uPA/SCID mouse model, NVR 3-778 monotherapy for 6 weeks was superior to PEG – IFN and the combination of NVR 3-778 with PEG – IFN reduced serum HBV DNA below the limit of quantitation in all treated animals but without affecting intrahepatic cccDNA and pgRNA levels (Petersen *et al.*, 2016). A phase 1a doseranging trial of orally administered NVR 3-778 in healthy adult volunteers [NVR3-778-101 Protocol, Clinicaltrials.org # NCT02112799] showed a good safety and tolerability profile (Petersen *et al.*, 2016). The phase 1b clinical trial enrolled 36 HBeAg positive chronic hepatitis B adult naïve patients with serum HBV DNA > 20,000 IU/ml and no history of clinical decompensation in 4 dosing cohorts [100, 200, 400 mg QD and 600 mg BD] (Petersen *et al.*, 2016). Safety and tolerability of NVR 3-778 was satisfactory for all cohorts, with no treatment – related discontinuations or serious adverse events with the exception of a severe hand and feet rash in one patient treated with the lowest dose. Significant HBV DNA reductions (1.72 log10, range 1.06-3.71 log10 IU/mL) were observed only with the 1200 mg (600 mg BD) daily dose (Petersen *et al.*, 2016). Additional clinical trials are currently testing NVR 3-778 combination with PEG – IFN (Petersen *et al.*, 2016) and higher NVR 3-778 doses as well as nucleoside combination regimens are planned. In an interim analysis, the combination of NVR 3-778 plus PEG – IFN led to some reduction of HBV DNA and HBeAg within the observation period of four weeks, but not to reduction of HBsAg (Petersen *et al.*, 2016).

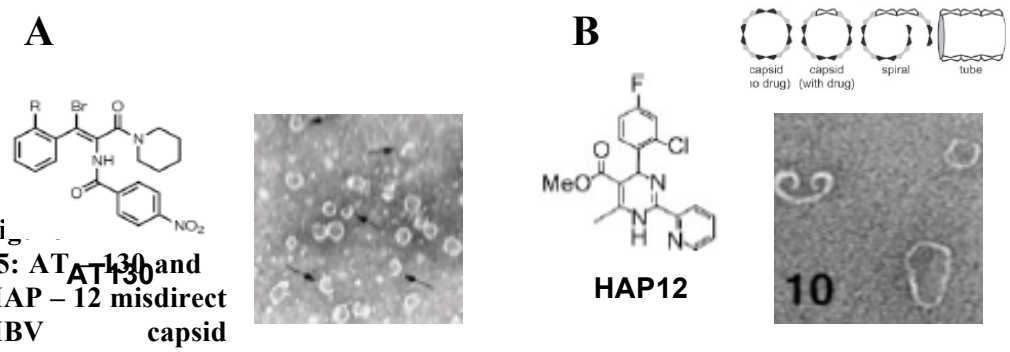

Figure 15: AT – 130 and HAP – 12 misdirect HBV capsid assembly and block HBV replication. Figure 15A. AT – 130 capsids are more "normalish" but inactive; Figure 15B. HAP – 12 leads to aberrant structures

Inhibition of HBV gene expression

Two main approaches have been developed to inhibit HBV gene expression at the post – transcriptional level in order to reduce the excess production of sub – viral particles and, by relieving HBsAg – mediated immunosuppression, aimed at restoring antiviral immunity.

HBV secretory pathways. α-Glucosidase inhibitors (Block *et al*., 1994); (Durantel *et al.*, 2007) and triazol – o – pyrimidine derivatives (Yu *et al.*, 2011) both inhibit HBV secretion. However, retention of HBV envelope proteins raises concerns about the activation of stress signals with oncogenic potential. More recently, the benzimidazole compound BM601 has been shown to inhibit selectively the intracellular relocalization of the HBV surface proteins to the Golgi leading to decreased HBsAg and HBV release without inducing ER stress or affecting cellular proteins and HBeAg secretion (Xu *et al.*, 2014). Nucleic Acid Polymers (NAPs), previously referred to as amphipathic DNA polymers (APs), are sequence – independent phosphorothioated oligonucleotides (PS – ONs) that display a broad spectrum of antiviral activity against several enveloped viruses, including HBV. Since NAPs activity is not sequence dependent they can be engineered to retain their antiviral potency without the pro – inflammatory activity and the off target effects common to other oligonucleotide – based drugs.

REP9-AC (REP 2055), a 40-nucleotide polycytidine amphipathic DNA polymer, inhibits DHBV infection (Noordeen *et al.*, 2013) and HBsAg release from infected hepatocytes, and prevents the establishment of DHBV infection *in vivo* (Petersen *et al.*, 2016). Recent *in vivo* data obtained in the DHBV model have validated new NAPs (REP 2165) with antiviral activity comparable to REP 2139 but significantly lower liver accumulation (Petersen *et al.*, 2016) and showed that an interferon free regimen of REP-2139 with TDF or ETV improved antiviral outcomes and allows the shortening of antiviral regimens in patients with chronic HBV infection (Petersen *et al.*, 2016). Larger controlled studies and a comprehensive evaluation of HBV and HDV virology in the liver of NAPs responder patients are needed together with immunological studies to evaluate the occurrence and the extent of immune restoration following NAPs induced HBsAg clearance (Petersen *et al.*, 2016).

RNA interference (RNAi). RNA interference describes the conserved biological pathway for sequence – specific mRNA cleavage and gene silencing (Randall and Rice, 2004); (Bernstein *et al.*, 2001); (Hammond *et al.*, 2000). HBV is an attractive therapeutic target for RNAi strategies the overlapping reading frame means that multiple transcripts can be targeted with a single siRNA sequence. The first wave of RNAi candidates were being developed to knock down production of HBV proteins with the intention of restoring the anti – HBV immune response. The primary focus is HBsAg, although HBeAg, HBcAg and HBxAg are all of interest. There have been a number of challenges: siRNA are not bioavailable and require parenteral (SC/IV) delivery, carrier molecules are necessary to prevent nuclease degradation and for hepatocyte targeting, but must have a good safety profile, and allergic reactions have been reported. Careful sequence design is necessary to limit the risk of off – target gene silencing. Multiple siRNA may be necessary to prevent the selection of resistant HBV mRNAs. Three candidates have entered clinical development. ARC-520 is composed of two cholesterol – conjugated siRNA duplexes directed against 2 different HBV sequences in the DR1-DR2 region that are highly conserved among all HBV genotypes (Wooddell *et al.*, 2013). An ongoing phase 2a single – dose – escalation study in humans has demonstrated reduction in serum HBsAg with mean nadir -51% (range -46 to -59)

with a mean change on day 85 of -22% (range -7 to -40). Reductions in HBeAg and HBV core – related antigen were also observed. ALN – HBV is a second siRNA formulation for the treatment of HBV infection that employs a lipid nanoparticle (LNP) formulation for drug delivery to the liver. This agent can be delivered subcutaneously. The target sequences have been designed to knock down all HBV transcripts (0.7 kb region overlapping all 4 transcripts and 1.4 kb region overlapping across 3 transcripts [S, pol, pregenomic RNA]). ALN – HBV has entered phase 1 development. TKM – HBV is a third RNAi therapeutic. TKM – HBV must be delivered intravenously, and also employs an LNP formulation for hepatocyte delivery (Petersen *et al.*, 2016). TKM – HBV simultaneously targets three sites on the HBV genome, including integrated S-ORF. In a humanized mouse model of HBV, TKM – HBV was associated with reductions in HBsAg, HBeAg, HBV DNA and cccDNA. A phase 1 program has been initiated in 2015 (Petersen *et al.*, 2016).

The early RNAi experience is also providing new insights into HBV biology and the potential utility of HBsAg as a treatment end – point. Emerging data from the Arrowhead chimp studies highlight the relative importance of integrated HBV DNA relative to cccDNA for HBsAg production. The data suggest that in HBeAg-negative animals a significant fraction of circulating HBsAg was derived from integrated HBV DNA producing Pre-S/S transcripts that had lost the ARC-520 target sites, rather than from cccDNA. The implication is that cccDNA could potentially be eradicated and yet an individual remain HBsAg – positive, due to HBsAg production from integrated HBV DNA. This is an important issue for the field, both for therapeutic development, as well as prognostication, particularly for individuals in the immune control phase of CHB. ARC-521, a 2nd generation RNAi agent, has been designed to knock down HBsAg derived from both integrated HBV DNA as well as cccDNA (Petersen *et al.*, 2016).

Cyclophilin inhibitors

Cyclophilins are cytoplasmic proteins involved in the life cycle of a wide range of viruses including HCV and human immunodeficiency virus. Alisporivir is a cyclophilin A inhibitor that was developed for the treatment of HCV. Recent

preclinical data suggest that alisporivir also inhibits HBV DNA replication as well as HBsAg production and release (Phillips *et al.*, 2015). A clinical program is planned. OCB-030 (formerly NVP018) is a second generation cyclophilin inhibitor. Preclinical data suggest that OCB-030 has dual anti – HBV activity including direct inhibition of HBV replication, as well as immune modulation via interferon regulatory factors (IRFs), including potent inhibition of an interaction between cyclophilin A and IRF9 (Petersen *et al.*, 2016).

Immunomodulation

Over the past decade, studies of the immunopathogenesis of HBV infection have identified a number of potential novel approaches.

Innate immune ligands

Toll – like Receptor (TLR) ligands. Toll – like receptors are pattern recognition receptors that play a key role in the recognition of foreign pathogens, both bacteria and viruses, as part of the innate immune response (Thompson and Locarnini, 2007). TLR expression and signalling is involved in the pathogenesis of HBV (Broering *et al.*, 2014); (Jiang *et al.*, 2014); (Wu *et al.*, 2010); (Wu *et al.*, 2007); (Thompson *et al*., 2009); TLR3, 7/8 and TLR9 represent a TLR subfamily that recognize endosomal viral nucleic acids (dsRNA, ssRNA and CpG motifs respectively), and induce a type-1 interferon response. Therapeutic manipulation of this pathway is possible and a TLR7 agonist is in clinical development (Lanford *et al.*, 2013); (Gane *et al*., 2015).

RIG-I ligands. Retinoic acid-inducible gene (RIG-I)-like RNA helicases (RLHs) are a second class of innate pattern recognition receptors. RLHs recognize dsRNA in the cytoplasm. RIG-I has been recently shown to have dual antiviral effect against HBV (Sato *et al*., 2015). RIG-I recognizes the 5′-ε region of HBV pgRNA leading to induction of a predominantly type III interferon response. RIG-I also counteracts the interaction of the HBV polymerase with pgRNA to directly suppress viral replication. SB-9200 is an oral dinucleotide prodrug; the active SB 9000 isomer products bind to RIG-I and NOD2 to stimulate an interferon response. SB-9200 has activity against both HCV and HBV in preclinical models

and was recently used sequentially in combination with ETV in the woodchuck model (Petersen *et al.*, 2016).

Check – point inhibitors

Chronic hepatitis B infection is associated with an "exhausted" HBV – specific CD8+ T cell phenotype, characterized by high levels of expression of inhibitory molecules including programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte antigen 4 (CTLA-4), lymphocyte activation gene 3 (LAG-3), T cell membrane 3 (Tim-3) and CD244 (2B4) (Chen and Flies, 2013); (Raziorrouh *et al.*, 2010); (Blackburn *et al.*, 2009). Collectively these molecules are termed 'checkpoints'. Checkpoint inhibitors, which block these inhibitory pathways, have been shown in experimental models to rescue virus specific CD8+ T cells by improving proliferation, cytotoxicity and cytokine production (Fisicaro *et al*., 2010); (Nebbia *et al*., 2012); (Schurich *et al.*, 2011); (Peppa *et al.*, 2010). Nivolumab (BMS-936558) and pembrolizumab (MK-3475) are both anti – PD-1 monoclonal antibodies that have progressed through phase 3 development for the treatment in oncology. Grade 3 – 4 adverse events have been seen in approximately 15% of patients treated with nivolumab in oncology patients, A phase 1 study of nivolumab for the treatment of advanced hepatocellular carcinoma that included patients with CHB has been presented in abstract form (Petersen *et al.*, 2016). Phase 1 studies of anti – PD-1 therapies for the treatment of CHB have not yet commenced, but are likely.

cIAP inhibitors

Cellular inhibitor of apoptosis proteins (cIAPs) have recently been shown to attenuate TNF signaling during HBV infection and restrict the death of infected hepatocytes, promoting viral persistence. Antagonizing the function of cIAPs may therefore promote the clearance of HBV infection. Birinipant is already in clinical development for the treatment of advanced malignancy. A phase 1 study of birinapant for the treatment of HBV was initiated in 2015 (Petersen *et al.*, 2016).

VACCINATION

Prophylactic vaccination

In view of the still unsatisfactory therapy of HBV infections prevention has highest priority. Besides obeying strict hygiene with all invasive procedures and a considerate life style, vaccination is the most important way to prevent hepatitis B diseases. In 1975 that Blumberg's concept of using purified 20 nm HBsAg particles from carrier plasma as HB vaccine was valid and could protect against an intravenous challenge with 3000 chimpanzee – infectious doses (Purcell and Gerin, 1975). Philippe Maupas and Alain Goudeau went one step further and used purified formalin – treated purified HBsAg as vaccine. In 1976 they reported good protection rates particularly in the staff of hemodialysis wards (Maupas *et al*., 1976). With the cloning of the HBV genome and the identification of the HBs gene in 1979 a new era of vaccine production was opened. It appeared logical to use the gene encoding the major HBs protein for production of the "recombinant" vaccine, i.e. using recombination of the gene with the DNA of expression vectors. The vaccine was generated thanks to yeast cell strains which expressed the major HBs protein in very large amounts at low cost (Harford *et al*., 1983). Thus, in 1986 yeast – derived HBsAg became the standard vaccine against HBV. Although these historical studies of therapeutic vaccination for HBV using traditional HBV protein vaccines have been disappointing, this approach is still being pursued with new vaccine candidates. ABX203 is a therapeutic vaccine composed of 2 recombinant proteins, HBsAg and HBcAg. A phase 2B/3 study of the efficacy of ABX203 for maintaining persistent HBV DNA suppression in HBeAg – negative patients after cessation of NA therapy is currently enrolling. Novel vaccine approaches currently in clinical development include GS-4774 (Gaggar *et al*., 2014), TG1050 (Martin *et al.*, 2015), and INO-1800 (Obeng-Adjei *et al.*, 2013). GS-4774 is a tarmogen vaccine made from genetically modified yeast expressing HBV core, X, and surface proteins. GS-4774 elicited HBV specific T cell immune responses in a phase 1 study (Gaggar *et al*., 2014). The results of a phase 2 dose-finding study evaluating GS-4774 for 24 weeks in patients virally suppressed by long – term NA have recently been presented. The primary endpoint was

reduction in HBsAg level. Modest reductions in HBsAg were observed; no patients lost HBsAg (Petersen *et al.*, 2016). GS-4774 appeared to be safe and well tolerated. A second phase 2 study evaluating the efficacy of combination therapy of GS-4774 plus tenofovir for treatment naïve patients is currently enrolling. TG1050 is an adenovirus – based immunotherapy encoding a unique large fusion protein composed of a truncated HBcAg domain, a modified HBV Polymerase domain and two HBsAg domains. In a murine model of HBV TG1050 induced a robust cross – reactive T cell response leading to reduction in HBV DNA and HBsAg levels (Martin *et al.*, 2015). A phase 1 program started in late 2015. INO-1800 is a DNA vaccine encoding HBsAg and HBcAg that generated robust cytotoxic and antibody responses in mice and Rhesus macaques. A phase 1 open-label dose escalation study of INO-1800 in combination with INO-9112 (DNA plasmid encoding human interleukin 12) delivered by electroporation in HBeAg – positive, HBV DNA – negative patients taking entecavir or tenofovir is planned. As a proof of concept study, in a preclinical transgenic mouse model, it was shown that reducing first viral antigens with an adeno-associated virus targeting HBV transcripts via RNAi, followed by a boost vaccination with an protein prime/modified vaccinia virus Ankara (MVA) virus was sufficient to induce some HBV specific T cell responses (Petersen *et al.*, 2016). Finally, preclinical data support the concept of T – cell therapies. Engineering HBV – specific T cells through transfer of HBV – specific T cell receptor or HBV – specific chimeric antigen receptors represent promising alternative strategies to construct an HBV-specific T – cell immunity in many CHB patients (Krebs *et al*., 2013); (Gehring *et al*., 2011).

HBV CORE PROTEIN BINDING TO HOST GENE PROMOTERS

HBV targets host genes that are involved in cell survival, and while this facilitates escape from immune surveillance and clearance, it also favors malignant transformation (Brechot, 2004). One of the possible mechanisms that contributes to malignant transformation involves HBV – encoded transactivating factors,

which influence particular intracellular signal transduction pathways by altering the host gene expression profile in hepatocytes (Lee and Lee, 2007). Several HBV protein products have been characterized as modulators of cellular growth, repair, and death, all of which are involved in oncogenesis (Murakami, 1999). Studies have shown that HBx can transactivate the expression of all HBV proteins, including HBc, which acts as the core antigen that stimulates the human immune response. The 21 – 22 kDa HBc protein has been detected in both the nuclear and cytoplasmic compartments of hepatocytes infected by HBV (Petit and Pillot, 1985). Subsequent studies have revealed pleiotropic functions of HBc that affect host processes, including the malignant transformation of chronically infected liver cells (Guo *et al.*, 2012). Moreover, this newly – recognized dual character of HBc as a novel regulator of the HBV life cycle and of hepatocellular carcinogenesis has been hypothesized to involve HBx, possibly through an inhibitory feedback mechanism (Kim *et al*., 2003). HBc was shown to repress the expression of the human tumorigenesis – associated genes, interferon (IFN) – β and p53 (Kwon and Rho, 2003); the latter of which is also modulated by HBx, via binding to the encoded protein and suppressing its activity (Elmore *et al.*, 1997). Additionally, recent studies have suggested that HBV down – regulates the human IFN inducible MxA promoter through direct interaction of precore/core proteins (Fernandez *et al*., 2003), and have shown that HBc inhibits apoptosis induced by the tumor necrosis factor family member, TRAIL, in hepatocytes by blocking gene expression of the pathway-related death receptor (Guo *et al.*, 2012). Collectively, these studies indicate that HBc may interact with the human genome to modulate normal functions of liver cells infected HBV (Guo *et al.*, 2012). Although HBc has been sufficiently demonstrated by many studies to functionally bind to both virus- and host- derived RNA and DNA, the way in which HBc interacts with the human genome to modulate normal hepatocyte function in HBV infection remains unknown. Based on the findings of *in vivo* studies that showed core particles binding specifically to the HBV pregenome and genome, we hypothesized that HBc may also bind specifically to certain human gene promoters, either through its C – terminal functional domain or its N – terminal assembling domain (Guo *et al.*, 2012). A recently developed high-throughput

strategy to perform targeted or genome – wide studies of transcription binding factors is the chromatin immunoprecipitation (ChIP) – coupled DNA microarray analysis. Known as ChIP – on – chip, this technique couples immunoprecipitation of chromatin – bound transcription factors with the identification of bound DNA sequences through hybridization on DNA microarrays (Guo *et al.*, 2012). In a recent study Guo Y. *et al*. used a combination of ChIP and location analysis with genome – wide tiling arrays to generate a human genome – wide binding profile of HBc. The human genes whose promoters were bound by HBc and had functions related to tumorigenesis were selected for verification by quantitative PCR (qPCR) and functional analysis by gene expression assays. Identification of the human gene targets of the HBV – encoded HBc protein provides further insights into HBV pathogenesis and potential new targets of molecular therapeutics against HBV – associated hepatocellular carcinogenesis (Guo *et al.*, 2012). We performed ChIP-on-chip analysis with the NimbleGen HG18 RefSeq Promoter Microarray consisting of 18028 of the best defined human transcript – associated promoters. Raw microarray data were extracted as pair files by the NimbleScan software. They performed median – centering, quantile normalization, and linear smoothing by using the Bioconductor packages, Ringo, Limma, and MEDME. The gene ontology (GO) and pathway analysis of promoter-related genes were carried out online (http:// www.geneontology.org). Gene pathway analysis was conducted with the Kyoto Encyclopedia of Genes and Gen- omes (KEGG) collection of online databases dealing with genomes, enzymatic pathways, and biological chemicals (Guo *et al.*, 2012). Hepatocytes infected with HBV and expressing HBV core protein were isolated from CHB patients, immunoprecipitated with HBc antibody, and investigated to determine the genome – wide profile of bound host gene promoters. In total, 3100 HBc – immunoprecipitated host promoter regions were found to be enriched by at least 2 – fold in HBc – infected tissues compared to the blank controls. They showed that the HBV core protein might bind to a broad spectrum of human gene promoters, especially those featuring high CpG density. The GO annotations for biological processes were found for 1933 of the genes related to the immunoprecipitated promoters, and annotations for molecular functions were

found for 2022. The biological processes represented within this dataset of putative HBc targets included: metabolic process 1404; primary metabolic process 1286; cellular metabolic process 1248; and biological regulation 1206 Figure 16. In addition, a remarkable amount of genes were found to be associated with dsRNA fragmentation, and intermediate filament cytoskeleton organization Figure 17. These results suggested that HBV core protein can bind to host gene promoters, especially those involved in tumor pathogenesis (Guo *et al.*, 2012).

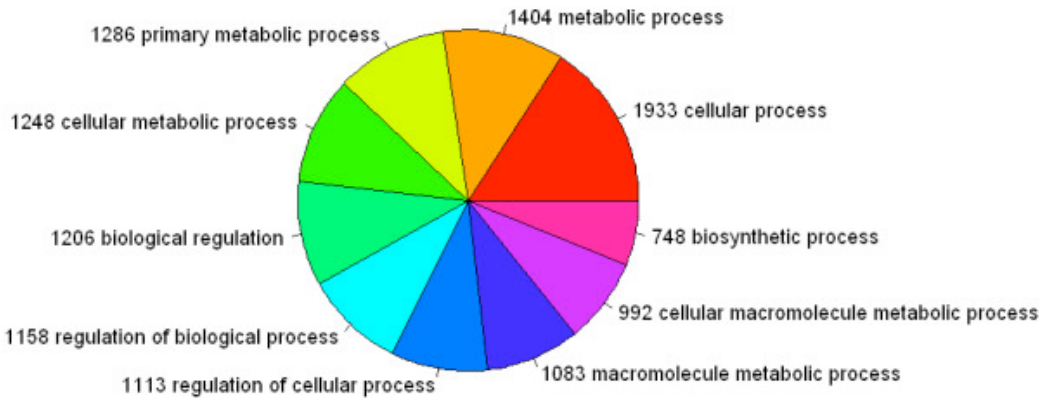

Figure 16: Count proportion pie of the GO analysis of Biological Process. The chart shows the top ten counts of the significant enrichment terms of designed gene involving Biological Process (Guo *et al.*, 2012).

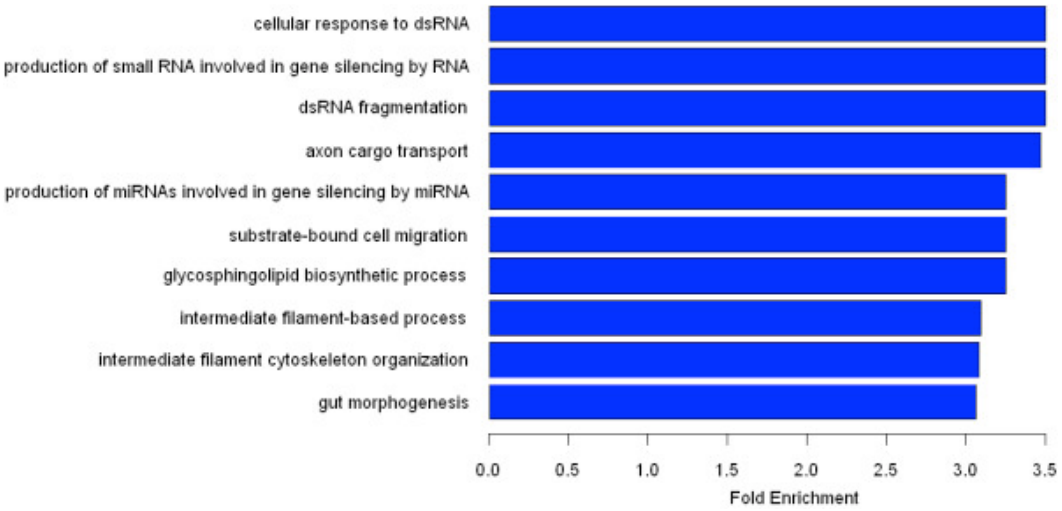

Figure 17: The bar plot of the GO analysis of Biological Process. The bar plot shows the top ten fold enrichment value of the significant enrichment terms of designed gene involving Biological Process (Guo *et al.*, 2012).

AIM

Infection with hepatitis B virus continues to be a major health problem with about 400 million people chronically infected worldwide who are at high risk of developing liver cirrhosis and hepatocellular carcinoma (HCC) (Guerrieri *et al.*, 2013). HCC is one of the most frequent solid tumors worldwide and represents the third cause of mortality among deaths from cancer (Zucman-Rossi and Laurent-Puig, 2007) and recent estimates attribute to HBV >50% of HCC cases worldwide. Currently available treatments in patients with chronic HBV hepatitis, based upon direct nucleos(t)ides analogs targeting the viral RT/Polimerase, suppress viral replication and halt disease progression in >98% of the patients. However, these treatments as well as Interferon based regimens, cannot completely eliminate the infection and the rate of "functional cure" (i.e. viral control without ongoing treatment) is as low as 10 – 15% (Vlachogiannakos and Papatheodoridis, 2013). There are two strategies to defeat HBV: eradication that means driving the virus to extinction from the earth (vaccination is the tool); cure that means eliminating the virus from the infected host (an effective treatment would be the tool). The barrier to cure chronic hepatitis B is the cccDNA. HBV cccDNA is the obligatory replicative intermediate responsible for persistent HBV infection and is the template for the transcription of all viral mRNAs, including the pregenomic RNA (pgRNA) which is reverse transcribed to produce the first HBV DNA strand. The accumulation and the maintenance of the cccDNA pool is ensured in infected hepatocytes by newly synthesized nucleocapsids which are not enveloped and secreted into the blood, but are transported into the nucleus. In quiescent cells the cccDNA is very stable and can persist throughout the life span of the hepatocytes without affecting its viability. Very low levels of cccDNA can persist indefinitely, possibly explaining lifelong immune responses to HBV despite clinical resolution of HBV infection. Many strategies can be applied to overcome this barrier: improve NAs potency, broaden viral targets (viral entry, nucleocapsid or virion assembly...), deplete or silence cccDNA.

HBV is a DNA virus organized into nucleosomal structures. HBV produces covalently closed double – stranded DNA (cccDNA) that is found in the nuclei of infected cells as a viral minichromosome (Levrero *et al.*, 2009). HBV encoded proteins HBx and HBc both bind the cccDNA early after infection (Belloni *et al.*, 2009); (Bock *et al.*, 2001). HBx is required for the inactivation of the HBV cellular restriction factor smc5/6 (decorsiere) and to establish and maintain cccDNA transcription (Levrero *et al.*, 2009); (Riviere *et al*., 2015). HBc binding to the cccDNA in thought to contribute to cccDNA chromatinization and nucleosome spacing (Bock *et al.*, 2001) whereas its impact on cccDNA transcription is still debated.

Increasing evidence indicate that HBV proteins regulating viral minichromosomes also interact with the cellular chromatin and chromatin modifying enzymes to target cellular genes expression through epigenetic modifications (Cougot *et al.*, 2007); (Cougot *et al.*, 2012). A ChIP – Seq genome wide analysis of HBx chromatin recruitment in HBV replicating cells has defined a broad repertoire cellular genes (~4.000) of and ncRNAs (75 miRNAs and 34 lncRNAs) potentially regulated by HBx with an enrichment in genes/ncRNAs involved in cell metabolism, chromatin dynamics and cancer but also in genes/ncRNAs that modulate HBV replication. HBc has also been shown to bind to cellular chromatin (~1000 genes) (Guo *et al.*, 2012) and to the promoters of a subset of cellular genes involved in inflammatory responses and innate immunity.

Core protein (Cp) represents an attractive new therapeutic specific target for HBV chronic infection. Cp is essential for HBV genome packaging, reverse transcription, intracellular trafficking and the reimport of encapsidated HBV genomes into the nucleus, but due to its nuclear functions drugs targeting the Cp may also impact on cccDNA transcriptional activity and host genes reprogramming with a great potential for enhanced antiviral activity. The elucidation of the HBc crystal structure and the clarification of core dimers assembly process has led to the development of several compounds that target Cp and capsid assembly inhibit HBV replication therapeutic potential (Zlotnick *et al.*, 2015). We focused on two different classes of Cp assembly modulators. Heteroaryldihydropyrimidines (HAPs) act as allosteric effectors to increase the

kinetics of assembly, strengthen dimer – dimer association and prevent the proper formation of viral capsids with the formation of aberrant core particles at high concentrations (Stray *et al.*, 2005). The phenyl – propenamide derivatives AT – 61 and AT – 130 increase capsid assembly reaction rate, interfere with HBV RNA packaging and to the formation of apparently normal empty capsids. Interestingly, compounds belonging to the HAP chemical class have moved into clinical trials.

In this study I will focus on the impact of two classes of capsid inhibitors HAP – 12 and AT – 130 on the formation, accumulation and transcription of cccDNA as part of their anti – HBV activity in HBV replicating cells, I will evaluate the activity of HAP – 12 on HBV replication in new, more relevant HBV infection models: NTCP – HepG2 (Na^+/taurocholate co – transporting polypeptide) infection system and primary human hepatocytes (PHH), and I will investigate the impact of HAP – 12 treatment on HBV Cp recruitment on the cccDNA and to expand these observation on the regulation of cellular genes targeted by Cp.
All relevant virological parameters will be evaluat: capsid – associated HBV – DNA, cccDNA levels (TaqMAN real – time PCR) and pgRNA levels (quantitative real – time PCR). Nuclear cccDNA will be visualiz by DNA Fluorescence In Situ Hybridization (FISH) using HBV specific probes encompassing the whole HBV genome. Anti-Hbc, anti-HBx and anti-AcH4 Chromatin Immuno Precipitations (ChIPs) and cccDNA – ChIP experiments will be performe in TET-released HepAD38 cells and in mock and HBV-infected NTCP – HepG2 cells and PHHs and analyzed by TaqMan real – time PCR using cccDNA and gene specific primers.

MATERIALS AND METHODS

MATERIALS

CHEMICALS AND REAGENTS

Dimethyl sulfoxide (DMSO)	Sigma-Aldrich
DNase I	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM)	Life technologies
ECL western blotting detection Reagents	GE Healthcare
EcoRI	New England biolabs
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Formaldehyde	Sigma-Aldrich
G-418	Life technologies
Glycogen	Roche
Hydrocortinson	Sigma-Aldrich
Insulin	Sigma-Aldrich

Isopropanol	Sigma-Aldrich
Maxima H minus first strand cDNA synthesis kit	Thermo scientific
MES SDS running buffer	Life technologies
$MgCl_2$	
milkpowder	Biorad
NaCl	Sigma-Aldrich
NP-40	Sigma-Aldrich
NuPage Bis-Tris Gel	Life technologies
PBS 1X	Life technologies
Penicilin-streptomycin	Sigma-Aldrich
Piperazine N,N bis zethone sulfonic acid (PIPES)	Sigma-Aldrich
Plasmid-Safe ATP dep. DNase	Epicentre
Polyethylenglycol (PEG) 8000	Sigma-Aldrich
Proteinase!inhibitor (PIC)	Sigma-Aldrich
Proteinase K	Sigma-Aldrich
PvuI	New England biolabs
RQ1 RNase-Free DNase	Promega

RQ1 RNase Free DNase stop solution	Promega
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
TransIT-LT1 transfection reagent	Mirus
Triton X-100	Sigma-Aldrich
Trizol	Life technologies
FISH kit	Life technologies
MTT	Sigma-Aldrich

CELLS

Name	Description
HepG2	These human hepatoma cells are derived from a 15 years old HCC patient from Argentina (Knowles *et al*., 1980); (Aden *et al*., 1979).
HepAD38	This human hepatoma cell line is derived from HepG2 cells which are stably transfected with a plasmid containing the HBV pgRNA under the control of a tetracycline-inducible promoter (Ladner *et al.*, 1997).
NTCP-HepG2	Stable clone expressing high levels of the HBV entry receptor Na^+/taurocholate co – transporting polypeptide (NTCP), that allow HBV infection *in vitro* (Ni *et al*., 2014).
PHHs	Primary human hepatocytes from multiple donors infeted with HBV inocula purified from the cell supernatants of HepAD38 stable cell lines.

CELL CULTURE MEDIA

Cells	Medium
HepG2	DMEM (Gibco); 10%FBS (Gibco); 2mM L-Glutamine; 50U/ml Penicillin; 50µg/ml Streptomycin
HepAD38	**Culture medium:** DMEM/F012; 10% FCS (not heat inactivated); 50U/ml Penicillin; 50µg/ml Streptomycin; 400µg/ml G418, 50µM Hydrocortisone-Hemisuccinate; 5µg/ml Insulin; 0,3µg/ml Tetracycline **Virus production medium:** culture medium without tetracycline
NTCP – HepG2	**Culture medium:** see HepG2 plus 5µg/ml Puromycin **Infection medium:** culture medium plus 2,5% DMSO
PHHs	Bought them on Life Technologies website; growth medium as indicated in their protocol **Infection medium:** culture medium plus 2,5% DMSO

ANTIBODIES

Anti-HBc	U.S. biological, cat no. H19015-15
Anti-HBx	Thermo Scientific, cat no. MA1-081
Anti-H4	Abcam, cat no. ab10158
Anti-AcH3	Upstate, cat no. 07-352
Anti-AcH4	Millipore, cat no. 06-866
Anti-ACTIN	Santa Cruz Biotechnology, cat. no. sc1616
Anti-LaminB	Santa Cruz Biotechnology, cat. no. sc6216
Donkey anti-goat IgG-HRP	Santa Cruz Biotechnology, cat. no. sc2020
Donkey anti-rabbit IgG-HRP	Santa Cruz Biotechnology, cat. no. sc2316
Donkey anti-mouse IgG-HRP	Santa Cruz Biotechnology, cat. no. sc2005

PRIMERS AND PROBES

cccDNA

Primers: FOR – CTCCCCGTCTGTGCCTTC

REV – GCCCCAAAGCCACCCAAG

Probes: GTTCACGGTGGTCTCCATGCAACGT-FL

R640-AGGTGAAGCGAAGTGCACACGGACC

HBV DNA

Primers: FOR – CTCGTGGTGGACTTCTCTC

REV – CAGCAGGATGAAGAGGAA

Probes: CACTCACCAACCTCCTGTCCTCCAA – FL

R640 – TGTCCTGGTTATCGCTGGATGTGTCT

β-GLOBIN

Primers: FOR – ACACAACTGTGTTCACTAGC

REV – CAACTTCATCCACGTTCACC

Probes: CAAACAGACACCATGGTGCACCTGACTCCTGAGGA – FL

Red640 – AAGTCTGCCGTTACTGCCCTGTGGGGCAA

pgRNA

Primers: FOR – GCCTTAGAGTCTCCTGAGCA

REV – GAGGGAGTTCTTCTTCTAGG

Probes: AGTGTGGATTCGCACTCCTCCAGC – FL

Red640 – ATAGACCACCAAATGCCCCTATCTTATCAAC

ACTIN-B

Primers: FOR – GCACTCTTCCAGCCTTCCT

REV – AGGTCTTTGCGGATGTCCAC

Promoter – EZH2

Primers: FOR – ACATAATCAAACAGGGCCCGGG

REV – TTGGTCAGGCTGGTCTCGAACTCC

Promoter – SRC

Primers: FOR – TCCTGTCGTGACCGTCTCCTCTCT

REV – CTTGATCTGCCTGGCCTGATGG

Promoter – E2F1

Primers: FOR – CGTTGGCTGTTGGAGATT

REV – AGAGCCCAAGCTGAAGA

Promoter – IL29

Primers: FOR – GCCCAGGGAGTTCTAAGGAT

REV – CTGATGAGGGAACAGGTGTG

Promoter – GAPDH

Primers: FOR – CTTCTCCCCATTCCGTCTTC

REV – CCCCAGCTACAGAAAGGTCA

Promoter – TP53

Primers: FOR – GAGTGCAGTGGCACGATTT

REV – GAATCGCTTTCAGCTCAGGA

Promoter – IL6

Primers: FOR – CAGAGGAGAACCTGCACTATTC

REV – CAGTTTCAGTGCAGGGTAGAA

METHODS

CULTIVATION OF EUCARYOTIC CELLS

The cultivation of eukaryotic cells was done - if not other stated otherwise at 37° C in the presence of 5% CO_2 and a humidity of 95%. Prior to use cells were thawed and cultivated with the respective medium in a flask or cell culture dish. During cultivation, the cells were split twice a week. Therefore, the cells were washed with 1x PBS and incubated with 1x trypsin to get detached from the surface. After 50 10 min of incubation, the cells were resuspended in new culture medium by pipetting up and down to obtain a single cell suspension. Cells were split depending on cell type and growth conditions.

VIRUS PRODUCTION

HepAD38 cells were cultivated in HepAD38 culture medium (supplemented with tetracycline). After two weeks, the culture medium was replaced by virus production medium (without tetracycline) to induce the production of HBV.

HBV VIRUS CONCENTRATION

The supernatants from induced HepAD38 cells were collected from day 9 to 14 and centrifugated at 4000 rpm for 10 min. The supernatants were mixed thoroughly with 40% PEG8000 to a concentration of 6% PEG8000 and incubated overnight at 4° C. The samples were centrifuged for 1h at 10000rpm, 4° C and the pellet was resuspended in 1/100 of the sample volume in PBS with 10% FCS. The samples were thoroughly vortexed overnight at 4° C and centrifuged at 8000 rpm, 5min at 4° C. The virus – containing supernatants were collected and stored at -80° C in aliquots for further use.

HBV INFECTION OF NTCP – HepG2 AND PHH CELLS

For HBV infection of NTCP – HepG2 and PHH cells, the cells were seeded in a density of 2 x 10^5 cells/well in a 6-well plate. 1 day after seeding the medium was exchanged and differentiation medium (supplemented with 2,5% DMSO) was added. Two days after seeding the cells were inoculated with 1,5 x 10^8 of the PEG – precipitated HBV virus mixed with 40% PEG 8000 and infection medium to a total volume of 800 µl. The medium of the cells was removed and the mixture was added to the cells. After at least 16 h of incubation, the cells were washed 3 times with 1x PBS and infection medium was! added. The medium was changed every second or third day, depending on the experiment. To verify the specificity of the infections, mock cells were inoculated with infection medium with 40% PEG 8000.

DNA AND RNA QUNATIFICATION

The nucleic acids concentration was measured with a UV-Vis Spectrophotometer at 260 nm and 280 nm against ddH_2O. An absorbance of 1 at 260 nm corresponds to a concentration of 50 µg/ml dsDNA and to a concentration of 40 µg/ml RNA. The quality of the DNA was examined by measurement of the absorbance at 280 nm (absorbance maximum of proteins) and therefore samples were considered to be pure when the ratio of OD 260/OD 280nm was between 1,8 for DNA and 2 for RNA.

TRANSIENT TRANFECTION OF FULL – LENGTH HBV DNA GENOMES

Monomeric linear full length wild – type (WT) genomes were released from the pCR.HBV.A.EcoRI using EcoRI – PvuI. Linear HBV monomers were transfected into HepG2 human hepatoma cells using the Mirus Bio trans IT-LT1. Briefly, HepG2 cells were seeded at a density of 2-3 millions of cells in 100 mm diameter Petri dishes and transfected 24 hours later with 1 mg to 2 mg of digested HBV DNA. Unless specified otherwise, culture medium was changed 1 day after transfection and cells were harvested after 48 hours. All transfection included 0,5

mg of green fluorescence protein expression vector (GFP) to assess transfection efficiency (HepG2 cells range 28-32%).

CELL CULTURES AND TREATMENTS

Four cellular models of HBV infection/replication:

HepG2 hepatoma cells were trasfected with a linear full – lenght genotype A HBV DNA (Gunther *et al.*, 1995), than were treated with the anticapsid HAP – 12 used at a final concentration 1 μM concentration and added directly to the culture medium, 30 min after transfection. The treatment was repeated daily. The cells were harvested 96 hours post treatment. Untreated cells were exposed to the same DMSO final concentration.

HepAD38 cells, a stable clones derived from the hepatocellular cell line HepG2, carry a complete integrated HBV genome under the control of a tetracycline – off inducible promoter. Upon tetracycline removal, the viral pgRNA and mRNAs are transcribed from the integrated HBV DNA leading to the accumulation of subviral particles in the cytoplasm and cccDNA in the nucleus and secretion HBV virions in the cell supernatant (Ladner *et al.*, 1997).

TET – off system: cells were induced and treated with core – inhibitors HAP – 12 and AT – 130 respectively 1 and 5 μM for 10 days, from the moment of the beginning of viral replication. Untreated cells were exposed to the respectively same DMSO final concentration.

TET – on system: after a first 6 days period in which confluent cells were allowed to replicate HBV from the integrated genome (TET – off) leading to the accumulation of a cccDNA pool into the nucleus, capsids containing pgRNA and mature viral particles into the cytoplasm, 0.3 μg/ml tetracycline was added daily to culture medium for 7 days to inhibit integrated HBV DNA replication and to allow cccDNA transcription. TET – on HapAD38 were treated for six days starting 1 day later the introduction of Tet in the medium with HAP – 12 at final concentration of 1 μM. Untreated cells were exposed to the same DMSO final concentration.

NTCP – HepG2 cells were infected as described and manteined in differentiation medium. HAP – 12 1 μM was added daily for 10 days 1 day after infection, or daily for 6 days after a period of 10 days to allow the accumulation of viral intermediates. Untreated cells were exposed to the same DMSO final concentration.

PHH cells were infected as described and manteined in differentiation medium. HAP – 12 1 μM was added daily for 6 days after a period of 10 days to allow the accumulation of viral intermediates. Untreated cells were exposed to the same DMSO final concentration.

PURIFICATION AND QUANTIFICATION OF CORE PARTICLES ASSOCIATED WITH HBV DNA FROM HBV – REPLICATING CELLS

To purify HBV DNA from intracellular core particles, the cells were washed once with ice-cold PBS and lysed in 50 mmol Tris-HCl, pH 7.4, 1 mmol EDTA, and 1% NP-40 (lysis buffer A). Nuclei were pelleted by centrifugation for 1 minute at 10.000 g. The supernatant was adjusted to 100 mmol $MgCl_2$ and treated with 100 mg/ml of DNase I for 60 minutes at 37° C. The reaction was stopped by adding EDTA to a final concentration of 25 mmol. Protein was digested with 0.5 mg/ml proteinase K and 1% SDS over night at 56° C. Nucleic acids were purified by phenolchloroform (1:1) extraction and isopropanol precipitation adding 0,1 M NaCl. HBV DNA was quantified by real – time PCR in a Light Cycler instrument (Roche) using specific primers and probes (described in section "primers and probes"). Amplifications were performed as follows: 95° C for 5 minutes followed by 45 cycles at 95° C for 10 seconds, 58° C for 10 seconds and 72° C for 20 seconds. Serial dilutions of a plasmid containing a monomeric genotype D HBV insert (Clonit Srl.) were used as quantification standards. The β – globin housekeeping gene was used to normalize the DNA samples. Amplifications were performed as follows: 95° C for 5 minutes followed by 45 cycles at 95° C for 10 seconds, 58° C for 10 seconds and 72° C for 20 seconds.

HBV cccDNA QUANTIFICATION

Cells were collected at the indicated times after transfection, resuspended in lysis buffer A (see above) and incubated 10 minutes at 4° C. Lysates were centrifuged 1 minute at 13.000 rpm, pelleted nuclei resuspended in lysis buffer B (10 mM Tris – HCL, 10 mM EDTA, 150 mM NaCl, 0,5% SDS and 0.5 mg/ml proteine K) and incubated overnight at 37° C. Nucleic acids were purified by phenolchloroform (1:1) extraction and ethanol precipitation. 500 ng aliquots of each extracted DNA were treated for 45 minutes at 37° C with 10 U Plasmid safe DNAse I. DNAse was inactivated by incubating the reactions for 30 minutes at 70° C. Real – time PCR experiments were performed using specific primers (described in "primers and probes" section) in a Light Cycler instrument (Roche) using 20 ng of DNA. Amplification was performed as follows: 95° C for 10 minutes then 45 cycles of 95° C for 10 seconds, 62° C for 10 seconds, and 72° C for 20 seconds. Serial dilutions of a plasmid containing a monomeric genotype D HBV insert (Clonit Srl.) were used as quantification standards. The β-globin housekeeping gene was used to normalize the DNA samples. Amplifications were performed as follows: 95° C for 5 minutes followed by 45 cycles at 95° C for 10 seconds, 58° C for 10 seconds and 72° C for 20 seconds.

HBV RNAs AND CELLULAR mRNA EXTRACTION

Total RNA was extracted from cells using the TRIzol reagent as recommended by the manufacturer. 4 μg of the RNA samples were treated with 1 U of RQ1 RNase – Free DNase for 60 minutes at 37° C, the reaction was stopped adding 1 μl of stop solution. RNA samples were stored until used.

REVERSE TRANSCRIPTION

2 μg of DNase – treated RNA was reverse transcribed and amplified by Maxima H minus first strand cDNA synthesis kit as recommended by the manufacturer. The reaction was done as follows: incubation for 10 min at 25° C followed by 30 min at 50° C. The reaction was terminate by heating at 85° C for 5 min. Then 1 μl

of each cDNA was quantified by real – time PCR analysis in a Light Cycler instrument (Roche) using the pgRNA specific primers and probes (described in section "primers and probes"). The actin housekeeping gene Light Cycler set was used to normalize the RNA samples.

HBV RNAs ANALYSIS

2 µl of each cDNA were quantified by real – time PCR analysis. The pgRNA was amplified with specific primers and probes (see above). The house – keeping gene ACTIN was use to normalized the RNA samples and was amplified with specific probes (see above). The reaction was performed as follows: 95° C for 10 minutes then 45 cycles of 95° C for 10 seconds, 62° C for 10 seconds, and 72° C for 20 seconds.

MTT ASSAY

The cytotoxicity of epigenetic compounds in replicating HepAD38 cells was measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The cells were incubated for 48 h with HAP – 12 and AT – 130 respectively at 1 and 5 µM final concentration in duplicate in a 6-well plate together with the respective control treatments (cells incubated with 2 µM doxorubicin (Sigma) only). The cells were then incubated with the MTT substrate (5 mg/mL in 0.1 M phosphate buffered saline (PBS) (GIBCO Life Technologies) for 4 h. Thereafter all supernatants were sucked, and DMSO (1 mL) was added to the wells. Finally the optical density was measured at 570 nm with a spectrophotometer (DU 530, Beckmann coulter). The data was translated to percentage cell viability.

FLOW CYTOMETRIC ANALYSIS

Flow cytometric analysis of the cell cycle, FACS analysis, was performed as described below: after 48 h incubation with DMSO (NT cells), 1 and 5 µM HAP – 12 and AT – 130 treatments and 50 µM cisplatin (Sigma) (positive control) cells

were washed with PBS and incubated with 0.25 % trypsin-EDTA (GIBCO Life Technologies) for 10 min at 37° C. Afterwards, cells were centrifuged and washed twice in PBS, fixed in ice-cold 70% ethanol and incubed 2 h at 4° C. The cells were centrifuged, washed twice in PBS and stained with a freshly made solution containing 50 μg/mL propidium iodide (PI) (Life technologies), 0.1% Triton x-100 (Sigma) and 40 μg/mL ribonuclease A (Sigma) in PBS. All samples were incubated for 30 min at room temperature in the dark. The percentages of cell cycle distribution were evaluated by PI staining by analytical DNA flow cytometer (Accuri C6, BD Biosciences) and data analysis was performed employing BD Accuri ^{TM} C6 software.

IMMUNOBLOTTING

PIERCE kit NE - PER® Nuclear and Cytoplasmic Extraction Reagents enable stepwise separation and preparation of cytoplasmic and nuclear extracts from mammalian cultured cells. HepAD38 pellet cells were resuspended and lysed in 500 μl of ice-cold CER I plus protease inhibitor cocktail (PIC) vortex on the highest setting 15 sec and leave 10 min on ice, than add 27,5 μl ice-cold CER II to the tube, vortex on the highest setting 5 sec and incubate on ice for1 min. The nuclei were separated by centrifuge 13.000 rpm for 10 min at 4° C. Immediatley transfer the supernatant (Cytoplasmic extract) fraction to a clean pre-chilled tube. After the nuclei were lysed in 250 μl of ice-cold NER plus PIC, vortex on the highest setting for 15 sec than return the sample to ice and continue vortexing for 15 sec every 10 min, for a total of 40 min. The proteins nuclear extract was separated by centrifuge 13.000 rpm for 10 min at 4° C and immediatley transfer the supernatant (Nuclear extract) to a clean pre – chilled tube. Protein concentration was determined by the BCA protein assay reagent. Protein lysates were transfered to a nitrocellulose membrane and incubated with CORE, LAMIN – B and ACTIN antibodies.

CHROMATIN IMMUNOPRECIPITATION ASSAY (ChIP)

Cells were resuspended in 1 ml of ChIP lysis buffer (50 mM TrisHCL pH 8; 0,5% NP40; 1 mM EDTA; and 100 mM NaCL) and incubated 10 minutes at 4° C. The lysate was centrifuged at 13.000 rpm for 2 minutes to pellet the nuclei. The supernatant was removed and the nuclei were fixed in 1% formaldehyde for 30 minutes at 4° C. Isolated cross – linked nuclei were extract with a 20 mM Tris pH 8, 3 mM $MgCl_2$, 20 mM KCl buffer containing protease inhibitors, pelleted by microcentrifugation and lysed by incubation in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris – chloride pH 8.1), containing protease inhibitors. The resulting chromatin solution was sonicated for 5 pulses of 45 seconds at 80% power to generate 300 – 1000 bp DNA fragments using a BioRuptor sonicator (Diagenode, Inc). After microcentrifugation, the supernatant was diluited 1:10 in a dilution buffer (0.01% sodium dodecyl sulfate, 1.1% Triton X – 100 , 1.2 mM EDTA, 16.7 mM Tris – chloride pH 8.1, 167 mM NaCl, containing protease inhibitors), precleared with blocked protein G plus (Pierce), and divided into aliquots. The chromatin was then subjected to immunoprecipitation for 14 – 16 hours at 4° C using antibodies specific to: HBc, HBx, H4, AcH3 and AcH4. Immunoprecipitations with non specific immunoglobulins were included in each experiment as a negative control. After the reverse cross – linking, immunoprecipitated chromatin was purified by phenol/chloroform (1:1) extraction and ethanol precipitation. Chipped DNA was analyzed by real – time PCR using specific cccDNA primers and probes, promoters of EZH2, SRC, E2F1, IL29, IL6, GAPDH and TP53 specific primers.

FISH ASSAY

12 day HBV – infected and HAP – 12 (1 μM) treated NTCP – Hepg2 cells were fixed with paraformaldehyde 4% in PBS 1x for 20 minutes at 4° C, then washed with PBS 1x three times and ultimately leaved in Et – OH 70% at 4° C. 500 ng of HBV genotype D linear genome labeled probe were precipitated in 17,5 γ of COT1 (1 mg/mL), 30 γ of salmon sperm DNA (10 mg/mL), NaAc 3 M pH 5,5 1/10 V and 3 V 100% EtOH for each microscope slide. The cells were incubated

for 1 h at -80° C and then they were centrifugated for half an hour 13000 rpm at 4° C; wash the pellet two times with1 mL EtOH 70%. The pellet was resuspended in 6 μl (Formamide 50%; SSC 2x; Dextransulphate 10%) hybridation solution. Cells were pre – treated in Triton 0,3% PBS for 10 minutes at room temperature. After washing in PBS 1x three times cells were incubated in glycerole 20% PBS for 20 min and then they were incubated in glycerole 50% PBS solution overnight at -20° C.

Microscope slides were equilibrated for 20 minutes at room temperature, incubated with glycerole 20% in PBS solution for 20 minutes and ice – cold freezed; the cells alternately were washed three times with PBS 1x and incubated with a diluition 1:100 of RNase A (10 mg/mL) in PBS for 1h at 37° C, with HCl 0,1M/H_2O for 30 minutes at room temperature and with Triton 0,3%/PBS for 20 minutes at room temperature. Microscope slides were washed at least three times in PBS then one time in SSC 2x solution. Incubate the slides with the pre – hybridation solution at least 30 minutes at room temperature. After that the slides were incubated with Et – OH 70, 90 and 100% for 3 minutes each. The containing precipitated probe hybridation solution was applied on dried slides. Finally the slides were placed at 78° C for 4,5 minutes and they were incubated overnight at 37° C. Wash two times the slides with warm SSC 2x for 5 minutes at 37° C, one time with SSC 1x and one time with SSC 4x 5 minutes at room temperature. Slides were equilibrated in PBS 1x, they were fixed with PFA 4% 5 minutes at room temperature. Before incubation of slides with diluited 1:300 anti – dig cy3 BSA 4% for 45 minutes wash them one time with PBS 1x and one time with SSC 4X for 5 minutes at room temperature. To visualize nuclei cells were incubated with DAPI for 4 minutes. The final observation was obtained with an A1R+ Nikon confocal microscope.

RESULTS

STEP I

cccDNA CHROMATINIZATION AND HBc RECRUITMENT ARE EARLY EVENTS

Several evidence support the notation that cccDNA bound histones. As shown in previous study (Bock *et al.*, 2001), Cp interact with histones and bind the nuclear cccDNA. Using the cccDNA ChIP assay, that couples a classical chromatin immunoprecipitation step with a cccDNA – specific real time PCR to selectively detect the immunoprecipitation of cccDNA – bound histone and non – histone proteins. We previously showed that cccDNA transcriptional activity and HBV replication are controlled by the acetilation status of cccDNA – bound H3 and H4 histones (Pollicino *et al.*, 2006). In previuses work we have shown the regulatory protein HBx is recruited onto the cccDNA minichromosome in HBV – replicating cells (Belloni *et al.*, 2009) and is required for both cccDNA transcription and HBV replication in the context of a natural HBV infection (Lucifora *et al.*, 2011). In the absence of HBx, cccDNA – bound histones are hypoacetylated, and the cccDNA transcribes significantly less pgRNA (Belloni *et al.*, 2009).

Firstly, to investigate when Cp is recruited on cccDNA and when cccDNA chromatinization begins, we performed a kinetics of HBV replication a cccDNA ChIP assay in infected NTCP – HepG2 cells with HBV MOI 500. We took advantage of an anti-HBc, anti-HBx, anti-H4 histone, a marker of chromatinization and an anti-acetylated-Lys9 of Histone 3, a marker of transcriptional activation. As shown in Figure 18A, already after 2 hours of infection it presents an enrichment of the cccDNA associated with the Cp, as in the case of the immunoprecipitate with anti-H4, whereas we observed, be

expected, an enrichment of cccDNA only after 4 – 8 hours in immunoprecipitates anti-HBx and anti-AcH4. To confirm this result we performed a similar kinetics of HBV replication cccDNA ChIP assay in infected PHHs with HBV MOI 200, we hybridized with an anti-HBc, we have shown that cccDNA accumulation was already in 2 hours Figure 18B.

These results indicate that cccDNA chromatinization and HBc recruitment onto the cccDNA occur early post – infection in both NTCP – HepG2 cells and PHHs Figure 18B.

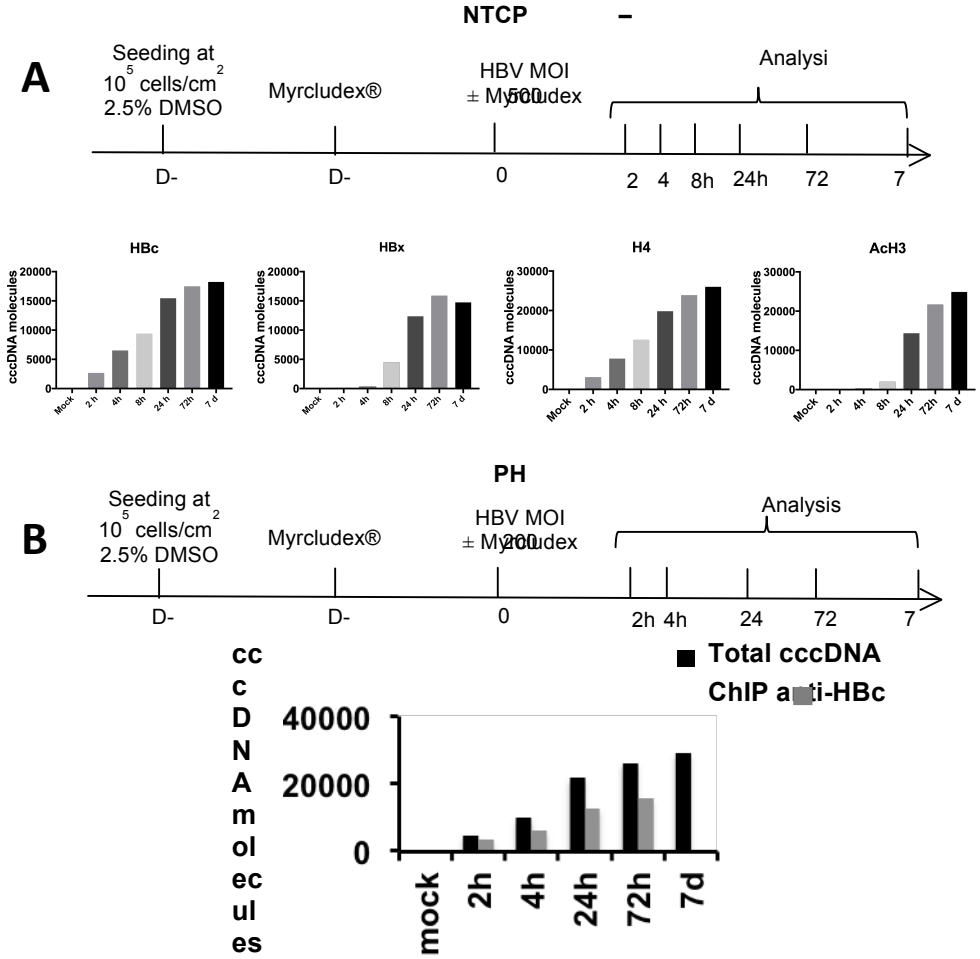

Figure 18: cccDNA chomatinization and HBc recruitment are early events.
(A) Cross – linked chromatin is extracted from HBV – infected NTCP – HepG2 cells, kinetic quantitative evaluation of HBc, HBx, H4 and AcH3 bound cccDNA was performed by real time PCR on the immunoprecipitation as detailed in the Matirials and Metods section and expressed as cccDNA molecules. (B) Cross – linked chromatin is extracted from HBV – infected PHHs, kinetic quantitative evaluation of HBc was performed by real time PCR on the immunoprecipitation and expressed as cccDNA molecules.

HBc BINDS TO THE PROMOTERS OF CELLULAR GENES

As mentioned in a previous study (Guo *et al.*, 2012) HBc interacts with several promoters of cellular genes implicate different kind of regulatory pathway. Guo and colleagues performed ChIP – on – chip analysis with the NimbleGen HG18 RefSeq Promoter Microarray consisting of 18028 of the best defined human transcript – associated promoters. Hepatocytes infected with HBV and expressing HBV core protein were isolated from CHB patients, immunoprecipitated with HBc antibody, and investigated to determine the genome – wide profile of bound host gene promoters. IgG pre – incubated with 100 – fold excess HBc antibody was used as a negative control, and immunoprecipitation of hepatocyte cells from healthy individuals was used as a blank control. In total, 3100 HBc – immunoprecipitated host promoter regions were found to be enriched by at least 2 – fold in HBc – infected tissues compared to the blank controls ($p < 0.05$). The GO analysis is a powerful tool by which the 3100 genes associated with the immunoprecipitated promoters may be correlated with biological processes or known protein functions. The 3100 genes were found to represent 55 pathways of the KEGG, according to the molecular interactions, reactions, and relations of the genes. They observed a further enrichment for pathways involved in tumor pathogenesis (Guo *et al.*, 2012).

In order to verify the reliability of the ChIP – on – chip results, we performed anti-HBc ChIP assay in Tet – released HepAD38 cells, HBV – infected NTCP – HepG2 cells and PHHs and we analyzed by TaqMan real time PCR using gene specific primers. We decided to investigate the genes those we found to be associated with HBV infection stages and we confirmed Cp bindings to the regulatory regions of the EZH2 histon methyl – transferase, cSRC proto – oncogene, that has been also shown to potentiate HBV replication, the E2F1 transcription factor and cell cycle regulator and the IL29/lamda3 interferon, whereas the IL6 and GAPDH promoters were consistently not enriched and

served as negative controls. Figure 19 shows the results obtained from ChIP on PHHs, similar result obtained in Tet – off HepAD38 cells HBV – infected NTCP – HepG2 cells.

The above results suggested that HBV core protein can bind to host gene promoters, especially those involved in HCC pathogenesis.

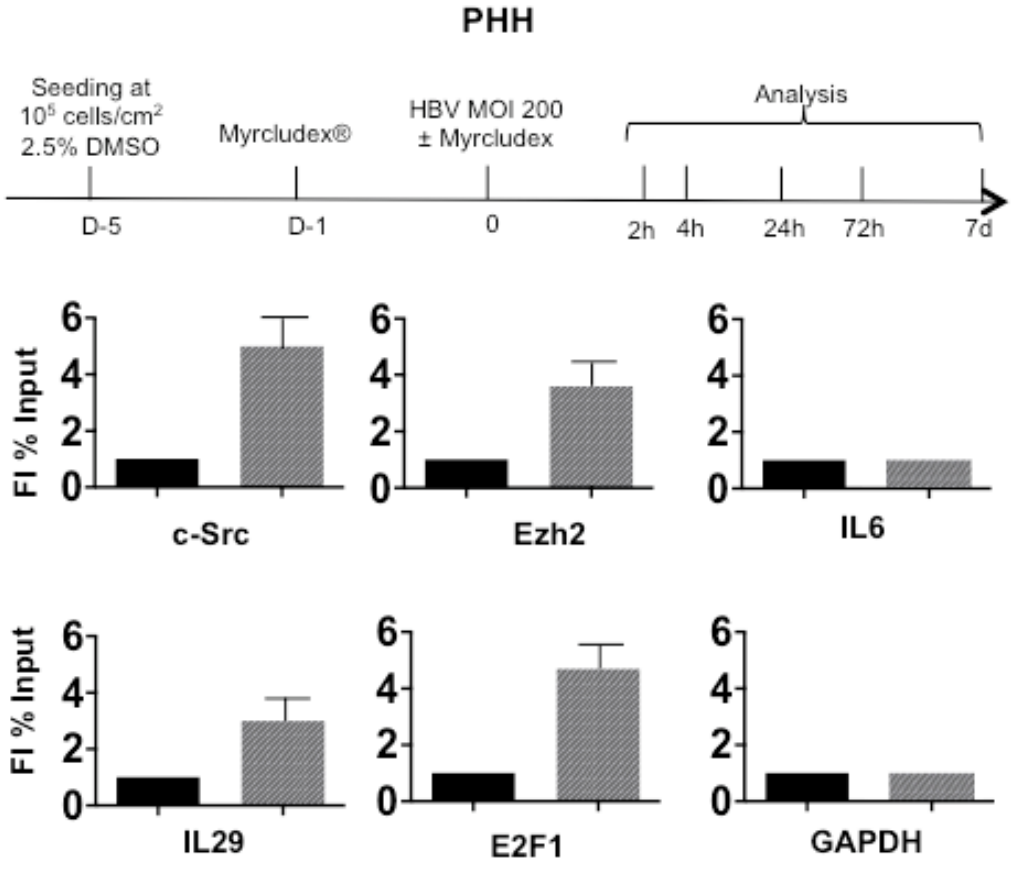

Figure 19: HBc binds on the cellular gene promoters. Cross – linked chromatin is extracted from HBV – infected PHHs after 6 days from the infection ChIP assay was performed with anti-HBc antibodies. Immunoprecipitated chromatin samples were analyzed by TaqMan real time PCR using c-SRC, EZH2, IL29 and E2F1 promoters specific primers. Results are expressed as a percentage of input. Data (mean ±SD) from 3 indipendent experiments are shown.

STEP II

CORE PROTEIN AND ANTI – CAPSID DRUGS HAP – 12 AND AT – 130

The viral capsid is formed by the HBc antigen, a small protein of 183 – 185 aminoacids. Two protein domains with specific functions can be distinguished: the N – terminal domain (NTD) and the C – terminal domain (CTD) (Chain and Myers, 2005). The NTD is involved in the formation of core dimers and in their self – assembly into capsids. The core protein (Cp) homodimers assembles in ichosaedrical capsids with a T=3 ot T=4 symmetry via oligomerization (Crowther *et al.*, 1994). *In vitro* HBV assembly is nucleated by a trimer of dimers and proceeds by the addition of individual dimeric subunits. Empty capsids assemble at a rate that is a function of protein concentration and ionic strength (Zlotnick *et al.*, 1999) The CTD domain is involved in pgRNA incapsidation and DNA replication (Gallina *et al.*, 1989). A basic sequence mostly composed of arginines and serines undergoes phosphorylation and lead to the interaction between capsid and pgRNA or viral DNA (Nassal *et al.*, 1990). The phosphorylation state of the core protein is associated with nucleocapsid maturation (Perlman *et al.*, 2005). Only mature capsids can interact with envelop proteins (Dryden *et al.*, 2006). Moreover core proteins have been shown to interact with histones and to bind the nuclear cccDNA, possibly contributing to the regulation of cccDNA function and the maintenance of the cccDNA stability (Bock *et al.*, 2001); (Pollicino *et al.*, 2006); (Guo *et al.*, 2011).

A molecule that modulates capsid assembly could interfere with the geometry of core protein interaction, packaging viral nucleic acid, and the stability of newly assembled virions (Zlotnick *et al.*, 1999). Recently, capsid assembly has also been targeted in other viral systems, including HIV and HCV (Kota *et al.*, 2010). A number of HBV assembly effectors have been investigated (Bourne *et al.*, 2006);

(Stray *et al.*, 2005). Phenyl – propenamide derivate AT – 130 and Heteroaryldihydropyrimidines (HAPs) are two new class of antivirals inhibiting HBV replication *in vitro* and *in vivo* (Deres *et al.*, 2003); (Stray *et al.*, 2005). AT – 130 increase capsid assembly reaction rate, interfere with HBV RNA packaging and to the formation of apparently normal empty capsids (Katen *et al.*, 2010). HAP – 12 have been shown to enhance the rate and the extent of core protein assembly over a broad concentration range and to act as allosteric effectors to induce an assembly – active state or, at high concentration, stabilize preferentially non – capsid polymers of Cp (Katen *et al.*, 2010); (Zlotnick *et al.*, 1999).

The antiviral activity was not related to the formation of polymer or intermediate structure. Normal capsid assembly is characterized by slow nucleation rate and weak pairwise dimer – dimer association energy. Therefore, changing either the strength or rate of association will affect the capsid production. Among others HAPs, HAP – 12 showed the strongest antiviral activity and also the greatest kinetic and thermodynamic effects on capsid assembly. Figure 20 shows the kinetic of the HAP – 12 activity.

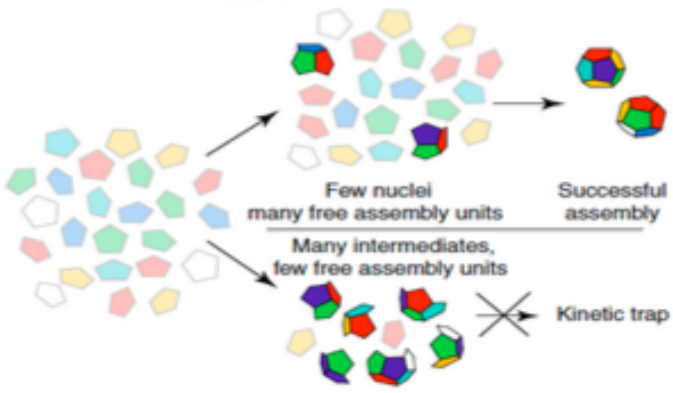

Figure 20: enhance the rate and the extent of core protein (Cp) assembly over a broad concentration range and to act as allosteric effectors to induce an assembly – active state or, at high concentration, stabilize preferentially non – capsid polymers of Cp.

CHARACTERIZATION OF HAP – 12 AND AT – 130 AND ACTIVITY

HAP – 12 AND AT – 130 DO NOT AFFECT CELL VIABILITY AND CELL CYCLE PROGRESSION

First of all we characterized core – inhibitors. We tested the cytotoxicity of HAP – 12 and AT – 130 compounds in HepAD38 inducible system.

We first performed a MTT assay on HepAD38 cells treated with anti – capsid HAP – 12 and AT – 130 respectively 1 and 5 μM concentration for 6 days. The graph in Figure 21B shows the fraction of living cells in HepAD38 samples treated with the anti – capsid is not significantly different from the untreated sample, meaning that the tested core – inhibitors drugs do not affect cell viability.

We then performed a FACS analysis Figure 21A using HepAD38 cells treated with HAP – 12 and AT – 130 respectively 1 and 5 μM for 6 days. The fraction of cell populations in G1, G2/M, S and sub-G1 phase in treated cells is not significantly different from the control, showing that the tested anti – capsid compounds do not alter the cell cycle progression.

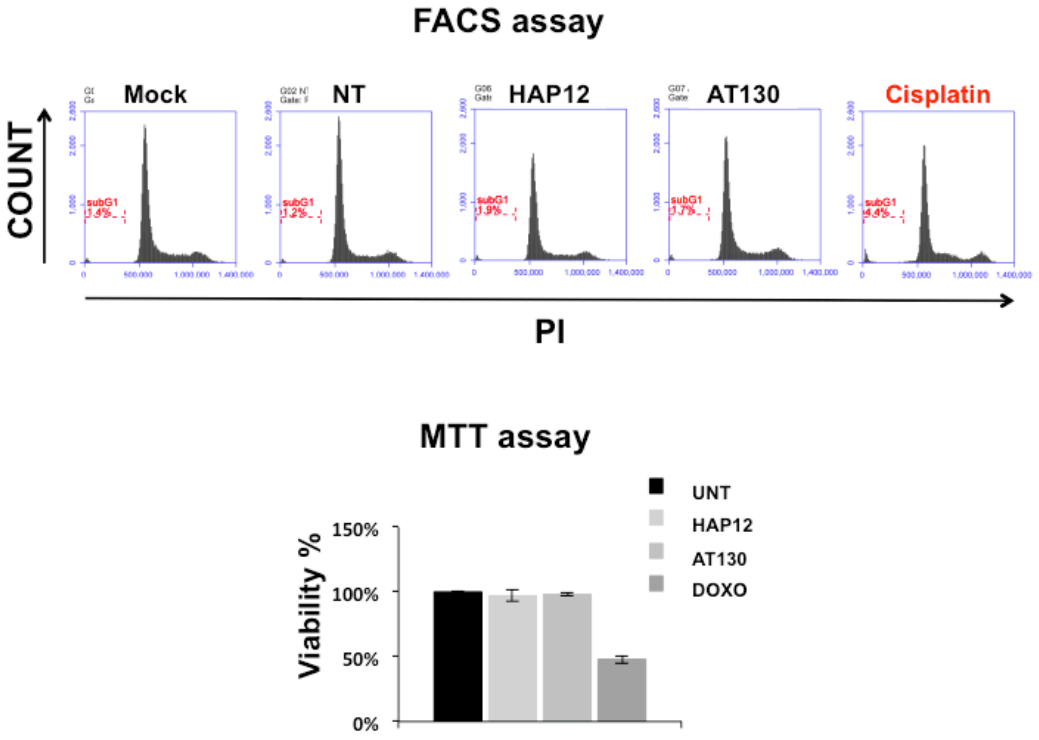

Figure 21: Effect of core – inhibitors on cycle progresson and cell viability. HepAD38 cells treated with anti – capsid HAP – 12 and AT – 130 respectively 1 and 5 µM concentration for 6 days. (A) Cell cycle analysis using propidium iodide (PI) staining and flow cytometry. HepAD38 cells were treated with DMSO (NT) 5 µM or HAP – 12 and AT – 130 drugs at the respectively concentration 1 and 5 µM for 48h. As control of apoptosis cells were treated with 50 µM cisplatin for 48h. Bars denote percentage of sub – diploid cells. Data from one representative experiment out of three are shown. (B) HepAD38 cells were incubated with HAP – 12 and AT – 130 drugs at the respectively concentration 1 and 5 µM or with DMSO (NT) 5 µM. As positive control HepAD38 cells were treated with 500 ng/mL doxorubicin. MTT assay was performed after 48h. the results are pictured as percentage of number of alive cells compared with the untreated sample (100% of living cells) and represent the mean ± SD of three independent experiments.

CORE – INHIBITORS HAVE A DIFFERENT EFFECT ON HBc EXPRESSION LEVELS

Secondly for further characterization we focused to evaluate the effect of core – inhibitors on HBc expression. HepAD38 cells were induced and treated with HAP – 12 and AT – 130 (respectively 1 and 5 μM), than we harvested the cells 10 days after treatment and we extracted the nuclear and cytoplasic fractions. We performed a Western Blot analysys and we found that HAP – 12 treatment lowers HBc protein levels in the cytoplasm, whereas AT – 130 does not have any effect on HBc protein levels, neither in the nucleus or in cytoplasm Figure 22.

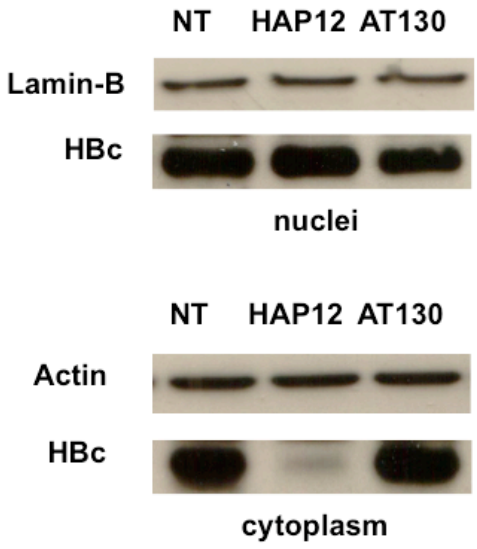

Figure 22: Effect of core – inhibitors on Cp expression. HepAD38 cells were induced and treated with HAP – 12 and AT – 130 (respectively 1 and 5 μM) for 10 days. Nuclear and cytoplasmatic proteins were extracted and analyzed by western blot analysis. 30 μg of nuclear and cytoplasmatic proteins were analyzed for HBc and lamin – B and actin as control.

HAP – 12 AND AT – 130 ANTIVIRAL ACTIVITY IN HepAD38 CELLS

HAP – 12 AND AT – 130 PREVENT CAPSID RECYCLING AND cccDNA ACCUMULATION IN HepAD38 CELLS

To investigate the impact of anti – capsid HAP – 12 and AT – 130 on cccDNA formation, HepAD38 cells were induced and treated with HAP – 12 and AT – 130 (1 and 5 μM) for 10 days. To characterize the effect of core – inhibitors on viral replication total and viral RNAs, HBV DNA and cccDNA were extracted and analyzed by real time PCR using specific primers and probes. As shown in Figure 23 anti – capsid drugs HAP – 12 and AT – 130 lead strong suppression of HBV replication that is accompanied by a 50% reduction of pgRNA transcription and by a 95% decrease in cccDNA levels.

These results suggest that core – inhibitors affect HBV replication and prevent cccDNA accumulation by blocking the recycling of mature core particles into the nucleus.

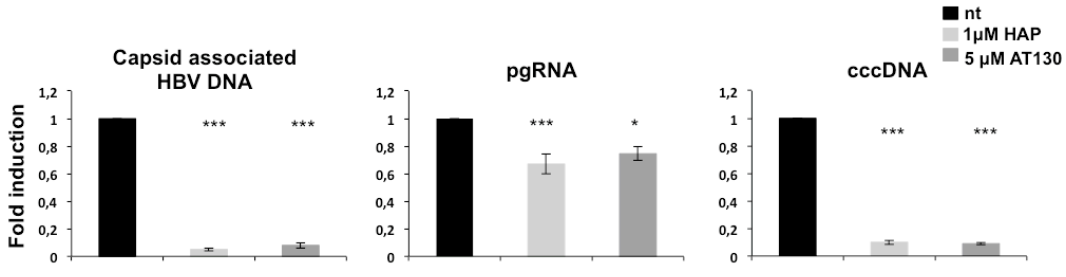

Figure 23: Core – inhibitors affects HBV DNA, pgRNA and cccDNA levels. Capsid associated (Cp) HBV DNA, pgRNA and cccDNA were extracted from stably replicating HepAD38 cells treated with HAP – 12 and AT – 130 for 10 days. The pgRNA, cccDNA and HBC DNA were extracted and quantificated by real time PCR using selective primers and probes. Results are expressed as fold induction (mean ± SD) from three independent experiments.

HAP – 12 REDUCES pgRNA TRANSCRIPTION FROM THE cccDNA IN HepAD38 CELLS

Previous experiment did not show a strong reduction of the pgRNA transcription as much as for HBV replication and cccDNA levels.

To better investigate if core – inhibitor HAP – 12 impacts on the pgRNA transcription, HepAD38 cells were inhibited to stably replicate reintroducing tetracycline in the medium, eliminating the contribution of the integrated HBV genome in HBV DNA and pgRNA; in these conditions the pool of cccDNA accumulated in the first phase of stably replication thanks to the recycling of mature capsids to the nucleus is the only source of pgRNA. Thus secondly HepAD38 cultured in presence of tetracycline were treated for 6 days whit HAP – 12 drugs and harvested to isolate pgRNA; as show by real time PCR assay in Figure 24, in agreement with previous experiment, HAP – 12, without interference of integrated HBV genome, leads to a strong suppression of HBV replication and reduction of pgRNA transcription.

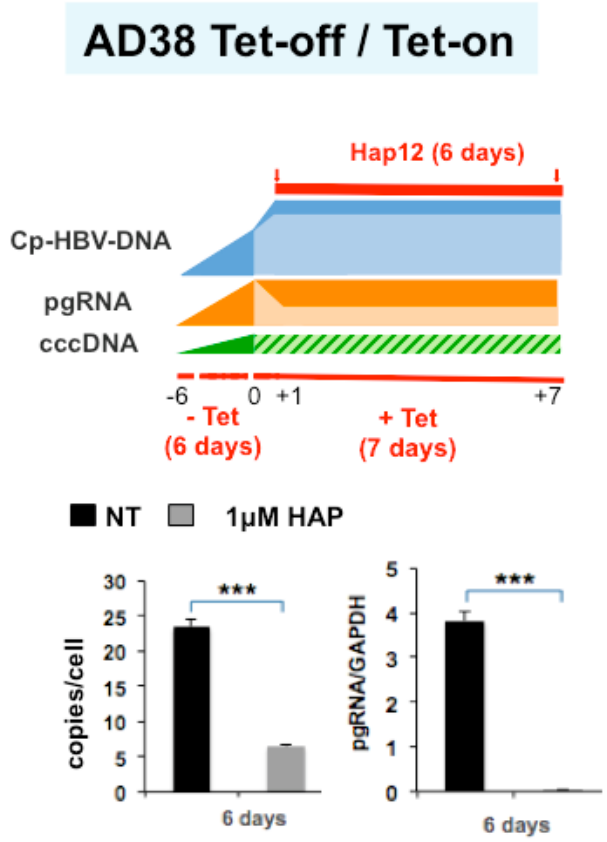

Figure 24: pgRNA transcription was analyzed in HepAD38 shutted down for HBV DNA integrated expression reintroducing Tetracycline in the mudium as described in Materials and Methods section. After allowing the cells to accumulate HBV intermediates in the nucleus and in the cytoplasm for 6 days with HAP – 12 drug, the pgRNA was extracted and quantificated by real time PCR using selective primers and probes and actin primers. Results are expressed as fold induction (mean ± SD) from three independent experiments.

STEP III

CHARACTERIZATION OF HAP – 12 ANTIVIRAL ACTIVITY IN HBV – INFECTED NTCP – HepG2 CELLS AND PHH

HAP – 12 AFFECTS HBV DNA, pgRNA AND cccDNA LEVELS IN NTCP – HepG2 INFECTED CELLS

To test the effect of HAP – 12 antiviral in modulating HBV replication and transcription, in a context of complete HBV infection cycle, we used NTCP – HepG2 cells. Recently, the sodium taurocholate co – transporting polypeptide (NTCP) membrane transporter was reported as an HBV entry receptor (Yan *et al.*, 2012); (Ni *et al.*, 2014). The NTCP – HepG2 cells can be efficiently infected with HBV and recapitulate all the steps of the viral cycle (see material and methods for procedure).

We infected NTCP – HepG2 cells and treated them with HAP – 12 (1 μM) for 12 days.

To characterize the effect of core – inhibitors on viral replication total and viral RNAs, HBV DNA and cccDNA were extracted and analyzed by real time PCR using specific primers and probes. In agreement with the results obtained in HepAD38 a HAP – 12 treatment started at the time of infection strongly suppresses of HBV replication, reduces pgRNA transcription and leads to a sharp reduction of cccDNA levels Figure 25.

These results indicate that in NTCP – HepG2 infected cells HAP – 12 efficiently inhibits HBV replication, pgRNA transcription and a drastic reduction of cccDNA formation, suggesting an important role of Cp for rcDNA release into the nucleus, conversion of rcDNA into cccDNA or cccDNA chromatinization.

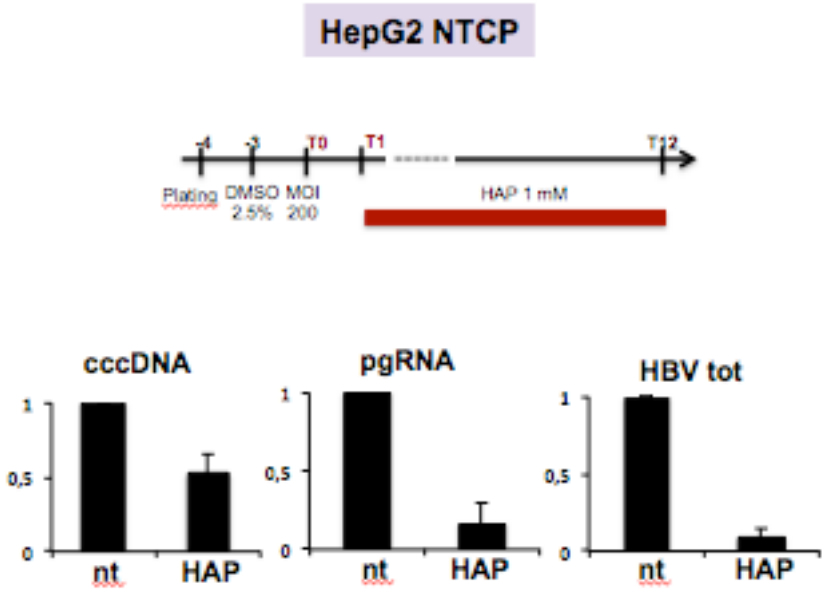

Figure 25: Core – inhibitors affects HBV DNA, pgRNA and cccDNA levels. cccDNA, pgRNA and capsid associated (Cp) were axtracted from NTCP – HepG2 treated with HAP – 12 for 12 days. The pgRNA, cccDNA and HBC DNA were extracted and quantificated by real time PCR using selective primers and probes. Results are expressed as fold induction (mean ± SD) from three independent experiments.

HAP – 12 REDUCES cccDNA TRANSCRIPTION AND HBV REPLICATION IN HBV – INFECTED NTCP – HepG2 CELLS AND PHHs

If HAP – 12 treatment is started at the time of HBV infection (NTCP – HepG2 cells) or at the time of HBV induction (HepAD38 cells), the effect seen on cccDNA levels could be due to the inhibition of cccDNA formation by the anti – capsid compounds that prevents the establishment ot cccDNA pool rather than a direct "destabilizing" effect on existing cccDNA.

To better investigate the HAP – 12 effects on cccDNA stability we also started to treat NTCP – HepG2 cells and PHHs after 10 days of infections, when cccDNA is accumulated in infected nuclei and cccDNA levels seem to be stable. As shown in Figure 26A-B, HAP – 12 treatment leads to a strong suppression of HBV replication and to a reduction of the 50% of pgRNA transcription, whereas no effect on cccDNA levels. These results suggest that the core – inhibitor blocks HBV replication and transcription, but HAP – 12 does not impact on cccDNA stability.

The reduction in cccDNA levels we observed both in HBV induction HepAD38 cells and in HBV – infected NTCP – HepG2 cells treated with HAP – 12 at the time of respectively induction or infection rather reflect a block of the drug on the formation of functional nucleocapsid and the block of the recycling of mature core particles in to the nucleus.

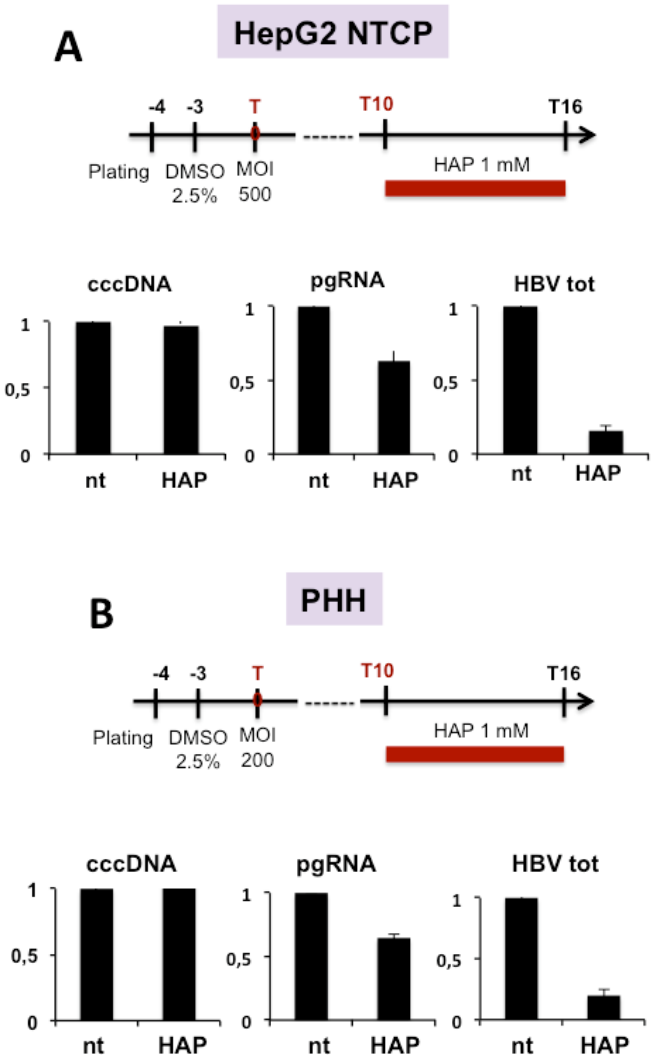

Figure 26: HAP – 12 does not impact on cccDNA stability. (A) cccDNA, pgRNA and capsid associated (Cp) were axtracted from NTCP – HepG2 cells treated with HAP – 12, after 10 days of HBV infection, for 6 days. (B) cccDNA, pgRNA and capsid associated (Cp) were axtracted from PHHs treated with HAP – 12, after 10 days of HBV infection, for 6 days. The pgRNA, cccDNA and HBC DNA from NTCP – HepG2 cells and PHHs were extracted and quantificated by real time PCR using selective primers and probes. Results are expressed as fold induction (mean ± SD) from three independent experiments.

HAP – 12 BLOCKS HBc RECRUITMENT ON THE cccDNA AND REDUCES cccDNA – BOUNDS HISTONES ACETILATION IN HBV INDUCTION HepAD38 AND HBV INFECTION NTCP – HepG2 CELLS

At this point we focused to understand how capsid – inhibitors inhibit HBV replication, prevent cccDNA accumulation and target cccDNA transcription.

In order to understand whether HAP – 12 antiviral activity involves an epigenetic mechanism mediated by the suppression of cccDNA transcription, we performed two ChIP assays, respectively, in stably replicating HepAD38 cells treated for 10 days and in HBV – infected NTCP – HepG2 cells treated for 6 days after 10 days of infection, with HAP – 12 (1 μM). As shown in Figure 27 A and B, in both induction and infection models, HAP – 12 decreases the relative amount acetylated H4 histone on the minichromosome, leading to a transcriptionally suppressed cccDNA. By ChIP assays we found, also, that HAP – 12 induces a strong reduction in Cp binding to the minichromosome Figure 27A-B. As show in Figure 22, HAP – 12 activity does not affect HBc protein levels in the nucleus.

Altogether, these results support a model in which the reduction of HBV replication after HAP – 12 treatment is due to both the epigenetic inhibition of cccDNA transcriptional activity (and consequently a reduction of pgRNA levels) and to an inappropriate assembly of Cp.

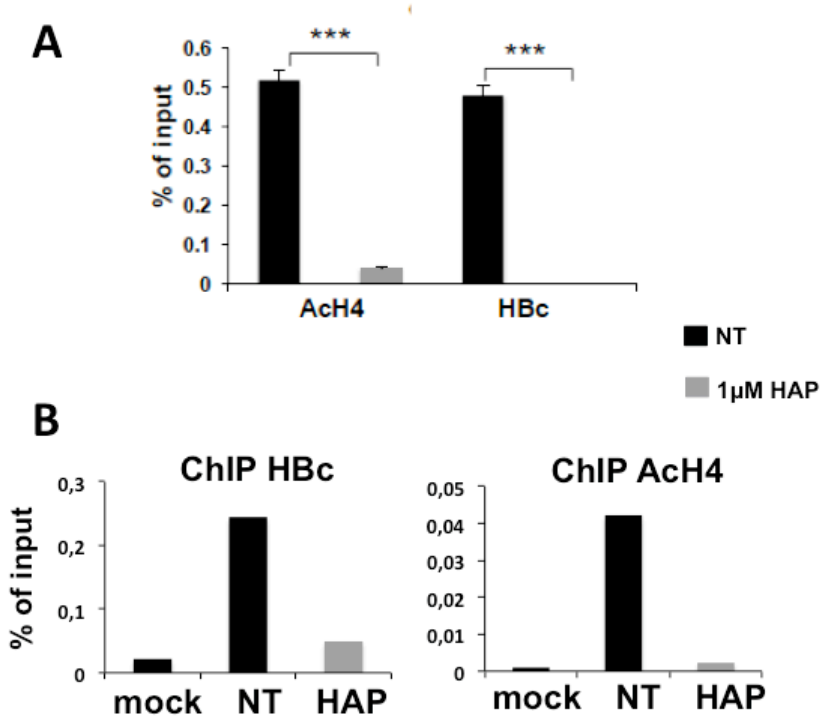

Figure 27: HAP – 12 Inhibits pgRNA transcription by epigenetic mechanisms. (A) Chromatin from stably replicating HepAD38 cells treated and non treated with HAP – 12 for 10 days was immunoprecipitated with anti-Acetylated H4 and anti-HBc antybodies; (B) Chromatin from HBV – infected NTCP – HepG2 cells treated and non treated with HAP – 12 for 6 days after 10 days of infection was immunoprecipitated with anti – Acetylated H4 and anti – HBc antybodies; Immunoprecipitated chromatin was analyzed by real time PCR with HBV cccDNA selective primers.

HBV cccDNA FISH

To visualized nuclear cccDNA, we performed a DNA FISH using genotype D specific probe encompassing the whole HBV genome in HBV – infected NTCP – HepG2 cells treated with HAP – 12 immediately after infection for 12 days. As shown in Figure 28 in agreement with results previously obtained, cccDNA was detected in untreated cells nuclei, whereas, as expected, no signal was seen in the HAP – 12 treated infected cells nuclei, exactly as in the mock cells nuclei.

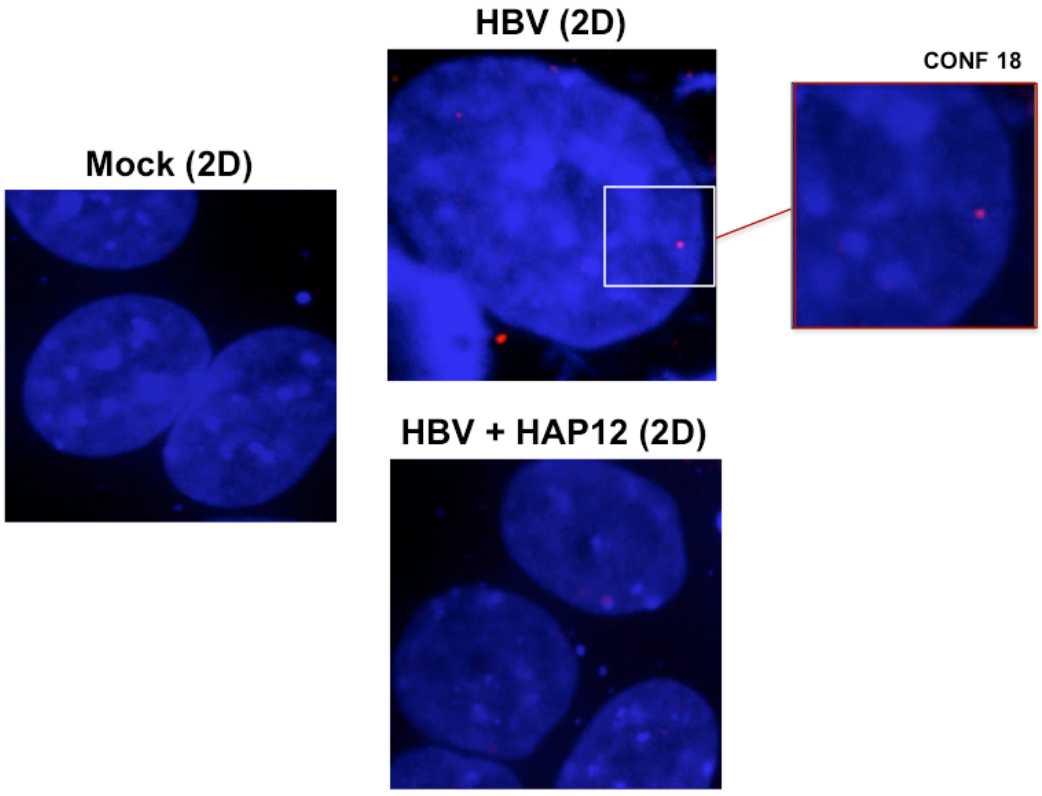

Figure 28: cccDNA does not detect in HAP – 12 treated nuclei. DNA FISH assay cccDNA expressed in NTCP – HepG2 infected with HBV at 500 moi treated with HAP – 12 at T0 post-infection for 12 days. DAPI staining, and their merged image are shown.

STEP IV

IMPACT OF HAP – 12 ON Cp RECRUITMENT ONTO PROMOTERS IN TET – OFF HepAD38 AND HBV – INFECTED NTCP – HepG2 CELLS

HAP – 12 TREATMENT BLOCKS HBc RECRUITMENT ON CELLULARE GENE PROMOTERS IN HBV INDUCTION HepAD38 AND HBV INFECTION NTCP – HepG2 CELLS

As seen before, in the recent work, Guo and colleagues have demonstrated that HBc bindings a large number of cellular gene promoters. Previously we have confirmed the results of Guo and we demonstrated that HBV core protein can bind to host gene promoters, especially those involved in HCC pathogenesis (see HBc binds to the promoters of cellular genes Figure 19).

Finally, we investigated the effect of HAP – 12 capsid – inhibitor onto Cp recruitment on cellular gene promoters. To this aim we performed anti – HBc ChIP assays in tet released HepAD38 cells and HBV – infected NTCP – HepG2 cells. We discovered that HAP – 12 treatment was able to block Cp recruitment to the regulatory regions of EZH2, cSRC, E2F1 and IL29/lamda3 in both tet – off HepAD38 and HBV – infected NTCP – HepG2 cells, wherase the TP53 and GAPDH promoters were consistently not enriched and served as negative controls Figure 29 A and B.

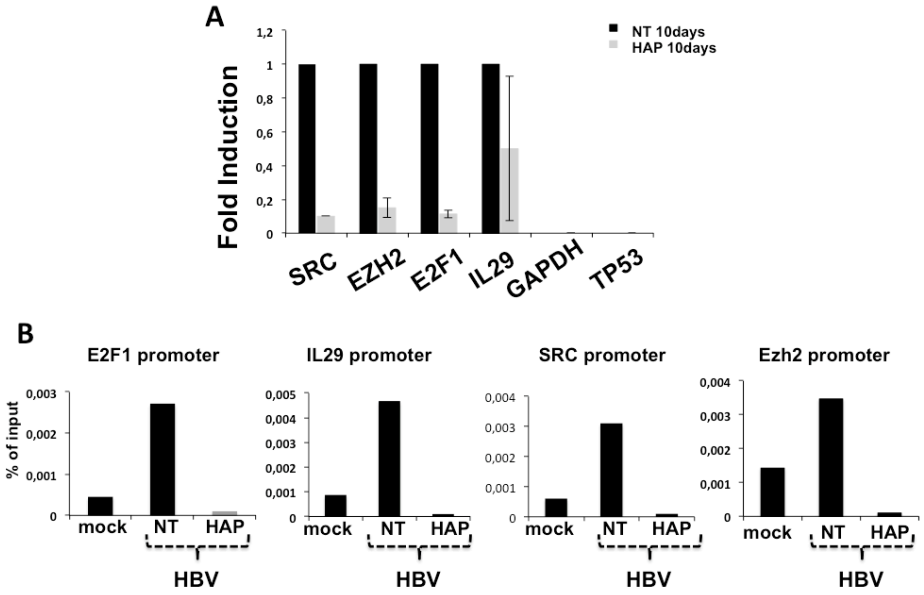

Figure 29: HAP12 blocks the binding of HBc to the cellular genes promoters.
(A) Stably replicating HepAD38 cells treated and non treated with HAP – 12 for 10 days and chromatin was immunoprecipitated with specific HBc antibody. ChIPed DNA was analyzed by TaqMan real time PCR using c-SRC, EZH2, IL29 and E2F1 promoters specific primers. TP53 and GAPDH promoters were served as negative controls. Results are expressed as a percentage of input. Data (mean ±SD) from three indipendent experiments are shown. (B) Chromatin from HBV – infected NTCP – HepG2 cells treated and non treated with HAP – 12 for 6 days after 10 days of infection was immunoprecipitated with anti-HBc. ChIPed DNA was analyzed by TaqMan real time PCR using c-SRC, EZH2, IL29 and E2F1 promoters specific primers. GAPDH promoters was served as negative controls. Results are expressed as a percentage of input.

DISCUSSION

The HBV nucleocapsid, that comprises the viral Cp assembled into the capsid proper, viral nucleic acids (pgRNA, converted ssDNA and than in dsDNA) and host, viral (DNA – Pol) proteins, represents an attractive target for anti – HBV therapy. It has been shown that the HBx regulatory protein is recruited onto the minichromosome cccDNA parallels the HBV replication. Results obtained in HBV mutant that does not express HBx show that p300 recruitment is severely impared, and cccDNA – bound histones era rapidly hypoacetylated, whereas the recruitment of the histones deacetylases hSIRT1 and HDAC1 is increased and occurs at earlier times (Belloni *et al.*, 2009), supporting the notation of an epigenetic mechanism underlying the ability of HBx to control HBV replication. Similarly to HBx protein, Cp has been considerate to be an excellent target for antiviral therapy due to its critical roles in multiple steps of virus replication. Indeed, proper assembly of the capsid is critical for RNA packaging, reverse transcription and intracellular trafficking (core nuclear delivery, core nuclear recycling). Moreover Cp have been shown to interact with histones and to the nuclear cccDNA (Bock *et al.*, 2001); (Pollicino *et al.*, 2006); (Guo *et al.*, 2011) Figure 30A. The results presented in my Thesis highlight the role of Cp, in the interaction with the cccDNA and cellular gene promoters, and the impact of a new class of antivirals that target the HBV capsid and inhibit HBV replication *in vitro* (Deres *et al.*, 2003); (Stray *et al.*, 2005).

In the step I by ChIP assay it has been shown that cccDNA chromatinization and HBc recruitment onto cccDNA occur early post – HBV infection. Moreover we verified the reliability of the ChIP – on – ChIP results that Guo and colleagues obtained. We confirmed that Cp binds some cellular gene promoters, EZH2 histon methyl – tranferase, cSRC proto – oncogene, that has been also shown to potentiate HBV replication, the E2F1 transcription factor and cell cycle regulator, and the IL29/lamda3 interferon, that are associated with HBV infection stages and involved in the development of HCC. Indicating that HBc recruitment onto

cccDNA occur in early stages of infection and suggesting that HBV core protein can bind to host gene promoters, especially those involved in HCC pathogenesis, these results confirm Cp as an attractive new therapeutic specific target for HBV chronic infection.

Several compound that target Cp and HBV capsid assembly have been shown to inhibit replication. In the step II we focused on two different classes of Cp assembly modulators. Heteroaryldihydropyrimidines (HAPs) have been shown to enhance the rate and extent of core protein assembly and to act as allosteric effectors to induce an assembly – active state (Stray *et al.*, 2005). Whereas the effect of phenyl – propenamide on capsid formation is described as forming apparently normal capsid that lack generic material (Feld *et al.*, 2007). It has been demonstrated, However, that AT – 130 behaves as an assembly and accelerator (Katen *et al.*, 2010), increasing the rate of assembly and generating empty capsids. At first we characterized HAP – 12 and AT – 130 activity. MTT assay indicated that the tested core – inhibitors drugs do not affect cell viability, than FACS analysis, also, show that the tested anti – capsid compounds do not alter the cell cycle progression. Finally, by western blot it has been shown that HAP – 12 treatment lowers HBc protein levels in the cytoplasm, whereas AT – 130 treatment does not have any effect on Cp levels, neither in the nucleus or in cytoplasm.
At a later time we focused HAP – 12 and AT – 130 antiviral activity in HepAD38 cells. We observed that core – inhibitor compounds reduce pgRNA transcription from the cccDNA, prevent capsid recycling and cccDNA accumulation in this cell based HBV replication model. These results show that core – inhibitors affect HBV replication and prevent cccDNA accumulation by blocking the recycle of mature core particles into the nucleus, suggesting an important role of Cp for rcDNA release into the nucleus, conversion of rcDNA into cccDNA or cccDNA chromatinization.

In the step III we better characterized HAP – 12 in HBV infection models and we proved that the reduction in cccDNA levels that we observed both in HBV

induction HepAD38 cells and in HBV – infected NTCP – HepG2 cells treated with HAP – 12 at the time of respectively induction or infection rather reflect a block of the drug on the formation of functional nucleocapsid and the block of the recycling of mature core particles in to the nucleus. Conversely, when HAP – 12 treatment was started 10 days post – infection, when the cccDNA pool is established and stable in HBV – infected cells we observed very little or no effect on cccDNA levels, a significant reduction of cccDNA transcription and pgRNA levels together with a very strong inhibition of total HBV DNA and viral replication. ChIP assay indicated that HAP – 12 inhibitory effect is induced by decreasing the acetylated histon H4 binging to the microchromosome.

Importantly, with the ChIP assays performed in the step IV we discovered that HAP – 12 treatment was able to blocks Cp recruitment on genes promoters.

Altogether these results identify capsid – inhibitors as the first class "virus specific" compounds capable to target the cccDNA functions and potentially counteract Cp pathogenicity in infected hepatocytes (Figure 30).

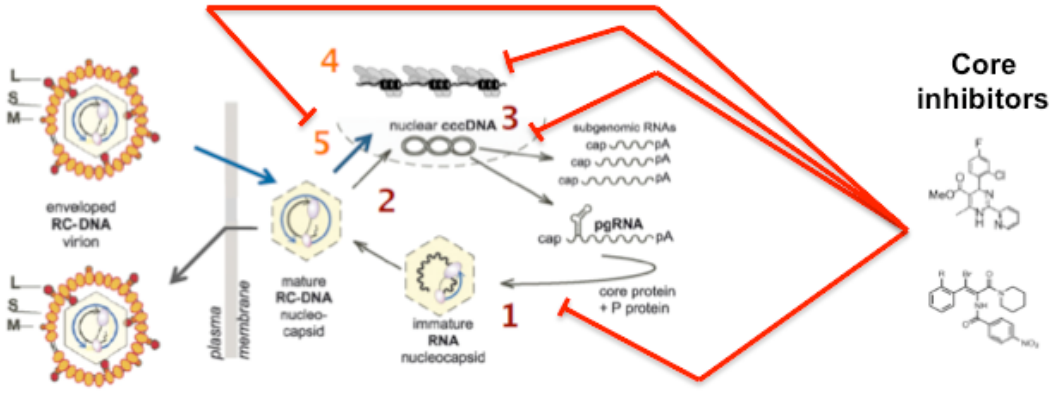

Figure 30: Core – inhibitors (HAP – 12, AT – 130) impact on Cp nuclear functions at multiple levels. Anti – capsid compounds block new cccDNA accumulation and they reduce viral RNAs transcription from an established cccDNA pool. HAP – 12 interferes with Cp recruitment onto minichromosome cccDNA and relevant host gene promoters. Figure modified from (Nassal, 2008).

Bibliografia

Aden, D.P., Fogel, A., Plotkin, S., Damjanov, I., and Knowles, B.B. (1979). Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature *282*, 615-616.

Alter, M.J., and Margolis, H.S. (1990). The emergence of hepatitis B as a sexually transmitted disease. Med Clin North Am *74*, 1529-1541.

Alward, W.L., McMahon, B.J., Hall, D.B., Heyward, W.L., Francis, D.P., and Bender, T.R. (1985). The long-term serological course of asymptomatic hepatitis B virus carriers and the development of primary hepatocellular carcinoma. J Infect Dis *151*, 604-609.

Arends, P., Sonneveld, M.J., Zoutendijk, R., Carey, I., Brown, A., Fasano, M., Mutimer, D., Deterding, K., Reijnders, J.G., Oo, Y.*, et al.* (2015). Entecavir treatment does not eliminate the risk of hepatocellular carcinoma in chronic hepatitis B: limited role for risk scores in Caucasians. Gut *64*, 1289-1295.

Bartenschlager, R., Junker-Niepmann, M., and Schaller, H. (1990). The P gene product of hepatitis B virus is required as a structural component for genomic RNA encapsidation. J Virol *64*, 5324-5332.

Bartenschlager, R., and Schaller, H. (1988). The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. EMBO J *7*, 4185-4192.

Bartenschlager, R., and Schaller, H. (1992). Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. EMBO J *11*, 3413-3420.

Basagoudanavar, S.H., Perlman, D.H., and Hu, J. (2007). Regulation of hepadnavirus reverse transcription by dynamic nucleocapsid phosphorylation. J Virol *81*, 1641-1649.

Beasley, R.P., Hwang, L.Y., Lin, C.C., Leu, M.L., Stevens, C.E., Szmuness, W., and Chen, K.P. (1982). Incidence of hepatitis B virus infections in preschool children in Taiwan. J Infect Dis *146*, 198-204.

Beck, J., and Nassal, M. (1997). Sequence- and structure-specific determinants in the interaction between the RNA encapsidation signal and reverse transcriptase of avian hepatitis B viruses. J Virol *71*, 4971-4980.

Belloni, L., Allweiss, L., Guerrieri, F., Pediconi, N., Volz, T., Pollicino, T., Petersen, J., Raimondo, G., Dandri, M., and Levrero, M. (2012). IFN-alpha inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. J Clin Invest *122*, 529-537.

Belloni, L., Pollicino, T., De Nicola, F., Guerrieri, F., Raffa, G., Fanciulli, M., Raimondo, G., and Levrero, M. (2009). Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. Proc Natl Acad Sci U S A *106*, 19975-19979.

Benhenda, S., Cougot, D., Buendia, M.A., and Neuveut, C. (2009). Hepatitis B virus X protein molecular functions and its role in virus life cycle and pathogenesis. Adv Cancer Res *103*, 75-109.

Benhenda, S., Ducroux, A., Riviere, L., Sobhian, B., Ward, M.D., Dion, S., Hantz, O., Protzer, U., Michel, M.L., Benkirane, M.*, et al.* (2013). Methyltransferase PRMT1 is a binding partner of HBx and a negative regulator of hepatitis B virus transcription. J Virol *87*, 4360-4371.
Benn, J., and Schneider, R.J. (1994). Hepatitis B virus HBx protein activates Ras-GTP complex formation and establishes a Ras, Raf, MAP kinase signaling cascade. Proc Natl Acad Sci U S A *91*, 10350-10354.
Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363-366.
Berting, A., Fischer, C., Schaefer, S., Garten, W., Klenk, H.D., and Gerlich, W.H. (2000). Hemifusion activity of a chimeric influenza virus hemagglutinin with a putative fusion peptide from hepatitis B virus. Virus Res *68*, 35-49.
Billioud, G., Pichoud, C., Puerstinger, G., Neyts, J., and Zoulim, F. (2011). The main hepatitis B virus (HBV) mutants resistant to nucleoside analogs are susceptible in vitro to non-nucleoside inhibitors of HBV replication. Antiviral Res *92*, 271-276.
Blackburn, S.D., Shin, H., Haining, W.N., Zou, T., Workman, C.J., Polley, A., Betts, M.R., Freeman, G.J., Vignali, D.A., and Wherry, E.J. (2009). Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol *10*, 29-37.
Blanpain, C., Knoop, C., Delforge, M.L., Antoine, M., Peny, M.O., Liesnard, C., Vereerstraeten, P., Cogan, E., Adler, M., and Abramowicz, D. (1998). Reactivation of hepatitis B after transplantation in patients with pre-existing anti-hepatitis B surface antigen antibodies: report on three cases and review of the literature. Transplantation *66*, 883-886.
Block, T.M., Lu, X., Platt, F.M., Foster, G.R., Gerlich, W.H., Blumberg, B.S., and Dwek, R.A. (1994). Secretion of human hepatitis B virus is inhibited by the imino sugar N-butyldeoxynojirimycin. Proc Natl Acad Sci U S A *91*, 2235-2239.
Bloom, K., Ely, A., Mussolino, C., Cathomen, T., and Arbuthnot, P. (2013). Inactivation of hepatitis B virus replication in cultured cells and in vivo with engineered transcription activator-like effector nucleases. Mol Ther *21*, 1889-1897.
Bock, C.T., Kubicka, S., Manns, M.P., and Trautwein, C. (1999). Two control elements in the hepatitis B virus S-promoter are important for full promoter activity mediated by CCAAT-binding factor. Hepatology *29*, 1236-1247.
Bock, C.T., Malek, N.P., Tillmann, H.L., Manns, M.P., and Trautwein, C. (2000). The enhancer I core region contributes to the replication level of hepatitis B virus in vivo and in vitro. J Virol *74*, 2193-2202.
Bock, C.T., Schwinn, S., Locarnini, S., Fyfe, J., Manns, M.P., Trautwein, C., and Zentgraf, H. (2001). Structural organization of the hepatitis B virus minichromosome. J Mol Biol *307*, 183-196.
Bogomolski-Yahalom, V., Klein, A., Greenblat, I., Haviv, Y., and Tur-Kaspa, R. (1997). The TATA-less promoter of hepatitis B virus S gene contains a TBP binding site and an active initiator. Virus Res *49*, 1-7.
Bond, W.W., Petersen, N.J., and Favero, M.S. (1977). Viral hepatitis B: aspects of environmental control. Health Lab Sci *14*, 235-252.

Bottcher, B., Wynne, S.A., and Crowther, R.A. (1997). Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. Nature *386*, 88-91.
Bouchard, M.J., and Schneider, R.J. (2004). The enigmatic X gene of hepatitis B virus. J Virol *78*, 12725-12734.
Bourne, C.R., Finn, M.G., and Zlotnick, A. (2006). Global structural changes in hepatitis B virus capsids induced by the assembly effector HAP1. J Virol *80*, 11055-11061.
Brandenburg, B., Stockl, L., Gutzeit, C., Roos, M., Lupberger, J., Schwartlander, R., Gelderblom, H., Sauer, I.M., Hofschneider, P.H., and Hildt, E. (2005). A novel system for efficient gene transfer into primary human hepatocytes via cell-permeable hepatitis B virus-like particle. Hepatology *42*, 1300-1309.
Brechot, C. (2004). Pathogenesis of hepatitis B virus-related hepatocellular carcinoma: old and new paradigms. Gastroenterology *127*, S56-61.
Bremer, C.M., Bung, C., Kott, N., Hardt, M., and Glebe, D. (2009). Hepatitis B virus infection is dependent on cholesterol in the viral envelope. Cell Microbiol *11*, 249-260.
Brezillon, N., Brunelle, M.N., Massinet, H., Giang, E., Lamant, C., DaSilva, L., Berissi, S., Belghiti, J., Hannoun, L., Puerstinger, G.*, et al.* (2011). Antiviral activity of Bay 41-4109 on hepatitis B virus in humanized Alb-uPA/SCID mice. PLoS One *6*, e25096.
Broering, R., Lutterbeck, M., Trippler, M., Kleinehr, K., Poggenpohl, L., Paul, A., Gerken, G., and Schlaak, J.F. (2014). Long-term stimulation of Toll-like receptor 3 in primary human hepatocytes leads to sensitization for antiviral responses induced by poly I:C treatment. J Viral Hepat *21*, 480-490.
Brunetto, M.R., Moriconi, F., Bonino, F., Lau, G.K., Farci, P., Yurdaydin, C., Piratvisuth, T., Luo, K., Wang, Y., Hadziyannis, S.*, et al.* (2009). Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. Hepatology *49*, 1141-1150.
Bruss, V., and Ganem, D. (1991). The role of envelope proteins in hepatitis B virus assembly. Proc Natl Acad Sci U S A *88*, 1059-1063.
Bruss, V., Hagelstein, J., Gerhardt, E., and Galle, P.R. (1996). Myristylation of the large surface protein is required for hepatitis B virus in vitro infectivity. Virology *218*, 396-399.
Busch, M.P., Kleinman, S.H., and Nemo, G.J. (2003). Current and emerging infectious risks of blood transfusions. JAMA *289*, 959-962.
Cai, D., Mills, C., Yu, W., Yan, R., Aldrich, C.E., Saputelli, J.R., Mason, W.S., Xu, X., Guo, J.T., Block, T.M.*, et al.* (2012). Identification of disubstituted sulfonamide compounds as specific inhibitors of hepatitis B virus covalently closed circular DNA formation. Antimicrob Agents Chemother *56*, 4277-4288.
Cao, F., and Tavis, J.E. (2006). Suppression of mRNA accumulation by the duck hepatitis B virus reverse transcriptase. Virology *350*, 475-483.
Cha, M.Y., Ryu, D.K., Jung, H.S., Chang, H.E., and Ryu, W.S. (2009). Stimulation of hepatitis B virus genome replication by HBx is linked to both nuclear and cytoplasmic HBx expression. J Gen Virol *90*, 978-986.
Chain, B.M., and Myers, R. (2005). Variability and conservation in hepatitis B virus core protein. BMC Microbiol *5*, 33.

Chamorro, A.J., Casado, J.L., Bellido, D., and Moreno, S. (2005). Reactivation of hepatitis B in an HIV-infected patient with antibodies against hepatitis B core antigen as the only serological marker. Eur J Clin Microbiol Infect Dis *24*, 492-494.
Chan, H.L., Thompson, A., Martinot-Peignoux, M., Piratvisuth, T., Cornberg, M., Brunetto, M.R., Tillmann, H.L., Kao, J.H., Jia, J.D., Wedemeyer, H.*, et al.* (2011). Hepatitis B surface antigen quantification: why and how to use it in 2011 - a core group report. J Hepatol *55*, 1121-1131.
Chen, L., and Flies, D.B. (2013). Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol *13*, 227-242.
Chen, M., and Ou, J.H. (1995). Cell type-dependent regulation of the activity of the negative regulatory element of the hepatitis B virus core promoter. Virology *214*, 198-206.
Chen, Y.C., Sheen, I.S., Chu, C.M., and Liaw, Y.F. (2002). Prognosis following spontaneous HBsAg seroclearance in chronic hepatitis B patients with or without concurrent infection. Gastroenterology *123*, 1084-1089.
Chu, C.J., Hussain, M., and Lok, A.S. (2002). Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. Gastroenterology *122*, 1756-1762.
Chu, C.J., Keeffe, E.B., Han, S.H., Perrillo, R.P., Min, A.D., Soldevila-Pico, C., Carey, W., Brown, R.S., Jr., Luketic, V.A., Terrault, N.*, et al.* (2003). Hepatitis B virus genotypes in the United States: results of a nationwide study. Gastroenterology *125*, 444-451.
Cirillo, L.A., Lin, F.R., Cuesta, I., Friedman, D., Jarnik, M., and Zaret, K.S. (2002). Opening of compacted chromatin by early developmental transcription factors HNF3 (FoxA) and GATA-4. Mol Cell *9*, 279-289.
Clippinger, A.J., and Bouchard, M.J. (2008). Hepatitis B virus HBx protein localizes to mitochondria in primary rat hepatocytes and modulates mitochondrial membrane potential. J Virol *82*, 6798-6811.
Cougot, D., Allemand, E., Riviere, L., Benhenda, S., Duroure, K., Levillayer, F., Muchardt, C., Buendia, M.A., and Neuveut, C. (2012). Inhibition of PP1 phosphatase activity by HBx: a mechanism for the activation of hepatitis B virus transcription. Sci Signal *5*, ra1.
Cougot, D., Wu, Y., Cairo, S., Caramel, J., Renard, C.A., Levy, L., Buendia, M.A., and Neuveut, C. (2007). The hepatitis B virus X protein functionally interacts with CREB-binding protein/p300 in the regulation of CREB-mediated transcription. J Biol Chem *282*, 4277-4287.
Coussens, L.M., and Werb, Z. (2002). Inflammation and cancer. Nature *420*, 860-867.
Crowther, R.A., Kiselev, N.A., Bottcher, B., Berriman, J.A., Borisova, G.P., Ose, V., and Pumpens, P. (1994). Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. Cell *77*, 943-950.
Cui, X., McAllister, R., Boregowda, R., Sohn, J.A., Cortes Ledesma, F., Caldecott, K.W., Seeger, C., and Hu, J. (2015). Does Tyrosyl DNA Phosphodiesterase-2 Play a Role in Hepatitis B Virus Genome Repair? PLoS One *10*, e0128401.
Dandri, M., and Locarnini, S. (2012). New insight in the pathobiology of hepatitis B virus infection. Gut *61 Suppl 1*, i6-17.

Dandri, M., and Petersen, J. (2016). Latest developments in the treatment of hepatitis B. Minerva Gastroenterol Dietol *62*, 88-102.
Dane, D.S., Cameron, C.H., and Briggs, M. (1970). Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet *1*, 695-698.
Davis, G.L., Hoofnagle, J.H., and Waggoner, J.G. (1984). Spontaneous reactivation of chronic hepatitis B virus infection. Gastroenterology *86*, 230-235.
Davis, L.G., Weber, D.J., and Lemon, S.M. (1989). Horizontal transmission of hepatitis B virus. Lancet *1*, 889-893.
De Falco, S., Ruvo, M., Verdoliva, A., Scarallo, A., Raimondo, D., Raucci, A., and Fassina, G. (2001). N-terminal myristylation of HBV preS1 domain enhances receptor recognition. J Pept Res *57*, 390-400.
de Franchis, R., Meucci, G., Vecchi, M., Tatarella, M., Colombo, M., Del Ninno, E., Rumi, M.G., Donato, M.F., and Ronchi, G. (1993). The natural history of asymptomatic hepatitis B surface antigen carriers. Ann Intern Med *118*, 191-194.
Decorsiere, A., Mueller, H., van Breugel, P.C., Abdul, F., Gerossier, L., Beran, R.K., Livingston, C.M., Niu, C., Fletcher, S.P., Hantz, O.*, et al.* (2016). Hepatitis B virus X protein identifies the Smc5/6 complex as a host restriction factor. Nature *531*, 386-389.
Delaney, W.E.t., Edwards, R., Colledge, D., Shaw, T., Furman, P., Painter, G., and Locarnini, S. (2002). Phenylpropenamide derivatives AT-61 and AT-130 inhibit replication of wild-type and lamivudine-resistant strains of hepatitis B virus in vitro. Antimicrob Agents Chemother *46*, 3057-3060.
Deres, K., Schroder, C.H., Paessens, A., Goldmann, S., Hacker, H.J., Weber, O., Kramer, T., Niewohner, U., Pleiss, U., Stoltefuss, J.*, et al.* (2003). Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. Science *299*, 893-896.
Di Marco, V., Lo Iacono, O., Camma, C., Vaccaro, A., Giunta, M., Martorana, G., Fuschi, P., Almasio, P.L., and Craxi, A. (1999). The long-term course of chronic hepatitis B. Hepatology *30*, 257-264.
Doitsh, G., and Shaul, Y. (2004). Enhancer I predominance in hepatitis B virus gene expression. Mol Cell Biol *24*, 1799-1808.
Dryden, K.A., Wieland, S.F., Whitten-Bauer, C., Gerin, J.L., Chisari, F.V., and Yeager, M. (2006). Native hepatitis B virions and capsids visualized by electron cryomicroscopy. Mol Cell *22*, 843-850.
Ducroux, A., Benhenda, S., Riviere, L., Semmes, O.J., Benkirane, M., and Neuveut, C. (2014). The Tudor domain protein Spindlin1 is involved in intrinsic antiviral defense against incoming hepatitis B Virus and herpes simplex virus type 1. PLoS Pathog *10*, e1004343.
Durantel, D., Alotte, C., and Zoulim, F. (2007). Glucosidase inhibitors as antiviral agents for hepatitis B and C. Curr Opin Investig Drugs *8*, 125-129.
El-Serag, H.B. (2012). Epidemiology of viral hepatitis and hepatocellular carcinoma. Gastroenterology *142*, 1264-1273 e1261.
Elmore, L.W., Hancock, A.R., Chang, S.F., Wang, X.W., Chang, S., Callahan, C.P., Geller, D.A., Will, H., and Harris, C.C. (1997). Hepatitis B virus X protein and p53 tumor suppressor interactions in the modulation of apoptosis. Proc Natl Acad Sci U S A *94*, 14707-14712.
Fattovich, G. (2003). Natural history of hepatitis B. J Hepatol *39 Suppl 1*, S50-58.

Fattovich, G., Brollo, L., Giustina, G., Noventa, F., Pontisso, P., Alberti, A., Realdi, G., and Ruol, A. (1991). Natural history and prognostic factors for chronic hepatitis type B. Gut *32*, 294-298.
Feitelson, M.A., Zhu, M., Duan, L.X., and London, W.T. (1993). Hepatitis B x antigen and p53 are associated in vitro and in liver tissues from patients with primary hepatocellular carcinoma. Oncogene *8*, 1109-1117.
Feld, J.J., Colledge, D., Sozzi, V., Edwards, R., Littlejohn, M., and Locarnini, S.A. (2007). The phenylpropenamide derivative AT-130 blocks HBV replication at the level of viral RNA packaging. Antiviral Res *76*, 168-177.
Fernandez, M., Quiroga, J.A., and Carreno, V. (2003). Hepatitis B virus downregulates the human interferon-inducible MxA promoter through direct interaction of precore/core proteins. J Gen Virol *84*, 2073-2082.
Fisicaro, P., Valdatta, C., Massari, M., Loggi, E., Biasini, E., Sacchelli, L., Cavallo, M.C., Silini, E.M., Andreone, P., Missale, G.*, et al.* (2010). Antiviral intrahepatic T-cell responses can be restored by blocking programmed death-1 pathway in chronic hepatitis B. Gastroenterology *138*, 682-693, 693 e681-684.
Forgues, M., Marrogi, A.J., Spillare, E.A., Wu, C.G., Yang, Q., Yoshida, M., and Wang, X.W. (2001). Interaction of the hepatitis B virus X protein with the Crm1-dependent nuclear export pathway. J Biol Chem *276*, 22797-22803.
Francis, D.P., Favero, M.S., and Maynard, J.E. (1981). Transmission of hepatitis B virus. Semin Liver Dis *1*, 27-32.
Fujii, R., Zhu, C., Wen, Y., Marusawa, H., Bailly-Maitre, B., Matsuzawa, S., Zhang, H., Kim, Y., Bennett, C.F., Jiang, W.*, et al.* (2006). HBXIP, cellular target of hepatitis B virus oncoprotein, is a regulator of centrosome dynamics and cytokinesis. Cancer Res *66*, 9099-9107.
Fukai, K., Takada, S., Yokosuka, O., Saisho, H., Omata, M., and Koike, K. (1997). Characterization of a specific region in the hepatitis B virus enhancer I for the efficient expression of X gene in the hepatic cell. Virology *236*, 279-287.
Fung, S.K., and Lok, A.S. (2004). Hepatitis B virus genotypes: do they play a role in the outcome of HBV infection? Hepatology *40*, 790-792.
Gaggar, A., Coeshott, C., Apelian, D., Rodell, T., Armstrong, B.R., Shen, G., Subramanian, G.M., and McHutchison, J.G. (2014). Safety, tolerability and immunogenicity of GS-4774, a hepatitis B virus-specific therapeutic vaccine, in healthy subjects: a randomized study. Vaccine *32*, 4925-4931.
Gallina, A., Bonelli, F., Zentilin, L., Rindi, G., Muttini, M., and Milanesi, G. (1989). A recombinant hepatitis B core antigen polypeptide with the protamine-like domain deleted self-assembles into capsid particles but fails to bind nucleic acids. J Virol *63*, 4645-4652.
Gane, E.J., Lim, Y.S., Gordon, S.C., Visvanathan, K., Sicard, E., Fedorak, R.N., Roberts, S., Massetto, B., Ye, Z., Pflanz, S.*, et al.* (2015). The oral toll-like receptor-7 agonist GS-9620 in patients with chronic hepatitis B virus infection. J Hepatol *63*, 320-328.
Ganem, D., and Prince, A.M. (2004). Hepatitis B virus infection--natural history and clinical consequences. N Engl J Med *350*, 1118-1129.
Gehring, A.J., Xue, S.A., Ho, Z.Z., Teoh, D., Ruedl, C., Chia, A., Koh, S., Lim, S.G., Maini, M.K., Stauss, H.*, et al.* (2011). Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. J Hepatol *55*, 103-110.

Gerelsaikhan, T., Tavis, J.E., and Bruss, V. (1996). Hepatitis B virus nucleocapsid envelopment does not occur without genomic DNA synthesis. J Virol *70*, 4269-4274.
Gerlich, W.H., and Robinson, W.S. (1980). Hepatitis B virus contains protein attached to the 5' terminus of its complete DNA strand. Cell *21*, 801-809.
Glebe, D., Aliakbari, M., Krass, P., Knoop, E.V., Valerius, K.P., and Gerlich, W.H. (2003). Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. J Virol *77*, 9511-9521.
Glebe, D., and Urban, S. (2007). Viral and cellular determinants involved in hepadnaviral entry. World J Gastroenterol *13*, 22-38.
Gripon, P., Le Seyec, J., Rumin, S., and Guguen-Guillouzo, C. (1995). Myristylation of the hepatitis B virus large surface protein is essential for viral infectivity. Virology *213*, 292-299.
Guan, H., Zhao, G., Chen, W., Wu, G., Liu, H., Jiang, X., Li, S., and Wang, L.L. (2015). The novel compound Z060228 inhibits assembly of the HBV capsid. Life Sci *133*, 1-7.
Guerrieri, F., Belloni, L., Pediconi, N., and Levrero, M. (2013). Molecular mechanisms of HBV-associated hepatocarcinogenesis. Semin Liver Dis *33*, 147-156.
Guerrieri, F., Belloni, L., D'Andrea, D. (2015). Genome-wide identification of direct HBx targets that control HBV replication. Gastroenterology.
Gunson, R.N., Shouval, D., Roggendorf, M., Zaaijer, H., Nicholas, H., Holzmann, H., de Schryver, A., Reynders, D., Connell, J., Gerlich, W.H*., et al.* (2003). Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections in health care workers (HCWs): guidelines for prevention of transmission of HBV and HCV from HCW to patients. J Clin Virol *27*, 213-230.
Gunther, S., Li, B.C., Miska, S., Kruger, D.H., Meisel, H., and Will, H. (1995). A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. J Virol *69*, 5437-5444.
Guo, H., Mao, R., Block, T.M., and Guo, J.T. (2010). Production and function of the cytoplasmic deproteinized relaxed circular DNA of hepadnaviruses. J Virol *84*, 387-396.
Guo, W.T., Bell, K.D., and Ou, J.H. (1991). Characterization of the hepatitis B virus EnhI enhancer and X promoter complex. J Virol *65*, 6686-6692.
Guo, Y., Kang, W., Lei, X., Li, Y., Xiang, A., Liu, Y., Zhao, J., Zhang, J., and Yan, Z. (2012). Hepatitis B viral core protein disrupts human host gene expression by binding to promoter regions. BMC Genomics *13*, 563.
Guo, Y.H., Li, Y.N., Zhao, J.R., Zhang, J., and Yan, Z. (2011). HBc binds to the CpG islands of HBV cccDNA and promotes an epigenetic permissive state. Epigenetics *6*, 720-726.
Habig, J.W., and Loeb, D.D. (2003). The conformation of the 3' end of the minus-strand DNA makes multiple contributions to template switches during plus-strand DNA synthesis of duck hepatitis B virus. J Virol *77*, 12401-12411.
Hadler, S.C., Doto, I.L., Maynard, J.E., Smith, J., Clark, B., Mosley, J., Eickhoff, T., Himmelsbach, C.K., and Cole, W.R. (1985). Occupational risk of hepatitis B infection in hospital workers. Infect Control *6*, 24-31.

Hadziyannis, S.J., Sevastianos, V., Rapti, I., Vassilopoulos, D., and Hadziyannis, E. (2012). Sustained responses and loss of HBsAg in HBeAg-negative patients with chronic hepatitis B who stop long-term treatment with adefovir. Gastroenterology *143*, 629-636 e621.
Hadziyannis, S.J., and Vassilopoulos, D. (2001). Hepatitis B e antigen-negative chronic hepatitis B. Hepatology *34*, 617-624.
Hagen, T.M., Huang, S., Curnutte, J., Fowler, P., Martinez, V., Wehr, C.M., Ames, B.N., and Chisari, F.V. (1994). Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. Proc Natl Acad Sci U S A *91*, 12808-12812.
Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293-296.
Harford, N., Cabezon, T., Crabeel, M., Simoen, E., Rutgers, A., and De Wilde, M. (1983). Expression of hepatitis B surface antigen in yeast. Dev Biol Stand *54*, 125-130.
Haruna, Y., Hayashi, N., Katayama, K., Yuki, N., Kasahara, A., Sasaki, Y., Fusamoto, H., and Kamada, T. (1991). Expression of X protein and hepatitis B virus replication in chronic hepatitis. Hepatology *13*, 417-421.
Heermann, K.H., Goldmann, U., Schwartz, W., Seyffarth, T., Baumgarten, H., and Gerlich, W.H. (1984). Large surface proteins of hepatitis B virus containing the pre-s sequence. J Virol *52*, 396-402.
Hodgson, A.J., Hyser, J.M., Keasler, V.V., Cang, Y., and Slagle, B.L. (2012). Hepatitis B virus regulatory HBx protein binding to DDB1 is required but is not sufficient for maximal HBV replication. Virology *426*, 73-82.
Hollinger, F.B., and Sood, G. (2010). Occult hepatitis B virus infection: a covert operation. J Viral Hepat *17*, 1-15.
Hoofnagle, J.H. (1981). Serologic markers of hepatitis B virus infection. Annu Rev Med *32*, 1-11.
Hoofnagle, J.H., Dusheiko, G.M., Seeff, L.B., Jones, E.A., Waggoner, J.G., and Bales, Z.B. (1981). Seroconversion from hepatitis B e antigen to antibody in chronic type B hepatitis. Ann Intern Med *94*, 744-748.
Hruska, J.F., Clayton, D.A., Rubenstein, J.L., and Robinson, W.S. (1977). Structure of hepatitis B Dane particle DNA before and after the Dane particle DNA polymerase reaction. J Virol *21*, 666-672.
Hsu, Y.S., Chien, R.N., Yeh, C.T., Sheen, I.S., Chiou, H.Y., Chu, C.M., and Liaw, Y.F. (2002). Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. Hepatology *35*, 1522-1527.
Hu, J., Toft, D.O., and Seeger, C. (1997). Hepadnavirus assembly and reverse transcription require a multi-component chaperone complex which is incorporated into nucleocapsids. EMBO J *16*, 59-68.
Hu, K.Q. (2002). Occult hepatitis B virus infection and its clinical implications. J Viral Hepat *9*, 243-257.
Huang, H.C., Chen, C.C., Chang, W.C., Tao, M.H., and Huang, C. (2012). Entry of hepatitis B virus into immortalized human primary hepatocytes by clathrin-dependent endocytosis. J Virol *86*, 9443-9453.
Huh, K.W., and Siddiqui, A. (2002). Characterization of the mitochondrial association of hepatitis B virus X protein, HBx. Mitochondrion *1*, 349-359.

Hui, C.K., Cheung, W.W., Zhang, H.Y., Au, W.Y., Yueng, Y.H., Leung, A.Y., Leung, N., Luk, J.M., Lie, A.K., Kwong, Y.L*., et al.* (2006). Kinetics and risk of de novo hepatitis B infection in HBsAg-negative patients undergoing cytotoxic chemotherapy. Gastroenterology *131*, 59-68.
Hui, C.K., Leung, N., Yuen, S.T., Zhang, H.Y., Leung, K.W., Lu, L., Cheung, S.K., Wong, W.M., Lau, G.K., and Hong Kong Liver Fibrosis Study, G. (2007). Natural history and disease progression in Chinese chronic hepatitis B patients in immune-tolerant phase. Hepatology *46*, 395-401.
Huovila, A.P., Eder, A.M., and Fuller, S.D. (1992). Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment. J Cell Biol *118*, 1305-1320.
Iloeje, U.H., Yang, H.I., Su, J., Jen, C.L., You, S.L., Chen, C.J., Risk Evaluation of Viral Load, E., and Associated Liver Disease/Cancer-In, H.B.V.S.G. (2006). Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. Gastroenterology *130*, 678-686.
Ishida, H., Ueda, K., Ohkawa, K., Kanazawa, Y., Hosui, A., Nakanishi, F., Mita, E., Kasahara, A., Sasaki, Y., Hori, M*., et al.* (2000). Identification of multiple transcription factors, HLF, FTF, and E4BP4, controlling hepatitis B virus enhancer II. J Virol *74*, 1241-1251.
Jaoude, G.A., and Sureau, C. (2005). Role of the antigenic loop of the hepatitis B virus envelope proteins in infectivity of hepatitis delta virus. J Virol *79*, 10460-10466.
Jeong, J.K., Yoon, G.S., and Ryu, W.S. (2000). Evidence that the 5'-end cap structure is essential for encapsidation of hepatitis B virus pregenomic RNA. J Virol *74*, 5502-5508.
Jiang, M., Broering, R., Trippler, M., Poggenpohl, L., Fiedler, M., Gerken, G., Lu, M., and Schlaak, J.F. (2014). Toll-like receptor-mediated immune responses are attenuated in the presence of high levels of hepatitis B virus surface antigen. J Viral Hepat *21*, 860-872.
Jun-Bin, S., Zhi, C., Wei-Qin, N., and Jun, F. (2003). A quantitative method to detect HBV cccDNA by chimeric primer and real-time polymerase chain reaction. J Virol Methods *112*, 45-52.
Junker-Niepmann, M., Bartenschlager, R., and Schaller, H. (1990). A short cis-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO J *9*, 3389-3396.
Kakimi, K., Isogawa, M., Chung, J., Sette, A., and Chisari, F.V. (2002). Immunogenicity and tolerogenicity of hepatitis B virus structural and nonstructural proteins: implications for immunotherapy of persistent viral infections. J Virol *76*, 8609-8620.
Kann, M., Schmitz, A., and Rabe, B. (2007). Intracellular transport of hepatitis B virus. World J Gastroenterol *13*, 39-47.
Katen, S.P., Chirapu, S.R., Finn, M.G., and Zlotnick, A. (2010). Trapping of hepatitis B virus capsid assembly intermediates by phenylpropenamide assembly accelerators. ACS Chem Biol *5*, 1125-1136.
Keasler, V.V., Hodgson, A.J., Madden, C.R., and Slagle, B.L. (2009). Hepatitis B virus HBx protein localized to the nucleus restores HBx-deficient virus replication in HepG2 cells and in vivo in hydrodynamically-injected mice. Virology *390*, 122-129.

Kennedy, E.M., Bassit, L.C., Mueller, H., Kornepati, A.V., Bogerd, H.P., Nie, T., Chatterjee, P., Javanbakht, H., Schinazi, R.F., and Cullen, B.R. (2015a). Suppression of hepatitis B virus DNA accumulation in chronically infected cells using a bacterial CRISPR/Cas RNA-guided DNA endonuclease. Virology *476*, 196-205.
Kennedy, E.M., Kornepati, A.V., and Cullen, B.R. (2015b). Targeting hepatitis B virus cccDNA using CRISPR/Cas9. Antiviral Res *123*, 188-192.
Kent, G.P., Brondum, J., Keenlyside, R.A., LaFazia, L.M., and Scott, H.D. (1988). A large outbreak of acupuncture-associated hepatitis B. Am J Epidemiol *127*, 591-598.
Kim, H., Lee, Y.H., Won, J., and Yun, Y. (2001). Through induction of juxtaposition and tyrosine kinase activity of Jak1, X-gene product of hepatitis B virus stimulates Ras and the transcriptional activation through AP-1, NF-kappaB, and SRE enhancers. Biochem Biophys Res Commun *286*, 886-894.
Kim, J.H., Kang, S., Kim, J., and Ahn, B.Y. (2003). Hepatitis B virus core protein stimulates the proteasome-mediated degradation of viral X protein. J Virol *77*, 7166-7173.
Kim, S., Kim, H.Y., Lee, S., Kim, S.W., Sohn, S., Kim, K., and Cho, H. (2007). Hepatitis B virus x protein induces perinuclear mitochondrial clustering in microtubule- and Dynein-dependent manners. J Virol *81*, 1714-1726.
Kimura, T., Ohno, N., Terada, N., Rokuhara, A., Matsumoto, A., Yagi, S., Tanaka, E., Kiyosawa, K., Ohno, S., and Maki, N. (2005). Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. J Biol Chem *280*, 21713-21719.
Knowles, B.B., Howe, C.C., and Aden, D.P. (1980). Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science *209*, 497-499.
Koff, R.S., Slavin, M.M., Connelly, J.D., and Rosen, D.R. (1977). Contagiousness of acute hepatitis B. Secondary attack rates in household contacts. Gastroenterology *72*, 297-300.
Koniger, C., Wingert, I., Marsmann, M., Rosler, C., Beck, J., and Nassal, M. (2014). Involvement of the host DNA-repair enzyme TDP2 in formation of the covalently closed circular DNA persistence reservoir of hepatitis B viruses. Proc Natl Acad Sci U S A *111*, E4244-4253.
Kota, S., Scampavia, L., Spicer, T., Beeler, A.B., Takahashi, V., Snyder, J.K., Porco, J.A., Hodder, P., and Strosberg, A.D. (2010). A time-resolved fluorescence-resonance energy transfer assay for identifying inhibitors of hepatitis C virus core dimerization. Assay Drug Dev Technol *8*, 96-105.
Kramvis, A., Kew, M., and Francois, G. (2005). Hepatitis B virus genotypes. Vaccine *23*, 2409-2423.
Kramvis, A., and Kew, M.C. (2005). Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. J Viral Hepat *12*, 456-464.
Krebs, K., Bottinger, N., Huang, L.R., Chmielewski, M., Arzberger, S., Gasteiger, G., Jager, C., Schmitt, E., Bohne, F., Aichler, M.*, et al.* (2013). T cells expressing a chimeric antigen receptor that binds hepatitis B virus envelope proteins control virus replication in mice. Gastroenterology *145*, 456-465.

Kwon, J.A., and Rho, H.M. (2003). Transcriptional repression of the human p53 gene by hepatitis B viral core protein (HBc) in human liver cells. Biol Chem *384*, 203-212.
Ladner, S.K., Otto, M.J., Barker, C.S., Zaifert, K., Wang, G.H., Guo, J.T., Seeger, C., and King, R.W. (1997). Inducible expression of human hepatitis B virus (HBV) in stably transfected hepatoblastoma cells: a novel system for screening potential inhibitors of HBV replication. Antimicrob Agents Chemother *41*, 1715-1720.
Lai, M.E., Solinas, A., Mazzoleni, A.P., Deplano, A., Farci, P., Lisci, V., Porru, A., Tocco, A., and Balestrieri, A. (1994). The role of pre-core hepatitis B virus mutants on the long-term outcome of chronic hepatitis B virus hepatitis. A longitudinal study. J Hepatol *20*, 773-781.
Lanford, R.E., Chavez, D., Brasky, K.M., Burns, R.B., 3rd, and Rico-Hesse, R. (1998). Isolation of a hepadnavirus from the woolly monkey, a New World primate. Proc Natl Acad Sci U S A *95*, 5757-5761.
Lanford, R.E., Guerra, B., Chavez, D., Giavedoni, L., Hodara, V.L., Brasky, K.M., Fosdick, A., Frey, C.R., Zheng, J., Wolfgang, G.*, et al.* (2013). GS-9620, an oral agonist of Toll-like receptor-7, induces prolonged suppression of hepatitis B virus in chronically infected chimpanzees. Gastroenterology *144*, 1508-1517, 1517 e1501-1510.
Le Seyec, J., Chouteau, P., Cannie, I., Guguen-Guillouzo, C., and Gripon, P. (1998). Role of the pre-S2 domain of the large envelope protein in hepatitis B virus assembly and infectivity. J Virol *72*, 5573-5578.
Le Seyec, J., Chouteau, P., Cannie, I., Guguen-Guillouzo, C., and Gripon, P. (1999). Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain. J Virol *73*, 2052-2057.
Lee, A.T., and Lee, C.G. (2007). Oncogenesis and transforming viruses: the hepatitis B virus and hepatocellularcarcinoma--the etiopathogenic link. Front Biosci *12*, 234-245.
Lee, D.K., Park, S.H., Yi, Y., Choi, S.G., Lee, C., Parks, W.T., Cho, H., de Caestecker, M.P., Shaul, Y., Roberts, A.B.*, et al.* (2001). The hepatitis B virus encoded oncoprotein pX amplifies TGF-beta family signaling through direct interaction with Smad4: potential mechanism of hepatitis B virus-induced liver fibrosis. Genes Dev *15*, 455-466.
Lee, T.H., Elledge, S.J., and Butel, J.S. (1995). Hepatitis B virus X protein interacts with a probable cellular DNA repair protein. J Virol *69*, 1107-1114.
Lee, Y.H., and Yun, Y. (1998). HBx protein of hepatitis B virus activates Jak1-STAT signaling. J Biol Chem *273*, 25510-25515.
Leistner, C.M., Gruen-Bernhard, S., and Glebe, D. (2008). Role of glycosaminoglycans for binding and infection of hepatitis B virus. Cell Microbiol *10*, 122-133.
Levrero, M., Pollicino, T., Petersen, J., Belloni, L., Raimondo, G., and Dandri, M. (2009). Control of cccDNA function in hepatitis B virus infection. J Hepatol *51*, 581-592.
Li, J., and Ou, J.H. (2001). Differential regulation of hepatitis B virus gene expression by the Sp1 transcription factor. J Virol *75*, 8400-8406.
Li, M., Xie, Y., Wu, X., Kong, Y., and Wang, Y. (1995). HNF3 binds and activates the second enhancer, ENII, of hepatitis B virus. Virology *214*, 371-378.

Liao, W., and Ou, J.H. (1995). Phosphorylation and nuclear localization of the hepatitis B virus core protein: significance of serine in the three repeated SPRRR motifs. J Virol *69*, 1025-1029.
Liaw, Y.F., Chen, Y.C., Sheen, I.S., Chien, R.N., Yeh, C.T., and Chu, C.M. (2004). Impact of acute hepatitis C virus superinfection in patients with chronic hepatitis B virus infection. Gastroenterology *126*, 1024-1029.
Liaw, Y.F., Sheen, I.S., Chen, T.J., Chu, C.M., and Pao, C.C. (1991). Incidence, determinants and significance of delayed clearance of serum HBsAg in chronic hepatitis B virus infection: a prospective study. Hepatology *13*, 627-631.
Liaw, Y.F., Tai, D.I., Chu, C.M., and Chen, T.J. (1988). The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. Hepatology *8*, 493-496.
Liaw, Y.F., Tai, D.I., Chu, C.M., Lin, D.Y., Sheen, I.S., Chen, T.J., and Pao, C.C. (1986). Early detection of hepatocellular carcinoma in patients with chronic type B hepatitis. A prospective study. Gastroenterology *90*, 263-267.
Liaw, Y.F., Yang, S.S., Chen, T.J., and Chu, C.M. (1985). Acute exacerbation in hepatitis B e antigen positive chronic type B hepatitis. A clinicopathological study. J Hepatol *1*, 227-233.
Limentani, A.E., Elliott, L.M., Noah, N.D., and Lamborn, J.K. (1979). An outbreak of hepatitis B from tattooing. Lancet *2*, 86-88.
Lin, W.J., Li, J., Lee, Y.F., Yeh, S.D., Altuwaijri, S., Ou, J.H., and Chang, C. (2003). Suppression of hepatitis B virus core promoter by the nuclear orphan receptor TR4. J Biol Chem *278*, 9353-9360.
Lin, Y.C., Hsu, E.C., and Ting, L.P. (2009). Repression of hepatitis B viral gene expression by transcription factor nuclear factor-kappaB. Cell Microbiol *11*, 645-660.
Liu, F., Campagna, M., Qi, Y., Zhao, X., Guo, F., Xu, C., Li, S., Li, W., Block, T.M., Chang, J.*, et al.* (2013). Alpha-interferon suppresses hepadnavirus transcription by altering epigenetic modification of cccDNA minichromosomes. PLoS Pathog *9*, e1003613.
Liu, N., Ji, L., Maguire, M.L., and Loeb, D.D. (2004). cis-Acting sequences that contribute to the synthesis of relaxed-circular DNA of human hepatitis B virus. J Virol *78*, 642-649.
Livingston, S.E., Simonetti, J.P., Bulkow, L.R., Homan, C.E., Snowball, M.M., Cagle, H.H., Negus, S.E., and McMahon, B.J. (2007). Clearance of hepatitis B e antigen in patients with chronic hepatitis B and genotypes A, B, C, D, and F. Gastroenterology *133*, 1452-1457.
Lok, A.S., Heathcote, E.J., and Hoofnagle, J.H. (2001). Management of hepatitis B: 2000--summary of a workshop. Gastroenterology *120*, 1828-1853.
Lok, A.S., and Lai, C.L. (1989). alpha-Fetoprotein monitoring in Chinese patients with chronic hepatitis B virus infection: role in the early detection of hepatocellular carcinoma. Hepatology *9*, 110-115.
Lopez-Cabrera, M., Letovsky, J., Hu, K.Q., and Siddiqui, A. (1991). Transcriptional factor C/EBP binds to and transactivates the enhancer element II of the hepatitis B virus. Virology *183*, 825-829.
Lu, C.C., Chen, M., Ou, J.H., and Yen, T.S. (1995). Key role of a CCAAT element in regulating hepatitis B virus surface protein expression. Virology *206*, 1155-1158.

Lucifora, J., Arzberger, S., Durantel, D., Belloni, L., Strubin, M., Levrero, M., Zoulim, F., Hantz, O., and Protzer, U. (2011). Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. J Hepatol *55*, 996-1003.
Lucifora, J., Xia, Y., Reisinger, F., Zhang, K., Stadler, D., Cheng, X., Sprinzl, M.F., Koppensteiner, H., Makowska, Z., Volz, T.*, et al.* (2014). Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. Science *343*, 1221-1228.
Lupberger, J., and Hildt, E. (2007). Hepatitis B virus-induced oncogenesis. World J Gastroenterol *13*, 74-81.
Lutgehetmann, M., Mancke, L.V., Volz, T., Helbig, M., Allweiss, L., Bornscheuer, T., Pollok, J.M., Lohse, A.W., Petersen, J., Urban, S.*, et al.* (2012). Humanized chimeric uPA mouse model for the study of hepatitis B and D virus interactions and preclinical drug evaluation. Hepatology *55*, 685-694.
Lutgehetmann, M., Volzt, T., Quaas, A., Zankel, M., Fischer, C., Dandri, M., and Petersen, J. (2008). Sequential combination therapy leads to biochemical and histological improvement despite low ongoing intrahepatic hepatitis B virus replication. Antivir Ther *13*, 57-66.
Mabit, H., and Schaller, H. (2000). Intracellular hepadnavirus nucleocapsids are selected for secretion by envelope protein-independent membrane binding. J Virol *74*, 11472-11478.
MacKellar, D.A., Valleroy, L.A., Secura, G.M., McFarland, W., Shehan, D., Ford, W., LaLota, M., Celentano, D.D., Koblin, B.A., Torian, L.V.*, et al.* (2001). Two decades after vaccine license: hepatitis B immunization and infection among young men who have sex with men. Am J Public Health *91*, 965-971.
Macovei, A., Petrareanu, C., Lazar, C., Florian, P., and Branza-Nichita, N. (2013). Regulation of hepatitis B virus infection by Rab5, Rab7, and the endolysosomal compartment. J Virol *87*, 6415-6427.
Macrae, D.R., Bruss, V., and Ganem, D. (1991). Myristylation of a duck hepatitis B virus envelope protein is essential for infectivity but not for virus assembly. Virology *181*, 359-363.
Marcellin, P., Wursthorn, K., Wedemeyer, H., Chuang, W.L., Lau, G., Avila, C., Peng, C.Y., Gane, E., Lim, S.G., Fainboim, H.*, et al.* (2015). Telbivudine plus pegylated interferon alfa-2a in a randomized study in chronic hepatitis B is associated with an unexpected high rate of peripheral neuropathy. J Hepatol *62*, 41-47.
Marion, P.L., Oshiro, L.S., Regnery, D.C., Scullard, G.H., and Robinson, W.S. (1980). A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. Proc Natl Acad Sci U S A *77*, 2941-2945.
Marsh, M., and Helenius, A. (2006). Virus entry: open sesame. Cell *124*, 729-740.
Martin, P., Dubois, C., Jacquier, E., Dion, S., Mancini-Bourgine, M., Godon, O., Kratzer, R., Lelu-Santolaria, K., Evlachev, A., Meritet, J.F.*, et al.* (2015). TG1050, an immunotherapeutic to treat chronic hepatitis B, induces robust T cells and exerts an antiviral effect in HBV-persistent mice. Gut *64*, 1961-1971.
Mason, W.S., Seal, G., and Summers, J. (1980). Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. J Virol *36*, 829-836.

Maupas, P., Goudeau, A., Coursaget, P., Drucker, J., and Bagros, P. (1976). Immunisation against hepatitis B in man. Lancet *1*, 1367-1370.
McMahon, B.J. (2004). The natural history of chronic hepatitis B virus infection. Semin Liver Dis *24 Suppl 1*, 17-21.
McMahon, B.J. (2009a). The influence of hepatitis B virus genotype and subgenotype on the natural history of chronic hepatitis B. Hepatol Int *3*, 334-342.
McMahon, B.J. (2009b). The natural history of chronic hepatitis B virus infection. Hepatology *49*, S45-55.
McMahon, B.J., Alward, W.L., Hall, D.B., Heyward, W.L., Bender, T.R., Francis, D.P., and Maynard, J.E. (1985). Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. J Infect Dis *151*, 599-603.
McMahon, B.J., Holck, P., Bulkow, L., and Snowball, M. (2001). Serologic and clinical outcomes of 1536 Alaska Natives chronically infected with hepatitis B virus. Ann Intern Med *135*, 759-768.
Meier, A., Mehrle, S., Weiss, T.S., Mier, W., and Urban, S. (2013). Myristoylated PreS1-domain of the hepatitis B virus L-protein mediates specific binding to differentiated hepatocytes. Hepatology *58*, 31-42.
Melegari, M., Wolf, S.K., and Schneider, R.J. (2005). Hepatitis B virus DNA replication is coordinated by core protein serine phosphorylation and HBx expression. J Virol *79*, 9810-9820.
Miyakawa, Y., and Mizokami, M. (2003). Classifying hepatitis B virus genotypes. Intervirology *46*, 329-338.
Moolla, N., Kew, M., and Arbuthnot, P. (2002). Regulatory elements of hepatitis B virus transcription. J Viral Hepat *9*, 323-331.
Moraleda, G., Saputelli, J., Aldrich, C.E., Averett, D., Condreay, L., and Mason, W.S. (1997). Lack of effect of antiviral therapy in nondividing hepatocyte cultures on the closed circular DNA of woodchuck hepatitis virus. J Virol *71*, 9392-9399.
Mueller-Hill, K., and Loeb, D.D. (2002). cis-Acting sequences 5E, M, and 3E interact to contribute to primer translocation and circularization during reverse transcription of avian hepadnavirus DNA. J Virol *76*, 4260-4266.
Mulrooney-Cousins, P.M., and Michalak, T.I. (2007). Persistent occult hepatitis B virus infection: experimental findings and clinical implications. World J Gastroenterol *13*, 5682-5686.
Murakami, S. (1999). Hepatitis B virus X protein: structure, function and biology. Intervirology *42*, 81-99.
Nassal, M. (2008). Hepatitis B viruses: reverse transcription a different way. Virus Res *134*, 235-249.
Nassal, M., Junker-Niepmann, M., and Schaller, H. (1990). Translational inactivation of RNA function: discrimination against a subset of genomic transcripts during HBV nucleocapsid assembly. Cell *63*, 1357-1363.
Nebbia, G., Peppa, D., Schurich, A., Khanna, P., Singh, H.D., Cheng, Y., Rosenberg, W., Dusheiko, G., Gilson, R., ChinAleong, J.*, et al.* (2012). Upregulation of the Tim-3/galectin-9 pathway of T cell exhaustion in chronic hepatitis B virus infection. PLoS One *7*, e47648.
Newbold, J.E., Xin, H., Tencza, M., Sherman, G., Dean, J., Bowden, S., and Locarnini, S. (1995). The covalently closed duplex form of the hepadnavirus

genome exists in situ as a heterogeneous population of viral minichromosomes. J Virol *69*, 3350-3357.
Nguyen, T., and Locarnini, S. (2009). Hepatitis: Monitoring drug therapy for hepatitis B--a global challenge? Nat Rev Gastroenterol Hepatol *6*, 565-567.
Ni, Y., Lempp, F.A., Mehrle, S., Nkongolo, S., Kaufman, C., Falth, M., Stindt, J., Koniger, C., Nassal, M., Kubitz, R.*, et al.* (2014). Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes. Gastroenterology *146*, 1070-1083.
Ning, Q., Han, M., Sun, Y., Jiang, J., Tan, D., Hou, J., Tang, H., Sheng, J., and Zhao, M. (2014). Switching from entecavir to PegIFN alfa-2a in patients with HBeAg-positive chronic hepatitis B: a randomised open-label trial (OSST trial). J Hepatol *61*, 777-784.
Nomura, T., Lin, Y., Dorjsuren, D., Ohno, S., Yamashita, T., and Murakami, S. (1999). Human hepatitis B virus X protein is detectable in nuclei of transfected cells, and is active for transactivation. Biochim Biophys Acta *1453*, 330-340.
Noordeen, F., Vaillant, A., and Jilbert, A.R. (2013). Nucleic acid polymers inhibit duck hepatitis B virus infection in vitro. Antimicrob Agents Chemother *57*, 5291-5298.
Nunez, E., Yelamos, B., Delgado, C., Gomez-Gutierrez, J., Peterson, D.L., and Gavilanes, F. (2009). Interaction of preS domains of hepatitis B virus with phospholipid vesicles. Biochim Biophys Acta *1788*, 417-424.
Obeng-Adjei, N., Hutnick, N.A., Yan, J., Chu, J.S., Myles, D.J., Morrow, M.P., Sardesai, N.Y., and Weiner, D.B. (2013). DNA vaccine cocktail expressing genotype A and C HBV surface and consensus core antigens generates robust cytotoxic and antibody responses in mice and Rhesus macaques. Cancer Gene Ther *20*, 652-662.
Oess, S., and Hildt, E. (2000). Novel cell permeable motif derived from the PreS2-domain of hepatitis-B virus surface antigens. Gene Ther *7*, 750-758.
Ori, A., Atzmony, D., Haviv, I., and Shaul, Y. (1994). An NF1 motif plays a central role in hepatitis B virus enhancer. Virology *204*, 600-608.
Ott, J.J., Stevens, G.A., Groeger, J., and Wiersma, S.T. (2012). Global epidemiology of hepatitis B virus infection: new estimates of age-specific HBsAg seroprevalence and endemicity. Vaccine *30*, 2212-2219.
Papatheodoridis, G.V., Manesis, E., and Hadziyannis, S.J. (2001). The long-term outcome of interferon-alpha treated and untreated patients with HBeAg-negative chronic hepatitis B. J Hepatol *34*, 306-313.
Patient, R., Hourioux, C., and Roingeard, P. (2009). Morphogenesis of hepatitis B virus and its subviral envelope particles. Cell Microbiol *11*, 1561-1570.
Penna, A., Artini, M., Cavalli, A., Levrero, M., Bertoletti, A., Pilli, M., Chisari, F.V., Rehermann, B., Del Prete, G., Fiaccadori, F.*, et al.* (1996). Long-lasting memory T cell responses following self-limited acute hepatitis B. J Clin Invest *98*, 1185-1194.
Peppa, D., Micco, L., Javaid, A., Kennedy, P.T., Schurich, A., Dunn, C., Pallant, C., Ellis, G., Khanna, P., Dusheiko, G.*, et al.* (2010). Blockade of immunosuppressive cytokines restores NK cell antiviral function in chronic hepatitis B virus infection. PLoS Pathog *6*, e1001227.

Perlman, D.H., Berg, E.A., O'Connor P, B., Costello, C.E., and Hu, J. (2005). Reverse transcription-associated dephosphorylation of hepadnavirus nucleocapsids. Proc Natl Acad Sci U S A *102*, 9020-9025.
Persing, D.H., Varmus, H.E., and Ganem, D. (1987). The preS1 protein of hepatitis B virus is acylated at its amino terminus with myristic acid. J Virol *61*, 1672-1677.
Petersen, J., Dandri, M., Mier, W., Lutgehetmann, M., Volz, T., von Weizsacker, F., Haberkorn, U., Fischer, L., Pollok, J.M., Erbes, B.*, et al.* (2008). Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. Nat Biotechnol *26*, 335-341.
Petersen, J., Thompson, A.J., and Levrero, M. (2016). Aiming for cure in HBV and HDV infection. J Hepatol *65*, 835-848.
Petit, M.A., and Pillot, J. (1985). HBc and HBe antigenicity and DNA-binding activity of major core protein P22 in hepatitis B virus core particles isolated from the cytoplasm of human liver cells. J Virol *53*, 543-551.
Phillips, S., Chokshi, S., Chatterji, U., Riva, A., Bobardt, M., Williams, R., Gallay, P., and Naoumov, N.V. (2015). Alisporivir inhibition of hepatocyte cyclophilins reduces HBV replication and hepatitis B surface antigen production. Gastroenterology *148*, 403-414 e407.
Pollack, J.R., and Ganem, D. (1994). Site-specific RNA binding by a hepatitis B virus reverse transcriptase initiates two distinct reactions: RNA packaging and DNA synthesis. J Virol *68*, 5579-5587.
Pollicino, T., Belloni, L., Raffa, G., Pediconi, N., Squadrito, G., Raimondo, G., and Levrero, M. (2006). Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. Gastroenterology *130*, 823-837.
Ponsel, D., and Bruss, V. (2003). Mapping of amino acid side chains on the surface of hepatitis B virus capsids required for envelopment and virion formation. J Virol *77*, 416-422.
Purcell, R.H., and Gerin, J.L. (1975). Hepatitis B subunit vaccine: a preliminary report of safety and efficacy tests in chimpanzees. Am J Med Sci *270*, 395-399.
Qin, J., Zhai, J., Hong, R., Shan, S., Kong, Y., Wen, Y., Wang, Y., Liu, J., and Xie, Y. (2009). Prospero-related homeobox protein (Prox1) inhibits hepatitis B virus replication through repressing multiple cis regulatory elements. J Gen Virol *90*, 1246-1255.
Qiu, Z., Lin, X., Zhou, M., Liu, Y., Zhu, W., Chen, W., Zhang, W., Guo, L., Liu, H., Wu, G.*, et al.* (2016). Design and Synthesis of Orally Bioavailable 4-Methyl Heteroaryldihydropyrimidine Based Hepatitis B Virus (HBV) Capsid Inhibitors. J Med Chem *59*, 7651-7666.
Radziwill, G., Tucker, W., and Schaller, H. (1990). Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. J Virol *64*, 613-620.
Rahmani, Z., Huh, K.W., Lasher, R., and Siddiqui, A. (2000). Hepatitis B virus X protein colocalizes to mitochondria with a human voltage-dependent anion channel, HVDAC3, and alters its transmembrane potential. J Virol *74*, 2840-2846.
Raimondo, G., Allain, J.P., Brunetto, M.R., Buendia, M.A., Chen, D.S., Colombo, M., Craxi, A., Donato, F., Ferrari, C., Gaeta, G.B.*, et al.* (2008a). Statements from

the Taormina expert meeting on occult hepatitis B virus infection. J Hepatol *49*, 652-657.
Raimondo, G., Navarra, G., Mondello, S., Costantino, L., Colloredo, G., Cucinotta, E., Di Vita, G., Scisca, C., Squadrito, G., and Pollicino, T. (2008b). Occult hepatitis B virus in liver tissue of individuals without hepatic disease. J Hepatol *48*, 743-746.
Ramanan, V., Shlomai, A., Cox, D.B., Schwartz, R.E., Michailidis, E., Bhatta, A., Scott, D.A., Zhang, F., Rice, C.M., and Bhatia, S.N. (2015). CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. Sci Rep *5*, 10833.
Ramiere, C., Scholtes, C., Diaz, O., Icard, V., Perrin-Cocon, L., Trabaud, M.A., Lotteau, V., and Andre, P. (2008). Transactivation of the hepatitis B virus core promoter by the nuclear receptor FXRalpha. J Virol *82*, 10832-10840.
Randall, G., and Rice, C.M. (2004). Interfering with hepatitis C virus RNA replication. Virus Res *102*, 19-25.
Raney, A.K., Easton, A.J., and McLachlan, A. (1994). Characterization of the minimal elements of the hepatitis B virus large surface antigen promoter. J Gen Virol *75 (Pt 10)*, 2671-2679.
Raney, A.K., Easton, A.J., Milich, D.R., and McLachlan, A. (1991). Promoter-specific transactivation of hepatitis B virus transcription by a glutamine- and proline-rich domain of hepatocyte nuclear factor 1. J Virol *65*, 5774-5781.
Raziorrouh, B., Schraut, W., Gerlach, T., Nowack, D., Gruner, N.H., Ulsenheimer, A., Zachoval, R., Wachtler, M., Spannagl, M., Haas, J.*, et al.* (2010). The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function. Hepatology *52*, 1934-1947.
Rehermann, B., Lau, D., Hoofnagle, J.H., and Chisari, F.V. (1996). Cytotoxic T lymphocyte responsiveness after resolution of chronic hepatitis B virus infection. J Clin Invest *97*, 1655-1665.
Ribeiro, R.M., Lo, A., and Perelson, A.S. (2002). Dynamics of hepatitis B virus infection. Microbes Infect *4*, 829-835.
Rieger, A., and Nassal, M. (1996). Specific hepatitis B virus minus-strand DNA synthesis requires only the 5' encapsidation signal and the 3'-proximal direct repeat DR1. J Virol *70*, 585-589.
Riviere, L., Gerossier, L., Ducroux, A., Dion, S., Deng, Q., Michel, M.L., Buendia, M.A., Hantz, O., and Neuveut, C. (2015). HBx relieves chromatin-mediated transcriptional repression of hepatitis B viral cccDNA involving SETDB1 histone methyltransferase. J Hepatol *63*, 1093-1102.
Rizzetto, M. (2016). The adventure of delta. Liver Int *36 Suppl 1*, 135-140.
Rodriguez-Crespo, I., Nunez, E., Yelamos, B., Gomez-Gutierrez, J., Albar, J.P., Peterson, D.L., and Gavilanes, F. (1999). Fusogenic activity of hepadnavirus peptides corresponding to sequences downstream of the putative cleavage site. Virology *261*, 133-142.
Roseman, A.M., Berriman, J.A., Wynne, S.A., Butler, P.J., and Crowther, R.A. (2005). A structural model for maturation of the hepatitis B virus core. Proc Natl Acad Sci U S A *102*, 15821-15826.
Sato, S., Li, K., Kameyama, T., Hayashi, T., Ishida, Y., Murakami, S., Watanabe, T., Iijima, S., Sakurai, Y., Watashi, K.*, et al.* (2015). The RNA sensor RIG-I

dually functions as an innate sensor and direct antiviral factor for hepatitis B virus. Immunity *42*, 123-132.
Sattler, F., and Robinson, W.S. (1979). Hepatitis B viral DNA molecules have cohesive ends. J Virol *32*, 226-233.
Schaefer, S. (2005). Hepatitis B virus: significance of genotypes. J Viral Hepat *12*, 111-124.
Schaefer, S. (2007). Hepatitis B virus taxonomy and hepatitis B virus genotypes. World J Gastroenterol *13*, 14-21.
Schreiber, G.B., Busch, M.P., Kleinman, S.H., and Korelitz, J.J. (1996). The risk of transfusion-transmitted viral infections. The Retrovirus Epidemiology Donor Study. N Engl J Med *334*, 1685-1690.
Schulze, A., Gripon, P., and Urban, S. (2007). Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. Hepatology *46*, 1759-1768.
Schurich, A., Khanna, P., Lopes, A.R., Han, K.J., Peppa, D., Micco, L., Nebbia, G., Kennedy, P.T., Geretti, A.M., Dusheiko, G.*, et al.* (2011). Role of the coinhibitory receptor cytotoxic T lymphocyte antigen-4 on apoptosis-Prone CD8 T cells in persistent hepatitis B virus infection. Hepatology *53*, 1494-1503.
Seeger, C., Ganem, D., and Varmus, H.E. (1986). Biochemical and genetic evidence for the hepatitis B virus replication strategy. Science *232*, 477-484.
Seeger, C., and Sohn, J.A. (2014). Targeting Hepatitis B Virus With CRISPR/Cas9. Mol Ther Nucleic Acids *3*, e216.
Seitz, S., Urban, S., Antoni, C., and Bottcher, B. (2007). Cryo-electron microscopy of hepatitis B virions reveals variability in envelope capsid interactions. EMBO J *26*, 4160-4167.
Sen, N., Cao, F., and Tavis, J.E. (2004). Translation of duck hepatitis B virus reverse transcriptase by ribosomal shunting. J Virol *78*, 11751-11757.
Shaul, Y., Garcia, P.D., Schonberg, S., and Rutter, W.J. (1986). Integration of hepatitis B virus DNA in chromosome-specific satellite sequences. J Virol *59*, 731-734.
Shepard, C.W., Simard, E.P., Finelli, L., Fiore, A.E., and Bell, B.P. (2006). Hepatitis B virus infection: epidemiology and vaccination. Epidemiol Rev *28*, 112-125.
Sir, D., Tian, Y., Chen, W.L., Ann, D.K., Yen, T.S., and Ou, J.H. (2010). The early autophagic pathway is activated by hepatitis B virus and required for viral DNA replication. Proc Natl Acad Sci U S A *107*, 4383-4388.
Sohn, J.A., Litwin, S., and Seeger, C. (2009). Mechanism for CCC DNA synthesis in hepadnaviruses. PLoS One *4*, e8093.
Sommer, G., van Bommel, F., and Will, H. (2000). Genotype-specific synthesis and secretion of spliced hepatitis B virus genomes in hepatoma cells. Virology *271*, 371-381.
Spandau, D.F., and Lee, C.H. (1992). Repression of the hepatitis B virus enhancer by a cellular factor. J Gen Virol *73 (Pt 1)*, 131-137.
Sprengel, R., Kaleta, E.F., and Will, H. (1988). Isolation and characterization of a hepatitis B virus endemic in herons. J Virol *62*, 3832-3839.
Stahl, M., Beck, J., and Nassal, M. (2007). Chaperones activate hepadnavirus reverse transcriptase by transiently exposing a C-proximal region in the terminal protein domain that contributes to epsilon RNA binding. J Virol *81*, 13354-13364.

Staprans, S., Loeb, D.D., and Ganem, D. (1991). Mutations affecting hepadnavirus plus-strand DNA synthesis dissociate primer cleavage from translocation and reveal the origin of linear viral DNA. J Virol *65*, 1255-1262.
Stoeckl, L., Funk, A., Kopitzki, A., Brandenburg, B., Oess, S., Will, H., Sirma, H., and Hildt, E. (2006). Identification of a structural motif crucial for infectivity of hepatitis B viruses. Proc Natl Acad Sci U S A *103*, 6730-6734.
Stray, S.J., Bourne, C.R., Punna, S., Lewis, W.G., Finn, M.G., and Zlotnick, A. (2005). A heteroaryldihydropyrimidine activates and can misdirect hepatitis B virus capsid assembly. Proc Natl Acad Sci U S A *102*, 8138-8143.
Su, Q., Schroder, C.H., Hofmann, W.J., Otto, G., Pichlmayr, R., and Bannasch, P. (1998). Expression of hepatitis B virus X protein in HBV-infected human livers and hepatocellular carcinomas. Hepatology *27*, 1109-1120.
Sumi, H., Yokosuka, O., Seki, N., Arai, M., Imazeki, F., Kurihara, T., Kanda, T., Fukai, K., Kato, M., and Saisho, H. (2003). Influence of hepatitis B virus genotypes on the progression of chronic type B liver disease. Hepatology *37*, 19-26.
Summers, J., O'Connell, A., and Millman, I. (1975). Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. Proc Natl Acad Sci U S A *72*, 4597-4601.
Summers, J., Smolec, J.M., and Snyder, R. (1978). A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc Natl Acad Sci U S A *75*, 4533-4537.
Sun, D., and Nassal, M. (2006). Stable HepG2- and Huh7-based human hepatoma cell lines for efficient regulated expression of infectious hepatitis B virus. J Hepatol *45*, 636-645.
Sunbul, M. (2014). Hepatitis B virus genotypes: global distribution and clinical importance. World J Gastroenterol *20*, 5427-5434.
Sung, J.J., Chan, H.L., Wong, M.L., Tse, C.H., Yuen, S.C., Tam, J.S., and Leung, N.W. (2002). Relationship of clinical and virological factors with hepatitis activity in hepatitis B e antigen-negative chronic hepatitis B virus-infected patients. J Viral Hepat *9*, 229-234.
Sung, J.J., Wong, M.L., Bowden, S., Liew, C.T., Hui, A.Y., Wong, V.W., Leung, N.W., Locarnini, S., and Chan, H.L. (2005). Intrahepatic hepatitis B virus covalently closed circular DNA can be a predictor of sustained response to therapy. Gastroenterology *128*, 1890-1897.
Sureau, C., and Salisse, J. (2013). A conformational heparan sulfate binding site essential to infectivity overlaps with the conserved hepatitis B virus a-determinant. Hepatology *57*, 985-994.
Tan, Z., Maguire, M.L., Loeb, D.D., and Zlotnick, A. (2013). Genetically altering the thermodynamics and kinetics of hepatitis B virus capsid assembly has profound effects on virus replication in cell culture. J Virol *87*, 3208-3216.
Tang, H., Delgermaa, L., Huang, F., Oishi, N., Liu, L., He, F., Zhao, L., and Murakami, S. (2005). The transcriptional transactivation function of HBx protein is important for its augmentation role in hepatitis B virus replication. J Virol *79*, 5548-5556.
Tassopoulos, N.C., Papaevangelou, G.J., Sjogren, M.H., Roumeliotou-Karayannis, A., Gerin, J.L., and Purcell, R.H. (1987). Natural history of acute

hepatitis B surface antigen-positive hepatitis in Greek adults. Gastroenterology *92*, 1844-1850.
Tavis, J.E., Massey, B., and Gong, Y. (1998). The duck hepatitis B virus polymerase is activated by its RNA packaging signal, epsilon. J Virol *72*, 5789-5796.
Tavis, J.E., Perri, S., and Ganem, D. (1994). Hepadnavirus reverse transcription initiates within the stem-loop of the RNA packaging signal and employs a novel strand transfer. J Virol *68*, 3536-3543.
Tedder, R.S., Ijaz, S., Gilbert, N., Barbara, J.A., Corden, S.A., Gilson, R.J., and Boxall, E.H. (2002). Evidence for a dynamic host-parasite relationship in e-negative hepatitis B carriers. J Med Virol *68*, 505-512.
Thio, C.L., Seaberg, E.C., Skolasky, R., Jr., Phair, J., Visscher, B., Munoz, A., Thomas, D.L., and Multicenter, A.C.S. (2002). HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). Lancet *360*, 1921-1926.
Thompson, A.J., Colledge, D., Rodgers, S., Wilson, R., Revill, P., Desmond, P., Mansell, A., Visvanathan, K., and Locarnini, S. (2009). Stimulation of the interleukin-1 receptor and Toll-like receptor 2 inhibits hepatitis B virus replication in hepatoma cell lines in vitro. Antivir Ther *14*, 797-808.
Thompson, A.J., and Locarnini, S.A. (2007). Toll-like receptors, RIG-I-like RNA helicases and the antiviral innate immune response. Immunol Cell Biol *85*, 435-445.
Tong, M.J., Blatt, L.M., Kao, J.H., Cheng, J.T., and Corey, W.G. (2007). Basal core promoter T1762/A1764 and precore A1896 gene mutations in hepatitis B surface antigen-positive hepatocellular carcinoma: a comparison with chronic carriers. Liver Int *27*, 1356-1363.
Torbenson, M., and Thomas, D.L. (2002). Occult hepatitis B. Lancet Infect Dis *2*, 479-486.
Tropberger, P., Mercier, A., Robinson, M., Zhong, W., Ganem, D.E., and Holdorf, M. (2015). Mapping of histone modifications in episomal HBV cccDNA uncovers an unusual chromatin organization amenable to epigenetic manipulation. Proc Natl Acad Sci U S A *112*, E5715-5724.
Tsuge, M., Hiraga, N., Akiyama, R., Tanaka, S., Matsushita, M., Mitsui, F., Abe, H., Kitamura, S., Hatakeyama, T., Kimura, T.*, et al.* (2010). HBx protein is indispensable for development of viraemia in human hepatocyte chimeric mice. J Gen Virol *91*, 1854-1864.
Tsukuda, S., Watashi, K., Iwamoto, M., Suzuki, R., Aizaki, H., Okada, M., Sugiyama, M., Kojima, S., Tanaka, Y., Mizokami, M.*, et al.* (2015). Dysregulation of retinoic acid receptor diminishes hepatocyte permissiveness to hepatitis B virus infection through modulation of sodium taurocholate cotransporting polypeptide (NTCP) expression. J Biol Chem *290*, 5673-5684.
Tuttleman, J.S., Pourcel, C., and Summers, J. (1986). Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell *47*, 451-460.
Urban, S. (2008). New insights into hepatitis B and hepatitis delta virus entry. Future Viral *3*, 253-264.
Urban, S., Bartenschlager, R., Kubitz, R., and Zoulim, F. (2014). Strategies to inhibit entry of HBV and HDV into hepatocytes. Gastroenterology *147*, 48-64.

Verrier, E.R., Colpitts, C.C., Bach, C., Heydmann, L., Weiss, A., Renaud, M., Durand, S.C., Habersetzer, F., Durantel, D., Abou-Jaoude, G.*, et al.* (2016). A targeted functional RNA interference screen uncovers glypican 5 as an entry factor for hepatitis B and D viruses. Hepatology *63*, 35-48.
Vlachogiannakos, J., and Papatheodoridis, G. (2013). Hepatocellular carcinoma in chronic hepatitis B patients under antiviral therapy. World J Gastroenterol *19*, 8822-8830.
Volz, T., Allweiss, L., Ben, M.M., Warlich, M., Lohse, A.W., Pollok, J.M., Alexandrov, A., Urban, S., Petersen, J., Lutgehetmann, M.*, et al.* (2013). The entry inhibitor Myrcludex-B efficiently blocks intrahepatic virus spreading in humanized mice previously infected with hepatitis B virus. J Hepatol *58*, 861-867.
Wang, G.H., and Seeger, C. (1993). Novel mechanism for reverse transcription in hepatitis B viruses. J Virol *67*, 6507-6512.
Wang, X.Y., Wei, Z.M., Wu, G.Y., Wang, J.H., Zhang, Y.J., Li, J., Zhang, H.H., Xie, X.W., Wang, X., Wang, Z.H.*, et al.* (2012). In vitro inhibition of HBV replication by a novel compound, GLS4, and its efficacy against adefovir-dipivoxil-resistant HBV mutations. Antivir Ther *17*, 793-803.
Waris, G., and Siddiqui, A. (2002). Interaction between STAT-3 and HNF-3 leads to the activation of liver-specific hepatitis B virus enhancer 1 function. J Virol *76*, 2721-2729.
Watanabe, T., Sorensen, E.M., Naito, A., Schott, M., Kim, S., and Ahlquist, P. (2007). Involvement of host cellular multivesicular body functions in hepatitis B virus budding. Proc Natl Acad Sci U S A *104*, 10205-10210.
Watashi, K. (2015). [HBV entry and its application to the drug development]. Nihon Rinsho *73 Suppl 9*, 372-376.
Watts, N.R., Conway, J.F., Cheng, N., Stahl, S.J., Steven, A.C., and Wingfield, P.T. (2011). Role of the propeptide in controlling conformation and assembly state of hepatitis B virus e-antigen. J Mol Biol *409*, 202-213.
Weber, N.D., Stone, D., Sedlak, R.H., De Silva Feelixge, H.S., Roychoudhury, P., Schiffer, J.T., Aubert, M., and Jerome, K.R. (2014). AAV-mediated delivery of zinc finger nucleases targeting hepatitis B virus inhibits active replication. PLoS One *9*, e97579.
Wei, X., Xiang, T., Ren, G., Tan, C., Liu, R., Xu, X., and Wu, Z. (2013). miR-101 is down-regulated by the hepatitis B virus x protein and induces aberrant DNA methylation by targeting DNA methyltransferase 3A. Cell Signal *25*, 439-446.
Werle-Lapostolle, B., Bowden, S., Locarnini, S., Wursthorn, K., Petersen, J., Lau, G., Trepo, C., Marcellin, P., Goodman, Z., Delaney, W.E.t.*, et al.* (2004). Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. Gastroenterology *126*, 1750-1758.
Williams, I.T., Perz, J.F., and Bell, B.P. (2004). Viral hepatitis transmission in ambulatory health care settings. Clin Infect Dis *38*, 1592-1598.
Wong, D.K., Yuen, M.F., Ngai, V.W., Fung, J., and Lai, C.L. (2006). One-year entecavir or lamivudine therapy results in reduction of hepatitis B virus intrahepatic covalently closed circular DNA levels. Antivir Ther *11*, 909-916.
Wooddell, C.I., Rozema, D.B., Hossbach, M., John, M., Hamilton, H.L., Chu, Q., Hegge, J.O., Klein, J.J., Wakefield, D.H., Oropeza, C.E.*, et al.* (2013). Hepatocyte-targeted RNAi therapeutics for the treatment of chronic hepatitis B virus infection. Mol Ther *21*, 973-985.

Wright, T.L., Mamish, D., Combs, C., Kim, M., Donegan, E., Ferrell, L., Lake, J., Roberts, J., and Ascher, N.L. (1992). Hepatitis B virus and apparent fulminant non-A, non-B hepatitis. Lancet *339*, 952-955.
Wu, G., Liu, B., Zhang, Y., Li, J., Arzumanyan, A., Clayton, M.M., Schinazi, R.F., Wang, Z., Goldmann, S., Ren, Q.*, et al.* (2013). Preclinical characterization of GLS4, an inhibitor of hepatitis B virus core particle assembly. Antimicrob Agents Chemother *57*, 5344-5354.
Wu, J., Lu, M., Meng, Z., Trippler, M., Broering, R., Szczeponek, A., Krux, F., Dittmer, U., Roggendorf, M., Gerken, G.*, et al.* (2007). Toll-like receptor-mediated control of HBV replication by nonparenchymal liver cells in mice. Hepatology *46*, 1769-1778.
Wu, J., Meng, Z., Jiang, M., Zhang, E., Trippler, M., Broering, R., Bucchi, A., Krux, F., Dittmer, U., Yang, D.*, et al.* (2010). Toll-like receptor-induced innate immune responses in non-parenchymal liver cells are cell type-specific. Immunology *129*, 363-374.
Wu, T.T., Coates, L., Aldrich, C.E., Summers, J., and Mason, W.S. (1990). In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. Virology *175*, 255-261.
Wursthorn, K., Lutgehetmann, M., Dandri, M., Volz, T., Buggisch, P., Zollner, B., Longerich, T., Schirmacher, P., Metzler, F., Zankel, M.*, et al.* (2006). Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. Hepatology *44*, 675-684.
Xia, Y., Stadler, D., Lucifora, J., Reisinger, F., Webb, D., Hosel, M., Michler, T., Wisskirchen, K., Cheng, X., Zhang, K.*, et al.* (2016). Interferon-gamma and Tumor Necrosis Factor-alpha Produced by T Cells Reduce the HBV Persistence Form, cccDNA, Without Cytolysis. Gastroenterology *150*, 194-205.
Xinfeng, H., Fengfeng, M., Zhiyi, J., Xiaohui, L., Wenhui L. (2015). Inactivation of NTCP causes gallbladder disease in mice. *2015 International Meeting on Molecular Biology of Hepatitis B Viruses, Bad Nauheim, Germany, October 4-8, 2015, Abstract O-177 (personal communication).*
Xu, Y.B., Yang, L., Wang, G.F., Tong, X.K., Wang, Y.J., Yu, Y., Jing, J.F., Feng, C.L., He, P.L., Lu, W.*, et al.* (2014). Benzimidazole derivative, BM601, a novel inhibitor of hepatitis B virus and HBsAg secretion. Antiviral Res *107*, 6-15.
Xu, Z.Y., Liu, C.B., Francis, D.P., Purcell, R.H., Gun, Z.L., Duan, S.C., Chen, R.J., Margolis, H.S., Huang, C.H., and Maynard, J.E. (1985). Prevention of perinatal acquisition of hepatitis B virus carriage using vaccine: preliminary report of a randomized, double-blind placebo-controlled and comparative trial. Pediatrics *76*, 713-718.
Yan, H., Zhong, G., Xu, G., He, W., Jing, Z., Gao, Z., Huang, Y., Qi, Y., Peng, B., Wang, H.*, et al.* (2012). Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. Elife *1*, e00049.
Yang, B., and Bouchard, M.J. (2012). The hepatitis B virus X protein elevates cytosolic calcium signals by modulating mitochondrial calcium uptake. J Virol *86*, 313-327.
Yang, H.I., Yeh, S.H., Chen, P.J., Iloeje, U.H., Jen, C.L., Su, J., Wang, L.Y., Lu, S.N., You, S.L., Chen, D.S.*, et al.* (2008). Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. J Natl Cancer Inst *100*, 1134-1143.

Yang, W., and Summers, J. (1995). Illegitimate replication of linear hepadnavirus DNA through nonhomologous recombination. J Virol *69*, 4029-4036.
Yim, H.J., and Lok, A.S. (2006). Natural history of chronic hepatitis B virus infection: what we knew in 1981 and what we know in 2005. Hepatology *43*, S173-181.
Yu, W., Goddard, C., Clearfield, E., Mills, C., Xiao, T., Guo, H., Morrey, J.D., Motter, N.E., Zhao, K., Block, T.M.*, et al.* (2011). Design, synthesis, and biological evaluation of triazolo-pyrimidine derivatives as novel inhibitors of hepatitis B virus surface antigen (HBsAg) secretion. J Med Chem *54*, 5660-5670.
Yu, X., and Mertz, J.E. (1996). Promoters for synthesis of the pre-C and pregenomic mRNAs of human hepatitis B virus are genetically distinct and differentially regulated. J Virol *70*, 8719-8726.
Yu, X., and Mertz, J.E. (1997). Differential regulation of the pre-C and pregenomic promoters of human hepatitis B virus by members of the nuclear receptor superfamily. J Virol *71*, 9366-9374.
Yu, X., and Mertz, J.E. (2003). Distinct modes of regulation of transcription of hepatitis B virus by the nuclear receptors HNF4alpha and COUP-TF1. J Virol *77*, 2489-2499.
Yuen, M.F., Yuan, H.J., Hui, C.K., Wong, D.K., Wong, W.M., Chan, A.O., Wong, B.C., and Lai, C.L. (2003). A large population study of spontaneous HBeAg seroconversion and acute exacerbation of chronic hepatitis B infection: implications for antiviral therapy. Gut *52*, 416-419.
Yuh, C.H., and Ting, L.P. (1990). The genome of hepatitis B virus contains a second enhancer: cooperation of two elements within this enhancer is required for its function. J Virol *64*, 4281-4287.
Zhang, Z., Protzer, U., Hu, Z., Jacob, J., and Liang, T.J. (2004). Inhibition of cellular proteasome activities enhances hepadnavirus replication in an HBX-dependent manner. J Virol *78*, 4566-4572.
Zheng, Y.W., Riegler, J., Wu, J., and Yen, T.S. (1994). Novel short transcripts of hepatitis B virus X gene derived from intragenic promoter. J Biol Chem *269*, 22593-22598.
Zhou, D.X., and Yen, T.S. (1991). The hepatitis B virus S promoter comprises A CCAAT motif and two initiation regions. J Biol Chem *266*, 23416-23421.
Zhou, X., Li, L., Deng, P., Chen, X., and Zhong, D. (2013). Characterization of metabolites of GLS4 in humans using ultrahigh-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. Rapid Commun Mass Spectrom *27*, 2483-2492.
Zhu, X., Zhao, G., Zhou, X., Xu, X., Xia, G., Zheng, Z., Wang, L., Yang, X., and Li, S. (2010). 2,4-Diaryl-4,6,7,8-tetrahydroquinazolin-5(1H)-one derivatives as anti-HBV agents targeting at capsid assembly. Bioorg Med Chem Lett *20*, 299-301.
Zlotnick, A., Johnson, J.M., Wingfield, P.W., Stahl, S.J., and Endres, D. (1999). A theoretical model successfully identifies features of hepatitis B virus capsid assembly. Biochemistry *38*, 14644-14652.
Zlotnick, A., Venkatakrishnan, B., Tan, Z., Lewellyn, E., Turner, W., and Francis, S. (2015). Core protein: A pleiotropic keystone in the HBV lifecycle. Antiviral Res *121*, 82-93.

Zucman-Rossi, J., and Laurent-Puig, P. (2007). Genetic diversity of hepatocellular carcinomas and its potential impact on targeted therapies. Pharmacogenomics *8*, 997-1003.