INSTITUTE OF BIOCHEMISTRY OF THE ROMANIAN ACADEMY

PhD THESIS SUMMARY

MECHANISM OF HEPATITIS B VIRUS PROTEINS DEGRADATION

SCIENTIFIC COORDINATOR:

DR. ȘTEFANA MARIA PETRESCU

PhD CANDIDATE:

UȚĂ MIHAELA ECATERINA

BUCHAREST

2018

Content

The purpose of the studies

Chronic hepatitis B virus (HBV) infection remain the primary risk factor for hepatocellular carcinoma (HCC), although an effective 95% HBV vaccine is available since 1982. Current antiviral treatments, inhibitors of viral polymerase or immunomodulators may delay progression of the disease and reduce HCC mortality. However, eradication of HBV infection is impossible because existing therapies do not target covalently closed circular DNA (cccDNA), the nuclear form of the viral genome responsible for virus persistence, latency and reactivation.

Viral surface glycoproteins are targets of the cell mediated immune response and must be degraded to be presented at the cell surface by the major histocompatibility complex I (CMHI) (Cascio *et al.*, 2001). A weak immune response against viral epitopes exposed to the surface of infected hepatocytes and the accumulation of cccDNA in the host cell nucleus are the main factors leading to chronic HBV infection. The complex process by which the synthesis and persistence of cccDNA it is realized in the infected cells is not fully understood. In an attempt to identify viral factors that can maintain the persistent infection, several *in vitro* studies suggest that viral surface proteins play an important role in regulating cccDNA synthesis (Summers, Smith and Horwich, 1990; Guo *et al.*, 2007; Lentz and Loeb, 2011). However, the lack of a natural and robust HBV infection system makes it difficult to determine the molecular pathways by which cccDNA formation and amplification is achieved. To address this issue, a thorough knowledge of the complex interactions between the virus and the host cell that lead to persistent infections is required. Better understanding of viral and cellular molecular mechanisms can contribute to the development of an effective antiviral therapy to eliminate chronic HBV infection.

The purpose of this paper is to study the role of ER-associated degradation (ERAD) pathway components in interaction with HBV surface proteins and the functional consequences of the virus and infected host cell.

This work aimed to: 1) develop and characterize *in vitro* cellular models susceptible and permissive to HBV infection for investigation of cellular and viral factors involved in the viral life cycle; 2) the identification and characterization of molecular mechanisms of HBV infection and activation of the ERAD pathway; 3) assess newly developed cellular models to identify possible HBV therapeutic strategies; 4) obtain an *in vitro* cell model in order to monitor cell differentiation in hepatocytes in real-time.

Chapter I. Hepatitis B Virus

HBV is a virus with a lipid envelope consisting of three surface proteins surrounding the nucleocapsid and the viral genome (figure 1). In fulminant infection with HBV, in addition to infectious particles, non-infectious viral particles are also presented as filamentous or spherical particles. These non-infectious particles (abbreviated SVP, from "**s**ub**v**iral **p**articles") are made up of viral surface proteins without nucleocapsid and viral DNA which during a natural HBV infection have a content up to 1,000-100,000 times higher compared to the number of detected infectious particles (Ganem and Prince, 2004; Chai *et al.*, 2008). The viral genome consists of a 3.2 kbp (kbp) double-stranded (dc) relaxed circular (rc) DNA with a complete negative strand and an incomplete positive strand. The viral genome encodes four overlapping open reading frames (abbreviated ORF, from the "**O**pen **R**eading **F**rame") important in the translation of viral proteins (Locarnini and Zoulim, 2010).

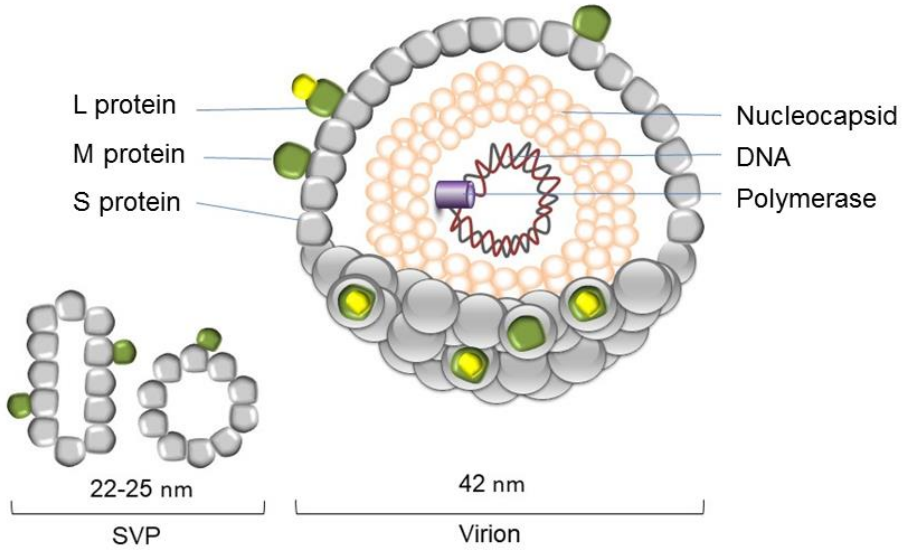


Figure 1 Schematic image of the infectious particle (virion) and non-infectious particles (SVP) of HBV. In the right part of the image, the 42 nm virion is represented consisting of a nucleocapsid containing the DNA and an envelope with three surface proteins S, M and L. To the left the two types of subviral particles, spheres and filaments, are represented.

Specific interaction of HBV with a functional receptor has been reported by Yan and colleagues who have identified a bile acid transport protein (abbreviated NTCP, from sodium-dependent Na + taurocholate co-transporting polypeptides) that specifically interact with a short fragment (3-77 aa) from the pre-S1 domain of the L protein (Yan *et al.*, 2013). One of the proposed mechanisms of receptor(s)-mediated entry of HBV through the plasma membrane is caveole/clatrine-dependent endocytosis (Macovei *et al.*, 2010; Huang *et al.*, 2012). According to this, HBV fuses at an endosomal compartment where surface proteins are processed for nucleocapsid release and nuclear transport (Watashi *et al.*, 2014). The nucleocapsid is "arrested" in the nuclear compartment and disassembled for the release of the HBV genome (Kann, Schmitz and Rabe, 2007). The biochemical process of cccDNA "repair" from rcDNA is mediated by cellular enzymes (Gao and Hu, 2007; Nassal, 2015). This replicative intermediate is organized into a minicromosome associated with histone proteins (15-16 nucleosomes of histones H2A, H2B, H3, H4 and a H1 linker) and non-histone proteins (viral proteins C and X), these epigenetic changes having an important contribution to the regulation of HBV replication (Pollicino *et al.*, 2006; Nassal, 2015). The cccDNA molecule (3,2-kpb) is transcribed by RNA polymerase II from the host cell to produce two classes of viral transcripts, genomic and subgenomic transcripts required for viral protein synthesis and VHB replication (Seeger and Mason, 2000). The transcription of the four open reading frames preC/C, P, S and X existing in the HBV genome is regulated by four different promoters (C, preS1, preS2 / S and X), two enhancers (Enh1 and Enh2) and encapsidation and polyadenylation signals (Seeger and Mason, 2000; Quasdorff and Protzer, 2010). Pregenomic RNA (pgRNA) resulting from initiation of transcription of the preC/C ORF plays a double role, functions both as mRNA encoding for structural proteins, capsid proteins (core or C protein) and viral polymerase (P protein), for a non-structural protein (HBe antigen) as well as a template for reverse transcription of the viral DNA negative strand. The preS1, S and X subgenomic transcripts function exclusively as mRNA encoding for surface protein (L, M, S) and protein X (Glebe and Bremer, 2013). The reverse transcription process of pgRNA is initiated simultaneously with the encapsidation process by P protein that interacts with a stem loop (ε) structure at the 5' end of the pregenomic RNA (Beck, Bartos and Nassal, 1997; Nassal, 2015). Concurrently with negative strand synthesis, pgRNA is degraded by the RNase H activity of viral polymerase (P) except for a short sequence (17 nucleotide RNAs) from the 5 'end of the pgRNA that will serve as a primer for the synthesis of the positive strand (Wang and Seeger, 1992; Jones and Hu, 2013). After the positive strand elongation, the mature capsid will contain DNA. These viral capsids will be transported to the nucleus or will be enveloped and exported to the extracellular environment.

Chapter II. HBV induces UPR, ERAD and autophagy

During a productive viral infection, the normal function of RE is disturbed by the synthesis of a large amount of viral proteins that must be monitored by protein quality control mechanisms. In order to counteract RE-related stress, cells induce the activation of an "Unfolding Protein Response" mechanism (abbreviated UPR) involving multiple signaling pathways that increase the capacity of folding proteins, blocks protein translation and degrades misfolded proteins (Lazar, Uta and Nichita, 2014). ERAD is associated at the functional level with the UPR to extract from the RE the misfolded proteins into the cytosol for proteasome degradation. Several studies suggest that viral infections can modulate UPR to initiate replication and viral persistence in infected cells (Urano *et al.*, 2000; Li *et al.*, 2007). HBV surface proteins activate UPR leading to the autophagy process, which is involved in nucleocapsid envelope and viral particle secretion. Activation of IRE1 pathways results in overexpression of EDEM proteins, reducing the level of viral surface proteins, which may contribute to persistence and chronic infection with HBV. Recent evidence gathered clearly demonstrates that HBV induces stress at RE by activating UPR, ERAD and autophagy, but the consequences of this activation on both the host cell and the viral life cycle require further investigations.

Chapter III: Materials and Methods

Stable cell lines HepaRG and Huh7 with constitutive or inducible expression of EDEM proteins were generated and characterized functionally and biochemically. The viral life cycle was investigated in the HepaRG^{EDEM3} cell line that overexpresses the EDEM3 protein and the HepaRG^{C} control cell line that expresses an endogenous level of EDEM proteins. HBV-infected cell lines were analyzed by transcripts, nucleocapsids and viral proteins quantification. During infection, time course experiments on the accumulation of cccDNA were realized in the presence or absence of kifunensine, a potent inhibitor of mannosidases.

For a real-time monitoring of cell differentiation, a new HepaRG^{DsRed} cell line was developed that expresses a fluorescence reporter gene under the control of a hepatocyte-specific promoter. Next, we used a gradient microstructured polydimethylsiloxane (PDMS) polymer to allow in-situ three-dimensional manipulation and monitoring the process of differentiation of HepaRG^{DsRed} cells in real time.

Chapter IV. Results and discussions

Study of HBV infection in EDEM modified cell lines

In a first approach, we analyzed the possible effects in establishing a productive infection of EDEM proteins on HBV surface proteins. A significant increase in viral transcriptions and nucleocapsids (figure 2A and B) was observed in EDEM3- overexpressing cells, correlated with accumulation of cccDNA (figure 3) and viral secretion.

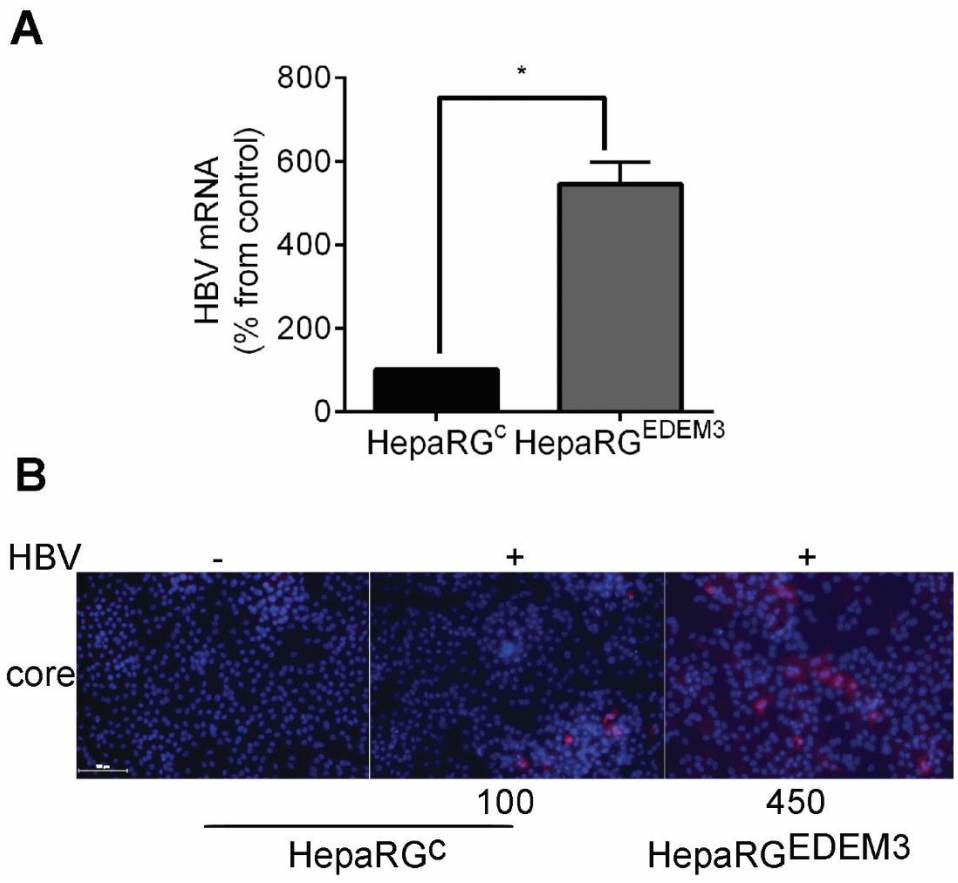


Figura 2. HBV infection in EDEM3-overexpressing HepaRG cells. (A) Total mRNA was purified from HBV-infected HepaRG^{EDEM3} and HepaRG^{C} cells at day 14 post-infection and quantified by reverse transcription real-time-PCR. The results from three independent experiments were normalized to GAPDH values and analyzed by the unpaired two-tailed Student t test (*, $P < 0.05$). (B) The level of core proteins was investigated in HBV-infected HepaRG^{EDEM3} and HepaRG^{C} cells by immunofluorescence microscopy, at day 14 pi. Images were taken with a Zeiss AxioImager.Z1 inverted microscope.

Quantification of core-positive cells was performed using the TissueQuest Cell Analysis software v4.0; the values obtained are shown below the panel. Scale bar is 50 µm.

To investigate the cccDNA accumulation in EDEM3-overexpressing cells in more detail, HepaRG^{EDEM3} and HepaRG^{C} cells were subjected to differentiation, followed by HBV inoculation and periodical monitoring of productive infection. The quantitative PCR indicated an important increase of the cccDNA copy numbers in EDEM3-overexpressing cells, before day 4 post-infection (figure 4A) and confirmed by the semi-quantitative cccDNA analysis (figure 4B). Interestingly, once reaching this level, the cccDNA form remained stable over the course of infection. This strongly suggests that events occurring at an incipient phase of infection are crucial for the cccDNA accumulation observed. In agreement with the strong cccDNA accumulation in the presence of EDEM3, secretion of both, SVPs and enveloped virions was significantly increased during infection in the same cells, confirming the important effect of EDEM3 in the HBV life-cycle observed previously (figure 4C and D).

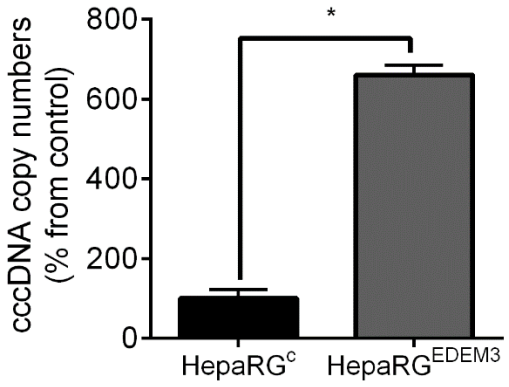


Figura 3. HBV cccDNA formation in EDEM3-overexpressing HepaRG cells. Hirt extraction of protein-free viral DNA from HBV-infected HepaRG^{EDEM3} and HepaRG^{C} cells was performed at day 14 pi. The DNA samples were quantified by real-time PCR using cccDNA-specific primers. Results from three independent experiments were analyzed using the unpaired two-tailed Student t test (*, $P < 0.05$).

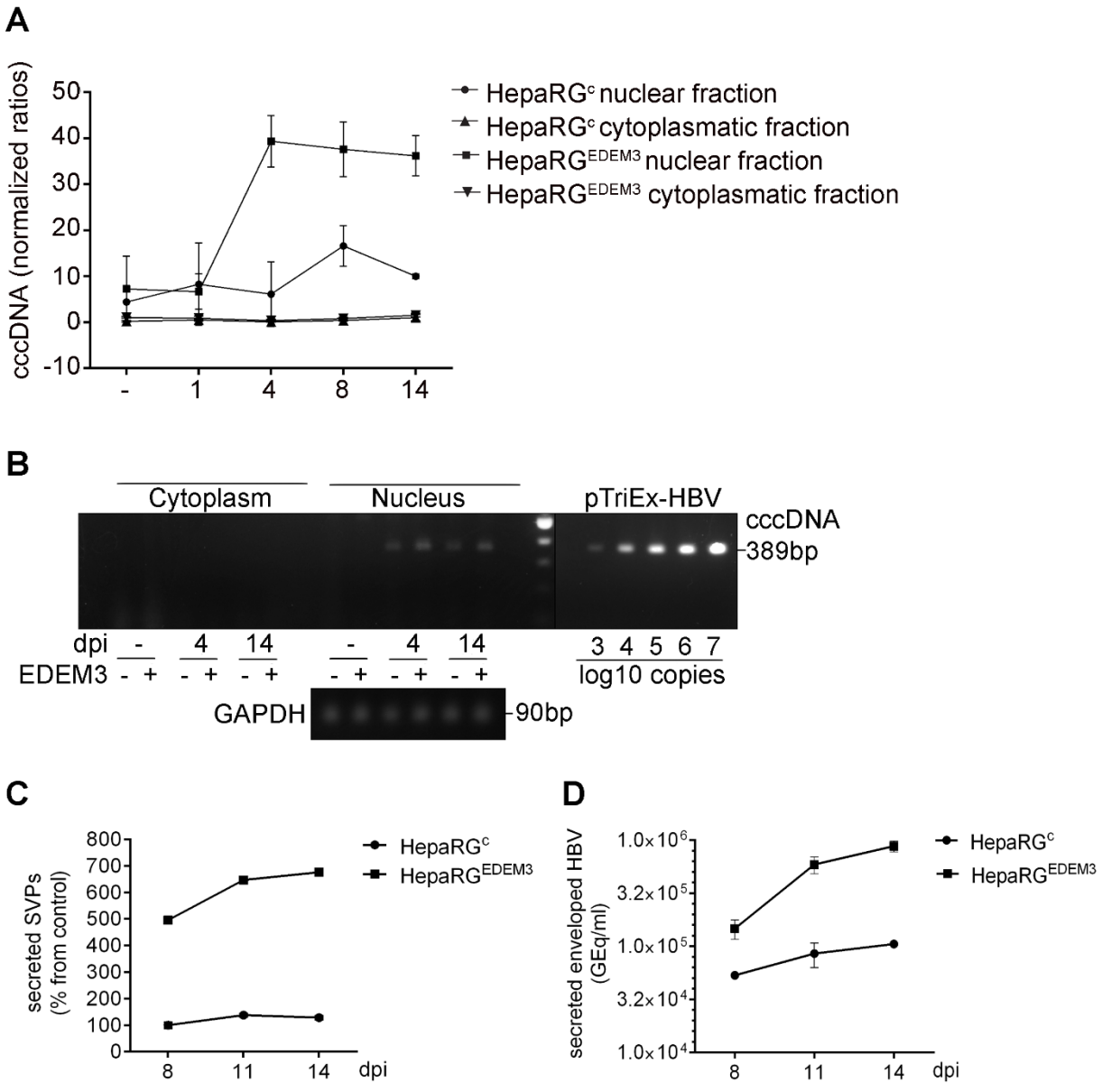


Figura 4. Kinetics of HBV infection in EDEM3 overexpressing HepaRG cells (A-D). Cytoplasmic and nuclear fractions were isolated from HBV-infected HepaRG^{EDEM3} and HepaRG^{C} cells at different points pi, as indicated in the figure. (A) HBV cccDNA formation was analyzed by real-time PCR or (B) semi-quantitative PCR, using GAPDH expression as control and serial dilutions known amounts of pTriex-HBV as standard curve (b). Secretion of SVPs (C) and enveloped virions (D) was determined by ELISA and immunoprecipitation followed by real-time PCR, respectively. The results represent the data from two independent experiments.

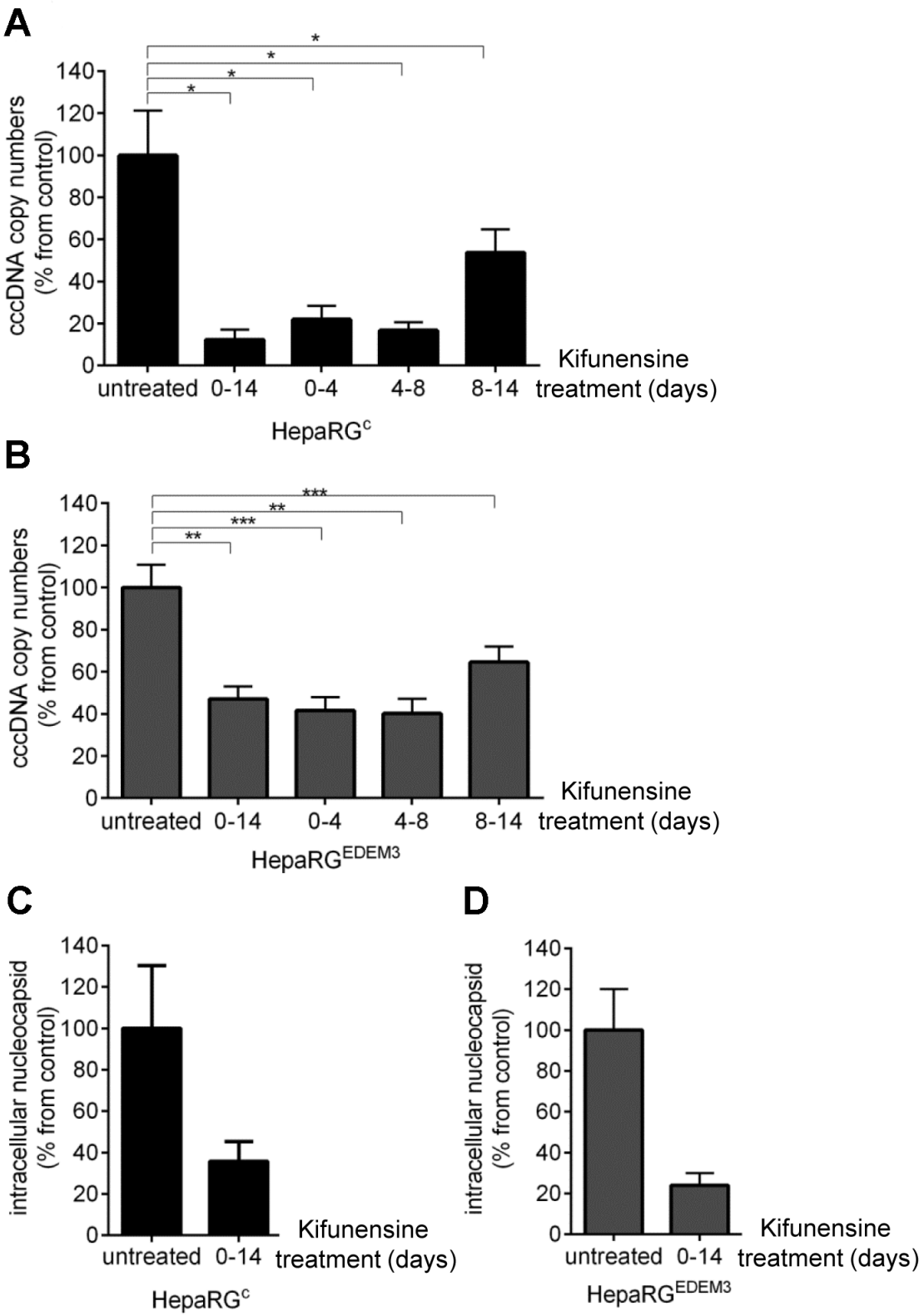


Figura 5. HBV infection in the presence of mannosidase inhibitors. HepaRG^{C} (A, C) and HepaRG^{EDEM3} (B, D) cells were infected with HBV and treated with 20 μM kifunensine for the times indicated, or

maintained untreated, as controls. Cells were collected at 14dpi. (A, B) Protein-free viral DNA was extracted from cells using the Hirt method. The DNA samples were quantified by real-time PCR using cccDNA-specific primers. Results from three independent experiments were analyzed using the unpaired two-tailed Student t test (**, P < 0.05; **, P < 0.01; ***, P <0,001*). (C, D) HBV nucleocapsids were purified from infected cells and the viral DNA was quantified by real-time PCR. The results represent the data and standard deviations from two independent experiments.

The accumulation of cccDNA was significantly inhibited by kifunensine treatment in the early stages of viral infection. An 80% reduction in the number of copies of the cccDNA in the control cell line and 60% in the EDEM3-overexpressing cell line versus untreated cells was observed (figures 5A and B). This effect was confirmed by quantification of HBV nucleocapsids in both cell lines (figures 5C and D). Treatment of cells with kifunensine in the 8-14 dpi inhibits less efficiently the accumulation of cccDNA, suggesting that the cccDNA formation process in the early stages of infection depends on α1,2-mannosidase activity.

Development of a DsRed-expressing HepaRG cell line for real-time monitoring of hepatocyte-like cell differentiation by fluorescence imaging, with application in screening of novel geometric microstructured cell growth substrates

In the second study, we obtained an *in vitro* cellular model (figure 6A and B) that allowed the controlled assembly of viable hepatocyte-like cells for functional studies that can be maintained in culture without loss of phenotype. Real-time monitoring of the HepaRG^{DsRed} cell line allowed the identification of topographic parameters that influence cell differentiation.

The intensity of fluorescence of HepaRG^{DsRed} cells seeded on microstructured PDMS was investigated by TissueFAXS analysis. This analysis indicates a preferential adhesion, cell spreading and clustering depend on both the structure of the topography (lines, pyramids, semispheres, cones) and the concave or convex characteristics of topography compared to unstructured PDMS or other standard substrates used (figure 7A). By microscopic analysis, the relationship between different topography and cell morphology was observed and some topographic forms were found to influence cell growth behavior. Reversed U lines structures (ABC/456) and V grooves (ABC/789) significantly favored cellular differentiation in comparison to the different controls in a diameter-dependent manner (figure 7B). This result suggests that a topography with a diameter larger than the size of a cell (approximately 15-20 µm) favors cell adhesion and cell activation by differentiation. On the other hand, smaller diameters can favor

cellular mobility if narrow geometry prevents cells from efficiently connecting to each other. Cells grown on reversed pyramidal structures with a depth of 5 µm and a diameter of 5, 10 and 25 µm (DEF/456) expressed an increase fluorescence intensity as the diameter decreases (figure 7B). In this case, the smaller diameters of reversed pyramids appear to support attachment of cells and junctions between cells over the edges of the structure. Larger diameters of reversed pyramids can induce cell capture within structures, thus reducing intercellular connectivity.

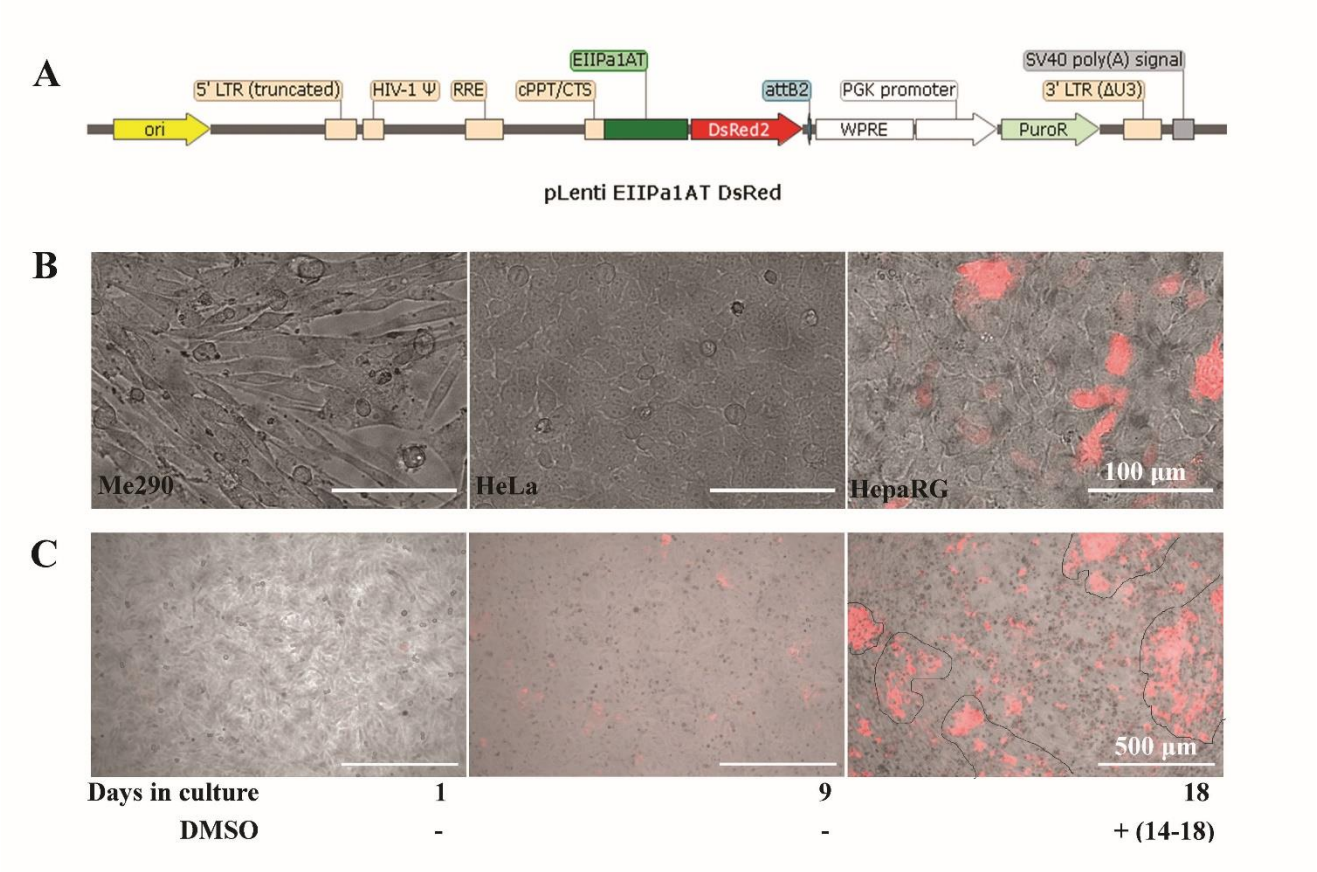


Figura 6. Generation of the HepaRG^{DsRed} stable cell line. (A) Schematic representation of the pLenti EIIPa1AT-DsRed construct used for transduction of HepaRG and control cells. (B) Me290, HeLa and HepaRG cells were transduced with pLenti EIIPa1AT-DsRed. Cells at 10 days post-transduction were scanned by TissueFAXS and representative images were taken with a 20x objective. Overlapped brightfield/TxRed images are shown. (C) HepaRGDsRed cells were subjected to differentiation for 18 days, in the absence (−), or the presence of 1.8% DMSO for the last 4 days (+). The same area of cells was monitored for changes in fluorescence during the course of differentiation and images were taken as above, using a 5x objective. Hepatocyte-like cell clusters are shown in surrounded areas.

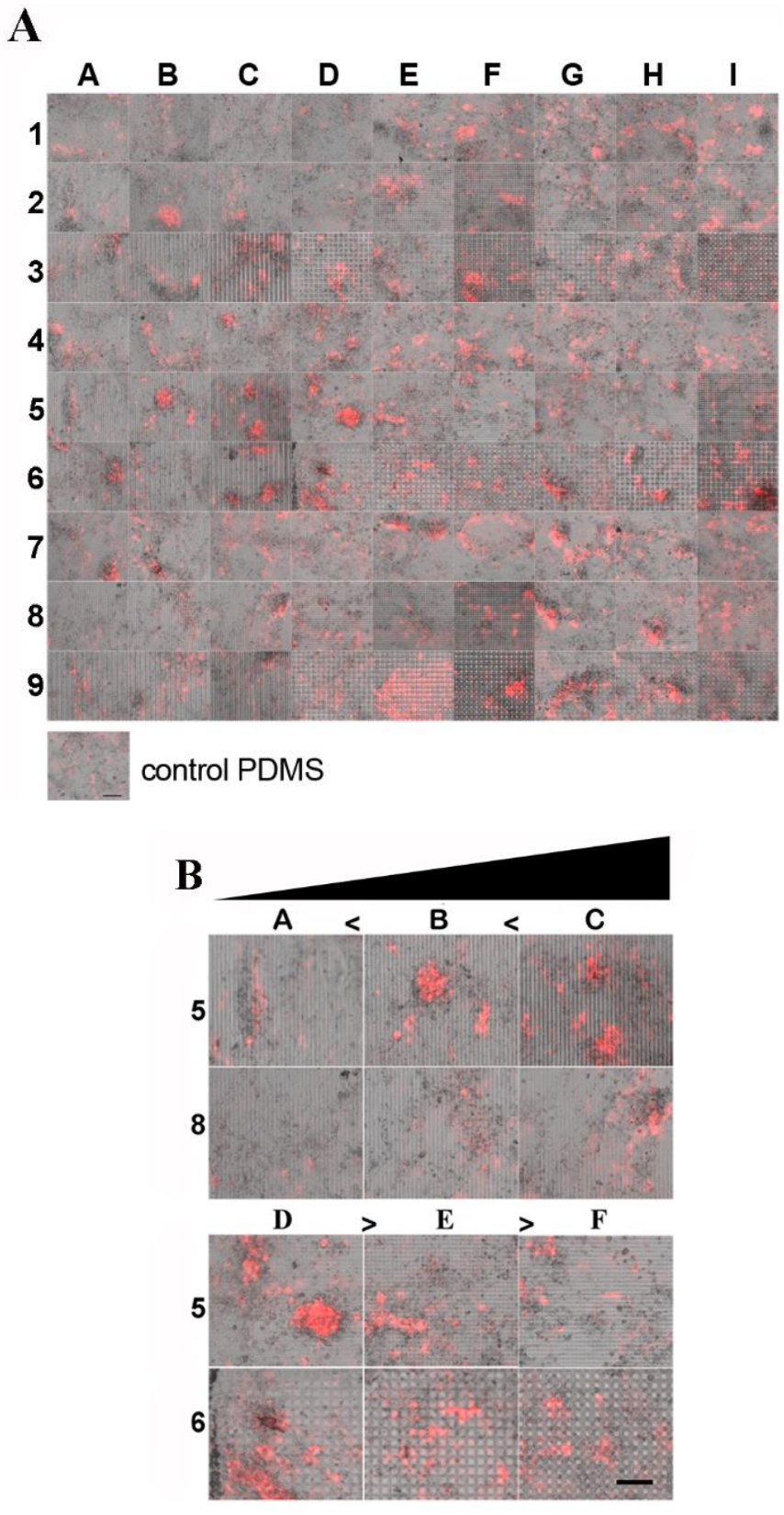


Figura 7. PDMS chip topography influences cell differentiation. (A) HepaRG^{DsRed} cells were seeded onto the microstructured substrate and grown for up to 25 days in the absence of DMSO with periodic

monitoring of cell fluorescence levels. (B) Overlapped images of representative brightfield and TxRed channel acquisitions captured by TissueFAXS automatic scanning and fields of stitching view of HepaRG^{DsRed} cells at day 14 after seeding are depicted. Black triangles indicate the gradients of the geometric pattern. Scale bar is 50 µm.

Conclusions and perspectives

In the present study we have characterized the molecular aspects of HBV infection by developing i*n vitro* cellular models. To study host-pathogen interactions, the expression of some proteins of interest was modulated in the HepaRG cell line permissive for HBV infection. The impact of these target proteins on HBV was highlighted by the analysis of each stage of the viral cycle.

The general conclusions in these studies are:

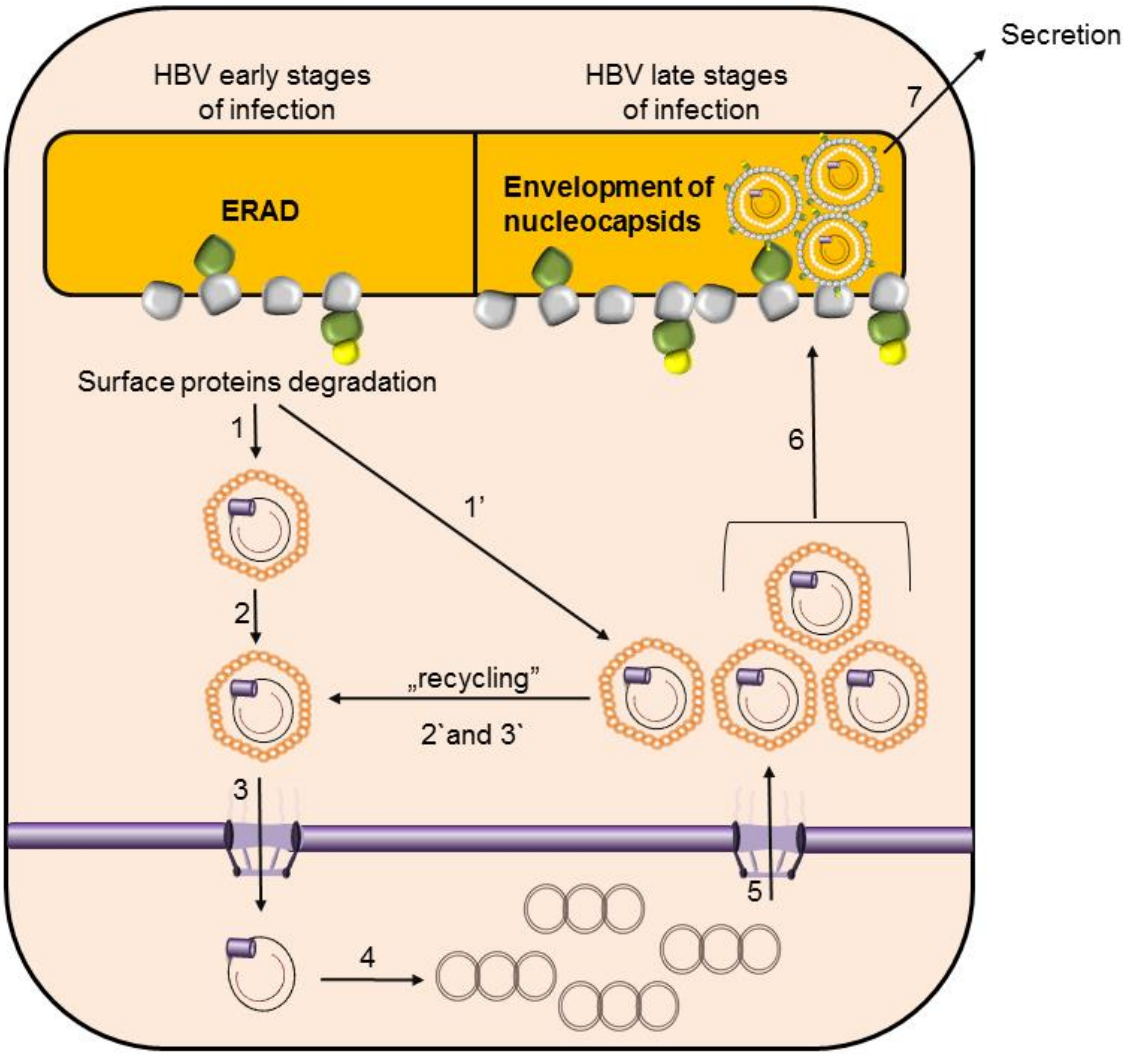
1. HepaRG cells expressing stable EDEM3 represent a robust and reproducible system that allowed the analysis of the first steps of the HBV life cycle. Significant degradation in these cells led to increased accumulation of transcriptions, nucleocapsids and viral proteins.
2. Degradation of EDEM-induced HBV surface proteins, irrespective of their folding stage, is an important cellular mechanism involved in cccDNA formation immediately after virus internalization, which can ensure the persistence of the infection. This hypothesis is supported by the reversal of this effect, by inhibiting degradation, thus identifying a potential antiviral agent in targeting cccDNA.
3. Real time monitoring of HepaRG^{DsRed} transgenic cell line has proven to be an extremely useful tool in identifying materials capable of inducing more efficient differentiation with a potential role in biomedical applications.

The study allowed:

1. To develop a model by which EDEM proteins could regulate HBV infection.

The proposed mechanism is the following: in the early stages of HBV infection, the level of surface proteins being low, a large part of these are removed from RE immediately after synthesis (1), thus favoring mature nucleocapsid trafficking and nuclear "recycling", (2) allowing formation of cccDNA molecules (3) ensuring the persistence of

the infection. When cccDNA molecules are amplified to a sufficient level (5), intracellular nucleocapsides will be enveloped (6) and secreted as infectious virions (7). In these late stages of infection, protein degradation can be alleviated or counterbalanced by increased surface protein synthesis as a result of a large number of cccDNA, so that the virus and production of SVPs are no longer affected.



2. Proposal for new therapeutic strategies for HBV infection

Treatment with kifunensin at the early stages of the infection resulted in significant inhibition of cccDNA formation. It is important to note that inhibitors of other RE-resident enzymes, such as α-glucosidases I and II, induce protein degradation, and their antiviral

activity on viruses, including HBV, is well known (Simsek et al., 2005). However, such inhibitors induce protein degradation by a different mechanism involving incorrect protein folding, which in the case of HBV is largely limited by the M protein, being the most dependent on the lectin chaperon proteins of RE for the correct folding of the three surface proteins (Prange, Werr and Löffler-Mary, 1999). This has consequences on the later stages of the viral life cycle, such as assembling and viral trafficking, leading to a significant reduction in HBV secretion and the formation of less infectious virions (Prange, Werr and Löffler-Mary, 1999). In a future perspective, studies that will address the effectiveness of other mannosidase inhibitors and their derivatives to block HBV infection are needed.

3. The identification of new PDMS based substrates that could accelerate the cell differentiation process.

   This paper, proposed the use of a hepatocytes specific fluorescent reporter in order to identify new cell substrates capable of increasing the efficiency of differentiation of HepaRG cells. This analysis was performed in real time by continuous monitoring of fluorescence levels in living cells, being an important advantage over other systems such as luciferase or β-galactosidase, which require cell lysis and enzyme activity measurement to determine the level of gene expression. Based on these properties, various PDMS microstructures that clearly favor cell clustering and differentiation into hepatocytes have been identified. Future studies will address larger-scale manufacture of these substrates to investigate the differentiation process at the molecular level.

List of publications

This PhD thesis is based on the following published studies:

I. Cătălin Lazăr, **Mihaela Uță** și Norica Brânză-Nichita. (2014) „Modulation of the unfolded protein response by the human hepatitis B virus", Frontiers in Microbiology, 5(AUG), pp. 1-9. doi: 10.3389/fmicb.2014.00433.

II. Cătălin Lazăr, **Mihaela Uță,** Ștefana Petrescu și Norica Brânză-Nichita. (2017) „Novel function of the endoplasmic reticulum degradation-enhancing α-mannosidase-like proteins in the human hepatitis B virus life cycle, mediated by the middle envelope protein", Cellular Microbiology, 19(2). doi: 10.1111/cmi.12653.

III. **Mihaela Uţă,** Livia Sima, Patrik Hoffmann, Valentina Dincă și Norica Brânză-Nichita. (2017) „Development of a DsRed-expressing HepaRG cell line for real-time monitoring of hepatocyte-like cell differentiation by fluorescence imaging, with application in screening of novel geometric microstructured cell growth substrates", Biomedical Microdevices. doi: 10.1007/s10544-016-0146-z.

IV. **Mihaela Uţă,** Cătălin Lazăr, Gabriela Chiriţoiu, Ștefana Petrescu și Norica Brânză-Nichita. "The Endoplasmic Reticulum-Associated Degradation pathway regulates formation of Hepatitis B virus covalently closed circular DNA in HepaRG cells", submitted to PlosOne.

No of citations (Scopus): 19
***h*-index: 2**

Poster presentations:

1. **Mihaela Uţă**, Cătălin Lazăr, Ştefana Petrescu, Norica Brânză-Nichita, "ER associated degradation of HBV surface protein", The IXth „Academician Nicolae Cajal" Symposium, Bucharest, 13-14 May 2014.
2. **Mihaela Uţă,** Cătălin Lazăr**,** Ştefana Petrescu, Norica Brânză-Nichita, "Mechanism of Hepatitis B Virus proteins degradation", The Annual International Conference of the Romanian Society of Biochemistry and Molecular Biology, Băile Felix, Oradea, 5-6 June 2014.
3. **Mihaela Uţă**, Cătălin Lazăr, Ştefana Petrescu, Norica Brânză-Nichita, "Calea de degradare a proteinelor de anvelopă ale virusului hepatitei B (VHB)", SIMPOZION ACADEMICIAN NICOLAE CAJAL - ediția a X-a -, București 1-4 Aprilie 2015.
4. **Mihaela Uţă**, Cătălin Lazăr**,** Ştefana Petrescu, Norica Brânză-Nichita, "The role of ERAD pathway in HBV life cycle", International Meeting on Molecular Biology of Hepatitis B Viruses, Bad Nauheim, Germany, 4-8 October 2015.

Acknowledgements

The realization of this work was possible with the help of several people, whom I would like to thank for their generous support throughout the years.

I would like to express my gratitude to Dr. Ştefana Petrescu and Norica Brânză-Nichita for giving me the opportunity to do my PhD studies by addressing such an interesting topic. Thank you very much for all the encouragement, sharing of knowledge and the scientific advice that helped me in the experimental approach.

It has always been a great pleasure to work with my colleagues at the Institute of Biochemistry and I would like to thank you very much for your help, support and very productive discussions. I would like to thank my current and former colleagues from the Viral Glycoprotein department, Dr. Cătălin Lazăr, Dr. Alina Macovei and Dr. Costin Ioan Popescu for the technical guidance and assistance they have given me at the beginning of my PhD.

I would like to thank my colleague, Dr Gabriela Chiriţoiu, for obtaining retroviral stocks containing cDNA over EDEM proteins. I would like to address special thanks to Dr. Livia Sima for the technical support offered in cell sorting and TissueFAXS data analysis. I would also like to thank Dr. Simona Ghenea for assisting with the purchase of microscopic images.

I would like to thank Dr. Valentina Dincă and Prof. Dr. Patrik Hoffmann for the preparation and characterization of PDMS microchips.

Finally, I thank and I am deeply grateful to my family and friends for understanding and permanent moral support.

References

Beck, J., Bartos, H. și Nassal, M. (1997) „Experimental Confirmation of a Hepatitis B Virus (HBV) ϵ-like Bulge-and-Loop Structure in Avian HBV RNA Encapsidation Signals", *Virology*, 227(2), pp. 500-504. doi: http://dx.doi.org/10.1006/viro.1996.8329.

Cascio, P. *et al.* (2001) „26S proteasomes and immunoproteasomes produce mainly N-extended versions of an antigenic peptide", *The EMBO Journal*. Oxford, UK: Oxford University Press, 20(10), pp. 2357-2366. doi: 10.1093/emboj/20.10.2357.

Chai, N. *et al.* (2008) „Properties of Subviral Particles of Hepatitis B Virus", *Journal of Virology*, 82(16), pp. 7812-7817. doi: 10.1128/JVI.00561-08.

Ganem, D. și Prince, A. M. (2004) „Hepatitis B Virus Infection — Natural History and Clinical Consequences", *New England Journal of Medicine*. Massachusetts Medical Society, 350(11), pp. 1118-1129. doi: 10.1056/NEJMra031087.

Gao, W. și Hu, J. (2007) „Formation of hepatitis B virus covalently closed circular DNA: removal of genome-linked protein.", *Journal of virology*, 81(12), pp. 6164-74. doi: 10.1128/JVI.02721-06.

Glebe, D. și Bremer, C. M. (2013) „The molecular virology of hepatitis B virus.", *Seminars in liver disease*. United States, 33(2), pp. 103-112. doi: 10.1055/s-0033-1345717.

Guo, H. *et al.* (2007) „Characterization of the Intracellular Deproteinized Relaxed Circular DNA of Hepatitis B Virus: an Intermediate of Covalently Closed Circular DNA Formation ", *Journal of Virology*. American Society for Microbiology (ASM), 81(22), pp. 12472-12484. doi: 10.1128/JVI.01123-07.

Huang, H.-C. *et al.* (2012) „Entry of hepatitis B virus into immortalized human primary hepatocytes by clathrin-dependent endocytosis.", *Journal of virology*, 86(17), pp. 9443-53. doi: 10.1128/JVI.00873-12.

Jones, S. A. și Hu, J. (2013) „Hepatitis B virus reverse transcriptase: diverse functions as classical and emerging targets for antiviral intervention", *Emerging Microbes & Infections*. Nature Publishing Group, 2(9), p. e56. doi: 10.1038/emi.2013.56.

Kann, M., Schmitz, A. și Rabe, B. (2007) „Intracellular transport of hepatitis B virus.", *World journal of gastroenterology : WJG*, 13(1), pp. 39-47. doi: 10.1016/j.jhep.2016.02.008.

Lentz, T. B. și Loeb, D. D. (2011) „Roles of the Envelope Proteins in the Amplification of Covalently Closed Circular DNA and Completion of Synthesis of the Plus-Strand DNA in Hepatitis B Virus ", *Journal of Virology*. 1752 N St., N.W., Washington, DC: American Society for Microbiology, 85(22), pp. 11916-11927. doi: 10.1128/JVI.05373-11.

Locarnini, S. și Zoulim, F. (2010) „Molecular genetics of HBV infection", *Antiviral Therapy*, 15(SUPPL. 3), pp. 3-14. doi: 10.3851/IMP1619.

Macovei, A. *et al.* (2010) „Hepatitis B Virus Requires Intact Caveolin-1 Function for Productive Infection in HepaRG Cells ", *Journal of Virology*. American Society for Microbiology (ASM), 84(1), pp. 243-253. doi: 10.1128/JVI.01207-09.

Nassal, M. (2008) „Hepatitis B viruses: Reverse transcription a different way", *Virus Research*, 134(1-2), pp. 235-249. doi: 10.1016/j.virusres.2007.12.024.

Nassal, M. (2015) „HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B”, *Gut*, 64(12), pp. 1972-1984. doi: 10.1136/gutjnl-2015-309809.

Pollicino, T. *et al.* (2006) „Hepatitis B Virus Replication Is Regulated by the Acetylation Status of Hepatitis B Virus cccDNA-Bound H3 and H4 Histones”, *Gastroenterology*. Elsevier, 130(3), pp. 823-837. doi: 10.1053/j.gastro.2006.01.001.

Prange, R., Werr, M. și Löffler-Mary, H. (1999) „Chaperones Involved in Hepatitis B Virus Morphogenesis”, *Biological Chemistry*, p. 305. doi: 10.1515/BC.1999.042.

Quasdorff, M. și Protzer, U. (2010) „Control of hepatitis B virus at the level of transcription”, *Journal of Viral Hepatitis*. Blackwell Publishing Ltd, 17(8), pp. 527-536. doi: 10.1111/j.1365-2893.2010.01315.x.

Seeger, C. și Mason, W. S. (2000) „Hepatitis B Virus Biology”, *Microbiology and Molecular Biology Reviews*, 64(1), pp. 51-68. doi: 10.1128/MMBR.64.1.51-68.2000.Updated.

Simsek, E. *et al.* (2005) „Hepatitis B virus large and middle glycoproteins are degraded by a proteasome pathway in glucosidase-inhibited cells but not in cells with functional glucosidase enzyme.”, *Journal of virology*. American Society for Microbiology, 79(20), pp. 12914-20. doi: 10.1128/JVI.79.20.12914-12920.2005.

Summers, J., Smith, P. M. și Horwich, A. L. (1990) „Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification.”, *Journal of Virology*, 64(6), pp. 2819-2824. Valabil la: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC249463/.

Urban, S. *et al.* (2010) „The replication cycle of hepatitis B virus”, *Journal of Hepatology*. Elsevier, 52(2), pp. 282-284. doi: 10.1016/j.jhep.2009.10.031.

Wang, G.-H. și Seeger, C. (1992) „The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis”, *Cell*. Elsevier, 71(4), pp. 663-670. doi: 10.1016/0092-8674(92)90599-8.

Watashi, K. *et al.* (2014) „NTCP and Beyond: Opening the Door to Unveil Hepatitis B Virus Entry”, *International Journal of Molecular Sciences*. Molecular Diversity Preservation International (MDPI), 15(2), pp. 2892-2905. doi: 10.3390/ijms15022892.

Yan, H. *et al.* (2013) „Molecular Determinants of Hepatitis B and D Virus Entry Restriction in Mouse Sodium Taurocholate Cotransporting Polypeptide”, *Journal of Virology*, 87(14), pp. 7977-7991. doi: Doi 10.1128/Jvi.03540-12.