

Université catholique de Louvain
IREC - Institut de Recherche Expérimentale et Clinique
Laboratory of Pediatric Hepatology and Cell Therapy



***Differentiated umbilical cord matrix stem cells
as a new in vitro model to study early events
during HBV infection***

Massimiliano Paganelli, MD

November 2012

Supervisors:

Prof. Etienne Sokal, MD, PhD

Dr. Mustapha Najimi, PhD

Dissertation submitted in fulfillment of the requirements
for the degree of Doctor of Medical Sciences (Ph.D.)
Specialization: Pediatric Hepatology

Université catholique de Louvain

Ph.D. in Medical Sciences

Examining committee

President: Prof. Isabelle Leclercq

Supervisor: Prof. Etienne Sokal

Co-supervisor: Dr. Mustapha Najimi

Committee members from the UCL:

- Prof. Patrick Goubau
- Prof. Peter Starkel
- Prof. Bertrand Tombal

External committee members:

- Prof. Johan Neyts (Katholieke Universiteit Leuven, Rega Institute, Leuven)
- Prof. David Durantel (INSERM U1052, Lyon, France)

to Claudia and Giulia

ACKNOWLEDGEMENTS

First and foremost I want to thank my Ph.D. supervisor, Prof. Etienne Sokal. It has been an honor to work with him both in the lab and in the clinical setting. He introduced me to basic research, and taught me the importance, for a modern clinician, to reason in terms of system biology. It is difficult to overstate my gratitude for his contribution of ideas, inspiration and funding, which made my Ph.D. experience productive and stimulating. With his enthusiasm, he drove me through the joys and sorrows of being a clinical scientist, teaching me, both consciously and unconsciously, how to conciliate scientific curiosity with a full commitment to the care of sick children.

It is a real pleasure to thank my Ph.D. co-supervisor, Dr. Mustapha Najimi, without whose daily advices this work would have not been possible. I truly appreciate the time he spent in teaching to a poor clinician what a research lab is: he guided me through this Ph.D. experience, from the first culture medium to data analysis and manuscript preparation. From him I learnt that asking the right question is sometimes harder than finding the relative answer. He provided encouragement, sound advice, and good company. With his passion for teaching, he is a living example that respect can be gained without renouncing to cheerfulness.

I am grateful to Prof. Patrick Goubau, who welcomed me in his lab and let me use his BSL-2 facility. He helped me along my entire project, providing suggestions and a precious “virological point of view”. I also thank all the people from his lab, who privileged me with their assistance and a friendly work environment. A special thank to Nordine Bakouche for our pleasant and stimulating conversations on the most disparate aspects of science, history and politics.

I am obliged towards Prof. Johan Neyts, without whose valuable contribution this work would have not been possible. He kindly supported me providing HepAD38 cells and welcoming me in his stimulating lab at Rega Institute. I was motivated and inspired by the extraordinary level of the research projects conducted by him and his group.

Thanks to the members of my advisory committee, Prof. Peter Starkel and Prof. Bertrand Tombal, who accompanied and guided me through this Ph.D. experience.

Their incisive questions and insightful remarks truly helped me improving my experimental work. A special thanks to the president, Prof. Isabelle Leclercq, for her commitment in assisting Ph.D. students resolve all kind of scientific and practical issues.

I wish to thank Prof. David Durantel for accepting to critically evaluate this work and participating as an external member to the advisory committee. His renown experience in the field will definitely help me improving this dissertation and will stimulate my future research.

I am grateful to my first mentors, Prof. Salvatore Cucchiara, of the Sapienza University of Rome, who introduced me into the world of pediatric gastroenterology, sparked in me the interest for research and funded my first year here at the UCL, and Prof. Raffaele Iorio, of the University of Naples “Federico II”, whose unique teachings and treasured friendship have always and will always guide my steps along the exciting journey.

I wish to thank Dr. Kai Dellmaier, from Rega Institute, who initiated me to the beauty of Southern blotting. His outstanding knowledge of hepatitis B virus, his passion for molecular biology and his love for science in general will always be of inspiration for me. He thought me that, for a scientist, knowledge and experience are much more powerful instruments than technology.

I would like to acknowledge all the junior and senior post-docs of my lab, and in particular Dr. Omar Nyabi, who significantly contributed to this work with his challenging questions and ingenious ideas, Dr. Catherine Lombard, who never got tired of explaining me (without much success) the mysteries of flow cytometry, Dr. Philippe Lysy, an example of scientific rigor and critical thinking, and Dr. Gisèle Deblandre, for her enthusiastic engagement in ameliorating the quality of everyone's research.

Big thanks to the clinical scientists Prof. Françoise Smets and Dr. Xavier Stephenne, with whom I was honored to work both in the lab and in the department of pediatric gastroenterology. From them I learnt hepatocyte isolation, scientific accuracy and plenty of tips and tricks on the management of children with gastrointestinal and liver diseases.

I wish to thank my colleagues Dr. Silvia Berardis, Dr. Dung Khuu, Cédric Maerckx, Dr. Gwenaelle Sana and Dr. Isabelle Scheers, who shared with me the daily difficulties of pursuing a Ph.D. project.

Thanks to Floriane André, Nawal Jazouli, Joachim Ravau and Jonathan Evraerts

and all the other people who work or worked in PEDI lab during this years (Julie, Julien, Ezhilarasan, Valérie, Astrid, Tatiana, Cédric, Charlotte, Sara, Mayuri): they have contributed immensely to my personal and professional time at the UCL.

I want to thank Camillo Sargiacomo, for his courage and enthusiasm in accepting to pursue this work. I expect great things from our collaboration in the future.

I would have been lost without the administrative support of Mrs. Ilse Van Elewijck, whose competence, kindness and humor have accompanied me along my time at the UCL. I truly thank her for the kind words and smiles that helped me getting through the hard days.

Thanks to all the people who in some way helped me along this Ph.D. experience: Dr. David Campard, without whose outstanding study on umbilical cord matrix stem cells this work would not have been possible; Dr. Benoit Kabamba, for his help on genotyping the virus; Dr. Patrick Van der Smissen, who initiated me to confocal laser microscopy; Dr. Sandrine Marie, for galactose quantification; Dr. Monique Bodéus for her help in viral antigens quantification; the research nurses Anne-Catherine Dubois, Danielle Arnold, Véronique Legros and Annick Burgois, for their kind help in collecting blood samples; Benjamin Verpeut and Dora Ourives Sereno, for their friendly administrative assistance.

I would like to thank my family and friends for all their love and support: my parents, who always sustained me in all my pursuits; my dearest friends Dr. Fabio Pierannunzi, for his precious encouragements, and Dr. Fabio Petrocca, for transmitting me his passion for research; and most of all for my loving and patient wife Claudia, whose help, both personal and scientific, has been invaluable.

Last, but not least, thanks to Giulia, my 4-year-old girl, who consciously renounced to some of our precious time together to let me finish this manuscript. She has been my strongest motive in getting through this project.

Grazie!

Massimiliano Paganelli
Université catholique de Louvain
November 2012

TABLE OF CONTENTS

Acknowledgements.....	7
Abbreviations.....	15
 Part 1. Background.....	 17
1.1. Hepatitis B infection: clinical aspects.....	19
1.1.1. Epidemiology.....	20
1.1.2. Transmission.....	22
1.1.3. Natural history.....	24
1.1.4. Vaccination and prevention.....	28
1.1.5. Who and when to treat.....	31
1.1.6. Treatment options.....	34
1.1.7. Proposition of a treatment scheme.....	41
1.1.8. Treatment failure and antiviral resistance.....	42
1.1.9. Coinfection with HIV, HCV or HDV.....	43
1.1.10. Conclusions.....	44
1.1.11. References.....	44
 1.2. Hepatitis B virus biology.....	 63
1.2.1. Hepadnaviridae.....	63
1.2.2. Structure of the HBV virion	64
1.2.3. HBe and HBx proteins	67
1.2.4. Viral entry into susceptible cells	68
1.2.5. Viral replication.....	72
1.2.6. References.....	77
 1.3. <i>In vitro</i> models of HBV infection.....	 83
1.3.1. Primary human hepatocytes.....	83
1.3.2. Primary hepatocytes of <i>T. belangeri</i>	85
1.3.3. Transfected and transduced hepatoma cell lines.....	86
1.3.4. HepaRG cells.....	87
1.3.5. References.....	89
 Part 2. Experimental work.....	 95

2.1. Aims	97
2.2. Materials and methods	99
2.2.1. Cell cultures.....	99
2.2.2. UCMSCs characterization and differentiation	100
2.2.3. Viral sources	102
2.2.4. Quantification of viral DNA and RNA	103
2.2.5. Assessment of viral proteins	107
2.2.6. Infection assays	109
2.2.7. Expression of asialoglycoprotein receptor	112
2.2.8. Statistical analysis	112
2.2.9. References	113
2.3. Development of a standardized strategy for <i>in vitro</i> infection studies	115
2.3.1. Introduction.....	115
2.3.2. Results.....	116
• Characterization of the viral source: HepAD38 culture for HBV production.....	116
• Assessment of biomolecular assays for evaluating infection efficiency.....	116
• Validation of infection and detection strategies on primary human hepatocytes.....	122
2.3.3. Discussion.....	124
2.3.4. References.....	128
2.4. Differentiated umbilical cord matrix stem cells as a new <i>in vitro</i> model to study early events during HBV infection	131
2.4.1. Introduction.....	131
2.4.2. Results.....	132
• Mesenchymal Stromal Cell identity of UCMSCs.....	132
• Hepatic differentiation of UCMSCs.....	133
• HBV binding to UCMSCs membranes.....	135
• Uptake of membrane-bound HBV.....	137
• Cell susceptibility to HBV replication.....	138
• Transcription of viral genes.....	143
• <i>De novo</i> synthesis of viral proteins.....	144
• Secretion of infectious progeny.....	146
2.4.3. Discussion.....	147
2.4.4. References.....	150

2.5. Asialoglycoprotein receptor mediates HBV entry into susceptible cells.....	153
2.5.1. Introduction.....	153
2.5.2. Results.....	154
• Asialoglycoprotein receptor is expressed by UCMSCs.....	154
• ASGPR saturation prevents HBV binding and uptake.....	154
• HBV controls ASGPR expression through a positive feedback mechanism.....	156
2.5.3. Discussion.....	158
2.5.4. References.....	159
 Part 3. General discussion and perspectives.....	 161
 Part 4. Annexes.....	 171
1. <i>Chronic hepatitis B infection in children and adolescents.....</i>	173
2. <i>Differentiation to hepatocyte-like cells determines umbilical cord matrix stem cells' susceptibility to hepatitis B virus entry and replication: a new in vitro model for HBV infection studies.....</i>	185

ABBREVIATIONS

ALT	alanine aminotransferase	IFN	interferon
AFP	alpha-fetoprotein	IMDM	Iscove's modified Dulbecco's medium
ASGPR	asialoglycoprotein receptor	ITS	insulin-transferrin-selenium
BM-MSC	bone marrow MSC	IU	international units
cccDNA	covalently closed circular DNA	LHBs	large HBV surface protein
CHB	chronic hepatitis B	LOD	limit of detection
CRD	carbohydrate recognition domain	MHBs	middle HBV surface protein
CYP3A4	cytochrome P450 3A4	MOI	multiplicity of infection
DAPI	4',6-diamidino-2-phenylindole	MSC	mesenchymal stem cells
DHBV	duck hepatitis B virus	OHb	occult hepatitis B
DIG	digoxigenin	OSM	oncostatin-M
DMEM	Dulbecco's modified Eagle's medium	pAM6	plasmid AM6
DMSO	dimethyl sulfoxide	PBS	phosphate buffered saline
DNA	deoxyribonucleic acid	PCR	polymerase chain reaction
dsDNA	double-stranded linear DNA	PEG	polyethylene glycol
D-UCMSC	differentiated UCMSC	PEG-INF	pegylated interferon
EC₅₀	half maximal effective concentration	pgRNA	pregenomic RNA
EDTA	ethylenediaminetetraacetic acid	PHH	primary human hepatocytes
EGF	epithelial growth factor	p.i.	post-infection
ELISA	enzyme-linked immunosorbent assay	PMMA	phosphonylmethoxypropyladenine (tenofovir)
FDA	Food and Drug Administration	PPIA	cyclophilin A
FGF	fibroblast growth factor	PS-DNase	plasmid-safe DNase
GSHV	ground squirrel hepatitis virus	PTH	primary hepatocytes of <i>T. belangeri</i>
HBIG	hepatitis B immunoglobulins	PVDF	polyvinylidene difluoride
HBcAg	HBV core antigen	qPCR	real time quantitative PCR
HBsAg	HBV e antigen	rcDNA	relaxed circular DNA
HBsAg	HBV surface antigen	RNA	ribonucleic acid
HBV	hepatitis B virus	RT-qPCR	reverse transcriptase qPCR
HBx	HBV X protein	SD	standard deviation
HCC	hepatocarcinoma	SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
HCV	hepatitis C virus	SEM	standard error of the mean
HDV	hepatitis D virus	SHBs	HBV small surface protein
HEV	hepatitis E virus	ssDNA	single-stranded DNA
HGF	hepatocyte growth factor	SVP	subviral particles
HIV	human immunodeficiency virus	TBE	tris-borate-EDTA
HNF1	hepatocyte nuclear factor 1	TBS	tris-buffered saline
HNF3	hepatocyte nuclear factor 3	Tet	tetracycline
HNF4α	hepatocyte nuclear factor 4- α	UCMSC	umbilical cord matrix stem cells
HRP	horseradish peroxidase		

UD-UCMSC	undifferentiated UCMSC
ULN	upper limit of normal
vge	viral genome equivalents
VR	virologic response
WHO	World Health Organization
WHV	woodchuck hepatitis virus

PART 1

BACKGROUND

1.1. Hepatitis B infection: clinical aspects

Hepatitis B Virus (HBV) infection is still one of the most important causes of liver disease, with 2 billion people having been infected worldwide and more than 600.000 deaths each year, of whom 94% dying from chronic infection-related cirrhosis and hepatocellular carcinoma (HCC) [1-3]. Incidence of HBV infection declined over the last two decades thanks to the introduction of universal immunization programs and the implementation of blood-donor screening. Nevertheless, a significant number of adults and children are still infected each year, the latter often developing chronic infection and requiring appropriate follow-up. The World Health Organization estimates more than 360 million persons being chronic carriers worldwide (6% of the world population). In spite of a rather benign course of chronic disease during childhood and adolescence, HBV chronic carriers have a lifetime risk of developing HCC up to 25%, and an incidence of cirrhosis of 2-3% per year [4]. Safe and partially effective antiviral therapies are available, but few are licensed for the use in children, and an accurate selection of subject to treat and of right timing for treatment is needed to optimize efficacy and reduce viral resistance.

This introduction treats clinical aspects of HBV infection, from epidemiology to treatment, focusing on pediatric patients. Nevertheless, although hepatitis B in children and adolescents presents some peculiar features, most of the concepts are valid for adult patients as well. Part of this introduction has been published as a review in the *Journal of Hepatology* (Paganelli M, Stephenne X, Sokal EM. *Chronic hepatitis B in children and adolescents*. J Hepatol 2012;57:885-896; see annex 1), and served as a basis for the official guidelines on the management of pediatric chronic hepatitis B of the European Society for Pediatric Gastroenterology,

Hepatology and Nutrition (ESPGHAN), which are in preparation.

1.1.1. Epidemiology

In countries where global immunization programs have been implemented, prevalence of HBV infection is declining. Many countries with high HBsAg seroprevalence ($\geq 8\%$) before vaccination programs (like China and Taiwan) are now considered at intermediate (2-7%) or low prevalence ($< 2\%$) [5-8]. The impact

Country [Ref.]	HBsAg prevalence %		Predominant genotypes	Adopted children per year			
	Total	<16 yr		United States ^a	Italy ^b	France ^c	Canada ^d
China [5]	7.2	1.8	B, C	3401	116	100	230
Russian Federation [207]	1.4-8	-	D	1079	707	301	66
Ethiopia [208]	4.7	-	A	2511	274	352	159
Haiti [209,210]	5	-	A	133	0	992	94
Korea [211,212]	5.9	0.9	C	865	4	6	87
Colombia [213]	5.7	3.1	F	235	592	369	25
Vietnam [214]	10	7.5	B, C	9	251	469	29
Ukraine [215]	1-2	-	D	450	426	59	35
Brazil [216,217]	1	0.8	A, D, F	26	318	13	1
Taiwan [7,41]	1.1*	0.5	B, C	282	3	3	6
India [218]	2-8	-	A, D	241	124	21	36
Philippines [210]	10	-	B, C	216	30	14	96
Poland [207,215]	1-2	-	A	50	193	26	0
Nigeria [219-221]	4-12	12.4	E	189	5	23	6
Kazakhstan [222]	3.8	-	D	181	28	46	45
Bulgaria [207]	2-7	-	D	40	128	8	2
Ghana [223]	11.4	-	A	117	0	2	9
Belarus [224]	4.8	-	D	0	99	0	1
Cambodia [225]	8	2-14	B, C	0	87	2	1
Peru [207]	0.8-5.2	-	F	35	83	1	3
Hungary [207]	<1	-	A, D	5	78	5	0
Others				993	584	692	261
Total				11,058	4130	3504	1192

Table 1.1.1. Estimated HBsAg seroprevalence and HBV genotype in countries of origin of internationally adopted children.

^a Data refer to year 2010 (Bureau of Consular Affairs, US Department of State. US Intercountry Adoption [Internet]. adoption.state.gov 2011;[cited 2012 Jan. 10] Available from: http://adoption.state.gov/about_us/statistics.php). HBsAg seroprevalence in the United States is 0.27% (0.03% in 6-12 years old) [12].

^b Data refer to year 2010 (Commission for intercountry adoptions. Data and perspectives in intercountry adoptions in Italy. Report on files from january 1 to december 31, 2010. Commission for intercountry adoptions; 2011. Available from: <http://commissioneadozioni.it/it/per-una-famiglia-adoattiva/rapporto-statistico.aspx>). HBsAg seroprevalence in Italy is 0.9% [226].

^c Data refer to year 2010 (Agence française de l'adoption. Statistiques de l'adoption internationale 2010. [Internet]. France Diplomatie 2011;[cited 2012 Jan. 10] Available from: <http://www.diplomatie.gouv.fr>). HBsAg seroprevalence in France is 0.65% [227].

^d Data refer to year 2008 (HCCH Hague Conference Statistics. Canada: annual adoption statistics 2005-2009. [Internet]. HCCH 2012;[cited 2012 Jan. 10] Available from: <http://www.hcch.net>). HBsAg seroprevalence in Canada is 0.3-0.6% (0.4% for preadolescents) [228].

* Prevalence obtained from a study on 18-year old freshmen.

of effective implementation of HBV vaccination programs is striking in term of both acute infections and HBV-related mortality. In Taiwan, overall prevalence of HBsAg between 18-year old subjects decreased from 14.3% in 1995 to 1.1% in 2009 [8]. Prevalence of HBsAg and anti-HBc positivity in children younger than 15 years of age decreased from 9.8% and 26%, respectively, before such a program was started, to 0.5% and 1% after 20 years of vaccination [7]. In China, HBsAg prevalence in children aged 1-14 years decreased by 83% since a national vaccination program was started (from 8% in 1992 to 1.36% in 2006) [9]. Acute hepatitis B incidence in 2007 (1.5/100,000) has been the lowest ever registered in the United States, corresponding to a decrease of 82% as compared to 1990 [10]. In children younger than 15 years of age, incidence was even lower (0.1/100,000 in 2004) [11]. In a recent study, 4.6% of the total population of the U.S. has been exposed to HBV (past or chronic infection), with up to a 50-fold variation as age, ethnicity and country of birth vary. Overall, the rate of chronic carriers is 0.27% of the total population aged 6 years or older, and much lower in children and adolescents aged 6 to 12 years (0.03%) and 13 to 17 years (0.02%). Prevalence of both exposure and chronic carrier state is higher between people coming from high or intermediate prevalence countries, between those with lower family income or with high-risk behavior (intravenous drug use, cocaine use, promiscuity) [12]. Although among children born in Western Europe and North America prevalence of HBV infection is low, pediatricians and hepatologists are confronted with an increasing number of children adopted from higher prevalence countries. Between 13% (in the United States) and 80% (France) of children adopted from families in Western countries come from outside their adopting country [13,14]. Many of these children come from areas with high or intermediate HBV prevalence (table 1.1.1). In high endemicity countries, the lifetime risk of infection is more than 60%, whereas it is between 20% and 60% in intermediate endemicity countries [15]. Although in most of these areas prevalence of HBV infection decreased dramatically after the implementation of global immunization programs [6,16,17], 2% to 5% of internationally adopted children, all coming from $\geq 2\%$ prevalence areas, are still infected with HBV [18,19]. CDC and American Academy of Pediatrics recommend screening all children adopted from these countries for HBV (HBsAg, HBsAb, HBcAb), at arrival and after 6 months, in order to provide

vaccination to those without protective antibody levels or diagnose a previously unknown infection and proceed to immunization of household contacts [20-22]. Eight genotypes (A to H) and several subtypes have been recognized for HBV, with distinctive geographic distribution (table 1.1.1). Some genotypes have been correlated with higher incidence of hepatocellular carcinoma and cirrhosis-related complications, and a different response to antiviral drugs [4]. Although in most of Europe and North America genotype A is predominant, many of the children coming from high or intermediate endemicity countries are infected with genotype B, C, D or F (table 1.1.1), are at increased risk of complications and may have a worse response to therapy [23-25].

1.1.2. Transmission

HBV is transmitted by percutaneous and mucous membrane exposure to infectious blood, semen, vaginal secretions and saliva [26]. In low prevalence countries, exposure to HBV occurs mostly in adulthood as a result of intravenous drug use or sexual intercourse, and leads to acute disease followed by clearance of the virus by most of the infected people [12]. The extreme resilience of HBV, allowing its survival for more than a week on dry surfaces, explains the increased risk of horizontal intrafamilial transmission and the need of carriers counseling and household members vaccination [20,27]. Surprisingly, although between 8% and 24% of household contacts of HBV infected subjects (children and adults) have been reported to be HBsAg-positive [28-32], immunization is not always implemented as suggested and vaccination coverage in this high-risk group is still low (15%-25%) even in developed countries [30,33-35]. Nonetheless, after almost 20 years from the introduction of universal immunization programs, most of the infants and children are protected against HBV [36], and chronic carriers should not be isolated in schools or prevented from practicing sports. Transmission occurs rarely in childcare settings [27], but the risk is higher in detention centers, jails, and prisons, where adolescents are less likely to be immunized and have high-risk behaviors [37,38].

In highly endemic areas, infection occurs mainly in infancy and during early childhood, with mother-to-child transmission accounting for more than half of chronic infections. After exposure, the risk of developing chronic HBV infection

is indeed higher for newborns (90%) than for infants and children of less than 5 years (25-30%), or adolescents and adults (<5%) [39,40]. The risk of vertical transmission is higher if the mother is HBeAg-positive: in Taiwan, newborns from HBeAg-positive mothers are at increased risk of HBV infection despite proper immunization (breakthrough infection), compared to babies born to HBeAg-negative mothers (16.8% vs. 1.6%), and are at increased risk for chronicity [41]. The risk of perinatal transmission depends also on maternal viral load, serum HBsAg titer and mode of delivery (10.5% for elective cesarean section vs. 28% for vaginal delivery) [42-44].

HBsAg and HBV DNA can be detected in breast milk of chronic carriers, but no increased risk of transmission to a breastfed infant has been shown, even when the carrier has high viral load, and breastfeeding is currently recommended after proper infant immunization [45-47].

Although, in countries where donor screening and blood testing have been implemented, current risk of acquiring HBV infection after blood transfusion is estimated at 1 in 500,000 per unit exposure [48,49], HBV nosocomial transmission can still be a problem for immunocompromised children, whose vaccination status need to be checked and all preventive measures to be strictly respected [50]. Treatment with lamivudine should be considered for HBsAg-positive children requiring immunosuppressive therapy (transplanted patients, patients undergoing cytotoxic chemotherapy or corticosteroids treatment), in order to prevent reactivation [51,52,246-249].

In spite of immunosuppressive regimens, transplantation of nonhepatic solid organs (such as kidneys, lungs or heart) from HBsAg-negative, anti-HBc-positive donors (i.e. with past HBV infection) is low (0-5%) if the recipient underwent proper immunization [53]. The risk of infection is higher after liver transplantation from anti-HBc-positive donors, with a 10% rate of *de novo* hepatitis in successfully vaccinated recipients (up to 78% in HBV naïve patients) and 69% recurrence rate in HBsAg-positive recipients [54-56]. In Taiwanese children, seropositivity to anti-HBs did not guarantee protection against *de novo* HBV infection, whereas the achievement of high anti-HBs titer (>200 mIU/ml) proved to be protective [57]. Therefore, immunization (with the achievement of an adequate anti-HBs titer) and prophylaxis with lamivudine (or other nucleoside analogs) for indefinite

period and/or hepatitis B immunoglobulins (HBIG) are recommended when transplanting an anti-HBc-positive liver to an HBV naïve recipient [58]. Anyway, because of the long post-transplant life expectancy, HBc-positive liver grafts should be discarded when transplanting pediatric patients.

1.1.3. Natural history

Patients infected with HBV can present with an acute or chronic infection. Acute infection is less frequent in pediatric age, and it can vary from subclinical to fulminant hepatitis. Classic symptoms are present in 30-50% of older children and adolescents with acute hepatitis B, include fever, jaundice, nausea and vomiting, abdominal pain, liver tenderness and fatigue, and last approximately 2-3 months. Less than 10% of infants born to HBeAg-positive mothers develop acute hepatitis, and jaundice may be the only sign [59,60]. Fulminant hepatitis is uncommon in infants and children but it is associated with more than 40% mortality rate without liver transplantation [61,62]. In high prevalence areas, before global vaccination programs, 65% of fulminant hepatitis was secondary to HBV infection [63]. After vaccination programs were implemented, HBV still caused 57% of fulminant hepatic failure in infants, but the mortality rate from fulminant hepatitis was reduced from 5.4 to 1.7 per 100,000 infants [64,65]. Several studies, conducted before and after universal vaccination programs, showed that fulminant hepatitis is more likely to affect infants born to HBsAg-positive, HBeAg-negative mothers [63,65,66].

Chronic infection is defined as a positive HBsAg for 6 months or longer. Chronic hepatitis B (CHB) in childhood is a mild disease, and infected children are mostly asymptomatic, have normal growth and a normal physical examination [67]. Most perinatally infected subjects present a positive HBeAg and high serum levels of HBV DNA, with normal or minimally elevated serum alanine aminotransferases (ALT). A transplacental transfer of maternal HBeAg has been proven both in mouse and in humans, and could elicit HBe/HBcAg-specific Th cell tolerance in utero [43,68-70]. Such an induced tolerance could explain the higher chronicity rate in babies born to HBeAg-positive mothers and the different rate of chronicity between neonatal and adult infection. The synthesis of large amount of HBeAg (which is not required for assembly, infection, or replication of HBV) by the wild

type virus could be necessary to maintain the tolerance [71]. This immunotolerant phase is characterized by high viral replication and little liver damage, although in Italian children, after 7 years of disease, 7-10% of subjects showed severe hepatitis at liver biopsy and 1.7-4.5% had cirrhosis [67,72]. This phase lasts 10-30 years when infection is acquired perinatally, whereas it is of minimal duration in subjects infected later in life.

Viral mutants not capable of secreting HBeAg are then slowly selected (precore mutants are detected in 89% of HBeAg-positive patients) [73]. Selection seems to be secondary to an advantage of such mutants over wild-type HBV, as hepatocytes infected by the latter are more susceptible to cytotoxic T-lymphocyte-mediated clearance (although HBeAg loss was shown to induce an increased T-cell response through the inhibition of programmed death 1 pathway) [74,75]. Precore mutants selection leads to a lower secretion of HBeAg. When the decreased amount of secreted tolerogen is no more sufficient to maintain immunotolerance, the immune system starts attacking infected hepatocytes [70,71]. ALT levels rise, reflecting the histologic finding of necroinflammation of liver parenchyma, and HBV DNA levels fluctuate. This phase of active hepatitis leads to seroconversion to anti-HBe antibodies in 60-95% of patients on long-term follow-up [72,76]. ALT levels significantly increase before HBeAg clearance and may remain elevated for 6 months after seroconversion [67,77-79]. Up to 20% of spontaneous seroconverters may have ALT flare-ups in the years following seroconversion, which seem to be more likely in subjects carrying a precore mutant [78]. Spontaneous seroconversion occurs earlier and more frequently in subjects who acquired HBV horizontally than in those infected perinatally (14-16% vs. 4-5% per year) [67,72,77,80]. Genotype C (60-70% vs. 80-90% for other genotypes) and maternal HBeAg-positive status (74% vs. 49% for HBeAg-negative mothers) are also associated with delayed spontaneous seroconversion [24,76,77,81-83]. Seroconversion occurs usually after puberty, with about 90% of children younger than 15 years of age still HBeAg positive [80]. It was shown that boys with earlier-onset puberty seroconvert at younger age (13 vs. 22 years), suggesting that androgens play a role in this process [79]. Environmental factors, such as nutritional status, have been suggested to influence immune response to HBV, accelerating seroconversion in children after adoption from developing countries [77].

HBsAg-positive, HBeAg-negative and anti-HBe-positive patients are considered inactive carriers, express absent or low viral replication, with low or undetectable HBV DNA, and have inactive liver histology, with normal ALT levels. On long-term follow-up inactive carriers with no signs of cirrhosis at seroconversion did not show progression to cirrhosis over 24-29 years follow-up [67,72].

On the contrary, 1-5% of HBeAg-positive children develops cirrhosis [67,72,84]. Between 0.01% and 0.03% of children with CHB develops HCC during childhood (32 per 100,000 person-year) [24,72,85]. Incidence of HCC in high prevalence areas was significantly reduced (from 0.5 per 100,000 children to 0.2, in Taiwan) after the implementation of global immunization programs [86]. Such an effect of vaccination was more pronounced in boys than in girls [87]. Children developing HCC are more likely to be males (70%), with cirrhosis (80%) and undergoing early seroconversion, suggesting that necroinflammation during seroconversion to anti-HBe may be severe and lead to cirrhosis and HCC [24,72,85]. Long-term risk of both HCC and cirrhosis is directly correlated to viral replication and serum HBV DNA levels [88,89]. The role of viral genotype on the risk of developing HCC is still to be defined: in children, 80% of HCC are in cirrhotic genotype B patients, whereas young adults with HCC are mostly non-cirrhotic [24,81]. In adults, genotypes C, which is associated with a higher viral load, and F are at increased risk for HCC [81,90-92]. Furthermore, the risk of HCC is higher in persons with a family history of HCC, and new a susceptibility locus has been recently described in 1p36.22 [93,94]. Seroconversion to anti-HBe (and the subsequent lower viremia) reduces the overall risk of developing HCC later in life, but an annual incidence of 0.2% has been described in HBeAg-negative adults (1.6% of asymptomatic HBsAg carriers) [95].

A small proportion of anti-HBe-positive subjects show an active viral replication with abnormal ALT levels and a histologically active hepatitis (HBeAg-negative chronic hepatitis). Such a state may be the consequence of a reactivation secondary to immunosuppression, as covalently closed circular DNA (cccDNA) persists in the liver. Low-level persistence of cccDNA has been demonstrated even after HBV clearance, and could account for such a risk of reactivation [241,242].

Indeed, reactivation may lead to HBeAg-negative chronic hepatitis when a precore mutant is selected (70-90% of adult carriers in Italy and France have HBeAg-

negative chronic hepatitis, with a 5-year cumulative incidence of cirrhosis higher than that of HBeAg-positive patients) or to seroreversion to HBeAg-positive status [96-98]. HBeAg-negative hepatitis affects about 10% of pediatric patients and is associated with lower serum HBV DNA levels and a more severe disease progression, more frequently leading to HCC [95]. A stronger immune response with a faster turnover of infected cells in these patients could account for such an increased severity, and could be secondary to the reduced secretion of the tolerogen HBeAg [69,99].

In long-term longitudinal studies, between 7 and 14% of inactive carriers lost HBsAg and became anti-HBs-positive. Spontaneous seroconversion to anti-HBs is a rare event in childhood (approximately 1%/year and 0.6%/year in horizontally and perinatally infected children, respectively) [67,72,77,98]. HBsAg clearance and anti-HBs seroconversion mark resolution of HBV infection, and are accompanied by improved liver histology. Long-term prognosis after HBsAg seroclearance is excellent if it occurs before the development of cirrhosis or HCC and in the absence of concomitant infections [100]. Nevertheless, as mentioned above, cccDNA persists indefinitely in hepatocytes, and low-level viral replication or reactivation upon immunosuppression are still possible [241,246-249].

HBsAg serum levels before treatment are currently being investigated as a possible predictor of long-term clearance of HBsAg in response to therapy with nucleoside analogs [101]. Reflecting the transcriptional activity of cccDNA, HBsAg levels were shown to decrease with age and disease progression, to be higher in immunotolerance phase, to lower after seroconversion to anti-HBe and to reach the lowest levels in inactive carriers [102,103].

Traces of HBV DNA persist for years after seroconversion to anti-HBe or anti-HBs, or after recovery from a self-limited acute infection [256]. Presence of HBV DNA in the liver, with or without HBV DNA in serum, with negative serum HBsAg, are the hallmarks of occult hepatitis B (OHB), which is typical of the early, pre-seroconversion window period of HBV infection, but has been described in healthy blood donors, as well as in patients with chronic liver disease or HCC [257-260]. Clinical significance of OHB in healthy, immunocompetent individuals is still debated, whereas it has an important role in reactivation of HBV infection upon immunosuppressive treatments and in patients infected by HIV or HCV [261-

263]. As stated above, liver grafts from donors who are HBsAg-negative but anti-HBc positive (i.e. OHB carriers) can transmit HBV to susceptible recipients after transplantation [54,56]. The risk of transmission is lower with other solid organs, but not negligible [53,55]. Detection of OHB requires assays with a lower limit of detection (LOD) ≤ 5 IU/ml HBV DNA, as median HBV DNA levels in OHB patients are between 5 and 10 IU/ml [258,264,265]. Only few, last-generation real-time polymerase chain reaction (PCR) assays, which allow a highly sensitive and specific quantification of HBV DNA [266-271], are suitable to detect OHB.

1.1.4. Vaccination and prevention

Vaccination is the most effective measure to prevent hepatitis B transmission. In highly endemic areas, it is also the most cost-effective medical intervention, offering the higher benefit-cost ratio, whereas in low-endemicity countries, such cost-effectiveness is not as clear [104-107].

Recombinant vaccine (made of spherical particles of HBsAg exposing the a determinant) induces a seroprotective response (anti-HBs > 10 mIU/ml) in about 95% of subjects vaccinated with three doses [41,108]. The first dose has to be administered at birth, within 24 hours, followed by at least 2 doses at 1 and 6 month of life [22,108,109]. The 3-dose regimen with the recombinant vaccine has a better long-term immunogenicity at 20-year follow up than a 2-dose strategy [110]. No difference was found comparing efficacy of recombinant and plasma-derived vaccines [111]. Only monovalent hepatitis B vaccine can be used for vaccination at birth, whereas combined vaccines can be used for subsequent doses [108,112]. Revaccination with further 3 doses induces protective anti-HBs response in the majority of non-responders [113-114].

WHO has recommended universal HBV vaccination since 1991 [115]. By the end of 2010, 179 of the 193 WHO member states had implemented a nationwide universal childhood HBV immunization program, global HBV vaccine coverage was estimated at 75% and the first dose of vaccine was administered at birth in 52% of countries [116]. A serum level of 10 mIU/ml of anti-HBs antibodies is considered to be protective [108,117]. After complete vaccination, waning anti-HBs titer has been noted over time [7,118-121]. Among children having received complete vaccination during infancy, 33%-63% had an anti-HBs titer <10 mIU/

ml after 10-20 years [110,122,123]. Nevertheless, long-term longitudinal studies showed vaccination to be protective for up to 20 years [7,110,119,124,125]. At the moment, there is no evidence for recommending administering a booster dose of hepatitis B vaccine in routine immunization programs [108,122,126,127]. Premature infants with low birth weight (<2000g) have decreased seroconversion rates after vaccination at birth, but they show an adequate response by the chronological age of 1 month [128-130]. According to current recommendations, these babies should receive the 3 doses of anti-HBV vaccine starting at 1 month of age. If the mother is HBsAg-positive, they should also receive an additional dose within 12 hours from birth [22,108,131]. As immunocompromised and coeliac patients are less likely to respond to vaccination, serological testing for anti-HBs antibodies and revaccination with a second 3-dose series should be considered [108,132,133].

Vaccine failure and mother-to-child transmission of hepatitis B affects 17% of infants born to HBeAg-positive mothers [41]. Several explanations have been suggested for breakthrough infection. The high viral load related to HBeAg-positivity seems to be the most important factor [41,43]. Infection is more likely to occur in children born to genotype C mothers, probably because immunization raises the threshold of maternal viral load required to have perinatal transmission, and genotype C infection is associated with higher viral loads. Universal vaccination programs are therefore changing HBV genotype distribution [134]. Intrauterine infection before vaccination can be administered and hyporesponsiveness to vaccine could also play a role [43,119]. Furthermore, vaccine escape mutants have been registered in 30% of children who acquired HBsAg-positivity despite proper immunization [41,135]. Nevertheless, overall prevalence of surface antigen mutant did not increase over time after the implementation of universal vaccination [135].

To improve response to vaccination, new vaccines (PreS/S, DNA, MVA) are currently being investigated, both for immunoprophylaxis and treatment of chronic HBsAg carriers (therapeutic vaccination) [136-139].

When the mother is a chronic carrier, vaccination at birth is not sufficient to avoid vertical transmission, and concurrent administration of HBIG (0.5 mL IM) is recommended by the WHO to give immediate passive immunity to the

newborn [27,108]. Administration of both the vaccine and HBIG to newborns of HBeAg-positive mothers within 24 hours from birth allows the achievement of 90% protection rate (98% when mothers are HBeAg-negative), compared to vaccine alone (RR 0.54) [41,111]. HBIG did not prove to increase protection rate in newborns of HBeAg-negative mothers (98% vs. 95% without HBIG), but their administration was shown to give better protection against fulminant hepatitis [41,65]. Overall, a cost benefit analysis showed an economic advantage in providing both vaccination and HBIG to all infants born to HBsAg-positive mothers in the US, regardless of the maternal HBeAg status [41]. Unfortunately, concomitant administration of vaccine and HBIG is not uniformly respected: in the US, in 2006, only 62% of infants born to HBsAg carrier mothers received it within 12 hours from birth and almost 20% did not receive HBIG at all [140].

Further risk reduction can be obtained by treating HBeAg-positive and highly viremic mothers during the last trimester of pregnancy [141]. No antiviral agent has been approved by the FDA for use in pregnancy. Lamivudine is classified pregnancy class C by the FDA, while both tenofovir and telbivudine are class B. Although effects on organogenesis secondary to the interference of the drug on replication of mitochondrial DNA cannot be excluded, data from the Antiretroviral Pregnancy Registry showed no increased incidence of birth defects with the use of lamivudine (3.1% when used during the first trimester and 2.8% during the second or third trimester) or tenofovir (2.2% and 2.1%, respectively) compared to CDC's population-based birth defects surveillance system (2.72% of total prevalence) [142]. Children of lamivudine-treated mothers have 13-23% lower incidence of intrauterine infection and 1-2% lower mother-to-child transmission rate [143,144]. Treatment with telbivudine during the third trimester of pregnancy proved to be effective in reducing maternal viral load and preventing perinatal transmission (0% vs. 8% in controls) [145]. No studies are yet available for tenofovir. Although no studies have been conducted in pregnant teens, the same recommendations for treatment in the third trimester of pregnancy may apply. Pediatricians and pediatric hepatologists should sensitize hepatologists and obstetricians caring for HBsAg-positive pregnant women on the need of lowering serum HBV DNA levels in the third trimester of pregnancy in order to reduce the risk of mother-to-child transmission.

1.1.5. Who and when to treat

Even though several guidelines have been published by major international societies on the management of adult patients with hepatitis B infection, treatment strategies for children are still evolving and mostly based on consensus of expert panels (figure 1.1.1) [52,146-148]. Decision to start a therapy must take into account the mild evolution of the disease during childhood for most of the patients and the potentially severe complications in few, not yet well identified cases, and it is complicated by the limited number of drugs labeled for use in children.

The goal of anti-HBV therapy, in children as in adults, is to reduce the risk of progressive liver disease, cirrhosis and HCC. It can be achieved by suppressing viral replication through durable HBeAg seroconversion and undetectable serum HBV DNA levels. Reduction of viremia levels leads to decreased liver

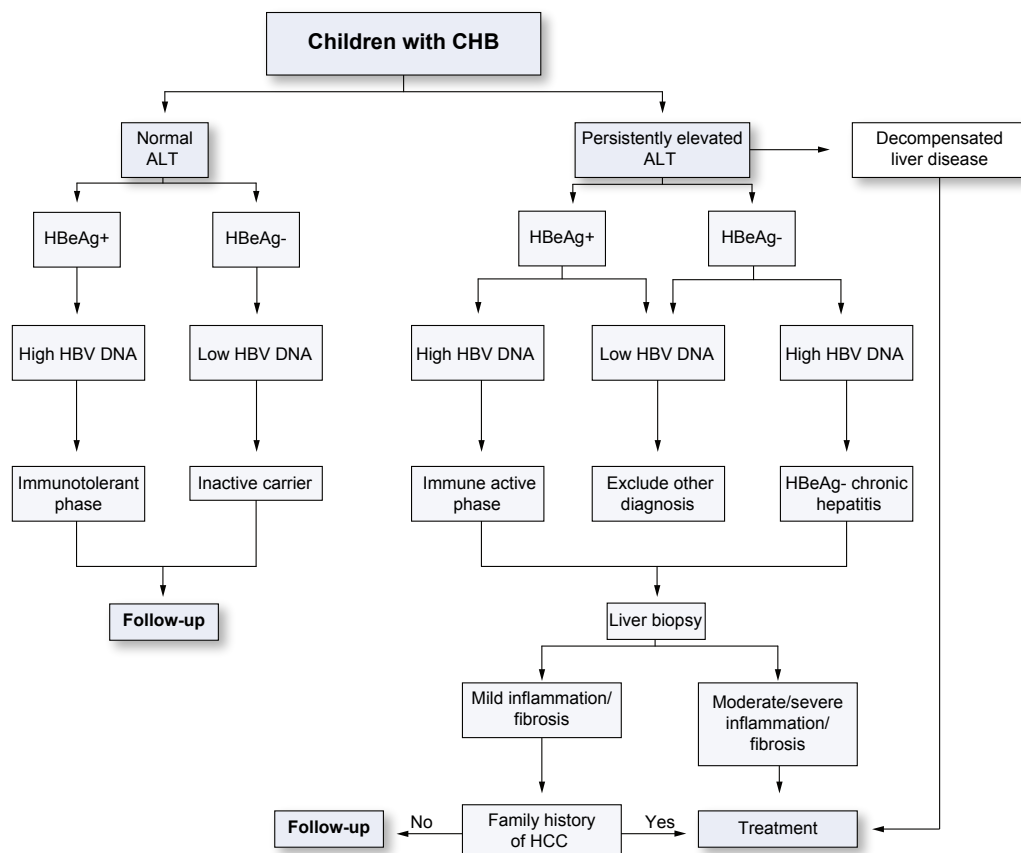


Figure 1.1.1. Who and when to treat: algorithm for treatment of children and adolescents with chronic hepatitis B.

inflammation and subsequent normalization of ALT levels. The final goal is anti-HBs seroconversion, as it stops disease progression and reduces the risk of HCC, although it occurs in a minority of treated subjects [149].

Necessity for treatment should be evaluated at each follow-up visit, in order to treat as soon as early signs of liver damage are detected. Children with CHB should undergo physical examination and measurement of serum ALT, HBeAg/anti-HBe and alpha-fetoprotein (AFP) levels every 6 months. ALT should be monitored every 3 months at diagnosis (for one year) and if persistently elevated, and every 12 months in HBeAg-negative patients with low viral load (<2000 IU/ml). HBV DNA levels should be assessed every 12 months, along with a complete blood count, a full liver panel and a liver ultrasound [52,147,150]. Lifetime follow-up is warranted even for inactive carriers, because of the risk of cirrhosis (7.8% of patients at 25 years), HCC (2.2%) and reactivation of HBV infection, with seroreversion to HBeAg positivity (1-4% of patients) or progression to HBeAg-negative hepatitis (15-24%) [95,151].

Decision to treat should be based on the following parameters: ALT levels, HBeAg positivity, HBV DNA levels, liver histology, family history of cirrhosis and/or HCC, coexisting liver diseases and patient's treatment history. Serum ALT level is the most useful marker of liver damage and should be used to identify patients who could be considered for possible antiviral therapy. Published evidence suggests that patients with normal or mildly elevated ALT respond poorly to available treatments [152-153]. Benefit of treating children with normal ALT has not been established, and the risk of developing antiviral resistance could turn it deleterious when considering the future of these patients [154]. Therefore, children in the immunotolerant phase should not be treated outside of clinical trials, and should be monitored and treated when the increase of ALT levels reveals immune activation. Unfortunately, the upper limit of normal (ULN) for ALT levels has not yet been established for children. In 2009, guidelines of the American Academy for the Study of Liver Disease, as well as a review from world-renowned experts, stated that, for a patient to be considered for antiviral treatment, ALT should be more than 2 times greater than the laboratory ULN [52,147]. A year later, an expert panel's consensus lowered the threshold to more than 1.5 times the laboratory ULN or more than 60 IU/L, whichever is lower [148]. This

value was chosen as it was used as the inclusion criteria for the three largest trials in children [152,153,155]. Nevertheless, a recent study showed that ULN values currently used in children hospitals are too high and underestimate the prevalence of liver disease in children [156]. Although these data need further validation, it is foreseeable that laboratories ULN, clinical trials inclusion and exclusion criteria and ALT levels chosen as indication for antiviral treatment will be lowered to account for biology-based thresholds derived from large cohort studies.

Whatever value will be chosen, for a child to be considered for treatment, serum ALT level has to stay elevated for at least 6 months (12 months if HBeAg-negative), to avoid treating patients who are undergoing spontaneous HBeAg seroconversion. Persistent ALT elevation mandates assessment of serum HBV DNA levels. High HBV DNA values warrant treatment, whereas a low HBV DNA level should lead to the exclusion of other causes of liver disease. The cutoff value for HBV DNA has not been defined, and arbitrary values are used in clinical trials and guidelines. As children have a higher HBV replication rate, a value of 20.000 IU/ml has been chosen by different authors [147,157]. However, lower values have been associated to progressive liver disease in adults and latest management guidelines identified 2.000 IU/ml to be a good cut off [52].

In adult patients younger than 40 years of age, treatment can be started solely on the detection of a high viral load, as it is an independent risk factor for cirrhosis and HCC [88,89]. No data exist in children to support such an approach. Therefore, as response to currently available treatments for children is partial and limited to specific subgroups, histologic assessment of the grade of inflammation and of the stage of fibrosis remains recommended before treating a child. Response to both interferon(IFN)-alfa and nucleos(t)ide analogues (NA) is more likely when at least moderate necroinflammation or moderate fibrosis are found at liver histology [152,158]. Although the benefit of treatment has not been established for children with mild inflammation or fibrosis, a family history of HCC may warrant a treatment even in children with mild histological changes, as they are at increased risk of developing HCC [93].

There are other special populations of children at increased risk for a rapid progression of liver disease (cirrhosis, HBV-related glomerulonephritis, coinfection with HDV, HCV or HIV). These patients could benefit from treatment even if

ALT, HBV DNA levels and liver histology do not match the cutoffs listed above. As previously mentioned, an antiviral treatment should be always considered in recipients of a liver graft from an anti-HBc-positive donor and to prevent (or treat) a recurrent HBV infection in children undergoing liver transplantation. Furthermore, prophylactic treatment should be administered to HBsAg-positive patients who have to receive immunosuppressive or cytotoxic therapy, as it decreases the risk of mortality and morbidity related to HBV reactivation [159]. Several studies conducted in adults showed a better response to interferon for viral genotypes A and B, compared to D and C respectively [25,160-162]. On the contrary, no significant difference was found when NA were used [163]. No studies have investigated the role of genotype on treatment response in pediatric patients, and genotype determination before treatment is not currently recommended [148]. Although confirmation of adult studies in children is needed, a future role of viral genotype in assigning children to treatment and in predicting response is conceivable.

1.1.6. Treatment options

The U.S. Food and Drug Administration approved four medications for treatment of children with CHB: IFN-alfa, lamivudine, adefovir and entecavir. IFN-alfa can be used in children older than 12 months of age, lamivudine starting at 3 years of age, adefovir in children aged 12 years and older and entecavir starting from 16 years of age. Each of these treatments, together with others currently studied or used off-label in reference centers, has advantages and disadvantages (table 1.1.2), and different response and resistance rates (figure 1.1.2).

Interferon-alfa

IFN-alfa-2b has been the first to be approved and it has been used to treat CHB in children for about 15 years. Efficacy in children is similar to that in adults. In the largest multinational randomized controlled trial of IFN-alfa therapy in children, 26% of patients achieved virologic response (VR: undetectable HBV DNA and loss of HBeAg) after 24 weeks of treatment, compared to 11% of untreated controls, and an additional 7% responded during the 24 weeks after cessation of treatment. Response rate rose to 35% when only patients with ALT at least twice

Treatment	Licensing	Dose	Duration	Advantages	Disadvantages
IFN α	≥ 12 mo	5-10 M units/m ² sc 3 x/wk	6 mo	No resistance Usable in young children Short treatment	Heavy side effects Parenteral administration Not usable if decompensated cirrhosis or transplantation
Lamivudine	≥ 3 yr	3 mg/kg po once daily (max 100 mg/d)	≥ 1 yr	Few side effects Usable in young children Oral administration Usable in 3 rd trimester of pregnancy	High resistance rate (increasing with time of treatment)
Adefovir	≥ 12 yr	10 mg po once daily	≥ 1 yr (+ 6 mo after HBeAg seroconversion)	Partially effective in lamivudine resistant patients Oral administration	Not approved for children <12 yr Emergence of resistant mutations
Entecavir	≥ 16 yr + Phase III (2-17 yr)	0.5 mg/d once daily (1 mg/d for lamivudine-resistant pts)	≥ 1 yr (+ 6 mo after HBeAg seroconversion)	Partially effective in lamivudine resistant patients Oral administration	Resistance rate on the long term Not approved for children <16 yr No available preparation for young children
PegIFN	Phase III (2-18 yr)	180 μ g/wk	6 mo	No resistance Once weekly administration Short treatment	Heavy side effects Parenteral administration Not usable if decompensated cirrhosis or transplantation
Telbivudine	Phase I (2-18 yr)	600 mg/d once daily	≥ 1 yr	Few side effects Oral administration Usable in 3 rd trimester of pregnancy	High resistance rate
Tenofovir	≥ 12 yr	300 mg/d once daily	≥ 1 yr	High response rate No resistance identified Few side effects Oral administration Usable in 3 rd trimester of pregnancy	No available preparation for young children Reduced mineral density in children

Table 1.1.2. Available treatments for chronic hepatitis B in pediatric age.

the ULN were considered. ALT level decreased in all responders, and they were normal in 44% at the end of treatment. Histological activity index improved in responders and loss of HBsAg occurred in 10% of treated children, as compared to 1.2% in the control group. The likeliness of a response was associated with low HBV DNA levels before treatment, younger age and female sex [152]. A better

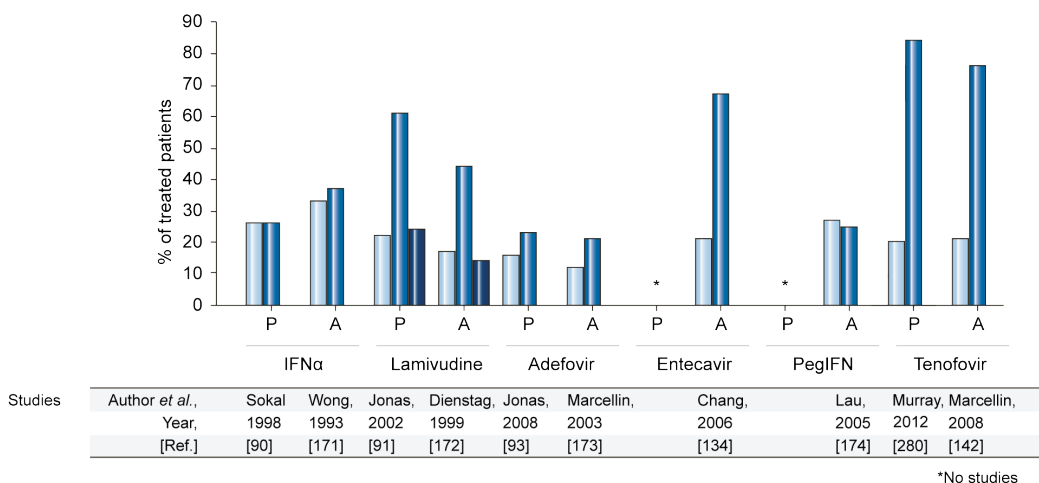


Figure 1.1.2. Comparison between available treatments in term of HBeAg seroconversion (light blue bars), undetectable HBV DNA (blue bars) and resistant mutations emergence (dark blue bars). Pediatric clinical trials (P) are compared to studies on adult patients (A).

response in young patients was subsequently confirmed in children younger than 5 years of age [164].

INF-alfa is thought to simply accelerate seroconversion, as many patients who do not respond to treatment may still seroconvert to anti-HBe later in life. In three different series of 108, 107 and 174 children with CHB, INF-alfa treatment was shown to accelerate HBeAg seroconversion, but treated and untreated patients had a similar rate of HBeAg clearance at long-term follow-up (figure 1.1.3) [67,77,165]. These data were confirmed in Asian children in a recent study [166]. Furthermore, children of Asian origin seroconvert slower than those of other

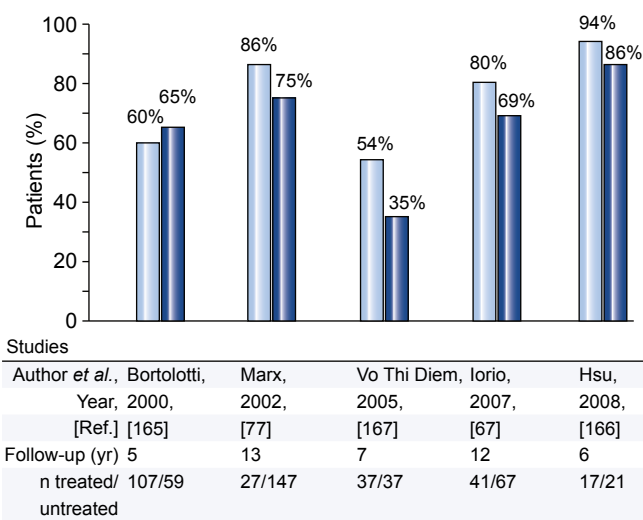


Figure 1.1.3. HBeAg seroconversion rates in children treated with INF-alfa (white bars) as compared to untreated subjects (gray bars). Differences between the two groups in each study are not statistically significant.

ethnic origin, but they reach the same proportion of seroconversion in the long term [77]. Otherwise, in a series of 74 children with CHB followed-up for 7 years, treated patients with elevated baseline ALT levels showed a higher long-term seroconversion rate, whereas elevated ALT at baseline did not significantly influenced the long-term natural seroconversion rate in untreated children [167].

HBsAg clearance was observed in 4-15% of children treated with INF (15-25% of responders), compared to 0-10% of controls [67,77,152,165-167]. The difference in HBsAg seroconversion rate between treated and untreated patients was shown to be significant by the randomized controlled trial, whereas none of long-term studies was able to find such a difference to be statistically significant. Nevertheless, children who responded promptly to INF were more likely to lose HBsAg than late or no-responders [165].

Overall, although pediatric studies failed to show an impact of treatment over the

time, large case-control studies and meta-analysis in adults showed that INF-alfa reduces the risk of cirrhosis (RR 0.65) and HCC (RR 0.59-0.66) in the long term [233-235].

European consensus recommendations for INF-alfa treatment suggest to treat HBeAg-positive children aged 2 years or older, with abnormal ALT and low-intermediate HBV DNA levels. The recommended regimen is 5 to 10 million units per square meter, three times weekly for 6 months [147,170]. The benefit of priming with corticosteroids has not been proven [171,172]. INF-alfa is contraindicated in children with decompensated cirrhosis, cytopenia, autoimmune disorders, cardiac or renal failure and in transplanted patients [170]. As VR may be achieved in the 6 months following the end of treatment, at least 6-12 months should pass before another therapy is started.

Side effects of INF-alfa treatment include fever and flu-like symptoms, behavioral disorders, gastrointestinal disorders and neutropenia [152]. Furthermore, INF-alfa was shown to temporarily affect growth [173]. All side effects resolve after treatment is stopped.

Pegylated interferon-alfa (PEG-INF) has not yet been approved for the treatment of CHB in children. Polyethylene glycol addition to INF-alfa enhances its half-life, allowing a once-weekly administration. Such a formulation is licensed for the use in children with chronic hepatitis C. In adults with CHB, PEG-INF proved to be superior to INF-alfa both in term of efficacy and compliance, with the same safety profile [174]. Clinical trials of PEG-INF in children with CHB are ongoing. Preliminary reports in children with HCV infection showed that it is well tolerated, while leading to better response rates than INF-alfa [175,176].

Lamivudine

Lamivudine is a pyrimidine NA approved for children aged 3 years and older. In the largest pediatric randomized, controlled study, VR was achieved by 23% of patients receiving lamivudine after 1-year treatment (as compared to 13% in the control group). Response increased to 31% when considering only children with ALT levels of at least twice the ULN [153]. An open label extension study was then carried out on the same cohort, reaching a total of 2 or 3 years of treatment. At the end of the extension period, response rate was 56% for children receiving

lamivudine in the absence of resistant mutations (being 5% in patients with a YMDD mutant HBV). Resistance rate increased over time (24% after 1 year of treatment, 49% at 2 years and 64% at 3 years) [177]. Likelihood of response was greater in patients with higher ALT levels and histologic activity index at baseline. In children, no data is yet available on long-term impact of treatment with lamivudine (or any other NA) on the natural history of the disease.

Lamivudine is well tolerated with no significant side effects. The recommended treatment dose is 3 mg/kg/day (maximum 100 mg/day), administered orally once daily. Such a dose provides levels of exposure and trough concentrations similar to the 100 mg dose in adults [178]. Optimal treatment duration is more difficult to determine. Published data suggest continuing treatment until VR is achieved, and possibly for 6 months after seroconversion [177]. As longer treatment duration leads to higher resistance rates, it is recommended to discontinue lamivudine after 6 months if a complete suppression of viral replication is not achieved or if YMDD mutations emerge. As post-treatment ALT flares are possible, children should be carefully followed and an alternative therapy should be started in the rare cases with severe and protracted ALT elevation [179]. Persisting cccDNA is thought to be responsible of viral relapse after withdrawal of antiviral therapy [242-244].

Combination of lamivudine with INF-alfa (either concurrent or sequential administration) proved to be more effective than the single drugs alone in adult patients with elevated ALT levels [180,181]. The two drugs were shown to have a synergistic effect on cells in vitro [182]. In children fewer data are available. Three studies investigated combined therapy in 32, 33 and 45 treatment-naïve children with elevated ALT levels. Although no difference was found between different combination strategies, the children reached 30-60% seroconversion to anti-HBe and 9-17% to anti-HBs [183-185]. An 8-week treatment with lamivudine followed by 10-month combination therapy with lamivudine and INF has been tested on 23 immunotolerant children: complete suppression of viral replication was achieved in 78% of subjects and 22% of children seroconverted to anti-HBe. The concurrent administration of INF-alfa appeared to be protective against mutations emergence [186]. No difference was seen on histological response between monotherapy and combine therapy [187]. As no large clinical trials have been conducted so far, advantages of combination therapy over monotherapy in children are still to be

confirmed.

Adefovir

Adefovir dipivoxil is a purine analogue approved to treat children with CHB aged 12 years and older. In a large pediatric randomized controlled trial, 23% of patients aged 12-17 years achieved VR after 48-week treatment with adefovir (compared to 0% of placebo-treated subjects). The efficacy on HBV DNA suppression and ALT normalization was less evident in younger children (15% vs. 3%), with differences between groups not being statistically significant [155]. Adefovir was well tolerated by children of all age groups, with no resistance-associated mutation registered. However, adefovir resistant mutations are reported in more than 20% of HBeAg-positive adults after 5 years of treatment [188]. In adults, a dose-dependent proximal renal tubular toxicity, with occasional Fanconi-like renal tubular acidosis, is a known side effect of adefovir, which has been rarely reported in adults with the 10 mg daily dose, but never in children. The nephrotoxicity is usually reversible if therapy is stopped rapidly [189,190].

Currently available antiviral drugs cannot prevent the initial formation of cccDNA and are not able to directly affect preformed cccDNA [254]. Nevertheless, cccDNA levels have been shown to decrease upon effective treatment with adefovir [241]. Such an indirect action of NA on cccDNA is believed to be secondary to a strong suppression of HBV DNA synthesis, which depletes the pool of mature cytoplasmic nucleocapsids available for the cccDNA amplification pathway.

The recommended dose for adefovir in 12-18 years old children is 10 mg orally once daily [191]. Treatment duration has not been established, but experts agree on continuing for at least 6 months after HBeAg seroconversion. Adefovir should be discontinued if a complete VR is not achieved after 24 weeks or if a resistant mutation emerge. As with other NA, patients should be monitored after discontinuation because post-treatment flares are not uncommon.

Entecavir

Entecavir is a carbocyclic analogue of 2'-deoxyguanosine that proved to be more potent than both lamivudine and adefovir in adult patients [192,193]. Furthermore, entecavir resistance is rare, even after 5 years of treatment, and entecavir is active

(although less effective) on lamivudine-resistant strains [194-196]. As with other NA, a close follow up should be insured at discontinuation because of the risk of flare ups [245]. Safety profile of entecavir is similar to that of lamivudine. Based on adult studies, entecavir has been approved by the FDA for treatment of adolescents aged 16 years or older. The recommended dose is 0.5 mg once daily for nucleoside-naïve patients and 1 mg/day for lamivudine-resistant patients. A phase III clinical trial in children as young as 2 years old is underway [197].

Tenofovir

Tenofovir disoproxil fumarate is a NA originally licensed for treatment of HIV infection which has recently been approved for adolescents with CHB (≥ 12 years old). Tenofovir is structurally similar to adefovir and of equal antiviral activity. Nevertheless, as it proved to be less nephrotoxic than adefovir, the approved dose for adults is higher than that of adefovir (300 mg/day). For such a reason, tenofovir was shown to have a greater antiviral activity than adefovir in clinical trials (undetectable HBV DNA in 76% of patients vs. 13% of adefovir-receiving subjects after 48 weeks of treatment) [200]. After three years of treatment, 72% of HBeAg-positive and 87% of HBeAg-negative patients have HBV DNA levels <400 copies/ml [201]. In the only available pediatric double-blind placebo controlled trial, after 72 weeks of treatment with tenofovir, 84.6% of 52 adolescents 12 to <18 years of age achieved HBV DNA levels <169 copies/ml, compared to 0% of controls [280]. Response reached 96% in those having ALT levels ≥ 1.5 times the ULN. HBeAg seroconversion was registered in 20.8% of originally HBeAg-positive patients (31% of patients with elevated ALT levels and 9% of those with low levels ALT). Overall, VR (plus normal ALT levels) was achieved by 14.9% of patients and HBsAg-loss by 1.9% of treated subjects. Safety profile of tenofovir is similar to that of adefovir, although associated with decreased bone mineral density in children [201,202]. In the pediatric trial, no patient experienced $\geq 6\%$ decrease of spine mineral density [280]. No genotypic resistance to tenofovir has yet been confirmed [201,203,280]. Tenofovir is currently used to treat HIV infection in children, and it proved to be well tolerated in phase I studies conducted in patients 4-18 years of age [204,205]. As there is no preparation suitable for young children, a phase III trial is underway on HBV patients 12-17 years old [206].

New drugs

Telbivudine is an L-NA with a potent antiviral activity and a safety profile similar to lamivudine (but myopathy and peripheral neuropathy have been reported in adults) [198]. Resistance rate is lower than lamivudine but higher than adefovir. For such a reason, telbivudine is only used in combination with other antiviral drugs. A phase I clinical trial is ongoing on children 2-18 years of age [199].

NA all act on viral replication, reducing the synthesis of new infectious viral particles. Viral replication can also be inhibited by inducing local production of antiviral cytokines through toll-like receptors (TLR) activation [272] or by preventing the proper formation of viral core particles with heteroaryldihydropyrimidines [273-274]. Such a non-nucleosidic inhibitor of HBV nucleocapsid maturation (BAY41-4109) proved effective both *in vitro* and *in vivo* [275]. Another possible therapeutic strategy is to prevent the spread of the infection to the neighbour hepatocytes [276-278]. Myrcludex-B is a peptide derived from the preS1 domain of the HBV large surface protein (see chapter 1.2.4, page 70), which was shown to competitively inhibit HBV infection [276,277]. A clinical trial is currently ongoing. Other compounds directly acting on cccDNA are being tested [279]. Preventing cccDNA formation, these molecules would prove useful in preventing the *de novo* hepatitis in recipient of liver grafts from anti-HBc-positive donors or relapse in HBV-infected recipients. Moreover, if given in combination with NA, they could presumably prevent the emergence of drug-resistant mutants, increasing the success rates of available antiviral drugs. Furthermore, they would virtually eliminate the well-known risk of viral flares upon suspension of NA. This would abolish the need for long-term, even life-long, antiviral treatments, reducing side effects and cutting costs.

1.1.7. Proposition of a treatment scheme

Outside clinical trials, until new drugs are licensed for pediatric patients, INF-alfa is still the drug of choice, unless decompensated cirrhosis is present. The high HBeAg seroconversion rate and the absence of genotypic resistance against such a drug are a big advantage, even though adverse effects are more pronounced and a clear benefit on the long-term remains to be confirmed. Furthermore, INF-alfa is the only treatment licensed for treating children younger than 3 years, although

patients in this age group requiring treatment are extremely rare. Currently available NA are second-line therapies, as the risk of emergence of resistant mutant strains is high. In adolescents older than 16 years of age, entecavir or tenofovir are the best choices, as less likely to induce antiviral resistance. In patients 12-18 years old tenofovir has to be preferred to lamivudine or adefovir, because of the lower response and higher resistance rates of the latter. The use of lamivudine is currently limited to young children unresponsive to INF-alfa, in special populations (coinfection with HIV, transplant recipients or patients with HBV-related glomerulonephritis) or to prevent reactivation in children receiving immunosuppressive therapies. Combination therapy is promising, but further data are needed in children.

PEG-INF is highly promising, and its licensing for children could change management of pediatric CHB.

1.1.8. Treatment failure and antiviral resistance

It is known that NA cannot prevent the formation of cccDNA [254]. Persistent viremia during antiviral therapy leads therefore to the infection of new hepatocytes. If a virus carrying a drug-resistant mutation is synthesized, it can be converted in cccDNA in newly infected cells [255]. The formation of new transcriptional templates of cccDNA from such traces of drug-resistant mutants is necessary for the selection of drug-resistant populations [154,252,253].

Partial response to NA or primary nonresponse are most often due to the emergence of genotypic resistant strains or to patient compliance. In nonresponders, HBV genotypic analysis is warranted in order to differentiate between resistance and patient noncompliance. In responders, virological breakthrough (which may be followed to biochemical breakthrough) is usually secondary to genotypic resistance. Likelihood of virological breakthrough depends on the intrinsic barrier to resistance of the specific NA (lamivudine > telbivudine > adefovir > entecavir > tenofovir). All children receiving NA should be monitored for virological breakthrough by measuring HBV DNA levels every 3 months.

When resistance to a NA develops, decision on therapy adjustment is based on liver biopsy. If mild hepatitis is present, treatment should be stopped and the child should be followed up in the eventuality of post-treatment flares. In case of

moderate hepatitis/fibrosis, a second antiviral can be added or the patient can be switched to IFN- α . If severe hepatitis is found at liver biopsy, a second antiviral should be added. *In vitro* phenotypic assays are becoming available to analyze the susceptibility of patient's mutant strain to other antivirals [250,251]. Although still expensive and not widely available, such tests will prove extremely useful to manage patients with cross and multi-resistance.

As the emergence of resistant mutant strains is becoming a major public health problem, pediatric practitioners should not treat children who are not likely to benefit from a licensed therapy and consider waiting for market approval of more effective drugs.

1.1.9. Coinfection with HIV, HCV or HDV

Treatment strategies of special populations of HBV infected children are rarely based on strong evidence. Indications and type of treatment are decided on the basis of few available case reports and are often extrapolated from evidence available for adult patients. Such children should be referred to highly experienced centers where individualized treatments (even with off-label newer antivirals) can be administered.

HIV infection should be ruled out in children coming from high-prevalence countries, as well as in adolescents who are injection drugs users. HBV/HIV-coinfected patients are at increased risk of disease progression [236]. Furthermore, they are at increased risk of developing a resistance against lamivudine or entecavir if they are used in monotherapy. Because of a risk of inducing HIV resistance, entecavir should only be used in patients receiving effective antiretroviral therapy [237]. In HBV/HIV-coinfected adult patients, the combination of tenofovir and lamivudine or emtricitabine is the recommended treatment. Until stronger pediatric evidence will be available, such a recommendation may be extrapolated to coinfecting children [238,239].

HBV/HCV coinfection is rare, and few data are available. IFN- α (at doses recommended for HBV treatment) and ribavirin may be a good option. IFN- α is also the drug of choice for HDV/HBV-coinfected children [240].

1.1.10. Conclusions

A dramatic reduction of pediatric HBV infection prevalence has been observed in countries where global immunization programs have been implemented. Nevertheless, an important number of children are still infected every year. Implementation of global immunization programs worldwide, respect of WHO recommendations on vaccination schedule and improvement of vertical transmission prevention protocols, could lead towards the elimination of HBV. Prevention and management of breakthrough infection, along with a better identification of children at higher risk for disease progression and complications, are the current challenges.

Overall, management of pediatric patients with CHB is satisfactory, but hampered by a chronic delay in licensing new drugs as compared to adults. As approval by regulatory agencies is based on the completion of appropriate clinical trials, studies on treatments that, in adults, have already been proved safe and more effective than available drugs should be conducted without undue delays. The current (2007) European regulation on medicines for children offers the appropriate regulatory framework to speed up this process.

1.1.11. References

- [1] World Health Organization. Hepatitis B [Internet]. WHO Website. 2008 [cited 2011 Oct. 25]; Available from: <http://www.who.int/mediacentre/factsheets/fs204/en/index.html>
- [2] Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004;11:97–107.
- [3] Goldstein ST, Zhou F, Hadler SC, Bell BP, Mast EE, Margolis HS. A mathematical model to estimate global hepatitis B disease burden and vaccination impact. *Int J Epidemiol* 2005;34:1329–1339.
- [4] McMahon BJ. The natural history of chronic hepatitis B virus infection. *Hepatology* 2009;49:S45–S55.
- [5] Liang X, Bi S, Yang W, Wang L, Cui G, Cui F, et al. Epidemiological serosurvey of Hepatitis B in China—Declining HBV prevalence due to Hepatitis B vaccination. *Vaccine* 2009;27:6550–6557.
- [6] Luo Z, Li L, Ruan B. Impact of the implementation of a vaccination strategy on hepatitis B virus infections in China over a 20-year period. *Int. J. Infect. Dis.* 2012;16:e82–8.
- [7] Ni Y-H, Huang LM, Chang M-H, Yen CJ, Lu CY, You S-L, et al. Two Decades of Universal Hepatitis B Vaccination in Taiwan: Impact and Implication for Future Strategies. *Gastroenterology* 2007;132:1287–1293.

- [8] Chen S-M, Kung C-M, Yang W-J, Wang H-L. Efficacy of the nationwide hepatitis B infant vaccination program in Taiwan. *J Clin Virol* 2011;52:11–16.
- [9] Zhang L, Xu A, Yan B, Song L, Li M, Xiao Z, et al. A significant reduction in hepatitis B virus infection among the children of Shandong Province, China: the effect of 15 years of universal infant hepatitis B vaccination. *International Journal of Infectious Diseases* 2010;14:e483–e488.
- [10] Centers for Disease Control and Prevention CDC. Surveillance for Acute Viral Hepatitis - United States, 2007. *MMWR Morb Mortal Wkly Rep* 2009;58:1–27.
- [11] Kim WR. Epidemiology of hepatitis B in the United States. *Hepatology* 2009;49:S28–S34.
- [12] Ioannou GN. Hepatitis B virus in the United States: infection, exposure, and immunity rates in a nationally representative survey. *Ann Intern Med* 2011;154:319–328.
- [13] Child Welfare Information Gateway. How Many Children Were Adopted in 2007 and 2008? Washington, DC: U.S. Department of Health and Human Services, Children's Bureau. 2011; Available from: <http://www.childwelfare.gov>
- [14] Agence Française de l'Adoption. Statistiques de l'adoption internationale 2010 [Internet]. France Diplomatie. 2008 [cited 2012 Jan. 10]; Available from: <http://www.diplomatie.gouv.fr/fr/enjeux-internationaux/adoption-internationale-2605/statistiques-de-l-adoption/article/2010-88914>
- [15] Te HS, Jensen DM. Epidemiology of Hepatitis B and C Viruses: A Global Overview. *Clin Liver Dis* 2010;14:1–21.
- [16] Chien YC. Nationwide Hepatitis B Vaccination Program in Taiwan: Effectiveness in the 20 Years After It Was Launched. *Epidemiologic Reviews* 2006;28:126–135.
- [17] Zanetti AR, van Damme P, Shouval D. The global impact of vaccination against hepatitis B: a historical overview. *Vaccine* 2008;26:6266–6273.
- [18] Stadler LP, Mezoff AG, Staat MA. Hepatitis B Virus Screening for Internationally Adopted Children. *Pediatrics* 2008;122:1223–1228.
- [19] Cai W, Poethko-Müller C, Hamouda O, Radun D. Hepatitis B Virus Infections Among Children and Adolescents in Germany. *Pediatr Infect Dis J* 2011;30:19–24.
- [20] Weinbaum CM, Williams I, Mast EE, Wang SA, Finelli L, Wasley A, et al. Recommendations for identification and public health management of persons with chronic hepatitis B virus infection. *MMWR Recomm Rep*. 2008;57:1–20.
- [21] American Academy of Pediatrics. Medical evaluation of internationally adopted children for infectious diseases. In: Pickering L, Baker C, Long S, MacMillan J, editors. *Red book: 2009 report of the Committee on Infectious Diseases*. Elk Grove Village, IL: American Academy of Pediatrics; 2009. p. 177–184.
- [22] National Center for Immunization and Respiratory Diseases. General recommendations on immunization - recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep*. 2011;60:1–64.
- [23] Liu HF, Sokal E, Goubau P. Wide variety of genotypes and geographic origins of hepatitis B virus in Belgian children. *J Pediatr Gastroenterol Nutr* 2001;32:274–277.
- [24] Ni Y-H, Chang M-H, Wang K-J, Hsu H-Y, Chen H-L, Kao J-H, et al. Clinical relevance of hepatitis B virus genotype in children with chronic infection and hepatocellular carcinoma. *Gastroenterology* 2004;127:1733–1738.
- [25] Lin C-L, Kao J-H. The clinical implications of hepatitis B virus genotype: Recent advances. *J Gastroenterol Hepatol* 2011;26:123–130.

- [26] Kidd-Ljunggren K, Holmberg A, Bläckberg J, Lindqvist B. High levels of hepatitis B virus DNA in body fluids from chronic carriers. *Journal of Hospital Infection* 2006;64:352–357.
- [27] Mast EE, Margolis HS, Fiore AE, Brink EW, Goldstein ST, Wang SA, et al. A Comprehensive Immunization Strategy to Eliminate Transmission of Hepatitis B Virus Infection in the United States - Recommendations of the Advisory Committee on Immunization Practices (ACIP) Part 1: Immunization of Infants, Children, and Adolescents. *MMWR Recomm Rep* 2005;54:1–23.
- [28] Sokal EM, Van Collie O, Buts JP. Horizontal transmission of hepatitis B from children to adoptive parents. *Archives of Disease in Childhood* 1995;72:191.
- [29] Martin A, Moyes C, Lucas C, Milne A. Hepatitis B infection in households of HBsAg positive New Zealand children. *N Z Med J* 1996;109:463–465.
- [30] Staff M, Angel P. Vaccination among household contacts of chronic hepatitis B carriers by general practitioners. *Aust Fam Physician* 2002;31:491–493.
- [31] Chakravarty R, Chowdhury A, Chaudhuri S, Santra A, Neogi M, Rajendran K, et al. Hepatitis B infection in Eastern Indian families: need for screening of adult siblings and mothers of adult index cases. *Public Health* 2005;119:647–654.
- [32] Kumar GT, Kazim SN, Kumar M, Hissar S, Chauhan R, Basir SF, et al. Hepatitis B virus genotypes and hepatitis B surface antigen mutations in family contacts of hepatitis B virus infected patients with occult hepatitis B virus infection. *J Gastroenterol Hepatol* 2009;24:588–598.
- [33] Richardson G, Evans MR, Westmoreland D. Hepatitis B immunisation of household contacts: retrospective study of vaccine coverage. *J Epidemiol Community Health* 2001;55:934–935.
- [34] Weinberg MS, Gunn RA, Mast EE, Gresham L, Ginsberg M. Preventing transmission of hepatitis B virus from people with chronic infection. *Am J Prev Med* 2001;20:272–276.
- [35] Scognamiglio P, Girardi E, Fusco M, Piselli P, Russo Spena S, Maione C, et al. Lack of implementation of Hepatitis B Virus (HBV) vaccination policy in household contacts of HBV carriers in Italy. *BMC Infect Dis* 2009;9:86.
- [36] Centers for Disease Control and Prevention CDC. Vaccination coverage among children in kindergarten--United States, 2009-10 school year. *MMWR Morb Mortal Wkly Rep* 2011;60:700–704.
- [37] Bartlett L, Kanellos-Sutton M, van Wylick R. Immunization Rates in a Canadian Juvenile Corrections Facility. *Journal of Adolescent Health* 2008;43:609–611.
- [38] Sneller VP, Fishbein DB, Weinbaum CM, Lombard A, Murray P, McLaurin JA, et al. Vaccinating Adolescents in High-Risk Settings: Lessons Learned From Experiences With Hepatitis B Vaccine. *Pediatrics* 2008;121:S55–S62.
- [39] McMahon BJ, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP, et al. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. *J. Infect. Dis.* 1985;151:599–603.
- [40] Tassopoulos NC, Papaevangelou GJ, Sjogren MH, Roumeliotou-Karayannis A, Gerin JL, Purcell RH. Natural history of acute hepatitis B surface antigen-positive hepatitis in Greek adults. *Gastroenterology* 1987;92:1844–1850.
- [41] Chen H-L, Lin L-H, Hu F-C, Lee J-T, Lin W-T, Yang Y-J, et al. Effects of Maternal Screening and Universal Immunization to Prevent Mother-To-Infant Transmission of HBV. *Gastroenterology* 2011; doi: 10.1053/j.gastro.2011.12.035.
- [42] Xu D-Z, Yan Y-P, Choi BCK, Xu J-Q, Men K, Zhang J-X, et al. Risk factors and mechanism of transplacental transmission of hepatitis B virus: A case-control

study. *J. Med. Virol.* 2002;67:20–26.

- [43] Wang Z, Zhang J, Yang H, Li X, Wen S, Guo Y, et al. Quantitative analysis of HBV DNA level and HBeAg titer in hepatitis B surface antigen positive mothers and their babies: HBeAg passage through the placenta and the rate of decay in babies. *J. Med. Virol.* 2003;71:360–366.
- [44] Yang J, Zeng X-M, Men Y-L, Zhao L-S. Elective caesarean section versus vaginal delivery for preventing mother to child transmission of hepatitis B virus – a systematic review. *Virol J* 2008;5:100.
- [45] World Health Organization. Hepatitis B and breastfeeding. World Health Organization. *J Int Assoc Physicians AIDS Care* 1998;4:20–21.
- [46] Shi Z, Yang Y, Wang H, Ma L, Schreiber A, Li X, et al. Breastfeeding of Newborns by Mothers Carrying Hepatitis B Virus: A Meta-analysis and Systematic Review. *Archives of Pediatrics and Adolescent Medicine* 2011;165:837–846.
- [47] Petrova M. Breastfeeding and chronic HBV infection: Clinical and social implications. *World J Gastroenterol* 2010;16:5042–5046.
- [48] Zou S, Stramer SL, Notari EP, Kuhns MC, Krysztof D, Musavi F, et al. Current incidence and residual risk of hepatitis B infection among blood donors in the United States. *Transfusion* 2009;49:1609–1620.
- [49] Perkins HA, Busch MP. Transfusion-associated infections: 50 years of relentless challenges and remarkable progress. *Transfusion* 2010;50:2080–2099.
- [50] Dumpis U, Kovalova A, Jansons J, upane L, Sominskaya I, Michailova M, et al. An outbreak of HBV and HCV infection in a paediatric oncology ward: Epidemiological investigations and prevention of further spread. *J. Med. Virol.* 2003;69:331–338.
- [51] Shapira R, Mor E, Bar-Nathan N, Sokal EM, Tur-Kaspa R, Dinari G, et al. Efficacy of Lamivudine for the Treatment of Hepatitis B Virus Infection After Liver Transplantation in Children. *Transplantation* 2001;72:333–336.
- [52] Lok ASF, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009;50:661–662.
- [53] Natov SN, Pereira BJG. Transmission of viral hepatitis by kidney transplantation: donor evaluation and transplant policies (Part 1: hepatitis B virus). *Transpl Infect Dis* 2002;4:117–123.
- [54] Dickson RC, Everhart JE, Lake JR, Wei Y, Seaberg EC, Wiesner RH, et al. Transmission of hepatitis B by transplantation of livers from donors positive for antibody to hepatitis B core antigen. The National Institute of Diabetes and Digestive and Kidney Diseases Liver Transplantation Database. *Gastroenterology* 1997;113:1668–1674.
- [55] Wachs ME, Amend WJ, Ascher NL, Bretan PN, Emond J, Lake JR, et al. The risk of transmission of hepatitis B from HBsAg(-), HBcAb(+), HBIgM(-) organ donors. *Transplantation* 1995;59:230–234.
- [56] Cholongitas E, Papatheodoridis GV, Burroughs AK. Liver grafts from anti-hepatitis B core positive donors: A systematic review. *J Hepatol* 2010;52:272–279.
- [57] Su W-J, Ho M-C, Ni Y-H, Chen H-L, Hu R-H, Wu Y-M, et al. High-titer antibody to hepatitis B surface antigen before liver transplantation can prevent de novo hepatitis B infection. *J Pediatr Gastroenterol Nutr* 2009;48:203–208.
- [58] Perrillo R. Hepatitis B virus prevention strategies for antibody to hepatitis B core antigen-positive liver donation: A survey of North American, European, and Asian-Pacific transplant programs. *Liver Transpl* 2009;15:223–232.
- [59] Shiraki K, Yoshihara N, Sakurai M, Eto T, Kawana T. Acute hepatitis B in

infants born to carrier mother with the antibody to hepatitis B e antigen. *J Pediatr* 1980;97:768–770.

- [60] Bortolotti F, Cadrobbi P, Bertaggia A, Rude L, Alberti A, Realdi G. A 7 year survey of acute hepatitis type B. *Archives of Disease in Childhood* 1983;58:993–996.
- [61] Squires RH, Shneider BL, Bucuvalas J, Alonso E, Sokol RJ, Narkewicz MR, et al. Acute liver failure in children: the first 348 patients in the pediatric acute liver failure study group. *J Pediatr* 2006;148:652–658.
- [62] Sundaram SS, Alonso EM, Narkewicz MR, Zhang S, Squires RH, Pediatric Acute Liver Failure Study Group. Characterization and outcomes of young infants with acute liver failure. *J Pediatr* 2011;159:813–818.
- [63] Chang MH, Lee CY, Chen DS, Hsu HC, Lai MY. Fulminant hepatitis in children in Taiwan: the important role of hepatitis B virus. *J Pediatr* 1987;111:34–39.
- [64] Kao J. Universal hepatitis B vaccination and the decreased mortality from fulminant hepatitis in infants in Taiwan. *J Pediatr* 2001;139:349–352.
- [65] Chen H-L, Chang C-J, Kong M-S, Huang F-C, Lee H-C, Lin C-C, et al. Pediatric fulminant hepatic failure in endemic areas of hepatitis B infection: 15 years after universal hepatitis B vaccination. *Hepatology* 2004;39:58–63.
- [66] Vanclaire J, Cornu C, Sokal EM. Fulminant hepatitis B in an infant born to a hepatitis Be antibody positive, DNA negative carrier. *Archives of Disease in Childhood* 1991;66:983–985.
- [67] Iorio R, Giannattasio A, Cirillo F, D'Alessandro L, Vegnente A. Long-term outcome in children with chronic hepatitis B: a 24-year observation period. *Clin Infect Dis* 2007;45:943–949.
- [68] Milich DR, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? *Proc Natl Acad Sci USA* 1990;87:6599–6603.
- [69] Chen MT, Billaud J-N, Sällberg M, Guidotti LG, Chisari FV, Jones J, et al. A function of the hepatitis B virus precore protein is to regulate the immune response to the core antigen. *Proc Natl Acad Sci USA* 2004;101:14913–14918.
- [70] Milich D. Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. *Hepatology* 2003;38:1075–1086.
- [71] Chen M, Sällberg M, Hughes J, Jones J, Guidotti LG, Chisari FV, et al. Immune tolerance split between hepatitis B virus precore and core proteins. *J Virol* 2005;79:3016–3027.
- [72] Bortolotti F, Guido M, Bartolacci S, Cadrobbi P, Crivellaro C, Noventa F, et al. Chronic hepatitis B in children after e antigen seroclearance: final report of a 29-year longitudinal study. *Hepatology* 2006;43:556–562.
- [73] Chu C-M, Yeh C-T, Lee C-S, Sheen I-S, Liaw Y-F. Precore stop mutant in HBeAg-positive patients with chronic hepatitis B: clinical characteristics and correlation with the course of HBeAg-to-anti-HBe seroconversion. *J Clin Microbiol* 2002;40:16–21.
- [74] Frelin L, Wahlstrom T, Tucker AE, Jones J, Hughes J, Lee BO, et al. A Mechanism To Explain the Selection of the Hepatitis e Antigen-Negative Mutant during Chronic Hepatitis B Virus Infection. *J Virol* 2009;83:1379–1392.
- [75] Evans A, Riva A, Cooksley H, Phillips S, Puranik S, Nathwani A, et al. Programmed death 1 expression during antiviral treatment of chronic hepatitis B: Impact of hepatitis B e-antigen seroconversion. *Hepatology* 2008;48:759–769.
- [76] Tseng Y-R, Wu J-F, Ni Y-H, Chen H-L, Chen C-C, Wen W-H, et al. Long-term

effect of maternal HBeAg on delayed HBeAg seroconversion in offspring with chronic hepatitis B infection. *Liver International* 2011;31:1373–1380.

- [77] Marx G, Martin SR, Chicoine J-F, Alvarez F. Long-term follow-up of chronic hepatitis B virus infection in children of different ethnic origins. *J. Infect. Dis.* 2002;186:295–301.
- [78] Ni Y-H, Chang M-H, Chen P-J, Tsai K-S, Hsu H-Y, Chen H-L, et al. Viremia Profiles in Children With Chronic Hepatitis B Virus Infection and Spontaneous e Antigen Seroconversion. *Gastroenterology* 2007;132:2340–2345.
- [79] Wu J-F, Su Y-R, Chen C-H, Chen H-L, Ni Y-H, Hsu H-Y, et al. Predictive Effect of Serial Serum Alanine Aminotransferase Levels on Spontaneous HBeAg Seroconversion in Chronic Genotype B and C HBV-Infected Children. *J Pediatr Gastroenterol Nutr* 2012;54:97–100.
- [80] Chu CM, Liaw YF. Chronic hepatitis B virus infection acquired in childhood: special emphasis on prognostic and therapeutic implication of delayed HBeAg seroconversion. *J Viral Hepat* 2007;14:147–152.
- [81] Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 2000;118:554–559.
- [82] Chu C-J, Hussain M, Lok AS. Hepatitis B Virus Genotype B Is Associated With Earlier HBeAg Seroconversion Compared With Hepatitis B Virus Genotype C. *Gastroenterology* 2002;122:1756–1762.
- [83] Livingston SE, Simonetti JP, Bulkow LR, Homan CE, Snowball MM, Cagle HH, et al. Clearance of Hepatitis B e Antigen in Patients With Chronic Hepatitis B and Genotypes A, B, C, D, and F. *Gastroenterology* 2007;133:1452–1457.
- [84] Chang M, Hsu H, Hsu H, Ni Y, Chen J, Chen D. The significance of spontaneous hepatitis B e antigen seroconversion in childhood: with special emphasis on the clearance of hepatitis B e antigen before 3 years of age. *Hepatology* 1995;22:1387–1392.
- [85] Wen W-H, Chang M-H, Hsu H-Y, Ni Y-H, Chen H-L. The development of hepatocellular carcinoma among prospectively followed children with chronic hepatitis B virus infection. *J Pediatr* 2004;144:397–399.
- [86] Chang MH, You SL, Chen CJ, Liu CJ, Lee CM, Lin SM, et al. Decreased Incidence of Hepatocellular Carcinoma in Hepatitis B Vaccinees: A 20-Year Follow-up Study. *JNCI Journal of the National Cancer Institute* 2009;101:1348–1355.
- [87] Chang MH, Shau WY, Chen CJ, Wu TC, Kong MS, Liang DC, et al. Hepatitis B vaccination and hepatocellular carcinoma rates in boys and girls. *JAMA: The Journal of the American Medical Association* 2000;284:3040–3042.
- [88] Iloeje UH, Yang H-I, Su J, Jen C-L, You S-L, Chen C-J. Predicting Cirrhosis Risk Based on the Level of Circulating Hepatitis B Viral Load. *Gastroenterology* 2006;130:678–686.
- [89] Chen C-J, Yang H-I, Su J, Jen C-L, You S-L, Lu S-N, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006;295:65–73.
- [90] Sánchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodés J. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. *Gastroenterology* 2002;123:1848–1856.
- [91] Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, et al. Hepatitis B Virus Genotype and DNA Level and Hepatocellular Carcinoma: A Prospective Study in Men. *JNCI Journal of the National Cancer Institute* 2005;97:265–272.

- [92] Livingston SE, Simonetti JP, McMahon BJ, Bulkow LR, Hurlburt KJ, Homan CE, et al. Hepatitis B virus genotypes in Alaska Native people with hepatocellular carcinoma: preponderance of genotype F. *J. Infect. Dis.* 2007;195:5–11.
- [93] Yu MW, Chang HC, Liaw YF, Lin SM, Lee SD, Liu CJ, et al. Familial risk of hepatocellular carcinoma among chronic hepatitis B carriers and their relatives. *J. Natl. Cancer Inst.* 2000;92:1159–1164.
- [94] Zhang H, Zhai Y, Hu Z, Wu C, Qian J, Jia W, et al. Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers. *Nat Genet* 2010;42:767–770.
- [95] Hsu Y-S, Chien R-N, Yeh C-T, Sheen I-S, Chiou H-Y, Chu C-M, et al. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology* 2002;35:1522–1527.
- [96] Gaeta G. Epidemiological and clinical burden of chronic hepatitis B virus/hepatitis C virus infection. A multicenter Italian study. *J Hepatol* 2003;39:1036–1041.
- [97] Ikeda K, Arase Y, Saitoh S, Kobayashi M, Someya T, Hosaka T, et al. Long-term outcome of HBV carriers with negative HBe antigen and normal aminotransferase. *The American Journal of Medicine* 2006;119:977–985.
- [98] Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. *J Hepatol* 2008;48:335–352.
- [99] Ribeiro RM, Germanidis G, Powers KA, Pellegrin B, Nikolaidis P, Perelson AS, et al. Hepatitis B Virus Kinetics under Antiviral Therapy Sheds Light on Differences in Hepatitis B e Antigen Positive and Negative Infections. *J. Infect. Dis.* 2010;202:1309–1318.
- [100] Arase Y, Ikeda K, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, et al. Long-term outcome after hepatitis B surface antigen seroclearance in patients with chronic hepatitis B. *The American Journal of Medicine* 2006;119:71.e9–16.
- [101] Wursthorn K, Jung M, Riva A, Goodman ZD, Lopez P, Bao W, et al. Kinetics of hepatitis B surface antigen decline during 3 years of telbivudine treatment in hepatitis B e antigen-positive patients. *Hepatology* 2010;52:1611–1620.
- [102] Jang JW, Yoo SH, Kwon JH, You CR, Lee S, Lee JH, et al. Serum hepatitis B surface antigen levels in the natural history of chronic hepatitis B infection. *Aliment Pharmacol Ther* 2011;34:1337–1346.
- [103] Chan HL-Y, Thompson A, Martinot-Peignoux M, Piratvisuth T, Cornberg M, Brunetto MR, et al. Hepatitis B surface antigen quantification: why and how to use it in 2011 - a core group report. *J Hepatol* 2011;55:1121–1131.
- [104] Beutels P, Edmunds WJ, Antóñanzas F, De Wit GA, Evans D, Feilden R, et al. Economic evaluation of vaccination programmes: a consensus statement focusing on viral hepatitis. In: *PharmacoEconomics*. 2002. p. 1–7.
- [105] Kim S-Y, Salomon JA, Goldie SJ. Economic evaluation of hepatitis B vaccination in low-income countries: using cost-effectiveness affordability curves. *Bull. World Health Organ.* 2007;85:833–842.
- [106] Hung H-F, Chen TH-H. Probabilistic cost-effectiveness analysis of the long-term effect of universal hepatitis B vaccination: An experience from Taiwan with high hepatitis B virus infection and Hepatitis B e Antigen positive prevalence. *Vaccine* 2009;27:6770–6776.
- [107] Siddiqui MR, Gay N, Edmunds WJ, Ramsay M. Economic evaluation of infant and adolescent hepatitis B vaccination in the UK. *Vaccine* 2011;29:466–475.
- [108] World Health Organization. Hepatitis B vaccines - WHO position paper. *Weekly*

epidemiological record 2009;89:405–420.

- [109] World Health Organization. Hepatitis B vaccines: WHO position paper—Recommendations. *Vaccine* 2010;28:589–590.
- [110] But DY-K, Lai C-L, Lim W-L, Fung J, Wong DK-H, Yuen M-F. Twenty-two years follow-up of a prospective randomized trial of hepatitis B vaccines without booster dose in children: Final report. *Vaccine* 2008;26:6587–6591.
- [111] Lee C, Gong Y, Brok J, Boxall EH, Gluud C. Hepatitis B immunisation for newborn infants of hepatitis B surface antigen-positive mothers. *Cochrane Database Syst Rev* 2006; Issue 2. Art. No.: CD004790. DOI: 10.1002/14651858.CD004790.pub2.
- [112] Zepp F, Knuf M, Heininger U, Jahn K, Collard A, Habermehl P, et al. Safety, reactogenicity and immunogenicity of a combined hexavalent tetanus, diphtheria, acellular pertussis, hepatitis B, inactivated poliovirus vaccine and Haemophilus influenzae type b conjugate vaccine, for primary immunization of infants. *Vaccine* 2004;22:2226–2233.
- [113] Cheng KF, Chang MH, Lee CY, Huang LM, Hsu HY, Lee PI, et al. Response to supplementary vaccination with recombinant or plasma hepatitis B vaccine in healthy non-responding children. *Vaccine* 1994;12:899–902.
- [114] Jafarzadeh A, Zarei S, Shokri F. Low dose revaccination induces robust protective anti-HBs antibody response in the majority of healthy non-responder neonates. *Vaccine* 2008;26:269–276.
- [115] World Health Organization. Expanded programme on immunization. Global Advisory Group--Part I. *Wkly. Epidemiol. Rec.* 1992;67:11–15.
- [116] World Health Organization. Global routine vaccination coverage, 2010. *Wkly. Epidemiol. Rec.* 2011;86:509–513.
- [117] Jack AD, Hall AJ, Maine N, Mendy M, Whittle HC. What level of hepatitis B antibody is protective? *J. Infect. Dis.* 1999;179:489–492.
- [118] Lin Y-C, Chang M-H, Ni Y-H, Hsu H-Y, Chen D-S. Long-term immunogenicity and efficacy of universal hepatitis B virus vaccination in Taiwan. *J. Infect. Dis.* 2003;187:134–138.
- [119] McMahon BJ, Bruden DL, Petersen KM, Bulkow LR, Parkinson AJ, Nainan O, et al. Antibody levels and protection after hepatitis B vaccination: results of a 15-year follow-up. *Ann Intern Med* 2005;142:333–341.
- [120] Dentinger CM, McMahon BJ, Butler JC, Dunaway CE, Zanis CL, Bulkow LR, et al. Persistence of Antibody to Hepatitis B and Protection From Disease Among Alaska Natives Immunized at Birth. *Pediatr Infect Dis J* 2005;24:786–792.
- [121] Bialek SR, Bower WA, Novak R, Helgenberger L, Auerbach SB, Williams IT, et al. Persistence of Protection Against Hepatitis B Virus Infection Among Adolescents Vaccinated With Recombinant Hepatitis B Vaccine Beginning at Birth. *Pediatr Infect Dis J* 2008;27:881–885.
- [122] Zanetti AR, Mariano A, Romanò L, D'Amelio R, Chironna M, Coppola RC, et al. Long-term immunogenicity of hepatitis B vaccination and policy for booster: an Italian multicentre study. *The Lancet* 2005;366:1379–1384.
- [123] Lu CY, Ni Y-H, Chiang BL, Chen P-J, Chang M-H, Chang LY, et al. Humoral and Cellular Immune Responses to a Hepatitis B Vaccine Booster 15–18 Years after Neonatal Immunization. *J. Infect. Dis.* 2008;197:1419–1426.
- [124] van der Sande MAB, Waight P, Mendy M, Rayco-Solon P, Hutt P, Fulford T, et al. Long-term protection against carriage of hepatitis B virus after infant vaccination. *J. Infect. Dis.* 2006;193:1528–1535.

- [125] Poovorawan Y, Chongsrisawat V, Theamboonlers A, Leroux-Roels G, Kuriyakose S, Leyssen M, et al. Evidence of protection against clinical and chronic hepatitis B infection 20 years after infant vaccination in a high endemicity region. *J Viral Hepat* 2011;18:369–375.
- [126] Yuen M-F, Lim W-L, Chan AO-O, Wong DK-H, Sum SS-M, Lai C-L. 18-year follow-up study of a prospective randomized trial of hepatitis B vaccinations without booster doses in children. *Clinical Gastroenterology and Hepatology* 2004;2:941–945.
- [127] European Consensus Group on Hepatitis B Immunity. Are booster immunisations needed for lifelong hepatitis B immunity? European Consensus Group on Hepatitis B Immunity. In: *Lancet*. 2000. p. 561–565.
- [128] Lau YL, Tam AY, Ng KW, Tsoi NS, Lam B, Lam P, et al. Response of preterm infants to hepatitis B vaccine. *J Pediatr* 1992;121:962–965.
- [129] Freitas da Motta MS, Mussi-Pinhata MM, Jorge SM, Tachibana Yoshida CF, Sandoval de Souza CB. Immunogenicity of hepatitis B vaccine in preterm and full term infants vaccinated within the first week of life. *Vaccine* 2002;20:1557–1562.
- [130] Kim SC, Chung EK, Hodinka RL, DeMaio J, West DJ, Jawad AF, et al. Immunogenicity of Hepatitis B Vaccine in Preterm Infants. *Pediatrics* 1997;99:534–536.
- [131] Saari TN, American Academy of Pediatrics Committee on Infectious Diseases. Immunization of preterm and low birth weight infants. American Academy of Pediatrics Committee on Infectious Diseases. *Pediatrics* 2003;112:193–198.
- [132] Park S-D, Markowitz J, Pettei M, Weinstein T, Sison CP, Swiss SR, et al. Failure to respond to hepatitis B vaccine in children with celiac disease. *J Pediatr Gastroenterol Nutr* 2007;44:431–435.
- [133] Zingone F, Morisco F, Zanetti A, Romanò L, Portella G, Capone P, et al. Long-term antibody persistence and immune memory to hepatitis B virus in adult celiac patients vaccinated as adolescents. *Vaccine* 2011;29:1005–1008.
- [134] Wen W-H, Chen H-L, Ni Y-H, Hsu H-Y, Kao J-H, Hu F-C, et al. Secular trend of the viral genotype distribution in children with chronic hepatitis B virus infection after universal infant immunization. *Hepatology* 2011;53:429–436.
- [135] Hsu H-Y, Chang M-H, Ni Y-H, Chiang C-L, Chen H-L, Wu J-F, et al. No Increase in Prevalence of Hepatitis B Surface Antigen Mutant in a Population of Children and Adolescents Who Were Fully Covered by Universal Infant Immunization. *J. Infect. Dis.* 2010;201:1192–1200.
- [136] Rendi-Wagner P, Shouval D, Genton B, Lurie Y, Rümke H, Boland G, et al. Comparative immunogenicity of a PreS/S hepatitis B vaccine in non- and low responders to conventional vaccine. *Vaccine* 2006;24:2781–2789.
- [137] Sylvan SPE, Madalinski K, Hellström UB. Anti-preS responses influence the anti-HBs response in newborns after vaccination with the third generation Sci-B-Vac™ vaccine. *Vaccine* 2009;28:446–451.
- [138] Mancini Bourguine M, Fontaine H, Bréchet C, Pol S, Michel M-L. Immunogenicity of a hepatitis B DNA vaccine administered to chronic HBV carriers. *Vaccine* 2006;24:4482–4489.
- [139] Cavanaugh JS, Awi D, Mendy M, Hill AVS, Whittle H, McConkey SJ. Partially Randomized, Non-Blinded Trial of DNA and MVA Therapeutic Vaccines Based on Hepatitis B Virus Surface Protein for Chronic HBV Infection. *PLoS ONE* 2011;6:e14626. doi:10.1371/journal.pone.0014626.
- [140] Willis BC, Wortley P, Wang SA, Jacques-Carroll L, Zhang F. Gaps in Hospital Policies and Practices to Prevent Perinatal Transmission of Hepatitis B Virus.

- [141] Bzowej NH. Hepatitis B Therapy in Pregnancy. *Curr Hepatitis Rep* 2010;9:197–204.
- [142] Antiretroviral Pregnancy Registry Steering Committee. Antiretroviral Pregnancy Registry International Interim Report for 1 January 1989 through 31 July 2011 [Internet]. Wilmington, NC: Registry Coordinating Center; 2011. Available from: <http://www.apregistry.com>
- [143] Xu WM, Cui YT, Wang L, Yang H, Liang ZQ, Li XM, et al. Lamivudine in late pregnancy to prevent perinatal transmission of hepatitis B virus infection: a multicentre, randomized, double-blind, placebo-controlled study. *J Viral Hepat* 2009;16:94–103.
- [144] Shi Z, Yang Y, Ma L, Li X, Schreiber A. Lamivudine in late pregnancy to interrupt in utero transmission of hepatitis B virus: a systematic review and meta-analysis. *Obstet Gynecol* 2010;116:147–159.
- [145] Han G-R, Cao M-K, Zhao W, Jiang H-X, Wang C-M, Bai S-F, et al. A prospective and open-label study for the efficacy and safety of telbivudine in pregnancy for the prevention of perinatal transmission of hepatitis B virus infection. *J Hepatol* 2011;55:1215–1221.
- [146] European Association for the Study of the Liver. EASL Clinical Practice Guidelines: Management of chronic hepatitis B. *J Hepatol* 2009;50:227–242.
- [147] Shah U, Kelly D, Chang M-H, Fujisawa T, Heller S, González-Peralta RP, et al. Management of chronic hepatitis B in children. *J Pediatr Gastroenterol Nutr* 2009;48:399–404.
- [148] Jonas MM, Block JM, Haber BA, Karpen SJ, London WT, Murray KF, et al. Treatment of children with chronic hepatitis B virus infection in the United States: Patient selection and therapeutic options. *Hepatology* 2010;52:2192–2205.
- [149] Simonetti J, Bulkow L, McMahon BJ, Homan C, Snowball M, Negus S, et al. Clearance of hepatitis B surface antigen and risk of hepatocellular carcinoma in a cohort chronically infected with hepatitis B virus. *Hepatology* 2010;51:1531–1537.
- [150] Haber BA, Block JM, Jonas MM, Karpen SJ, London WT, McMahon BJ, et al. Recommendations for Screening, Monitoring, and Referral of Pediatric Chronic Hepatitis B. *Pediatrics* 2009;124:e1007–e1013.
- [151] Fattovich G, Olivari N, Pasino M, D’Onofrio M, Martone E, Donato F. Long-term outcome of chronic hepatitis B in Caucasian patients: mortality after 25 years. *Gut* 2008;57:84–90.
- [152] Sokal EM, Conjeevaram HS, Roberts EA, Alvarez F, Bern EM, Goyens P, et al. Interferon alfa therapy for chronic hepatitis B in children: a multinational randomized controlled trial. *Gastroenterology* 1998;114:988–995.
- [153] Jonas MM, Kelly DA, Mizerski J, Badia IB, Areias JA, Schwarz KB, et al. Clinical trial of lamivudine in children with chronic hepatitis B. *N Engl J Med* 2002;346:1706–1713.
- [154] Zoulim F, Locarnini S. Hepatitis B Virus Resistance to Nucleos(t)ide Analogues. *Gastroenterology* 2009;137:1593–1608.
- [155] Jonas MM, Kelly D, Pollack H, Mizerski J, Sorbel J, Frederick D, et al. Safety, efficacy, and pharmacokinetics of adefovir dipivoxil in children and adolescents (age 2 to <18 years) with chronic hepatitis B. *Hepatology* 2008;47:1863–1871.
- [156] Schwimmer JB, Dunn W, Norman GJ, Pardee PE, Middleton MS, Kerkar N, et al. SAFETY Study: Alanine Aminotransferase Cutoff Values Are Set Too High for Reliable Detection of Pediatric Chronic Liver Disease. *Gastroenterology*

2010;138:1357–1364.

- [157] Hsu EK, Murray KF. Hepatitis B and C in children. *Nature clinical practice Gastroenterology & hepatology* 2008;5:311–320.
- [158] Hom X, Little NR, Gardner SD, Jonas MM. Predictors of Virologic Response to Lamivudine Treatment in Children With Chronic Hepatitis B Infection. *Pediatr Infect Dis J* 2004;23:441–445.
- [159] Katz LH, Fraser A, Gaftor-Gvili A, Leibovici L, Tur-Kaspa R. Lamivudine prevents reactivation of hepatitis B and reduces mortality in immunosuppressed patients: systematic review and meta-analysis. *J Viral Hepat* 2007;0:070924202706003–???
- [160] Wai CT, Chu C-J, Hussain M, Lok ASF. HBV genotype B is associated with better response to interferon therapy in HBeAg(+) chronic hepatitis than genotype C. *Hepatology* 2002;36:1425–1430.
- [161] Erhardt A. Response to interferon alfa is hepatitis B virus genotype dependent: genotype A is more sensitive to interferon than genotype D. *Gut* 2005;54:1009–1013.
- [162] Flink HJ, van Zonneveld M, Hansen BE, de Man RA, Schalm SW, Janssen HLA, et al. Treatment with Peg-Interferon alpha-2b for HBeAg-Positive Chronic Hepatitis B: HBsAg Loss Is Associated with HBV Genotype. *Am J Gastroenterol* 2006;101:297–303.
- [163] Raimondi S, Maisonneuve P, Bruno S, Mondelli MU. Is response to antiviral treatment influenced by hepatitis B virus genotype? *J Hepatol* 2010;52:441–449.
- [164] Kobak GE, MacKenzie T, Sokol RJ, Narkewicz MR. Interferon treatment for chronic hepatitis B: enhanced response in children 5 years old or younger. *J Pediatr* 2004;145:340–345.
- [165] Bortolotti F, Jara P, Barbera C, Gregorio GV, Vegnente A, Zancan L, et al. Long term effect of alpha interferon in children with chronic hepatitis B. *Gut* 2000;46:715–718.
- [166] Hsu H-Y, Tsai H-Y, Wu T-C, Chiang C-L, Ni Y-H, Chen P-J, et al. Interferon- α treatment in children and young adults with chronic hepatitis B: a long-term follow-up study in Taiwan. *Liver International* 2008;28:1288–1297.
- [167] Vo Thi Diem H, Bourgois A, Bontems P, Goyens P, Buts J-P, Nackers F, et al. Chronic hepatitis B infection: long term comparison of children receiving interferon alpha and untreated controls. *J Pediatr Gastroenterol Nutr* 2005;40:141–145.
- [168] Yuen M. Long-term follow-up of interferon alfa treatment in chinese patients with chronic hepatitis B infection: The effect on hepatitis B e antigen seroconversion and the development of cirrhosis-related complications. *Hepatology* 2001;34:139–145.
- [169] Cammà C, Giunta M, Andreone P, Craxi A. Interferon and prevention of hepatocellular carcinoma in viral cirrhosis: an evidence-based approach. *J Hepatol* 2001;34:593–602.
- [170] Jara P, Bortolotti F. Interferon-alpha treatment of chronic hepatitis B in childhood: a consensus advice based on experience in European children. *J Pediatr Gastroenterol Nutr* 1999;29:163–170.
- [171] Boxall EH, Sira J, Ballard AL, Davies P, Kelly DA. Long-term follow-up of hepatitis B carrier children treated with interferon and prednisolone. *J. Med. Virol.* 2006;78:888–895.
- [172] Gregorio GV, Jara P, Hierro L, Diaz C, La Vega de A, Vegnente A, et al. Lymphoblastoid interferon alfa with or without steroid pretreatment in children with chronic hepatitis B: a multicenter controlled trial. *Hepatology* 1996;23:700–

- [173] Comanor L, Minor J, Conjeevaram HS, Roberts EA, Alvarez F, Bern EM, et al. Impact of chronic hepatitis B and interferon-alpha therapy on growth of children. *J Viral Hepat* 2001;8:139–147.
- [174] Reddy K. Efficacy and safety of pegylated (40-kd) interferon α -2a compared with interferon α -2a in noncirrhotic patients with chronic hepatitis C. *Hepatology* 2001;33:433–438.
- [175] Wirth S, Pieper-Boustani H, Lang T, Ballauff A, Kullmer U, Gerner P, et al. Peginterferon alfa-2b plus ribavirin treatment in children and adolescents with chronic hepatitis C. *Hepatology* 2005;41:1013–1018.
- [176] Sokal EM, Bourgois A, Stephenne X, Silveira T, Porta G, Gardovska D, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection in children and adolescents. *J Hepatol* 2010;52:827–831.
- [177] Sokal EM, Kelly DA, Mizerski J, Badia IB, Areias JA, Schwarz KB, et al. Long-term lamivudine therapy for children with HBeAg-positive chronic hepatitis B. *Hepatology* 2006;43:225–232.
- [178] Sokal EM, Roberts EA, Mieli-Vergani G, McPhillips P, Johnson M, Barber J, et al. A dose ranging study of the pharmacokinetics, safety, and preliminary efficacy of lamivudine in children and adolescents with chronic hepatitis B. *Antimicrob Agents Chemother* 2000;44:590–597.
- [179] Jonas MM, Little NR, Gardner SD, International Pediatric Lamivudine Investigator Group. Long-term lamivudine treatment of children with chronic hepatitis B: durability of therapeutic responses and safety. *J Viral Hepat* 2008;15:20–27.
- [180] Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, et al. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. *Gut* 2000;46:562–568.
- [181] Sarin SK, Kumar M, Kumar R, Kazim SN, Guptan RC, Sakhuja P, et al. Higher Efficacy of Sequential Therapy with Interferon-alpha and Lamivudine Combination Compared to Lamivudine Monotherapy in HBeAg Positive Chronic Hepatitis B Patients. *Am J Gastroenterol* 2005;100:2463–2471.
- [182] Korba B, Cote P, Hornbuckle W, Schinazi R, Gangemi J, Tennant B, et al. Enhanced antiviral benefit of combination therapy with lamivudine and alpha interferon against WHV replication in chronic carrier woodchucks. *Antivir Ther* 2000;5:95–104.
- [183] Dikici B, Bosnak M, Bosnak V, Dagli A, Ece A, Yagci RV, et al. Combination therapy for children with chronic hepatitis B virus infection. *J Gastroenterol Hepatol* 2002;17:1087–1091.
- [184] Yilmaz A, Akcam M, Gelen T, Artan R. Lamivudine and high-dose interferon alpha 2a combination treatment in naïve HBeAg-positive immunoreactive chronic hepatitis B in children: an East Mediterranean center's experience. *Eur J Pediatr* 2006;166:195–199.
- [185] Akman SA, Okcu SC, Halicioglu O, Sutcuoglu S, Anil M, Kizilgunesler A, et al. Therapeutic efficacy of sequential and simultaneous treatments with interferon-alpha and lamivudine in children with chronic hepatitis B. *Pediatr Int* 2007;49:848–852.
- [186] D'Antiga L, Aw M, Atkins M, Moorat A, Vergani D, Mieli-Vergani G. Combined lamivudine/interferon-alpha treatment in “immunotolerant” children perinatally infected with hepatitis B: a pilot study. *J Pediatr* 2006;148:228–233.
- [187] Kuloglu Z, Krsacoglu CT, Kansu A, Erden E, Girgin N. Liver histology of children with chronic hepatitis treated with interferon-alpha alone or in combination with

lamivudine. *J Pediatr Gastroenterol Nutr* 2007;45:564–568.

- [188] Marcellin P, Chang T-T, Lim SGL, Sievert W, Tong M, Arterburn S, et al. Long-term efficacy and safety of adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *Hepatology* 2008;48:750–758.
- [189] Fontana RJ. Side effects of long-term oral antiviral therapy for hepatitis B. *Hepatology* 2009;49:S185–S195.
- [190] Viganò M, Lampertico P, Colombo M. Drug safety evaluation of adefovir in HBV infection. *Expert Opinion on Drug Safety* 2011;10:809–818.
- [191] Sokal EM, Kelly D, Wirth S, Mizerski J, Dhawan A, Frederick D. The pharmacokinetics and safety of adefovir dipivoxil in children and adolescents with chronic hepatitis B virus infection. *Journal of clinical pharmacology* 2008;48:512–517.
- [192] Chang T-T, Gish RG, de Man R, Gadano A, Sollano J, Chao Y-C, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006;354:1001–1010.
- [193] Leung N, Peng C-Y, Hann H-W, Sollano J, Lao-Tan J, Hsu C-W, et al. Early hepatitis B virus DNA reduction in hepatitis B e antigen-positive patients with chronic hepatitis B: A randomized international study of entecavir versus adefovir. *Hepatology* 2009;49:72–79.
- [194] Colonno RJ, Rose R, Baldick CJ, Levine S, Pokornowski K, Yu CF, et al. Entecavir resistance is rare in nucleoside naïve patients with hepatitis B. *Hepatology* 2006;44:1656–1665.
- [195] Tenney DJ, Rose RE, Baldick CJ, Pokornowski KA, Eggers BJ, Fang J, et al. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. *Hepatology* 2009;49:1503–1514.
- [196] Sherman M, Yurdaydin C, Simsek H, Silva M, Liaw Y-F, Rustgi VK, et al. Entecavir therapy for lamivudine-refractory chronic hepatitis B: improved virologic, biochemical, and serology outcomes through 96 weeks. *Hepatology* 2008;48:99–108.
- [197] Bristol-Myers Squibb. A Phase III Study of the Safety and Efficacy of Entecavir in Pediatric Patients With Chronic HBV-Infection. In: *ClinicalTrials.gov* [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 Sep 18]. Available from: <http://clinicaltrials.gov/show/NCT01079806> NLM Identifier: NCT01079806.
- [198] Liaw YF, Gane E, Leung N, Zeuzem S, Wang Y, Lai CL, et al. 2-Year GLOBE Trial Results: Telbivudine Is Superior to Lamivudine in Patients With Chronic Hepatitis B. *Gastroenterology* 2009;136:486–495.
- [199] Novartis Pharmaceuticals. Pharmacokinetics and Safety of Single-Dose Telbivudine in Children and Adolescents With Chronic Hepatitis B. In: *ClinicalTrials.gov* [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 Sep 18]. Available from: <http://clinicaltrials.gov/show/NCT00907894> NLM Identifier: NCT00907894.
- [200] Marcellin P, Heathcote EJ, Buti M, Gane E, de Man RA, Krastev Z, et al. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. *N Engl J Med* 2008;359:2442–2455.
- [201] Heathcote EJ, Marcellin P, Buti M, Gane E, de Man RA, Krastev Z, et al. Three-year efficacy and safety of tenofovir disoproxil fumarate treatment for chronic hepatitis B. *Gastroenterology* 2011;140:132–143.
- [202] Purdy JB, Gafni RI, Reynolds JC, Zeichner S, Hazra R. Decreased bone mineral density with off-label use of tenofovir in children and adolescents infected with

human immunodeficiency virus. *J Pediatr* 2008;152:582–584.

- [203] Snow-Lampart A, Chappell B, Curtis M, Zhu Y, Myrick F, Schawalter J, et al. No resistance to tenofovir disoproxil fumarate detected after up to 144 weeks of therapy in patients monoinfected with chronic hepatitis B virus. *Hepatology* 2011;53:763–773.
- [204] Hazra R, Balis FM, Tullio AN, DeCarlo E, Worrell CJ, Steinberg SM, et al. Single-dose and steady-state pharmacokinetics of tenofovir disoproxil fumarate in human immunodeficiency virus-infected children. *Antimicrob Agents Chemother* 2004;48:124–129.
- [205] Bouazza N, Urien S, Hirt D, Frange P, Rey E, Benaboud S, et al. Population Pharmacokinetics of Tenofovir in HIV-1 Infected Pediatric Patients. *Journal of acquired immune deficiency syndrome* 2011;58:283–288.
- [206] Gilead Sciences. Evaluation of Tenofovir Disoproxil Fumarate in Adolescents With Chronic Hepatitis B Infection. In: *ClinicalTrials.gov* [Internet]. Bethesda (MD): National Library of Medicine (US). 2000- [cited 2011 Sep 18]. Available from: <http://clinicaltrials.gov/show/NCT00734162> NLM Identifier: NCT00734162.
- [207] Custer B, Sullivan SD, Hazlet TK, Iløje U, Veenstra DL, Kowdley KV. Global epidemiology of hepatitis B virus. *J Clin Gastroenterol* 2004;38:S158–68.
- [208] Tessema B, Yismaw G, Kassu A, Amsalu A, Mulu A, Emmrich F, et al. Seroprevalence of HIV, HBV, HCV and syphilis infections among blood donors at Gondar University Teaching Hospital, Northwest Ethiopia: declining trends over a period of five years. *BMC Infect Dis* 2010;10:111.
- [209] Andernach IE, Nolte C, Pape JW, Muller CP. Slave Trade and Hepatitis B Virus Genotypes and Subgenotypes in Haiti and Africa. *Emerg. Infect. Dis.* 2009;15:1222–1228.
- [210] Mitchell T, Armstrong GL, Hu DJ, Wasley A, Painter JA. The Increasing Burden of Imported Chronic Hepatitis B — United States, 1974–2008. *PLoS ONE* 2011;6:e27717.
- [211] Juon H-S, Choi K, Park E-C, Kwak M-S, Lee S. Hepatitis B vaccinations among Koreans: Results from 2005 Korea National Cancer Screening Survey. *BMC Infect Dis* 2009;9:185.
- [212] Chang M-H. Impact of hepatitis B vaccination on hepatitis B disease and nucleic acid testing in high-prevalence populations. *J Clin Virol* 2006;36 Suppl 1:S45–50.
- [213] Alvarado-Mora MV, Gutierrez Fernandez MF, Gomes-Gouvêa MS, de Azevedo Neto RS, Carrilho FJ, Pinho JRR. Hepatitis B (HBV), Hepatitis C (HCV) and Hepatitis Delta (HDV) Viruses in the Colombian Population—How Is the Epidemiological Situation? *PLoS ONE* 2011;6:e18888.
- [214] Nguyen VTT, Law MG, Dore GJ. An enormous hepatitis B virus-related liver disease burden projected in Vietnam by 2025. *Liver International* 2008;28:525–531.
- [215] Magdzik WW. Hepatitis B epidemiology in Poland, Central and Eastern Europe and the newly independent states. *Vaccine* 2000;18 Suppl 1:S13–6.
- [216] Pereira LMMLB, Martelli CMT, Merchán-Hamann E, Montarroyos UR, Braga MC, de Lima MLC, et al. Population-based multicentric survey of hepatitis B infection and risk factor differences among three regions in Brazil. *Am. J. Trop. Med. Hyg.* 2009;81:240–247.
- [217] Livramento AD, Cordova CMM de, Spada C, Treitinger A. Seroprevalence of hepatitis B and C infection markers among children and adolescents in the southern Brazilian region. *Rev. Inst. Med. Trop. Sao Paulo.* 2011;53:13–17.

- [218] Datta S. An overview of molecular epidemiology of hepatitis B virus (HBV) in India. *Virol J* 2008;5:156.
- [219] Ugwuja E, Ugwu N. Seroprevalence of Hepatitis B Surface Antigen and Liver Function Tests among Adolescents in Abakaliki, South Eastern Nigeria. *Internet Journal of Tropical Medicine* 2010; Volume 6, Number 2.
- [220] Eke AC, Eke UA, Okafor CI, Ezebialu IU, Ogbuagu C. Prevalence, correlates and pattern of hepatitis B surface antigen in a low resource setting. *Virol J* 2011;8:12.
- [221] Alikor E, Erhabor O. Seroprevalence of hepatitis B surface antigenaemia in children in a tertiary health institution in the Niger Delta of Nigeria. *Niger J Med* 2007;16:326–329.
- [222] Nurgalieva ZZ, Hollinger FB, Graham DY, Zhangabylova S, Zhangabylov A. Epidemiology and transmission of hepatitis B and C viruses in Kazakhstan. *World J Gastroenterol* 2007;13:1204–1207.
- [223] Allain JP. The risk of hepatitis B virus infection by transfusion in Kumasi, Ghana. *Blood* 2003;101:2419–2425.
- [224] Olinger CM, Lazouskaya NV, Eremin VF, Muller CP. Multiple genotypes and subtypes of hepatitis B and C viruses in Belarus: similarities with Russia and western European influences. *Clin Microbiol Infect* 2008;14:575–581.
- [225] Thüring E, Joller-Jemelka H, Sereth H, Reth C, Grob P. Prevalence of markers of hepatitis viruses A, B, C and of HIV in healthy individuals and patients of a Cambodian province. *Southeast Asian J Trop Med Public Health* 1993;24:239–249.
- [226] Bonanni P, Pesavento G, Bechini A, Tiscione E, Mannelli F, Benucci C, et al. Impact of universal vaccination programmes on the epidemiology of hepatitis B: 10 years of experience in Italy. *Vaccine* 2003;21:685–691.
- [227] Meffre C, Le Strat Y, Delarocque-Astagneau E, Dubois F, Antona D, Lemasson J-M, et al. Prevalence of hepatitis B and hepatitis C virus infections in France in 2004: Social factors are important predictors after adjusting for known risk factors. *J. Med. Virol.* 2010;82:546–555.
- [228] Zhang J, Zou S, Giulivi A. Epidemiology of hepatitis B in Canada. *Can J Infect Dis* 2001;12:345–350.
- [229] Wong D, Cheung A, O'Rourke K, Naylor C, Detsky A, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Ann Intern Med* 1993;119:312–323.
- [230] Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999;341:1256–1263.
- [231] Marcellin P, Chang T-T, Lim S-G, Tong MJ, Sievert W, Shiffman ML, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003;348:808–816.
- [232] Lau GKK, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, Cooksley G, et al. Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2005;352:2682–2695.
- [233] Lin SM, Yu ML, Lee CM, Chien RN, Sheen IS, Chu CM, et al. Interferon therapy in HBeAg positive chronic hepatitis reduces progression to cirrhosis and hepatocellular carcinoma. *J Hepatol* 2007;46:45–52.
- [234] Yang YF, Zhao W, Zhong YD, Xia HM, Shen L, Zhang N. Interferon therapy in chronic hepatitis B reduces progression to cirrhosis and hepatocellular carcinoma: a meta-analysis. *J Viral Hepat* 2009;16:265–271.

- [235] Sung JY, Tsoi KKF, Wong VWS, Li KCT, Chan HLY. Meta-analysis: treatment of hepatitis B infection reduces risk of hepatocellular carcinoma. *Aliment Pharmacol Ther* 2008;28:1067–1077.
- [236] Thio CL, Seaberg EC, Skolasky R Jr, Phair J, Visscher B, Muñoz A, et al. HIV-1, hepatitis B virus, and risk of liver-related mortality in the Multicenter Cohort Study (MACS). *Lancet* 2002;360:1921–1926.
- [237] McMahon MA, Jilek BL, Brennan TP, Shen L, Zhou Y, Wind-Rotolo M, et al. The HBV drug entecavir - effects on HIV-1 replication and resistance. *N Engl J Med*. 2007;356:2614-2621.
- [238] Soriano V, Puoti M, Bonacini M, Brook G, Cargnel A, Rockstroh J, et al. Care of patients with chronic hepatitis B and HIV co-infection: recommendations from an HIV-HBV International Panel. *AIDS* 2005;19:221-240.
- [239] Mofenson LM, Brady MT, Danner SP, Dominguez KL, Hazra R, Handelsman E, et al. Guidelines for the Prevention and Treatment of Opportunistic Infections among HIV-exposed and HIV-infected children: recommendations from CDC, the National Institutes of Health, the HIV Medicine Association of the Infectious Diseases Society of America, the Pediatric Infectious Diseases Society, and the American Academy of Pediatrics. *MMWR Recomm Rep*. 2009;58(RR-11):1-166.
- [240] Rizzetto M. Hepatitis D: thirty years after. *J Hepatol* 2009;50:1043-1050.
- [241] Werle-Lapostolle B, Bowden S, Locarnini S, Wurstthorn K, Petersen J, Lau G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004;126:1750-1758.
- [242] Zoulim F. New insight on hepatitis B virus persistence from the study of intrahepatic viral cccDNA. *J Hepatol* 2005;42:302-308.
- [243] Honkoop P, de Man RA, Niesters HG, Zondervan PE, Schalm SW. Acute exacerbation of chronic hepatitis B virus infection after withdrawal of lamivudine therapy. *Hepatology* 2000;32:635-639.
- [244] Santantonio T, Mazzola M, Iacovazzi T, Miglietta A, Guastadisegni A, Pastore G. Long-term follow-up of patients with anti-HBe/HBV DNA-positive chronic hepatitis B treated for 12 months with lamivudine. *J Hepatol* 2000;32:300-306.
- [245] Shouval D, Lai CL, Chang TT, Cheinquer H, Martin P, Carosi G, et al. Relapse of hepatitis B in HBeAg-negative chronic hepatitis B patients who discontinued successful entecavir treatment: the case for continuous antiviral therapy. *J Hepatol*. 2009;50:289-295.
- [246] Lok AS, Liang RH, Chiu EK, Wong KL, Chan TK, Todd D. Reactivation of hepatitis B virus replication in patients receiving cytotoxic therapy. Report of a prospective study. *Gastroenterology* 1991;100:182-188.
- [247] Yeo W, Chan PK, Zhong S, Ho WM, Steinberg JL, Tam JS, et al. Frequency of hepatitis B virus reactivation in cancer patients undergoing cytotoxic chemotherapy: a prospective study of 626 patients with identification of risk factors. *J Med Virol*. 2000;62:299-307.
- [248] Yeo W, Johnson PJ. Diagnosis, prevention and management of hepatitis B virus reactivation during anticancer therapy. *Hepatology* 2006;43:209-220.
- [249] Yeo W, Chan TC, Leung NW, Lam WY, Mo FK, Chu MT, et al. Hepatitis B virus reactivation in lymphoma patients with prior resolved hepatitis B undergoing anticancer therapy with or without rituximab. *J Clin Oncol* 2009;27:605-611.
- [250] Durantel D, Carrouée-Durantel S, Werle-Lapostolle B, Brunelle MN, Pichoud C, Trépo C, Zoulim F. A new strategy for studying in vitro the drug susceptibility of clinical isolates of human hepatitis B virus. *Hepatology* 2004;40:855-864.

- [251] Durantel D, Brunelle MN, Gros E, Carrouée-Durantel S, Pichoud C, Villet S, et al. Resistance of human hepatitis B virus to reverse transcriptase inhibitors: from genotypic to phenotypic testing. *J Clin Virol* 2005;34 Suppl 1:S34-43.
- [252] Zhang YY, Summers J. Low dynamic state of viral competition in a chronic avian hepadnavirus infection. *J Virol.* 2000;74:5257-5265.
- [253] Locarnini S, Mason WS. Cellular and virological mechanisms of HBV drug resistance. *J Hepatol* 2006;44:422-431.
- [254] Köck J, Baumert TF, Delaney WE 4th, Blum HE, von Weizsäcker F. Inhibitory effect of adefovir and lamivudine on the initiation of hepatitis B virus infection in primary tupaia hepatocytes. *Hepatology.* 2003;38:1410-1418.
- [255] Zhou T, Saputelli J, Aldrich CE, Deslauriers M, Condreay LD, Mason WS. Emergence of drug-resistant populations of woodchuck hepatitis virus in woodchucks treated with the antiviral nucleoside lamivudine. *Antimicrob Agents Chemother* 1999;43:1947-1954.
- [256] Rehmann B, Ferrari C, Pasquinelli C, Chisari FV. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nat Med.* 1996;2:1104-1108.
- [257] Brechot C. Persistent hepatitis B virus infection in subjects without hepatitis B surface antigen: Clinically significant or purely "occult?" *Hepatology (Baltimore, Md).* 2001;34:194-203.
- [258] Raimondo G, Navarra G, Mondello S, Costantino L, Colloredo G, Cucinotta E, et al. Occult hepatitis B virus in liver tissue of individuals without hepatic disease. *J Hepatol.* 2008;48:743-746.
- [259] Raimondo G, Allain J-P, Brunetto MR, Buendia M-A, Chen D-S, Colombo M, et al. Statements from the Taormina expert meeting on occult hepatitis B virus infection. *J Hepatol.* 2008;49:652-657.
- [260] Hollinger FB, Sood G. Occult hepatitis B virus infection: a covert operation. *J Viral Hepat.* 2010;17:1-15.
- [261] Hui C-K, Cheung WWW, Zhang HY, Au WY, Yueng YH, Leung AYH, et al. Kinetics and Risk of De Novo Hepatitis B Infection in HBsAg-Negative Patients Undergoing Cytotoxic Chemotherapy. *Gastroenterology.* 2006;131:59-68.
- [262] Fukushima N, Mizuta T, Tanaka M, Yokoo M, Ide M, Hisatomi T, et al. Retrospective and prospective studies of hepatitis B virus reactivation in malignant lymphoma with occult HBV carrier. *Annals of Oncology.* 2009;20:2013-2017.
- [263] Shetty K, Hussain M, Nei L, Reddy KR, Lok ASF. Prevalence and significance of occult hepatitis B in a liver transplant population with chronic hepatitis C. *Liver Transpl.* 2008;14:534-540.
- [264] Hollinger FB. Hepatitis B virus infection and transfusion medicine: science and the occult. *Transfusion.* 2008;48:1001-1026.
- [265] Raimondo G, Pollicino T, Romanò L, Zanetti AR. A 2010 update on occult hepatitis B infection. *Pathol Biol.* 2010;58:254-257.
- [266] Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *J Clin Microbiol.* 1999;37:2899-2903.
- [267] Pas SD, Fries E, De Man RA, Osterhaus AD, Niesters HG. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. *J Clin Microbiol.* 2000;38:2897-2901.
- [268] Chen RW, Piiparinen H, Seppänen M, Koskela P, Sarna S, Lappalainen M. Real-

- time PCR for detection and quantitation of hepatitis B virus DNA. *J. Med. Virol.* 2001;65:250–256.
- [269] Jardi R, Rodriguez F, Buti M, Costa X, Cotrina M, Valdes A, et al. Quantitative detection of hepatitis B virus DNA in serum by a new rapid real-time fluorescence PCR assay. *J Viral Hepat.* 2001;8:465–471.
- [270] Leb V, Stöcher M, Valentine-Thon E, Hölzl G, Kessler H, Stekel H, et al. Fully automated, internally controlled quantification of hepatitis B Virus DNA by real-time PCR by use of the MagNA Pure LC and LightCycler instruments. *J Clin Microbiol.* 2004;42:585–590.
- [271] Liu Y, Hussain M, Wong S, Fung SK, Yim HJ, Lok ASF. A Genotype-Independent Real-Time PCR Assay for Quantification of Hepatitis B Virus DNA. *J Clin Microbiol.* 2007;45:553–558.
- [272] Isogawa M, Robek MD, Furuichi Y, Chisari FV. Toll-Like Receptor Signaling Inhibits Hepatitis B Virus Replication In Vivo. *J Virol* 2005;79: 7269–7272.
- [273] Deres K, Schröder CH, Paessens A, Goldmann S, Hacker HJ, Weber O, et al. Inhibition of hepatitis B virus replication by drug-induced depletion of nucleocapsids. *Science* 2003;299:893-896.
- [274] Billioud G, Pichoud C, Puerstinger G, Neyts J, Zoulim J. The main Hepatitis B virus (HBV) mutants resistant to nucleoside analogs are susceptible in vitro to non-nucleoside inhibitors of HBV replication. *Antiviral Res.* 2011;92:271-276.
- [275] Brezillon N, Brunelle MN, Massinet H, Giang E, Lamant C, DaSilva L, et al. Antiviral activity of Bay 41-4109 on hepatitis B virus in humanized Alb-uPA/SCID mice. *PLoS One.* 2011;6:e25096.
- [276] Petersen J, Dandri M, Mier W, Lutgehetmann M, Volz T, et al. Prevention of hepatitis B infection in vivo by entry inhibitors derived from the large envelope protein. *Nat Biotechnol* 2008;26:335-341.
- [277] Lütgehetmann M, Mancke LV, Volz T, Helbig M, Allweiss L, Bornscheuer T, et al. Humanized chimeric uPA mouse model for the study of hepatitis B and D virus interactions and preclinical drug evaluation. *Hepatology* 2012;55:685-694.
- [278] Krepstakies M, Lucifora J, Nagel CH, Zeisel MB, Holstermann B, Hohenberg H, et al. A New Class of Synthetic Peptide Inhibitors Blocks Attachment and Entry of Human Pathogenic Viruses. *J. Infect. Dis.* 2012;205:1654–1664.
- [279] Cai D, Mills C, Yu W, Yan R, Aldrich CE, Saputelli JR, et al. Identification of disubstituted sulfonamide compounds as specific inhibitors of hepatitis B virus covalently closed circular DNA formation. *Antimicrob Agents Chemother* 2012;56:4277-4288.
- [280] Murray KF, Wysocki J, Rossi S, Corsa AC, Dihn P, McHutchison J, et al. Randomized, placebo-controlled trial of tenofovir disoproxil fumarate in adolescents with chronic hepatitis B. *Hepatology* 2012; Epub ahead of print, doi: 10.1002/hep.25818.

1.2. Hepatitis B virus biology

Hepatitis B virus (HBV) is a member of *Hepadnaviridae* family causing transient and chronic infection in man. As described in the previous chapter, available antiviral treatments are only partially effective, but development of new drugs has been hampered by a still incomplete knowledge of the viral life cycle [1]. As all hepadnaviruses, HBV is indeed hepatotropic and highly species-specific, which has limited research possibilities because of the lack of a suitable *in vitro* model. The reasons of such specificity have not been fully clarified yet. Comprehension of HBV biology is the starting point for understanding HBV hepatotropism, which would in turn lead the development of new antiviral drugs directed against new targets.

1.2.1. Hepadnaviridae

Hepadnaviruses are a family of small, enveloped DNA viruses that replicate through reverse transcription of a RNA intermediate [2]. Such viruses are all hepatotropic and can result in different outcomes, from subclinical transient infection to chronic hepatitis leading to cirrhosis and hepatocellular carcinoma [3]. Viruses infecting mammals, such as HBV, woodchuck hepatitis virus (WHV) and ground squirrel hepatitis virus (GSHV), are grouped in the genus Orthohepadnavirus, and share similarities in DNA sequence and genome organization [4,5]. Duck hepatitis B virus (DHBV) is more distantly related, and belongs to the genus Avihepadnavirus [6]. All these viruses share many structural and biological characteristics, although they have a distinct species-specificity [7]. As HBV can infect only man and the great apes, and hepatocytes are the only cells susceptible of infection, other viruses have been used to clarify hepadnaviral life cycle. WHV has been studied

as a model for chronic liver disease and infection-related liver cancer, whereas DHBV allowed unveiling how such viruses replicate. Nevertheless, existing differences with HBV preclude a complete transposition of such discoveries to the human virus, obliging researchers to develop new models to study HBV life cycle directly.

1.2.2. Structure of the HBV virion

The HBV virion (called Dane particle from the author who described its appearance in 1970) is a sphere measuring 42 nm in diameter [8], and is formed by an enveloped icosahedral capsid containing the 3.2 Kb partially double-stranded DNA coupled to a RNA-dependent DNA polymerase (figure 1.2.1). The envelope is formed by 3 different glycosylated, type II transmembrane surface (HBsAg) proteins, which are the products of a single (preS/S) open reading frame. The most represented is the small (SHBs) protein (70%), followed by large (LHBs; 20%) and middle (MHBs; 10%) proteins. They share a common C-terminal S domain (226 amino acids, 24-27 KDa), with 4 transmembrane-spanning alpha helices and a potential N-glycosylation site [9-11], but differ in their N termini (figures 1.2.2 - 1.2.3) . Amino acids 120-160 of the S domain (which constitutes the entire SHBs protein) are the most exposed on the surface of

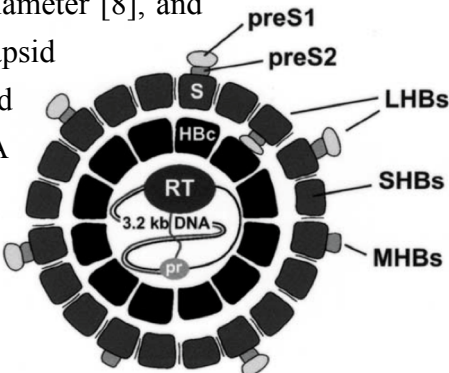


Figure 1.2.1. Schematic representation of Dane particle (from Glebe 2007).

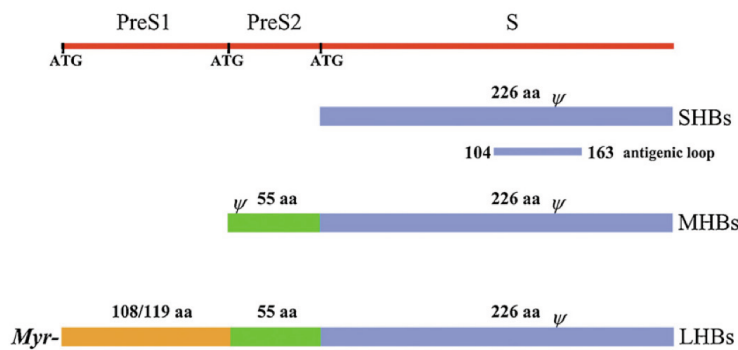


Figure 1.2.2. Schematic representation of HBV envelope proteins (modified from Xie 2010). SHBs, MHBs and LHBs are translated from distinct start codons in the same open reading frame (Ψ marks N-glycosylations sites).

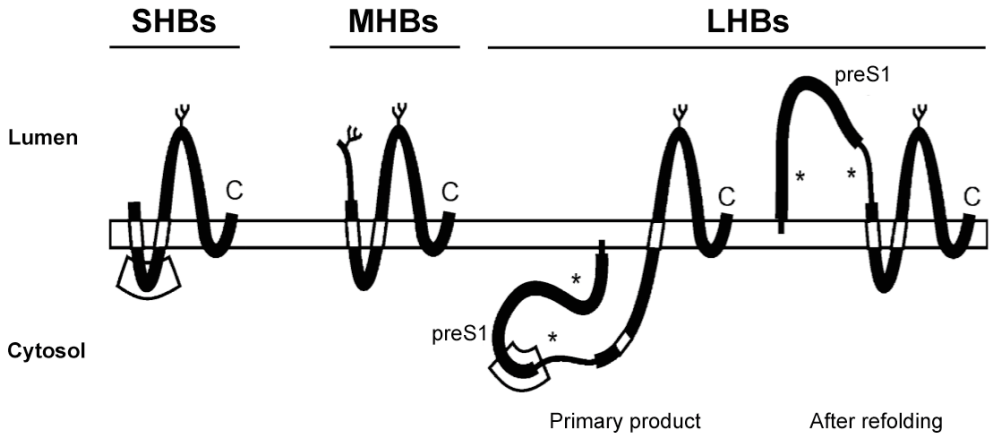


Figure 1.2.3. Transmembrane conformation the HBV envelope proteins (modified from Bruss 2004). The C terminus (C) is oriented towards the ER lumen (and then the external surface of the virion). N-linked glycans residues bound to the luminal loop are shown as branched structures. The MHBs protein has a folding similar to SHBs, and the N-glycosylated preS2 domain (thinner line) is located in the lumen. The LHBs protein may have two different conformations. In the primary folding the preS domains are located in the cytosol. Part of LHBs chains refold and expose their pre-S domains at the luminal side of the membrane (i.e. outside the virus). Boxes identify cytosolic domains that have been suggested to interact with the capsid during virion assembly.

viral particles and determine HBs antigenicity [12]. HBsAg exists in different serological subtypes [13]. Purified SHBs particles of adw2 subtype, produced in yeast cells, are the most widely used recombinant hepatitis B vaccine [14]. SHBs are highly cross-linked by disulfide-bonds and do not disassemble in the presence of detergents. A preS2 domain (55 amino acids) is present in both MHBs and LHBs proteins, and contains N-glycosylation and O-glycosylation sites. The LHBs protein contains an additional preS1 domain of 108 or 119 amino acids, depending on the genotype [15]. As it will be discussed in the following paragraphs, preS1 has been shown to be essential for viral binding to the cell membrane [16-22]. A fraction of LHBs proteins maintain their preS1 domain on the cytosolic side of endoplasmic reticulum membrane, providing the ligand for core particles, being therefore necessary for virion envelopment [23]. The N-terminus of preS1 domain is posttranslationally modified by addition of a myristic acid [24], which is not necessary to assemble the viral particle, but is required for infection [25,26]. The viral core is composed by either 90 or 120 dimers of core proteins (HBcAg), assembled to form an icosahedron of 36 nm, with a triangulation number (T) of 3 or 4, respectively (figure 1.2.4). HBV core proteins (183 amino acids, 21

KDa), which have a C-terminal region capable of interacting with viral RNA and DNA, form 4-helix spikes on the surface of the shell [27-29]. Such spikes interact with the cytoplasmic domains of envelope proteins [30].

In Dane particles, viral partially double-stranded relaxed circular DNA (rcDNA) is located inside the core (figure 1.2.5). As described in detail in the following paragraphs, once the virus enters a susceptible cell, rcDNA is converted in covalently closed circular DNA (cccDNA), which in turn serves as template for the transcription of viral genes. Among different mRNAs coding for viral proteins, the terminally

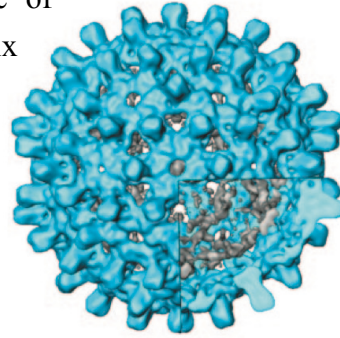


Figure 1.2.4: Map of a T=4 DNA-containing mature core (from Roseman 2005). The outer protein parts are colored blue and the inner shell comprising DNA and C-terminal tails is colored gray.

redundant pregenomic RNA (pgRNA) is synthesized.

pgRNA is then linked to the viral polymerase and

assembled in the capsid. Eventually, pgRNA

is reverse transcribed into new rcDNA.

Only minus-strand of the 3.2 Kb rcDNA is

completed, and is covalently linked to the viral DNA polymerase at its 5' end [31].

Plus-strand DNA is incomplete, with a heterogeneous length, and has a 5'-linked RNA oligonucleotide serving as primer.

Polymerase is coded by the P open reading frame, which covers nearly 80% of the hepadnaviral genome. Its N-terminal domain (TP) has a major role in pgRNA packaging and in priming of minus-strand DNA, and is separated by the reverse transcriptase (RT) and RNase H (RH) domains by a variable and dispensable spacer [32].

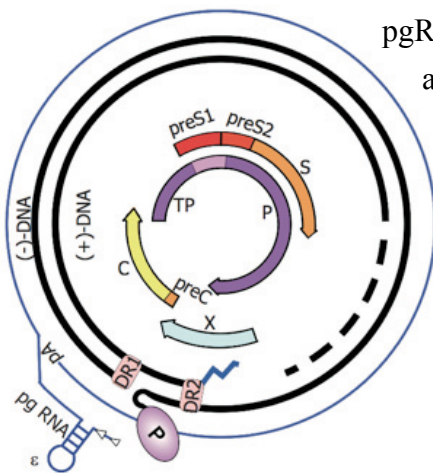


Figure 1.2.5. HBV genome organization (from Beck & Nassal 2007). Plus and minus strand of rcDNA are indicated by thick black lines, with the polymerase (P) covalently linked to the 5' end of the (-)-DNA, and the RNA primer (zigzag line) at the 5' end of (+)-DNA. (+)-strands has a variable length (dashed line). The outer circle symbolizes pgRNA. The four open reading frames for core (C), P, preS/S, and X are shown inside.

1.2.3. HBe and HBx proteins

The viral genome encodes for two other proteins that are not included in the virion and whose role is still unclear. HBeAg, which is translated from the precore mRNAs and shares substantial sequence overlap with HBcAg, is not required for virus assembly, replication or infection [33]. Nevertheless, it has been shown to induce T-cell tolerance in newborns of carrier mothers (see page 24) [34,35]. Secretion of a great amount of HBeAg is needed to maintain tolerance and promotes chronicity of the infection [36]. On the other way, HBeAg may also work as an immunogen (mostly in immunocompetent adults), eliciting a cytotoxic T cell response. Such a role of HBeAg in the clearance of infected cells may explain the selective advantage of HBeAg-negative mutants [37].

HBx protein is a 154-amino acids polypeptide (17 KDa) encoded in small quantities by the smallest open reading frame of HBV genome. It is present in orthohepadnaviruses but absent in avihepadnaviruses, and is localized mainly in the nucleus, where it indirectly transactivates several host as well as viral genes. As shown in figure 1.2.6, HBx interacts with mitochondria, p53, proteasome subunits and DDB1, and stimulates signal transduction [1,38]. HBx is necessary for an effective viral replication both in vitro and in vivo [39-43], and it was shown to regulate cell cycle and induce G1 phase arrest of hepatocytes, regulate apoptosis and play a role in HBV-induced development of liver cancer [44-46]. However, its exact mechanism of action is still unclear.

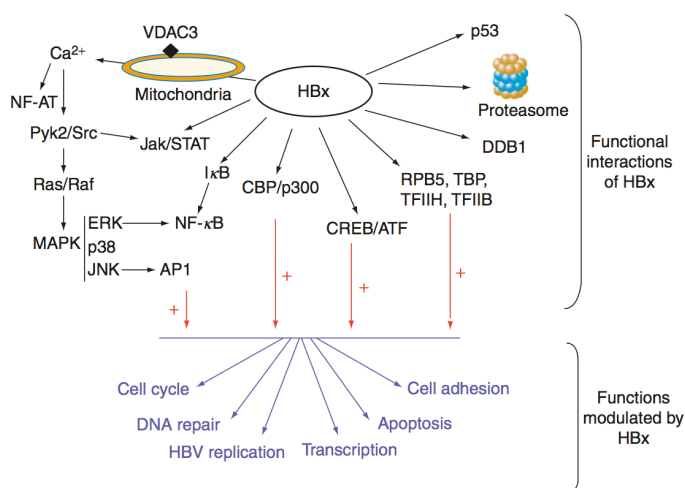


Figure 1.2.6. Multiple functions of HBx (from Benhenda 2009)

1.2.4. Viral entry into susceptible cells

HBV binding and entry into susceptible cells are poorly understood, and seem to be the most important determinants of HBV hepatotropism. Comprehension of these mechanisms has greatly advanced in the last decades thanks to primary Tupaia hepatocytes and HepaRG cells, two new in vitro models of HBV infection that will be discussed in next chapter. The quest for the HBV receptor(s) is justified far beyond scientific curiosity, as it could lead to the development of new drugs to prevent HBV infection and spread.

In 2002, Ying and colleagues showed that sulphated polyanions (such heparin or dextran sulfate) inhibit HBV binding to primary human hepatocytes and HepG2 cell membrane [47]. Nevertheless, heparan sulfate at cell surface (which is structurally similar to heparin) was shown not to be responsible for HBV initial attachment to the cell membrane by demonstrating that digestion with heparinase before inoculation did not affect HBV binding. Some years later, using HepaRG cell model, the inhibitory effect of sulfated polyanions was confirmed (although heparin inhibition was dependent on the presence of polyethylene glycol during infection; figure 1.2.7A), sulfation of cellular glycosaminoglycans was shown to be necessary for infection, and neutralization of negative charges on the cell membrane was demonstrated to inhibit infection [48]. Contrarily to what observed by Ying and colleagues, the authors demonstrated that heparinase digestion decreased viral attachment, suggesting a prominent role of heparan sulfate as the initial attachment site of HBV L protein (figure 1.2.7B). As polyethylene glycol (PEG) is known to enhance HBV binding to glycosaminoglycans on cell membrane, such a difference between the two studies could be explained by the presence of PEG during the infection experiments with HepaRG. Such an effect of PEG on viral attachment was shown to be independent of cell differentiation state [48]. Inhibition of HBV infection with highly sulfated polyanions, as well as the importance of sulfation for infection, was further confirmed on primary Tupaia hepatocytes and HepG2 cells, using purified HBV from human plasma, in the absence of PEG [49]. Nevertheless, in this study, a complete inhibition of viral binding with polyanions could not be achieved. The difference with Ying's study was attributed to the possible contamination of the inoculum used by the latter with partially or non-enveloped core particles. Moreover, Leistner et al. showed

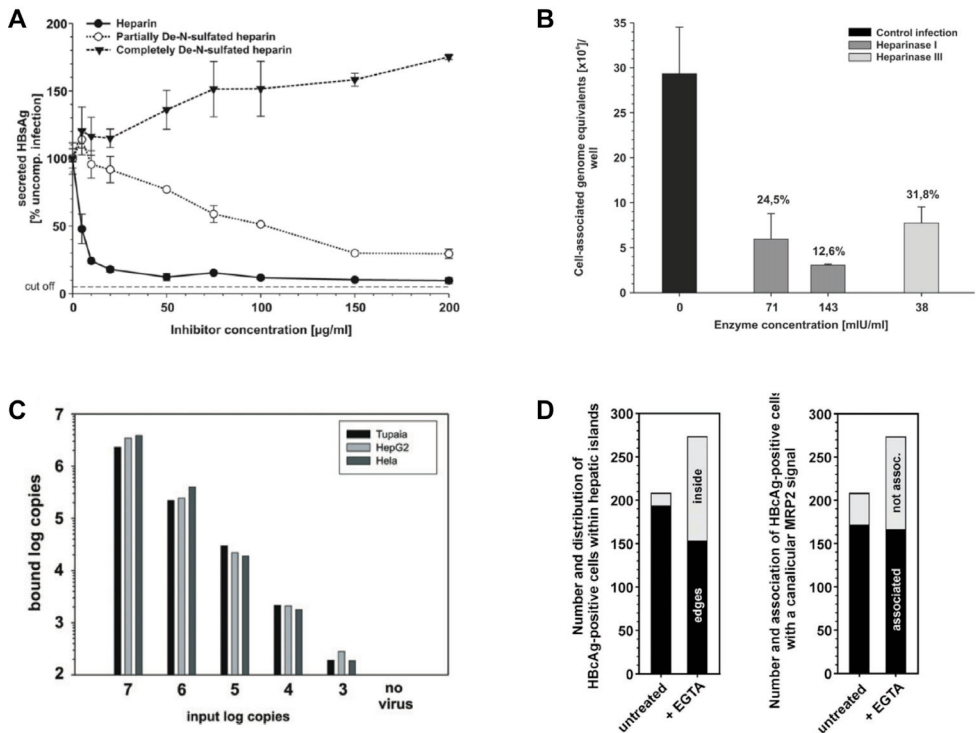


Figure 1.2.7. A) Sulphated polyanions inhibit HBV infection (from Schulze 2007): HBV infection is specifically inhibited by heparin, and the degree of sulfation correlates with the ability to interfere with infection. **B) Cell-surface glycosaminoglycans are implicated in HBV binding (from Schulze 2007):** pretreatment of HepaRG cells with heparinase reduces HBV binding through enzymatic removal of glycosaminoglycans. **C) Binding of HBV to different cell lines (from Leistner 2008):** HBV binding is equivalent between susceptible (primary Tupaia hepatocytes) and non-susceptible cells (HepG2 and Hela). **D) Cell polarization is needed for HBV infection (from Schulze 2012):** HepaRG cells susceptible to HBV infection are located at the edges of hepatic islands (left panel), and are for the most part associated with a canalicular MRP2 signal (right panel). Pretreatment with EGTA allows the infection of cells in internal parts of an island, which do not form canalicular structure and whose infection is otherwise restricted by physical barriers.

that HBV binding was equivalent between susceptible and non-susceptible cells (figure 1.2.7C), and suggested that an initial, non cell-specific attachment of HBV to heparan sulfate proteoglycans could be followed by a more specific subsequent binding to an unidentified hepatocyte-specific receptor. Recently, Schulze et al. demonstrated that hepatocyte polarization is necessary for HBV entry (figure 1.2.7D), suggesting that the putative HBV receptor should be localized on the basolateral membrane of susceptible cells [50]. Although such a receptor is still to be defined, viral structures responsible for the attachment of HBV to the cell membrane have been well described. Neurath et al. showed that the attachment

of HBV to HepG2 cells is mediated by amino acids 21-47 of preS1 domain (corresponding to amino acids 10-36 of genotype D) of LHBs protein [21]. The importance of such a domain (figure 1.2.8) for HBV binding was confirmed by a later study by Petit and colleagues [17]. Amino acids 3-77 of preS1 (genotype D)

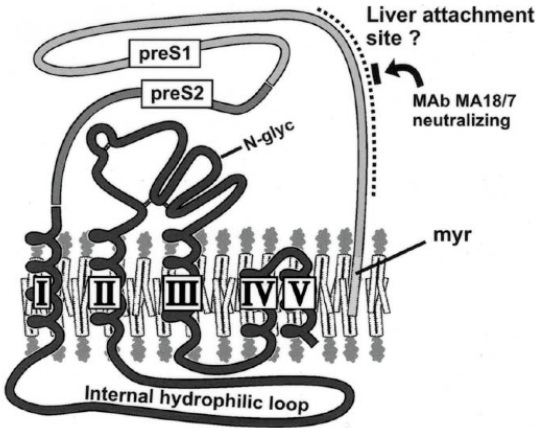


Figure 1.2.8. Schematic representation of LHBs preS1 domain (from Glebe 2005).

proved to be necessary for the virus to bind to PHHs [18]. Importance of preS1 domain (amino acids 2-48, genotype D) was further confirmed by the demonstration that its inhibition with monoclonal antibodies and specific lipopeptides prevents HBV attachment to cell membrane [19,20,22]. A clinical trial with myristoylated preS/2-48 peptides (Myrccludex-B) is currently ongoing (see chapter 1.1.6, page

41). Glebe et al. showed that amino acids 2-18 of preS1 (genotype D) are essential to inhibit HBV infection in PTHs (figure 1.2.9) [20], whereas amino acids 5-21 (genotype D) are required for the inhibition of HBV infection of HepaRG cells [51]. Although such a dissimilarity could be explained by the diverse approaches used in the two studies, a difference in HBV binding mechanism between PTHs and HepaRG cannot be excluded.

Many different receptors have been described to recognize preS1 domain. Among the many candidate receptors for HBV, asialoglycoprotein receptor (ASGPR) is the only to be expressed almost exclusively by hepatocyte (table 1.2.1). ASGPR is a Ca^{2+} -dependent lectin expressed on the sinusoidal and basolateral hepatocellular membrane,

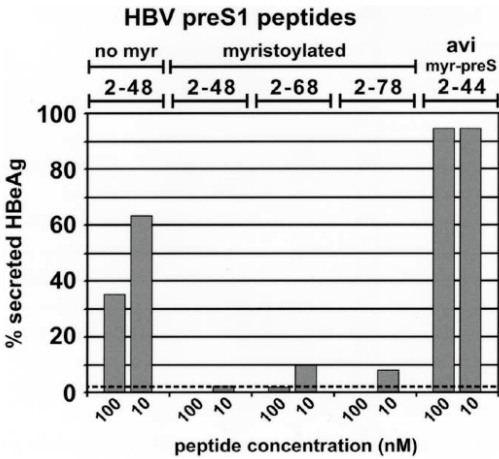


Figure 1.2.9. preS1 domain is required for HBV infection (from Glebe 2005): inhibition of infection with myristoylated HBV preS1 peptides.

Protein	Binding site on HBV envelope	Subcellular localization	Liver specificity
IL-6	PreS1 21–47	Serum	No
SCAA1	PreS1 21–47	Plasma membrane	No
Lipoprotein lipase (LPL)	PreS1 21–47	Serum	Targeted to hepatocytes during lipid metabolism
IgA receptor	PreS1 21–32	Plasma membrane	No
Asialoglycoprotein receptor (ASGPR)	PreS1	Plasma membrane	Yes
Transferrin receptor	PreS2	Plasma membrane	No
Apolipoprotein H (ApoH)	PreS1, phospholipids	Serum, associated with chylomicrons and high-density lipoproteins	Targeted to hepatocytes during lipid metabolism
Polymerized serum albumin (pHSA)	PreS2	Serum	No
Annexin V	S	Plasma membrane	No
Fibronectin	PreS2	Extracellular matrix	No
p31	PreS1 21–47	Intracellular	n.d.
50-kDa serum glycoprotein	PreS1 and PreS2	Serum	n.d.
p80	PreS1 12–20, 82–90	Plasma membrane	No

Table 1.2.1. Host proteins potentially involved in HBV entry into susceptible cells (from Xie 2010).

which is responsible for the uptake of desialylated glycoproteins [52,53]. Basma and colleagues showed that ASGPR is upregulated in embryonic stem cells upon hepatic differentiation [54]. Treichel and colleagues showed that ASGPR mediates HBV binding and endocytosis and that such a binding is inhibited by ASGPR-specific ligands (asialofetuin and asialoorosomucoid), as well as by calcium chelator EDTA (table 1.2.2) and by anti-ASGPR antibodies [55]. ASGPR was shown to recognize

viral preS1 domain, and this interaction was inhibited by anti-preS1 monoclonal antibodies. ASGPR-preS1 interaction was subsequently demonstrated by a yeast two-hybrid system [56]. Owada et al. confirmed that asialofetuin and EDTA inhibited HBV

Inhibitor	Inhibition	
	100 µg /well	500 µg /well
Asialoorosomucoid	67	100
Orosomucoid	0	31
Asialofetuin	56	90
Fetuin	0	37
Bovine serum albumin	3.6	5.3
Human serum albumin	0	0
Bovine haemoglobin	0	4.3
Human immunoglobulin	0	5.1
EDTA (10mM)	98	98

Table 1.2.2. Inhibition of HBV attachment to human ASGPR (modified from Treichel 1994).

binding to HepG2 cells and demonstrated that desialylation of HBV improved its binding to ASGPR [57]. Polyions were shown not to influence such an interaction. The authors also speculated on a possible role of sialidase present in sinusoidal endothelial cells in processing HBV particles to improve their binding to hepatocytes. Furthermore, ASGPR was shown to be overexpressed in HepG2.2.15 cells (a transformed cell line stably transduced with HBV genome), and its downregulation with antisense oligonucleotides was shown to reduce HBV

DNA levels in HepG2.2.15 culture media [58,59].

Other receptors have been implicated in HBV uptake. Budowska and colleagues showed that fibronectin present in liver sinusoids is able to bind HBV recognizing the preS2 domain of LHBs and MHBs surface proteins, and suggested that it could play a role in viral entry [60]. Circulating fibronectin was also shown to be a ligand of ASGPR [61]. Yang et al. demonstrated by co-immunoprecipitation that ASGPR, fibronectin and HBsAg bind to each other in HepG2.2.15 cells [62]. According to the presented results, fibronectin might interact with HBsAg through its heparin-binding domain or through the N-terminus of its cell-binding domain, and with ASGPR with its cell-binding domain. The authors confirmed that HBsAg binds carbohydrate recognition domain (CRD) of ASGPR. While ASGPR overexpression enhanced HBsAg binding to HepG2 cells, the preincubation of the cells with fibronectin had no effect on such a binding. Nevertheless, preincubation of HBsAg with fibronectin slightly, but significantly, increased HBsAg binding to HepG2 cells.

Overall, the exact mechanism of HBV binding and entry into susceptible cells is still to be unrevealed. ASGPR is a promising candidate receptor, but a clear demonstration of its mechanism of action in a non-transformed human model is lacking. Whether viral entry is a double-step process involving a low-affinity attachment to heparan sulfate proteoglycans followed by a high-affinity binding to a liver-specific receptor, and whether the latter is indeed ASGPR, is still to be confirmed in a human model free of confounding variables (such as the use of PEG). Furthermore, the role of co-players such as endogenous sialidase or fibronectin, is to be clarified.

1.2.5. Viral replication

Once the HBV virion has bound to the putative cell receptor, the viral envelope fuses with the cell membrane and the capsid is released in the cytosol [11]. It is then transferred to the cell nucleus using microtubule transport system and nuclear pore complexes (figure 1.2.10) [63,64]. The first event of hepadnaviral replication is the conversion of rcDNA into supercoiled covalently closed circular DNA (cccDNA). cccDNA is formed in the nucleus and serves as template for

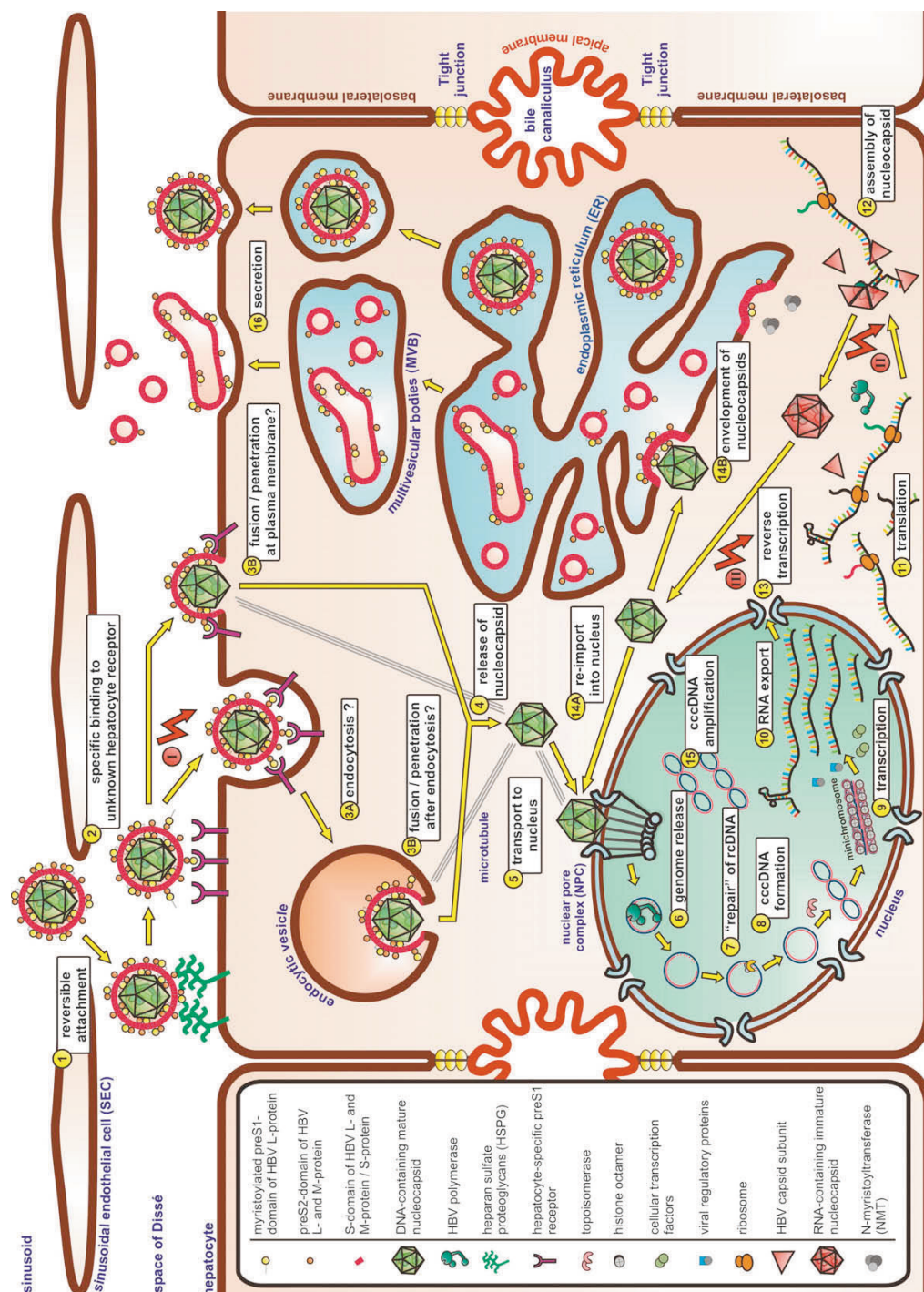


Figure 1.2.10. HBV replication cycle (from Urban 2010)

viral genes transcription [65]. Its persistence inside the infected hepatocytes as a stable episome is at the basis of HBV chronicity [75,76], and its elimination is the ultimate goal of antiviral therapy. Conversion of rcDNA into cccDNA (figure 1.2.11) requires the removal of the covalently attached reverse transcriptase (which is cleaved in the capsid), the completion of plus-strand DNA and the ligation of the ends of the two DNA strands. Whereas the latter are mechanisms typical of DNA repair and are supposed to use cellular machinery, the role of viral polymerase (which was shown to be necessary for cccDNA formation) and how it is cleaved from the 5' end of rcDNA have not been clarified yet [66]. To maximize transcription of viral genes and to ensure that viral genome is not lost during cell division, several copies (1-50) of cccDNA accumulate in the nucleus through an amplification loop (figure 1.2.10) that drives part of newly formed mature capsid containing rcDNA back to nucleus [65]. HBV, unlike all other retroviruses, brings a DNA molecule in the virion, which gives it the specific evolution advantage of allowing such a cccDNA amplification loop.

Viral mRNAs are transcribed from cccDNA by the cellular RNA polymerase II. HBV genome encodes for three subgenomic mRNAs (preS1 and preS2/S for the surface proteins and X for the HBx protein) and two greater-than-genome length mRNAs (precore RNA and pgRNA). The latter is terminally redundant, with a second copy of the direct repeats (DR1 and DR2) and the ϵ signal, plus the poly-A tail. It encodes for the core protein and viral polymerase. Furthermore, pgRNA has a major role in HBV replication, as it is the template used for reverse transcription. The precore RNA (preC/C) is nearly identical to pgRNA, except it starts slightly upstream and encodes for the precore precursor protein giving rise to HBeAg.

Each gene of HBV has one or more promoters regulating its activity and such promoters are in turn regulated by one or both of the viral enhancer elements (EnI

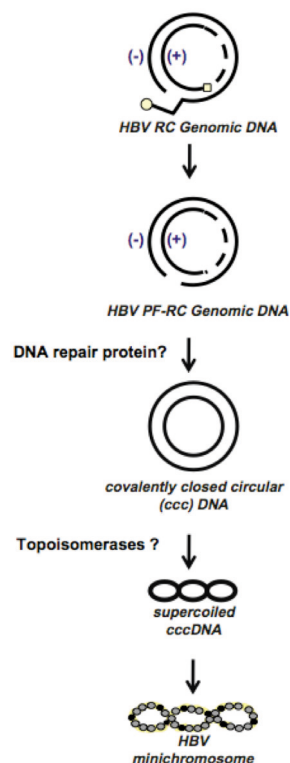


Figure 1.2.11. Synthesis of cccDNA from rcDNA (from Levrero 2009)

and En2), that are located upstream of the core promoter. Viral promoters and enhancers are regulated by the expression of liver-enriched transcription factors. The activity of preS1 and preC/C promoters is governed by multiple liver-enriched transcription factors synergistically cooperating to control pgRNA transcription and viral replication [67-69]. Hepatocyte nuclear factor 4 α (HNF4 α), whose expression is restricted to the liver, kidney, and intestine, is essential for pgRNA synthesis (figure 1.2.12) [70,71]. HNF4 α is essential for morphological and functional differentiation of hepatocytes [72,73], and has been demonstrated to link the degree of hepatocyte differentiation to the efficiency of HBV replication [71].

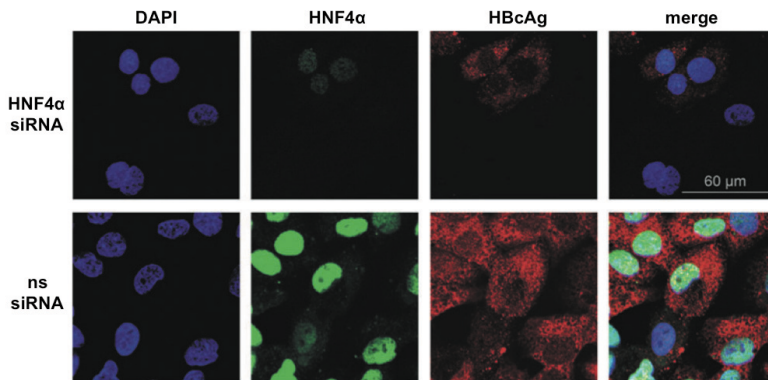


Figure 1.2.12. Analysis of HBcAg and HNF4 α expression in single cells (from Quasdorff 2008). Immunofluorescence staining of HNF4 α (AlexaFluor488TM, green), HBcAg (Alexa-Fluor568TM, red) or cell nuclei (DAPI, blue) in HepG2-H1.3 cells (a cell line containing one copy of a 1.3-fold overlenght HBV genome) transfected with either HNF4 α -specific or ns siRNA at day 7 post transfection.

Hepatocyte nuclear factor 1 (HNF1), as well as retinoid X receptor α (RXR α) and peroxisome proliferator-activated receptor α (PPAR α), have also been suggested to play a role in HBV replication control [70,71,74]. Hepatocyte nuclear factor 3 (HNF3) antagonizes the nuclear hormone receptor-mediated viral replication by inhibiting pgRNA synthesis [70]. The level of viral replication in a cell depends not only on nuclear hormone receptors but on their relative abundance compared with HNF3. Overall, the dependence of HBV replication on nuclear hormone receptors is a major determinant of viral tropism.

The existence of 4 open reading frames (figure 1.2.5) allows the translation of all viral proteins from the mRNAs described above: preS/S for surface proteins (figure 1.2.2), preC/C for HBeAg and core protein, P for polymerase and X for

HBx protein. Efficiency of reading varies between different open reading frames, and allows further regulation of protein synthesis at the level of translation.

The next step in hepadnaviral replication is the specific packaging of pgRNA and reverse transcriptase into newly forming capsids (figure 1.2.10). Key actors are the encapsidation signal ϵ on the pgRNA and the polymerase, which binds to ϵ . Although still poorly understood, this interaction mediates the recruitment of core protein dimers and leads to packaging of the immature core. At this point, the viral polymerase starts reverse transcription of pgRNA. After the first nucleotide is covalently linked to P protein, the minus-strand DNA is completed, giving rise to the single-stranded DNA (ssDNA), and pgRNA is degraded. Then the partial second DNA strand is synthesized, leading to a new molecule of rcDNA [32].

A full-length linear double-stranded DNA (dslDNA) is formed as a by-product of viral genome synthesis (in about 5% of the nucleocapsids) [78-79]. Such viral DNA molecules may undergo recombination with cellular DNA in a small fraction of infected hepatocytes and be integrated in the cellular genome (one viral genome per 10^3 to 10^4 cells) [78]. These integrations contain enhancers that can activate cellular promoter which are normally silent and can have oncogenic consequences for the host [80-82].

After rcDNA is completed, cores are finally mature and interact with cytosolic preS domains of LHBs (figure 1.2.3), budding to the lumen of a post-endoplasmic reticulum, pre-Golgi compartment [10]. Envelopment does not occur until plus-strand DNA is synthesized. According to current knowledge, a signal is propagated from the interior of the core upon completion of DNA synthesis and originates core maturation, which in turn triggers envelopment [30].

Along with Dane particles, the HBV replication cycle produces a great amount of nucleocapsid-free subviral particles (SVP). These SVPs are mainly composed of the SHBs protein (% of MHBs and LHBs), and form 22 nm spheres and filaments, which are secreted from the infected cell in 10000-fold excess over virions. Such particles interact with antibodies and are probably responsible for the immune complex syndromes sometimes occurring during transient infection.

1.2.6. References

- [1] Seeger C, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000;64:51-68.
- [2] Summers J, Mason WS. Replication of the genome of a hepatitis B--like virus by reverse transcription of an RNA intermediate. *Cell* 1982;29:403-415.
- [3] Ganem D, Schneider RJ. (2001) in *Fields Virology*, eds. Fields BN, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Straus SE, Knipe DM. Lippincott Williams & Wilkins, Philadelphia, 4th Ed., Vol. 2, pp. 2923–2969.
- [4] Summers J, Smolec JM, Snyder R. A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. *Proc Natl Acad Sci USA* 1978;75:4533-4537.
- [5] Marion PL, Oshiro LS, Regnery DC, Scullard GH, Robinson WS. A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. *Proc Natl Acad Sci USA* 1980;77:2941-2945.
- [6] Mason WS, Seal G, Summers J. Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. *J Virol* 1980;36:829-836.
- [7] Summers J. Three recently described animal virus models for human hepatitis B virus. *Hepatology* 1981;1:179-183.
- [8] Dane DS, Cameron CH, Briggs M. Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. *Lancet* 1970;1(7649):695-698.
- [9] Berting A, Hahnen J, Kröger M, Gerlich WH. Computer-aided studies on the spatial structure of the small hepatitis B surface protein. *Intervirology* 1995;38:8-15.
- [10] Bruss V. Envelopment of the hepatitis B virus nucleocapsid. *Virus Research* 2004;106:199–209.
- [11] Glebe D, Urban S. Viral and cellular determinants involved in hepadnaviral entry. *World J Gastroenterol* 2007;13:22–38.
- [12] Heermann KH, Kruse F, Seifer M, Gerlich WH. Immunogenicity of the gene S and Pre-S domains in hepatitis B virions and HBsAg filaments. *Intervirology* 1987;28:14-25.
- [13] Kramvis A, Kew M, François G. Hepatitis B virus genotypes. *Vaccine* 2005;23:2409-2423.
- [14] McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR. Human hepatitis B vaccine from recombinant yeast. *Nature* 1984;307:178-180.
- [15] Heermann KH, Goldmann U, Schwartz W, Seyffarth T, Baumgarten H, Gerlich WH. Large surface proteins of hepatitis B virus containing the pre-s sequence. *J Virol* 1984;52:396-402.
- [16] Neurath AR, Kent SB, Strick N, Parker K. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. *Cell* 1986;46:429-436.
- [17] Petit MA, Dubanchet S, Capel F, Voet P, Dauguet C, Hauser P. HepG2 cell binding activities of different hepatitis B virus isolates: inhibitory effect of anti-HBs and anti-preS1(21-47). *Virology* 1991;180:483-491.
- [18] Le Seyec J, Chouteau P, Cannie I, Guguen-Guillouzo C, Gripon P. Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain. *J Virol* 1999;73:2052-2057.
- [19] Glebe D, Aliakbari M, Krass P, Knoop EV, Valerius KP, Gerlich WH. Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B

virus. *J Virol* 2003;77:9511-9521.

- [20] Glebe D, Urban S, Knoop EV, Cag N, Krass P, Grün S, et al. Mapping of the Hepatitis B Virus Attachment Site by Use of Infection-Inhibiting preS1 Lipopeptides and Tupaia Hepatocytes. *Gastroenterology* 2005;129:234–245.
- [21] Neurath AR, Kent SB, Strick N, Parker K. Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. *Cell* 1986;46:429-436.
- [22] Schulze A, Schieck A, Ni Y, Mier W, Urban S. Fine mapping of pre-S sequence requirements for hepatitis B virus large envelope protein-mediated receptor interaction. *J Virol* 2010;84:1989-2000.
- [23] Bruss V, Lu X, Thomssen R, Gerlich WH. Post-translational alterations in transmembrane topology of the hepatitis B virus large envelope protein. *EMBO J* 1994;13:2273-2279.
- [24] Persing DH, Varmus HE, Ganem D. The preS1 protein of hepatitis B virus is acylated at its amino terminus with myristic acid. *J Virol* 1987;61:1672-1677.
- [25] Bruss V, Hagelstein J, Gerhardt E, Galle PR. Myristylation of the large surface protein is required for hepatitis B virus in vitro infectivity. *Virology* 1996;218:396-399.
- [26] Gripon P, Le Seyec J, Rumin S, Guguen-Guillouzo C. Myristylation of the hepatitis B virus large surface protein is essential for viral infectivity. *Virology* 1995;213:292-299.
- [27] Crowther RA, Kiselev NA, Böttcher B, Berriman JA, Borisova GP, Ose V, Pumpens P. Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. *Cell* 1994;77:943-950.
- [28] Böttcher B, Wynne SA, Crowther RA. Determination of the fold of the core protein of hepatitis B virus by electron cryomicroscopy. *Nature* 1997;386:88-91.
- [29] Conway JF, Cheng N, Zlotnick A, Wingfield PT, Stahl SJ, Steven AC. Visualization of a 4-helix bundle in the hepatitis B virus capsid by cryo-electron microscopy. *Nature* 1997;386:91-94.
- [30] Roseman AM, Berriman JA, Wynne SA, Butler PJG, Crowther RA. A structural model for maturation of the hepatitis B virus core. *Proc Natl Acad Sci USA* 2005;102:15821–15826.
- [31] Summers J, O'Connell A, Millman, I. Genome of hepatitis B virus: restriction enzyme cleavage and structure of DNA extracted from Dane particles. *Proc Natl Acad Sci USA* 1975;72:4597–4601.
- [32] Beck J, Nassal M. Hepatitis B virus replication. *World J Gastroenterol* 2007;13:48–64.
- [33] Milich D, Liang TJ. Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. *Hepatology* 2003;38:1075–1086.
- [34] Milich DR, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? *Proc Natl Acad Sci USA* 1990;87:6599–6603.
- [35] Chen MT, Billaud JN, Sällberg M, Guidotti LG, Chisari FV, Jones J, Hughes J, Milich DR. A function of the hepatitis B virus precore protein is to regulate the immune response to the core antigen. *Proc Natl Acad Sci USA* 2004;101:14913-14918.
- [36] Chen M, Sällberg M, Hughes J, Jones J, Guidotti LG, Chisari FV, et al. Immune tolerance split between hepatitis B virus precore and core proteins. *J Virol* 2005;79:3016-3027.

- [37] Frelin L, Wahlström T, Tucker AE, Jones J, Hughes J, Lee BO, et al. A Mechanism To Explain the Selection of the Hepatitis e Antigen-Negative Mutant during Chronic Hepatitis B Virus Infection. *J Virol* 2009;83:1379–1392.
- [38] Benhenda S, Cougot D, Buendia MA, Neuveut C. Hepatitis B virus X protein molecular functions and its role in virus life cycle and pathogenesis. *Adv Cancer Res* 2009;103:75-109.
- [39] Zoulim F, Saputelli J, Seeger C. Woodchuck hepatitis virus X protein is required for viral infection in vivo. *J Virol* 1994;68:2026-30.
- [40] Keasler VV, Hodgson AJ, Madden CR, Slagle BL. Enhancement of hepatitis B virus replication by the regulatory X protein in vitro and in vivo. *J Virol* 2007;81:2656-2662.
- [41] Keasler VV, Hodgson AJ, Madden CR, Slagle BL. 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* 2009;390:122-129.
- [42] Zhang Z, Torii N, Hu Z, Jacob J, Liang TJ. X-deficient woodchuck hepatitis virus mutants behave like attenuated viruses and induce protective immunity in vivo. *J Clin Invest* 2001;108:1523-1531.
- [43] Lucifora J, Arzberger S, Durantel D, Belloni L, Strubin M, Levrero M. Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. *J Hepatol* 2011;55:996-1003.
- [44] Gearhart TL, Bouchard MJ. Replication of the hepatitis B virus requires a calcium-dependent HBx-induced G1 phase arrest of hepatocytes. *Virology*. 2010;407:14-25.
- [45] Gearhart TL, Bouchard MJ. The hepatitis B virus HBx protein modulates cell cycle regulatory proteins in cultured primary human hepatocytes. *Virus Res* 2011;155:363-367.
- [46] Benhenda S, Cougot D, Buendia MA, Neuveut C. Hepatitis B virus X protein molecular functions and its role in virus life cycle and pathogenesis. *Adv Cancer Res* 2009;103:75-109.
- [47] Ying C, Van Pelt JF, Van Lommel A, Van Ranst M, Leyssen P, De Clercq E, Neyts J. Sulphated and sulphonated polymers inhibit the initial interaction of hepatitis B virus with hepatocytes. *Antivir Chem Chemother* 2002;13:157-164.
- [48] Schulze A, Gripon P, Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. *Hepatology*. 2007;46:1759-1768.
- [49] Leistner CM, Gruen-Bernhard S, Glebe D. Role of glycosaminoglycans for binding and infection of hepatitis B virus. *Cell Microbiol* 2008;10:122-133.
- [50] Schulze A, Mills K, Weiss TS, Urban S. Hepatocyte polarization is essential for the productive entry of the hepatitis B virus. *Hepatology* 2012;55:373-383.
- [51] Engelke M, Mills K, Seitz S, Simon P, Gripon P, Schnölzer M, Urban S. Characterization of a hepatitis B and hepatitis delta virus receptor binding site. *Hepatology* 2006;43:750-760.
- [52] Harford J, Ashwell GG. Immunological evidence for the transmembrane nature of the rat liver receptor for asialoglycoproteins. *Proc Natl Acad Sci USA* 1981;78:1557-1561.
- [53] Spiess M. The asialoglycoprotein receptor: a model for endocytic transport receptors. *Biochemistry* 1990;29:10009-10018
- [54] Basma H, Soto-Gutiérrez A, Yannam GR, Liu L, Ito R, Yamamoto T, et al.

Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. *Gastroenterology* 2009;136:990-999.

- [55] Treichel U, Meyer zum Büschenfelde KH, Stockert RJ, Poralla T, Gerken G. The asialoglycoprotein receptor mediates hepatic binding and uptake of natural hepatitis B virus particles derived from viraemic carriers. *J Gen Virol* 1994;75:3021-3029.
- [56] Zhang X, Lin SM, Chen TY, Liu M, Ye F, Chen YR. Asialoglycoprotein receptor interacts with the preS1 domain of hepatitis B virus in vivo and in vitro. *Arch Virol* 2011;156:637-645.
- [57] Owada T, Matsubayashi K, Sakata H, Ihara H, Sato S, Ikebuchi K, et al. Interaction between desialylated hepatitis B virus and asialoglycoprotein receptor on hepatocytes may be indispensable for viral binding and entry. *J Viral Hepat* 2006;13:11-18.
- [58] Yang J, Bo XC, Yao J, Yang NM, Wang SQ. Differentially expressed cellular genes following HBV: potential targets of anti-HBV drugs? *J Viral Hepat* 2005;12:357-363.
- [59] Yang J, Bo XC, Ding XR, Dai JM, Zhang ML, Wang XH, Wang SQ. Antisense oligonucleotides targeted against asialoglycoprotein receptor 1 block human hepatitis B virus replication. *J Viral Hepat* 2006;13:158-165.
- [60] Budkowska A, Bedossa P, Groh F, Louise A, Pillot J. Fibronectin of human liver sinusoids binds hepatitis B virus: identification by an anti-idiotypic antibody bearing the internal image of the pre-S2 domain. *J Virol* 1995;69:840-848.
- [61] Rotundo RF, Rebres RA, Mckeown-Longo PJ, Blumenstock FA, Saba TM. Circulating cellular fibronectin may be a natural ligand for the hepatic asialoglycoprotein receptor: possible pathway for fibronectin deposition and turnover in the rat liver. *Hepatology* 1998;28:475-485.
- [62] Yang J, Wang F, Tian L, Su J, Zhu X, Lin L, et al. Fibronectin and asialoglycoprotein receptor mediate hepatitis B surface antigen binding to the cell surface. *Arch Virol* 2010;155:881-888.
- [63] Rabe B, Glebe D, Kann M. Lipid-mediated introduction of hepatitis B virus capsids into nonsusceptible cells allows highly efficient replication and facilitates the study of early infection events. *J Virol* 2006;80:5465-5473.
- [64] Schmitz A, Schwarz A, Foss M, Zhou L, Rabe B, Hoellenriegel J, et al. Nucleoporin 153 arrests the nuclear import of hepatitis B virus capsids in the nuclear basket. *PLoS Pathog* 2010;6:e1000741.
- [65] Tuttleman JS, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* 1986;47:451-460.
- [66] Gao W, Hu J. Formation of hepatitis B virus covalently closed circular DNA: removal of genome-linked protein. *J Virol* 2007;81:6164-6174.
- [67] Moolla N, Kew M, Arbuthnot P. Regulatory elements of hepatitis B virus transcription. *J Viral Hepat* 2002;9:323-331.
- [68] Zhang P, McLachlan A. Differentiation-specific transcriptional regulation of the hepatitis B virus nucleocapsid gene in human hepatoma cell lines. *Virology*. 1994;202:430-440.
- [69] Raney AK, Johnson JL, Palmer CN, McLachlan A. Members of the nuclear receptor superfamily regulate transcription from the hepatitis B virus nucleocapsid promoter. *J Virol* 1997;71:1058-1071.
- [70] Tang H, McLachlan A. Transcriptional regulation of hepatitis B virus by nuclear hormone receptors is a critical determinant of viral tropism. *Proc Natl Acad Sci USA* 2001;98:1841-1846.

- [71] Quasdorff M, Hösel M, Odenthal M, Zedler U, Bohne F, Gripon P, et al. A concerted action of HNF4alpha and HNF1alpha links hepatitis B virus replication to hepatocyte differentiation. *Cell Microbiol* 2008;10:1478-1490.
- [72] Li J, Ning G, Duncan SA. Mammalian hepatocyte differentiation requires the transcription factor HNF-4alpha. *Genes Dev* 2000;14:464-474.
- [73] Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, et al. Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. *Nat Genet.* 2003;34:292-296.
- [74] Zheng Y, Li J, Ou JH. Regulation of hepatitis B virus core promoter by transcription factors HNF1 and HNF4 and the viral X protein. *J Virol.* 2004;78:6908-6914.
- [75] Werle-Lapostolle B, Bowden S, Locarnini S, Wurstthorn K, Petersen J, Lau G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004;126:1750-1758.
- [76] Zoulim F. New insight on hepatitis B virus persistence from the study of intrahepatic viral cccDNA. *J Hepatol* 2005;42:302-308.
- [77] Xie, Y, Li J, Ou JH. Entry of hepatitis B virus: mechanism and new therapeutic target. *Pathol Biol* 2010;58:301-307.
- [78] Yang W, Summers J. Integration of hepadnavirus DNA in infected liver: evidence for a linear precursor. *J Virol* 1999;73:9710-9717
- [79] Gong SS, Jensen AD, Chang CJ, Rogler CE. Double-stranded linear duck hepatitis B virus (DHBV) stably integrates at a higher frequency than wild-type DHBV in LMH chicken hepatoma cells. *J Virol.* 1999 Feb;73(2):1492-502.
- [80] Shafritz DA, Shouval D, Sherman HI, Hadziyannis SJ, Kew MC. Integration of hepatitis B virus DNA into the genome of liver cells in chronic liver disease and hepatocellular carcinoma. Studies in percutaneous liver biopsies and post-mortem tissue specimens. *N Engl J Med.* 1981;29;305:1067-1073.
- [81] Fourel G, Trepo C, Bougueleret L, Hengelein B, Ponzetto A, Tiollais P, Buendia MA. Frequent activation of N-myc genes by hepadnavirus insertion in woodchuck liver tumors. *Nature* 1990;347:294-298.
- [82] Fourel G, Conturier J, Wei Y, Apiou F, Tiollais P, Buendia MA. Evidence for long-range oncogene activation by hepadnavirus insertion. *EMBO J* 1994;13:2526-2534.

1.3. *In vitro* models of HBV infection

HBV exhibits a restricted host range, being capable to infect only man and the great apes. Furthermore, the virus shows a strong tropism for liver parenchymal cells, further limiting experimental possibilities. The reasons of such species- and organ-specificity are currently unknown, but viral entry seems to be the most important limiting step (see previous chapter). Human primary hepatocytes cultures represent the gold standard to study the viral life cycle, but they are extremely difficult to use because of scarce availability, rapid dedifferentiation *in vitro* and extreme variability between donors [1]. A few human hepatoma-derived cell lines have been transfected or transduced with the HBV genome. They support HBV replication and are available to study responses to antiviral treatments, but cannot be used to assess the complete viral life cycle as they are not susceptible to HBV infection [2,3]. In the last decade two interesting models emerged (primary hepatocytes of *Tupaia belangeri* and the HepaRG cell line), which are permissive towards viral entry and proved extremely useful to evaluate early infection events [4-6].

1.3.1. Primary human hepatocytes

Primary human hepatocytes (PHH) are still the ideal tissue culture model to study HBV infection. They can be isolated by two-step collagenase perfusion of liver tissue obtained from one of two sources: whole livers from multi-organ donors or an encapsulated end wedge removed from patients undergoing resection for liver tumors [7]. Organ shortage, new techniques of tumor resection and ablation, and the necessity of respecting the priority of pathologic analysis of specimens make obtaining PHHs extremely difficult.

Isolated cells are cultured in fetal calf serum-free medium supplemented with dexamethasone. All experiments need to be performed on freshly isolated cells, as PHHs are poorly resistant to cryopreservation, which induces significant alteration both at the morphological and functional levels [8,9]. Only a limited number of cells is infected *in vitro*. The reasons of such a

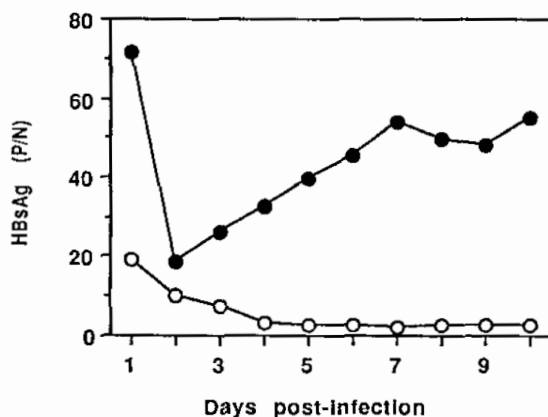


Figure 1.3.1. Kinetic of HBsAg secretion by PHH culture in the presence (solid circles) or absence (empty circles) of PEG (from Gripon 1993).

low efficiency are still unknown, but have been suggested to be secondary to an innate cellular response that could limit virus replication [10,11]. Moreover, it is well known that susceptibility of PHHs to HBV infection is rapidly lost during prolonged culture [1]. Such a loss in susceptibility has been correlated to the dedifferentiation process to which PHHs rapidly undergo *in vitro*, and that already starts during the isolation procedure [65]. The role of cell differentiation state on HBV replication has been extensively studied and explained in chapter 1.2, whereas how it determines cell susceptibility to HBV entry is far less understood. The addition of dimethyl sulfoxide (DMSO) to the culture medium was proven to maintain the differentiated state of rat and human hepatocyte, and to prolong and improve their susceptibility to HBV infection [10,12]. DMSO has been shown to induce G1 phase arrest, which has been suggested to be necessary for HBV replication [13-16]. DMSO could therefore facilitate infection letting the virus find the cell already blocked in G1 phase. Unfortunately, DMSO's mechanism of action on cell cycle and differentiation has not been fully clarified yet. Furthermore, DMSO has a multitude of effects on different cellular compartments and functions, most of which are not completely understood [17]. It has been shown to affect cell membrane integrity [18], alter intracellular signaling [19,20], and modify cellular alternative splicing [21], among others.

In vitro infection of PHHs by HBV is difficult, and its efficiency varies widely

between different donors [1]. The addition of polyethylene glycol (PEG) was shown to improve the infection yield, increasing reproducibility of infection between different PHHs donors (figure 1.3.1) [22]. Although it was shown to preserve HBV species and tissue specificity, it acts by increasing the attachment of the virus to membrane proteoglycans by a yet unspecified mechanism (see page 68) [23], and could therefore represent a confounding factor for studies on viral entry.

Overall, the use of PHHs is limited by the difficulty in obtaining the cells and maintaining them in culture. The use of adjuvant molecules such DMSO and PEG can improve infection efficiency and reduce donor-related variability, but it can as well alter the mechanism by which HBV enters the cell, reducing the representativeness of the model.

Human liver slices have been described as an interesting *ex vivo* model for the evaluation of adenovirus and HCV infection [66-68]. Such a culture system has the advantage of maintaining hepatocytes in their physiological 3D environment, allowing to study virus-cell interactions in conditions far closer to those found in the human body. Suitability of this model to study HBV infection is presumable, but has not been demonstrated yet. uPA/SCID mice repopulated with human hepatocytes have been shown to be susceptible to HBV infection [69], and proved to be a useful *in vivo* model for preclinical studies [29,70].

1.3.2. Primary hepatocytes of *T. belangeri*

The tree shrew *Tupaia belangeri* has a close phylogenetic relationship with primates. Primary cultures of its hepatocytes (PTH) were shown to fully support HBV replication, although with low efficiency (figure 1.3.2) [4]. Efficiency was increased by purifying HBV in the inoculum, but replication rate was confirmed to be low [5]. Compared to human hepatocytes, PTHs have the advantage of being readily available from in-house-bred animals, therefore allowing a greater reproducibility of infection. PTHs have been used to study early infection events [24-27], and to repopulate immunodepressed mice livers for *in vivo* infection studies and antiviral development [28,29]. Unfortunately, they share some of the limitations of PHHs in terms of low infection efficiency and dedifferentiation upon prolonged culture.

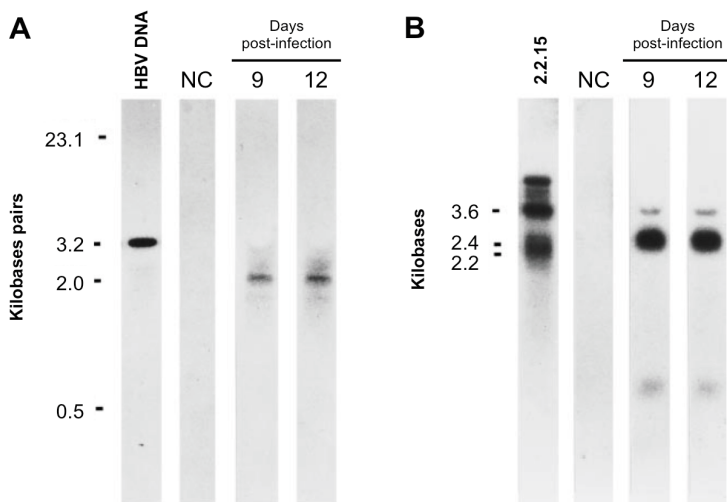


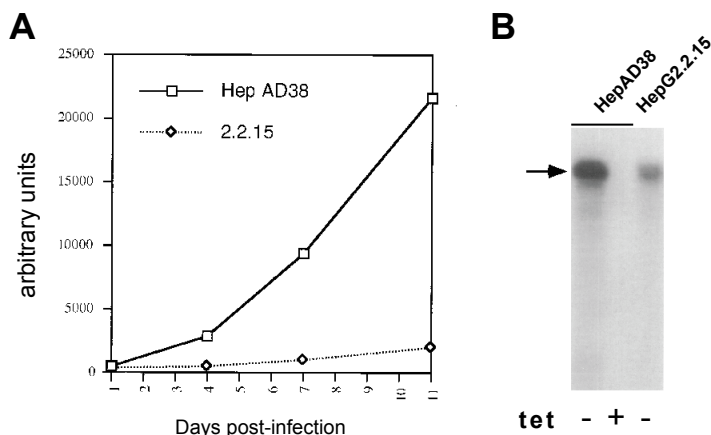
Figure 1.3.2. HBV DNA and RNA synthesis in primary tupaia hepatocytes after HBV infection *in vitro* (modified from Walter 1996). A) Southern blot hybridization of DNA isolated from PTH. **B)** Northern blot hybridization of RNA isolated from PTH. NC: negative control.

1.3.3. Transfected and transduced hepatoma cell lines

The low infection efficiency obtained in PHHs and PTHs complicates research on viral replication and antiviral drugs development. For such a reason, transiently transfected HepG2 and Huh7 cells, two well-differentiated human hepatoma-derived cell lines, remain the best available systems to analyze HBV replication, and its inhibition. Such systems are usually employed to study HBV resistance to antiviral drugs [30-33], although they are only effective to study HBV strains with a high replication rate, showing limitations for those having a low replication rate. Such transient transfections are also inadequate for large-scale drug screening, which requires highly standardized conditions. HepG2.2.15, a stably HBV producing cell line, has therefore been established [2]. Such a model derives from HepG2 cells by transfection of a four-copy HBV construct. HepG2.2.15 cells are easily maintained in culture and expanded, produce modest levels of virus (about 1×10^7 viral genome equivalents/ml), and their use minimizes inter-experiment variation [34-36].

Another transfected cell line (HepAD38) has been then established from HepG2 [3]. In such cells, transcription of viral genes is controlled by a TetOff system: it is

Figure 1.3.3. Production of HBV DNA by HepAD38 and 2.2.15 cells. (from Ladner 1997). A) Amount of HBV DNA in the conditioned medium as determined by quantitative dot blot analysis. **B)** Intracellular HBV DNA by Southern blot analysis.



repressed by the presence of tetracycline in culture medium, whereas tetracycline removal activates the synthesis of pgRNA and viral replication. HepAD38 cells produce larger quantities of HBV compared to HepG2.2.15 (figure 1.3.3), and can be formatted into a high-throughput assay for large-scale screening of antiviral compounds [3,37,38]. Both HepAD38 and HepG2.2.15 have been used to produce HBV particles to use for infection studies on other systems [34,39-42]. Furthermore, stable cell lines can be designed for HBV mutants, in order to study resistance to antiviral drugs [34,43].

Another possibility for in vitro studies on HBV replication (and its inhibition) is to transduce HepG2 cells with recombinant adenovirus or baculovirus carrying the HBV genome [44-46]. Such a strategy has led to higher HBV replication levels compared with both transient and stable transfection.

None of these models based on transfected/transduced hepatoma cells is susceptible to HBV entry (although HBV binds to the cell membrane). Therefore they are not suitable for studies on viral binding or uptake [26].

1.3.4. HepaRG cells

In 2002, Gripon and colleagues described the HepaRG, a cell line derived from a differentiated liver tumor of a HCV-infected female patient [6]. HepaRG cells are capable of acquiring hepatocyte-like morphology (forming bile canalicules) and functions (with high-level expression of detoxifying enzymes) upon treatment with corticosteroids and DMSO (figure 1.3.4) [6,47]. Such a

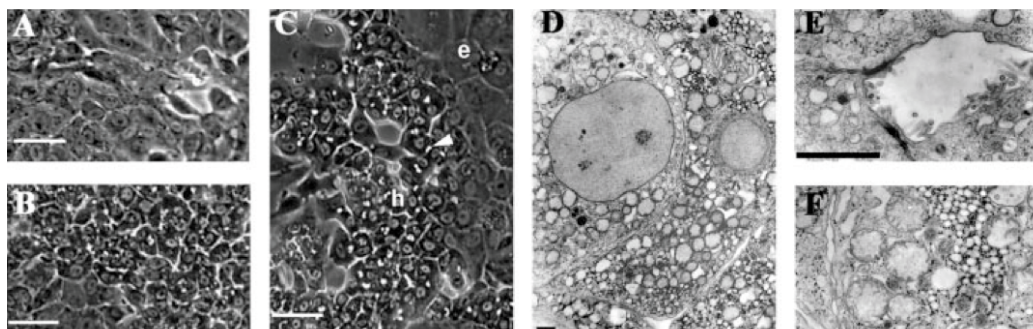


Figure 1.3.4. Morphology of HepaRG cells (from Gripon 2002). Phase contrast micrographs of HepaRG cells under proliferating conditions (A), maintained in culture for 30 days without DMSO (B), and maintained for 15 days without DMSO, then treated with 2% DMSO for 15 days (C). Hepatocyte-like cells and epithelium-like cells are indicated, respectively, by “h” and “e.” A bile canaliculus is indicated by a white triangle. (Bars 50 μm .) Electron micrographs of HepaRG cells: low magnification view of HepaRG cells (D) and higher magnification views (E and F), showing a typical bile canaliculus-like structure and glycogen accumulation, respectively. (Bars 2 μm .)

4-week *in vitro* differentiation protocol turns HepaRG cells permissive towards HBV infection. Interestingly, the cells are susceptible to viral binding and capable of viral uptake, and support the full HBV replication cycle. Infection efficiency of HepaRG cells, which is strictly correlated to their differentiation, is not very high, although enhanced by the use of PEG. Among many compounds known to induce hepatocyte differentiation, such as EGF, HGF and oncostatin M [48,49], only DMSO proved to induce HepaRG susceptibility to HBV infection [41]. Although PEG is not essential for viral entry in HepaRG, infection efficiency in its absence decreases by 97% [6]. Only a subpopulation of the cells undergoing *in vitro* differentiation (about 10%) is susceptible to HBV infection. Such cells express albumin and remain infected for more than 100 days [41]. This susceptible subpopulation is composed of hepatocyte-like cells located at the edges of PHH-like islands. Cells located within the islands are resistant to HBV infection until cell-cell junctions are disrupted, proving a basolateral localization of the putative HBV receptor (see page 69) [42].

HepaRG cells produce complete infectious viral particles, but almost no spread of the infection is noted, and the infection itself is a slow process. Initial conversion of rcDNA to cccDNA was demonstrated, whereas the cccDNA amplification loop was absent [41]. DMSO-induced G1 phase arrest was shown to inhibit cccDNA

amplification in the duck model [50,51], and could explain the absence of such an important step of HBV life cycle.

This model has proven extremely useful to study HBV life cycle, from viral entry [23,52-54], to replication [41,55,56], and antiviral resistant mutants [33,57]. Furthermore, it has been widely used to study other hepatotropic viruses [58,59], as well as hepatocyte differentiation and liver regeneration [60-62], and hepatotoxicology [63,64]. Nevertheless, the low infection efficiency and the dependence of such an infection upon the use of DMSO and PEG, together with controverted HepaRG transformed nature, are important limitations to the use of this model. Although it proved extremely useful to study viral entry kinetics, allowing researchers to obtain interesting results, the validity of such results for PHHs cannot be *a priori* assumed.

1.3.5. References

- [1] Mabit H, Vons C, Dubanchet S, Capel F, Franco D, Petit MA. Primary cultured normal human hepatocytes for hepatitis B virus receptor studies. *J Hepatol* 1996;24:403-412.
- [2] Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci USA* 1987;84:1005-1009.
- [3] Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, Seeger C, King RW. 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* 1997;41:1715-1720.
- [4] Walter E, Keist R, Niederöst B, Pult I, Blum HE. Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. *Hepatology* 1996;24:1-5.
- [5] Köck J, Nassal M, MacNelly S, Baumert TF, Blum HE, von Weizsäcker F. Efficient infection of primary tupaia hepatocytes with purified human and woolly monkey hepatitis B virus. *J Virol* 2001;75:5084-5089.
- [6] Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, Guguen-Guillouzo C. Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci USA* 2002;99:15655-15660.
- [7] Guguen-Guillouzo C, Campion JP, Brissot P, Glaise D, Launois B, Bourel M, Guillouzo A. High yield preparation of isolated human adult hepatocytes by enzymatic perfusion of the liver. *Cell Biol Int Rep* 1982;6:625-628.
- [8] Stéphenne X, Najimi M, Ngoc DK, Smets F, Hue L, Guigas B, Sokal EM. Cryopreservation of human hepatocytes alters the mitochondrial respiratory chain complex 1. *Cell Transplant* 2007;16:409-419.
- [9] Stéphenne X, Najimi M, Sokal EM. Hepatocyte cryopreservation: is it time to change the strategy? *World J Gastroenterol.* 2010;16:1-14.
- [10] Gripon P, Diot C, Thézé N, Fourel I, Loreal O, Brechot C, Guguen-Guillouzo C. Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl

sulfoxide. *J Virol* 1988;62:4136-4143.

- [11] Ait-goughoulte M, Lucifora J, Zoulim F, Durantel D. Innate antiviral immune responses to hepatitis B virus. *Viruses* 2010;2:1394-1410.
- [12] Isom HC, Secott T, Georgoff I, Woodworth C, Mummaw J. Maintenance of differentiated rat hepatocytes in primary culture. *Proc. Natl. Acad. Sci. USA* 1985;82:3252-3256.
- [13] Takase K, Sawai M, Yamamoto K, Yata J, Takasaki Y, Teraoka H, Tsukada K. Reversible G1 arrest induced by dimethyl sulfoxide in human lymphoid cell lines: kinetics of the arrest and expression of the cell cycle marker proliferating cell nuclear antigen in Raji cells. *Cell Growth Differ* 1992;3:515-521.
- [14] Huang YQ, Wang LW, Yan SN, Gong ZJ. Effects of cell cycle on telomerase activity and on hepatitis B virus replication in HepG2 2.2.15 cells. *Hepatobiliary Pancreat Dis Int* 2004;3:543-547.
- [15] Ozer A, Khaoustov VI, Mearns M, Lewis DE, Genta RM, Darlington GJ, Yoffe B. Effect of hepatocyte proliferation and cellular DNA synthesis on hepatitis B virus replication. *Gastroenterology* 1996;110:1519-1528.
- [16] Gearhart TL, Bouchard MJ. Replication of the hepatitis B virus requires a calcium-dependent HBx-induced G1 phase arrest of hepatocytes. *Virology* 2010;407:14-25.
- [17] Santos NC, Figueira-Coelho J, Martins-Silva J, Saldanha C. Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects. *Biochem Pharmacol* 2003;65:1035-1041.
- [18] Melkonyan H, Sorg C, Klempt M. Electroporation efficiency in mammalian cells is increased by dimethyl sulfoxide (DMSO). *Nucleic Acids Res* 1996;24:4356-4357.
- [19] Makowske M, Ballester R, Cayre Y, Rosen OM. Immunochemical evidence that three protein kinase C isozymes increase in abundance during HL-60 differentiation induced by dimethyl sulfoxide and retinoic acid. *J Biol Chem.* 1988;263:3402-3410.
- [20] Fiore M, Degraffi F. Dimethyl sulfoxide restores contact inhibition-induced growth arrest and inhibits cell density-dependent apoptosis in hamster cells. *Exp Cell Res* 1999;251:102-110.
- [21] Bolduc L, Labrecque B, Cordeau M, Blanchette M, Chabot B. Dimethyl sulfoxide affects the selection of splice sites. *J Biol Chem* 2001;276:17597-17602.
- [22] Gripon P, Diot C, Guguen-Guillouzo C. Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. *Virology* 1993;192:534-540.
- [23] Schulze A, Gripon P, Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. *Hepatology.* 2007;46:1759-1768.
- [24] Glebe D, Aliakbari M, Krass P, Knoop EV, Valerius KP, Gerlich WH. Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. *J Virol.* 2003;77:9511-9521.
- [25] Glebe D, Urban S, Knoop EV, Cag N, Krass P, Grün S, et al. Mapping of the Hepatitis B Virus Attachment Site by Use of Infection-Inhibiting preS1 Lipopeptides and Tupaia Hepatocytes. *Gastroenterology* 2005;129:234-245.
- [26] Leistner CM, Gruen-Bernhard S, Glebe D. Role of glycosaminoglycans for binding and infection of hepatitis B virus. *Cell Microbiol* 2008;10:122-133.
- [27] Köck J, Baumert TF, Delaney WE 4th, Blum HE, von Weizsäcker F. Inhibitory effect of adefovir and lamivudine on the initiation of hepatitis B virus infection in primary tupaia hepatocytes. *Hepatology* 2003;38:1410-1418.
- [28] Dandri M, Burda MR, Zuckerman DM, Wursthorn K, Matschl U, Pollok JM. Chronic

- infection with hepatitis B viruses and antiviral drug evaluation in uPA mice after liver repopulation with tupaia hepatocytes. *J Hepatol* 2005;42:54-60.
- [29] Petersen J, Dandri M, Mier W, Lütgehetmann M, Volz T, von Weizsäcker F, Haberkorn U, Fischer L, Pollok JM, Erbes B, Seitz S, Urban S. Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. *Nat Biotechnol* 2008;26:335-341.
 - [30] Durantel D, Carrouée-Durantel S, Werle-Lapostolle B, Brunelle MN, Pichoud C, Trépo C, Zoulim F. A new strategy for studying in vitro the drug susceptibility of clinical isolates of human hepatitis B virus. *Hepatology*. 2004;40:855-864.
 - [31] Brunelle MN, Jacquard AC, Pichoud C, Durantel D, Carrouée-Durantel S, Villeneuve JP, et al. Susceptibility to antivirals of a human HBV strain with mutations conferring resistance to both lamivudine and adefovir. *Hepatology* 2005;41:1391-1398.
 - [32] Durantel D, Brunelle MN, Gros E, Carrouée-Durantel S, Pichoud C, Villet S, et al. Resistance of human hepatitis B virus to reverse transcriptase inhibitors: from genotypic to phenotypic testing. *J Clin Virol*. 2005;34 Suppl 1:S34-43.
 - [33] Billioud G, Pichoud C, Parent R, Zoulim F. Decreased infectivity of nucleoside analogs-resistant hepatitis B virus mutants. *J Hepatol* 2012;56:1269-1275.
 - [34] Sun D, Nassal M. Stable HepG2- and Huh7-based human hepatoma cell lines for efficient regulated expression of infectious hepatitis B virus. *J Hepatol* 2006;45:636-645.
 - [35] Glebe D, Berting A, Broehl S, Naumann H, Schuster R, Fiedler N, Tolle TK, Nitsche S, Seifer M, Gerlich WH, Schaefer S. Optimised conditions for the production of hepatitis B virus from cell culture. *Intervirology* 2001;44:370-378.
 - [36] Ying C, De Clercq E, Neyts J. Lamivudine, adefovir and tenofovir exhibit long-lasting anti-hepatitis B virus activity in cell culture. *J Viral Hepat* 2000;7:79-83.
 - [37] Ying C, De Clercq E, Nicholson W, Furman P, Neyts J. Inhibition of the replication of the DNA polymerase M550V mutation variant of human hepatitis B virus by adefovir, tenofovir, L-FMAU, DAPD, penciclovir and lobucavir. *J Viral Hepat* 2000;7:161-165.
 - [38] Zhou T, Guo H, Guo JT, Cuconati A, Mehta A, Block TM. Hepatitis B virus e antigen production is dependent upon covalently closed circular (ccc) DNA in HepAD38 cell cultures and may serve as a cccDNA surrogate in antiviral screening assays. *Antiviral Res* 2006;72:116-124.
 - [39] Acs G, Sells MA, Purcell RH, Price P, Engle R, Shapiro M, Popper H. Hepatitis B virus produced by transfected Hep G2 cells causes hepatitis in chimpanzees. *Proc Natl Acad Sci USA* 1987;84:4641-4644.
 - [40] Ying C, Van Pelt JF, Van Lommel A, Van Ranst M, Leyssen P, De Clercq E, Neyts J. Sulphated and sulphonated polymers inhibit the initial interaction of hepatitis B virus with hepatocytes. *Antivir Chem Chemother* 2002;13:157-164.
 - [41] Hantz O, Parent R, Durantel D, Gripon P, Guguen-Guillouzo C, Zoulim F. Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells. *J Gen Virol*. 2009 Jan;90(Pt 1):127-35.
 - [42] Schulze A, Mills K, Weiss TS, Urban S. Hepatocyte polarization is essential for the productive entry of the hepatitis B virus. *Hepatology* 2012;55:373-383.
 - [43] Qi X, Xiong S, Yang H, Miller M, Delaney WE 4th. In vitro susceptibility of adefovir-associated hepatitis B virus polymerase mutations to other antiviral agents. *Antivir Ther*. 2007;12:355-362.
 - [44] Delaney WE 4th, Isom HC. Hepatitis B virus replication in human HepG2 cells mediated by hepatitis B virus recombinant baculovirus. *Hepatology*. 1998;28:1134-1146.
 - [45] Ren S, Nassal M. Hepatitis B virus (HBV) virion and covalently closed circular DNA

formation in primary tupaia hepatocytes and human hepatoma cell lines upon HBV genome transduction with replication-defective adenovirus vectors. *J Virol* 2001;75:1104-16.

- [46] Lucifora J, Durantel D, Belloni L, Barraud L, Villet S, Vincent IE, Margeridon-Thermet S, Hantz O, Kay A, Levrero M, Zoulim F. Initiation of hepatitis B virus genome replication and production of infectious virus following delivery in HepG2 cells by novel recombinant baculovirus vector. *J Gen Virol* 2008;89:1819-1828.
- [47] Marion MJ, Hantz O, Durantel D. The HepaRG cell line: biological properties and relevance as a tool for cell biology, drug metabolism, and virology studies. *Methods Mol Biol* 2010;640:261-72.
- [48] Lee KD, Kuo TK, Whang-Peng J, Chung YF, Lin CT, Chou SH, Chen JR, Chen YP, Lee OK. In vitro hepatic differentiation of human mesenchymal stem cells. *Hepatology* 2004;40:1275-84.
- [49] Campard D, Lysy PA, Najimi M, Sokal EM. Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. *Gastroenterology* 2008;134:833-848.
- [50] Borel C, Schorr O, Durand I, Zoulim F, Kay A, Trepo C, Hantz O. Initial amplification of duck hepatitis B virus covalently closed circular DNA after in vitro infection of embryonic duck hepatocytes is increased by cell cycle progression. *Hepatology* 2001;34:168-179.
- [51] Schorr O, Borel C, Trepo C, Zoulim F, Hantz O. Effects of liver growth factors on hepadnavirus replication in chronically infected duck hepatocytes. *J Hepatol* 2006;44:842-847.
- [52] Engelke M, Mills K, Seitz S, Simon P, Gripon P, Schnölzer M, Urban S. Characterization of a hepatitis B and hepatitis delta virus receptor binding site. *Hepatology* 2006;43:750-760.
- [53] Macovei A, Radulescu C, Lazar C, Petrescu S, Durantel D, Dwek RA, et al. Hepatitis B virus requires intact caveolin-1 function for productive infection in HepaRG cells. *J Virol* 2010;84:243-253.
- [54] Schulze A, Schieck A, Ni Y, Mier W, Urban S. Fine mapping of pre-S sequence requirements for hepatitis B virus large envelope protein-mediated receptor interaction. *J Virol* 2010;84:1989-2000.
- [55] Lucifora J, Durantel D, Testoni B, Hantz O, Levrero M, Zoulim F. Control of hepatitis B virus replication by innate response of HepaRG cells. *Hepatology* 2010;51:63-72.
- [56] Lucifora J, Arzberger S, Durantel D, Belloni L, Strubin M, Levrero M. Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. *J Hepatol* 2011;55:996-1003.
- [57] Villet S, Billioud G, Pichoud C, Lucifora J, Hantz O, Sureau C, Dény P, Zoulim F. In vitro characterization of viral fitness of therapy-resistant hepatitis B variants. *Gastroenterology* 2009;136:168-176.
- [58] Ndongo-Thiam N, Berthillon P, Errazuriz E, Bordes I, De Sequeira S, Trépo C, Petit MA. Long-term propagation of serum hepatitis C virus (HCV) with production of enveloped HCV particles in human HepaRG hepatocytes. *Hepatology* 2011;54:406-417.
- [59] Sureau C. The use of hepatocytes to investigate HDV infection: the HDV/HepaRG model. *Methods Mol Biol* 2010;640:463-473.
- [60] Cerec V, Glaise D, Garnier D, Morosan S, Turlin B, Drenou B, et al. Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor. *Hepatology* 2007;45:957-967.
- [61] Sirma H, Kumar M, Meena JK, Witt B, Weise JM, Lechel A, et al. The promoter of human telomerase reverse transcriptase is activated during liver regeneration and hepatocyte

proliferation. *Gastroenterology* 2011;141:326-337.

- [62] Nibourg GA, Chamuleau RA, van der Hoeven TV, Maas MA, Ruiter AF, Lamers WH, et al. Liver progenitor cell line HepaRG differentiated in a bioartificial liver effectively supplies liver support to rats with acute liver failure. *PLoS One* 2012;7:e38778.
- [63] Andersson TB, Kanebratt KP, Kenna JG. The HepaRG cell line: a unique in vitro tool for understanding drug metabolism and toxicology in human. *Expert Opin Drug Metab Toxicol* 2012;8:909-920.
- [64] Anthérieu S, Chesné C, Li R, Guguen-Guillouzo C, Guillouzo A. Optimization of the HepaRG cell model for drug metabolism and toxicity studies. *Toxicol In Vitro* 2012 May 27.
- [65] Elaut G, Henkens T, Papeleu P, Snykers S, Vinken M, Vanhaecke T, Rogiers V. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. *Curr Drug Metab* 2006;7:629-660.
- [66] Kirby TO, Rivera A, Rein D, Wang M, Ulasov I, Breidenbach M, et al. A novel ex vivo model system for evaluation of conditionally replicative adenoviruses therapeutic efficacy and toxicity. *Clin Cancer Res* 2004;10:8697-8703.
- [67] Lagaye S, Shen H, Saunier B, Nascimbeni M, Gaston J, Bourdoncle P, et al. Efficient replication of primary or culture hepatitis C virus isolates in human liver slices: A relevant ex vivo model of liver infection. *Hepatology* 2012;56:861-872.
- [68] Nascimbeni M, Pol S, Saunier B. Distinct CD4+CD8+ Double-Positive T Cells in the Blood and Liver of Patients during Chronic Hepatitis B and C. *PLoS ONE* 2011;6:e20145.
- [69] Dandri M, Burda MR, Török E, Pollok JM, Iwanska A, Sommer G, et al. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. *Hepatology* 2001;33:981–988.
- [70] Lütgehetmann M, Mancke LV, Volz T, Helbig M, Allweiss L, Bornscheuer T, et al. Humanized chimeric uPA mouse model for the study of hepatitis B and D virus interactions and preclinical drug evaluation. *Hepatology* 2012;55:685-694.

PART 2

EXPERIMENTAL WORK

2.1. Aims

As described in chapter 1.2, the reasons of HBV hepatotropism have not been clarified yet. Viral entry seems to be the most important determinant, but the responsible receptor has not been identified. Understanding of HBV entry into susceptible cells could lead to the development of new drugs to prevent the spread of HBV infection among hepatocytes. Unfortunately, the study of early infection events has been limited by the lack of suitable *in vitro* models (see chapter 1.3). Each of the three cellular models permissive towards HBV entry (PHH, PTH and HepaRG) has some major limitations. The perfect model should be of human origin, non-transformed, easily available and easy to expand and maintain in culture, and it should allow studying the role of cellular determinants of HBV hepatotropism (such as the state of differentiation).

This experimental work aimed at establishing a new *in vitro* model with such characteristics. First of all we focused on standardizing *in vitro* infection conditions and developing tailored biomolecular assays in order to allow comparing the new potential model to the gold standard constituted by PHHs. We chose umbilical cord matrix stem cells (UCMSCs) because of their easy availability, self-renewal potential and ability of acquiring mature hepatocytes functions upon *in vitro* differentiation. These cells were assessed, before and after hepatogenic differentiation, for their susceptibility to HBV infection, setting up experimental conditions in order to discretely analyze each step of the viral life cycle, from binding to the cell membrane to secretion of infectious progeny. Having demonstrated that this new model was capable of supporting viral replication and, most notably, viral entry upon hepatogenic differentiation, we tested its suitability to study early infection events, focusing on a receptor (the asialoglycoprotein receptor) that had proven a promising candidate in other models.

2.2. Materials and methods

The present study has been approved by the ethical committee of the Université catholique de Louvain and the Cliniques Universitaires Saint-Luc.

2.2.1. Cell cultures

Isolation and culture of human hepatocytes

Primary human hepatocytes (PHHs) were obtained from the Hepatocytes and Stem Cells Bank of Cliniques Universitaires Saint-Luc (Brussels, Belgium). They were isolated from deceased donors' livers by two-step collagenase perfusion as previously described [24,25]. PHHs were seeded at a density of 1.5×10^5 cells/cm² in tissue culture plates coated with 5 µg/cm² of rat tail collagen type I (BD Biosciences) and cultured in serum-free Williams' E medium (Invitrogen) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen), 10^{-6} M dexamethasone (Organon SA, Belgium) and 10 µg/ml insulin (Lilly Benelux SA, Belgium), at 37°C in a 5% CO₂, fully humidified atmosphere. Infection experiments were performed 24h after seeding. Medium was changed twice weekly.

HepAD38 cells

As described in chapter 1.3, HepAD38 cells are transfected hepatoma cells in which viral genes are controlled by a TetOff system [1]. HepAD38 were received as a kind gift from Prof. J. Neyts (Rega Institute for Medical Research, Leuven, Belgium). They synthesize covalently closed circular DNA (cccDNA), produce high amounts of HBV virions (as well as subviral particles and partially or

non-enveloped core particles) and have been used for antiviral screening [2-4]. were cultured in 175 cm² flasks in Dulbecco's modified Eagle's/F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 µg/mL streptomycin, 400 µg/mL G418 (Invitrogen) and 0.3 µg/mL tetracycline (Sigma). Once confluence was reached, HBV replication was induced by removing tetracycline from culture medium [1].

Isolation and culture of UCMSCs

Umbilical cords have been obtained from healthy donors after signature of an informed consent. Umbilical cord matrix stem cells (UCMSCs) have been isolated by collagenase type I digestion of Wharton's jelly according to a well standardized method we described before [13]. Cells were grown on plastic tissue culture flasks (Greiner Bio-One BVBA/SPRL, Belgium) and cultured in expansion medium at 37°C in a 5% CO₂, fully humidified atmosphere. Expansion medium was composed of Dulbecco's modified Eagle medium low glucose (1 g/L D-glucose) with sodium pyruvate and L-glutamine (Invitrogen, Life Technologies, Belgium) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Austria), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen). At 80%-90% of confluence, cells were detached from the flask using 0.05% trypsin-EDTA solution (Invitrogen), viable cells were counted using trypan blue exclusion assay and seeded at a density of 7500 cells/cm² in expansion medium. Medium was changed twice a week. Cells were used between passages 4 and 10.

2.2.2. UCMSCs characterization and differentiation

Flow cytometry

Undifferentiated UCMSCs have been characterized before each infection experiment and before hepatic differentiation procedure. Cells were trypsinized, suspended in 0.5% bovine serum albumin (Sigma-Aldrich NV/SA, Belgium) phosphate-buffered saline (PBS) at a concentration of 1×10^6 cells/mL and incubated with antibodies against hematopoietic cell markers (CD45-phycoerythrin cyanin7 [PE-Cy7], CD117-allophycocyanin [APC], CD133-fluorescein isothiocyanate

[FITC]), and mesenchymal cell markers (CD90-APC, CD73-phycoerythrin [PE], CD105-FITC). All antibodies were provided by BD Biosciences except CD105-FITC (Ansell Corp). After 20 minutes cells were washed and resuspended in 300 μ L Cell Fix (BD Biosciences). Unstained cells and cells incubated with PE-Cy7, APC, FITC and PE-coupled respective isotypes (BD Biosciences) served as negative controls. Cells were analyzed by FACS Canto II flow cytometer (BD Biosciences), and at least 10,000 events were acquired for each sample.

Differentiation procedures

Osteogenic differentiation has been carried out on UCMSCs at passage 3 as described before [13].

Hepatic differentiation has been induced on UCMSCs from passage 4 to passage 10 according to the procedure we previously described [13], with some modifications [14-16]. Briefly, cells were seeded at 1×10^4 cells/cm² in 6-well and 24-well plates in expansion medium for 48 hours. Culture medium was then switched to IMDM containing 1% penicillin/streptomycin, 20 ng/mL epithelial growth factor (EGF, Peprotech EC Ltd, United Kingdom) and 10 ng/mL basic fibroblast growth factor (bFGF; Peprotech) for 2 days. Then differentiation was induced culturing UCMSCs in IMDM supplemented with 1% penicillin/streptomycin, 20 ng/mL hepatocyte growth factor (HGF; Peprotech EC Ltd), 10 ng/mL bFGF, 0.61 g/L nicotinamide (Sigma) and 1% insulin-transferrin-selenium premix (ITS; Gibco, Life Technologies, Belgium) for 10 days. Subsequently, bFGF was replaced by 20 ng/mL oncostatin M (OSM; Peprotech) for 10 additional days. UCMSCs maturation was then induced by a 14-day final treatment with IMDM containing 1% penicillin/streptomycin, 20 ng/mL OSM, 1 μ mol/L dexamethasone and 1% ITS (differentiation medium). HGF (20 ng/ml) was added at the last medium change.

For differentiation of UD-UCMSCs 24 hours after HBV infection, cells were cultured in differentiation medium plus 20 ng/mL HGF for 7 days.

Undifferentiated UCMSCs used as controls were cultured in IMDM supplemented with 1% penicillin/streptomycin and 2% fetal bovine serum (control medium). Medium was changed twice weekly.

Cytochrome P450 3A4 metabolic activity assay

Cytochrome P450 3A4 (CYP3A4) metabolic activity of UCMSCs was assessed with P450-Glo CYP3A4 Assay (Promega) at the end of hepatic differentiation protocol, according to manufacturer's instructions. Briefly, 4×10^5 cells were incubated with luciferin-IPA substrate for 4 hours at 37°C and luminescence of D-luciferin specifically produced by CYP3A4 was measured with a Victor X4 luminometer (PerkinElmer). Samples were analyzed in duplicate. IMDM supplemented with luciferin-IPA substrate without cells served as negative control.

2.2.3. Viral sources

HBV from infected serum

Serum from 3 HBeAg-positive, immunotolerant, treatment-naïve patients was collected after having obtained an informed consent. A viral load of $1.1 \pm 0.6 \times 10^{10}$ IU/ml was measured. All patients were infected with wild-type HBV of genotype D.

HBV production from HepAD38 cells and concentration

As a great variability in infection efficiency was to be expected [17], we attempted to standardize infection conditions using HBV produced *in vitro* by HepAD38 cells. After 10-day culture in the absence of tetracycline (three medium changes), conditioned medium of HepAD38 was collected and centrifuged at 3500g for 15 min to remove cells and cellular remnants. The virus was then concentrated 30 times by polyethylene glycol 6000 (PEG; Sigma) precipitation. Briefly, PEG was added to the conditioned medium to a final concentration of 8% (wt/vol) and the resulting solution stored overnight at 4°C to allow virus precipitation. The solution was then centrifuged at 600g for 45 min at room temperature and the supernatant discarded. The pellet was then resuspended in PBS and stored at -80°C until use.

2.2.4. Quantification of viral DNA and RNA

Design of real time PCR assays for HBV DNA and cccDNA quantification

A TaqMan real-time polymerase chain reaction (qPCR) assay (RC01; Applied Biosystems ID: AIS07DM) has been designed with BLASTN (version 2.2.26+, National Center for Biotechnology Information, Bethesda, MD, USA) and Custom TaqMan Assay Design Tool (Applied Biosystems) software, based on HBV genotype D sequence (subtype ayw, GenBank accession number U95551.1) [5]. RC01 specifically amplify a 106 bp region of the precore/core protein gene of HBV genome (5'-TGTCCTACTGTTCAAGCC-3', corresponding to position 1853-1870 based on position 1 starting at the *EcoRI* site of HBV genome). The probe is 5'-labelled with FAM (6-carboxyfluoresceine) and 3'-labelled with NFQ-MGB (nonfluorescent quencher). The forward (5'-CAGCACCATGCAACTTTTTCAC-3') and reverse (5'-ATCAATGTCCATGCCCAAA-3') primers correspond to position 1807-1828 and 1912-1893, respectively (figure 2.2.1A).

A second TaqMan assay was designed for specific detection of cccDNA (CC02; Applied Biosystems ID: AICSVC2). CC02 amplifies a 337 bp region of HBV genome across the nick between DR1 and DR2 on the minus-strand (figure 2.2.1A-B). The hybridization probe and primers were the following: 5'-CATACTTCGAAGACTGTTTG-3' (FAM- and NFQ-MGB-labelled probe,

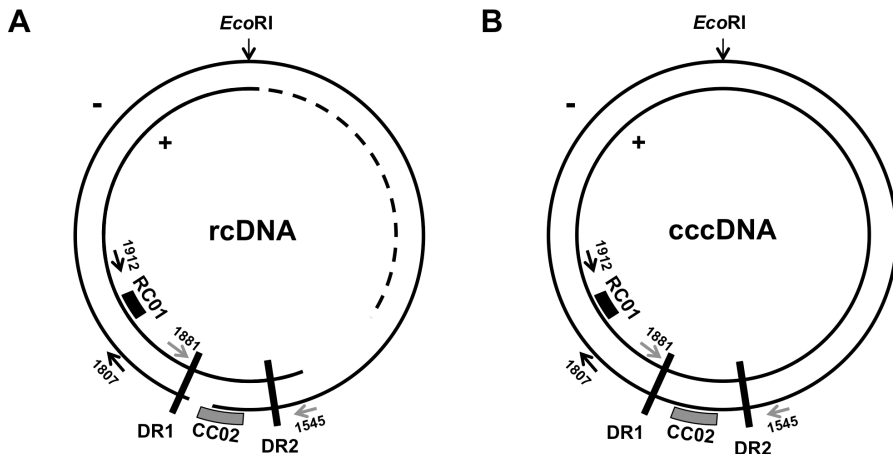


Figure 2.2.1. Schematic representation of RC01 and CC02 probes and primers on rcDNA (A) and cccDNA (B).

position 1702-1721), 5'-CTCCCCGTCTGTGCCTTCTCAT-3' (forward primer, position 1545-1566) and 5'-CACAGCTTGGAGGCTTGAACAGTAG-3' (reverse primer, 1881-1857).

In silico analysis of RC01 assay

HBV genotypes A (NCBI reference sequence NC_003977.1 and GenBank accession numbers X02763, X51970 and AF090842), B (D00329, AF100309, AB033554, D00330 and AB073858), C (X04615, AB014381, AY123041 and AB033556), D (X65259, M32138, X85254 and X02496), E (X75657 and AB032431), F (X69798 and AB036910), G (AF160501, AB064310 and AF405706) and H (AY090454, AY090457 and AY090460) were aligned with ClustalX software (version 2.1 for Mac OS). A sequence logo was created with WebLogo software (version 3.2) [6,7].

DNA extraction

DNA was extracted from cells using DNA Blood Mini kit (Qiagen) according to manufacturer's instructions. For binding assay, the cells were treated with lysis buffer (containing protease) directly on the plate, whereas, for all other experiments, prior detachment with trypsin was carried out. RNA digestion was carried out with RNase A (Qiagen). DNA was eluted in 100 µl of AE buffer (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0) and quantified using a Nanodrop 2000 (Thermo Fisher Scientific).

Plasmid and standard preparation

WHO 2nd HBV International Standard (97/750, NCBSC), containing 10⁶ IU/ml HBV genotype A2 (subtype *adw2*), was used to build the standard curve and determine HBV quantity in IU [8]. Five 200-µl samples containing from 2x10⁵ to 20 IU HBV were prepared through four 10-fold serial dilutions in PBS. DNA was extracted from each sample as described above. Five µl DNA were used in triplicate for real-time PCR amplification to build a standard curve. Plasmid AM6 (pAM6; ATCC #45020D) containing HBV complete genome (genotype A, GenBank AF462041.1) was amplified in Top10 chemically competent *E. coli* (Invitrogen) and quantified with Nanodrop 2000 after DNA extraction. Ten 10-

fold serial dilutions (from 10 ng to 0.1 fg) of pAM6 were calibrated against the WHO standard in order to validate the plasmid as an internal standard to quantify HBV in IU. Carrier DNA (Applied Biosystems) was added to diluted plasmid DNA (10 µg/ml) and 20 µl aliquots were prepared for each dilution and stored at -20°C until use.

HBV genotype determination and POL sequencing

HBV genotype of viral sources used was determined with the help of the Microbiology Lab of Cliniques Universitaires Saint-Luc (Prof. P. Goubau, Dr B. Kabamba). A 703 bp fragment in the overlapping region of S and P HBV genes by PCR (DNA Engine Dyad thermal cycler) was amplified, followed by sequencing (Applied Biosystems 3730xl DNA Analyzer), in order to simultaneously detect resistance mutations and determine HBV genotype.

Total HBV DNA quantification

For each sample, a 25 µl reaction mixture was prepared with 12.5 µl TaqMan GeneExpression Mastermix (Applied-Biosystems), 1 µl primers/probe mix, 5 µl sample DNA and 6.5 µl DEPC-treated water (Invitrogen). All samples were analyzed in triplicate with a TaqMan standard 40-cycle amplification program (10 min at 50°C, 10 min at 95°C and 40 cycles of 15 seconds at 95°C and 90 seconds at 60°C). Each assay included pAM6 standards, a positive control (DNA from HepAD38 culture), two negative controls (DNA from healthy, HBsAg-negative, HBcAg-negative subjects) and water. StepOne Software (version 2.2; Applied Biosystems) was used to analyze the results. Conversion to viral genome equivalents (vge) was obtained multiplying IU by 5.4 [8,9].

cccDNA detection

Ten µl of DNA from each sample were digested with 30 IU of Plasmid-Safe ATP-dependent DNase (PS-DNase, Epicentre Biotechnologies), in 50 µl solution, for 1h at 37°C. PS-DNase selectively and efficaciously degrades linear DNA or circular single-stranded DNA without affecting cccDNA [10]. We used both RC01 and CC02 assays to quantify cccDNA by qPCR. Results were normalized vs. β-actin amplification in undigested samples diluted 10 times in DEPC-treated. All

samples were analyzed in triplicate with a TaqMan standard 40-cycle amplification program as described above.

Exonuclease III (Promega) was used to digest DNA obtained after treatment with PS-DNase. Ten ng of PS-DNase-digested DNA from HepAD38 cells was incubated with 10 units of the enzyme for 30 min at room temperature, and then heat inactivated at 75°C for 10 min. qPCR was performed with RC01 assay as described above.

Southern blot for intracellular viral DNAs

DNA extracted from D-UCMSCs at 1, 3 and 10 days post-infection were analyzed by Southern blot. The very same samples used for qPCR experiments were pooled and DNA was precipitated. 3 µg of cellular total DNA were separated by 1% agarose gel electrophoresis in 0.5x TBE buffer containing 0.5 µg per mL ethidium bromide, and transferred on a positively charged nylon membrane in 20x SSC. HBV replicative intermediates were detected by a digoxigenin (DIG) labeled DNA probe corresponding nt 1500-2000 of HBV ayw genotype D (Genbank V01460.1) as described before [18,19]. A 3.2 kb supercoiled bacterial plasmid (pJet/HBVccc, a derivative of pJet1.2, Fermentas) containing HBV nt 1500-1707 served as a size marker for HBV cccDNA. Plasmid pJet/HBVccc nicked with Nt.BbvCI (New England Biolabs) served as marker for rcDNA; pJet/HBVccc digested with *Pst*I as marker for double-stranded linear DNA (dsDNA) and, after heat denaturation, for single-stranded DNA (ssDNA).

RNA extraction and Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from cells using the TriPure isolation reagent (Roche Diagnostics). RNA was quantified using a Nanodrop 2000 (Thermo Fisher Scientific, Belgium). Residual contaminating DNA was digested using 1U DNase I amplification grade (Invitrogen) per µg of RNA, for 15 min at room temperature. Complementary DNA was generated from 2 µg RNA using the Thermoscript RT kit (Invitrogen) according to the manufacturer's instructions. Real time reverse transcriptase polymerase chain reaction (RT-qPCR) was carried out on 10 ng cDNA using specific TaqMan Gene Expression Assays (Applied-Biosystems, Life Technologies Europe BV, Belgium) for CYP3A4 (ID:Hs00604506_m1),

albumin (ID:Hs00910225_m1), and hepatocyte nuclear factor 4- α (HNF4 α ; ID:Hs00230853_m1). Cyclophilin A (PPIA; ID:Hs99999904_m1) was used as reference gene to normalize the result. All samples were analyzed in triplicate using a standard TaqMan 40-cycle amplification program (10 minutes at 50°C, 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 90 seconds at 60°C) by a Step One Plus real time PCR machine (Applied–Biosystems).

Viral RNA assessment

RNA was extracted from D-UCMSCs at day 1, 3 and 7 post-infection. After DNase I treatment and reverse transcription, pregenomic (pg) and precore RNAs were measured by TaqMan qPCR using RC01 assay, as described above. A sample containing 1 ng plasmid pAM6 (corresponding to 34.6x10⁹ IU HBV DNA/ml) was added to each experiment as internal control for efficient DNA digestion, and results were excluded if amplification was detected in such a control.

Efficacy of DNase I digestion to assure specificity of RT-qPCR quantification of viral RNAs

Efficacy of DNase treatment was assessed by digesting 7 ten-fold dilutions of pAM6 (from 0.1 pg to 100 ng) with 1U DNase I as described above. Real-time PCR with RC01 assay was then performed on digested samples. DNase I digestion potential was further assessed by adding the same increasing amounts of pAM6 to 1x10⁶ uninfected HepG2 cells (ATCC #HB-8065). RNA was then extracted and, after DNase I treatment, 2 μ g were reverse transcribed to cDNA as described above. Real-time PCR was performed with RC01 assay. PPIA was used as reference gene to evaluate homogeneity of sample distribution.

2.2.5. Assessment of viral protein synthesis

Immunofluorescence

Infected cells were extensively washed with PBS and fixed with 4% paraphormaldehyde (Sigma), for 10 min at 37°C. After permeabilization with 0.2% Triton X-100 (Roche Diagnostics) and blocking of non-specific binding

sites with horse serum (Sigma), a mouse monoclonal antibody against HBcAg (NCL-HBcAg-506, Leica) was used to stain the cells (1:100 in 2% bovine serum albumin for 1h at room temperature). Donkey anti-mouse AlexaFluor 594-labeled antibody (Invitrogen, A21203) was used for secondary staining (1:200 for 45 min at room temperature). DAPI (4',6-diamidino-2-phenylindole; Invitrogen) was used to stain the nuclei (1:500 for 10 minutes at room temperature). Results were analyzed with the Cell Observer SD laser confocal microscope (Carl Zeiss).

Western blot analysis for HBcAg

Cell pellets were lysed in 300 mM NaCl, 50 mM Tris pH 7.4, and 5 mM EDTA pH 7.4 (Sigma), 1% Triton X-100 (Roche), supplemented with protease inhibitors (Complete Mini, Roche). After sonication (Branson Sonifier 250, Branson Ultrasonics) and centrifugation, proteins were dosed following BCA method (Pierce BCA protein assay kit, Thermo Scientific) and stored at -80°C until use. Twenty µg of proteins were separated by SDS-PAGE in Laemmli buffer with dithiothreitol. Electrophoretic separation was conducted on Mini-Protean TGX precast gels (Bio-Rad Laboratories N.V., Nazareth Eke, Belgium) at 150V for 45 minutes. Proteins were then transferred to PVDF membranes (Immobilon-FL, Merck Millipore) through semi-dry transfer (Bio-Rad) at 200 mA for 20 min. After blocking (StartingBlock, Thermo Scientific), a mouse monoclonal antibody against HBcAg (NCL-HBcAg-506, Leica) was used for overnight incubation at 4°C (1:2000 in TBS-tween). A goat polyclonal antibody anti- α -actin (1:2000; I-19, Santa Cruz Biotechnology) served as internal control. Fluorescent secondary antibodies (goat anti-mouse CF770, and donkey anti-goat CF680, Biotium) were used (1:10000 for 1h at room temperature in blocking buffer). Results were read with the Odyssey infrared imaging system (Li-Cor Biosciences).

Immunoprecipitation and western blot analysis for HBcAg

Cells well lysed as described above. One ml lysate from 10⁶ cells was pre-cleared incubating with unbound protein A Sepharose CL-4B beads (GE Healthcare) for 30 min. For each sample, after 50% dilution in 0.1% BSA PBS, 150 µl of protein A Sepharose beads were incubated with 5 µg polyclonal rabbit anti-HBc antibody (B0586, Dako) in 500 µl PBS for 1.5h at 4°C. After centrifugation, the

beads were washed in non-denaturing lysis buffer. Pre-cleared cell lysate was then incubated with charged beads for 2 hours at 4°C. Beads were then washed 4 times with wash buffer and one time with PBS. Laemmli buffer with dithiothreitol was added to the final pellet, heated at 95°C for 5 min and separated by SDS-PAGE as described in chapter 5. After semi-dry transfer to PVDF membranes, staining was obtained by incubation with a mouse monoclonal antibody against HBcAg (NCL-HBcAg-506, Leica), overnight at 4°C (1:2000 in TBS-tween). The use of an antibody from a different species allowed avoiding staining of light and heavy chains of the IgGs used for immunoprecipitation. A goat anti-mouse CF770 secondary antibody was used as described above. Subsequent incubation of the membrane with the same antibody used for immunoprecipitation (B0586, Dako) allowed verifying homogeneity between samples through visualization of IgG heavy chains (55 KDa).

ELISA for HBsAg and HBeAg in supernatant

Collected samples of conditioned medium from a culture of 150,000 cells were analyzed for HBsAg and HBeAg (Monolisa HBsAg Ultra and HBeAg/Ab Plus, Bio-Rad) according to manufacturer's instructions. Reading of the optical densities at 450/620 nm was carried out with an absorbance reader (Reader 250, BioMérieux). Results were expressed as optical density/cut-off (OD/CO).

To improve the sensitivity of the assay, conditioned media were concentrated ten times by centrifugation at 4000g for 15 minutes in 10 KDa Amicon Ultra centrifugal filter tubes (Millipore). Resulting samples were analyzed by ELISA for HBeAg and HBsAg as described above.

2.2.6. HBV infection assays

All experiments were carried out using HepAD38-derived HBV. Such a viral source was further verified by comparing the results with those obtained using HBV from patients' sera.

HBV binding assay

PHHs and UCMSCs, cultured in 6-well plates, were incubated with HBV from HepAD38. Multiplicity of infection (MOI) was calculated assuming that one viral genome equivalent (vge) corresponds to one infectious particle. Inoculation with HBV was carried out for 2 hours at 4°C. After 2 hours, as large amounts of viral particles are known to remain non-specifically bound to the cell membrane [11], the cells were extensively washed with cold PBS. Thereafter, DNA was extracted as described above, without previous treatment with trypsin to avoid detachment of receptor-bound HBV.

Soluble horseradish peroxidase (HRP) uptake assay

Uptake of HRP was determined as previously described [12]. Briefly, PHHs and UD-UCMSCs between passage 4 and 8 were plated on collagen-coated 24-well plates and incubated with 0.1 mg/ml HRP in DMEM low glucose for 4h, either at 37°C, 15°C or 4°C. Excess HRP was removed by four washes with ice-cold DMEM. Cells were scraped and suspended in PBS containing 0.1% Triton X-100. After 10 min at 4°C, insoluble cellular debris were removed by centrifugation at 5000 g for 10 min at 4°C. Soluble cellular extracts were assayed for HRP activity by adding an o-phenylenediamine/H₂O₂ substrate (tablets from Dako Belgium nv/sa), for 15 min at room temperature. The amount of coloured product was quantified determining the optical density at 492 nm (OD₄₉₂) with Reader 250 (BioMérieux). Values were normalized for cell number (10⁵ cells).

HBV uptake and viral replication kinetics

After incubation with HBV for 2h at 4°C and extensive washing, PHHs and UCMSCs were cultured in normal conditions. DNA was extracted after 1, 4, and 24 hours and after 3 and 7 days. A 10-minute treatment with 0.05% trypsin-EDTA (Invitrogen) solution, followed by centrifugation and supernatant discard, were carried out before DNA extraction, in order to detach all particles still bound to the cell membrane. Conditioned medium was collected at days 1, 3 and 7 and stored at -20°C until use.

Binding and uptake inhibition

D-UCMSCs were pre-incubated with either 5 mM EDTA, 1 mg/ml thyroglobulin (with and without EDTA) or 100 µg/ml suramin (with and without EDTA), for 1h at 37°C. Such doses of ligands (all from Sigma) have previously proved effective towards ASGPR inhibition in other models [20-23]. Cells were then inoculated at a MOI of 10^3 for 2h at 4°C, and then extensively washed with cold PBS. DNA was extracted and membrane-bound HBV DNA quantified by qPCR.

For uptake inhibition, D-UCMSCs were pretreated with increasing concentrations of D-galactose (0.03-100 µM; Sigma) for 1 hour at 37°C, and then inoculated at a MOI of 10^3 , in the presence of the inhibitor, for 4h at 37°C. DNA was extracted after extensive washing and trypsin treatment.

Viral replication inhibition assay

D-UCMSCs from 3 different donors (5 experiments) were pre-incubated with 0.07-2.5 µg/ml tenofovir (phosphonylmethoxypropyladenine [PMPA, Rega Institute, Leuven, Belgium]), for 1h. They were then inoculated at a MOI of 10^5 for 4h at 37°C, washed and cultured in differentiation medium supplemented with PMPA over the full course of the experiment. DNA was extracted 7 days post-infection, and intracellular HBV DNA was quantified by qPCR. Anti-HBV activity of PMPA was also tested on HepAD38 cells as described before [16,20]. Viral RNA (pg and precore) was measured in 2.5 µg/ml PMPA-treated D-UCMSCs at 1, 3 and 7 days post-infection, and compared to untreated cells as described above.

Infectivity assay

Conditioned medium was collected from D-UCMSCs culture 14 days post-infection. After centrifugation to remove cellular debris, HBV was concentrated by PEG precipitation. Pellets were resuspended in PBS and used to infect PHHs by overnight incubation at 37°C. After extensive washing, PHHs were cultured as described above. DNA was extracted after 24h and 7 days to test for viral replication.

2.2.7. Expression of asialoglycoprotein receptor

Reverse-Transcription Polymerase Chain Reaction

RNA extraction and reverse transcription were performed as described above. qPCR was carried out on 10 ng cDNA using specific TaqMan Gene Expression Assays for ASGPR (Hs01005019_m1). PPIA was used as reference gene to normalize the result. All samples were analyzed in triplicate using a standard TaqMan 40-cycle amplification program (10 minutes at 50°C, 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 90 seconds at 60°C) by a Step One Plus real time PCR machine.

Western blot analysis for ASGPR

Cell lysis, electrophoretic separation and transfer to PVDF membranes was performed as described above for HBc protein. Membranes were subsequently blocked with blocking buffer for 1 hour. A rabbit polyclonal antibody against ASGPR (HPA011954, Sigma) was used for overnight incubation at 4°C (1:5000 in TBS-tween). A goat polyclonal antibody anti- α -actin served as internal control. Fluorescent secondary antibodies as described above. Results were read with the Odyssey infrared imaging system.

Immunofluorescence analysis

Non-infected PHHs, UD- and D-UCMSCs were stained for ASGPR, after pre-blocking with goat serum, with a rabbit polyclonal antibody (1:400 in 2% bovine serum albumin for 1 hour at room temperature; HPA011954, Sigma). Alexa Fluor 594 was used for secondary staining as described above. DAPI was used to stain the nuclei. Results were analyzed with the Cell Observer SD laser confocal microscope.

2.2.8. Statistical analysis

Statistical analysis was performed with PRISM 4 (GraphPad Software). Mann-

Whitney U test, one sample two-tailed *t*-test and Wilcoxon signed-rank test were used as appropriate. A *p* value ≤ 0.05 was considered significant. Values are expressed as mean $\pm$ standard deviation (SD) or standard error of the mean (SEM).

2.2.9. References

- [1] Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, et al. 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.* 1997;41:1715-1720.
- [2] Sun D, Nassal M. Stable HepG2- and Huh7-based human hepatoma cell lines for efficient regulated expression of infectious hepatitis B virus. *J Hepatol* 2006;45:636-645.
- [3] Zhou T, Guo H, Guo J-T, Cuconati A, Mehta A, Block TM. Hepatitis B virus e antigen production is dependent upon covalently closed circular (ccc) DNA in HepAD38 cell cultures and may serve as a cccDNA surrogate in antiviral screening assays. *Antiviral Res* 2006;72:116-124.
- [4] Ying C, de Clercq E, Nicholson W, Furman P, Neyts J. Inhibition of the replication of the DNA polymerase M550V mutation variant of human hepatitis B virus by adefovir, tenofovir, L-FMAU, DAPD, penciclovir and lobucavir. *J Viral Hepat* 2000;7:161-165.
- [5] Okamoto H, Imai M, Shimozaaki M, Hoshi Y, Iizuka H, Gotanda T, et al. Nucleotide sequence of a cloned hepatitis B virus genome, subtype ayr: comparison with genomes of the other three subtypes. *J Gen Virol* 1986;67:2305-2314.
- [6] Schneider TD, Stephens RM. Sequence logos: a new way to display consensus sequences. *Nucleic Acids Res* 1990;18:6097-6100.
- [7] Crooks GE, Hon G, Chandonia J-M, Brenner SE. WebLogo: a sequence logo generator. *Genome Res* 2004;14:1188-1190.
- [8] Baylis SA, Heath AB, Chudy M, Pisani G, Klotz A, Kerby S, et al. An international collaborative study to establish the 2nd World Health Organization International Standard for hepatitis B virus DNA nucleic acid amplification technology-based assays. *Vox Sang* 2008;94:358-362.
- [9] Saldanha J, Gerlich W, Lelie N, Dawson P, Heermann K, Heath A, et al. An international collaborative study to establish a World Health Organization international standard for hepatitis B virus DNA nucleic acid amplification techniques. *Vox Sang* 2001;80:63–71.
- [10] Werle-Lapostolle B, Bowden S, Locarnini S, Wurstthorn K, Petersen J, Lau G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004;126:1750-1758.
- [11] Gripon P, Diot C, Guguen-Guillouzo C. Reproducible High Level Infection of

Cultured Adult Human Hepatocytes by Hepatitis B Virus: Effect of Polyethylene Glycol on Adsorption and Penetration. *Virology* 1993;192:534-540.

- [12] Leistner CM, Gruen-Bernhard S, Glebe D. Role of glycosaminoglycans for binding and infection of hepatitis B virus. *Cell Microbiol.* 2008;10:122-133.
- [13] Campard D, Lysy PA, Najimi M, Sokal EM. Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. *Gastroenterology.* 2008;134:833–848.
- [14] Najimi M, Khuu DN, Lysy PA, Jazouli N, Abarca J, Sempoux C, et al. Adult-derived human liver mesenchymal-like cells as a potential progenitor reservoir of hepatocytes? *Cell Transplant.* 2007;16:717–728.
- [15] Lysy PA, Smets F, Sibille C, Najimi M, Sokal EM. Human skin fibroblasts: From mesodermal to hepatocyte-like differentiation. *Hepatology.* 2007;46:1574–1585.
- [16] Khuu DN, Scheers I, Ehnert S, Jazouli N, Nyabi O, Buc-Calderon P, et al. In vitro differentiated adult human liver progenitor cells display mature hepatic metabolic functions: a potential tool for in vitro pharmacotoxicological testing. *Cell Transplant.* 2011;20:287–302.
- [17] Mabit H, Vons C, Dubanchet S, Capel F, Franco D, Petit MA. Primary cultured normal human hepatocytes for hepatitis B virus receptor studies. *J Hepatol.* 1996;24:403–412.
- [18] Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. *Nature.* 1979;281:646-650.
- [19] Dallmeier K, Schultz U, Nassal M. Heterologous replacement of the supposed host determining region of avihepadnaviruses: high in vivo infectivity despite low infectivity for hepatocytes. *PLoS Pathog.* 2008;4: e1000230.
- [20] Treichel U, Meyer zum Büschenfelde KH, Stockert RJ, Poralla T, Gerken G. The asialoglycoprotein receptor mediates hepatic binding and uptake of natural hepatitis B virus particles derived from viraemic carriers. *J Gen Virol.* 1994;75:3021-3029.
- [21] Saunier B, Triyatni M, Ulianich L, Maruvada P, Yen P, Kohn LD. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. *J Virol.* 2003;77:546-559.
- [22] Offensperger WB, Offensperger S, Walter E, Blum HE & Gerok W. Suramin prevents duck hepatitis B virus infection in vivo. *Antimicrob Agents Chemother.* 1993;37:1539-1542.
- [23] Schulze A, Gripon P & Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. *Hepatology.* 2007;46:1759-1768.
- [24] Seglen P. Preparation of isolated rat liver cells. *Methods Cell Biol.* 1976;13:29–83.
- [25] Sokal EM, Smets F, Bourgois A, van Maldergem L, Buts J-P, Reding R, et al. Hepatocyte transplantation in a 4-year-old girl with peroxisomal biogenesis disease: technique, safety, and metabolic follow-up. *Transplantation.* 2003;76:735–738.

2.3. Development of a standardized strategy for *in vitro* infection studies

2.3.1. Introduction

Development of new *in vitro* models of HBV infection relies on standardization of the viral source, reproducibility of infection conditions and performance of methods used for assessment of infection efficiency. It is known that *in vitro* HBV infection efficiency varies widely when infected sera from different donors are used as source of HBV [1]. Furthermore, accurate assessment of cellular susceptibility to HBV infection requires evaluation of each step of virus life cycle, from binding to secretion of newly formed virions and viral proteins. Therefore, standardization of the viral source, definition of infection conditions suitable to follow the virus along its life cycle in the studied cell, and highly sensitive and specific quantitation assays adequately tailored to such conditions are required to develop and compare different *in vitro* models.

Using HBV from a well-characterized source, standardizing infection conditions and assessing efficiency of infection with tailored assays, we developed a complete strategy to assess and compare candidate cellular models of HBV infection, and tested it on PHHs, which are considered the gold standard.

Such strategy has been then used to assess and characterize our new *in vitro* model of HBV infection (see chapter 2.4). Part of results from the present study have been published as supplementary material in the following article: Paganelli M, et al. Differentiated umbilical cord matrix stem cells as a new *in vitro* model to study early events during HBV infection. Hepatology 2012, in press (DOI: 10.1002/hep.26006; see annex 2).

2.3.2. Results

In order to develop a standardized strategy to evaluate new models for HBV infection studies, we characterized the viral source, designed and validated specifically tailored real-time PCR assays and designed infection conditions in order to follow the virus along its life cycle. Eventually, we validated such a strategy by infecting PHHs.

Characterization of the viral source: HepAD38 culture for HBV production

When cultured in presence of tetracycline, HepAD38 reached 9.1 ± 0.3 cumulative population doublings in 28 days (figure 2.3.1B). After the induction of HBV genes' transcription by tetracycline removal, we did not observe any morphological changes (figure 2.3.1Ab) and cell proliferation was unaffected (figure 2.3.1B).

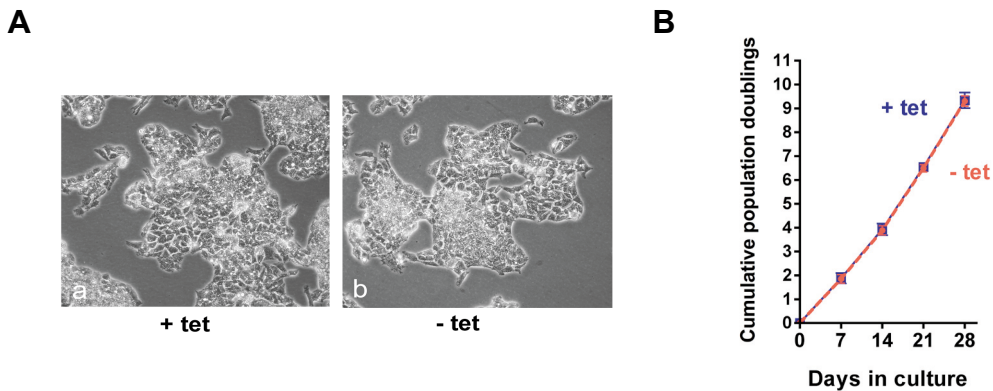


Figure 2.3.1. HepAD38 characterization. **A)** Morphology of a HepAD38 cell culture: culture on plastic support under standard conditions, in presence of tetracycline (a) and ten days after tetracycline removal from culture medium (b). **B)** Exponential growth of HepAD38 in culture: population doubling time in the presence (blue solid line) and absence (red dashed line) of tetracycline (each point representing a passage; N=3).

After 10-day culture without tetracycline, HBV DNA in supernatant reached $6.2 \pm 2.7 \times 10^8$ IU/ml. We genotyped the produced virus, which proved to be a wild-type genotype D, subtype *ayw*, with no genotypic resistance to lamivudine, entecavir, adefovir or telbivudine identified (figure 2.3.2).

Overall, a total of 8.7 ± 2 copies of cccDNA per cell were detected in HepAD38 10 days after tetracycline removal. At the same time point, synthesis of viral proteins

HepAD38 HBV P/S nucleotide sequence:

GTTGGTCTTCTGGACTATCAGGTATGTTGCCGCTTTGTCCTCT
AATTCAGGATCCTCAACCACGACGCGGACCATGCCGAACC
TGCATGACTACTGCTCAAGGAACCTYTATGTATCCCTCMRTT
GCTGTACCAACCTTCGGACGGAATTCACCTGTATCCCAT
CCCATCATCCTGGGCTTCGGAAAAATTCATGCGAGTGGGCC
TCAGCCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTGTTT
AGTGGTTCGTAGGGCTTCCGCCACTGTTGGCTTTCAGTTAT
ATGGATGATGGTATTGGGGCCCAAGTCTGACAGCATCTTG
AGTCCCTTTTACCGCTGTTACCAATTTCTTTTGTCTTTGGG
TATACATTTAAACCTAACAAAAAAGAGATGGGGTTACTCT
CTGAATTTTATGGGTTATGTCAATTGGAAGTATGGGTCCTGC
CACAAGAACACATCATACAAAAATCAAGAATGTTTAGAAA
ACTTCTTATTAACAGGCCTATTGATTGGAAGTATGCAACGA
ATTGTGGGCTTTTGGGTTTGTGCCCCATTTACACATGTG
GTTATCCTGCGTTAATGCCCTGTATGCATGTATTCAATCTAA
GCAGGCTTTCCTTTCTGCCCACTTACAAGGCCTTTCTGTGT
AAACAATACCTGAACCTT

Genotype D

HepAD38 HBV POL sequencing

FRKPMGVGLSPFLAQFTS
AICSVVRRAFPCHLAFSYMD
DVVLGAKSVQHLESLEFATV
NFLLSLGLHLPNPKTKRWGY
SLNFGYVIG

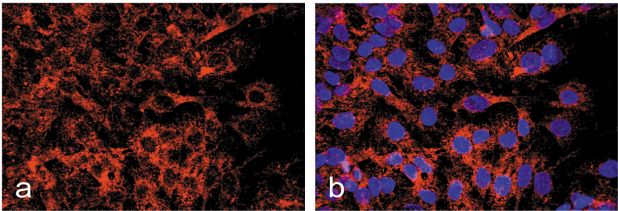
rtI169T
rtV173L
rtL180M
rtA181V/T
rtT184G
rtS202G/I
rtM204I/V
rtI233V
rtN236T
rtM250V

Wild type

Figure 2.3.2. HepAD38 produce wild-type genotype D HBV: sequencing of a 703 bp fragment overlapping S and P genes of HBV obtained from HepAD38 after 10 days from tetracycline removal (genotype D, 99% homology). Polymerase gene sequencing revealed no mutations conferring resistance to nucleos(t)ide analogs.

by HepAD38 cells was evident by immunofluorescence (figure 2.3.3A). At least 7 days were needed to reach adequate HBcAg synthesis (figure 2.3.3B). HBV from HepAD38's conditioned medium was further concentrated 30 times by PEG precipitation, obtaining a final HBV concentration of 1.7×10^{10} IU/ml.

A



B

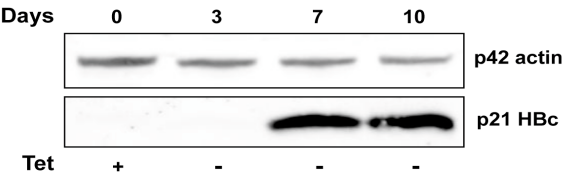


Figure 2.3.3. A) Synthesis of HBV core antigen by HepAD38 10 days from tetracycline removal (a: mouse anti-HBc/Alexa Fluor 594; b: costaining with DAPI for nuclei; laser confocal microscopy, 400x original magnification). **B)** Western blot analysis for HBcAg: at least 7 days from tetracycline removal are needed for HepAD38 to synthesize adequate amounts of core protein (20 μ g of total protein, α -actin served as internal control; mouse monoclonal anti-HBc antibody)

Assessment of biomolecular assays for evaluating infection efficiency

In-house TaqMan real-time PCR assay for HBV DNA quantitation

RC01 assay was designed on the basis of genotype D HBV (subtype *ayw*), which is produced by both HepAD38 (figure 2.3.2) and HepG2.2.15 cell lines (GenBank U95551.1). We performed an *in silico* analysis of this assay by aligning 26 different isolates (HBV genotypes A to H), and built a sequence logo (figure 2.3.4). Both the probe and the forward primer proved to hybridize to sequences that are well conserved across all genotypes. Reverse primer corresponded to a less conserved nucleotide sequence including positions 1896 and 1899, known to be the sites of precore mutations leading to HBe-negative chronic hepatitis B [14,15]. Two mismatches were noted comparing reverse primer to reference genotype A sequence. Overall, based on *in silico* analysis, RC01 assay was able to recognize HBV genotype A-F.

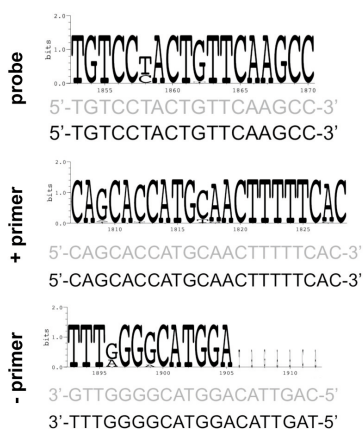


Figure 2.3.4. Consensus sequences (WebLogo) of 26 HBV isolates (genotypes A through H) and alignment with HBV genotype A reference sequence (gray) and RC01 probe, forward and reverse primers (black).

Serial dilutions of WHO 2nd HBV international standard were used to assess amplification efficiency with RC01 TaqMan probe ($R^2=0.996$, PCR efficiency: 98.8%, $p<0.0001$; figure 2.3.5A). WHO 2nd HBV international standard was used to quantify HBV DNA in five 10-fold dilutions of pAM6 DNA (from 100 pg to 10 fg), in order to calibrate it as an internal standard ($R^2=0.999$, $p<0.0001$, figure 2.3.5B).

To determine dynamic range and lower limit of detection (LOD) of the assay,

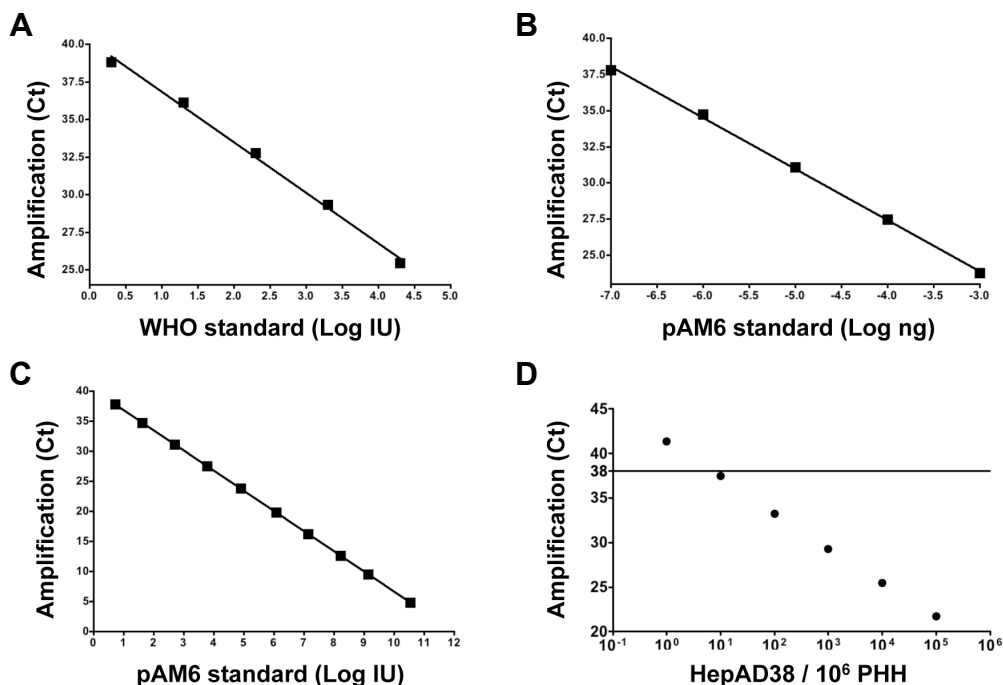


Figure 2.3.5. Characterization of RC01 assay for real-time PCR. **A)** Amplification efficiency of RC01 assay using WHO 2nd HBV international standard ($R^2=0.996$, PCR efficiency: 98.8%, $p<0.0001$; 40-cycle standard TaqMan real-time PCR). **B)** Lower amount of HBV DNA detectable by RC01 assay: 5-fold dilutions of pAM6 DNA (from 100 pg to 10 fg; $R^2=0.999$, $p<0.0001$) were measured by a 40-cycle standard TaqMan real-time PCR. **C)** Dynamic range and lower limit of detection of the assay in IU: ten 10-fold dilutions of pAM6 DNA (from 10 ng to 0.1 fg DNA) were used in a 40-cycle standard TaqMan real-time PCR. A linear relationship was observed over the range of 5.3 to 3.5×10^{10} IU ($R^2=0.999$, PCR efficiency: 98.8%, $p<0.0001$). **D)** Lower limit of detection of RC01 expressed as infected cells per million PHHs: HBV DNA was quantified in serial dilutions of HepAD38 cells (from 1×10^5 to 1) per million PHHs (45-cycle TaqMan real-time PCR; Ct=38 is marked by the horizontal line mark).

ten 10-fold dilutions of pAM6 DNA (from 10 ng to 0.1 fg DNA) were tested in triplicate (figure 2.3.5C). A linear relationship was observed over the range of 5.3 to 3.5×10^{10} IU ($R^2=0.999$, PCR efficiency: 98.8%, $p<0.0001$). The lower LOD with a Ct <38 was 4.5 IU (hit rate 100% on 12 tests). As low as 2 and 1 IU were detected with a hit rate of 100% (10 tests) and 90% (9/10 tests), respectively. Lower LOD and specificity of RC01 were further assessed measuring the minimum number of HBV infected cells detectable in a fixed amount of PHHs. HBV DNA was quantified in serial dilutions of HepAD38 cells (from 1×10^5 to 1) per million PHHs. RC01 assay proved to be specific (no amplification in PHHs alone) and sensitive enough to detect 10 infected cells per million hepatocytes with a Ct

<38 (figure 2.3.5D). When the number of PCR cycles was increased to 45, the probe was able to identify 1 infected cell in 1 million PHHs (Ct 41.32±0.28, corresponding to 2.5 vge).

cccDNA quantification by real-time PCR

We designed primers and a TaqMan probe to specifically amplify a 337 bp region of genotype D HBV genome across the nick between DR1 and DR2 (CC02 assay; figure 2.2.1A-B). Such an assay is theoretically capable of amplifying only cccDNA, as the nick and gap present in rcDNA would prevent amplification. We tested both RC01 and CC02 assays’ specificity for cccDNA by pre-digesting DNA samples with plasmid-safe DNase (PS-DNase), which is known to selectively and efficiently digesting linear DNA or circular single-stranded DNA without affecting cccDNA [11,16].

When used according to manufacturer recommendations (10 IU for 30 minutes for up to 1µg DNA), PS-DNase failed to get rid of all non-circular DNA (table 2.3.1). Although designed to be cccDNA-specific, CC02 assay cross-amplified viral DNA other than cccDNA. As shown in figure 2.3.6, when analyzing a sample of HepAD38-derived DNA (corresponding to 5.8x10⁶ IU HBV DNA/ml or 1 pg HBV DNA) by qPCR with CC02 probe, a 2-Ct variation was noted after the first

		Ct			
	DNase	100 ng DNA	10 ng DNA	1 ng DNA	WHO standard
β-actin	-	26.33±0.03	30.08±0.07	33.51±0.44	UD
	+	35.18±0.28	37.14±0.12	UD	UD
RC01	-	20.22±0.11	23.54±0.04	26.91±0.09	25.45±0.15
	+	32.37±0.04	35.06±0.24	38.09±0.36	UD
CC02	-	28.78±0.23	32.48±0.06	36.01±0.15	UD
	+	35.3±0.17	37.8±0.26	UD	UD

Table 2.3.1. ATP-dependant Plasmid-Safe DNase digestion for specific cccDNA amplification. Digestion of DNA extracted from HepAD38 with ATP-dependent Plasmid-Safe DNase following manufacturer’s instructions (10 IU for 30 minutes for up to 1 µg DNA) was not sufficient to get rid of all non-circular DNA, as proved by positive β-actin amplification for total DNA concentrations higher than 1 ng. PS-DNase digestion resulted in an important Ct increase for both RC01 (recognizing all forms of viral DNA) and CC02 (designed to be cccDNA-specific) probes. Such an effect was more pronounced for RC01 probe (about 12 Ct increase) than CC02 probe (5-7 Ct increase). Cross-amplification of RC-DNA by CC02 probe was greater at higher DNA concentrations. As expected, CC02 did not amplify HBV DNA in WHO standard (1x10⁶ IU/ml), which is intended to contain only RC-DNA.

1-hour digestion with 20 IU PS-DNase ($p<0.01$), whereas a 4-Ct increase was registered with RC01 probe ($p<0.0001$).

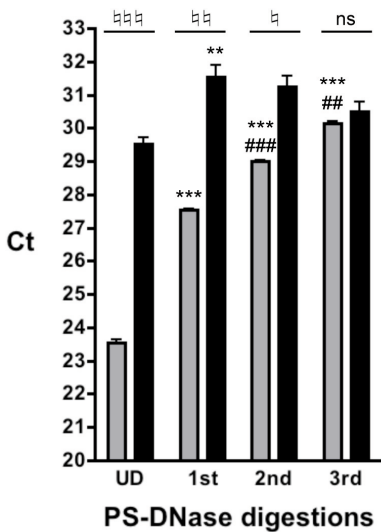


Figure 2.3.6. Sequential PS-DNase digestions of HepAD38 DNA (10 ng) before HBV DNA amplification (40-cycle standard TaqMan real-time PCR; ** $p<0.01$ and *** $p<0.001$ compared to undigested samples; ## $p<0.01$ and ### $p<0.001$ compared to the sample after the previous digestion; ‡ $p<0.05$, ‡‡ $p<0.01$, ‡‡‡ $p<0.001$ compared to RC01). CC02 assay (black bars) was specific for cccDNA after the first 20-IU, 1-hour digestion, whereas 3 sequential digestions were needed to specifically detect cccDNA with RC01 assay (gray bars).

No significant change was observed after a subsequent second digestion before amplification with CC02, suggesting that one 20-IU, 1-hour digestion was sufficient for this assay to be cccDNA-specific, whereas a further 1.5-Ct variation was noted with RC01 probe ($p<0.0001$). RC01 was specific for cccDNA when samples were digested 3 subsequent times with 20 IU for 1 hour before amplification, as no further Ct increase was seen over subsequent digestions. The same result (i.e. a complete digestion of non-circular HBV DNA) was obtained with a single 30-IU digestion for 1 hour (Ct 30.2 ± 0.2 and 30.5 ± 0.1 before and after a second subsequent digestion).

To further assess specificity of cccDNA detection, after treatment with PS-DNase, HepAD38-derived DNA was digested with exonuclease III. After digestion, no significant change in qPCR amplification was seen with RC01 assay (Ct 30.5 ± 0.1 and 30.7 ± 0.1 before and after digestion, respectively).

Validation of infection and detection strategies on primary human hepatocytes

To validate HepAD38s as a standardized source of HBV for *in vitro* infection studies, as well as the described methods for HBV total DNA, cccDNA and protein synthesis detection and quantification, we infected PHHs *in vitro*. Cells from four different donors were inoculated with HepAD38-derived HBV at a MOI of 10^5 , and HBV DNA and proteins were analyzed at different time points to study viral life cycle.

Binding

To assess binding of HBV to cell membrane, we incubated PHHs with HBV for 2h at 4°C. It is known that, at a temperature below 18°C, endocytosis is inhibited (figure 2.3.7A), whereas binding of viral particles to membrane receptors continues to be active [13]. After incubation, cells were washed extensively before DNA extraction in order to achieve HBV DNA levels <100 IU/ml in the last washing (figure 2.3.7B).

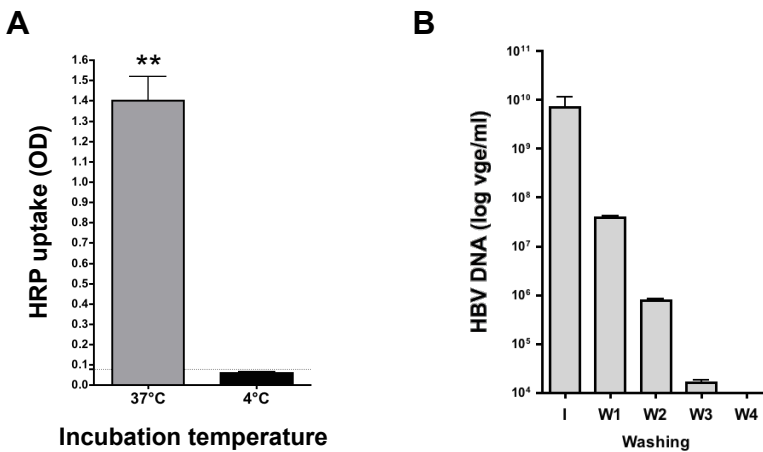


Figure 2.3.7. A) Inhibition of endocytosis at 4°C: soluble horseradish peroxidase (HRP) uptake assay (4h incubation at 4°/37°C; PHHs from 3 donors, 6 independent experiments; ** $p < 0.01$). **B) HBV DNA in conditioned medium after sequential washes:** 4 sequential washes with ice-cold PBS were necessary to reach a concentration of HBV DNA <100 vge/ml (real-time PCR with RC01 assay; N=4).

An average of 68.4 ± 18.4 vge/cell remained bound to the cell membrane (figure 2.3.8).

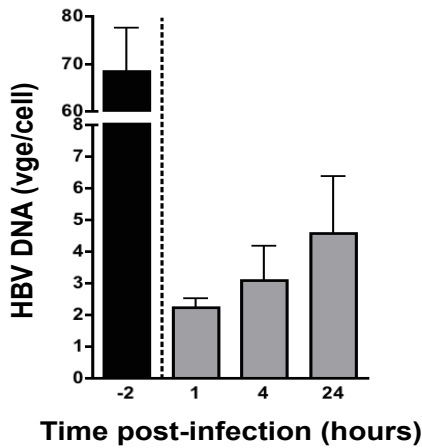


Figure 2.3.8. HBV entry in PHHs: binding of HBV to cellular membrane after 2h incubation at 4°C (black bar) and viral entry after 1, 4 and 24 hours at 37°C (gray bars) expressed as vge/cell (real-time PCR with RC01 assay; N=4).

Uptake

To assess viral entry, after the 2h incubation at 4°C and extensive washing, PHHs were moved to a 37°C environment. They were cultured under standard conditions and DNA was extracted after 1, 4 and 24 hours (figure 2.3.8). Trypsin was applied before DNA extraction, in order to detach all particles still bound to cell membrane and extract only intracellular DNA. After 1h at 37°C, PHHs were able to internalize 2.2 ± 0.5 vge/cell ($3.6 \pm 1.6\%$ of membrane-bound HBV). The proportion of viral uptake increased at 4 and 24 hours (3.1 ± 2.2 and 4.6 ± 3.6 vge/cell, respectively; $p=ns$).

Viral replication

To assess viral replication, intracellular total HBV DNA was quantified by qPCR at 3 and 7 days post-infection, and compared to the amount of viral DNA present inside the cells at 24h post-incubation (3 ± 0.6 vge/cell). Intracellular HBV DNA levels increased along time in PHHs at day 3 (4.6 ± 1.7 vge/cell; $p=ns$) and 7 (6.5 ± 3.6 vge/cell; $p<0.05$), suggesting viral replication (figure 2.3.9). We did not test hepatocytes beyond 7 days post-infection to avoid possible experimental artifacts secondary to dedifferentiation.

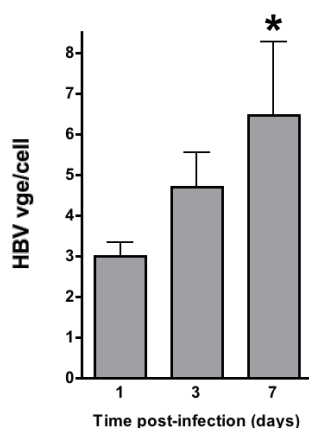


Figure 2.3.9. Viral replication in PHHs: HBV replication at 1, 3 and 7 days post-infection expressed as vge/cell (qPCR with RC01 assay; N=4, $p < 0.05$ vs. 1 day post-infection).

Synthesis of viral proteins

PHHs' ability to synthesize viral proteins was evident using immunofluorescence at day 7 post-infection (figure 2.3.10A). Anyway, a great variability among different donors was noted when analyzing secretion of HBV proteins by ELISA (figure 2.3.10B-C). Secretion of newly synthesized HBsAg and HBeAg was clearly evident in 2 donors (PHH-3 and PHH-4) and borderline in one (PHH-1). As shown in figure 2.3.10D, PHHs from PHH-3 and PHH-4 donors showed a higher expression of Cyp3A4 and albumin than PHH-1 and PHH-2, suggesting that PHHs from PHH-1 and PHH-2 might have undergone dedifferentiation during isolation and/or first 24-hour culture.

2.3.3. Discussion

In order to develop a reproducible strategy to assess our candidate *in vitro* model for HBV infection studies, we used HepAD38 cells as a source of HBV, characterized the produced virus, and prepared the inoculum by a simple virus concentration method. We designed real-time PCR assays tailored to such a viral source, and validated them using international standards. We then designed infection experiments in order to follow the virus along its life cycle inside the host cell, and used concentrated HepAD38-derived HBV to infect PHHs (the gold

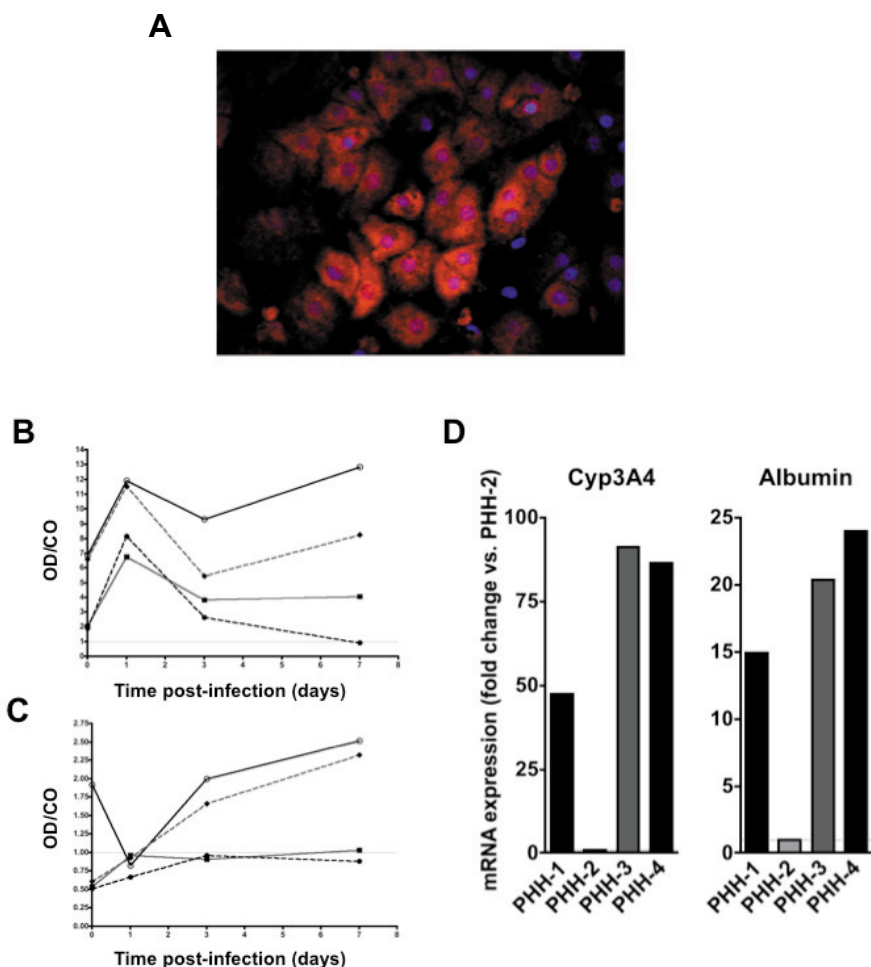


Figure 2.3.10. Synthesis of viral proteins: **A)** HBcAg synthesis by PHHs: at 7 days post-infection, synthesis of HBV core protein was evident at immunofluorescence (mouse anti-HBc/Alexa Fluor 594/DAPI staining; laser confocal microscopy, 250x original magnification). **B-C)** Secretion of viral proteins by infected PHHs is widely variable between donors: after a known release of non-internalized viral particles at day 1, donors PHH-3 (black continuous line) and PHH-4 (gray dotted line) secreted increasing amounts of HBsAg (**B**) and HBeAg (**C**), whereas donors PHH-1 (gray continuous line) and PHH-2 (black dotted line) were not able to synthesize significant quantities of viral proteins (ELISA on conditioned medium). **D)** Variability of mRNA expression of Cyp3A4 and albumin between the four PHHs donors: both Cyp3A4 and albumin appeared to be more expressed in donors PHH-3 and PHH-4 as compared to PHH-1 and PHH-2 (real-time RT-PCR on RNA extracted 24h post plating; fold change vs. donor PHH-2; PPIA served as reference gene).

standard). Analysis of infection efficiency in PHHs with our biomolecular assays, as well as with other tests already used in clinical diagnostics, allowed us to test such an infection and detection strategy before using it to assess a new cellular model.

Infection efficiency varies widely when patients' sera are used as inoculum [1]. Human serum was also reported to inhibit infection of primary tupaia hepatocytes [17]. The use of HepAD38-derived HBV allows having an unlimited amount of inoculum virtually free of antibodies and other active molecules present in human serum, thus eliminating the variability related to the viral source. As described in chapter 1.3 (page 86), HepAD38 are able to synthesize larger quantities of viral particles than the more widely used HepG2.2.15 cell line [2], and have previously been employed as a source of HBV for infection studies [18,19]. We show here that HBV produced by such cells is a genotype D, subtype *ayw*, and does not carry any of the known mutations conferring resistance to antiviral drugs, thus being perfectly suitable as inoculum for *in vitro* studies. HepG2.2.15 cells were shown to produce partially and non-enveloped cores, besides HBV virions [3]. Such nude nucleocapsids could bind to the cell membrane and enter via a different pathway than complete Dane particles [34,35]. It is unknown whether HepAD38 secrete non-enveloped cores, and whether such a phenomenon could affect the infection process. Concentration of viral particles via ultracentrifugation through a sucrose gradient, instead of PEG precipitation, would allow purification of the inoculum and elimination of both subviral particles and potential non-enveloped capsids. Infection conditions were framed in order to allow the analysis of different steps of the viral cycle, while avoiding the addition of adjuvant molecules (such as DMSO or PEG) that could alter cellular responses and the permeability of the cell membrane (see pages 68 and 84).

We used WHO HBV international standard, pAM6 and HepAD38 cells to validate specifically tailored assays for total HBV DNA, cccDNA and protein synthesis assessment and quantitation. Several real-time PCR assays for HBV DNA quantification have been published [20-25]. RC01 TaqMan assay was specifically designed to amplify genotype D HBV derived from HepAD38 (while being capable of recognizing also HBV genotype A-F and HBV produced by HepG2.2.15). It proved to have a very low LOD and a dynamic range wide enough to cover the highly variable viral loads typical of HBV infection, while being highly reproducible and 100% specific for HBV DNA. Furthermore, using a standard 40-cycle TaqMan amplification protocol, this assay can be coupled to any other TaqMan assay on the same plate, allowing important cost and time

savings in the research environment. Although such an assay would be suitable to follow the highly variable levels of viremia typical of HBV infected patients, and is sensible enough to detect occult hepatitis B (see page 27), its *in silico*-predicted ability to amplify different genotypes should be properly tested with actual samples before suggesting its use in the clinical setting.

As cccDNA sequence is identical to partially double-stranded rcDNA, specific detection of cccDNA is complicated and has been based on Southern blot analysis until recently. PCR with primers designed across the gap region of rcDNA is supposed to allow selective cccDNA amplification [26]. Digestion with mung bean nuclease, which removes single-stranded DNA in rcDNA but preserves cccDNA, further increased specificity [27]. Real-time PCR assays allowed an increase in sensitivity and are now considered the gold standard in the clinical setting [28-31]. Here we show that Plasmid-Safe DNase treatment is necessary even for presumptive specific assays in order to amplify only cccDNA. Furthermore, as compared to manufacturer instruction and previously published papers, higher enzyme concentration and longer digestion times are needed for complete non-cccDNA digestion. RC01 assay proved to be highly specific for cccDNA after adequate DNase digestion, eliminating the need of using a different probe for cccDNA detection and allowing further time and cost savings. Southern blot analysis, which allows the evaluation of HBV replicative intermediates, is still considered the most informative method to assess HBV replication in *in vitro* models. Nevertheless, it is challenging, time consuming, and requires great amount of DNA to allow the visualization of cccDNA. RC01 assay, as other qPCR assays, is more sensible than Southern blotting, and allows an easy, rapid and reproducible quantification of even few copies of HBV DNA and cccDNA, proving useful to evaluating low-efficiency *in vitro* models.

Commercially available anti-HBcAg antibody and ELISA kits for HBsAg and HBeAg proved to be suitable for detection of viral protein synthesis in this research setting, although preventive protein concentration might be necessary when testing cellular models with an infection efficiency lower than PHHs.

We tested this infection and detection strategy on PHHs as they are the gold standard for HBV infection studies (see page 83). Unfortunately, it is well known that PHHs' susceptibility to HBV infection varies between donors and is

rapidly lost during prolonged culture [1]. Such an acquired resistance has been correlated to the dedifferentiation process to which hepatocytes rapidly undergo *in vitro* [32,33]. Here, using standardized infection conditions, we confirm such variability between different donors and show how it is correlated to the state of differentiation of PHHs.

In conclusion, the use of concentrated HBV from HepAD38 cells allows the standardization of the inoculum to be used for *in vitro* infection studies. Such a stable viral source, together with a stepwise design of infection experiments, allows the discrete assessment of each step of viral life cycle, from binding to protein excretion. Infection efficiency can be assessed by real-time PCR with RC01 assay, which has been specifically tailored for this particular viral source and proved to be among the most sensible assays ever described, allowing rapid, sensitive and cost-effective quantification of total HBV DNA and cccDNA. Standardized viral source and infection conditions, along with such biomolecular assays and previously validated tests for viral proteins assessment, constitute a complete strategy for evaluating new cellular models of HBV infection.

2.3.4. References

- [1] Mabit H, Vons C, Dubanchet S, Capel F, Franco D, Petit MA. Primary cultured normal human hepatocytes for hepatitis B virus receptor studies. *J Hepatol* 1996;24:403-412.
- [2] Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, et al. 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*. 1997;41:1715-1720.
- [3] Sun D, Nassal M. Stable HepG2- and Huh7-based human hepatoma cell lines for efficient regulated expression of infectious hepatitis B virus. *J Hepatol* 2006;45:636-645.
- [4] Zhou T, Guo H, Guo J-T, Cuconati A, Mehta A, Block TM. Hepatitis B virus e antigen production is dependent upon covalently closed circular (ccc) DNA in HepAD38 cell cultures and may serve as a cccDNA surrogate in antiviral screening assays. *Antiviral Res* 2006;72:116-124.
- [5] Ying C, de Clercq E, Nicholson W, Furman P, Neyts J. Inhibition of the replication of the DNA polymerase M550V mutation variant of human hepatitis B virus by adefovir, tenofovir, L-FMAU, DAPD, penciclovir and lobucavir. *J Viral Hepat* 2000;7:161-165.
- [6] Okamoto H, Imai M, Shimozaki M, Hoshi Y, Iizuka H, Gotanda T, et al. Nucleotide

- sequence of a cloned hepatitis B virus genome, subtype ayr: comparison with genomes of the other three subtypes. *J Gen Virol* 1986;67:2305-2314.
- [7] Schneider TD, Stephens RM. Sequence logos: a new way to display consensus sequences. *Nucleic Acids Res* 1990;18:6097-6100.
 - [8] Crooks GE, Hon G, Chandonia J-M, Brenner SE. WebLogo: a sequence logo generator. *Genome Res* 2004;14:1188-1190.
 - [9] Baylis SA, Heath AB, Chudy M, Pisani G, Klotz A, Kerby S, et al. An international collaborative study to establish the 2nd World Health Organization International Standard for hepatitis B virus DNA nucleic acid amplification technology-based assays. *Vox Sang* 2008;94:358-362.
 - [10] Saldanha J, Gerlich W, Lelie N, Dawson P, Heermann K, Heath A, et al. An international collaborative study to establish a World Health Organization international standard for hepatitis B virus DNA nucleic acid amplification techniques. *Vox Sang* 2001;80:63–71.
 - [11] Werle-Lapostolle B, Bowden S, Locarnini S, Wursthorn K, Petersen J, Lau G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004;126:1750-1758.
 - [12] Gripon P, Diot C, Guguen-Guillouzo C. Reproducible High Level Infection of Cultured Adult Human Hepatocytes by Hepatitis B Virus: Effect of Polyethylene Glycol on Adsorption and Penetration. *Virology* 1993;192:534-540.
 - [13] Leistner CM, Gruen-Bernhard S, Glebe D. Role of glycosaminoglycans for binding and infection of hepatitis B virus. *Cell Microbiol.* 2008;10:122-133.
 - [14] Carman W, Hadziyannis S, Mcgarvey M, Jacyna M, Karayiannis P, Makris A, et al. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet.* 1989;2(8663):588-591.
 - [15] Chen RYM, Edwards R, Shaw T, Colledge D, Delaney WE, Isom H, et al. Effect of the G1896A precore mutation on drug sensitivity and replication yield of lamivudine-resistant HBV in vitro. *Hepatology.* 2003;37:27–35.
 - [16] Pollicino T, Belloni L, Raffa G, Pediconi N, Squadrito G, Raimondo G, et al. Hepatitis B virus replication is regulated by the acetylation status of hepatitis B virus cccDNA-bound H3 and H4 histones. *Gastroenterology.* 2006;130:823-837.
 - [17] Köck J, Nassal M, MacNelly S, Baumert TF, Blum HE, Weizsäcker von F. Efficient infection of primary tupaia hepatocytes with purified human and woolly monkey hepatitis B virus. *J Virol* 2001;75:5084–5089.
 - [18] Ying C, Van Pelt J, Van Lommel A, Van Ranst M, Leyssen P, de Clercq E, et al. Sulphated and sulphonated polymers inhibit the initial interaction of hepatitis B virus with hepatocytes. *Antivir Chem Chemother.* 2002;13(3):157–64.
 - [19] Schulze A, Mills K, Weiss TS, Urban S. Hepatocyte polarization is essential for the productive entry of the hepatitis B virus. *Hepatology* 2012;55:373-383.
 - [20] Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R, et al. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *J Clin Microbiol.* 1999;37:2899–2903.
 - [21] Pas SD, Fries E, De Man RA, Osterhaus AD, Niesters HG. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. *J Clin Microbiol.* 2000;38:2897-2901.
 - [22] Chen RW, Piiparinen H, Seppänen M, Koskela P, Sarna S, Lappalainen M. Real-time PCR for detection and quantitation of hepatitis B virus DNA. *J. Med. Virol.* 2001;65:250-256.

- [23] Jardi R, Rodriguez F, Buti M, Costa X, Cotrina M, Valdes A, et al. Quantitative detection of hepatitis B virus DNA in serum by a new rapid real-time fluorescence PCR assay. *J Viral Hepat.* 2001;8:465-471.
- [24] Leb V, Stöcher M, Valentine-Thon E, Hölzl G, Kessler H, Stekel H, et al. Fully automated, internally controlled quantification of hepatitis B Virus DNA by real-time PCR by use of the MagNA Pure LC and LightCycler instruments. *J Clin Microbiol.* 2004;42:585-590.
- [25] Liu Y, Hussain M, Wong S, Fung SK, Yim HJ, Lok ASF. A Genotype-Independent Real-Time PCR Assay for Quantification of Hepatitis B Virus DNA. *J Clin Microbiol.* 2007;45:553-558.
- [26] Köck J, Schlicht HJ. Analysis of the earliest steps of hepadnavirus replication: genome repair after infectious entry into hepatocytes does not depend on viral polymerase activity. *J Virol.* 1993;67:4867-4874.
- [27] Stoll-Becker S, Repp R, Glebe D, Schaefer S, Kreuder J, Kann M, et al. Transcription of hepatitis B virus in peripheral blood mononuclear cells from persistently infected patients. *J Virol.* 1997;71:5399-5407.
- [28] He M-L, Wu J, Chen Y, Lin MC, Lau GKK, Kung H-F. A new and sensitive method for the quantification of HBV cccDNA by real-time PCR. *Biochemical and Biophysical Research Communications.* 2002;295:1102-1107.
- [29] Jun-Bin S, Zhi C, Wei-Qin N, Jun F. A quantitative method to detect HBV cccDNA by chimeric primer and real-time polymerase chain reaction. *J Virol Methods.* 2003;112:45-52.
- [30] Wong DK-H, Yuen M-F, Yuan H, Sum SS-M, Hui C-K, Hall J, et al. Quantitation of covalently closed circular hepatitis B virus DNA in chronic hepatitis B patients. *Hepatology.* 2004;40:727-737.
- [31] Takkenberg R, Zaaijer H, Molenkamp R, Menting S, Terpstra V, Weegink C, et al. Validation of a sensitive and specific real-time PCR for detection and quantitation of hepatitis B virus covalently closed circular DNA in plasma of chronic hepatitis B patients. *J. Med. Virol.* 2009;81:988-995.
- [32] Elaut G, Henkens T, Papeleu P, Snykers S, Vinken M, Vanhaecke T, et al. Molecular Mechanisms Underlying the Dedifferentiation Process of Isolated Hepatocytes and Their Cultures. *curr drug metab.* Bentham Science Publishers; 2006;7:629-660.
- [33] Richert L, Liguori MJ, Abadie C, Heyd B, Manton G, Halkic N, et al. Gene expression in human hepatocytes in suspension after isolation is similar to the liver of origin, is not affected by hepatocyte cold storage and cryopreservation, but is strongly changed after hepatocyte plating. *Drug Metabolism and Disposition.* 2006;34:870-879.
- [34] Vanlandschoot P, Van Houtte F, Serruys B, Leroux-Roels G. The arginine-rich carboxy-terminal domain of the hepatitis B virus core protein mediates attachment of nucleocapsids to cell-surface-expressed heparan sulfate. *J Gen Virol.* 2005;86(Pt 1):75-84.
- [35] Cooper A, Shaul Y. Clathrin-mediated endocytosis and lysosomal cleavage of hepatitis B virus capsid-like core particles. *J Biol Chem.* 2006;281:16563-16569.

2.4. Differentiated umbilical cord matrix stem cells as a new *in vitro* model to study early events during HBV infection

2.4.1. Introduction

As described in chapter 1.2, the reasons of HBV species- and organ-specificity have not been clarified yet. The role of cell differentiation state on HBV replication has been well demonstrated [1-3], whereas how it determines cell susceptibility to HBV entry is far less understood. Although hepatoma cell lines can replicate the virus efficiently after transfection of the viral genome, they are not permissive to HBV entry (see page 86) [4-6]. Thus, viral entry seems to be the most important determinant of HBV hepatotropism, but the responsible receptor has not been identified yet [7]. Study of early infection events has been limited by the lack of suitable models. PHHs represent the gold standard, but their use is significantly hampered by limited availability, rapid dedifferentiation *in vitro* and very high variability between donors [8]. PTHs and the HepaRG cell line have been shown as being susceptible to HBV infection [9-11], but they share the limitations of PHHs in terms of accessibility, reproducibility and low yield of infection (see chapter 1.3).

As stated above, the perfect *in vitro* model to study HBV life cycle should be of human origin, non-transformed, easily available and easy to expand and maintain in culture. Furthermore, in order to understand hepatotropism, it should allow studying the role of the state of differentiation on cellular susceptibility to HBV entry. Previous publications from our laboratory showed that mesenchymal stem cells (MSCs) from different sources can be *in vitro* differentiated to hepatocyte-

like cells [12-15]. Such a potential, together with their self-renewal ability, makes MSCs good candidates to study HBV infection and the role of differentiation on HBV-cell interactions.

In the current study we used the infection and detection strategy described in chapter 2.3 to assess whether umbilical cord matrix stem cells (UCMSCs) could serve as a new *in vitro* model of HBV infection. UCMSCs were chosen among other MSCs because they were easily available and differentiated well to hepatocyte-like cells [12]. Being of non-hepatic origin, such cells were supposed being resistant to HBV infection in their basal, undifferentiated state. If they proved to become susceptible to viral entry upon hepatogenic differentiation, they could be used to understand the role of the state of differentiation in determining viral hepatotropism.

This study has been published as “Paganelli M, et al. Differentiated umbilical cord matrix stem cells as a new *in vitro* model to study early events during HBV infection. *Hepatology* 2012, in press” (DOI: 10.1002/hep.26006; see annex 2).

2.4.2. Results

Mesenchymal Stromal Cell identity of UCMSCs

UCMSCs obtained from 6 healthy donors grew rapidly on plastic, showing a fibroblast-like morphology (figure 2.4.1), and needed to be split every 3-7 days up to 12 passages, without slowing proliferation.

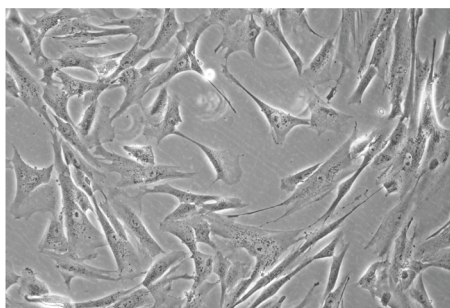


Figure 2.4.1. Fibroblast-like morphology of UCMSCs under expansion conditions (original magnification 200x).

According to the International Society for Cellular Therapy definition of mesenchymal stromal cells (MSC), UCMSCs expressed CD90 (99.4 ± 0.2), CD73 (98.2 ± 0.7) and CD105 (90.5 ± 3) surface markers and were negative for CD45 (4.4 ± 1.6), CD117 (1.3 ± 0.6) and CD133 (3.2 ± 2.1), as previously described (figure 2.4.2) [12,21].

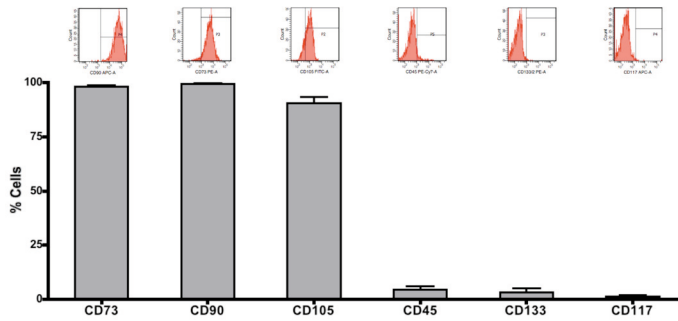


Figure 2.4.2. Mesenchymal stromal cell identity of UCMSCs. Flow cytometric characterization of UCMSCs' surface markers: representative analysis and mean of rate of positive cells (%) for 5 independent experiments $\pm$ SEM.

Besides self-renewal properties, their multipotency was confirmed by a good osteogenic *in vitro* differentiation potential (figure 2.4.3).

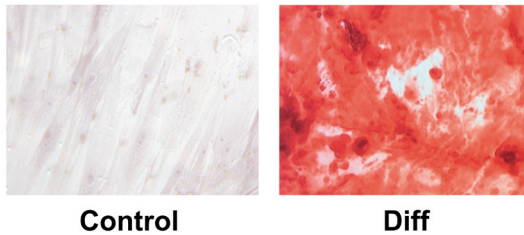


Figure 2.4.3. UCMSCs' osteogenic differentiation potential: alizarin red staining after osteogenic differentiation protocol, as compared to UCMSCs cultured in control medium.

Hepatic differentiation of UCMSCs

UCMSCs at passage 4 to 10 were differentiated *in vitro* to hepatocyte-like cells according to a well established 5-week multistep protocol [12-15]. At the end of the differentiation process, cells changed morphology and showed a polygonal shape with granular cytoplasm (figure 2.4.4A). As shown in figure 2.4.4B, quality of differentiation was proved by the increased gene expression of cytochrome

P450 3A4 (CYP3A4; 354.7 ± 109.8 fold change, $N=6$, $p=0.0156$), hepatocyte nuclear factor 4 (HNF4; 9.34 ± 0.89 fold change, $N=5$, $p=0.0312$) and albumin (32.2 ± 17.2 fold change, $N=6$, $p=0.0156$). HNF4 α mRNA expression in PHHs was higher than in D-UCMSCs ($N=3$ for PHHs and $N=5$ for D-UCMSCs, $p=0.036$; figure 2.4.4C). The wide error bars show the variability of differentiation quality between different donors.

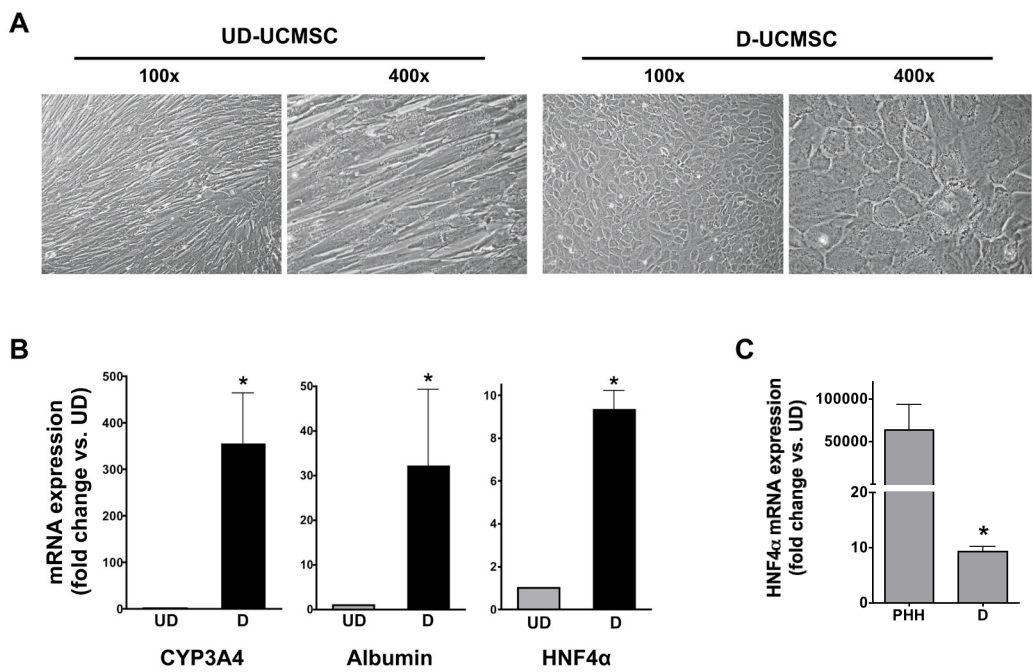


Figure 2.4.4. Hepatogenic differentiation potential of UCMSCs. **A)** Morphology of UD-UCMSCs and D-UCMSCs at confluence, before infection experiments. At the end of the differentiation protocol, D-UCMSCs show a polygonal shape with granular cytoplasm. Original magnification 100x and 400x. **B)** Increased expression of CYP3A4 (354.7 ± 109.8 , $N=6$, $p=0.016$), albumin (32.2 ± 17.2 , $N=6$, $p=0.016$) and HNF4 α (9.34 ± 0.89 , $N=5$, $p=0.031$) mRNAs, in D-UCMSCs compared to UD-UCMSCs (Wilcoxon signed rank test). **C)** HNF4 α mRNA expression in PHHs is much higher than in D-UCMSCs (RT-qPCR results expressed as fold change vs. UD-UCMSCs; $N=3$ for PHHs and $N=5$ for D-UCMSCs, $p=0.0357$).

Efficiency of differentiation was also evaluated at the functional level. D-UCMSCs showed a significantly greater activity of CYP3A4 isoenzyme (18.3 ± 7.2 fold change) compared to UD-UCMSCs ($p=0.031$; figure 2.4.5A). Such activity was evident without the need of phenobarbital stimulation. As we previously described [12], activity of glucose-6-phosphatase was noted in few UD-UCMSCs (figure 2.4.5B left), and was up-regulated in D-UCMSCs (figure 2.4.5B right).

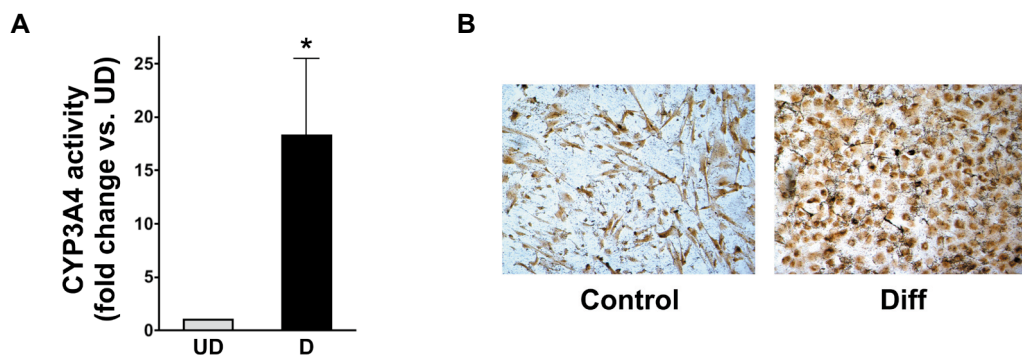


Figure 2.4.5. A) Increased CYP3A4 activity (luciferin-IPA test) in D-UCMSCs as compared to UD-UCMSCs (N=5; $p=0.031$, Wilcoxon signed rank test). **B)** UCMSCs show an increased glucose-6-phosphatase activity after *in vitro* differentiation to hepatocyte-like cells, compared to UCMSCs cultured in control medium.

HBV binding to UCMSCs membranes

To study the effect of differentiation state on HBV-cell interactions, we first assessed viral attachment to the cell membranes. At a temperature below 18°C , endocytosis is inhibited (figure 2.4.6A), whereas binding of viral particles to membrane receptors remains active [17]. We incubated PHHs, UD-UCMSCs and D-UCMSCs with HBV at a MOI of $1.0 \pm 0.8 \times 10^5$, for 2h at 4°C . Under these conditions, endocytosis was totally inhibited while cellular viability was not affected (figure 2.4.6B-C).

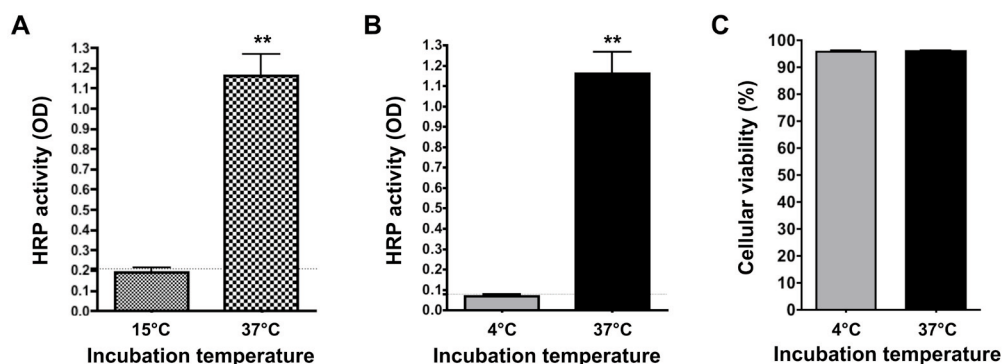
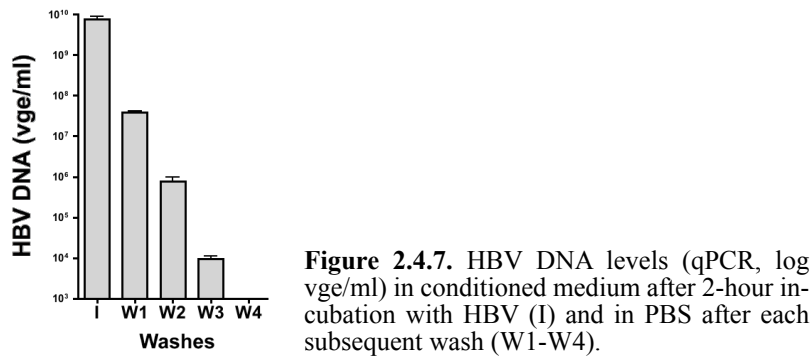


Figure 2.4.6. Endocytosis inhibition and cellular viability. A-B) Soluble horseradish peroxidase (HRP) uptake assay at 15°C (A; UD-UCMSC) and 4°C (B; D-UCMSC) showing that endocytosis is inhibited in D-UCMSCs at temperatures below 18°C (optical density at 492nm ; N=6 for each experiment; $p=0.002$ [**]). **C)** Cellular viability by trypan blue exclusion assay after 2-hour incubation with concentrated HBV either at 37°C or 4°C (D-UCMSC, N=4; $p=\text{ns}$).

After 2h, as large amounts of viral particles are known to remain non-specifically bound to cell membrane [22], the cells were extensively washed with cold PBS. At the fourth washing, viral DNA in supernatant was <100 vge/ml (figure 2.4.7).



After extensive washing, the amount of membrane-bound HBV DNA was similar for PHHs and D-UCMSCs, but lower for UD-UCMSCs ($p=0.052$; figure 2.4.8A). To prove that binding of viral particles on cell membrane was receptor-mediated, after incubation with HBV at 4°C and extensive washing, cells where treated with trypsin before DNA extraction. Protease detached 95% of the viral particles, without any difference between cell types (figure 2.4.8B), indicating that proteinaceous structures were involved in HBV binding.

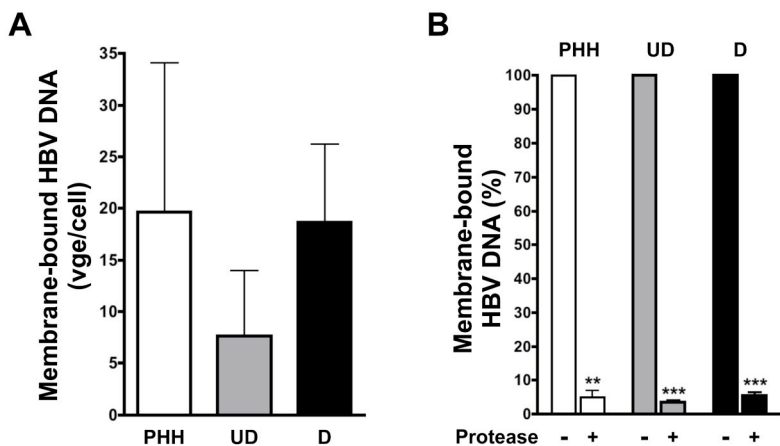


Figure 2.4.8. D-UCMSCs are permissive to HBV binding. **A)** Binding of HBV on UCMSCs after 2-hour incubation at 4°C compared to PHHs. Results are expressed as vge/cell (N=6 for UD- and D-UCMSCs, N=3 for PHHs; $p=ns$). **B)** Protease sensitive binding of HBV to the cell surface: treatment with 0.25% trypsin-EDTA at the end of incubation and before DNA extraction, detached 95% of membrane-bound HBV (N=5 for UD- and D-UCMSCs, N=3 for PHHs; $p<0.001$ [$**$] and $p<0.0001$ [$***$] as compared to no protease treatment, one sample two-sided t test).

Uptake of membrane-bound HBV

To assess whether viral particles attached to membrane receptors could be internalized, after the 2-hour incubation at 4°C and extensive washing, cells were moved to a 37°C environment. They were cultured under standard conditions and DNA was extracted after 1, 4 and 24h. To make sure to extract only intracellular DNA, trypsin was applied before DNA extraction, in order to detach all particles still bound to the cell membrane. After 1 hour at 37°C, PHHs, UD-UCMSCs, and D-UCMSCs were able to internalize $4.9\pm0.7\%$, $6.3\pm1.5\%$ and $5.5\pm1.3\%$ of membrane-bound HBV, respectively ($p=ns$; figure 2.4.9A). The proportion of viral uptake increased at 4 and 24h for PHHs ($p=ns$) and D-UCMSCs ($p=0.016$), but remained stable for UD-UCMSCs. HBV uptake after 24h was significantly greater in D-UCMSCs than in UD-UCMSCs ($p=0.004$). No difference was found in viral uptake capacity of UD- and D-UCMSCs when HBV from serum of HBeAg-positive, treatment-naïve patients or HepAD38 culture were compared (figure 2.4.9B).

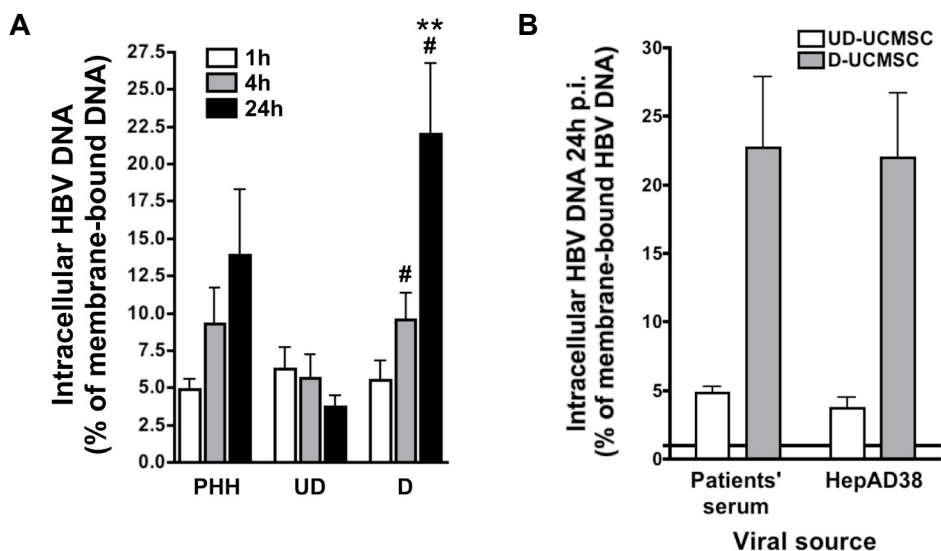


Figure 2.4.9. D-UCMSCs are capable of HBV uptake. A) D-UCMSCs were as efficient as PHHs in internalizing membrane-bound HBV after temperature shift to 37°C. Results are expressed as percentage of the internalized (i.e. protease-protected) versus cell membrane-attached (protease-sensitive) HBV DNA after a given incubation period (N=5 for UD- and D-UCMSCs, N=3 for PHHs; $p=0.016$ [#] compared to 1h post-infection and $p=0.004$ [**] compared to UD-UCMSCs, Mann-Whitney test). **B)** No difference was found in UD- (white bars) and D-UCMSCs (black bars) uptake capabilities when HBV from serum of HBeAg-positive, treatment-naïve patients or HepAD38 culture were compared. Results are expressed as percentage of the amount of HBV DNA attached to the cell membrane after the incubation period (solid line) (N=2 with patients serum, N=5 with HepAD38-derived HBV; $p=ns$).

The amount of virus taken up by D-UCMSCs at 24h increased with the increase of MOI (figure 2.4.10). Little increase was seen for MOI $>10^3$, suggesting saturation of the receptor(s).

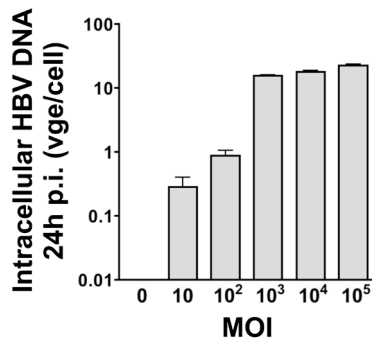


Figure 2.4.10. A MOI of 100 was needed to achieve at least 1 vge/cell in D-UCMSCs at 24h post-infection. No further increase was observed for MOIs $>10^3$ (N=3, $p=ns$).

Viral entry after 24h at 37°C was confirmed by immunofluorescence. Both PHHs and D-UCMSCs, but not UD-UCMSCs, showed a positive staining for intracellular HBcAg (figure 2.4.11).

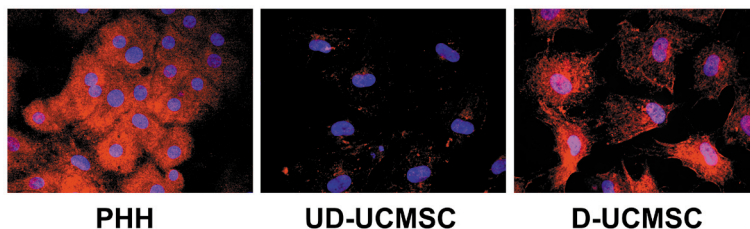


Figure 2.4.11. Immunofluorescence for HBcAg in PHHs, UD-UCMSCs and D-UCMSCs 24h post-infection (confocal laser microscopy, AlexaFluor-594/DAPI staining; original magnification 400x).

Cell susceptibility to HBV replication

Total HBV DNA was quantified by qPCR at 3 and 7 days post-infection, and compared to the amount of viral DNA present inside the cells at 24h post-incubation (figure 2.4.12A). Intracellular HBV DNA levels decreased along time in UD-UCMSCs ($p<0.0001$), whereas they increased in PHHs ($p=ns$) and D-UCMSCs ($p=0.016$) at day 3 and 7, suggesting productive viral replication. At 7 days post-infection, intracellular HBV DNA levels did not differ between D-UCMSCs

and PHHs, but were significantly higher in D-UCMSCs than in UD-UCMSCs ($p<0.01$). Further increase of HBV DNA levels was seen at 10 days post-infection in D-UCMSCs ($p=0.029$; figure 2.4.12B). PHHs were not tested beyond 7 days post-infection to avoid experimental artifacts due to dedifferentiation [8].

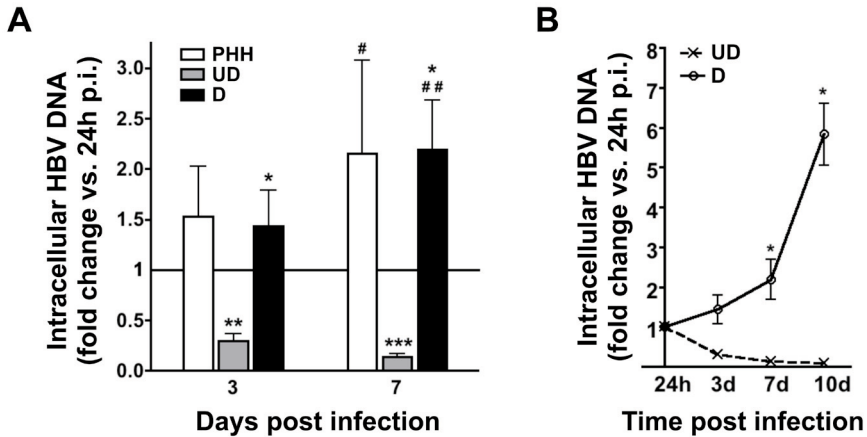


Figure 2.4.12. HBV replication in UCMSCs. A) Total intracellular HBV DNA at 3 and 7 days post-infection (qPCR), as compared to the amount of HBV DNA internalized by the cells at 24 hours post-infection (represented by the horizontal line; N=6 for D-UCMSCs, N=4 for UD-UCMSCs and N=3 for PHH; $p<0.05$ [*], $p<0.01$ [**] and $p<0.001$ [***] compared to 24h, one sample two-sided t test; $p<0.05$ [#] and $p<0.01$ [##] compared to UD-UCMSCs; Mann-Whitney test). **B)** At 10 days post-infection, intracellular HBV DNA further increased in D-UCMSCs but not in UD-UCMSCs (qPCR, N=6 for D-UCMSCs and N=4 for UD-UCMSCs, $p=0.029$; Mann-Whitney test).

In D-UCMSCs, at least a MOI of 100 was needed to yield detectable viral replication (figure 2.4.13). No difference in replication efficiency was found when comparing HBV-infected serum and HepAD38-derived HBV as inocula ($p=ns$).

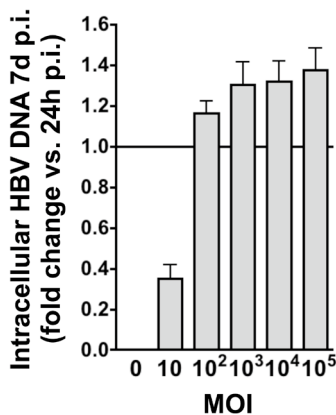


Figure 2.4.13. In D-UCMSCs, a MOI of at least 100 was needed to have detectable viral replication (expressed as fold change of intracellular HBV DNA levels at 7 days post-infection vs. 24h post-infection; N=3, $p=ns$).

To prove HBV replication in D-UCMSCs, we assessed HBV DNA replication intermediates by Southern blot on the very same samples used for qPCR experiments (Figure 2.4.12). As shown in figure 2.4.14, rcDNA increases along time. dsIDNA, which was absent from the inoculum and at day 1, increased at day 3 and 10. ssDNA was absent from the inoculum, increased at day 1 and 3 and was not detected anymore at day 10 (when it possibly had been converted into rcDNA). cccDNA levels were below the detection limit of the technique. However, establishment of an initial cccDNA pool has to be anticipated otherwise no synthesis of replicative intermediates could be observed [23].

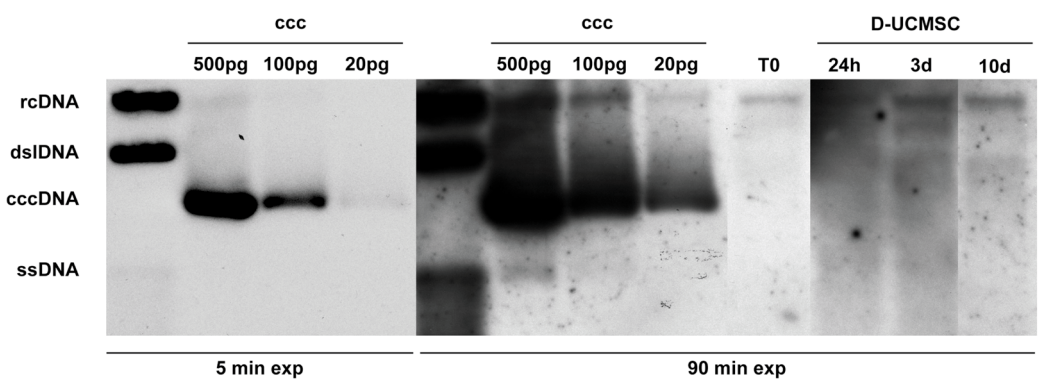


Figure 2.4.14. Southern blot analysis for HBV replicative forms. Southern blot on 3 µg total cellular DNA from a pool of the very same samples as used for qPCR experiments shown in figure 6.9A-B. All the lanes were on the same membrane. The film was exposed 90 minutes (same exposure for all the lanes). A shorter exposure (5 min) of size markers is shown for clarity. T0 represents DNA from the inoculum. ccc, 3.2 kb supercoiled bacterial plasmid standard cross-reacting with the HBV specific DIG-probe.

We subsequently measured cccDNA by qPCR (figure 2.4.15). In D-UCMSCs, cccDNA levels increased along time up to 0.03 ± 0.04 copies/cell at 7 days post-infection, corresponding to approximately every 30th cell containing at least 1 copy of cccDNA. By contrast, 0.7 ± 0.8 copies/per cell were found in PHHs and no cccDNA was detected in UD-UCMSCs. Controls to prove specificity of cccDNA detection by qPCR have been described in chapter 2.3 (see page 120).

To further confirm the effect of differentiation on cellular susceptibility to viral replication, we infected UD-UCMSCs as described above and induced differentiation after 24h. For this post-infection differentiation we used a serum-free medium corresponding to the final step of the differentiation protocol described

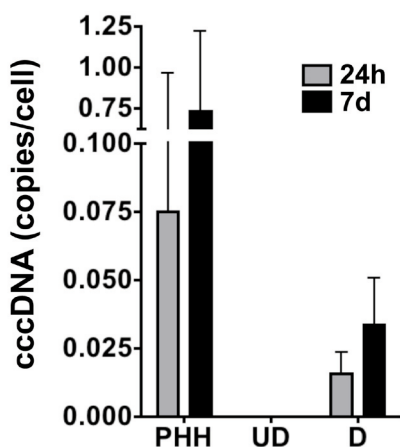


Figure 2.4.15. cccDNA in D-UCMSCs, 7 days post-infection, expressed as copies/cell (qPCR, N=5 for D-UCMSCs, N=4 for UD-UCMSCs and N=3 for PHH; $p=ns$, Mann-Whitney test).

above. HBV DNA levels in UCMSCs undergoing post-infection differentiation increased along time as compared to UD-UCMSCs ($p=0.018$), suggesting replication of the small quantity of HBV (0.22 ± 0.32 vge/cell) internalized by undifferentiated cells (figure 2.4.16A).

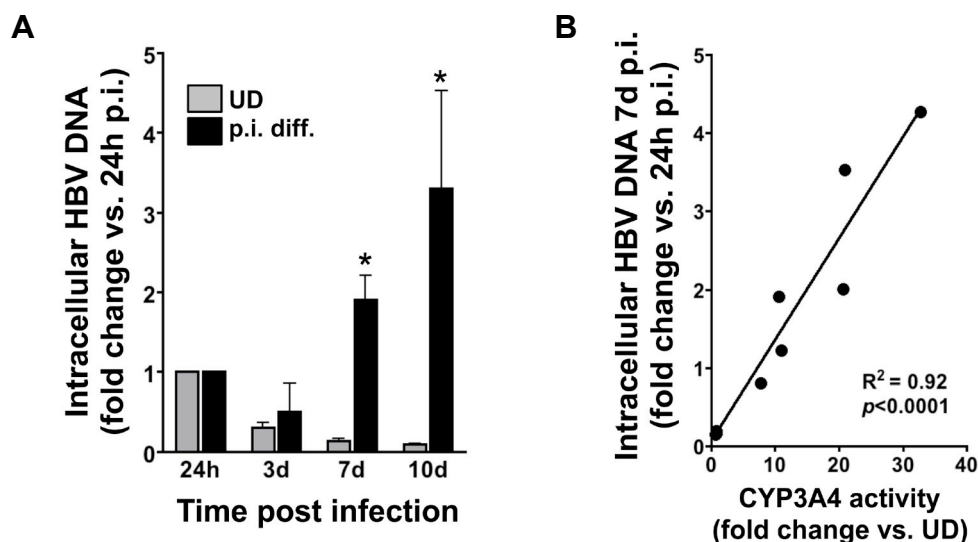


Figure 2.4.16. A) Post-infection differentiation assay: differentiation of UD-UCMSCs 24 hours post-infection led to viral replication of the small amount of HBV already internalized (N=3 for post-infection diff. UCMSCs [black bars], N=4 for UD-UCMSCs [gray bars]; $p=0.018$ [*] as compared to UD-UCMSCs; one sample t test). **B) Correlation between differentiation status and cell susceptibility to viral replication.** The state of differentiation is expressed as relative activity of CYP3A4 as compared to the average activity in UD-UCMSCs. Ability of the cells to support viral replication is expressed as relative increase of intracellular HBV DNA levels at day 7 post-infection as compared to the virus internalized after 24 hours at 37°C. Both UD- and D-UCMSCs are considered in this analysis ($R^2=0.92$, $p<0.0001$, N=9: N=6 for D-UCMSCs and N=3 for UD-UCMSCs).

HBV replication efficiency in UCMSCs varied among different donors, and was directly correlated with the quality of differentiation ($R^2=0.92$, $p<0.0001$; figure 2.4.16B).

To assess whether known antivirals could inhibit viral replication in D-UCMSCs, the cells were inoculated at a MOI of 10^5 in the presence of increasing concentration of PMPA (0-2.5 $\mu\text{g/mL}$; figure 2.4.17A). A dose-dependent inhibition of HBV replication was shown after 7 days of PMPA treatment (figure 2.4.17B). EC_{50} for PMPA was 0.21 $\mu\text{g/mL}$ (95% confidence interval, 0.12-0.39) in D-UCMSCs, as compared to 0.12 $\mu\text{g/mL}$ (95% confidence interval, 0.11-0.14) in HepAD38 (figure 2.4.17C).

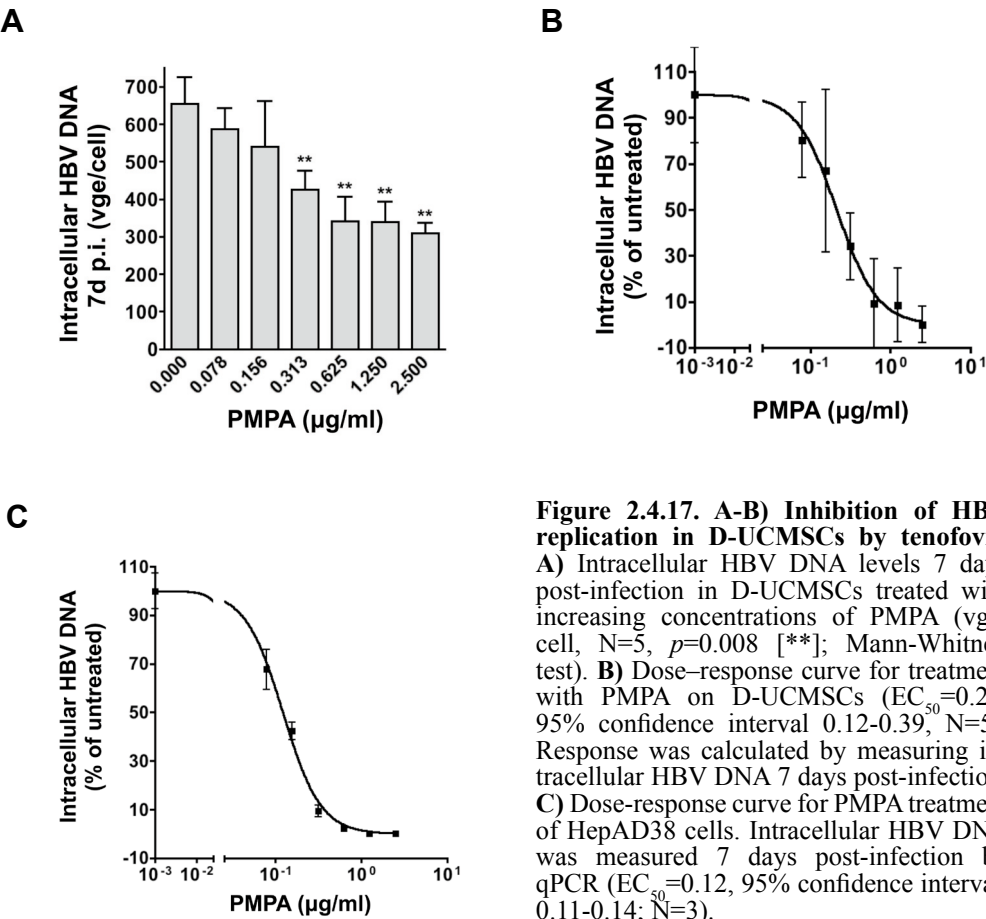


Figure 2.4.17. A-B) Inhibition of HBV replication in D-UCMSCs by PMPA. **A)** Intracellular HBV DNA levels 7 days post-infection in D-UCMSCs treated with increasing concentrations of PMPA (vge/cell, $N=5$, $p=0.008$ [**]; Mann-Whitney test). **B)** Dose-response curve for treatment with PMPA on D-UCMSCs ($EC_{50}=0.21$, 95% confidence interval 0.12-0.39, $N=5$). Response was calculated by measuring intracellular HBV DNA 7 days post-infection. **C)** Dose-response curve for PMPA treatment of HepAD38 cells. Intracellular HBV DNA was measured 7 days post-infection by qPCR ($EC_{50}=0.12$, 95% confidence interval, 0.11-0.14; $N=3$).

Transcription of viral genes

Viral RNAs (pg and precore) were quantified in D-UCMSCs by RT-qPCR. As shown in figure 2.4.18A, viral RNAs increased in D-UCMSCs along time, reaching 0.103 ± 0.023 copies/cell at day 7 post-infection. Treatment with $2.5 \mu\text{g/ml}$ PMPA reduced the amount of viral RNAs found in D-UCMSCs by 30%, 81% and 97% at 1, 3 and 7 days post-infection, respectively ($p=\text{ns}$; figure 2.4.18B).

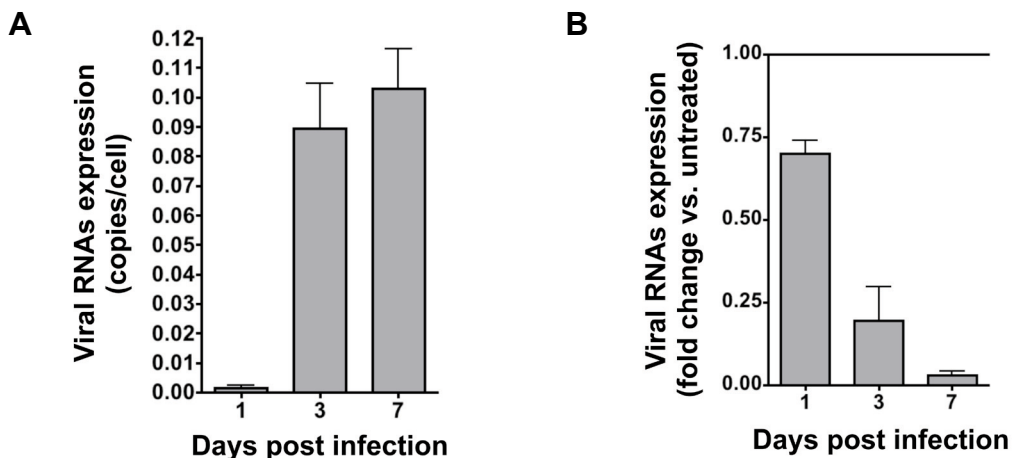


Figure 2.4.18. Transcription of viral RNAs. **A)** Viral RNAs (pg and preC) measured in D-UCMSCs by RT-qPCR at 1, 3 and 7 days post-infection (copies/cell ; $N=3$, $p=\text{ns}$). **B)** Viral RNAs measured in D-UCMSCs after $2.5 \mu\text{g/ml}$ PMPA treatment (RT-qPCR). Results are expressed as fold change vs. no treatment (horizontal line).

Specificity of viral RNA quantification by RT-qPCR was assessed by evaluating efficacy of DNase I treatment. Increasing concentrations of plasmid pAM6 (from $0.1 \mu\text{g}$ to 100 ng) were treated with 1 U DNase I . HBV DNA concentration was then measured by qPCR with the RC01 assay (figure 2.4.19). HBV DNA was undetectable in samples containing less than $10 \mu\text{g}$ pAM6 (corresponding to $2.5 \times 10^8 \text{ IU/ml}$). Further evaluation of DNase I efficiency was carried out by adding increasing amounts of pAM6 to 1×10^6 uninfected HepG2 cells (from $0.1 \mu\text{g}$ to 100 ng) and extracting RNA as described above. After DNase I treatment and reverse transcription, no HBV DNA was detectable by qPCR anymore in samples containing less than 10 ng pAM6 (corresponding to $2.8 \times 10^{11} \text{ IU/ml}$; figure 2.4.19). Specificity of viral RNA quantification by RT-qPCR was confirmed at each experiment.

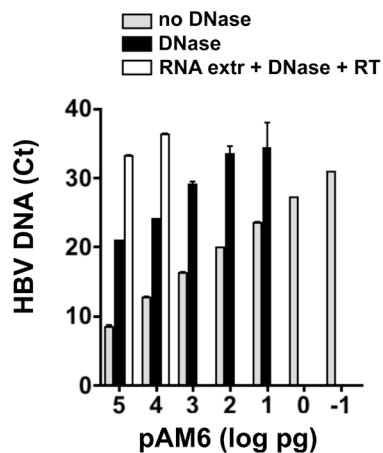


Figure 2.4.19. Specificity of viral RNAs quantitation by RC01 assay. Efficacy of DNase I treatment was assessed by treating decreasing concentrations of pAM6 (from 100 ng to 0.1 pg) with 1U DNase I before qPCR (black bars). The same amounts of pAM6 were added to 1×10^6 uninfected HepG2 cells before RNA extraction and RT-qPCR (white bars). Non digested pAM6 samples (100 ng - 0.1 pg) served as controls (gray bars). Results are expressed as Ct.

De novo synthesis of viral proteins

We assessed synthesis of viral proteins in UCMSCs by immunofluorescence at day 10 post-infection. A staining for HBcAg was shown in D-UCMSC, whereas it was absent in UD-UCMSCs (figure 2.4.20).

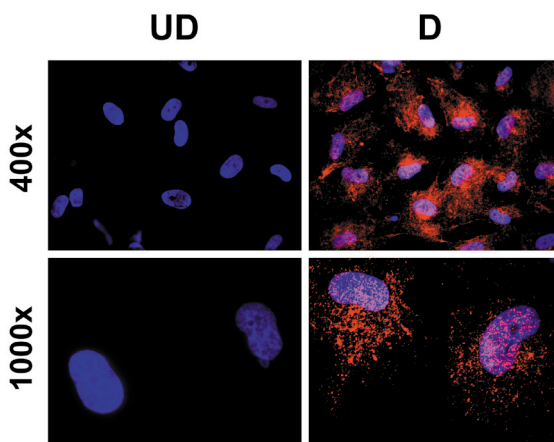


Figure 2.4.20. Synthesis of viral proteins. Immunofluorescence for HBcAg in UD-UCMSCs and D-UCMSCs 10 days post-infection (AlexaFluor-594/DAPI staining). Original magnifications: 400x and 1000x (confocal laser microscopy).

Secretion of HBsAg and HBeAg was measured by ELISA at different time points post-infection. PHHs secreted increasing amounts of HBeAg from day 3 post-infection (figure 2.4.21A), as expected [8,27]. As the amount of both HBsAg and HBeAg secreted by D-UCMSCs was too close to the limit of detection of the technique, we concentrated proteins from conditioned medium by ultrafiltration before ELISA. A significant increase of both viral antigens was detected over time

in D-UCMSCs supernatant ($p<0.05$; figure 2.4.21B-C), whereas they remained negative in UD-UCMSCs.

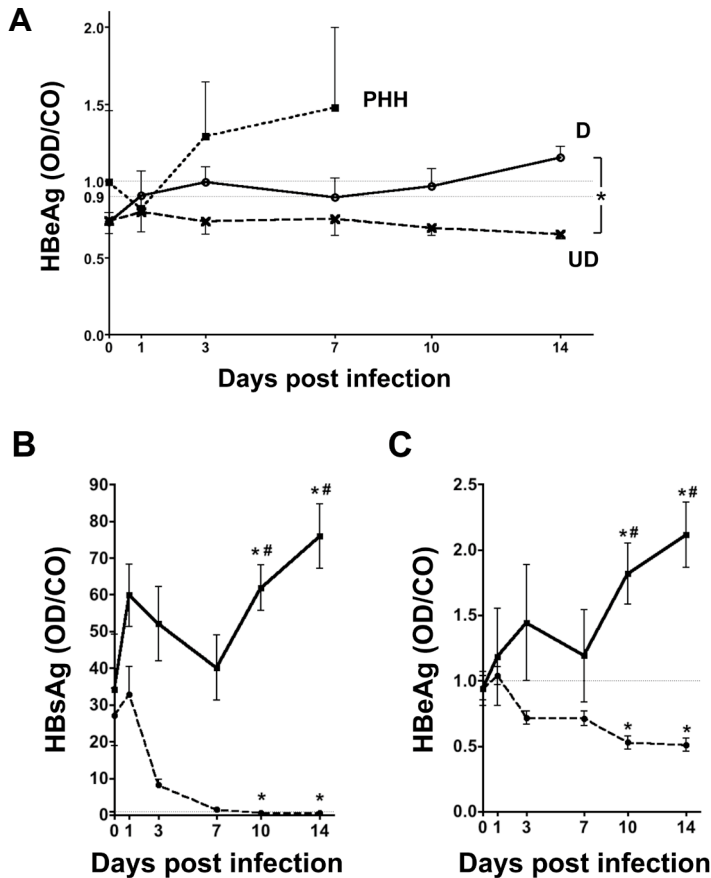


Figure 2.4.21. Secretion of viral proteins. **A)** Synthesis of HBeAg measured in conditioned medium of D-UCMSCs (○), PHHs (■), and UD-UCMSCs (×) by ELISA (N=3 for PHHs, UD- and D-UCMSC; $p<0.05$ (*) as compared to UD-UCMSCs). **B-C)** Synthesis of HBsAg (B) and HBeAg (C) measured in conditioned medium of UD- (dotted line) and D-UCMSCs (solid line) by ELISA after 10x concentration via ultrafiltration (N=4; $p<0.05$ [*] as compared to 7 days post-infection; $p<0.05$ [#] as compared to UD-UCMSCs). Cut-off was calculated according to manufacturer's instructions. Values are expressed as optical density/cut-off (OD/CO ≥ 1 considered positive, OD/CO ≥ 0.9 considered as borderline).

Low-level, yet clearly detectable synthesis of viral proteins was confirmed in D-UCMSCs (but not in UD-UCMSCs) by western blotting for HBeAg after immunoprecipitation (figure 2.4.22).

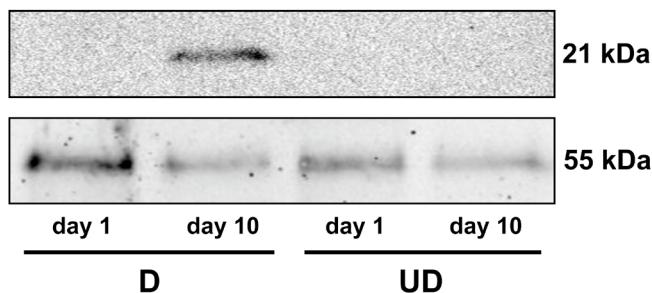


Figure 2.4.22. Western blot analysis for HBcAg (21 KDa) on UD- and D-UCMSCs 10 days post-infection, after immunoprecipitation. As internal control, IgG heavy chains are shown at 55 KDa (subsequent staining with the same antibody as used for immunoprecipitation).

Secretion of infectious progeny

We assessed the infectivity of viral particles secreted by D-UCMSCs on PHHs. PHHs from one donor were inoculated with D-UCMSCs-derived HBV (3 donors, MOI 21.1 ± 26.6) for 16h at 37°C. Intracellular HBV DNA levels increased in PHHs at day 7 post-infection as compared to 24h post-infection (8 ± 1.8 fold change, $p=0.06$; figure 2.4.23A), suggesting productive viral replication and confirming

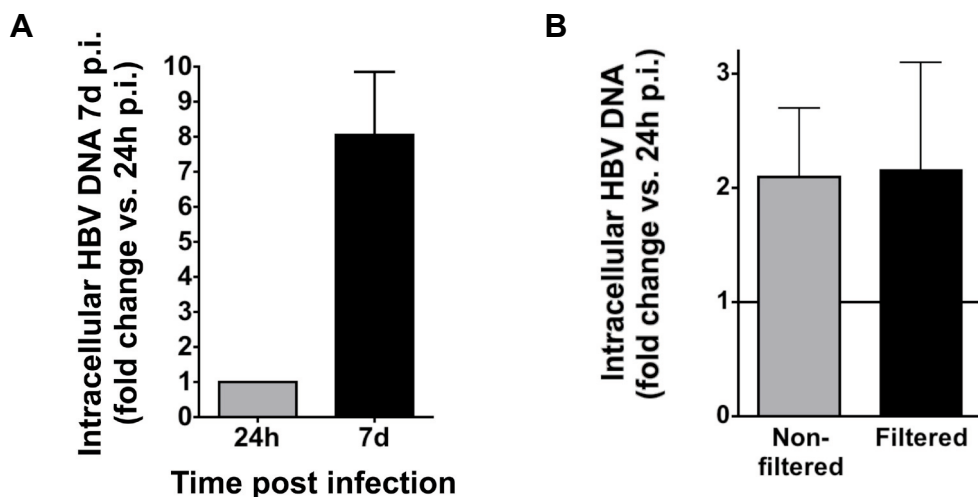


Figure 2.4.23. Passage of infectious progeny virus. **A)** D-UCMSCs proved to produce fully competent viral particles, as HBV secreted in conditioned medium by D-UCMSCs 14 days post-infection was able to infect PHHs. Three independent infection assays were performed using PHHs from the same donor and concentrated HBV from 3 different D-UCMSCs' infection experiments ($p=0.06$). **B)** Conditioned medium from D-UCMSCs cultures collected 14 days post-infection was used to infect another population of D-UCMSCs (two independent experiments). Filtration of such a medium with 200 nm filters before HBV PEG precipitation and infection had did not modify infection efficiency (intracellular HBV DNA expressed as fold change as compared to 24h post-infection).

the ability of D-UCMSCs to synthesize infectious HBV particles, completing the full viral life cycle.

To rule out the possibility of a contamination due to the carry over of infected cells from the original culture, infectivity assay was repeated filtering the HBV-containing conditioned medium through a 200 nm filter before PEG precipitation. Such an inoculum was used to infect a different D-UCMSCs population. Such a second-line infection was carried out as described for PHHs. The same replication efficiency was observed at 7 days post infection when HBV from filtered and non-filtered supernatant was used (figure 2.4.23B).

2.4.3. Discussion

We describe here a new *in vitro* non-transformed human model of HBV infection. We show that non-liver derived mesenchymal stem cells (UCMSCs) are turned permissive to the entire HBV life cycle upon *in vitro* hepatogenic differentiation. UCMSCs are widely characterized MSCs that are easy to obtain and to expand in culture. As we previously demonstrated, UCMSCs are capable of exhibiting many of the markers and functions typical of mature hepatocyte upon *in vitro* differentiation [12]. We set up infection conditions in order to analyze the different steps of the viral cycle, and demonstrated that, although replication efficiency downstream of viral entry was quite low, HBV uptake was fully supported by D-UCMSCs, and comparable to PHHs. To our knowledge, this is the first human, non-transformed *in vitro* model, other than primary hepatocytes, to be fully capable of HBV uptake.

Few studies have been conducted on the susceptibility of MSCs to HBV infection, none on UCMSCs. In a first report, undifferentiated bone marrow MSCs (BM-MSCs) have been shown to be partially permissive to HBV entry (although the possibility of nonspecific attachment not leading to endocytosis was not ruled out), but resistant to viral replication [24]. On the contrary, Xie and colleagues demonstrated that BM-MSCs were resistant to HBV infection, before and after differentiation to hepatocyte-like cells [25]. Eventually, in a recent paper, undifferentiated BM-MSCs were surprisingly, but not unequivocally, shown to support HBV replication for some days [30]. As none of these conflicting studies

evaluated binding or uptake kinetics, used a validated model for control, nor analyzed the relationship between the differentiation status and the susceptibility to infection, it is difficult to draw conclusions. Here, using PHHs as control, we analyzed in detail the different steps of HBV cycle, and showed that undifferentiated MSCs are susceptible to HBV attachment, almost completely resistant to viral entry and definitely resistant to viral replication, whereas they become capable to support the full viral life cycle if efficiently differentiated to hepatocyte-like cells. As described in chapter 1.2 and 1.3, HBV extremely restrictive tropism has complicated the understanding of viral life cycle and has hampered new antiviral drug development for decades. PHHs are the preferred tissue culture model, but they are difficult to obtain, maintain in culture and infect *in vitro* [8]. PTHs are an interesting alternative [9,10]. Unfortunately, it is well known that susceptibility of primary hepatocytes to HBV infection is rapidly lost during prolonged culture (see chapter 1.3). Such a loss in susceptibility has been correlated to the dedifferentiation process to which hepatocytes rapidly undergo *in vitro* [26]. The significant direct correlation between CYP3A4 activity (a surrogate marker of the state of differentiation) and UCMSCs susceptibility to viral replication described here further highlights the central role of differentiation status in determining HBV hepatotropism, confirming in a non-transformed cellular model of extrahepatic origin what had been observed in hepatocytes and demonstrated in HepaRG cells. As variability in the quality of differentiation of UCMSCs obtained from different donors was observed, CYP3A4 activity could be used as a marker to predict suitability of each UCMSCs population for infection studies.

HBV is highly infectious *in vivo*, but only a small proportion of the cells are infected *in vitro* [27]. PTHs and HepaRG cells share the same disadvantages of PHHs in terms of low replication efficiency and high MOI needed to infect a reasonable proportion of cells [9-11]. UCMSCs were even less efficient than PHHs in replicating HBV, showed a low-level protein synthesis and a MOI of at least 100 was needed to achieve a productive infection. Nevertheless, infection efficiency of D-UCMSCs was comparable to other widely used models. A MOI of 100 is indeed commonly used to infect primary duck hepatocytes with DHBV [19], whereas a MOI of 200 is required to infect HepaRG cells in the presence of PEG (and an 8-time higher MOI is needed in the absence of PEG) [11]. It has

been shown that transcription of HBV genes and subsequent viral replication is dependent upon the degree of differentiation of the host cell (see page 75) [1]. Liver-enriched transcription factors such as HNF4 α , HNF1 α and HNF3 β have been demonstrated to have a central role in regulating viral promoters and enhancers [2,3]. Here we show that UCMSCs express HNF4 α mRNA upon differentiation, but at much lower level than PHHs, which could account for the observed lower intracellular replication efficiency. Furthermore, a difference in the mechanism of cccDNA formation between PHHs and D-UCMSCs cannot be *a priori* excluded and could account for the lower levels of cccDNA measured in the latter.

As our aim was to create an *in vitro* model as “physiological” as possible, and not to maximize infection efficiency, we decided to avoid the use of all adjuvant molecules that could cause possible experimental artifacts. Although no PEG was added during infection, a small carryover secondary to the HBV concentration method used cannot be excluded. Therefore, confirmation of HBV uptake efficiency of D-UCMSCs need to be confirmed using viral particles purified through ultracentrifugation.

Of the growth factor used to achieve hepatogenic differentiation of UCMSCs, only EGF has an effect on viral replication (although probably indirect, through the induction of cell cycle progression), stimulating cccDNA synthesis and repressing transcription of viral genes [28,29]. Anyway, none of the growth factors added during the last steps of differentiation or during the infection experiments is known to have a direct regulatory effect on HBV cycle. Therefore, their effect on viral entry, cccDNA amplification and transcription is probably secondary only to the induction of cellular differentiation. According to the presented data, it is conceivable that an improvement of the quality of differentiation would allow improving infection efficiency of this model. The successful inhibition of viral replication by tenofovir demonstrated here suggests that, upon achievement of higher infection efficiency, UCMSCs could also be suitable for antiviral drug testing development.

It is known that infection efficiency varies widely when infected sera from different donors are used [8]. Furthermore, human serum was reported to inhibit infection of HBV to primary tupaia hepatocytes [10]. We chose to use sera from treatment-naïve patients in the immunotolerant phase of the disease (all infected by wild-

type genotype D HBV) in order to avoid having antibodies or resistant mutants. Moreover, to further standardize infection conditions, we also used the virus produced by HepAD38 cells (see page 116). Interestingly, when immunotolerant carriers were used as donors, we found no difference in infection efficiency and reproducibility between patient-derived and HepAD38-derived HBV.

In chapter 1.2 we described HBV binding and uptake as the most important determinants of HBV hepatotropism. As stated above, the role of the differentiation state on cell susceptibility to HBV entry has not been clarified yet, but very few *in vitro* models are capable of viral uptake and suitable to study this problem [9-11]. UCMSCs susceptibility to viral entry, dependency of such permissiveness upon hepatogenic differentiation, and a HBV uptake efficiency comparable to PHHs, represent the most exciting characteristics of new model described here. This unique human, easily available, non-transformed, *in vitro* model of HBV infection could prove extremely useful to study early infection events and the role of cell differentiation state on such events.

2.4.4. References

- [1] Quasdorff M, Protzer U. Control of hepatitis B virus at the level of transcription. *J Viral Hep.* 2010;17:527-536.
- [2] Tang H, McLachlan A. Transcriptional regulation of hepatitis B virus by nuclear hormone receptors is a critical determinant of viral tropism. *Proc Natl Acad Sci USA.* 2001;98:1841-1846.
- [3] Quasdorff M, Hösel M, Odenthal M, Zedler U, Böhne F, Gripon P, et al. A concerted action of HNF4 α and HNF1 α links hepatitis B virus replication to hepatocyte differentiation. *Cell Microbiol.* 2008;10:1478-1490.
- [4] Sells MA, Zelent AZ, Shvartsman M, Acs G. Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. *J Virol.* 1988;62:2836-2844.
- [5] Sun D, Nassal M. Stable HepG2- and Huh7-based human hepatoma cell lines for efficient regulated expression of infectious hepatitis B virus. *J Hepatol.* 2006;45:636-645.
- [6] Rabe B, Glebe D, Kann M. Lipid-Mediated Introduction of Hepatitis B Virus Capsids into Nonsusceptible Cells Allows Highly Efficient Replication and Facilitates the Study of Early Infection Events. *J Virol.* 2006;80:5465–5473.
- [7] Glebe D & Urban S. Viral and cellular determinants involved in hepadnaviral entry. *World J Gastroenterol.* 2007;13:22-38.
- [8] Mabit H, Vons C, Dubanchet S, Capel F, Franco D, Petit MA. Primary cultured normal human hepatocytes for hepatitis B virus receptor studies. *J Hepatol.* 1996;24:403–412.

- [9] Walter E, Keist R, Niederöst B, Pult I, Blum HE. Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. *Hepatology*. 1996;24:1–5.
- [10] Köck J, Nassal M, MacNelly S, Baumert TF, Blum HE, Weizsäcker von F. Efficient infection of primary tupaia hepatocytes with purified human and woolly monkey hepatitis B virus. *J Virol*. 2001;75:5084–5089.
- [11] Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci USA*. 2002;99:15655–15660.
- [12] Campard D, Lysy PA, Najimi M, Sokal EM. Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. *Gastroenterology*. 2008;134:833–848.
- [13] Najimi M, Khuu DN, Lysy PA, Jazouli N, Abarca J, Sempoux C, et al. Adult-derived human liver mesenchymal-like cells as a potential progenitor reservoir of hepatocytes? *Cell Transplant*. 2007;16:717–728.
- [14] Lysy PA, Smets F, Sibille C, Najimi M, Sokal EM. Human skin fibroblasts: From mesodermal to hepatocyte-like differentiation. *Hepatology*. 2007;46:1574–1585.
- [15] Khuu DN, Scheers I, Ehnert S, Jazouli N, Nyabi O, Buc-Calderon P, et al. In vitro differentiated adult human liver progenitor cells display mature hepatic metabolic functions: a potential tool for in vitro pharmacotoxicological testing. *Cell Transplant*. 2011;20:287–302.
- [16] Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, et al. 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*. 1997;41:1715–1720.
- [17] Leistner CM, Gruen-Bernhard S, Glebe D. Role of glycosaminoglycans for binding and infection of hepatitis B virus. *Cell Microbiol*. 2008;10:122–133.
- [18] Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. *Nature*. 1979;281:646–650.
- [19] Dallmeier K, Schultz U, Nassal M. Heterologous replacement of the supposed host determining region of avihepadnaviruses: high in vivo infectivity despite low infectivity for hepatocytes. *PLoS Pathog*. 2008;4: e1000230.
- [20] Ying C, de Clercq E, Nicholson W, Furman P & Neyts J. Inhibition of the replication of the DNA polymerase M550V mutation variant of human hepatitis B virus by adefovir, tenofovir, L-FMAU, DAPD, penciclovir and lobucavir. *J Viral Hepat* 2000;7:161-165.
- [21] Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8:315–317.
- [22] Gripon P, Diot C, Guguen-Guillouzo C. Reproducible High Level Infection of Cultured Adult Human Hepatocytes by Hepatitis B Virus: Effect of Polyethylene Glycol on Adsorption and Penetration. *Virology*. 1993;192:534–540.
- [23] Tuttleman JS, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell*. 1986;47:451-460.
- [24] Rong Q, Zhang L, Su E, Li J, Liu Z, Huang Z, et al. Bone marrow-derived mesenchymal stem cells are capable of mediating hepatitis B virus infection in injured tissues. *J Viral Hepat*. 2008;15:607-614.
- [25] Xie C, Zheng Y-B, Zhu H-P, Peng L, Gao Z-L. Human bone marrow mesenchymal

stem cells are resistant to HBV infection during differentiation into hepatocytes in vivo and in vitro. *Cell Biology International*. 2009;33:493-500.

- [26] Elaut G, Henkens T, Papeleu P, Snykers S, Vinken M, Vanhaecke T, et al. Molecular Mechanisms Underlying the Dedifferentiation Process of Isolated Hepatocytes and Their Cultures. *curr drug metab*. 2006;7:629-660.
- [27] Ochiya T, Tsurimoto T, Ueda K, Okubo K, Shiozawa M, Matsubara K. An in vitro system for infection with hepatitis B virus that uses primary human fetal hepatocytes. *Proc Natl Acad Sci USA*. 1989;86:1875-1879.
- [28] Schorr O, Borel C, Trepo C, Zoulim F, Hantz O. Effects of liver growth factors on hepadnavirus replication in chronically infected duck hepatocytes. *J Hepatol* 2006;44:842-847.
- [29] Hantz O, Parent R, Durantel D, Gripon P, Guguen-Guillouzo C, Zoulim F. Persistence of the hepatitis B virus covalently closed circular DNA in HepaRG human hepatocyte-like cells. *J Gen Virol* 2009;90:127-135.
- [30] Ma R, Xing Q, Shao L, Wang D, Hao Q, Li X, et al. Hepatitis B virus infection and replication in human bone marrow mesenchymal stem cells. *Virol J*. 2011;8:486.

2.5. Asialoglycoprotein receptor mediates HBV entry into susceptible cells

2.5.1. Introduction

In the previous chapter, we demonstrated that UCMSCs are able of supporting the full HBV life cycle upon *in vitro* differentiation to hepatocyte-like cells. Viral replication in D-UCMSCs, although not very efficient, was confirmed by (i) the increase of intracellular HBV DNA at qPCR, (ii) increased transcription of viral RNAs along time, (iii) a dose-dependent inhibition by tenofovir, (iv) *de novo* synthesis of viral proteins and (v) the secretion of infectious viral progeny. Most importantly, D-UCMSCs were shown to be susceptible to viral entry, taking up HBV as efficiently as PHHs. Being human and non-transformed, and not requiring the use of adjuvant molecules to achieve a productive infection, this new model proved suitable to study early events during HBV infection.

As described in chapter 1.2, preS1 domain of HBV L surface protein is necessary for binding to the cell membrane. Asialoglycoprotein receptor (ASGPR) is the only preS1-recognizing receptor to be expressed almost exclusively by hepatocytes [1], and it has been suggested to play a major role in HBV binding and uptake (see page 70) [2-4]. In the present study we used D-UCMSCs to evaluate ASGPR as a possible candidate receptor for HBV. Part of the results presented here served as a proof of concept for the suitability of D-UCMSCs for studies on HBV entry, and were published in the article describing the model (see annex 2).

2.5.2. Results

Asialoglycoprotein receptor is expressed by UCMSCs

ASGPR, an hepatocyte-specific lectin [2], was significantly more expressed by D-UCMSCs than UD-UCMSCs, both at mRNA (N=6, $p=0.16$) and protein levels (figure 2.5.1A). Immunofluorescence analysis confirmed the expression of such a receptor by UCMSCs upon hepatogenic differentiation (figure 2.5.1B). In this regard, D-UCMSCs resembled PHHs serving as positive controls.

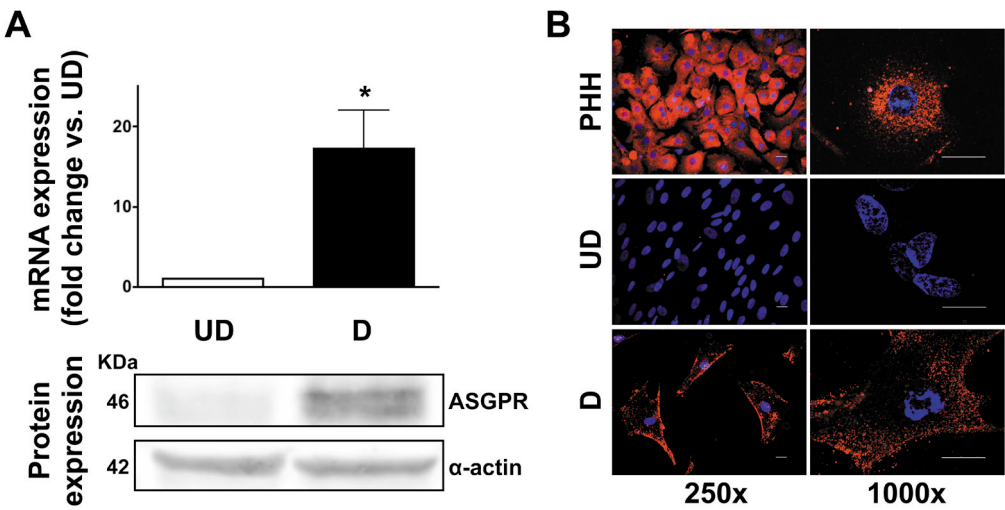


Figure 2.5.1. A) ASGPR was more strongly expressed by D-UCMSCs than by UD-UCMSCs both at mRNA (N=6, $p=0.16$, Wilcoxon signed rank test; RT-qPCR) and protein (western blot analysis) levels. **B)** Immunofluorescence for ASGPR on uninfected PHHs, UD-UCMSCs and D-UCMSCs (confocal laser microscopy, AlexaFluor-594/DAPI staining; original magnifications 250x and 1000x).

Interestingly, HBV uptake by D-UCMSCs was directly correlated to ASGPR mRNA expression ($R^2=0.924$, $p<0.01$; figure 2.5.2), suggesting that ASGPR could play a role in viral entry into these cells.

ASGPR saturation prevents HBV binding and uptake

Binding of ligands to ASGPR is known to be Ca^{2+} -dependent [1]. In figure 2.5.3, we show that HBV binding to D-UCMSCs may be partially inhibited by Ca^{2+} chelation ($21.1\pm 2.5\%$ inhibition). Thyroglobulin addition, a known ASGPR-

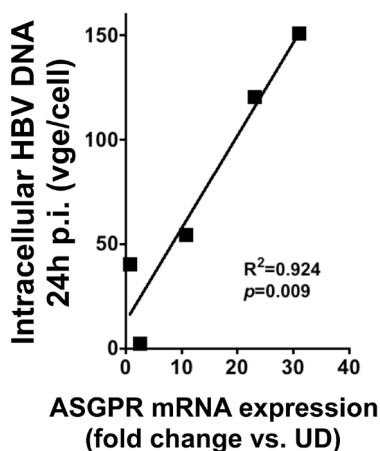


Figure 2.5.2. Correlation between ASGPR mRNA expression and D-UCMSCs susceptibility to viral uptake ($R^2=0.924$, $p<0.009$, $N=5$). Uptake capacity is expressed as intracellular HBV DNA (vge/cell) at 24h post-infection. ASGPR expression is expressed as fold change as compared to UD-UCMSCs.

specific ligand [5], reduced viral binding by $77.8\pm1\%$. Suramin, which does not bind ASGPR, but has been proposed to mimick highly sulfated cellular structures that are required for attachment [6,7], inhibited $87.4\pm1\%$ of HBV binding to D-UCMSCs.

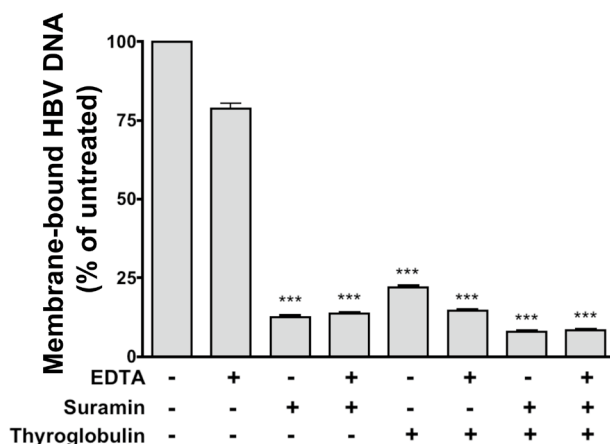


Figure 2.5.3. HBV binding inhibition. Incubation of D-UCMSCs before and during HBV infection ($\text{MOI } 10^3$ for 2h at 4°C) with either 5mM EDTA, 1mg/ml thyroglobulin (with and without EDTA), or 100 $\mu\text{g/ml}$ suramin (with and without EDTA) led to inhibition of HBV binding (membrane-bound HBV DNA measured by qPCR, $N=3$, $p<0.0001$, one sample two-sided t test).

The addition of increasing concentrations of D-galactose (0.03-100 μM), before and during inoculation of D-UCMSCs, resulted in a dose-dependent inhibition of HBV uptake (up to $79.3\pm0.7\%$, $p<0.0001$; figure 2.5.4A). An $\text{EC}_{50}=0.2 \mu\text{M}$ (95%

confidence interval, 0.17-0.23) was calculated for D-galactose by dose-response analysis (figure 2.5.4B).

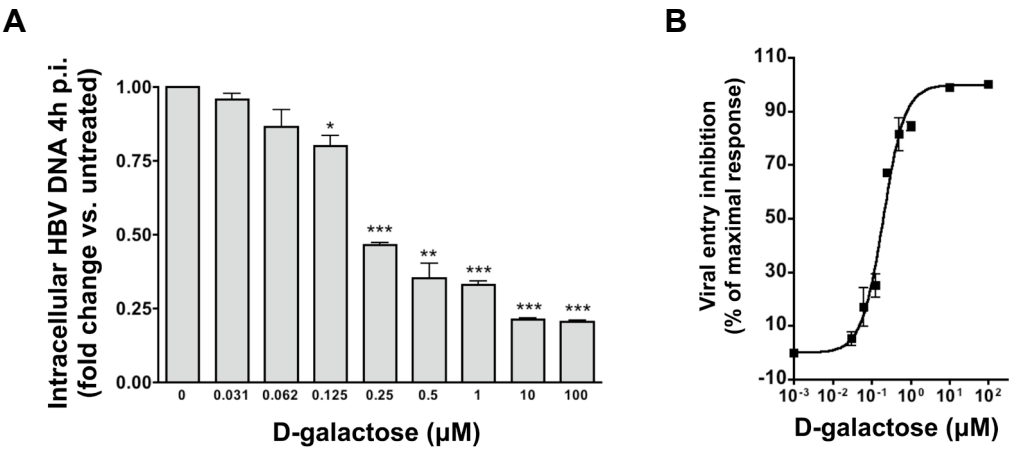


Figure 2.5.4. Inhibition of viral uptake. **A)** HBV uptake by D-UCMSCs was inhibited by D-galactose in a dose-dependent manner (treatment before and during 4h of inoculation with HBV at a MOI of 10³). Intracellular HBV DNA levels measured by qPCR are shown (expressed as fold change vs. no galactose; N=3; $p=0.03$ [*], $p<0.01$ [**], $p<0.001$ [***], one sample two sided t test). **B)** Inhibition of viral uptake upon saturation of ASGPR with D-galactose: dose-response curve for D-galactose treatment (0.03-100 μM) of D-UCMSCs. Intracellular HBV DNA was measured after 4h post-infection by qPCR and compared to cells incubated with HBV in absence of the ligand ($EC_{50}=0.2\mu\text{M}$, 95% confidence interval, 0.17-0.23; N=3).

HBV controls ASGPR expression through a positive feedback mechanism

Eventually, we used HepAD38 cells to evaluate a potential viral control over ASGPR expression. As described in chapter 1.3, transcription of viral genes in HepAD38 model is controlled by a TetOff system, and the full viral life cycle is hence supported upon removal of tetracycline from the culture media. We measured ASGPR expression in HepAD38 before and 3, 7 and 10 days after tetracycline removal. Induction of viral genes transcription led to an overexpression of ASGPR by the cells (figure 2.5.5).

ASGPR upregulation by tetracycline removal was evident at protein level as well (figure 2.5.6A-B). It was an early event, as it was already present at day 3, preceding the increase in HBV DNA levels, which was evident from day 7 (figure 2.3.3B, page 117).

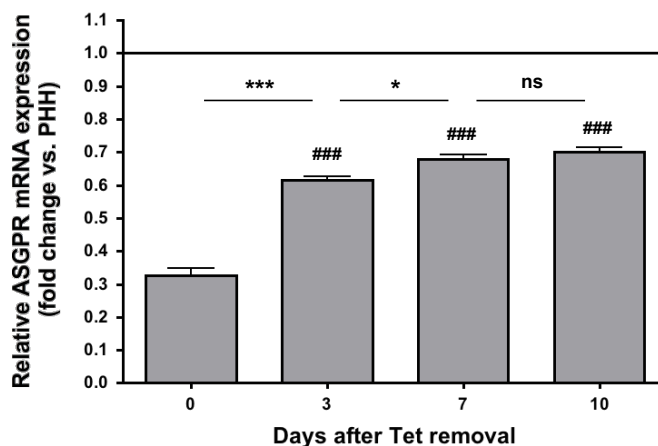
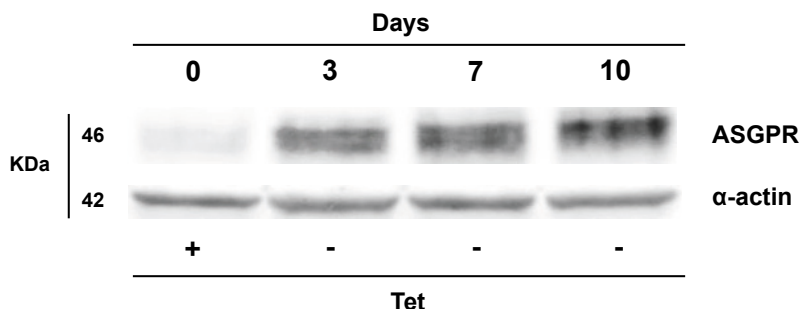


Figure 2.5.5. ASGPR mRNA expression in HepAD38 cells upon induction of viral replication through tetracycline removal from culture medium (fold change vs. PHH; N=3; * $p=0.121$, *** $p<0.001$ compared to the previous sample, ### $p<0.001$ compared to day 0).

A



B

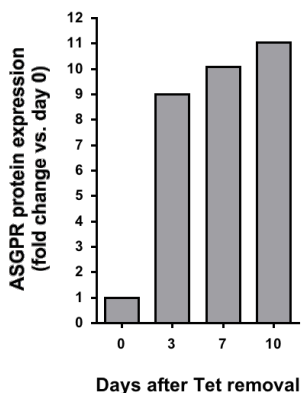


Figure 2.5.6. A) Western blot analysis showing ASGPR protein expression in HepAD38 cells upon induction of viral replication through tetracycline removal from culture medium (representative image of two experiments). **B)** Densitometric analysis of ASGPR bands obtained by western blotting, normalized for α -actin (N=2).

2.5.3. Discussion

HBV binding and entry into susceptible cells is believed to be a multistep process (see chapter 1.2, page 68). The initial attachment of the virus to the cell membrane could be mediated by heparan sulfate proteoglycans [7], and does not seem to be specific of susceptible cells [8]. A more specific binding to one or multiple receptors, expressed mainly on hepatocytes, and localized on the basolateral membrane, is probably responsible of restricted HBV host range [9]. Among the many candidate receptors, ASGPR is the only to be mostly hepatocyte-specific. It is a lectin expressed on the sinusoidal and basolateral hepatocellular membrane, which is responsible for the uptake of desialylated glycoproteins [1]. Basma and colleagues showed that ASGPR is upregulated in embryonic stem cells upon hepatic differentiation [10]. As described in chapter 1.2, different studies have proved it to be necessary for HBV binding and uptake [2-4]. Furthermore, ASGPR was shown to be overexpressed in HepG2.2.15 cells (see page 71), and its downregulation with antisense oligonucleotides was shown to reduce HBV DNA levels [4,11].

Here we show that ASGPR is upregulated in UCMSCs upon hepatogenic differentiation. We also show a dose-dependent inhibition of HBV binding and uptake when ASGPR is saturated with known specific ligands. Taken together, these preliminary results suggest that HBV might regulate ASGPR expression through one of its proteins, predisposing the host cell to favor viral binding and uptake, and thus regulating its own entry through a positive feedback mechanism. Nevertheless, viral control of cellular ASGPR expression needs confirmation in a non-transformed model, and further study to understand the exact mechanism. Overall, ASGPR is a promising candidate receptor, but further verification is unequivocally necessary to definitely prove the role of this receptor in HBV entry. Whether viral binding is a double-step process effectively involving heparan sulfate proteoglycans, and how the latter interacts with the high-affinity, liver-specific receptor(s) responsible of HBV uptake, is still to be confirmed in an unbiased human model. The experiments presented here are a proof of concept that UCMSCs may be a suitable model to study early infection events and could be used to address such questions.

2.5.4. References

- [1] Spiess M. The asialoglycoprotein receptor: a model for endocytic transport receptors. *Biochemistry*. 1990;29:10009-10018.
- [2] Treichel U, Meyer zum Büschenfelde KH, Stockert RJ, Poralla T, Gerken G. The asialoglycoprotein receptor mediates hepatic binding and uptake of natural hepatitis B virus particles derived from viraemic carriers. *J Gen Virol*. 1994;75:3021-3029.
- [3] Owada T, Matsubayashi H, Sakata H, Ihara S, Sato K, Ikebuchi, et al. Interaction between desialylated hepatitis B virus and asialoglycoprotein receptor on hepatocytes may be indispensable for viral binding and entry. *J Viral Hepat*. 2006;13:11-18.
- [4] Yang J, Bo XC, Ding XR, Dai JM, Zhang ML, Wang XH, et al. Antisense oligonucleotides targeted against asialoglycoprotein receptor 1 block human hepatitis B virus replication. *J Viral Hepat*. 2006;13:158-165.
- [5] Saunier B, Triyatni M, Ulianich L, Maruvada P, Yen P, Kohn LD. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. *J Virol*. 2003;77:546-559.
- [6] Offensperger WB, Offensperger S, Walter E, Blum HE & Gerok W. Suramin prevents duck hepatitis B virus infection in vivo. *Antimicrob Agents Chemother*. 1993;37:1539-1542.
- [7] Schulze A, Gripon P & Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. *Hepatology*. 2007;46:1759-1768.
- [8] Leistner CM, Gruen-Bernhard S, Glebe D. Role of glycosaminoglycans for binding and infection of hepatitis B virus. *Cell Microbiol*. 2008;10:122-133.
- [9] Schulze A, Mills K, Weiss TS, Urban S. Hepatocyte polarization is essential for the productive entry of the hepatitis B virus. *Hepatology* 2012;55:373-383.
- [10] Basma H, Soto-Gutiérrez A, Yannam GR, Liu L, Ito R, Yamamoto T, et al. Differentiation and Transplantation of Human Embryonic Stem Cell-Derived Hepatocytes. *Gastroenterology*. 2009;136:990-999.
- [11] Yang J, Bo XC, Yao J, Yang NM, Wang SQ. Differentially expressed cellular genes following HBV: potential targets of anti-HBV drugs? *J Viral Hepat*. 2005;12:357-363.

PART 3

GENERAL DISCUSSION AND PERSPECTIVES

HBV infection is still one of the most important causes of liver disease worldwide. Although the incidence of HBV infection dramatically declined thanks to the introduction of universal immunization programs and the implementation of blood-donor screening, a significant number of subjects are still infected each year. In spite of a rather benign course of chronic disease during childhood and adolescence, HBV chronic carriers are at risk of developing infection-related HCC and cirrhosis. Available antiviral therapies, of which only few are licensed for children, are mostly safe but partially effective.

Such treatments act either stimulating the host immune system or inhibiting viral replication. In order to develop new antiviral drugs against new targets, an in-depth understanding of each step of the viral life cycle is required. Although in the last decades our knowledge of HBV biology has significantly improved, there are some huge gaps in our comprehension of its life cycle. Most notably, the way by which HBV enters susceptible cells, and the determinants of its hepatotropism and strict species-specificity are still poorly understood. We know the viral structures responsible of such events (the preS1 domain of LHBs), whereas their cellular counterparts have not been identified yet.

Unfortunately, research on receptors and pathways responsible for cell susceptibility to HBV infection has been hampered by the lack of a suitable models. The *ex vivo* model constituted by human liver slices, although promising, has not been tested for HBV infection yet. PHHs still represent the gold standard, whereas their use is limited by scarce availability, rapid dedifferentiation *in vitro* and high variability between donors. PTHs and the HepaRG cell line are the only other models susceptible to HBV infection, but each presents some major disadvantages hampering its definitive adoption to study viral entry, besides sharing some of the limitations of PHHs in terms of accessibility, reproducibility and low yield of infection.

The ideal *in vitro* model, besides being capable of supporting the full HBV life cycle, should be of human origin, non-transformed, easily available and easy to expand and maintain in culture. We studied umbilical cord matrix stem cells, which are widely characterized MSCs capable of acquiring hepatocytes functions upon *in vitro* differentiation, and assessed their suitability for such a purpose. We demonstrate here that UCMSCs are turned permissive to support the entire

HBV life cycle upon *in vitro* hepatogenic differentiation. We inoculated UCMSCs with a standardized source of HBV and studied infection kinetics comparing this new potential model to PHHs. UD-UCMSCs, although being permissive to viral binding, had a very limited uptake capacity, whereas D-UCMSCs showed binding and uptake capabilities similar to PHHs. Replication efficiency was low compared to PHHs, but was confirmed by (i) cccDNA synthesis, (ii) dose-dependent inhibition by specific antiviral treatment, (iii) synthesis of viral RNAs, (iv) *de novo* synthesis of viral proteins and (v) secretion of infectious viral progeny. D-UCMSCs proved to be the first human, non-transformed *in vitro* model, other than PHHs, to be fully capable of HBV uptake. Although some variability among different donors was noted, this model allows more reproducible results than primary hepatocytes. Furthermore, UCMSCs are much more easily available than PHHs and, unlike HepaRG cells, their infection does not depend on the use of adjuvant molecules (such as PEG or DMSO), that could cause experimental artifacts.

Among the several different receptors described to recognize HBV preS1 domain, asialoglycoprotein receptor is the only to be expressed almost exclusively by hepatocytes. ASGPR is upregulated in embryonic stem cells upon hepatic differentiation, and it has been shown to be involved in HBV entry into PTHs and HepaRG. ASGPR is a promising candidate receptor, but a clear demonstration of its mechanism of action in a non-transformed human model is lacking. We used UCMSCs to study the receptor, and confirmed in this new model that ASGPR is more strongly expressed upon hepatogenic differentiation, that its expression is correlated to viral uptake, and that the latter is inhibited in a dose-dependent manner upon addition of ASGPR-specific ligands. Although further verification is unequivocally necessary to prove the role of this receptor in HBV binding and uptake, these data are a proof of concept that UCMSCs may be a suitable model to study viral entry. Such cells could constitute a unique instrument to study early infection events and the role of cell differentiation state on such events.

The exciting qualities and limitations of this new model allow rising several puzzling questions that open the way for plenty of possibilities for future research (figure 3.1).

The high efficiency of viral uptake constitutes the most noteworthy feature of this model. It permits to plan studies that could eventually lead to the definition of the

receptor responsible for HBV entry, unveiling the reasons of HBV hepatotropism and, potentially, leading to the discovery of new antiviral drugs. Treatments acting on viral entry could be extremely effective in preventing HBV infection. As a proof

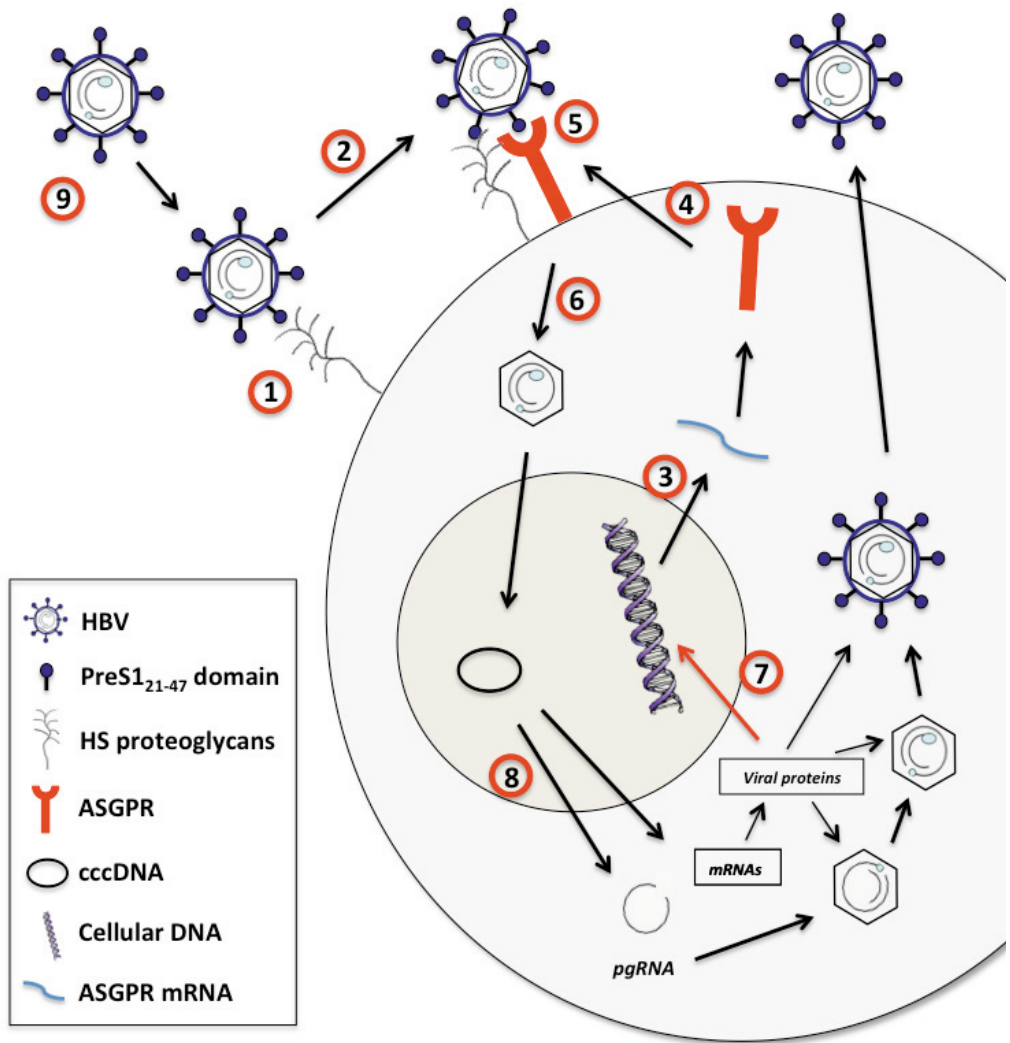


Figure 3.1. Possibilities for future research on HBV-cell interaction using UCMSCs. This model could allow assessing the role of heparan sulfate proteoglycans (1) and the double-step hypothesis of viral binding (2). Comparison between UD- and D-UCMSCs in terms of gene expression (3) and membrane proteins (4) could lead to the identification of cellular determinants of HBV hepatotropism. Studies of candidate receptors in this model could prove the major role of ASGPR, from its interaction with the virus (5) to the mechanism of uptake and trafficking of viral particles inside the cell (6). UCMSCs could also allow to confirm that HBV can control the expression of cellular genes (including ASGPR) through a positive feedback mechanism (7). Improvement of replication efficiency (8) through the amelioration of the quality of differentiation, could make UCMSCs suitable for antiviral screening assays and phenotypic testing of resistant viral strains. Eventually, D-UCMSCs could prove susceptible to the infection by several other hepatotropic viruses (9), thus opening the way for further exciting possibilities.

of concept, even low subcutaneous doses of acylated preS1-derived peptides, which are supposed to compete with HBV for binding to liver cells, were shown to efficaciously prevent HBV infection in immunodeficient mice repopulated with PHHs [1]. Therapies addressing viral entry would be of great utility for acute hepatitis B (reducing viral spread across the liver) and to prevent HBV infection in patients undergoing liver transplantation or reactivation in those receiving immunosuppressive treatments. They could also be useful in chronic hepatitis B, in combination with interferon or nucleos(t)ide analogues, avoiding reinfection of neighbor hepatocytes and thus maximizing the effect of other antivirals.

We carefully set up infection experiments to study each step of the viral life cycle discretely. This allowed the demonstration that UCMSCs are a suitable model to analyze HBV binding and uptake in detail, and could be used to study the mechanism of action of candidate receptors, from viral attachment, to internalization and intracellular trafficking. This model could allow to understand whether viral entry is a double-step process involving a low-affinity attachment to heparan sulfate proteoglycans followed by a high-affinity binding to a liver-specific receptor, as suggested [2-3]. The data previously obtained on HepaRG cells and PTHs could indeed suffer from the limitations of the model used (such as the origin of the cells and, most notably, the use of PEG) and might not reflect the real circumstances. Being human, non-transformed cells not needing adjuvant molecules for infection, UCMSCs are suitable to overcome such limitations, and could allow clarifying the role of heparan sulfate proteoglycans. For the same reasons, the role of co-players, such as endogenous sialidase or fibronectin, on HBV infection, could be assessed in this model.

We showed that UCMSCs' susceptibility to HBV infection is inducible upon hepatogenic differentiation. This finding is extremely exciting, as it could facilitate identifying the specific determinants of such a susceptibility, and hence of HBV hepatotropism. Previous assays to identify such determinants comparing whole genome gene expression profiles of permissive and non-permissive cells failed because of the wide differences between the models used [4]. On the contrary, with UCMSCs, we could compare exactly the same cell type, from the same donor, in two different state of differentiation, thus increasing the chances of identifying the variations significant for susceptibility (and, by contrast, for

resistance) to infection. Moreover, as the hepatocyte-like phenotype is achieved through a stepwise process, a comparison of the cells at each step of differentiation could allow to detailed tracking of the events leading to such a permissiveness. Likewise, it is also conceivable to use this model to perform a screening of membrane receptors and their modulation upon hepatogenic differentiation, directly identifying receptors more susceptible to have a role in HBV entry. As we showed above, UCMSCs would then be suitable to pursue the study on such receptors to assess their functional involvement in viral binding and uptake.

Our preliminary results on the study of the receptor responsible for viral entry are promising. Nevertheless they need to be confirmed and clarified in order to understand the mechanism of HBV-cell interaction. Such results were indeed obtained using concentrated HBV produced by HepAD38 cells. As it cannot not be excluded that HepAD38 cells could release a significant amount of non-enveloped capsids [5], which could bind to the cell by a mechanism different from complete viral particles, these results need to be repeated using purified HBV particles before inoculation.

As described above, to assess UCMSCs suitability for studies on candidate receptors for HBV, we focused on ASGPR because of its prevalent liver-specificity, its upregulation upon hepatogenic differentiation shown in other models, and the suggestive clues available on its ability to interact with HBV. Our results show that ASGPR could have a major role in HBV binding and uptake. Future studies would have to prove unequivocally that this receptor is necessary for HBV infection, and characterize in detail its interaction with HBV, assessing whether preS1 domain of LHBs is indeed recognized by one of ASGPR domains (as CRD domain). Moreover, following the hypothesis of a double-step binding, ASGPR potential collaboration with heparan sulfate proteoglycans should be tested and defined. The mechanism of viral uptake, and ASGPR exact role in viral entry and intracellular trafficking of viral particles, could be therefore elucidated. Furthermore, in-depth evaluation of ASGPR expression before, during and after *in vitro* hepatogenic differentiation of UCMSCs could offer important insight on HBV hepatotropism.

Our preliminary results on positive feedback control of ASGPR expression by HBV reveal a new kind of interaction between the virus and its host. A previous

study on experimentally infected chimpanzee suggested that HBV does not modify host cellular gene transcription [6], whereas *in vitro* studies on HepG2.2.15 demonstrated a direct action of the virus on cellular transcription profile [7]. We observed such an exciting phenomenon in HepAD38 cells. It needs therefore to be confirmed in a non-transformed model such as UCMSCs, and deserves further study to understand its exact mechanism.

Although susceptibility of differentiated UCMSCs to infection is the most important finding of this work, resistance of undifferentiated cells deserves great attention. If such a feature would prove valid for liver stem cells as well, it could have interesting repercussions on the management of patients with chronic hepatitis B. Liver cells would indeed be infected by the virus released by neighbour hepatocytes only upon their differentiation. During the natural history of CHB, after the loss of immunotolerance, the immune system constantly attacks infected liver cells, which leads to liver regeneration (and, eventually to cirrhosis). Resistance of the stem cell population would mean that a treatment preventing such an infection, even though ineffective on already infected cells, would efficaciously protect newly differentiated hepatocytes. Such drugs could either be addressed against viral entry, as suggested above, or act preventing cccDNA formation, as the recently described disubstituted sulfonamides [8]. Combination of this treatments with available antiviral drugs could change the fate of patients affected by CHB.

The improvement of the quality of differentiation or the development of methods of enrichment of well-differentiated cells could ameliorate replication efficiency of the model presented here, leading to its use for antiviral screening assays or for *in vitro* phenotypic assays on patients' mutant strains [9]. CYP3A4 activity at the end of the differentiation protocol could hypothetically be used to select populations more likely to have good infection efficiency. Otherwise, D-UCMSCs could be enriched for ASGPR by fluorescence-activated cell sorting, as previously described for other *in vitro*-differentiated cells [10].

Based on the results obtained with HBV, it is conceivable that D-UCMSCs could be used to study other hepatotropic viruses. Recently, Roelandt and colleagues demonstrated that human embryonic stem cells could be infected with HCV *in vitro* upon differentiation to hepatocyte-like cells [11]. Such results constitute a

proof of concept of the feasibility of infecting an *in vitro* differentiated human stem cell with HCV. Nevertheless, our model has some advantages over the one described by Roelandt et al., as it is based on UCMSCs, which are much more easily available than embryonic stem cells, and of uncontroversial origin. D-UCMSCs could therefore be suitable to study other major hepatotropic viruses, such as HAV, HDV, HEV and HCV. Many other systemic viruses may cause liver injury, ranging from mild elevation of aminotransferases to acute hepatitis and occasionally acute liver failure. Cytomegalovirus, as many of such viruses, replicates in liver cells and may cause severe hepatitis with life-threatening consequences (prevalently, but not exclusively, in immunocompromized patients). Unfortunately, virus-hepatocyte interactions have not been clarified because of the difficulty in using PHHs described above [12,13]. As UCMSCs express most of mature hepatocytes markers and functions upon *in vitro* differentiation, it is likely that they could be susceptible to many other viruses such as adenoviruses, herpesviruses (cytomegalovirus), flaviviruses (dengue virus and yellow fever virus), among others, representing an interesting instrument for drug development.

Conclusions

Overall, we showed that UCMSCs represent a precious new *in vitro* model to study HBV infection. Besides supporting the full viral life cycle, they are the first human, non-transformed *in vitro* model, other than PHHs, to be fully permissive to viral entry. Their easy availability and expansion potential, together with the straightforwardness of the protocol used for their hepatogenic differentiation, could allow their widespread use for both basic research and drug development. Through the understanding of virus biology and virus-cell interactions, such a model could allow the discovery of new targets for antiviral treatment, answering to the need for new drugs to prevent and cure hepatitis B infection and, hypothetically, many other viral liver diseases.

References

- [1] Petersen J, Dandri M, Mier W, Lütgehetmann M, Volz T, von Weizsäcker F, et al. Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein. *Nat Biotechnol.* 2008;26:335-341.
- [2] Schulze A, Gripon P, Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. *Hepatology.* 2007;46:1759-1768.
- [3] Leistner CM, Gruen-Bernhard S, Glebe D. Role of glycosaminoglycans for binding and infection of hepatitis B virus. *Cell Microbiol* 2008;10:122-133.
- [4] Hart SN, Li Y, Nakamoto K, Subileau EA, Steen D, Zhong XB. A comparison of whole genome gene expression profiles of HepaRG cells and hepG2 Cells to primary human hepatocytes and human liver tissues. *Drug Metabolism and Disposition* 2010;38, 988–994.
- [5] Sun D, Nassal M. Stable HepG2- and Huh7-based human hepatoma cell lines for efficient regulated expression of infectious hepatitis B virus. *J Hepatol.* 2006;45:636-645.
- [6] Wieland S, Thimme R, Purcell RH, Chisari FV. Genomic analysis of the host response to hepatitis B virus infection. *Proc. Natl. Acad. Sci. USA* 2004;101:6669-6674.
- [7] Yang J, Bo XC, Yao J, Yang NM, Wang SQ. Differentially expressed cellular genes following HBV: potential targets of anti-HBV drugs? *J Viral Hepat* 2005;12:357-363.
- [8] Cai D, Mills C, Yu W, Yan R, Aldrich CE, Saputelli JR, et al. Identification of disubstituted sulfonamide compounds as specific inhibitors of hepatitis B virus covalently closed circular DNA formation. *Antimicrob. Agents Chemother.* 2012;56:4277-4288.
- [9] Durantel D, Brunelle MN, Gros E, Carrouée-Durantel S, Pichoud C, Villet S, et al. Resistance of human hepatitis B virus to reverse transcriptase inhibitors: from genotypic to phenotypic testing. *J Clin Virol.* 2005;34 Suppl 1:S34-43.
- [10] Basma H, Soto-Gutiérrez A, Yannam GR, Liu L, Ito R, Yamamoto T, et al. Differentiation and Transplantation of Human Embryonic Stem Cell-Derived Hepatocytes. *Gastroenterology.* 2009;136:990-999.
- [11] Roelandt P, Obeid S, Paeshuyse J, Vanhove J, Van Lommel A, Nahmias Y, et al. Human pluripotent stem cell-derived hepatocytes support complete replication of hepatitis C virus. *J Hepatol.* 2012;57:246-251.
- [12] Plachter B, Sinzger C, Jahn G. Cell types involved in replication and distribution of human cytomegalovirus. *Adv Virus Res.* 1996;46:195-261.
- [13] Sacher T, Mohr CA, Weyn A, Schlichting C, Koszinowski UH, Ruzsics Z. The role of cell types in cytomegalovirus infection in vivo. *Eur J Cell Biol.* 2012;91:70-77.

PART 4

ANNEXES

Chronic hepatitis B in children and adolescents

Massimiliano Paganelli, Xavier Stephenne, Etienne M. Sokal*

Pediatric Gastroenterology and Liver Unit, Cliniques St Luc, Université Catholique de Louvain, Brussels, Belgium

Introduction

Hepatitis B virus (HBV) infection is still one of the most important causes of liver disease, with 2 billion people infected worldwide and more than 600,000 deaths each year, caused in 94% of cases by chronic infection-related cirrhosis and hepatocellular carcinoma (HCC) [1–3]. The World Health Organization (WHO) estimates more than 360 million persons being chronic carriers worldwide (6% of the world population). The incidence of HBV infection has been declining over the last two decades thanks to the introduction of universal immunization programs and the implementation of blood-donor screening. Nevertheless, a significant number of adults and children are still infected each year, the latter often developing chronic infection and requiring an appropriate follow-up. In spite of a rather benign course of chronic disease during childhood and adolescence, HBV chronic carriers have a lifetime risk of developing HCC up to 25%, and an incidence of cirrhosis of 2–3% per year [4]. Safe and partially effective antiviral therapies are available, but few are licensed for use in children, and an accurate selection of subjects to treat and of the right timing for treatment is needed to optimize efficacy and reduce viral resistance.

© 2012 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Epidemiology

HBV is transmitted by percutaneous and mucous membrane exposure to infectious blood, semen, vaginal secretions and saliva [5]. The extreme resilience of HBV, allowing its survival for more than a week on dry surfaces, explains the increased risk of horizontal intrafamilial transmission and the need of carriers counseling and household members vaccination [6–9]. Nonetheless, after 20 years from the introduction of immunization programs,

most infants and children are protected against HBV [10], and chronic carriers should not be isolated in schools or prevented from practicing sports. Transmission occurs rarely in childcare settings [7], but the risk is higher in detention centers, where adolescents are less likely to be immunized and have high-risk behaviors [11,12].

In highly endemic areas, infection occurs mainly in infancy and early childhood, with mother-to-child transmission accounting for more than half of chronic infections. After exposure, the risk of developing CHB is indeed higher for newborns (90%) than for infants and children <5 years of age (25–30%) or adolescents and adults (<5%) [13,14]. To further reduce mother-to-child transmission, WHO recommends the administration of both the vaccine and hepatitis B immunoglobulins (HBIG) to newborns of HBsAg-positive mothers within 24 h from birth (90–98% protection rate) [7,15–17]. HBIG administration to newborns of HBeAg-negative mothers does not change the overall protection rate but decreases the risk of fulminant hepatitis (higher in this group), proving to be cost-effective [16,18–20].

Newborns of highly viremic HBeAg-positive mothers are at increased risk of HBV infection despite proper immunization (breakthrough infection), compared to babies born to HBeAg-negative mothers (16.8% vs. 1.6%), and are at increased risk for chronicity [16,21]. Breakthrough infection is more likely to occur in newborns of mothers with genotype C, who have higher viral loads. Vaccination programs are therefore changing the HBV genotype distribution [22]. Intrauterine infection, hyporesponsiveness to vaccine, and vaccine escape mutants could play a role as well [16,21,23,24]. The risk of mother-to-child transmission also depends on maternal serum HBsAg titer and mode of delivery (10.5% for elective cesarean section vs. 28% for vaginal delivery) [21,25,26]. Vertical transmission can be further reduced by treating highly viremic mothers during the last trimester of pregnancy with either lamivudine, telbivudine, or tenofovir (1–2% risk reduction for lamivudine and 8% for telbivudine) [27–30].

HBsAg and HBV DNA can be detected in breast milk of chronic carriers, but no increased risk of transmission to a breastfed infant has been shown and breastfeeding is currently recommended after proper infant immunization [31–33].

WHO has recommended universal HBV vaccination since 1991, with the first dose to be administered within 24 h from birth, followed by at least two doses at 1 and 6 months of life [15,34–36]. By the end of 2010, 179 of the 193 WHO member states had implemented a nationwide childhood HBV immunization program and global HBV vaccine coverage was estimated at 75% [37]. In countries where such programs have been imple-

Keywords: Chronic hepatitis B; Children; Epidemiology; Natural history; Vaccination; Prevention; Treatment.

Received 29 October 2011; received in revised form 16 March 2012; accepted 20 March 2012

* Corresponding author. Address: Department of Paediatrics, Université Catholique de Louvain, 10, Av. Hippocrate, 1200 Brussels, Belgium. Tel.: +32 2 764 1386/87.

E-mail address: etienne.sokal@uclouvain.be (E.M. Sokal).

Abbreviations: HBV, hepatitis B virus; HCC, hepatocarcinoma; HBIG, hepatitis B immunoglobulins; CHB, chronic hepatitis B; ALT, alanine aminotransferases; cc-cDNA, covalently closed circular DNA; WHO, World Health Organization; ULN, upper limit of normal; IFN, interferon; FDA, Food and Drug Administration; VR, virologic response; PegIFN, pegylated interferon.



ELSEVIER

Review

Key Points

- Incidence of HBV infection declined over the last two decades in countries where universal immunization programs have been implemented
- Newborns, infants and young children are at higher risk for chronicity
- To reduce mother-to-child transmission, newborns of HBsAg-positive mothers should receive both the vaccine and HBIG at birth
- Vertical transmission of HBV to properly immunized newborns (breakthrough infection) is an unsolved problem, which is changing HBV genotype distribution
- CHB in childhood is a mild disease but can lead to cirrhosis and HCC in few, not yet well identified cases
- The goal of antiviral therapy is to reduce the risk of infection-related complications
- Response to the few treatments currently available for children is partial and limited to specific subgroups
- Decision to treat should weight the risk of disease progression against side effects and the risk of developing antiviral resistance
- A chronic delay in licensing new drugs for children has limited treatment options
- Treatment with IFN α should be considered for patients with elevated serum ALT, high HBV DNA and moderate to severe fibrosis/inflammation at liver biopsy
- Children in the immunotolerant phase should not be treated outside of clinical trials
- Pediatric clinical trials for telbivudine, PegIFN and tenofovir are currently ongoing

mented, prevalence of HBV infection decreased dramatically [38–40]. Many countries with high HBsAg seroprevalence ($\geq 8\%$) before vaccination programs (like China and Taiwan) are now considered at intermediate (2–7%) or low prevalence ($< 2\%$) [38,41–43]. In Taiwan, prevalence of HBsAg in children younger than 15 years of age decreased from 9.8% to 0.5% after 20 years of vaccination [42]. In China, HBsAg prevalence in children aged 1–14 years decreased by 83% (from 8% in 1992 to 1.36% in 2006) [44]. In a recent study, 4.6% of the total population of the United States has been exposed to HBV (past or chronic infection), with up to a 50-fold variation as age, ethnicity and country of birth vary. HBsAg seroprevalence in children and adolescents aged 6–12 years and 13–17 years is 0.03% and 0.02%, respectively. Prevalence of both exposure and chronic carrier state is higher among people coming from high or intermediate prevalence countries, and among those with lower family income or with high-risk behavior [45].

Although, among children born in Western Europe and North America, prevalence of HBV infection is low, pediatricians and

hepatologists are confronted with an increasing number of children adopted from higher prevalence countries (Table 1). Between 2% and 5% of all internationally adopted children, all coming from $\geq 2\%$ prevalence areas, are still infected with HBV [46,47]. CDC and American Academy of Pediatrics recommend screening all children adopted from these countries for HBV (HBsAg, HBsAb, HBcAb), at arrival and after 6 months, in order to provide vaccination to those without protective antibody levels (anti-HBs ≥ 10 mIU/ml) [15,48] or diagnose a previously unknown infection and proceed to immunization of household contacts [6,34,49].

Ten genotypes (A–J) and several subtypes have been described for HBV, with a distinctive geographic distribution. Although in most of Europe and North America, genotype A is predominant, many of the children coming from high or intermediate endemicity countries are infected with genotype B, C, D, or F (Table 1), are at increased risk of complications and may have a worse response to therapy [50–52].

Natural history

Chronic HBV infection is defined as a positive HBsAg for 6 months or longer. Chronic hepatitis B (CHB) in childhood is a mild disease, and infected children are mostly asymptomatic, have normal growth and a normal physical examination [53]. Most perinatally infected subjects present a positive HBeAg and high serum levels of HBV DNA, with normal or minimally elevated serum alanine aminotransferases (ALT). A transplacental transfer of maternal HBeAg has been proved both in mice and in humans, and could elicit HBe/HBcAg-specific Th cell tolerance in utero [40,54–56]. Such an induced tolerance could explain the higher chronicity rate in babies born to HBeAg-positive mothers and the different rate of chronicity between neonatal and adult infection. The synthesis of large amounts of HBeAg by the wild type virus could be necessary to maintain the tolerance [57]. This immunotolerant phase is characterized by high viral replication and little liver damage, although in Italian children, after 7 years of disease, 7–10% of subjects showed severe hepatitis at liver biopsy and 1.7–4.5% had cirrhosis [53,58]. It lasts 10–30 years when infection is acquired perinatally, whereas it is of minimal duration in subjects infected later in life.

Viral mutants not capable of secreting HBeAg are then slowly selected (pre-core mutants are detected in 89% of HBeAg-positive patients) [59]. Selection seems to be secondary to an advantage of such mutants over wild type HBV, as hepatocytes infected by the latter are more susceptible to cytotoxic T lymphocyte-mediated clearance [60]. Pre-core mutants selection leads to a lower secretion of HBeAg. When the decreased amount of secreted tolerogen is no more sufficient to maintain immunotolerance, the immune system starts attacking infected hepatocytes [56,57]. ALT levels rise, reflecting the biptic finding of necroinflammation of liver parenchyma, and HBV DNA levels fluctuate. This phase of active hepatitis leads to seroconversion to anti-HBe antibodies in 60–95% of patients on long-term follow-up [58,61]. ALT levels significantly increase before HBeAg clearance and may remain elevated for 6 months after seroconversion [53,62–64]. Up to 20% of spontaneous seroconverters may have ALT flare-ups in the years following seroconversion, which seems to be more likely in subjects carrying a pre-core mutant [63]. Spontaneous seroconversion occurs earlier and more frequently in subjects who have

Table 1. Estimated HBsAg seroprevalence and HBV genotype in countries of origin of internationally adopted children. (See below-mentioned references for further information.)

Country [Ref.]	HBsAg prevalence %		Predominant genotypes	Adopted children per year			
	Total	<16 yr		United States ^a	Italy ^b	France ^c	Canada ^d
China [41]	7.2	1.8	B, C	3401	116	100	230
Russian Federation [149]	1.4-8	-	D	1079	707	301	66
Ethiopia [150]	4.7	-	A	2511	274	352	159
Haiti [151,152]	5	-	A	133	0	992	94
Korea [153,154]	5.9	0.9	C	865	4	6	87
Colombia [155]	5.7	3.1	F	235	592	369	25
Vietnam [156]	10	7.5	B, C	9	251	469	29
Ukraine [157]	1-2	-	D	450	426	59	35
Brazil [158,159]	1	0.8	A, D, F	26	318	13	1
Taiwan [16,42]	1.1*	0.5	B, C	282	3	3	6
India [160]	2-8	-	A, D	241	124	21	36
Philippines [152]	10	-	B, C	216	30	14	96
Poland [149,157]	1-2	-	A	50	193	26	0
Nigeria [161-163]	4-12	12.4	E	189	5	23	6
Kazakhstan [164]	3.8	-	D	181	28	46	45
Bulgaria [149]	2-7	-	D	40	128	8	2
Ghana [165]	11.4	-	A	117	0	2	9
Belarus [166]	4.8	-	D	0	99	0	1
Cambodia [167]	8	2-14	B, C	0	87	2	1
Peru [149]	0.8-5.2	-	F	35	83	1	3
Hungary [149]	<1	-	A, D	5	78	5	0
Others				993	584	692	261
Total				11,058	4130	3504	1192

*Prevalence obtained from a study on 18-year old freshmen.

^aData refer to year 2010 (Bureau of Consular Affairs, US Department of State. US Intercountry Adoption [Internet]. adoption.state.gov 2011; [cited 10.01.12] Available from: http://adoption.state.gov/about_us/statistics.php). HBsAg seroprevalence in the United States is 0.27% (0.03% in 6–12 years old) [45].

^bData refer to year 2010 (Commission for intercountry adoptions. Data and perspectives in intercountry adoptions in Italy. Report on files from January 1 to December 31, 2010. Commission for intercountry adoptions; 2011. Available from: <http://commissioneadozioni.it/per-una-famiglia-adottiva/rapporto-statistico.aspx>). HBsAg seroprevalence in Italy is 0.9% [168].

^cData refer to year 2010 (Agence française de l'adoption. Statistiques de l'adoption internationale 2010. [Internet]. France Diplomatie 2011; [cited 10.01.12] Available from: <http://www.diplomatie.gouv.fr>). HBsAg seroprevalence in France is 0.65% [169].

^dData refer to year 2008 (HCCH Hague Conference Statistics. Canada: annual adoption statistics 2005–2009. [Internet]. HCCH 2012; [cited 10.01.12] Available from: <http://www.hcch.net>). HBsAg seroprevalence in Canada is 0.3–0.6% (0.4% for preadolescents) [170].

acquired HBV horizontally than in those infected perinatally (14–16% vs. 4–5% per year) [53,58,62,65]. It usually occurs after puberty, with about 90% of children <15 years of age still HBeAg positive [65]. It was shown that boys with earlier-onset puberty seroconverted at younger age (13 vs. 22 years), suggesting that androgens play a role in this process [64]. Environmental factors, such as nutritional status, have been suggested to influence immune response to HBV, accelerating seroconversion in children after adoption from developing countries [62]. Genotype C and maternal HBeAg-positive status were also associated with delayed spontaneous seroconversion [24,61,62,66–68].

HBsAg-positive, HBeAg-negative, and anti-HBe-positive patients are considered inactive carriers, express absent or low viral replication, with low or undetectable HBV DNA, and have inactive liver histology, with normal ALT levels. Inactive carriers with no signs of cirrhosis at seroconversion did not show progression to cirrhosis over 24–29 year follow-up [53,58].

On the contrary, 1–5% of HBeAg-positive children developed cirrhosis [53,58,69]. Between 2% and 5% of children with CHB developed HCC during childhood [24,58,70]. Incidence of HCC in high prevalence areas was significantly reduced after the implementation of immunization programs [71]. Children developing HCC are more likely to be males (70%), with cirrhosis (80%) and undergoing early seroconversion, suggesting that necroinflammation during seroconversion to anti-HBe may be severe and lead to cirrhosis and HCC [24,58,70]. Long-term risk of both HCC and cirrhosis is directly correlated to viral replication and serum HBV DNA levels [72,73]. The role of viral genotype on the risk of developing HCC is still to be defined: in children, 80% of HCCs are present in cirrhotic genotype B patients, whereas young adults with HCC are mostly non-cirrhotic [24,66]. In adults, genotypes C and F increase the risk for HCC [66,74–76]. Furthermore, such a risk is higher in persons with a family history of HCC, and a new susceptibility locus has been recently

Review

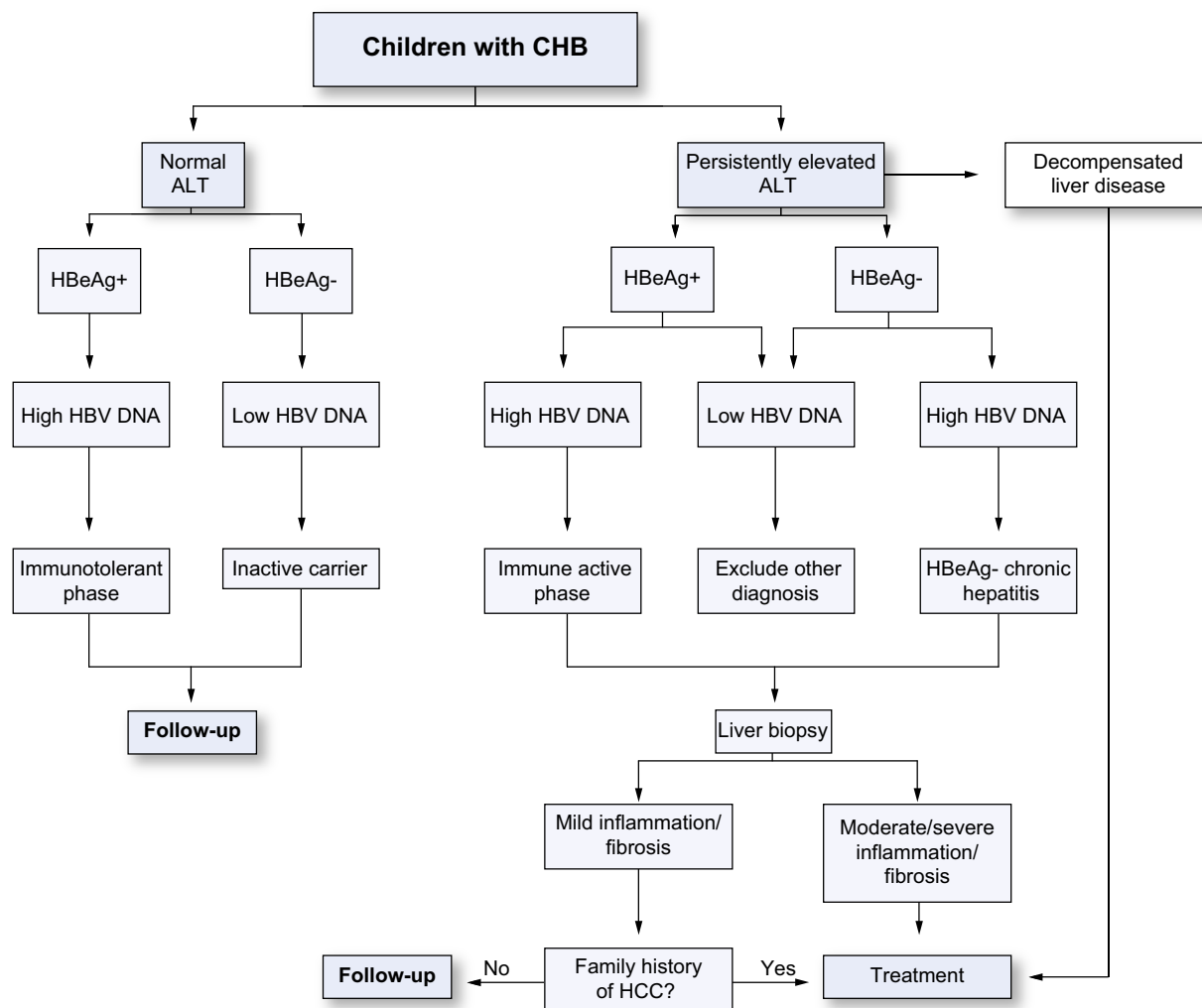


Fig. 1. Who and when to treat: algorithm for treatment of children and adolescents with chronic hepatitis B.

described in 1p36.22 [77,78]. Seroconversion to anti-HBe reduces the overall risk of developing HCC later in life, but an annual incidence of 0.2% has been described in HBeAg-negative adults (1.6% of asymptomatic HBsAg carriers) [79].

In 5% of anti-HBe-positive children, a pre-core mutant is selected, with persistent viral replication, abnormal ALT levels and histologically active hepatitis (HBeAg-negative chronic hepatitis). The latter progresses slowly in children, whereas adult patients are at increased risk of cirrhosis and HCC [53,58,79,80]. A stronger immune response with a faster turnover of infected cells could account for such an increased severity, and could be secondary to the reduced secretion of the tolerogen HBeAg [55,81].

In long-term longitudinal studies, it was shown that 7–14% of inactive carriers lost HBsAg and became anti-HBs-positive. Spontaneous seroconversion to anti-HBs is a rare event in childhood (1%/year and 0.6%/year in horizontally and perinatally infected children, respectively) [53,58,62,80]. HBsAg clearance and anti-HBs seroconversion mark the resolution of HBV infection, and are accompanied by improved liver histology. Long-term prognosis after HBsAg seroclearance is excellent if it occurs before the development of cirrhosis or HCC and in the absence of concomi-

tant infections [82]. Nevertheless, cccDNA persists indefinitely in hepatocytes, and low-level viral replication or reactivation upon immunosuppression are still possible.

Who and when to treat

Even though several guidelines have been published by major international societies on the management of adult patients with CHB, treatment strategies for children are still evolving and are mostly based on consensus of expert panels (Fig. 1) [83–86]. Decision to start a therapy must take into account the mild evolution of the disease during childhood for most of the patients and the potentially severe complications in few, not yet well identified cases, and it is complicated by the limited number of drugs labeled for use in children.

The goal of the anti-HBV therapy, in children as in adults, is to reduce the risk of progressive liver disease, cirrhosis, and HCC. It can be achieved by suppressing viral replication through durable HBeAg seroconversion and undetectable serum HBV DNA levels. Reduction of viremia levels leads to decreased liver inflammation

and subsequent normalization of ALT levels. The final goal is anti-HBs seroconversion, as it stops disease progression and reduces the risk of HCC, although it occurs in a minority of treated subjects [87].

Need for treatment should be evaluated at each follow-up visit, in order to treat as soon as early signs of liver damage are detected. Children with CHB should undergo physical examination and measurement of serum ALT, HBeAg/anti-HBe, and alpha-fetoprotein levels every 6 months. ALT should be monitored every 3 months at diagnosis (for 1 year) and if persistently elevated, and every 12 months in HBeAg-negative patients with low viral load (<2000 IU/ml). HBV DNA levels should be assessed every 12 months, along with a complete blood count, a full liver panel and a liver ultrasound [83,85,88]. Lifetime follow-up is warranted even for inactive carriers, because of the risk of cirrhosis (7.8% of patients at 25 years), HCC (2.2%) and reactivation of HBV infection, with seroreversion to HBeAg positivity (1–4% of patients) or progression to HBeAg-negative hepatitis (15–24%) [79,89].

Decision to treat should be based on the following parameters: ALT levels, HBeAg positivity, HBV DNA levels, liver histology, family history of cirrhosis, and/or HCC, co-existing liver diseases, and patient's treatment history. Serum ALT level is the most useful marker of liver damage and should be used to identify patients who could be considered for possible antiviral therapy. Published evidence suggests that patients with normal or mildly elevated ALT respond poorly to available treatments [90,91]. Benefit of treating children with normal ALT has not been established, and the risk of developing antiviral resistance could turn it deleterious when considering the future of these patients [92]. Therefore, children in the immunotolerant phase should not be treated outside of clinical trials, and should be monitored and treated when the increase of ALT levels reveals immune activation. The three largest clinical trials of antiviral drugs in children used ALT levels higher than 1.5 times the laboratory ULN or more than 60 IU/L, whichever was lower, as inclusion criteria [90,91,93]. Unfortunately, the upper limit of normal (ULN) for ALT levels has not yet been established for children [94].

Serum ALT level has to be elevated for at least 6 months (12 months if HBeAg-negative), in order to avoid treating patients who are undergoing spontaneous HBeAg seroconversion. Persistent ALT elevation mandates assessment of serum HBV DNA levels. High HBV DNA values warrant treatment, whereas low HBV DNA levels should lead to the exclusion of other causes of liver disease. The cut-off value for HBV DNA has not been defined, and arbitrary values are used in clinical trials and guidelines. As children have a higher HBV replication rate, a value of 20,000 IU/ml has been chosen by different authors [85,95]. However, lower values have been associated with progressive liver disease in adults and latest management guidelines identified 2,000 IU/ml to be a good cut-off [83].

In adult patients older than 40 years of age, treatment can be started solely upon detection of a high viral load, as it is an independent risk factor of cirrhosis and HCC [72,73]. No data exist in children to support such an approach. Therefore, as response to currently available treatments for children is partial and limited to specific subgroups, histologic assessment of the grade of inflammation and the stage of fibrosis remains recommended before treating a child. Response to both interferon(IFN)-alpha and nucleos(t)ide analogues is more likely when at least moderate necroinflammation or moderate fibrosis are found at liver his-

tology [90,96]. Although the benefit of treatment has not been established for children with mild inflammation or fibrosis, a family history of HCC may warrant treatment even in children with mild histological changes, as they are at increased risk of developing HCC [77].

There are other special populations of children at increased risk of rapid disease progression (cirrhosis, HBV-related glomerulonephritis, co-infection with HDV, HCV, or HIV). These patients could benefit from treatment even if ALT, HBV DNA levels, and liver histology do not match the above listed cut-offs. An antiviral treatment should be always considered in recipients of a liver graft from an anti-HBc-positive donor and to prevent (or treat) a recurrent HBV infection in children undergoing liver transplantation [83,97–99]. Furthermore, prophylactic treatment should be administered to HBsAg-positive patients who have to receive immunosuppressive or cytotoxic therapy, as it decreases the risk of mortality and morbidity related to HBV reactivation [100].

Several studies conducted in adults showed a better response to interferon for viral genotypes A and B, compared to D and C, respectively [25,101–103]. On the contrary, no significant difference was found when nucleoside analogues were used [104]. No studies have investigated the role of genotype on treatment response in pediatric patients, and genotype determination before treatment is not currently recommended [86].

Treatment options

The US Food and Drug Administration (FDA) approved four medications for treatment of children with CHB: IFN α , lamivudine, adefovir, and entecavir. IFN α can be used in children older than 12 months of age, lamivudine starting at 3 years of age, adefovir in children aged 12 years and older and entecavir starting from 16 years of age. Each of these treatments, together with others currently studied or used off-label in reference centers, has advantages and disadvantages (Table 2), and different response and resistance rates (Fig. 2).

Interferon-alfa

IFN α -2b has been the first drug to be approved and it has been used to treat CHB in children for about 15 years. Efficacy in children is similar to that in adults. In the largest multinational randomized controlled trial of IFN α therapy in children, 26% of patients achieved virologic response (VR: undetectable HBV DNA and loss of HBeAg) after 24 weeks of treatment, compared to 11% of untreated controls, and an additional 7% responded during the 24 weeks after treatment cessation. Response rate rose to 35% when only patients with ALT at least twice the ULN were considered. ALT levels decreased in all responders, and they were normal in 44% of cases at the end of treatment. Histological activity index improved in responders and loss of HBsAg occurred in 10% of treated children, as compared to 1.2% in the control group. Likelihood of a response was associated with low HBV DNA levels before treatment, younger age and female sex [90]. A better response in young patients was subsequently confirmed in children younger than 5 years of age [105].

IFN α is thought to simply accelerate seroconversion, as many patients who do not respond to treatment may still seroconvert to anti-HBe later in life. In three different series of 108, 107, and 174 children with CHB, IFN α treatment was shown to

Review

Table 2. Available treatments for chronic hepatitis B in pediatric age.

Treatment	Licensing	Dose	Duration	Advantages	Disadvantages
IFN α	≥ 12 mo	5-10 M units/m ² sc 3 x/wk	6 mo	No resistance Usable in young children Short treatment	Heavy side effects Parenteral administration Not usable if decompensated cirrhosis or transplantation
Lamivudine	≥ 3 yr	3 mg/kg po once daily (max 100 mg/d)	≥ 1 yr	Few side effects Usable in young children Oral administration Usable in 3 rd trimester of pregnancy	High resistance rate (increasing with time of treatment)
Adefovir	≥ 12 yr	10 mg po once daily	≥ 1 yr (+ 6 mo after HBeAg seroconversion)	Partially effective in lamivudine resistant patients Oral administration	Not approved for children <12 yr Emergence of resistant mutations
Entecavir	≥ 16 yr + Phase III (2-17 yr)	0.5 mg/d once daily (1 mg/d for lamivudine-resistant pts)	≥ 1 yr (+ 6 mo after HBeAg seroconversion)	Partially effective in lamivudine resistant patients Oral administration	Resistance rate on the long term Not approved for children <16 yr No available preparation for young children
PegIFN	Phase III (2-18 yr)	180 μ g/wk	6 mo	No resistance Once weekly administration Short treatment	Heavy side effects Parenteral administration Not usable if decompensated cirrhosis or transplantation
Telbivudine	Phase I (2-18 yr)	600 mg/d once daily	≥ 1 yr	Few side effects Oral administration Usable in 3 rd trimester of pregnancy	High resistance rate
Tenofovir	Phase III (12-17 yr)	300 mg/d once daily	≥ 1 yr	High response rate No resistance identified Few side effects Oral administration Usable in 3 rd trimester of pregnancy	No available preparation for young children Reduced mineral density in children

pts, Patients.

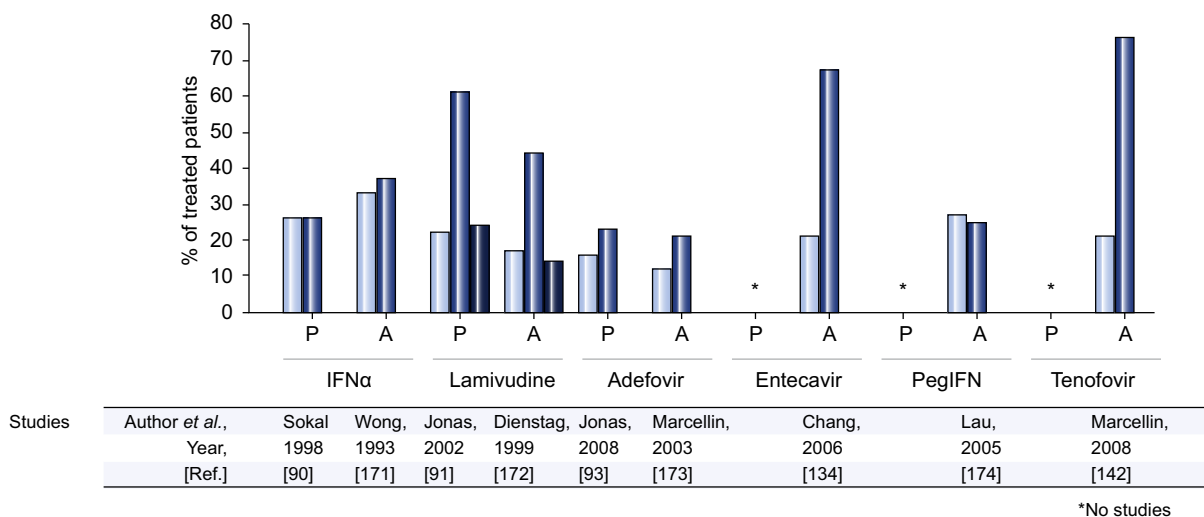


Fig. 2. Comparison between available treatments in term of HBeAg seroconversion (light blue bars), undetectable HBV DNA (blue bars) and resistant mutations emergence (dark blue bars). Pediatric clinical trials (P) are compared to studies on adult patients (A). (See below-mentioned references for further information.)

accelerate HBeAg seroconversion, but treated and untreated patients had a similar rate of HBeAg clearance at long-term follow-up (Fig. 3) [53,62,106]. These data were confirmed in a recent study on Asian children [107]. Furthermore, children of

Asian origin seroconverted slower than those of other ethnic origin, but they reached the same proportion of seroconversion in the long-term [62]. In a series of 74 children followed-up for 7 years, treated patients with elevated baseline ALT levels

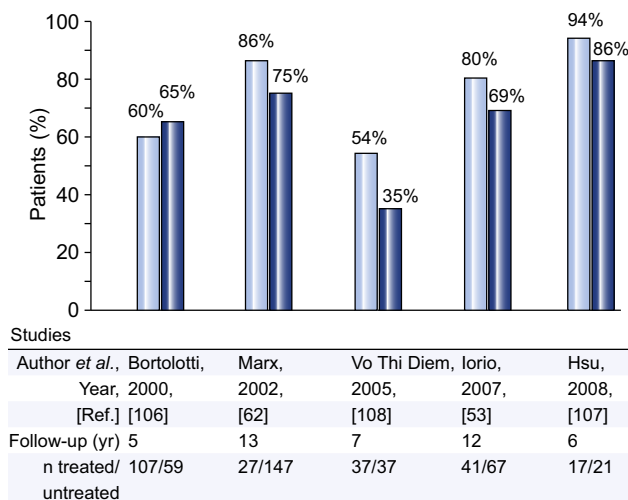


Fig. 3. HBsAg seroconversion rates in children treated with IFN-alfa (light blue bars) as compared to untreated subjects (dark blue bars). Differences between the two groups in each study are not statistically significant.

showed a higher long-term seroconversion rate, whereas elevated ALT at baseline did not significantly influence the long-term natural seroconversion rate in untreated children [108].

HBsAg clearance was observed in 4–15% of children treated with IFN (15–25% of responders), compared to 0–10% of controls [53,62,90,106–108]. The difference in HBsAg seroconversion rate between treated and untreated patients was shown to be significant in the randomized controlled trial, whereas none of long-term studies was able to find such a difference to be statistically significant. Nevertheless, children who responded promptly to IFN were more likely to lose HBsAg than late or no-responders [106].

Overall, although pediatric studies failed to show an impact of treatment over time, large case-control studies and meta-analyses in adults showed that IFN α reduced the risk of cirrhosis (RR 0.65) and HCC (RR 0.59–0.66) in the long-term [109–111].

European consensus recommendations for IFN α treatment suggest to treat HBsAg-positive children aged 2 years or older, with abnormal ALT and low-intermediate HBV DNA levels. The recommended regimen is 5–10 million units per square meter, three times weekly for 6 months [86,112]. The benefit of priming with corticosteroids has not been proven [113,114]. IFN α is contraindicated in children with decompensated cirrhosis, cytopenia, autoimmune disorders, cardiac or renal failure, and in transplanted patients [112]. As VR may be achieved in the 6 months following the end of treatment, at least 6–12 months should pass by before another therapy is started.

Side effects of IFN α treatment included fever and flu-like symptoms, behavioral disorders, gastrointestinal disorders and neutropenia [90]. Furthermore, IFN α was shown to temporarily affect growth [115]. All side effects resolve after treatment is stopped.

Pegylated interferon-alfa (PegIFN) has not yet been approved for the treatment of CHB in children. Polyethylene glycol addition to IFN α enhances its half-life, allowing a once-weekly administration. Such a formulation is licensed for the use in children with chronic hepatitis C. In adults with CHB, PegIFN proved to have superior efficacy than conventional IFN α , with the same safety profile [116]. Clinical trials of PegIFN in children with CHB are

ongoing. Preliminary reports in children with HCV infection showed that it was well tolerated, while leading to better response rates than IFN α [117,118].

Lamivudine

Lamivudine is a pyrimidine nucleoside analogue approved for children aged 3 years and older. In the largest pediatric randomized, controlled trial, VR was achieved by 23% of patients receiving lamivudine after 1-year treatment (compared to 13% in the control group). Response increased to 35% when considering only children with ALT levels of at least twice the ULN [91]. An open label extension study was then carried out on the same cohort, reaching a total of 2 or 3 years of treatment. At the end of the extension period, response rate was 56% for children receiving lamivudine in the absence of resistant mutations (being 5% in patients with a YMDD mutant HBV). Resistance rate increased over time (24% after 1 year of treatment, 49% at 2 years, and 64% at 3 years) [119]. Likelihood of response was greater in patients with higher ALT levels and histologic activity index at baseline. In children, no data is yet available on the long-term impact of treatment with lamivudine (or any other nucleoside analogue) on the natural history of the disease.

Lamivudine is well tolerated with no significant side effects. The recommended treatment dose is 3 mg/kg/day (maximum 100 mg/day), administered orally once daily. Such a dose provides levels of exposure and trough concentrations similar to the 100 mg dose in adults [120]. Optimal treatment duration is more difficult to determine. Published data suggest continuing treatment until VR is achieved, and possibly for 6 months following seroconversion [119]. As a longer treatment duration leads to higher resistance rates, it is recommended to discontinue lamivudine after 6 months if a complete suppression of viral replication is not achieved or if YMDD mutations emerge. As post-treatment ALT flares are possible, children should be carefully followed and an alternative therapy should be started in the rare cases of severe and protracted ALT elevation [121].

Combination of lamivudine with IFN α (either concurrent or sequential) proved to be more effective than the single drugs alone in adult patients with elevated ALT levels [122,123]. The two drugs were shown to have a synergistic effect on cells *in vitro* [124]. In children, fewer data are available. Three studies investigated combined therapy in 32, 33, and 45 treatment-naïve children with elevated ALT levels. Although no difference was found between different combination strategies, the children reached 30–60% seroconversion to anti-HBe and 9–17% to anti-HBs [125–127]. An 8-week treatment with lamivudine followed by 10-month combination therapy with lamivudine and IFN has been tested on 23 immunotolerant children: complete suppression of viral replication was achieved in 78% of subjects and 22% of children seroconverted to anti-HBe. The concurrent administration of IFN α appeared to be protective against mutations emergence [128]. No difference was seen on histological response between monotherapy and combined therapy [129]. As no large clinical trials have been conducted so far, advantages of combination therapy over monotherapy in children are still to be confirmed.

Adefovir

Adefovir dipivoxil is a purine analogue approved to treat children with CHB aged 12 years and older. In a large pediatric

Review

randomized controlled trial, 23% of patients aged 12–17 years achieved VR after 48-week treatment with adefovir (compared to 0% of placebo-treated subjects). The efficacy on HBV DNA suppression and ALT normalization was less evident in younger children (15% vs. 3%), with differences between groups not being statistically significant [93]. Adefovir was well tolerated by children of all age groups, with no resistance-associated mutation registered. However, adefovir resistant mutations are reported in more than 20% of HBeAg-positive adults after a 5-year treatment [130]. A dose-dependent proximal renal tubular toxicity, with occasional Fanconi-like renal tubular acidosis, is a known side effect of adefovir, which has been rarely reported in adults, but never in children. The nephrotoxicity is usually reversible if the therapy is rapidly stopped [131,132]. The recommended dose in patients 12–18 years of age is 10 mg orally once daily [133]. Treatment duration has not been established, but experts agree to continue for at least 6 months after HBeAg seroconversion. Adefovir should be discontinued if a complete VR is not achieved after 24 weeks or if a resistant mutation emerges. As with other nucleos(t)ide analogues, patients should be monitored after discontinuation because post-treatment flares are not uncommon.

Entecavir

Entecavir is a carbocyclic analogue of 2'-deoxyguanosine that proved to be more potent than both lamivudine and adefovir in adult patients [134,135]. Furthermore, entecavir resistance is rare, even after 5 years of treatment, and entecavir is active (although less effective) on lamivudine-resistant strains [136–138]. The safety profile of entecavir is similar to that of lamivudine. Based on adult studies, entecavir has been approved by the FDA for treatment of adolescents aged 16 years or older. The recommended dose is 0.5 mg once daily for nucleoside-naïve patients and 1 mg/day for lamivudine-resistant patients. A phase III clinical trial in children as young as 2 years old is underway [139].

New drugs

Telbivudine is an L-nucleoside analogue with a potent antiviral activity and a safety profile similar to lamivudine (although myopathy and peripheral neuropathy were reported in adults) [140]. Resistance rate is lower than lamivudine but higher than adefovir. For such a reason, telbivudine is only used in combination with other antiviral drugs. A phase I clinical trial is ongoing on children 2–18 years of age [141].

Tenofovir disoproxil fumarate is a nucleos(t)ide analogue originally licensed for treatment of HIV infection, which is structurally similar to adefovir and of equal antiviral activity. Nevertheless, as it proved to be less nephrotoxic than adefovir, the approved dose for adults is higher than that of adefovir (300 mg/day). For such a reason, tenofovir was shown to have a greater antiviral activity than adefovir in clinical trials (undetectable HBV DNA in 76% of patients vs. 13% of adefovir-receiving subjects after 48 weeks of treatment) [142]. After three years of treatment, 72% of HBeAg-positive and 87% of HBeAg-negative patients have HBV DNA levels <400 copies/ml [143]. The safety profile of tenofovir is similar to that of adefovir, although associated with decreased bone mineral density in children with HIV [144]. No genotypic resistance to tenofovir has yet been confirmed [143,145]. Tenofovir is currently used to treat HIV infection in children, and it proved to be well tolerated in phase I

studies conducted in patients 4–18 years of age [146,147]. As there is no preparation suitable for young children, a phase III trial is ongoing on 12–17 year old CHB patients [148].

Proposition of a treatment scheme

Outside clinical trials, until new drugs are licensed for pediatric patients, IFN α is still the drug of choice, unless decompensated cirrhosis is present. The impossibility of developing a genotypic resistance against such a drug is a big advantage, even though adverse effects are more pronounced and a clear benefit on the long-term period remains to be confirmed. Furthermore, IFN α is the only treatment licensed for treating children younger than 3 years of age, although patients in this age group requiring treatment, are extremely rare. Currently available nucleos(t)ide analogues are second-line therapies, as the risk of emergence of resistant mutant strains is high. In adolescents older than 16 years of age, entecavir is the best choice, as resistance is less likely. Such a risk is higher with both lamivudine and adefovir, the latter being preferable in children aged 12–15 years. The use of lamivudine is currently limited to young children unresponsive to IFN α , in special populations (co-infection with HIV, transplant recipients or patients with HBV-related glomerulonephritis) or to prevent reactivation in children receiving immunosuppressive therapies. Combination therapy is promising, but further data are needed in children.

Newer drugs such as PegIFN and tenofovir are extremely promising and their licensing for children could change management of pediatric CHB. As the emergence of resistant mutant strains is becoming a major public health problem, pediatric practitioners should refrain from treating children who are not likely to benefit from a licensed therapy and consider waiting for safer and more effective drugs made available through clinical trials or future market approval.

Conclusions

A dramatic reduction of pediatric HBV infection prevalence has been observed in countries where global immunization programs have been implemented. Nevertheless, an important number of children are still infected every year. Prevention and management of breakthrough infection, along with a better identification of children at higher risk for disease progression and complications, are the current challenges.

Overall, management of pediatric patients with CHB is satisfactory, but hampered by a chronic delay in licensing new drugs as compared to adults. As the approval by regulatory agencies is based on the completion of appropriate clinical trials, studies on treatments that, in adults, have already been proved safe and more effective than available drugs should be conducted without undue delays. The current (2007) European regulation on medicines for children offers the appropriate regulatory framework to speed up this process.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

References

- [1] World Health Organization. Hepatitis B [Internet]. WHO Website. 2008 [cited 25.10.11]; Available from: <http://www.who.int/mediacentre/factsheets/fs204/en/index.html>.
- [2] Lavanchy D. Hepatitis B. Virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J Viral Hepat* 2004;11:97–107.
- [3] Goldstein ST, Zhou F, Hadler SC, Bell BP, Mast EE, Margolis HS. A mathematical model to estimate global hepatitis B disease burden and vaccination impact. *Int J Epidemiol* 2005;34:1329–1339.
- [4] McMahon BJ. The natural history of chronic hepatitis B virus infection. *Hepatology* 2009;49:S45–S55.
- [5] Kidd-Ljunggren K, Holmberg A, Bläckberg J, Lindqvist B. High levels of hepatitis B virus DNA in body fluids from chronic carriers. *J Hosp Infect* 2006;64:352–357.
- [6] Weinbaum CM, Williams I, Mast EE, Wang SA, Finelli L, Wasley A, et al. Recommendations for identification and public health management of persons with chronic hepatitis B virus infection. *MMWR Recomm Rep* 2008;57:1–20.
- [7] Mast EE, Margolis HS, Fiore AE, Brink EW, Goldstein ST, Wang SA, et al. A comprehensive immunization strategy to eliminate transmission of hepatitis B virus infection in the United States – recommendations of the advisory committee on immunization practices (ACIP) Part 1: Immunization of infants, children, and adolescents. *MMWR Recomm Rep* 2005;54:1–23.
- [8] Sokal EM, Van Collie O, Buts JP. Horizontal transmission of hepatitis B from children to adoptive parents. *Arch Dis Child* 1995;72:191.
- [9] Martin A, Moyes C, Lucas C, Milne A. Hepatitis B infection in households of HBsAg positive New Zealand children. *N Z Med J* 1996;109:463–465.
- [10] Centers for Disease Control and Prevention CDC. Vaccination coverage among children in kindergarten—United States, 2009–10 school year. *MMWR Morb Mortal Wkly Rep* 2011;60:700–704.
- [11] Bartlett L, Kanellos-Sutton M, van Wylick R. Immunization rates in a Canadian juvenile corrections facility. *J Adolesc Health* 2008;43:609–611.
- [12] Sneller VP, Fishbein DB, Weinbaum CM, Lombard A, Murray P, McLaurin JA, et al. Vaccinating adolescents in high-risk settings: lessons learned from experiences with hepatitis B vaccine. *Pediatrics* 2008;121:S55–S62.
- [13] McMahon BJ, Alward WL, Hall DB, Heyward WL, Bender TR, Francis DP, et al. Acute hepatitis B virus infection: relation of age to the clinical expression of disease and subsequent development of the carrier state. *J Infect Dis* 1985;151:599–603.
- [14] Tassopoulos NC, Papaevangelou GJ, Sjogren MH, Roumeliotou-Karayannis A, Gerin JL, Purcell RH. Natural history of acute hepatitis B surface antigen-positive hepatitis in Greek adults. *Gastroenterology* 1987;92:1844–1850.
- [15] World Health Organization. Hepatitis B vaccines – WHO position paper. *Weekly epidemiological record* 2009;89:405–420.
- [16] Chen H-L, Lin L-H, Hu F-C, Lee J-T, Lin W-T, Yang Y-J, et al. Effects of maternal screening and universal immunization to prevent mother-to-infant transmission of HBV. *Gastroenterology* 2011. <http://dx.doi.org/10.1053/j.gastro.2011.12.035>.
- [17] Lee C, Gong Y, Brok J, Boxall EH, Gluud C. Hepatitis B immunisation for newborn infants of hepatitis B surface antigen-positive mothers. *Cochrane Database Syst Rev* 2006;Issue 2. Art. No.: CD004790. <http://dx.doi.org/10.1002/14651858.CD004790.pub2>.
- [18] Chang MH, Lee CY, Chen DS, Hsu HC, Lai MY. Fulminant hepatitis in children in Taiwan: the important role of hepatitis B virus. *J Pediatr* 1987;111:34–39.
- [19] Chen H-L, Chang C-J, Kong M-S, Huang F-C, Lee H-C, Lin C-C, et al. Pediatric fulminant hepatic failure in endemic areas of hepatitis B infection: 15 years after universal hepatitis B vaccination. *Hepatology* 2004;39:58–63.
- [20] Vanclaire J, Cornu C, Sokal EM. Fulminant hepatitis B in an infant born to a hepatitis Be antibody positive, DNA negative carrier. *Arch Dis Child* 1991;66:983–985.
- [21] Wang Z, Zhang J, Yang H, Li X, Wen S, Guo Y, et al. Quantitative analysis of HBV DNA level and HBeAg titer in hepatitis B surface antigen positive mothers and their babies: HBeAg passage through the placenta and the rate of decay in babies. *J Med Virol* 2003;71:360–366.
- [22] Wen W-H, Chen H-L, Ni Y-H, Hsu H-Y, Kao J-H, Hu F-C, et al. Secular trend of the viral genotype distribution in children with chronic hepatitis B virus infection after universal infant immunization. *Hepatology* 2011;53:429–436.
- [23] McMahon BJ, Bruden DL, Petersen KM, Bulkow LR, Parkinson AJ, Nainan O, et al. Antibody levels and protection after hepatitis B vaccination: results of a 15-year follow-up. *Ann Intern Med* 2005;142:333–341.
- [24] Hsu H-Y, Chang M-H, Ni Y-H, Chiang C-L, Chen H-L, Wu J-F, et al. No increase in prevalence of hepatitis B surface antigen mutant in a population of children and adolescents who were fully covered by universal infant immunization. *J Infect Dis* 2010;201:1192–1200.
- [25] Xu D-Z, Yan Y-P, Choi BCK, Xu J-Q, Men K, Zhang J-X, et al. Risk factors and mechanism of transplacental transmission of hepatitis B virus: a case-control study. *J Med Virol* 2002;67:20–26.
- [26] Yang J, Zeng X-M, Men Y-L, Zhao L-S. Elective caesarean section versus vaginal delivery for preventing mother to child transmission of hepatitis B virus – a systematic review. *Virol J* 2008;5:100.
- [27] Bzowej NH. Hepatitis B. Therapy in pregnancy. *Curr Hepatitis Rep* 2010;9:197–204.
- [28] Xu WM, Cui YT, Wang L, Yang H, Liang ZQ, Li XM, et al. Lamivudine in late pregnancy to prevent perinatal transmission of hepatitis B virus infection: a multicentre, randomized, double-blind, placebo-controlled study. *J Viral Hepat* 2009;16:94–103.
- [29] Shi Z, Yang Y, Ma L, Li X, Schreiber A. Lamivudine in late pregnancy to interrupt in utero transmission of hepatitis B virus: a systematic review and meta-analysis. *Obstet Gynecol* 2010;116:147–159.
- [30] Han G-R, Cao M-K, Zhao W, Jiang H-X, Wang C-M, Bai S-F, et al. A prospective and open-label study for the efficacy and safety of telbivudine in pregnancy for the prevention of perinatal transmission of hepatitis B virus infection. *J Hepatol* 2011;55:1215–1221.
- [31] World Health Organization. Hepatitis B and breastfeeding. *World Health Organization. J Int Assoc Physicians AIDS Care* 1998;4:20–21.
- [32] Shi Z, Yang Y, Wang H, Ma L, Schreiber A, Li X, et al. Breastfeeding of newborns by mothers carrying hepatitis B virus: a meta-analysis and systematic review. *Arch Pediatr Adolesc Med* 2011;165:837–846.
- [33] Petrova M. Breastfeeding and chronic HBV infection: clinical and social implications. *World J Gastroenterol* 2010;16:5042–5046.
- [34] National Center for Immunization and Respiratory Diseases. General recommendations on immunization – recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2011;60:1–64.
- [35] World Health Organization. Hepatitis B vaccines: WHO position paper—recommendations. *Vaccine* 2010;28:589–590.
- [36] World Health Organization. Expanded programme on immunization. *Global Advisory Group—Part I. Wkly Epidemiol Rec* 1992;67:11–15.
- [37] World Health Organization. Global routine vaccination coverage, 2010. *Wkly Epidemiol Rec* 2011;86:509–513.
- [38] Luo Z, Li L, Ruan B. Impact of the implementation of a vaccination strategy on hepatitis B virus infections in China over a 20-year period. *Int J Infect Dis* 2012;16:e82–8.
- [39] Chien YC. Nationwide hepatitis B Vaccination program in Taiwan: effectiveness in the 20 years after it was launched. *Epidemiol Rev* 2006;28:126–135.
- [40] Zanetti AR, van Damme P, Shouval D. The global impact of vaccination against hepatitis B: a historical overview. *Vaccine* 2008;26:6266–6273.
- [41] Liang X, Bi S, Yang W, Wang L, Cui G, Cui F, et al. Epidemiological serosurvey of Hepatitis B in China—declining HBV prevalence due to Hepatitis B vaccination. *Vaccine* 2009;27:6550–6557.
- [42] Ni Y-H, Huang LM, Chang M-H, Yen CJ, Lu CY, You S-L, et al. Two decades of universal hepatitis B vaccination in Taiwan: impact and implication for future strategies. *Gastroenterology* 2007;132:1287–1293.
- [43] Chen S-M, Kung C-M, Yang W-J, Wang H-L. Efficacy of the nationwide hepatitis B infant vaccination program in Taiwan. *J Clin Virol* 2011;52:11–16.
- [44] Zhang L, Xu A, Yan B, Song L, Li M, Xiao Z, et al. A significant reduction in hepatitis B virus infection among the children of Shandong Province, China: the effect of 15 years of universal infant hepatitis B vaccination. *Int J Infect Dis* 2010;14:e483–e488.
- [45] Ioannou GN. Hepatitis B. Virus in the United States: infection, exposure, and immunity rates in a nationally representative survey. *Ann Intern Med* 2011;154:319–328.
- [46] Stadler LP, Mezoff AG, Staat MA. Hepatitis B virus screening for internationally adopted children. *Pediatrics* 2008;122:1223–1228.
- [47] Cai W, Poethko-Müller C, Hamouda O, Radun D. Hepatitis B virus infections among children and adolescents in Germany. *Pediatr Infect Dis J* 2011;30:19–24.
- [48] Jack AD, Hall AJ, Maine N, Mendy M, Whittle HC. What level of hepatitis B antibody is protective? *J Infect Dis* 1999;179:489–492.

Review

- [49] American Academy of Pediatrics. Medical evaluation of internationally adopted children for infectious diseases. In: Pickering L, Baker C, Long S, MacMillan J, editors. Red book: 2009 report of the Committee on Infectious Diseases. Elk Grove Village, IL: American Academy of Pediatrics; 2009. p. 177–184.
- [50] Liu HF, Sokal E, Goubau P. Wide variety of genotypes and geographic origins of hepatitis B virus in Belgian children. *J Pediatr Gastroenterol Nutr* 2001;32:274–277.
- [51] Ni Y-H, Chang M-H, Wang K-J, Hsu H-Y, Chen H-L, Kao J-H, et al. Clinical relevance of hepatitis B virus genotype in children with chronic infection and hepatocellular carcinoma. *Gastroenterology* 2004;127:1733–1738.
- [52] Lin C-L, Kao J-H. The clinical implications of hepatitis B virus genotype: recent advances. *J Gastroenterol Hepatol* 2011;26:123–130.
- [53] Iorio R, Giannattasio A, Cirillo FD, Cirillo F, Alessandro L, Vegnente A. Long-term outcome in children with chronic hepatitis B: a 24-year observation period. *Clin Infect Dis* 2007;45:943–949.
- [54] Milich DR, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? *Proc Natl Acad Sci U S A* 1990;87:6599–6603.
- [55] Chen MT, Billaud J-N, Sällberg M, Guidotti LG, Chisari FV, Jones J, et al. A function of the hepatitis B virus pre-core protein is to regulate the immune response to the core antigen. *Proc Natl Acad Sci U S A* 2004;101:14913–14918.
- [56] Milich D. Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. *Hepatology* 2003;38:1075–1086.
- [57] Chen M, Sällberg M, Hughes J, Jones J, Guidotti LG, Chisari FV, et al. Immune tolerance split between hepatitis B virus pre-core and core proteins. *J Virol* 2005;79:3016–3027.
- [58] Bortolotti F, Guido M, Bartolacci S, Cadrobbi P, Crivellaro C, Noventa F, et al. Chronic hepatitis B in children after e antigen seroclearance. final report of a 29-year longitudinal study. *Hepatology* 2006;43:556–562.
- [59] Chu C-M, Yeh C-T, Lee C-S, Sheen I-S, Liaw Y-F. Pre-core stop mutant in HBeAg-positive patients with chronic hepatitis B: clinical characteristics and correlation with the course of HBeAg-to-anti-HBe seroconversion. *J Clin Microbiol* 2002;40:16–21.
- [60] Frelin L, Wahlstrom T, Tucker AE, Jones J, Hughes J, Lee BO, et al. A mechanism to explain the selection of the hepatitis e antigen-negative mutant during chronic hepatitis B virus infection. *J Virol* 2009;83:1379–1392.
- [61] Tseng Y-R, Wu J-F, Ni Y-H, Chen H-L, Chen C-C, Wen W-H, et al. Long-term effect of maternal HBeAg on delayed HBeAg seroconversion in offspring with chronic hepatitis B infection. *Liver Int* 2011;31:1373–1380.
- [62] Marx G, Martin SR, Chicoine J-F, Alvarez F. Long-term follow-up of chronic hepatitis B virus infection in children of different ethnic origins. *J Infect Dis* 2002;186:295–301.
- [63] Ni Y-H, Chang M-H, Chen P-J, Tsai K-S, Hsu H-Y, Chen H-L, et al. Viremia profiles in children with chronic hepatitis B virus infection and spontaneous e antigen seroconversion. *Gastroenterology* 2007;132:2340–2345.
- [64] Wu J-F, Su Y-R, Chen C-H, Chen H-L, Ni Y-H, Hsu H-Y, et al. Predictive effect of serial serum alanine aminotransferase levels on spontaneous HBeAg seroconversion in chronic genotype B and C HBV-infected children. *J Pediatr Gastroenterol Nutr* 2012;54:97–100.
- [65] Chu CM, Liaw YF. Chronic hepatitis B virus infection acquired in childhood: special emphasis on prognostic and therapeutic implication of delayed HBeAg seroconversion. *J Viral Hepat* 2007;14:147–152.
- [66] Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 2000;118:554–559.
- [67] Chu C-J, Hussain M, Lok AS. Hepatitis B virus genotype B is associated with earlier hbeag seroconversion compared with hepatitis B virus genotype C. *Gastroenterology* 2002;122:1756–1762.
- [68] Livingston SE, Simonetti JP, Bulkow LR, Homan CE, Snowball MM, Cagle HH, et al. Clearance of hepatitis B e Antigen in patients with chronic hepatitis B and genotypes A, B, C, D, and F. *Gastroenterology* 2007;133:1452–1457.
- [69] Chang M, Hsu H, Hsu H, Ni Y, Chen J, Chen D. The significance of spontaneous hepatitis B e antigen seroconversion in childhood: with special emphasis on the clearance of hepatitis B e antigen before 3 years of age. *Hepatology* 1995;22:1387–1392.
- [70] Wen W-H, Chang M-H, Hsu H-Y, Ni Y-H, Chen H-L. The development of hepatocellular carcinoma among prospectively followed children with chronic hepatitis B virus infection. *J Pediatr* 2004;144:397–399.
- [71] Chang MH, You SL, Chen CJ, Liu CJ, Lee CM, Lin SM, et al. Decreased incidence of hepatocellular carcinoma in hepatitis B vaccinees: A 20-year follow-up study. *JNCI J Natl Cancer Inst* 2009;101:1348–1355.
- [72] Iloeje UH, Yang H-I, Su J, Jen C-L, You S-L, Chen C-J. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. *Gastroenterology* 2006;130:678–686.
- [73] Chen C-J, Yang H-I, Su J, Jen C-L, You S-L, Lu S-N, et al. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006;295:65–73.
- [74] Sánchez-Tapias JM, Costa J, Mas A, Bruguera M, Rodés J. Influence of hepatitis B virus genotype on the long-term outcome of chronic hepatitis B in western patients. *Gastroenterology* 2002;123:1848–1856.
- [75] Yu MW, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, et al. Hepatitis B Virus Genotype and DNA Level and Hepatocellular Carcinoma: A Prospective Study in Men. *JNCI J Natl Cancer Inst* 2005;97:265–272.
- [76] Livingston SE, Simonetti JP, McMahon BJ, Bulkow LR, Hurlburt KJ, Homan CE, et al. Hepatitis B virus genotypes in Alaska native people with hepatocellular carcinoma: preponderance of genotype F. *J Infect Dis* 2007;195:5–11.
- [77] Yu MW, Chang HC, Liaw YF, Lin SM, Lee SD, Liu CJ, et al. Familial risk of hepatocellular carcinoma among chronic hepatitis B carriers and their relatives. *J Natl Cancer Inst* 2000;92:1159–1164.
- [78] Zhang H, Zhai Y, Hu Z, Wu C, Qian J, Jia W, et al. Genome-wide association study identifies 1p36.22 as a new susceptibility locus for hepatocellular carcinoma in chronic hepatitis B virus carriers. *Nat Genet* 2010;42:767–770.
- [79] Hsu Y-S, Chien R-N, Yeh C-T, Sheen I-S, Chiou H-Y, Chu C-M, et al. Long-term outcome after spontaneous HBeAg seroconversion in patients with chronic hepatitis B. *Hepatology* 2002;35:1522–1527.
- [80] Fattovich G, Bortolotti F, Donato F. Natural history of chronic hepatitis B: special emphasis on disease progression and prognostic factors. *J Hepatol* 2008;48:335–352.
- [81] Ribeiro RM, Germanidis G, Powers KA, Pellegrin B, Nikolaidis P, Perelson AS, et al. Hepatitis B virus kinetics under antiviral therapy sheds light on differences in hepatitis B e antigen positive and negative infections. *J Infect Dis* 2010;202:1309–1318.
- [82] Arase Y, Ikeda K, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, et al. Long-term outcome after hepatitis B surface antigen seroclearance in patients with chronic hepatitis B. *Am J Med* 2006;119:71e9–71e16.
- [83] Lok ASF, McMahon BJ. Chronic hepatitis B: update 2009. *Hepatology* 2009;50:661–662.
- [84] European Association for the Study of the Liver. EASL clinical practice guidelines: management of chronic hepatitis B. *J Hepatol* 2009;50:227–242.
- [85] Shah U, Kelly D, Chang M-H, Fujisawa T, Heller S, González-Peralta RP, et al. Management of chronic hepatitis B in children. *J Pediatr Gastroenterol Nutr* 2009;48:399–404.
- [86] Jonas MM, Block JM, Haber BA, Karpen SJ, London WT, Murray KF, et al. Treatment of children with chronic hepatitis B virus infection in the United States: patient selection and therapeutic options. *Hepatology* 2010;52:2192–2205.
- [87] Simonetti J, Bulkow L, McMahon BJ, Homan C, Snowball M, Negus S, et al. Clearance of hepatitis B surface antigen and risk of hepatocellular carcinoma in a cohort chronically infected with hepatitis B virus. *Hepatology* 2010;51:1531–1537.
- [88] Haber BA, Block JM, Jonas MM, Karpen SJ, London WT, McMahon BJ, et al. Recommendations for screening, monitoring, and referral of pediatric chronic hepatitis B. *Pediatrics* 2009;124:e1007–e1013.
- [89] Fattovich G, Olivari N, Pasino M, D'Onofrio M, Martone E, Donato F. Long-term outcome of chronic hepatitis B in Caucasian patients: mortality after 25 years. *Gut* 2008;57:84–90.
- [90] Sokal EM, Conjeevaram HS, Roberts EA, Alvarez F, Bern EM, Goyens P, et al. Interferon alfa therapy for chronic hepatitis B in children: a multinational randomized controlled trial. *Gastroenterology* 1998;114:988–995.
- [91] Jonas MM, Kelly DA, Mizerski J, Badia IB, Areias JA, Schwarz KB, et al. Clinical trial of lamivudine in children with chronic hepatitis B. *N Engl J Med* 2002;346:1706–1713.
- [92] Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* 2009;137:1593–1608.
- [93] Jonas MM, Kelly D, Pollack H, Mizerski J, Sorbel J, Frederick D, et al. Safety, efficacy, and pharmacokinetics of adefovir dipivoxil in children and adolescents (age 2 to <18 years) with chronic hepatitis B. *Hepatology* 2008;47:1863–1871.
- [94] Schwimmer JB, Dunn W, Norman GJ, Pardee PE, Middleton MS, Kerkar N, et al. SAFETY study: alanine aminotransferase cut-off values are set too high for reliable detection of pediatric chronic liver disease. *Gastroenterology* 2010;138:1357–1364.

- [95] Hsu EK, Murray KF. Hepatitis B and C in children. *Nature Clin Prac Gastroenterol Hepatol* 2008;5:311–320.
- [96] Hom X, Little NR, Gardner SD, Jonas MM. Predictors of virologic response to lamivudine treatment in children with chronic hepatitis B infection. *Pediatr Infect Dis J* 2004;23:441–445.
- [97] Shapira R, Mor E, Bar-Nathan N, Sokal EM, Tur-Kaspa R, Dinari G, et al. Efficacy of lamivudine for the treatment of hepatitis B virus infection after liver transplantation in children. *Transplantation* 2001;72:333–336.
- [98] Cholongitas E, Papatheodoridis GV, Burroughs AK. Liver grafts from anti-hepatitis B core positive donors: a systematic review. *J Hepatol* 2010;52:272–279.
- [99] Perrillo R, Hepatitis B. Virus prevention strategies for antibody to hepatitis B core antigen-positive liver donation: a survey of North American, European, and Asian-Pacific transplant programs. *Liver Transpl* 2009;15:223–232.
- [100] Katz LH, Fraser A, Gaftner-Gvili A, Leibovici L, Tur-Kaspa R. Lamivudine prevents reactivation of hepatitis B and reduces mortality in immunosuppressed patients: systematic review and meta-analysis. *J Viral Hepat* 2008;15:89–102.
- [101] Wai CT, Chu C-J, Hussain M, Lok ASF. HBV genotype B is associated with better response to interferon therapy in HBeAg(+) chronic hepatitis than genotype C. *Hepatology* 2002;36:1425–1430.
- [102] Erhardt A. Response to interferon alfa is hepatitis B virus genotype dependent: genotype A is more sensitive to interferon than genotype D. *Gut* 2005;54:1009–1013.
- [103] Flink HJ, van Zonneveld M, Hansen BE, de Man RA, Schalm SW, Janssen HLA, et al. Treatment with peg-interferon alpha-2b for HBeAg-positive chronic hepatitis B: HBsAg loss is associated with HBV genotype. *Am J Gastroenterol* 2006;101:297–303.
- [104] Raimondi S, Maisonneuve P, Bruno S, Mondelli MU. Is response to antiviral treatment influenced by hepatitis B virus genotype? *J Hepatol* 2010;52:441–449.
- [105] Kobak GE, MacKenzie T, Sokol RJ, Narkewicz MR. Interferon treatment for chronic hepatitis B: enhanced response in children 5 years old or younger. *J Pediatr* 2004;145:340–345.
- [106] Bortolotti F, Jara P, Barbera C, Gregorio GV, Vegnente A, Zancan L, et al. Long-term effect of alpha interferon in children with chronic hepatitis B. *Gut* 2000;46:715–718.
- [107] Hsu H-Y, Tsai H-Y, Wu T-C, Chiang C-L, Ni Y-H, Chen P-J, et al. Interferon- α treatment in children and young adults with chronic hepatitis B: a long-term follow-up study in Taiwan. *Liver Int* 2008;28:1288–1297.
- [108] Vo Thi Diem H, Bourgois A, Bontemps P, Goyens P, Buts J-P, Nackers F, et al. Chronic hepatitis B infection: long-term comparison of children receiving interferon alpha and untreated controls. *J Pediatr Gastroenterol Nutr* 2005;40:141–145.
- [109] Lin SM, Yu ML, Lee CM, Chien RN, Sheen IS, Chu CM, et al. Interferon therapy in HBeAg positive chronic hepatitis reduces progression to cirrhosis and hepatocellular carcinoma. *J Hepatol* 2007;46:45–52.
- [110] Yang YF, Zhao W, Zhong YD, Xia HM, Shen L, Zhang N. Interferon therapy in chronic hepatitis B reduces progression to cirrhosis and hepatocellular carcinoma: a meta-analysis. *J Viral Hepat* 2009;16:265–271.
- [111] Sung JJY, Tsoi KKF, Wong VWS, Li KCT, Chan HLY. Meta-analysis: treatment of hepatitis B infection reduces risk of hepatocellular carcinoma. *Aliment Pharmacol Ther* 2008;28:1067–1077.
- [112] Jara P, Bortolotti F. Interferon-alpha treatment of chronic hepatitis B in childhood: a consensus advice based on experience in European children. *J Pediatr Gastroenterol Nutr* 1999;29:163–170.
- [113] Boxall EH, Sira J, Ballard AL, Davies P, Kelly DA. Long-term follow-up of hepatitis B carrier children treated with interferon and prednisolone. *J Med Virol* 2006;78:888–895.
- [114] Gregorio GV, Jara P, Hierro L, Diaz C, La Vega de A, et al. Lymphoblastoid interferon alfa with or without steroid pretreatment in children with chronic hepatitis B: a multicenter controlled trial. *Hepatology* 1996;23:700–707.
- [115] Comanor L, Minor J, Conjeevaram HS, Roberts EA, Alvarez F, Bern EM, et al. Impact of chronic hepatitis B and interferon-alpha therapy on growth of children. *J Viral Hepat* 2001;8:139–147.
- [116] Cooksley WG, Piratvisuth T, Lee SD, Mahachai V, Chao YC, Tanwandee T, et al. Peginterferon alpha-2a (40 kDa): an advance in the treatment of hepatitis B e antigen-positive chronic hepatitis B. *J Viral Hepat* 2003;10:298–305.
- [117] Wirth S, Pieper-Boustani H, Lang T, Ballauff A, Kullmer U, Gerner P, et al. Peginterferon alfa-2b plus ribavirin treatment in children and adolescents with chronic hepatitis C. *Hepatology* 2005;41:1013–1018.
- [118] Sokal EM, Bourgois A, Stephenne X, Silveira T, Porta G, Gardovska D, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection in children and adolescents. *J Hepatol* 2010;52:827–831.
- [119] Sokal EM, Kelly DA, Mizerski J, Badia IB, Areias JA, Schwarz KB, et al. Long-term lamivudine therapy for children with HBeAg-positive chronic hepatitis B. *Hepatology* 2006;43:225–232.
- [120] Sokal EM, Roberts EA, Mieli-Vergani G, McPhillips P, Johnson M, Barber J, et al. A dose ranging study of the pharmacokinetics, safety, and preliminary efficacy of lamivudine in children and adolescents with chronic hepatitis B. *Antimicrob Agents Chemother* 2000;44:590–597.
- [121] Jonas MM, Little NR, Gardner SD. International Pediatric Lamivudine Investigator Group. Long-term lamivudine treatment of children with chronic hepatitis B: durability of therapeutic responses and safety. *J Viral Hepat* 2008;15:20–27.
- [122] Schalm SW, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, et al. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. *Gut* 2000;46:562–568.
- [123] Sarin SK, Kumar M, Kumar R, Kazim SN, Guptan RC, Sakhuja P, et al. Higher efficacy of sequential therapy with interferon-alpha and lamivudine combination compared to lamivudine monotherapy in HBeAg positive chronic hepatitis B patients. *Am J Gastroenterol* 2005;100:2463–2471.
- [124] Korba B, Cote P, Hornbuckle W, Schinazi R, Gangemi J, Tennant B, et al. Enhanced antiviral benefit of combination therapy with lamivudine and alpha interferon against WHV replication in chronic carrier woodchucks. *Antivir Ther* 2000;5:95–104.
- [125] Dikici B, Bosnak M, Bosnak V, Dagli A, Ece A, Yagci RV, et al. Combination therapy for children with chronic hepatitis B virus infection. *J Gastroenterol Hepatol* 2002;17:1087–1091.
- [126] Yilmaz A, Akcam M, Gelen T, Artan R. Lamivudine and high-dose interferon alpha 2a combination treatment in naïve HBeAg-positive immunoreactive chronic hepatitis B in children: an East Mediterranean center's experience. *Eur J Pediatr* 2006;166:195–199.
- [127] Akman SA, Okcu SC, Halicioğlu O, Sutcuoglu S, Anil M, Kizilgunesler A, et al. Therapeutic efficacy of sequential and simultaneous treatments with interferon-alpha and lamivudine in children with chronic hepatitis B. *Pediatr Int* 2007;49:848–852.
- [128] D'Antiga L, Aw M, Atkins M, Moorat A, Vergani D, Mieli-Vergani G. Combined lamivudine/interferon-alpha treatment in "immunotolerant" children perinatally infected with hepatitis B: a pilot study. *J Pediatr* 2006;148:228–233.
- [129] Kuloglu Z, Krsacoglu CT, Kansu A, Erden E, Girgin N. Liver histology of children with chronic hepatitis treated with interferon-alpha alone or in combination with lamivudine. *J Pediatr Gastroenterol Nutr* 2007;45:564–568.
- [130] Marcellin P, Chang T-T, Lim SGL, Sievert W, Tong M, Arterburn S, et al. Long-term efficacy and safety of adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *Hepatology* 2008;48:750–758.
- [131] Fontana RJ. Side effects of long-term oral antiviral therapy for hepatitis B. *Hepatology* 2009;49:S185–S195.
- [132] Viganò M, Lampertico P, Colombo M. Drug safety evaluation of adefovir in HBV infection. *Expert Opin Drug Safety* 2011;10:809–818.
- [133] Sokal EM, Kelly D, Wirth S, Mizerski J, Dhawan A, Frederick D. The pharmacokinetics and safety of adefovir dipivoxil in children and adolescents with chronic hepatitis B virus infection. *J Clin Pharmacol* 2008;48:512–517.
- [134] Chang T-T, Gish RG, de Man R, Gadano A, Sollano J, Chao Y-C, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006;354:1001–1010.
- [135] Leung N, Peng C-Y, Hann H-W, Sollano J, Lao-Tan J, Hsu C-W, et al. Early hepatitis B virus DNA reduction in hepatitis B e antigen-positive patients with chronic hepatitis B: a randomized international study of entecavir versus adefovir. *Hepatology* 2009;49:72–79.
- [136] Colonna RJ, Rose R, Baldick CJ, Levine S, Pokornowski K, Yu CF, et al. Entecavir resistance is rare in nucleoside naïve patients with hepatitis B. *Hepatology* 2006;44:1656–1665.
- [137] Tenney DJ, Rose RE, Baldick CJ, Pokornowski KA, Eggers BJ, Fang J, et al. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. *Hepatology* 2009;49:1503–1514.
- [138] Sherman M, Yurdaydin C, Simsek H, Silva M, Liaw Y-F, Rustgi VK, et al. Entecavir therapy for lamivudine-refractory chronic hepatitis B: improved virologic, biochemical, and serology outcomes through 96 weeks. *Hepatology* 2008;48:99–108.
- [139] Bristol-Myers Squibb. A Phase III Study of the Safety and Efficacy of Entecavir in Pediatric Patients With Chronic HBV-Infection. In: *ClinicalTri-*

Review

- als.gov [Internet]. Bethesda (MD): National Library of Medicine (US). 2000 – [cited 18.09.11]. Available from: <http://clinicaltrials.gov/show/NCT01079806> NLM Identifier: NCT01079806.
- [140] Liaw YF, Gane E, Leung N, Zeuzem S, Wang Y, Lai CL, et al. 2-year GLOBE trial results: telbivudine is superior to lamivudine in patients with chronic hepatitis B. *Gastroenterology* 2009;136:486–495.
- [141] Novartis Pharmaceuticals. Pharmacokinetics and Safety of Single-Dose Telbivudine in Children and Adolescents With Chronic Hepatitis B. In: *ClinicalTrials.gov* [Internet]. Bethesda (MD): National Library of Medicine (US). 2000 – [cited 18.09.11]. Available from: <http://clinicaltrials.gov/show/NCT00907894> NLM Identifier: NCT00907894.
- [142] Marcellin P, Heathcote EJ, Buti M, Gane E, de Man RA, Krastev Z, et al. Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. *N Engl J Med* 2008;359:2442–2455.
- [143] Heathcote EJ, Marcellin P, Buti M, Gane E, de Man RA, Krastev Z, et al. Three-year efficacy and safety of tenofovir disoproxil fumarate treatment for chronic hepatitis B. *Gastroenterology* 2011;140:132–143.
- [144] Purdy JB, Gafni RI, Reynolds JC, Zeichner S, Hazra R. Decreased bone mineral density with off-label use of tenofovir in children and adolescents infected with human immunodeficiency virus. *J Pediatr* 2008;152:582–584.
- [145] Snow-Lampart A, Chappell B, Curtis M, Zhu Y, Myrick F, Schawwalder J, et al. No resistance to tenofovir disoproxil fumarate detected after up to 144 weeks of therapy in patients monoinfected with chronic hepatitis B virus. *Hepatology* 2011;53:763–773.
- [146] Hazra R, Balis FM, Tullio AN, DeCarlo E, Worrell CJ, Steinberg SM, et al. Single-dose and steady-state pharmacokinetics of tenofovir disoproxil fumarate in human immunodeficiency virus-infected children. *Antimicrob Agents Chemother* 2004;48:124–129.
- [147] Bouazza N, Urien S, Hirt D, Frange P, Rey E, Benaboud S, et al. Population pharmacokinetics of tenofovir in HIV-1 infected pediatric patients. *J Acquir Immune Defic Syndr* 2011;58:283–288.
- [148] Gilead Sciences. Evaluation of Tenofovir Disoproxil Fumarate in Adolescents With Chronic Hepatitis B Infection. In: *ClinicalTrials.gov* [Internet]. Bethesda (MD): National Library of Medicine (US). 2000 – [cited 18.09.11]. Available from: <http://clinicaltrials.gov/show/NCT00734162> NLM Identifier: NCT00734162.
- [149] Custer B, Sullivan SD, Hazlet TK, Iloeje U, Veenstra DL, Kowdley KV. Global epidemiology of hepatitis B virus. *J Clin Gastroenterol* 2004;38:S158–68.
- [150] Tessema B, Yismaw G, Kassu A, Amsalu A, Mulu A, Emmrich F, et al. Seroprevalence of HIV, HBV, HCV and syphilis infections among blood donors at Gondar University Teaching Hospital, Northwest Ethiopia: declining trends over a period of five years. *BMC Infect Dis* 2010;10:111.
- [151] Andernach IE, Nolte C, Pape JW, Muller CP. Slave Trade and Hepatitis B Virus Genotypes and Subgenotypes in Haiti and Africa. *Emerg Infect Dis* 2009;15:1222–1228.
- [152] Mitchell T, Armstrong GL, Hu DJ, Wasley A, Painter JA. The increasing burden of imported chronic hepatitis B – United States, 1974–2008. *PLoS ONE* 2011;6:e27717.
- [153] Juon H-S, Choi K, Park E-C, Kwak M-S, Lee S. Hepatitis B vaccinations among Koreans: results from 2005 Korea National Cancer Screening Survey. *BMC Infect Dis* 2009;9:185.
- [154] Chang M-H. Impact of hepatitis B vaccination on hepatitis B disease and nucleic acid testing in high-prevalence populations. *J Clin Virol* 2006;36:S45–S50.
- [155] Alvarado-Mora MV, Gutierrez Fernandez MF, Gomes-Gouvêa MS, de Azevedo Neto RS, Carrilho FJ, Pinho JRR. Hepatitis B (HBV), hepatitis C (HCV) and hepatitis delta (HDV) viruses in the Colombian population—how is the epidemiological situation? *PLoS ONE* 2011;6:e18888.
- [156] Nguyen VTT, Law MG, Dore GJ. An enormous hepatitis B virus-related liver disease burden projected in Vietnam by 2025. *Liver Int* 2008;28:525–531.
- [157] Magdzik WW. Hepatitis B. *Epidemiology in Poland, Central and Eastern Europe and the newly independent states*. *Vaccine* 2000;18:S13–6.
- [158] Pereira LMMB, Martelli CMT, Merchán-Hamann E, Montarroyos UR, Braga MC, de Lima MLC, et al. Population-based multicentric survey of hepatitis B infection and risk factor differences among three regions in Brazil. *Am J Trop Med Hyg* 2009;81:240–247.
- [159] Livramento AD, de Cordova CMM, Spada C, Treitinger A. Seroprevalence of hepatitis B and C infection markers among children and adolescents in the southern Brazilian region. *Rev Inst Med Trop São Paulo* 2011;53:13–17.
- [160] Datta S. An overview of molecular epidemiology of hepatitis B virus (HBV) in India. *Virol J* 2008;5:156.
- [161] Ugwuja E, Ugwu N. Seroprevalence of hepatitis B surface antigen and liver function tests among adolescents in Abakaliki, South Eastern Nigeria. *Internet J Trop Med* 2010;6, Number 2.
- [162] Eke AC, Eke UA, Okafor CI, Ezebialu IU, Ogbuagu C. Prevalence, correlates and pattern of hepatitis B surface antigen in a low resource setting. *Virol J* 2011;8:12.
- [163] Alikor E, Erhabor O. Seroprevalence of hepatitis B surface antigenaemia in children in a tertiary health institution in the Niger Delta of Nigeria. *Niger J Med* 2007;16:326–329.
- [164] Nurgalieva ZZ, Hollinger FB, Graham DY, Zhangabylova S, Zhangabylov A. Epidemiology and transmission of hepatitis B and C viruses in Kazakhstan. *World J Gastroenterol* 2007;13:1204–1207.
- [165] Allain JP. The risk of hepatitis B virus infection by transfusion in Kumasi, Ghana. *Blood* 2003;101:2419–2425.
- [166] Olinger CM, Lazouskaya NV, Eremin VF, Muller CP. Multiple genotypes and subtypes of hepatitis B and C viruses in Belarus: similarities with Russia and western European influences. *Clin Microbiol Infect* 2008;14:575–581.
- [167] Thüring E, Joller-Jemelka H, Sereth H, Reth C, Grob P. Prevalence of markers of hepatitis viruses A, B, C and of HIV in healthy individuals and patients of a Cambodian province. *Southeast Asian J Trop Med Public Health* 1993;24:239–249.
- [168] Bonanni P, Pesavento G, Bechini A, Tiscione E, Mannelli F, Benucci C, et al. Impact of universal vaccination programmes on the epidemiology of hepatitis B: 10 years of experience in Italy. *Vaccine* 2003;21:685–691.
- [169] Meffre C, Le Strat Y, Delarocque-Astagneau E, Dubois F, Antona D, Lemasson J-M, et al. Prevalence of hepatitis B and hepatitis C virus infections in France in 2004: Social factors are important predictors after adjusting for known risk factors. *J Med Virol* 2010;82:546–555.
- [170] Zhang J, Zou S, Giulivi A. Epidemiology of hepatitis B in Canada. *Can J Infect Dis* 2001;12:345–350.
- [171] Wong D, Cheung A, O'Rourke K, Naylor C, Detsky A, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Ann Intern Med* 1993;119:312–323.
- [172] Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999;341:1256–1263.
- [173] Marcellin P, Chang T-T, Lim S-G, Tong MJ, Sievert W, Shiffman ML, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003;348:808–816.
- [174] Lau GKK, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, Cooksley G, et al. Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2005;352:2682–2695.

Differentiated Umbilical Cord Matrix Stem Cells as a New *In Vitro* Model to Study Early Events During Hepatitis B Virus Infection

Massimiliano Paganelli,¹ Kai Dallmeier,² Omar Nyabi,¹ Isabelle Scheers,¹ Benoît Kabamba,³ Johan Neyts,² Patrick Goubau,³ Mustapha Najimi,¹ and Etienne M. Sokal¹

The role of cell differentiation state on hepatitis B virus (HBV) replication has been well demonstrated, whereas how it determines cell susceptibility to HBV entry is far less understood. We previously showed that umbilical cord matrix stem cells (UCMSC) can be differentiated towards hepatocyte-like cells *in vitro*. In this study we infected undifferentiated (UD-) and differentiated (D-) UCMSCs with HBV and studied the infection kinetics, comparing them to primary human hepatocytes (PHHs). UD-UCMSCs, although permissive to viral binding, had a very limited uptake capacity, whereas D-UCMSCs showed binding and uptake capabilities similar to PHHs. Likewise, asialoglycoprotein receptor (ASGPR) was up-regulated in UCMSCs upon differentiation. In D-UCMSCs, a dose-dependent inhibition of HBV binding and uptake was observed when ASGPR was saturated with known specific ligands. Subsequent viral replication was shown in D-UCMSCs but not in UD-UCMSCs. Susceptibility of UCMSCs to viral replication correlated with the degree of differentiation. Replication efficiency was low compared to PHHs, but was confirmed by (1) a dose-dependent inhibition by specific antiviral treatment using tenofovir; (2) the increase of viral RNAs along time; (3) *de novo* synthesis of viral proteins; and (4) secretion of infectious viral progeny. **Conclusion:** UCMSCs become supportive of the entire HBV life cycle upon *in vitro* hepatic differentiation. Despite low replication efficiency, D-UCMSCs proved to be fully capable of HBV uptake. Overall, UCMSCs are a unique human, easily available, nontransformed, *in vitro* model of HBV infection that could prove useful to study early infection events and the role of the cell differentiation state on such events. (HEPATOLOGY 2012;00:000–000)

Abbreviations: ASGPR, asialoglycoprotein receptor; cccDNA, covalently closed circular DNA; CYP3A4, cytochrome P450 3A4; HBV, hepatitis B virus; HNF4 α , hepatocyte nuclear factor 4- α ; MOI, multiplicity of infection; MSC, mesenchymal stem cells; PEG, polyethylene glycol; PHHs, primary human hepatocytes; PTH, primary Tupaia hepatocytes; qPCR, real time quantitative polymerase chain reaction; UD- and D-UCMSCs, undifferentiated and differentiated umbilical cord matrix stem cells; vge, viral genome equivalents.

From the ¹Pediatric Gastroenterology & Hepatology Unit, Université catholique de Louvain and Cliniques Saint Luc, Brussels, Belgium; ²Laboratory of Virology, Institut de Recherche Expérimentale et Clinique, Université catholique de Louvain and Cliniques Saint Luc, Brussels, Belgium; and ³Katholieke Universiteit Leuven, Rega Institute for Medical Research, Department of Virology, Leuven, Belgium.

Received March 25, 2012; accepted July 30, 2012.

Address reprint requests to: Etienne M. Sokal, Laboratoire d'hépatologie pédiatrique et service de gastroentérologie et hépatologie pédiatrique, Cliniques Saint-Luc, Université Catholique de Louvain, Av. Hippocrate 10, 1200 Brussels, Belgium. E-mail: etienne.sokal@uclouvain.be.

Copyright © 2012 by the American Association for the Study of Liver Diseases.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.26006

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Hepatitis B virus (HBV), as all hepadnaviruses, is hepatotropic and highly species-specific. The reasons for such specificity have not been clarified yet. The role of the cell differentiation state on HBV replication has been well demonstrated,^{1–3} whereas how it determines cell susceptibility to HBV entry is far less understood.

Although hepatoma cell lines can replicate the virus efficiently after transfection of the viral genome, they are not supportive of HBV entry.^{4–6} Thus, viral entry seems to be the most important determinant of HBV hepatotropism, but the responsible receptor(s) has not been identified yet.⁷ Asialoglycoprotein receptor (ASGPR), a hepatocyte-specific lectin, is one of the candidate membrane proteins that have been suggested to play a role in HBV binding and uptake.^{8–10}

Study of early infection events has been limited by the lack of suitable models. Primary human hepatocytes (PHHs) represent the gold standard, but their

use is significantly hampered by limited availability, rapid dedifferentiation *in vitro*, and very high variability between donors.¹¹ Hepatocytes of the tree shrew *Tupaia belangeri* and the HepaRG cell line have been shown as being infectable by HBV,¹²⁻¹⁴ but they share limitations of PHHs in terms of accessibility, reproducibility, and low yield of infection.

We previously showed that mesenchymal stem cells (MSCs) from different sources can be differentiated *in vitro* to hepatocyte-like cells.¹⁵⁻¹⁷ Such a potential, together with their self-renewal ability, make MSCs good candidates to study the role of differentiation of HBV-cell interactions.

In the current study we used umbilical cord matrix stem cells (UCMSCs) and showed that they can support the entire HBV life cycle upon hepatogenic differentiation. Although they were shown to replicate the virus with limited efficiency, they proved to be fully capable of HBV uptake. We analyzed the expression of ASGPR by UCMSCs, and its role in viral uptake, as a proof of concept for the use of this easily accessible, nontransformed human model for studies on early HBV infection events.

Materials and Methods

A detailed description of all methods used is provided in the Supporting Material.

UCMSC and Human Hepatocytes. The present study was approved by the local ethical committee. Umbilical cords were obtained from healthy donors after an informed consent was signed. UCMSCs were isolated by collagenase type I digestion of Wharton's jelly according to a well-standardized method we described before and cultured in standard conditions.¹⁶ Cells were used between passages 4 and 10. PHHs were obtained from the Hepatocytes and Hepatic Stem Cells Bank of Cliniques Saint-Luc (Brussels, Belgium). They were isolated from deceased donors' livers by two-step collagenase perfusion as described,¹⁸ and cultured in serum-free Williams' E medium (Invitrogen) supplemented with 100 U/mL penicillin / 100 μ g/mL streptomycin (Invitrogen), 10^{-6} M dexamethasone (Organon), and 10 μ g/mL insulin (Lilly Benelux).

Differentiation Procedure. Hepatic differentiation ~~were~~ induced on UCMSCs (D-UCMSCs) at passages 4-10 according to the described procedure.^{15,16}

Undifferentiated UCMSCs (UD-UCMSCs) used as controls were cultured in Iscove's modified Dulbecco's medium (IMDM, Invitrogen) supplemented with 1% penicillin/streptomycin and 2% fetal bovine serum (control medium) in standard conditions.

Viral Sources. Serum from three HBeAg-positive, immunotolerant, treatment-naïve patients was collected after informed consent. A viral load of $1.1 \pm 0.6 \times 10^{10}$ IU/mL was measured. All patients were infected with wildtype HBV of genotype D. As a great variability in infection efficiency was to be expected,¹¹ we attempted to standardize infection conditions using HBV produced *in vitro* by HepAD38 cells. These cells were cultured as described (detailed description and characterization in the Supporting Material).¹⁹ Under such conditions, HepAD38s produced $6.2 \pm 2.7 \times 10^8$ IU HBV/mL. Such a virus resulted in a wildtype genotype D, subtype *ayw*, with no genotypic resistance to lamivudine, entecavir, or adefovir identified (Supporting Fig. 2). The virus was concentrated 30 times by polyethylene glycol 6000 (PEG) precipitation, reaching a final concentration of 1.7×10^{10} IU/mL of HBV and stored at -80°C until use.

HBV Binding Assay. D-UCMSCs, UD-UCMSCs, and PHHs, cultured in 6-well plates, were incubated with concentrated HBV from HepAD38 in IMDM supplemented with penicillin/streptomycin. Incubation with HBV was carried out for 2 hours at 4°C . Multiplicity of infection (MOI) was calculated assuming that one viral genome equivalent (vge) corresponds to one infectious particle. After 2 hours, as large amounts of viral particles are known to remain nonspecifically bound to cell membrane,²⁰ the cells were extensively washed with cold phosphate-buffered saline (PBS). After the fourth washing, viral DNA in supernatant was <100 vge/mL (Supporting Fig. 4D). Thereafter, DNA was extracted without previous treatment with trypsin to avoid detachment of receptor-bound HBV.

Protease protection assay was carried out after washing by adding 1 mL of 0.25% trypsin to each well and incubation for 10 minutes at 37°C . DNA was then extracted after stopping the reaction and pelleting the cells.

HBV Uptake and Viral Replication Kinetics. After incubation with HepAD38-derived HBV for 2 hours at 4°C and extensive washing, the cells were moved to a 37°C environment and cultured as described above. DNA was extracted after 1, 4, and 24 hours and after 3, 7, and 10 days. A 10-minute treatment with 0.05% trypsin-EDTA solution, followed by pelleting, was carried out before DNA extraction in order to detach all viral particles still bound to the cell membrane. Conditioned medium was collected at days 1, 3, 7, 10, and 14 and stored at -20°C until use. All experiments were repeated using HBV from patients' sera and no difference in HBV uptake or replication was found (Supporting Fig. 6A,B).

Binding and Uptake Inhibition. D-UCMSCs were preincubated with either 5 mM EDTA, 1 mg/mL thyroglobulin (with and without EDTA), or 100 μ g/mL suramin (with and without EDTA) for 1 hour at 37°C. Such doses of ligands (all from Sigma) have previously proved effective towards ASGPR inhibition in other models.^{8,21-23} Cells were then inoculated at an MOI of 10^3 for 2 hours at 4°C, then extensively washed with cold PBS. DNA was extracted and membrane-bound HBV DNA quantified by quantitative polymerase chain reaction (qPCR).

For uptake inhibition, D-UCMSCs were pretreated with increasing concentrations of D-galactose (0.03-100 μ M; Sigma) for 1 hour at 37°C, then inoculated at an MOI of 10^3 , in the presence of the inhibitor, for 4 hours at 37°C. DNA was extracted after extensive washing and trypsin treatment.

Total HBV DNA Quantitation. We designed a TaqMan assay (RC01; Applied Biosystems ID: AIS07DM) able to specifically amplify a 106-bp region of the precore/core protein gene of genotype D HBV genome (which was common to all our viral sources). All samples were analyzed in triplicate by qPCR with a TaqMan standard 40-cycle amplification program with both annealing and elongation performed at 60°C. Beta-actin was used as the reference gene (Applied Biosystems). The assay proved to have a very low limit of detection (4.5 IU with a Ct <38, hit rate 100% on 12 tests) and a wide dynamic range (up to 3.5×10^{10} IU, $R^2 = 0.999$, PCR efficiency = 98.8%, $P < 0.0001$), while being highly reproducible and 100% specific for HBV DNA (Supporting Fig. 3). Full details on RC01 assay's *in silico* analysis, validation with WHO 2nd HBV International Standard, and determination of sensibility and specificity are available in the Supporting Material.

Covalently Closed Circular DNA (cccDNA) Quantitation. Ten μ L of extracted DNA from each sample was digested with 30 IU of Plasmid-Safe DNase (PS-DNase, Epicentre Biotechnologies) in a total volume of 50 μ L, for 60 minutes at 37°C. PS-DNase selectively and efficaciously degrades linear DNA or circular single-stranded DNA without affecting cccDNA.²⁴ We used RC01 assay to quantify cccDNA by qPCR. The results were normalized versus β -actin amplification in undigested samples. Detailed evaluation of PS-DNase digestion efficacy and RC01 specificity for cccDNA detection is available in the Supporting Material.

Viral RNA Assessment. RNA was extracted from D-UCMSCs at days 1, 3, and 7 postinfection. After DNase I treatment and reverse transcription, pregenomic (pg) and precore (preC) RNAs were measured

by TaqMan qPCR using the RC01 assay as described above. A sample containing 1 ng plasmid pAM6 (corresponding to 34.6×10^3 IU HBV DNA/mL) was added to each experiment as internal control for efficient DNA digestion and results were excluded if amplification was detected in such a control. Experiments performed to assess specificity of reverse transcription (RT)-qPCR for viral RNAs are described in the Supporting Material.

Viral Replication Inhibition Assay. D-UCMSCs from three different donors (five experiments) were preincubated with 0.07-2.5 μ g/mL tenofovir (phosphonomethoxypropyladenine [PMPA], Rega Institute, Leuven, Belgium), for 1 hour. They were then inoculated at an MOI of 10^5 for 4 hours at 37°C, washed, and cultured in differentiation medium supplemented with PMPA over the full course of the experiment. DNA was extracted 7 days postinfection and intracellular HBV DNA was quantified by qPCR. Anti-HBV activity of PMPA was also tested on HepAD38 cells as described.^{19,25}

Viral RNA (pg and preC) was measured in 2.5 μ g/mL PMPA-treated D-UCMSCs at 1, 3, and 7 days postinfection and compared to untreated cells as described above.

Immunofluorescence Analysis. To assess HBV uptake by the cells studied, and cellular ability to synthesize viral proteins, PHHs, UD- and D-UCMSCs were inoculated at an MOI of 10^5 for 4 hours at 37°C. After extensive washing they were cultured in standard conditions for 24 hours. After fixation, permeabilization, and preblocking, a mouse monoclonal antibody against HBcAg (NCL-HBcAg-506, Leica) was used (1:100). Staining for ASGPR was obtained on noninfected cells with a rabbit polyclonal antibody (1:400; HPA011954, Sigma). Alexa Fluor 594-labeled antibodies (A11012, A21203, Invitrogen) were used for secondary staining (1:200). DAPI was used to stain the nuclei. Results were analyzed with the Cell Observer SD laser confocal microscope (Carl Zeiss).

Enzyme-Linked Immunosorbent Assay (ELISA) for HBeAg and HBsAg. Conditioned media from a culture of 150,000 cells were concentrated 10 times by centrifugation at 4,000g for 15 minutes in 10 kDa Amicon Ultra centrifugal filter tubes (Millipore). The resulting samples were analyzed for HBeAg and HBsAg (Monolisa HBeAg/Ab Plus and HBsAg Ultra, Bio-Rad) according to the manufacturer's instructions. Reading of the optical densities at 450/620 nm was carried out with an absorbance reader (Reader 250, BioMérieux).

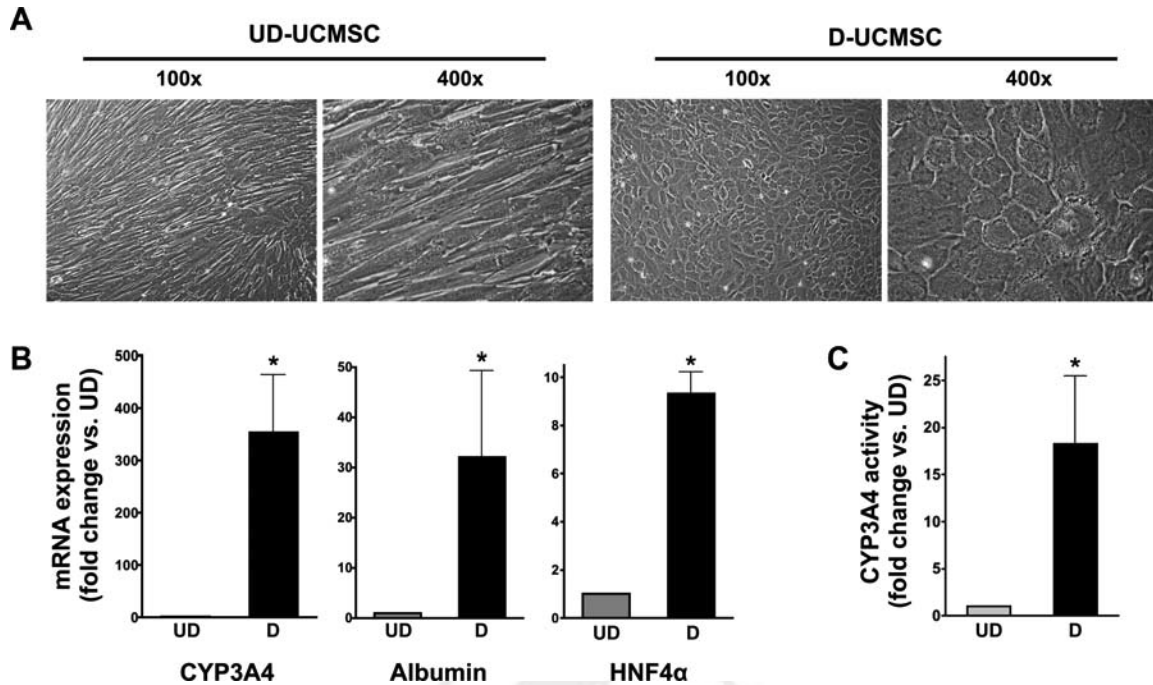


Fig. 1. Hepatogenic differentiation potential of UCMSCs. (A) Morphology of UD-UCMSCs and D-UCMSCs at confluence, before infection experiments. At the end of the differentiation protocol, D-UCMSCs show a polygonal shape with granular cytoplasm. Original magnification 100 $\times$ and 400 $\times$. (B) Increased expression of CYP3A4 (354.7 ± 109.8 , $n = 6$, $P = 0.016$), albumin (32.2 ± 17.2 , $n = 6$, $P = 0.016$), and HNF4 α (9.34 ± 0.89 , $n = 5$, $P = 0.031$) mRNAs, in D-UCMSCs compared to UD-UCMSCs (Wilcoxon signed rank test). (C) Increased CYP3A4 activity (luciferin-IPA test) in D-UCMSCs as compared to UD-UCMSCs ($n = 5$; $P = 0.031$, Wilcoxon signed rank test).

Western Blot Analysis for ASGPR and HBcAg.

For ASGPR detection, 20 μ g of total cellular proteins was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes through semidry transfer. After blocking, a rabbit polyclonal antibody against ASGPR (HPA011954, Sigma) was used for overnight incubation at 4 $^{\circ}$ C (1:5,000). A goat polyclonal anti- α -actin antibody (1:2,000; sc-1616, Santa Cruz Biotechnology) served as internal control. Fluorescent secondary antibodies (Biotium) were used (1:10,000) and the results were read with the Odyssey infrared imaging system (Li-Cor Biosciences).

For HBcAg detection, an immunoprecipitation was performed on lysate from 10^6 cells (10 days postinfection) using protein A Sepharose beads (GE Healthcare) bound to a rabbit polyclonal anti-HBc antibody (B0586, Dako). After SDS-PAGE separation and transfer, staining was obtained incubating the membrane with a mouse monoclonal antibody against HBcAg (1:2,000; NCL-HBcAg-506, Leica).

Infectivity Assay. Conditioned medium was collected from D-UCMSCs culture 14 days postinfection. After centrifugation to remove cellular debris, HBV was concentrated by PEG precipitation. Pellets were resuspended in PBS and used to infect PHHs by

overnight incubation at 37 $^{\circ}$ C. After extensive washing, PHHs were cultured as described above. DNA was extracted after 24 hours and 7 days to test for viral replication.

Statistical Analysis. Statistical analysis was performed with PRISM 4 (GraphPad Software). Mann-Whitney U test, Wilcoxon signed-rank test, and one-sample two-tailed t test were used as appropriate. $P \leq 0.05$ was considered significant. Values are expressed as mean $\pm$ standard deviation or standard error of the mean.

Results

Hepatic Differentiation of UCMSC. UCMSCs were isolated from Wharton's jelly of six healthy donors. Their identity was confirmed as described by us and others (Supporting Material and Supporting Fig. 1).¹⁶ They were differentiated *in vitro* to hepatocyte-like cells at passages 4 to 10 (Fig. 1A), according to a well-established multistep protocol.^{15,16} Quality of differentiation was proven by an increased gene expression of cytochrome P450 3A4 (CYP3A4; $P = 0.016$), hepatocyte nuclear factor 4- α (HNF4 α ; $P = 0.031$), and albumin ($P = 0.016$), and the exhibition of functions typical of mature hepatocytes, such as CYP3A4

F1

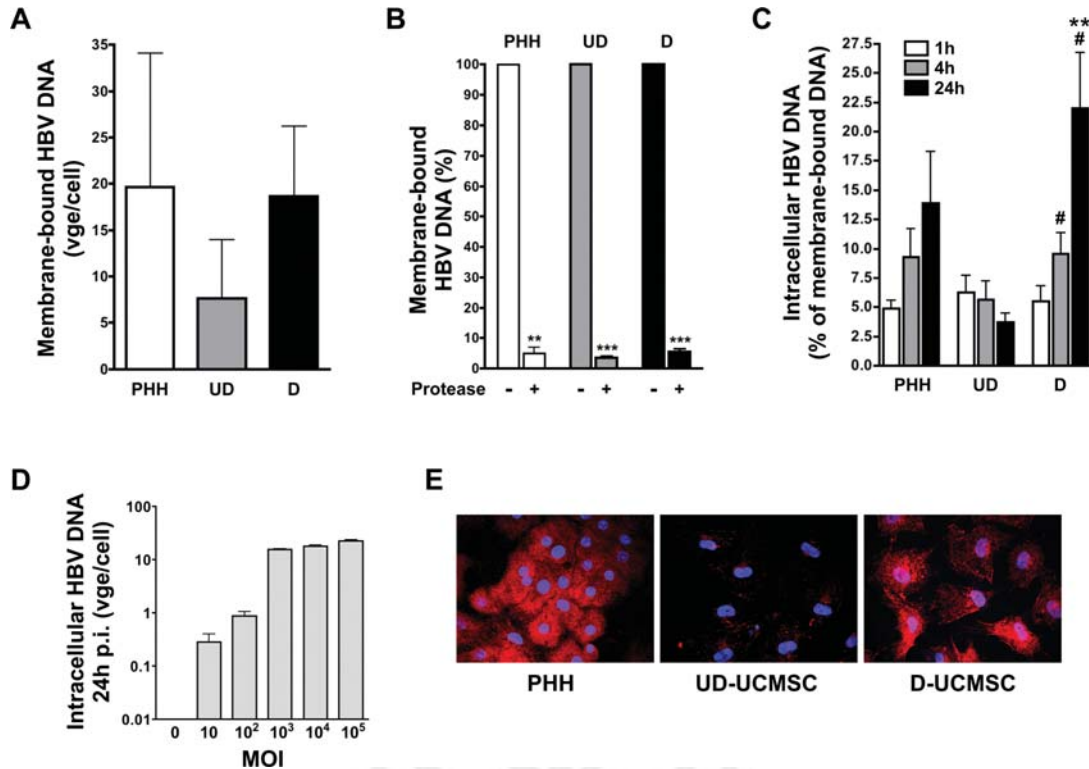


Fig. 2. D-UCMSCs permit HBV binding and uptake. (A) Binding of HBV on UCMSCs after 2-hour incubation at 4°C compared to PHHs. Results are expressed as vge/cell ($n = 6$ for UD- and D-UCMSCs, $n = 3$ for PHHs; $P = ns$). (B) Protease sensitive binding of HBV to the cell surface: treatment with 0.25% trypsin-EDTA for 10 minutes, at the end of incubation and before DNA extraction, detached 95% of membrane-bound HBV ($n = 5$ for UD- and D-UCMSCs, $n = 3$ for PHHs; $**P < 0.001$ and $***P < 0.0001$ as compared to no protease treatment, one-sample two-sided t test). (C) D-UCMSCs were as efficient as PHHs in internalizing membrane-bound HBV after temperature shift to 37°C. Results are expressed as percentage of the internalized (i.e., protease-protected) versus cell membrane-attached (protease-sensitive) HBV DNA after a given incubation period ($n = 5$ for UD- and D-UCMSCs, $n = 3$ for PHHs; $#P = 0.016$ compared to 1 hour postinfection and $**P = 0.004$ compared to UD-UCMSCs, Mann-Whitney test). (D) An MOI of 100 was needed to achieve at least 1 vge/cell in D-UCMSCs at 24 hours postinfection. No further increase was observed for MOIs $>10^3$ ($n = 3$, $P = ns$). (E) Immunofluorescence for HBcAg on PHHs, UD-UCMSCs, and D-UCMSCs 24 hours postinfection (confocal laser microscopy, AlexaFluor-594/DAPI staining; original magnification 400 $\times$).

activity ($P = 0.031$; Fig. 1B,C). Variability in differentiation quality among different donors is shown in Supporting Fig. 1E.

HBV Binding to UCMSC Membranes. To study the effect of differentiation state on HBV-cell interactions, we first assessed viral attachment to the cell membranes. At a temperature below 18°C, endocytosis is inhibited (Supporting Fig. 4A), whereas binding of viral particles to membrane receptors remains active.²⁶ We incubated PHHs, UD-UCMSCs, and D-UCMSCs with HBV at an MOI of $1.0 \pm 0.8 \times 10^5$, for 2 hours at 4°C. Under these conditions, endocytosis was totally inhibited while cellular viability was not affected (Supporting Fig. 4B,C). After extensive washing (Supporting Fig. 4D), the amount of membrane-bound HBV DNA was similar for PHHs and D-UCMSCs, but lower for UD-UCMSCs F2 ($P = 0.052$; Fig. 2A).

To prove that binding of viral particles on cell membrane was receptor-mediated, after incubation

with HBV at 4°C and extensive washing, cells were treated with trypsin before DNA extraction. Protease detached 95% of the viral particles, without any difference between cell types (Fig. 2B), indicating that proteinaceous structures were involved in HBV binding.

Uptake of Membrane-Bound HBV. To assess whether viral particles attached to membrane receptors could be internalized, after the 2-hour incubation at 4°C and extensive washing cells were moved to a 37°C environment. They were cultured under standard conditions and DNA was extracted after 1, 4, and 24 hours. To make sure to extract only intracellular DNA, trypsin was applied before DNA extraction, in order to detach all particles still bound to the cell membrane.

After 1 hour at 37°C, PHHs, UD-UCMSCs, and D-UCMSCs were able to internalize $4.9 \pm 0.7\%$, $6.3 \pm 1.5\%$, and $5.5 \pm 1.3\%$ of membrane-bound HBV, respectively ($P = ns$; Fig. 2C). The proportion of viral uptake increased at 4 and 24 hours for PHHs

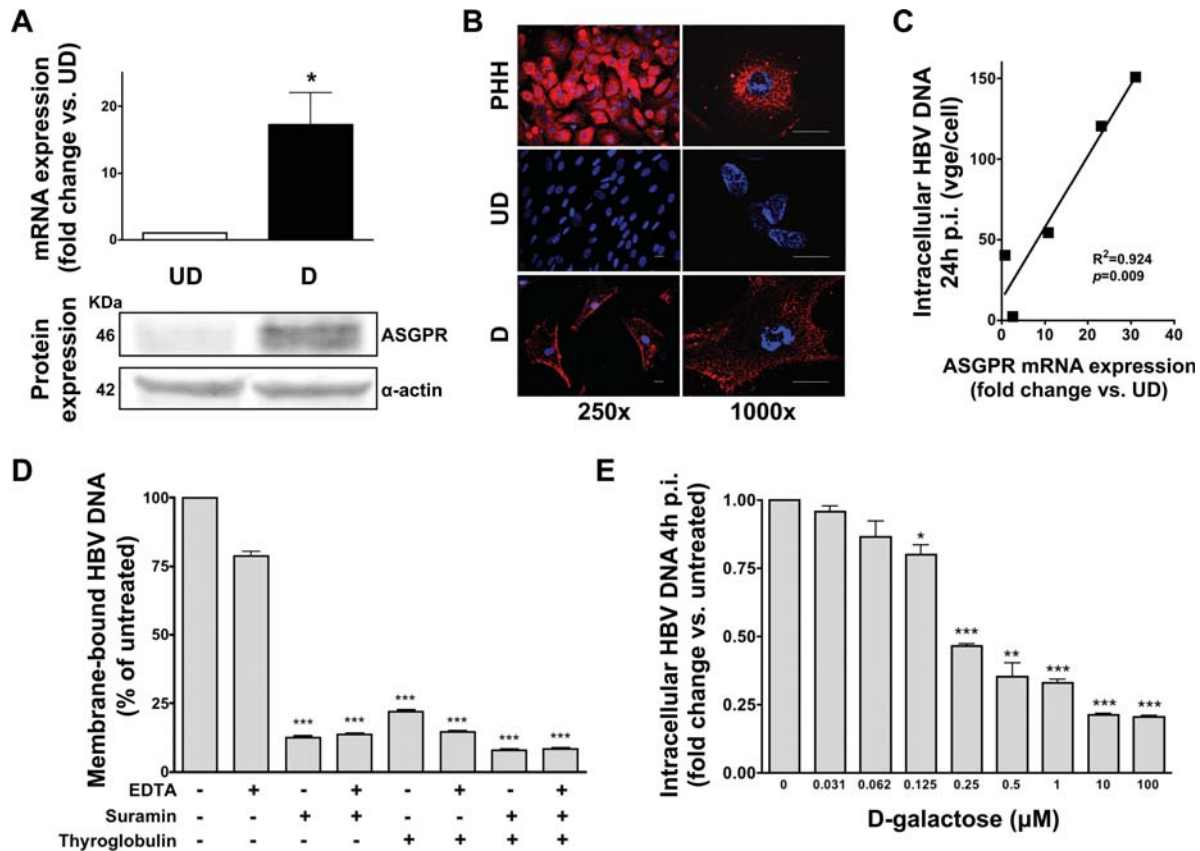


Fig. 3. Role of ASGPR in HBV binding to D-UCMSCs. (A) ASGPR was more strongly expressed by D-UCMSCs than by UD-UCMSCs both at mRNA ($n = 6$, $P = 0.16$, Wilcoxon signed rank test; RT-qPCR) and protein (western blot analysis) levels. (B) Immunofluorescence for ASGPR on uninfected PHHs, UD-UCMSCs, and D-UCMSCs (confocal laser microscopy, AlexaFluor-594/DAPI staining; original magnifications 250 $\times$ and 1,000 $\times$). (C) Correlation between ASGPR mRNA expression and D-UCMSCs susceptibility to viral uptake ($R^2 = 0.924$, $P < 0.009$, $n = 5$). Uptake capacity is expressed as intracellular HBV DNA (vge/cell) at 24 hours postinfection. ASGPR expression is expressed as fold change as compared to UD-UCMSCs. (D) Incubation of D-UCMSCs before and during HBV infection (MOI 10^3 for 2 hours at 4 $^{\circ}$ C) with either 5 mM EDTA, 1 mg/mL thyroglobulin (with and without EDTA), or 100 μ g/mL suramin (with and without EDTA) led to inhibition of HBV binding (membrane-bound HBV DNA measured by qPCR, $n = 3$, $P < 0.0001$, one-sample two-sided t test). (E) HBV uptake by D-UCMSCs was inhibited by D-galactose in a dose-dependent manner ($EC_{50} = 0.2 \mu$ M; treatment before and during 4 hours of inoculation with HBV at an MOI of 10^3). Intracellular HBV DNA levels measured by qPCR are shown (expressed as fold change versus no galactose; $n = 3$; * $P = 0.03$, ** $P < 0.01$, *** $P < 0.001$, one-sample two-sided t test).

($P = ns$) and D-UCMSCs ($P = 0.016$), but remained stable for UD-UCMSCs. HBV uptake after 24 hours was significantly greater in D-UCMSCs than in UD-UCMSCs ($P = 0.004$). The amount of virus taken up by D-UCMSCs at 24 hours increased with the increase of MOI (Fig. 2D). Little increase was seen for MOI $> 10^3$, suggesting saturation of the receptor(s).

Viral entry after 24 hours at 37 $^{\circ}$ C was confirmed by immunofluorescence. Both PHHs and D-UCMSCs, but not UD-UCMSCs, showed a positive staining for intracellular HBcAg (Fig. 2E).

ASGPR Is Expressed by UCMSCs and Its Saturation by Competitive Inhibitors Prevents HBV Binding and Uptake. ASGPR, a Ca^{2+} -dependent hepatocyte-specific lectin,²⁷ was significantly more expressed by D-UCMSCs than by UD-UCMSCs, both at messenger RNA (mRNA) and protein levels

(Fig. 3A,B). In this regard, D-UCMSCs resembled PHHs serving as positive controls.

ASGPR has been suggested to play a role in HBV binding and uptake.⁸⁻¹⁰ HBV uptake by D-UCMSCs was directly correlated with ASGPR mRNA expression ($R^2 = 0.924$, $P < 0.01$; Fig. 3C). In Fig. 3D we show that HBV binding to D-UCMSCs may be partially inhibited by Ca^{2+} chelation ($21.1 \pm 2.5\%$ inhibition) and thyroglobulin addition ($77.8 \pm 1\%$), the latter being one known ASGPR-specific ligand. Suramin, which is known to block HBV attachment,^{22,23} inhibited $87.4 \pm 1\%$ of HBV binding to D-UCMSCs. The addition of increasing concentrations of D-galactose (0.03-100 μ M), before and during inoculation of D-UCMSCs, resulted in a dose-dependent inhibition of HBV uptake (up to $79.3 \pm 0.7\%$, $P < 0.0001$; Fig. 3E). The median effective concentration (EC_{50}) = 0.2 μ M (95% confidence

F3

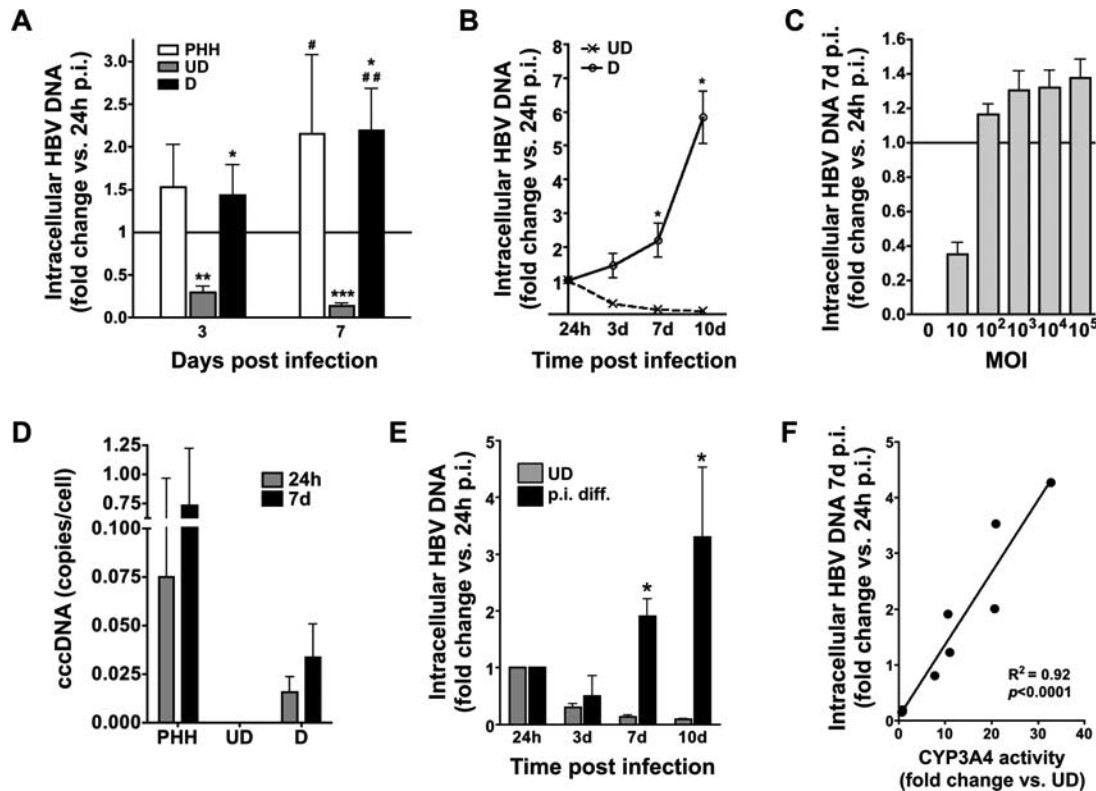


Fig. 4. HBV replication in UCMSCs. (A) Total intracellular HBV DNA at 3 and 7 days postinfection (qPCR), as compared to the amount of HBV DNA internalized by the cells at 24 hours postinfection (represented by the horizontal line; $n = 6$ for D-UCMSCs, $n = 4$ for UD-UCMSCs and $n = 3$ for PHH; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared to 24 hours, one-sample two-sided t test; $\#P < 0.05$ and $##P < 0.01$ compared to UD-UCMSCs; Mann-Whitney test). (B) At 10 days postinfection, intracellular HBV DNA further increased in D-UCMSCs but not in UD-UCMSCs (qPCR, $n = 6$ for D-UCMSCs and $n = 4$ for UD-UCMSCs, $P = 0.029$; Mann-Whitney test). (C) In D-UCMSCs, an MOI of at least 100 was needed to have detectable viral replication (expressed as fold change of intracellular HBV DNA levels at 7 days postinfection versus 24 hours postinfection; $n = 3$, $P = ns$). (D) cccDNA at 7 days postinfection, expressed as copies/cell (qPCR, $n = 5$ for D-UCMSCs, $n = 4$ for UD-UCMSCs and $n = 3$ for PHH; $P = ns$, Mann-Whitney test). (E) Postinfection differentiation assay: differentiation of UD-UCMSCs 24 hours postinfection led to viral replication of the small amount of HBV already internalized ($n = 3$ for postinfection diff. UCMSCs [black bars], $n = 4$ for UD-UCMSCs [gray bars]; $*P = 0.018$ as compared to UD-UCMSCs; one-sample t test). (F) Correlation between differentiation status and cell susceptibility to viral replication. The state of differentiation is expressed as relative activity of CYP3A4 as compared to the average activity in UD-UCMSCs. Ability of the cells to support viral replication is expressed as relative increase of intracellular HBV DNA levels at day 7 postinfection as compared to the virus internalized after 24 hours at 37°C . Both UD- and D-UCMSCs are considered in this analysis ($R^2 = 0.92$, $P < 0.0001$, $n = 9$: $n = 6$ for D-UCMSCs and $n = 3$ for UD-UCMSCs).

interval [CI], 0.17-0.23) was calculated for D-galactose by dose-response analysis (Supporting Fig. 4F).

Cell Susceptibility to HBV Replication. Total HBV DNA was quantified by qPCR at 3 and 7 days postinfection and compared to the amount of viral DNA present inside the cells at 24 hours postincubation (Fig. 4A). Intracellular HBV DNA levels decreased along time in UD-UCMSCs ($P < 0.0001$), whereas they increased in PHHs ($P = ns$) and D-UCMSCs ($P = 0.016$) at day 3 and 7, suggesting productive viral replication. At 7 days postinfection, intracellular HBV DNA levels did not differ between D-UCMSCs and PHHs, but were significantly higher in D-UCMSCs than in UD-UCMSCs ($P < 0.01$, Fig. 4B). A further increase of HBV DNA levels was seen at 10 days postinfection in D-UCMSCs ($P = 0.029$;

Fig. 4B). PHHs were not tested beyond 7 days postinfection to avoid biases due to dedifferentiation.¹¹ In D-UCMSCs, an MOI of at least 100 was needed to yield detectable viral replication (Fig. 4C). Increase of HBV DNA replication intermediates along time was confirmed at Southern blotting on the same samples used for qPCR (Supporting Fig. 5C).

We subsequently measured cccDNA by qPCR (Fig. 4D). In D-UCMSCs, cccDNA levels increased along time up to 0.03 ± 0.04 copies/cell at 7 days postinfection, corresponding to approximately every 30th cell containing at least one copy of cccDNA. By contrast, 0.7 ± 0.8 copies/per cell were found in PHHs and no cccDNA was detected in UD-UCMSCs. Controls to prove specificity of cccDNA detection by qPCR are shown in the Supporting Data.

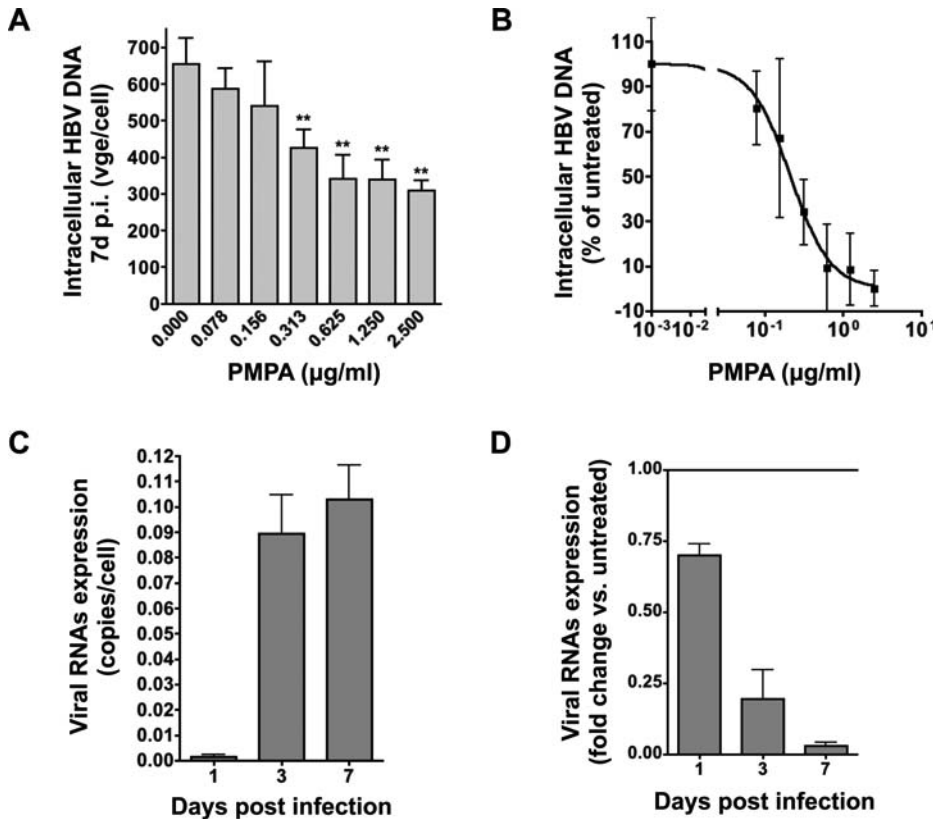


Fig. 5. (A,B) Inhibition of HBV replication in D-UCMSCs by tenofovir. (A) Intracellular HBV DNA levels 7 days postinfection in D-UCMSCs treated with increasing concentrations of PMPA (vge/cell, $n = 5$, $**P = 0.008$; Mann-Whitney test). (B) Dose-response curve for treatment with PMPA on D-UCMSCs ($EC_{50} = 0.21$, 95% CI, 0.12-0.39, $n = 5$). Response was calculated by measuring intracellular HBV DNA 7 days postinfection. (C,D) Transcription of viral RNAs. (C) Viral RNAs (pg and preC) measured in D-UCMSCs by RT-qPCR at 1, 3, and 7 days postinfection (copies/cell; $n = 3$, $P = ns$). (D) Viral RNAs measured in D-UCMSCs after 2.5 µg/mL PMPA treatment (RT-qPCR). Results are expressed as fold change versus no treatment (horizontal line).

To further confirm the effect of differentiation on cellular susceptibility to viral replication, we infected UD-UCMSCs as described above and induced differentiation after 24 hours. For this postinfection differentiation we used a serum-free medium corresponding to the final step of the differentiation protocol described above. HBV DNA levels in UCMSCs undergoing postinfection differentiation increased along time as compared to UD-UCMSCs ($P = 0.018$), suggesting replication of the small quantity of HBV (0.22 ± 0.32 vge/cell) internalized by undifferentiated cells (Fig. 4E).

HBV replication efficiency in UCMSCs varied among different donors, and was directly correlated with the quality of differentiation ($R^2 = 0.92$, $P < 0.0001$; Fig. 4F).

To assess whether known antivirals could inhibit viral replication in D-UCMSCs, the cells were inoculated at an MOI of 10^5 in the presence of an increasing concentration of PMPA (0-2.5 µg/mL; Fig. 5A,B). A dose-dependent inhibition of HBV replication was shown after 7 days of PMPA treatment (Fig. 5B). EC_{50} for PMPA was 0.21 µg/mL (95% CI, 0.12-0.39) in D-UCMSCs, as compared to 0.12 µg/mL (95% CI, 0.11-0.14) in HepAD38 (Supporting Fig. 5A).

Transcription of Viral Genes. Viral RNAs (pg and preC) were quantified in D-UCMSCs by RT-qPCR. As shown in Fig. 5C, viral RNAs increased in D-UCMSCs along time, reaching 0.103 ± 0.023 copies/cell at day 7 postinfection. Treatment with 2.5 µg/mL PMPA reduced the amount of viral RNAs found in D-UCMSCs by 30%, 81%, and 97% at 1, 3, and 7 days postinfection, respectively ($P = ns$; Fig. 5D). Specificity of viral RNA quantification by RT-qPCR was carefully assessed (Supporting Material) and confirmed at each experiment.

De Novo Synthesis of Viral Proteins. We assessed synthesis of viral proteins in UCMSCs by immunofluorescence at day 10 postinfection. A staining for HBeAg was shown in D-UCMSC, whereas it was absent in UD-UCMSCs (Fig. 6A). Secretion of HBsAg and HBeAg was measured by ELISA at different timepoints postinfection. PHHs secreted increasing amounts of HBeAg from day 3 postinfection (Supporting Fig. 5B). To increase sensibility of the technique, we concentrated proteins from conditioned medium by ultrafiltration before ELISA. A significant increase of both viral antigens was detected over time in D-UCMSCs supernatant ($P < 0.05$; Fig. 6B,C), whereas they remained negative in UD-UCMSCs. Low-level, yet clearly detectable synthesis of viral

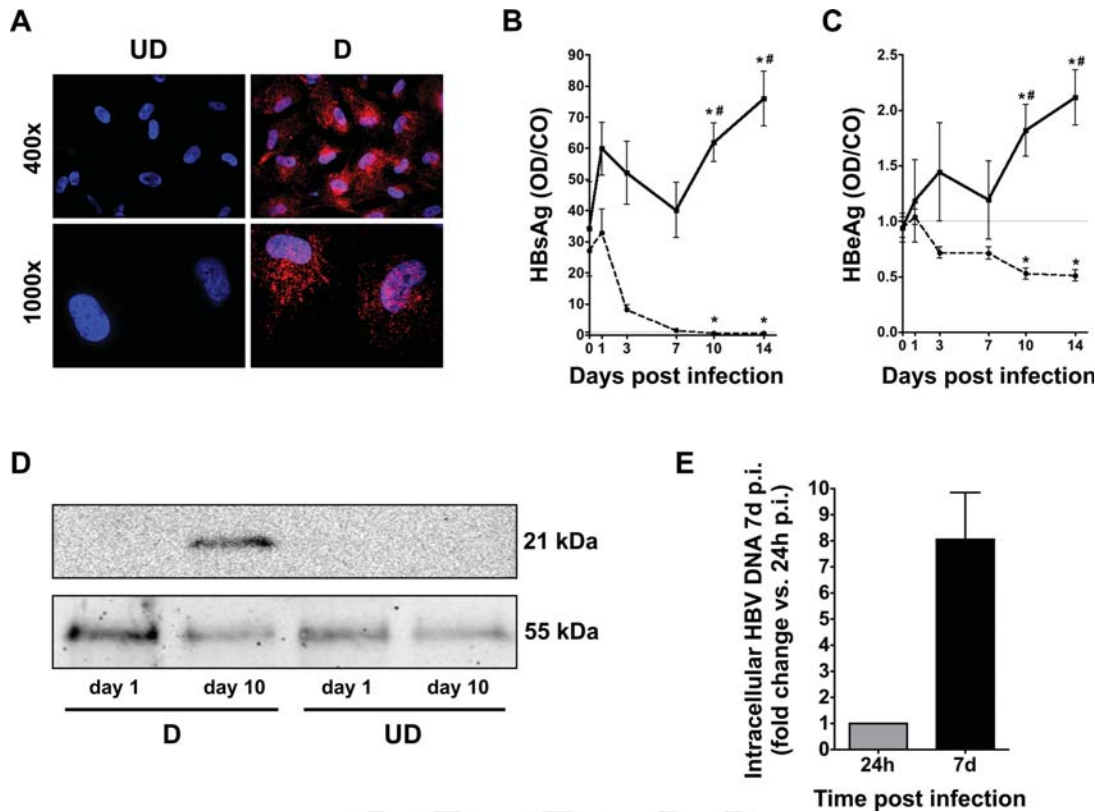


Fig. 6. Synthesis of viral proteins and passage of infectious progeny virus. (A) Immunofluorescence for HBcAg in UD-UCMSCs and D-UCMSCs 10 days postinfection (AlexaFluor-594/DAPI staining). Original magnifications: 400 $\times$ and 1,000 $\times$ (confocal laser microscopy). (B,C) Synthesis of HBsAg (B) and HBeAg (C) measured in conditioned medium of UD- (dotted line) and D-UCMSCs (solid line) by ELISA after 10 $\times$ concentration via ultrafiltration. Cutoff was calculated according to the manufacturer's instructions. Values are expressed as optical density/cutoff (OD/CO ≥ 1 considered positive; $n = 4$; * $P < 0.05$ as compared to 7 days postinfection; # $P < 0.05$ as compared to UD-UCMSCs). (D) Western blot analysis for HBcAg (21 kDa) on UD- and D-UCMSCs 10 days postinfection, after immunoprecipitation. As internal control, IgG heavy chains are shown at 55 kDa (subsequent staining with the same antibody as used for immunoprecipitation). (E) D-UCMSCs proved to produce fully competent viral particles, as HBV secreted in conditioned medium by D-UCMSCs 14 days postinfection was able to infect PHHs. Three independent infection assays were performed using PHHs from the same donor and concentrated HBV from three different D-UCMSC infection experiments ($P = 0.06$).

proteins was confirmed in D-UCMSCs (but not in UD-UCMSCs) by western blotting for HBcAg after immunoprecipitation (Fig. 6D).

Infectivity Assay. We assessed the infectivity of viral particles secreted by D-UCMSCs on PHHs. PHHs from one donor were inoculated with D-UCMSCs-derived HBV (three donors, $\text{MOI } 21.1 \pm 26.6$) for 16 hours at 37°C. Intracellular HBV DNA levels increased in PHHs at day 7 postinfection as compared to 24 hours postinfection (8 ± 1.8 -fold change, $P = 0.06$; Fig. 6E), suggesting productive viral replication and confirming the ability of D-UCMSCs to synthesize infectious HBV particles, completing the full viral life cycle.

Discussion

We describe here a new *in vitro* nontransformed human model of HBV infection. We show that non-liver-derived mesenchymal stem cells (UCMSCs) are

turned permissive to the entire HBV life cycle upon *in vitro* hepatogenic differentiation. None of the few studies conducted on *in vitro* infection of other MSCs evaluated binding and uptake kinetics.^{28,29} We set up infection conditions in order to analyze the different steps of the viral cycle and demonstrated that, although replication efficiency downstream of viral entry was quite low, HBV uptake was fully supported by D-UCMSCs and comparable to PHHs. To our knowledge, this is the first human, nontransformed *in vitro* model, other than primary hepatocytes, to be fully capable of HBV uptake.

UCMSCs are widely characterized MSCs that are easy to obtain and to expand in culture. As we previously demonstrated, UCMSCs are capable of exhibiting many of the markers and functions typical of mature hepatocytes.¹⁶ Nevertheless, we observed variability in the quality of differentiation among donors. As shown by the direct correlation between CYP3A4 activity (a surrogate marker of differentiation state)

and susceptibility to viral replication, quality of differentiation seems to be a key factor influencing efficiency of infection in D-UCMSCs. CYP3A4 activity could therefore be used as a marker to predict suitability of each UCMSCs population for infection studies.

The understanding of the viral life cycle has been hampered by the extremely restrictive tropism of HBV. PHHs are the preferred tissue culture model, but they are difficult to obtain, maintain in culture, and infect *in vitro*.¹¹ Primary *Tupaia* hepatocytes (PTH) are an interesting alternative.^{12,13} Unfortunately, it is well known that susceptibility of primary hepatocytes to HBV infection is rapidly lost during prolonged culture.¹¹ Such a loss in susceptibility has been correlated to the dedifferentiation process to which hepatocytes rapidly undergo *in vitro*.³⁰

It has been shown that transcription of HBV genes and subsequent viral replication is dependent upon the degree of differentiation of the host cell.¹ Liver-enriched transcription factors such as HNF4 α , HNF1 α , and HNF3 β have been shown to have a central role in regulating viral promoters and enhancers.^{2,3} Here we show that UCMSCs express HNF4 α mRNA upon differentiation, but at a much lower level than PHHs, which could account for the observed lower intracellular replication efficiency.

The role of the differentiation state on cell susceptibility to HBV entry is far less understood. HBV binding and uptake seem to be the most important determinants of HBV hepatotropism. Although many different hepatoma-derived cell lines have been demonstrated to be capable of viral replication after transfection of the viral genome,⁴⁻⁶ very few *in vitro* models, besides PHHs, permit viral uptake.¹²⁻¹⁴ D-UCMSCs susceptibility to HBV uptake therefore makes them a highly promising model.

HBV binding and entry into susceptible cells is a multistep process which is poorly understood. The initial attachment of the virus to the cell membrane is mediated by heparan sulfate proteoglycans,²³ and does not seem to be specific for susceptible cells.²⁶ A more specific binding to one or multiple receptors, expressed mainly on hepatocytes, and localized on the basolateral membrane, is probably responsible for the restricted HBV host range.³¹ Among the many candidate receptors, ASGPR is the only one that is strictly hepatocyte-specific. It is a lectin expressed on the sinusoidal and basolateral hepatocellular membrane, which is responsible for the uptake of desialylated glycoproteins.²⁷ Basma et al.³² showed that ASGPR is up-regulated in embryonic stem cells upon hepatic differentiation. Different studies have proved it to be necessary for HBV

binding and uptake.⁸⁻¹⁰ Here we show that ASGPR is up-regulated in UCMSCs upon differentiation. We also show a dose-dependent inhibition of HBV binding and uptake when ASGPR is saturated with known specific ligands. Although further verification would be necessary to definitely prove the role of this receptor, these experiments are a proof of concept that UCMSCs may be a suitable model to study early infection events.

HBV is highly infectious *in vivo*, but only a small proportion of the cells are infected *in vitro*.³³ PTH and HepaRG share the same disadvantages of PHHs in terms of low replication efficiency and high MOI needed to infect a reasonable proportion of cells.¹²⁻¹⁴ UCMSCs were even less efficient than PHHs in replicating HBV, showed a low-level protein synthesis, and a high MOI was indeed needed to achieve a productive infection. Nevertheless, viral entry was as efficient as in PHHs.

As our aim was to create an *in vitro* model as "physiological" as possible, and not to maximize infection efficiency, we decided to avoid the use of all adjuvant molecules (such as dimethyl sulfoxide or polyethylene glycol) that could cause possible experimental artifacts. Improvement of the quality of differentiation would be needed to improve infection efficiency of this model.

Taken together, these data show that UCMSCs are a unique human, easily available, nontransformed, *in vitro* model of HBV infection. Such cells could prove useful to study early infection events and the role of the cell differentiation state on such events.

Acknowledgment: We thank Dr. Patrick Van Der Smissen (de Duve Institute, Cellular Biology Unit), Mrs. Nawal Jazouli, Mrs. Floriane André, Mr. Joachim Ravau, and Mr. Jonathan Evraerts (Pediatric Hepatology and Cell Therapy Lab) for technical assistance.

References

1. Quasdorff M, Protzer U. Control of hepatitis B virus at the level of transcription. *J Viral Hep* 2010;17:527-536.
2. Tang H, McLachlan A. Transcriptional regulation of hepatitis B virus by nuclear hormone receptors is a critical determinant of viral tropism. *Proc Natl Acad Sci U S A* 2001;98:1841-1846.
3. Quasdorff M, Hösel M, Odenthal M, Zedler U, Böhne F, Gripon P, et al. A concerted action of HNF4 α and HNF1 α links hepatitis B virus replication to hepatocyte differentiation. *Cell Microbiol* 2008;10:1478-1490.
4. Sells MA, Zelent AZ, Shvartsman M, Acs G. Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions. *J Virol* 1988;62:2836-2844.
5. Sun D, Nassal M. Stable HepG2- and Huh7-based human hepatoma cell lines for efficient regulated expression of infectious hepatitis B virus. *J Hepatol* 2006;45:636-645.

6. Rabe B, Glebe D, Kann M. Lipid-mediated introduction of hepatitis B virus capsids into nonsusceptible cells allows highly efficient replication and facilitates the study of early infection events. *J Virol* 2006;80:5465-5473.
7. Glebe D, Urban S. Viral and cellular determinants involved in hepadnaviral entry. *World J Gastroenterol* 2007;13:22-38.
8. Treichel U, Meyer zum Büschenfelde KH, Stockert RJ, Poralla T, Gerken G. The asialoglycoprotein receptor mediates hepatic binding and uptake of natural hepatitis B virus particles derived from viraemic carriers. *J Gen Virol* 1994;75:3021-3029.
9. Owada T, Matsubayashi H, Sakata H, Ihara S, Sato K, Ikebuchi et al. Interaction between desialylated hepatitis B virus and asialoglycoprotein receptor on hepatocytes may be indispensable for viral binding and entry. *J Viral Hepat* 2006;13:11-18.
10. Yang J, Bo XC, Ding XR, Dai JM, Zhang ML, Wang XH, et al. Antisense oligonucleotides targeted against asialoglycoprotein receptor 1 block human hepatitis B virus replication. *J Viral Hepat* 2006;13:158-165.
11. Mabit H, Vons C, Dubanchet S, Capel F, Franco D, Petit MA. Primary cultured normal human hepatocytes for hepatitis B virus receptor studies. *J Hepatol* 1996;24:403-412.
12. Walter E, Keist R, Niederöst B, Pult I, Blum HE. Hepatitis B virus infection of tupaia hepatocytes in vitro and in vivo. *HEPATOLOGY* 1996;24:1-5.
13. Köck J, Nassal M, MacNelly S, Baumert TF, Blum HE, Weizsäcker von F. Efficient infection of primary tupaia hepatocytes with purified human and woolly monkey hepatitis B virus. *J Virol* 2001;75:5084-5089.
14. Gripon P, Rumin S, Urban S, Le Seyec J, Glaire D, Cannie I, et al. Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci U S A* 2002;99:15655-15660.
15. Najimi M, Khuu DN, Lysy PA, Jazouli N, Abarca J, Sempoux C, et al. Adult-derived human liver mesenchymal-like cells as a potential progenitor reservoir of hepatocytes? *Cell Transplant* 2007;16:717-728.
16. Campard D, Lysy PA, Najimi M, Sokal EM. Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. *Gastroenterology* 2008;134:833-848.
17. Lysy PA, Smets F, Sibille C, Najimi M, Sokal EM. Human skin fibroblasts: From mesodermal to hepatocyte-like differentiation. *HEPATOLOGY* 2007;46:1574-1585.
18. Sokal EM, Smets F, Bourgeois A, van Maldergem L, Buts J-P, Reding R, et al. Hepatocyte transplantation in a 4-year-old girl with peroxisomal biogenesis disease: technique, safety, and metabolic follow-up. *Transplantation* 2003;76:735-738.
19. Ladner SK, Otto MJ, Barker CS, Zaifert K, Wang GH, Guo JT, et al. 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* 1997;41:1715-1720.
20. Gripon P, Diot C, Guguen-Guillouzo C. Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. *Virology* 1993;192:534-540.
21. Saunier B, Triyatni M, Ulianich L, Maruvada P, Yen P, Kohn LD. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. *J Virol* 2003;77:546-559.
22. Offensperger WB, Offensperger S, Walter E, Blum HE, Gerok W. Suramin prevents duck hepatitis B virus infection in vivo. *Antimicrob Agents Chemother* 1993;37:1539-1542.
23. Schulze A, Gripon P, Urban S. Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans. *HEPATOLOGY* 2007;46:1759-1768.
24. Werle-Lapostolle B, Bowden S, Locarnini S, Würsthorst K, Petersen J, Lau G, et al. Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004;126:1750-1758.
25. Ying C, de Clercq E, Nicholson W, Furman P, Neyts J. Inhibition of the replication of the DNA polymerase M550V mutation variant of human hepatitis B virus by adefovir, tenofovir, L-FMAU, DAPD, penciclovir and lobucavir. *J Viral Hepat* 2000;7:161-165.
26. Leistner CM, Gruen-Bernhard S, Glebe D. Role of glycosaminoglycans for binding and infection of hepatitis B virus. *Cell Microbiol* 2008;10:122-133.
27. Spiess M. The asialoglycoprotein receptor: a model for endocytic transport receptors. *Biochemistry* 1990;29:10009-10018.
28. Rong Q, Zhang L, Su E, Li J, Liu Z, Huang Z, et al. Bone marrow-derived mesenchymal stem cells are capable of mediating hepatitis B virus infection in injured tissues. *J Viral Hepat* 2008;15:607-614.
29. Xie C, Zheng Y-B, Zhu H-P, Peng L, Gao Z-L. Human bone marrow mesenchymal stem cells are resistant to HBV infection during differentiation into hepatocytes in vivo and in vitro. *Cell Biol Int* 2009;33:493-500.
30. Elaut G, Henkens T, Papeleu P, Snykers S, Vinken M, Vanhaecke T, et al. Molecular mechanisms underlying the dedifferentiation process of isolated hepatocytes and their cultures. *Curr Drug Metab* 2006;7:629-660.
31. Schulze A, Mills K, Weiss TS, Urban S. Hepatocyte polarization is essential for the productive entry of the hepatitis B virus. *HEPATOLOGY* 2012;55:373-383.
32. Basma H, Soto-Gutiérrez A, Yannam GR, Liu L, Ito R, Yamamoto T, et al. Differentiation and transplantation of human embryonic stem cell-derived hepatocytes. *Gastroenterology* 2009;136:990-999.
33. Ochiya T, Tsurimoto T, Ueda K, Okubo K, Shiozawa M, Matsubara K. An in vitro system for infection with hepatitis B virus that uses primary human fetal hepatocytes. *Proc Natl Acad Sci U S A* 1989;86:1875-1879.