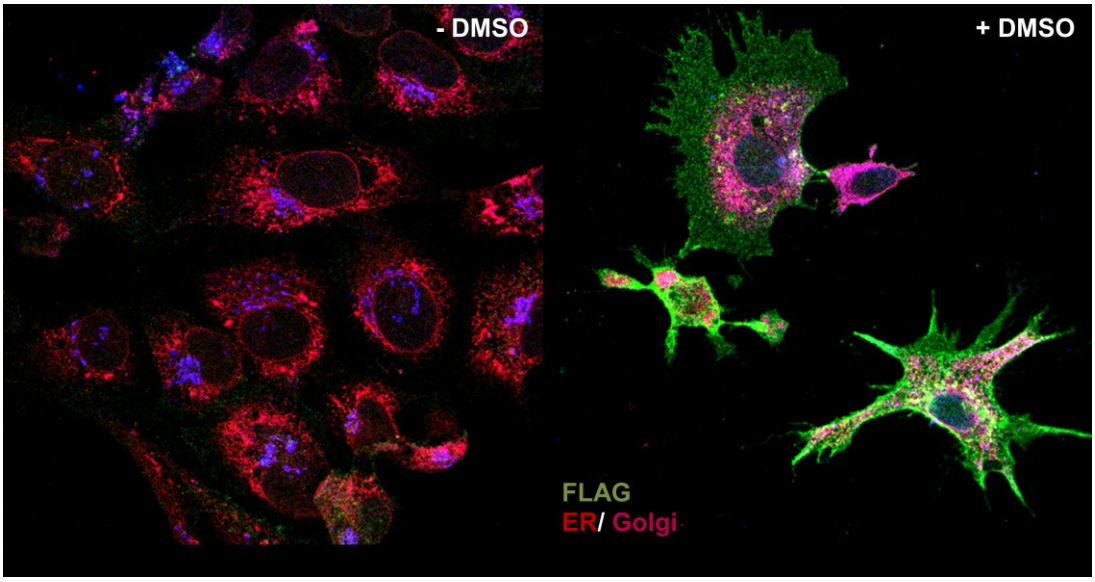
Dissection of the molecular determinants and pathways involved in Hepatitis B virus (HBV) entry into hepatocytes



A thesis submitted to Imperial College London

for the degree of PhD

by

Antonia Alexandra Evripioti

BSc (Hons), MSc, MPhill

Imperial College London

Department of Medicine

Section of Virology

St' Mary's Campus

Declaration of Originality

I, Antonia Alexandra Evripioti, hereby certify that this thesis, which is submitted for assessment as part of a PhD research project, has been written by me and is a record of work carried out by me, unless otherwise stated.



Copyright Declarations

‘The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work’.

Acknowledgements

My sincere thanks go to my primary supervisor, Dr. Marcus Dorner, and my co-supervisor, Professor Peter O'Hare. I would especially like to thank Dr. Marcus Dorner for trusting in me and offering me the funding to complete this project. Thank you for giving me this fantastic opportunity, while I am very grateful to both, Dr. Marcus Dorner and Professor Peter O'Hare, for your time, valuable support, feedback, and guidance throughout this project.

My heartfelt thanks go to Dr. Quentin Bazot for his persistent technical and experimental help and advice, particularly in regards to human NTCP protein expression. Without your assistance, constant support and encouragement this project could have possibly had a very different ending. So, thank you very much for everything.

I would like to offer my sincere thanks to Professor Paul Farrell and his group for offering me a space to work in and generally for being very welcoming and very friendly, as well as offering me experimental help and advice when needed. Sam and Paulo, thank you for all the fun and for always keeping a positive outlook on every situation.

I would also like to acknowledge everyone else from within the department (Aisling, Claudio and Gillian, amongst others), who spent time discussing with me whenever I needed some extra advice or just someone to talk to. Unfortunately, I cannot mention everyone by name but I do thank you all for offering me your expertise, it was and is highly appreciated.

Finally, my deepest thanks go to my parents, Betty and Tasos, and my partner, Hugh Cannon, for their continuous support, understanding, and encouragement. Thank you for always being there for me and for putting up with me during the good and bad times.

Abstract (295 words)

HBV entry into cells is mediated through a precisely orchestrated interaction of the pre-S1 glycoprotein domain of the virus principally with the sodium taurocholate cotransporting polypeptide (NTCP) receptor on the surface of hepatocytes. However, *in vitro* this typically relies on high multiplicities of infections, and even in this case a very small majority of the hepatocytes become infected with the virus. Here we describe a mechanism by which DMSO facilitates overexpression of human NTCP, resulting in the bulk trafficking of the receptor to the cell surface and hence enhancing susceptibility of these cells to HBV infection. This mechanism is an intricate network of intracellular interactions, with the main one being the inhibition of particular phosphodiesterases (PDEs), including PDE4, by DMSO. This outcome can hopefully now open the door for the study of new small molecule inhibitors to prevent HBV entry into hepatocytes by specifically targeting the levels of NTCP on the cells' surface.

It is highly likely, however, that HBV entry into hepatocytes additionally relies on other, yet not fully characterised, cell surface receptors or host factors. Such an example may be the asialoglycoprotein receptor (ASGPR), which our data suggests may possibly be important and play a role during HBV entry. In particular, we found that the simultaneous presence of NTCP and ASGPR enhanced entry of HBV-mimicking particles into hepatocytes, more so than when only the NTCP receptor was overexpressed on those cells.

This is the first study to have established positive HBV infection of a mouse hepatic cell line overexpressing a chimeric form of the NTCP receptor. This outcome should hopefully now open the road towards further research and understanding of the HBV interspecies transmission barrier, as well as identification of those host factors vital for productive HBV infection *in vitro* and *in vivo*.

Table of Contents

Title Page……1
Declaration of Originality....2
Copyright Declarations....2
Acknowledgements....3
Abstract (295 words)....4
Table of Contents....5
List of Figures....13
List of Tables....22
Abbreviations....24
1. Introduction....30
1.1 Viral Hepatitis....30
1.2 Hepatitis B virus (HBV)....32
1.2.1 History of HBV identification....32
1.2.2 Epidemiology of HBV, prevalence of its different serotypes and genotypes, and mode of transmission....33
1.2.3 HBV pathogenesis, clinical manifestation and persistence....37
1.2.4 Current HBV treatments....43
1.2.5 HBV taxonomy, genomic structure and organisation....44
1.2.6 HBV life cycle and replication....49
1.2.6.1 HBV entry into host cells....51
1.2.6.2 HBV replication cycle....56
1.2.7 The HBV liver-specific receptor, NTCP....62
1.2.7.1 The rat NTCP....70
1.2.8 Previously reported HBV receptors....72
1.2.9 Protein trafficking mechanisms of the predicted HBV receptors....76
1.3 Hepatitis Delta virus (HDV)....81
1.4 Current systems for studying HBV and HDV infections....87
1.4.1 *In vitro*, cell culture systems....88
1.4.1.1 HBV-mimicking pseudoparticles and recombinant virus....95
1.4.1.2 Effect of DMSO in the cell culture systems, and particularly on NTCP expression and receptor functionality....100

1.4.2 *In vivo*, animal models 103
1.5 Project Aims 110
2. Materials and Methods 112
2.1 Materials 112
2.1.1 Reagents 112
2.1.2 Solutions and Buffers 118
2.1.3 Sequencing and Cloning Primers 121
2.1.4 DNA constructs 124
2.1.5 Cell lines 127
2.1.6 Equipment 128
2.1.7 Computer Programs 129
2.2 Methods 130
2.2.1 Molecular techniques 130
2.2.1.1 General Cloning Strategy 130
2.2.1.1.1 Summary of Gateway® Cloning 131
2.2.1.1.2 Example of a cloning strategy designed for the construction of replication-competent HBV genomes encoding reporter genes 132
2.2.1.2 Restriction digest of DNA fragments 137
2.2.1.3 Agarose gel electrophoresis 138
2.2.1.4 Gel extraction of DNA fragments from agarose gels 138
2.2.1.5 Ligation of the DNA fragments of interest into the appropriate vectors and Transformation into bacteria cells 139
2.2.1.6 DNA plasmid preparation 140
2.2.1.7 DNA sequencing 140
2.2.1.8 PCR amplification 140
2.2.1.8.1 Bacteria Colony PCR 141
2.2.1.8.2 Detection of HBV cccDNA formation 142
2.2.1.9 Quantitative PCR amplification 142
2.2.1.10 Analysis of mRNA expression by qRT-PCR 144
2.2.1.11 Analysis of protein expression by Immunoblotting 146
2.2.1.11.1 Deglycosylation/ Dephosphorylation of protein samples prior to Immunoblotting 148

2.2.1.12 Analysis of protein expression by Immunofluorescence ... 148
2.2.1.13 Analysis of protein expression by Flow Cytometry ... 150
2.2.1.14 ELISA analysis ... 151
2.2.2 Cell-based techniques ... 152
2.2.2.1 General Cell Culture ... 152
2.2.2.2 Optimised cell culture for cellular differentiation and polarisation ... 152
2.2.2.3 Electroporation of cells with plasmids of interest ... 153
2.2.2.4 Transfection of cells with plasmids of interest ... 153
2.2.2.4.1 Production of lentiviral pseudoparticles ... 154
2.2.2.4.1.1 Production of lentiviral pseudoparticles expressing HBV-related receptors ... 159
2.2.2.4.1.2 Production of lentiviral pseudoparticles expressing the HBV-mimicking lentiviral particles ... 160
2.2.2.4.1.3 Production of LMN8-Huh7.5 lentiviral cDNA library, for use during the cyclic repackaging rescue screen ... 163
2.2.2.4.1.3.1 Lentiviral cyclic repackaging screening for the identification of novel host factors required for HBV entry ... 164
2.2.2.4.2 Concentration of lentiviral pseudoparticles ... 167
2.2.2.5 Transduction of cells with lentiviral pseudoparticles ... 168
2.2.2.5.1 Creation of cell lines stably overexpressing the HBV-related receptors ... 168
2.2.2.5.2 Transduction of cells overexpressing the HBV-related receptors with HBSpp ... 170
2.2.2.6 Infection of cells with infectious HBV supernatants ... 171
2.2.2.6.1 Production of infectious HBV supernatants ... 171
2.2.2.6.2 Concentration of recombinant HBV virus through an Iodixanol Gradient ... 171
2.2.2.6.3 HBV infection of cell lines stably overexpressing the HBV-related receptor, NTCP ... 172
2.2.2.6.3.1 Treatment of cells with various drugs, and their optimum concentrations ... 173

2.2.2.7 Cell viability assay 174
2.2.3 Statistics 175
3. Results 176
3.1 HBV entry into human cells 176
3.1.1 Lentiviral overexpression of human NTCP does not readily result in functional protein expression of the receptor, further impacting on cells susceptibility to HBV infection 176
3.1.1.1 Effect of lentiviral overexpression of human NTCP on the mRNA and protein levels 176
3.1.1.1.1 Effect of antibiotic selection on functional human NTCP (FLAG-tag) protein expression during the production of stable cell lines 181
3.1.1.1.2 Culturing single cell clones from the hepatic cell line overexpressing human NTCP, so as to select a stable monoclonal cell population positive for functional hNTCP protein expression 184
3.1.1.2 Effect of human NTCP overexpression on susceptibility of cells to HBV infection 187
3.1.2 DMSO differentiation of certain human cells is required for functional human NTCP protein expression and subsequent susceptibility of cells to HBV infection 189
3.1.2.1 Effect of DMEM versus William's E base media on human NTCP mRNA expression 189
3.1.2.2 Effect of DMSO differentiation on human NTCP mRNA and protein expression 191
3.1.2.2.1 Characteristics of DMSO-induced human NTCP expression and the reversibility of this effect 194
3.1.2.2.2 Effect of DMSO on the CMV promoter driving expression of genes 202
3.1.2.2.3 Effect of DMSO on human NTCP expression in other stable human hepatic cell lines 204
3.1.2.3 Glycosylation of the human NTCP receptor following induction with DMSO 206
3.1.2.4 mRNA and protein stability of the human NTCP receptor 209

3.1.2.5 Susceptibility of cells to HBV infection upon DMSO differentiation 214
3.1.3 DMSO results in human NTCP expression via cAMP-mediated restructuring of actin filaments, which further enhances susceptibility of cells to HBV 216
3.1.3.1 DMSO-induced changes in DMSO-treated cells suggest epigenetic and cytoskeletal modifications could be affecting human NTCP expression 216
3.1.3.1.1 Impact of epigenetic regulation on human NTCP expression 217
3.1.3.1.2 Impact of drugs targeting the cell's cytoskeleton on human NTCP expression 219
3.1.3.1.3 Disrupting the actin and tubulin cytoskeleton and its subsequent effect on susceptibility of cells to HBV infection 224
3.1.3.2 DMSO results in accumulation of cAMP, which in turn may drive remodelling of actin filaments 228
3.1.3.3 The second messenger cAMP increases human NTCP protein levels on the plasma membrane and thus renders cells more susceptible to HBV infection 230
3.1.4 Intracellular cAMP levels regulate the expression of human NTCP on the plasma membrane, as well as the resulting susceptibility of cells to HBV infection 238
3.1.4.1 Modulating intracellular cAMP levels by targeting PDEs or adenylyl cyclase greatly affects human NTCP expression on the plasma membrane and subsequent HBV susceptibility of cells 238
3.1.4.2 PDE 4 and 12 are important players in suppressing baseline human NTCP expression and subsequent susceptibility of cells to HBV 247
3.1.4.3 Targeting PDE4 greatly enhances human NTCP expression and subsequent susceptibility of cells to HBV infection 250

3.1.5 Characterising another HBV-related receptor; Lentiviral overexpression of human ASGPR and subsequent susceptibility of cells to HBV infection 255
3.1.5.1 Effect of lentiviral overexpression of human ASGPR on the mRNA and protein levels 255
3.1.5.2 The expression of the human ASGPR receptor may be regulated in a similar manner to the expression of the human NTCP receptor during DMSO differentiation 257
3.1.5.2.1 DMSO has a broad, but variable, effect on the expression of different receptor proteins 263
3.1.5.3 Infection of cells with HBV- & HDV- mimicking particles appears to require expression of both the human NTCP and ASGPR receptors 266
3.1.5.3.1 Generation and characterisation of HBV- & HDV-pseudotyped lentiviral particles 266
3.1.5.3.2 Effect of ASGPR overexpression on susceptibility of cells to HBV- & HDV- mimicking particles 268
3.2 HBV entry into mouse cells, and the interspecies restriction of HBV infection 272
3.2.1 Human NTCP protein expression in some mouse cell lines is regulated similarly to human cells 272
3.2.2 A mouse/human chimeric NTCP receptor renders murine cells susceptible to HBV infection 275
3.2.3 Expression of the chimeric NTCP receptor renders mice susceptible to low-level HBV infection 284
3.3 Enhancing HBV susceptibility by identifying novel host factors restricting HBV replication in low-susceptibility or altogether non-susceptible hosts, *in vitro* 289
3.3.1 Generation and characterisation of recombinant HBV viruses 289
3.3.2.1 Characterisation of a pCMV_HBV plasmid 289
3.3.2.2 Characterisation of the newly created HBV constructs encoding transgenes 292

3.3.2.3 Further evaluation of virus production from the newly created HBV constructs encoding transgenes....295
3.3.2.3.1 Additional attempts to produce functional recombinant HBV virus encoding transgenes....301
3.3.2 Isolation and clonal analysis of cells supporting HBV infection 306
4. Discussion 313
4.1 Importance of this study....313
4.2 Results of this study....317
4.2.1 HBV tools generated for this study....319
4.2.1.1 HBV-mimicking lentiviral pseudoparticles....319
4.2.1.2 HBV recombinant viruses encoding reporter genes 319
4.2.2 Properties of the human NTCP receptor, and how they affect susceptibility of cells to HBV infection 324
4.2.2.1 Human NTCP protein analysis; Technical & Experimental difficulties faced particularly during western blot analysis.350
4.2.3 Effect of DMSO on the human ASGPR receptor, and how this further affects susceptibility of cells overexpressing this receptor to HBV infection....353
4.2.3.1 The human EGFR and TfR receptors, and their differential regulation by DMSO 357
4.2.4 Properties of the chimeric NTCP receptor, its particular regulation by DMSO, and subsequent susceptibility of cells overexpressing this receptor to HBV infection....358
4.2.5 The cyclic repackaging rescue screen for identification of additional factors vital for HBV entry and subsequent infection 361
4.3 Future work....364
4.4 Conclusions 368
Bibliography....374
Appendix & Supplementary Data....395
2. Materials and Methods 395
Vector Maps....395
Purchased siRNAs....433

Analysis of ECL versus LICOR® technology 437
Analysis of the generated hNTCP lentiviral pseudoparticles 439
3. Results.....440
3.I Flow cytometry analysis of hNTCP_FLAG-tag in stable hepatic and non-hepatic cell lines 440
3.II Baseline hNTCP expression in a stable human hepatic cell line (HepG2), in the absence of DMSO 441
3.III hNTCP dephosphorylation by a Lambda phosphatase enzyme, illustrating particular differences between hNTCP protein band levels and patterns.....442
3.IV Effect of various drugs on cAMP levels within cells 443
3.V PNGaseF deglycosylation of stable human and mouse cell lines overexpressing hNTCP.....444
3.VI The result of Amicon® filter concentration on HepG2.2.15 infectious sups 445
3.VII FACS sorting protocol of single cell clones positive for GFP expression 446
3.VIII *Cont.* Single cell clones that have been infected with the Type I HBSpp and FACS sorted for GFP expression 447
4. Discussion 450
4.I Human NTCP protein expression differences between conventional western blotting procedures and the NuPAGE® technology (*ThermoFisher Scientific*). 450
In addition; Human NTCP protein expression differences when utilising different protein extraction procedures, as well as the effect of heat denaturation on the protein's expression. 450
4.II Analysis of the anti-hNTCP antibody specificity by conventional western blotting.....451

List of Figures

Figure 1.1; The natural history of chronic liver disease. 31
Figure 1.2; HBV-associated viral particles as seen by their negative staining with the use of electron microscopy. 33
Figure 1.3; Geographic distribution and prevalence of HBV infections. 34
Figure 1.4; Geographical distribution pattern of HBV genotypes. 35
Figure 1.5; Summary of routes of HBV transmission; vertical and horizontal pathways. 36
Figure 1.6; Natural history of HBV progression and persistence. 37
Figure 1.7; The clinical course and serological profiles of acute (a) and chronic (b) HBV infections. 38
Figure 1.8; The HBV virion and a schematic representation of the virus' compact coding genomic organisation. 45
Figure 1.9; Organisation of the HBV rcDNA genome, and representation of the transcriptional regulatory elements. 46
Figure 1.10; Structural organization and expression of the HBV pre-S1/S2/S genes. 49
Figure 1.11; Schematic diagram of the myristoylated pre-S1 domain of the HBsAg. 52
Figure 1.12; Model of HBV entry into hepatocytes. 54
Figure 1.13; HBV genome entry into the nucleus of a host cell via the NPC. 55
Figure 1.14; (A) HBV replication via reverse transcription of the viral DNA. (B) The products of cccDNA transcription and the respective proteins produced. 57
Figure 1.15; The constant cycle of HBV genome replication. 60
Figure 1.16; HBV life cycle, and the virus' replication strategy. 61
Figure 1.17; (A) Schematic of the multiple transmembrane transporter, NTCP; (B) Representation of the location and function of the NTCP receptor. 63
Figure 1.18; RNA and protein expression summary of the *SLC10A1* gene. ..65
Figure 1.19; Sequence homology analysis between human and *Tupaia* NTCP. 66
Figure 1.20; The pre-S1 domain of HBV competing with bile acids for entry through the NTCP receptor. 68

Figure 1.21; Sequence homology analysis between human, mouse and rat NTCP. 69
Figure 1.22; Proposed mechanism of cAMP-mediated rNTCP dephosphorylation and translocation to the plasma membrane. 71
Figure 1.23; The ASGPR receptor functions by degrading asialoglycoproteins via endocytosis. 73
Figure 1.24; Function of the TfR in uptake of transferrin-bound iron molecules. 74
Figure 1.25; Simplified schematic of the EGFR signalling cascade. 75
Figure 1.26; (A) Schematic of the overall cellular sites associated with protein synthesis and trafficking; (B) Schematic of the endosomal trafficking/ sorting pathway of newly synthesised proteins. 78
Figure 1.27; Endosomal trafficking/ sorting pathway of plasma membrane proteins after endocytosis. 79
Figure 1.28; (A) Geographic distribution and prevalence of HDV infections and its different viral genotypes. (B) The clinical course and serological profiles of HDV and HBV infections. 83
Figure 1.29; Structural organisation of the infectious HDV virion. 85
Figure 1.30; Schematic representation of the HDV infection and replication cycle. 86
Figure 1.31; Examples of the key human cell culture systems for studying HBV infection. 90
Figure 1.32; Example of a MPCC *in vitro* system model. 92
Figure 1.33; Example of genomic organisation of wild-type and recombinant HBV transcripts. 98
Figure 1.34; Summary of cyclic repackaging rescue screen procedure. 99
Figure 1.35; Role of cellular polarisation for HBV infection *in vitro*. 101
Figure 1.36; Examples of the key mouse models for studying HBV infection. 104
Figure 1.37; The human liver chimeric uPA mouse model. 107
Figure 2.1; The Gateway® reaction. 130
Figure 2.2; Visualisation of the HBV core and polymerase PCR products on a typical agarose gel. 133

Figure 2.3; Visualisation of the Gateway® destination cassette PCR product on a typical agarose gel. 134
Figure 2.4; Visualisation of the reporter PCR products on a typical agarose gel. 135
Figure 2.5; Diagrammatic overview of the cloning strategy for the construction of HBV genomes encoding transgenes. 136
Figure 2.6; Schematic of typical lentiviral pseudoparticles produced. 155
Figure 2.7; Production of lentiviral pseudoparticles by performing a $CaPO_4$ precipitation technique. 157
Figure 2.8; Lentiviral vector production and Cell transduction. 158
Figure 2.9; Production of lentiviral pseudoparticles expressing the hNTCP. 160
Figure 2.10; Production of two types of HBV-mimicking pseudoparticles. ... 161
Figure 2.11; Production of two types of HBV-mimicking pseudoparticles. ... 161
Figure 2.12; Production of non-infectious HBV-mimicking lentiviral particles. 162
Figure 2.13; Diagrammatic summary of the “cyclic repackaging rescue screen” (CRRS). 165
Figure 2.14; Diagrammatic summary of an altered version of the CRRS. 166
Figure 2.15; Example of a titration kill curve of HepG2 cells treated with G418 antibiotic. 169
Figure 2.16; Timeline of HBV infection experiments. 173
Figure 3.1; Human NTCP mRNA overexpression in stable human hepatic cells. 176
Figure 3.2; (A) Absence of hNTCP protein detection on a western blot, using samples isolated from stable, receptor overexpressing cell lines; (B, C) Detection of a recombinant hNTCP protein by western blot when using anti-NTCP-specific antibodies. 177
Figure 3.3; Human NTCP (FLAG-tag) protein overexpression in a transiently-transfected non-hepatic cell line 179
Figure 3.4; Human NTCP (FLAG-tag) mRNA overexpression in stable human hepatic and non-hepatic cells. 180
Figure 3.5; Effect of antibiotic selection on hNTCP (FLAG-tag) protein expression during the generation of stable cell lines. 183

Figure 3.6; Examples of stable HepG2_hNTCP monoclonal cell populations cultured in optimised media (HMM) versus complete media (DMEM). 185
Figure 3.7; Human NTCP (FLAG-tag) mRNA overexpression in a selection of stable HepG2_hNTCP polyclonal and monoclonal cell populations. 186
Figure 3.8; Differential morphology of stable HepG2_hNTCP cell populations when cultured in complete (DMEM) versus optimised (HMM) media conditions.....187
Figure 3.9; Representative example of an infection experiment of HepG2_hNTCP cells with infectious HBV.....188
Figure 3.10; Effect of DMEM versus William's E optimised media on hNTCP (FLAG-tag) in stable hepatic and non-hepatic cell lines.....190
Figure 3.11; Effect of DMSO on hNTCP (FLAG-tag) mRNA (A) and protein (B) overexpression in the stable cell lines created, as well as on the endogenous levels of the receptor expressed in the parental human hepatic cells (C). ...192
Figure 3.12; Effect of different culturing conditions on the expression of hNTCP (FLAG-tag) in stable cell lines, as seen by immunofluorescence. ...193
Figure 3.13; Kinetic analysis of the DMSO-induced effect on hNTCP (FLAG-tag) expression in the stable human hepatic cell line.....195
Figure 3.14; (A) Shuttling of hNTCP towards the cell surface over time and in the presence of DMSO; (B) Subcellular fractionation experiment for the expression of the hNTCP receptor.....197
Figure 3.15; Testing varying concentrations of DMSO and their effect on hNTCP (FLAG-tag) overexpression on the mRNA (A) and protein (B) level, as well as on cell viability (C).....199
Figure 3.16; Reversibility of the DMSO-induced effect on hNTCP (FLAG-tag) mRNA (A) and protein (B) expression levels in the stable cell lines.....201
Figure 3.17; Effect of DMSO on the CMV promoter driving expression of a GFP sequence.....203
Figure 3.18; The effect of DMSO on hNTCP (FLAG-tag) expression in another stable human hepatic cell line.....205
Figure 3.19; Analysis of the hNTCP glycosylation status upon induction with DMSO and treatment with a PNGaseF glycosidase enzyme.....207
Figure 3.20; Analysis of the hNTCP glycosylation status upon induction with DMSO and treatment with a Endo H glycosidase enzyme.....208

Figure 3.21; Kinetic analysis of the hNTCP glycosylation status upon induction with DMSO and treatment with Tunicamycin. 209
Figure 3.22; Effect of Actinomycin D on *de novo* transcription of hNTCP after induction with DMSO. 211
Figure 3.23; Effect of Cycloheximide on *de novo* protein synthesis of hNTCP after induction with DMSO and subsequent withdrawal of the agent. 213
Figure 3.24; Enhanced susceptibility of human liver cells overexpressing hNTCP to HBV infection in the presence of DMSO. 215
Figure 3.25; The effect of epigenetic modification drugs on hNTCP (FLAG-tag) protein expression. 218
Figure 3.26; Actin (Phalloidin) stain of HepG2_hNTCP cells in the presence or absence of DMSO.....219
Figure 3.27; The effect of actin and tubulin polymerisation inhibitors or active depolymerisers on the expression of hNTCP (FLAG-tag) protein, and comparison with the effect of DMSO.....224
Figure 3.28; Analysing the susceptibility of HepG2_hNTCP cells to HBV infection in the presence of actin and tubulin polymerisation inhibitors, as well as DMSO. 228
Figure 3.29; Analysis of the endogenous cAMP levels in HepG2_hNTCP cells treated with DMSO.....229
Figure 3.30; The effect of exogenous cAMP on the expression of hNTCP (FLAG-tag) protein. 231
Figure 3.31; The effect of Wortmannin on the protein expression of hNTCP (FLAG-tag), after induction by DMSO and/or exogenous cAMP. 235
Figure 3.32; The effect of exogenous cAMP and wortmannin, as well as DMSO, on the susceptibility of HepG2_hNTCP cells to HBV infection. 236
Figure 3.33; Analysing the susceptibility of HepG2_hNTCP cells to HBV infection, in the presence of exogenous cAMP, Wortmannin, and DMSO, by staining for HBcAg and visualising the cells by immunofluorescence. 237
Figure 3.34; The effect of MDL12 or Caffeine on the protein expression of hNTCP (FLAG-tag), after induction by DMSO and/or exogenous cAMP. 243
Figure 3.35; The effect of MDL12 and caffeine, as well as DMSO, on the susceptibility of HepG2_hNTCP cells to HBV infection. 244

Figure 3.36; Analysing the susceptibility of HepG2_hNTCP cells to HBV infection, in the presence of MDL12, Caffeine, and DMSO, by staining for HBcAg and visualising the cells by immunofluorescence. 245
Figure 3.37; Degree of silencing of the different PDEs via siRNAs within the stable hepatic cells overexpressing hNTCP.....248
Figure 3.38; Effect of silencing of the various PDE(s) on hNTCP (FLAG-tag) protein expression in the stable human hepatic cell line. 249
Figure 3.39; The effect of Roflumilast on inhibition of PDE4, resulting in indirect stimulation of hNTCP protein expression on the plasma membrane. 251
Figure 3.40; The effect of Roflumilast on susceptibility of HepG2_hNTCP cells to HBV infection. 253
Figure 3.41; Human ASGPR mRNA overexpression in stable human hepatic cell lines. 256
Figure 3.42; The effect of DMSO on hASGPR expression in a stable human hepatic and non-hepatic cell line.....259
Figure 3.43; Comparison of the effect of DMSO on hASGPR and hNTCP expression in stable human hepatic (HepG2) cells, when cultured under varying DMSO conditions. 261
Figure 3.44; Effect of DMSO on hASGPR expression in stable human hepatic cell lines. 262
Figure 3.45; The effect of DMSO on expression of human EGFR and TfR in stable human hepatic and non-hepatic cell lines.....265
Figure 3.46; Characterisation of Type I HBSpp. 267
Figure 3.47; Infection of human hepatic cell lines, overexpressing two HBV-related receptors, with HBSpp. 269
Figure 3.48; Infection of non-hepatic and hepatic cell lines overexpressing the HBV-related receptors with HBSpp.....271
Figure 3.49; Human NTCP mRNA overexpression in stable mouse hepatic cells.....272
Figure 3.50; The effect of DMSO on hNTCP (FLAG-tag) expression in stable mouse hepatic cell lines.....274
Figure 3.51; Sequence alignment analysis of the human, mouse and newly synthesised chimeric form of the NTCP receptor.....276

Figure 3.52; Effect of DMSO on the expression of a chimNTCP in stable non-hepatic and hepatic cell lines. 278
Figure 3.53; Susceptibility of human and mouse hepatic cells expressing two different forms of the NTCP receptor to HBV infection, *in vitro*. 280
Figure 3.54; Signalling pathway analysis activated in either a human or a mouse cell line, overexpressing different forms of the NTCP receptor, upon infection with HBV. 284
Figure 3.55; Weight chart of mice into which two forms of the NTCP receptor were separately hydrodynamically delivered, a human and a chimeric form, so as to evaluate if they would facilitate HBV entry further allowing the mice to support viral infection. 285
Figure 3.56; Evaluating the susceptibility of mice, overexpressing either the human NTCP receptor or the chimNTCP form of the receptor, to HBV infection (day 5 and 10). 286
Figure 3.57; Evaluating the susceptibility of mice, overexpressing either the human NTCP receptor or the chimNTCP form of the receptor, to HBV infection (day 11). 288
Figure 3.58; Characterisation of a 1.3kb overlength HBV plasmid. 291
Figure 3.59; Characterisation of two newly generated recombinant HBV genomes encoding transgenes. 293
Figure 3.60; Immunofluorescence analysis of the HepG2 cells after transfection with the HBV plasmid encoding for BFP. 294
Figure 3.61; Characterisation of the BFP-expressing HBV genome in Huh7.5 and stable Huh7.5 cell lines overexpressing HBV-related receptors.....296
Figure 3.62; Characterisation of the BFP- and BsdR- expressing HBV genomes in HepG2.2.15 cells. 298
Figure 3.63; Additional characterisation of the BsdR-expressing HBV genome in HepG2.2.15 cells.....300
Figure 3.64; Analysis of the effect of additional HBsAg being co-transfected with the different HBV genomes, wild-type (wt) or reporter-expressing, on virus production.....302
Figure 3.65; Analysis of the effect of different plasmid and FBS concentrations during HBV plasmid electroporation on virus production. 303

Figure 3.66; Analysis of the levels of secreted HBsAg and HBV DNA copies expressed in twenty fractions collected after an iodixanol gradient of supernatants collected from cells transfected with the BSD-expressing HBV plasmid. The samples regarded as infectious and thus collected for future use were fractions 9 to 13.....304
Figure 3.67; Analysing the effect of antibiotic selection on the replication and production of HBV virus from cells transfected with reporter-expressing HBV plasmids.....305
Figure 3.68; GFP-positive single cell clones of stable Huh7.5 cells overexpressing hNTCP/hASGPR, further transduced with a particular lentiviral cDNA library and infected with Type I HBSpp.....307
Figure 3.69; Single cell clones of stable Huh7.5 cells overexpressing hNTCP/hASGPR, further transduced with a particular lentiviral cDNA library and infected with initially Type I HBSpp and then HepG2.2.15 infectious supernatants. 308
Figure 3.70; Analysis of the expression levels of hNTCP (A) and hASGPR (B) in stable Huh7.5 cells overexpressing hNTCP/hASGPR, further transduced with a particular lentiviral cDNA library and infected with Type I HBSpp and HepG2.2.15 infectious supernatants.....310
Figure 3.71; Two blasticidin-positive cell colonies observed after clone #33 was infected with HBV virus expressing BsdR and subsequently selected with the particular antibiotic. 312
Figure 4.1; Possible model of DMSO's mechanism of action, and comparison to the effect of Cycloheximide in inducing hNTCP overexpression and translocation to the cell surface. 337
Figure 4.2; DMSO's mechanism of action #1; Induction of cytoskeletal modifications further permit direct hNTCP translocation to the plasma membrane.....340
Figure 4.3; DMSO and cAMP are involved in the same regulatory pathway resulting in hNTCP translocation to the plasma membrane. 342
Figure 4.4; DMSO's hypothetical mechanism of action #2; DMSO targets and blocks a PDE, which consequently permits cAMP to perform its function and promote hNTCP translocation to the plasma membrane. 343

Figure 4.5; Effect of the MDL12 inhibitor on the subsequent hNTCP receptor translocation to the plasma membrane. 344
Figure 4.6; Effect of Caffeine on the subsequent hNTCP receptor translocation to the plasma membrane. 345
Figure 4.7; DMSO's mechanism of action #2; DMSO targets PDE4(D), eventually resulting in indirect translocation of hNTCP to the plasma membrane. 348

List of Tables

Table 1.1; Summary of the geographical distribution of the different HBV serotypes, genotypes and subgenotypes. 35
Table 1.2; Assessment markers for patients with chronic HBV infection. 42
Table 1.3; Antiviral therapies currently used for treatment of chronic HBV infections. 43
Table 1.4; HDV diagnostic markers. 84
Table 1.5; Examples of current and most widely used cell culture systems for the study of HBV. 95
Table 1.6; Examples of current and most widely used mouse models for the study of HBV. 109
Table 2.1; Consumables used during the course of the project. 117
Table 2.2; Solutions and Buffers used during the experimental procedures. 120
Table 2.3; List of primers used for sequencing or cloning of various DNA plasmids. 123
Table 2.4; List of DNA plasmids utilised during the experimental procedures. 126
Table 2.5; Various cell lines utilised during the course of the project. 127
Table 2.6; The apparatus used during the experimental procedures. 129
Table 2.7; Computer software programs used for experimental analysis of the data. 129
Table 2.8; Example of a standard BP Gateway® reaction. 131
Table 2.9; Example of a standard LR Gateway® reaction. 131
Table 2.10; Example of a standard restriction digest reaction. 137
Table 2.11; Example of a standard DNA ligation reaction. 139
Table 2.12; Example of a standard PCR method. 141
Table 2.13; Example of the usual cycling conditions followed for a standard PCR. 141
Table 2.14; Example of a standard Taqman® qPCR method. 143
Table 2.15; Example of the usual cycling conditions for a standard Taqman® qPCR reaction 144
Table 2.16; Example of a standard SYBR® qPCR method. 145

Table 2.17; Example of the usual cycling conditions for a standard SYBR® qPCR reaction.....145
Table 2.18; Commonly used antibodies for immunofluorescence detection of the expression of particular proteins. 149
Table 2.19; Typical number of cells seeded per cell culture dish or plate. ...153
Table 2.20; Typical preparation of a transfection mixture for a $CaPO_4$ precipitation protocol. 156
Table 2.21; Different reagents/ drugs/ inhibitors utilised during various experimental procedures and their optimum concentrations. 174
Table 3.1; Proteomics analysis of DMSO-treated cells revealed upregulation of a number of epigenetic and cytoskeletal factors. 216
Table 3.2; Epigenetic modification drugs and their particular functions. 217
Table 3.3; Actin and Tubulin polymerisation inhibitors and active depolymerisers.....220
Table 3.4; Phospho-kinase signalling factors activated in either a human or a mouse cell line, overexpressing different forms of the NTCP receptor, upon infection with HBV. 281
Table 3.5; Genes, Names and Uniprot Accession numbers of the different phospho-kinase signalling factors activated in either a human or a mouse cell line, overexpressing different forms of the NTCP receptor, upon infection with HBV. 282
Table 3.6; Summary of the single cell clones selected according to their GFP expression (indicating infection with HBSpp) and levels of HBsAg/ HBeAg secretion (after infection with HepG2.2.15 supernatants). 309
Table 3.7; Summary of the single cell clones selected according to their expression of hNTCP and hASGPR receptors. 311

Abbreviations

^{o}C	Degrees Celsius
aa	Amino acid
AAV	Adeno-associated virus
AB	Applied Biosystems
ALT	Alanine transaminase
APS	Ammonium persulfate
ASGPR	Asialoglycoprotein receptor
ATP	Adenosine triphosphate
AV	Adenovirus
BC	Bacteria culture
BFP	Blue fluorescent protein
BIX-01294	2-(Hexahydro-4-methyl-1H-1,4-diazepin-1-yl)-6,7-dimethoxy-N-[1-(phenylmethyl)-4-piperidinyl]-4-quinazolinamine trihydrochloride hydrate
bp	Base pair
BSA	Bovine serum albumin
BsdR/BSD	Blasticidin selection cassette/ resistance
C	Carboxyl
C646	4-[4-[[5-(4,5-Dimethyl-2-nitrophenyl)-2-furanyl]methylene]-4,5-dihydro-3-methyl-5-oxo-1H-pyrazol-1-yl]benzoic acid
cAMP	Cyclic adenosine monophosphate
CC	Cell culture
cccDNA	Covalently closed circular DNA
cDNA	Complementary DNA
chimNTCP	Chimeric NTCP
CIP	Calf intestinal
CMV	Cytomegalovirus
CRRS	Cyclic repackaging rescue screen
DAPI	4',6-Diamidino-2-Phenylindole
dd	Double-distilled
DMEM	Dulbecco's modified eagle's medium

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleotide
DR	Direct repeat
ds	Double-stranded
DTT	Dithiothreitol
EASL	European association for the study of liver disease
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Endo H	Endoglycosidase H
ER	Endoplasmic reticulum
Fah	Fumaryl acetoacetate hydrolase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
Fluc	Firefly luciferase
Fw	Forward
g	Grams
G418	Geneticin/Neomycin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
Gluc	Gaussia luciferase
GPC5	Glypican 5
hASGPR	Human ASGPR
HBcAg	Hepatitis B core antigen
HBeAg	Hepatitis B e antigen
HBS	HEPES-buffered saline
HBsAg	Hepatitis B surface antigen
HBSS	Hank’s balanced salt solution
HBV	Hepatitis B virus
HBV/Spp	HBV-mimicking lentiviral pseudoparticles
HCC	Hepatocellular carcinoma

HCl	Tris- Hydrogen Chloride
HCV	Hepatitis C virus
HCVpp	HCV-mimicking lentiviral pseudoparticles
HDV	Hepatitis delta virus
hEGFR	Human EGFP
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hiPSC	Human induced pluripotent stem cell
HIV	Human immunodeficiency virus
hNTCP	Human NTCP
HPA	Helix pomatia agglutinin
HPV	Human papillomavirus
HRP	Horseradish peroxidise
HSPG	Heparan sulfate proteoglycan
hTfR	Human TfR
IF	Immunofluorescence
IFN-α	Interferon-alpha
IgG	Immunoglobulin
IRES	Internal ribosome entry site
kb	Kilobase
kDa	Kilodalton
L	Litre
L (pre-S1/S2/S)	Large HBV envelope glycoprotein
LB	Luria-Bertani
L-HDAg	Large hepatitis delta antigen
M	Molar
M (pre-S2/S)	Middle HBV envelope glycoprotein
MB-3	(2R,3S)-rel-4-Methyl-5-oxo-2-propyl tetrahydrofuran-3-carboxylic acid
MCS	Multiple cloning sequence
MEM	Minimum essential medium
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar

mg	Milligram
mkNTCP	*Macaca fascicularis* NTCP
ml	Millilitre
mM	Millimolar
mNTCP	Mouse NTCP
MOI	Multiplicity of infection
MPCC	Micro-patterned co-culture
mRNA	Messenger RNA
MRP2	Multidrug resistance-associated protein 2
NE	Non-envelope
NEB	New England Biolabs
NE pp	NE lentiviral pseudoparticles
ng	Nanogram
NLS	Nuclear localisation signal
nm	Nanometer
nM	Nanomolar
NP40	Nonidet P40
NPC	Nuclear pore complexes
nt	Nucleotide
NTBC	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione
NTC	Non-template control
NTCP	Sodium taurocholate co-transporting polypeptide
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	PBS, Tween
PCR	Polymerase chain reaction
PCNA	Proliferating Cell Nuclear Antigen
PDE	Phosphodiesterase
PE	Phycoerythrin
PEG	Polyethylene glycol
PEV	Position-effect variegation
PFA	Paraformaldehyde
pg	Pregenomic

PHH	Primary human hepatocyte
PHMM	Primary hepatocyte maintenance media
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PKCζ	Protein kinase C zeta
PMSF	Phenylmethylsulfonyl Fluoride
PNGaseF	Peptide *N*-glycosidase F
PP2B	Protein phosphatise 2B
PTH	Primary tupaia hepatocyte
qRT-PCR	Quantitative real-time or reverse-transcription PCR
Rbm3	RNA-binding motif protein 3
rc	Relaxed circular
Rev	Reverse
RFP	Red fluorescent protein
RIG-1	Retinoic acid-inducible gene 1
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
rNTCP	Rat NTCP
RPS11	Ribosomal protein S11
RTK	Receptor tyrosine kinase
sg	Subgenomic
S (pre-S)	Small HBV envelope glycoprotein
SCID	Severe combined immunodeficiency
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SG-PERT	SYBR-green, product-enhanced reverse transcriptase
S-HDAg	Small hepatitis delta antigen
siRNA	Small Interference RNA
SLC10A1	Solute carrier family 10 member 1
SRPK1/2	SR protein-specific kinase 1/2
STD	Sexually transmitted disease
TAE	Tris-Acetate EDTA
TB	Terrific broth

TC	Tissue culture
TEMED	Tetramethylethylenediamine
Tet	Tetracycline
TfR	Transferrin receptor
tsNTCP	*Tupaia* NTCP
TUDC	Tauroursodeoxycholate
uPA	Plasminogen activator
UV	Ultraviolet
VSVg	Vesicular stomatitis envelope glycoproteins
VSVg pp	VSVg lentiviral pseudoparticles
v/v	Volume/volume
WE	William's E
WGA	Wheat germ agglutinin
WHO	World Health Organisation
w/v	Weight/volume
X-gal	5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside

1. Introduction

1.1 Viral Hepatitis

Infectious viral hepatitis, the leading cause of liver inflammation from a viral origin, is most commonly caused by either one of five hepatotropic viruses; Hepatitis A, B, C, D or E. Of these five unrelated hepatitis viruses, three of them may cause a serious chronic infection: B, C and D viruses, while hepatitis A and E cause mostly acute infections that are self-limiting [1-3]. Chronic infections typically result in liver fibrosis, cirrhosis and higher risks of developing liver cancer or hepatocellular carcinoma (HCC). HCC is currently a very important socioeconomic burden particularly as it is a leading cause of cancer-related deaths worldwide (*Figure 1.1)* [4-7]. In 2013, approximately 1.5 million people died as a result of viral hepatitis with the majority of deaths being attributed to hepatitis B and C viruses, making them the seventh leading cause of deaths worldwide. However there is scope for prevention or treatment; for example, hepatitis B can be prevented by vaccination, while hepatitis C can be successfully treated [8]. Unfortunately, though, treatment still remains very expensive and hence is not available to all [9, 10]. In the cases where patients have already developed advanced irreversible liver disease, liver transplantation is still the only available treatment option [11].

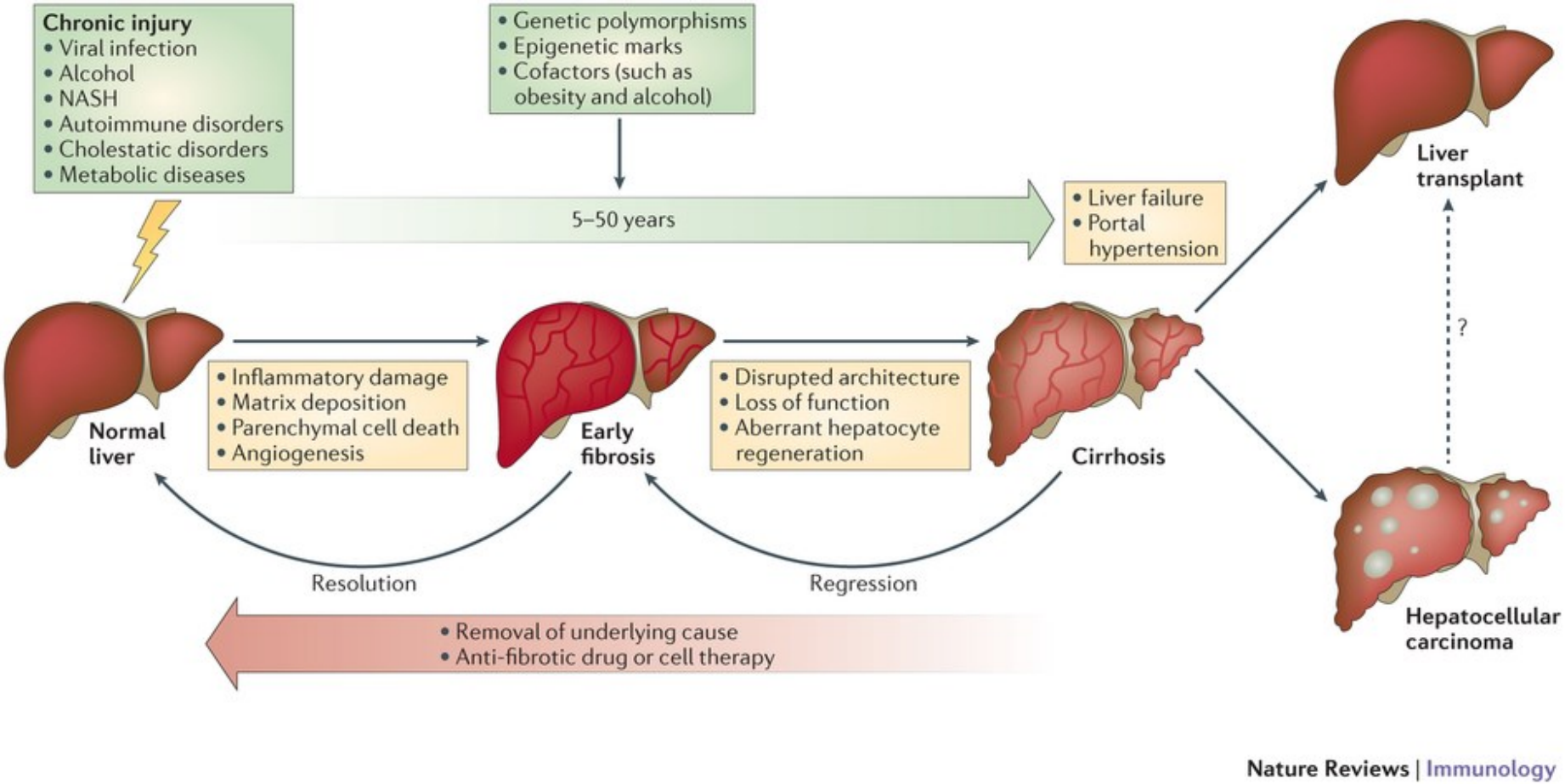


Figure 1.1; The natural history of chronic liver disease.

A number of factors can cause chronic liver injury, one of them being viral infections. However, the progression of disease is similar regardless of the reason that caused it. After the initial damage, and if there is no resolution, the liver becomes fibrotic. Slowly this results in cirrhosis and liver failure, with the final stages being liver cancer and the development of HCC. This can be resolved by transplanting a new liver into the patient(s). *Source:* [12].

1.2 Hepatitis B virus (HBV)

1.2.1 History of HBV identification

In brief, since the 1940s it has been known that blood and plasma samples can contain viruses responsible for causing transient and chronic hepatitis infections. These types of infections have primarily been known as post-transfusion hepatitis, and around 40% of transfusion recipients were affected in those days. In 1947, the thus far uncharacterised infectious virus was named HBV, primarily so as to distinguish it from infections associated with hepatitis A, while in the 1950s an association of this newly discovered virus with HCC was already noted. However, regardless of the high incidence of hepatitis cases due to HBV infections, there were still no means of properly recognising or testing for the virus. This changed in the 1960s when Blumberg and his colleagues first discovered what they named the “Australian antigen” as a component of HBV [13, 14]. This component belonging to the infectious virus is now known as the hepatitis B surface antigen (HBsAg), and its identification opened the door for the further study of HBV. Specifically, ten years later the first infectious virions were visualised by electron microscopy and were named Dane particles after the scientist who discovered them (*Figure 1.2*) [15]. In the same year the first assays for screening HBsAg were developed, resulting in a 2-3 fold reduction in hepatitis cases as a result of blood transfusions while these assays also confirmed the link between HBV and HCC. Not long after this, the first HBsAg vaccine was developed, which was specifically purified from infected carriers [16, 17].

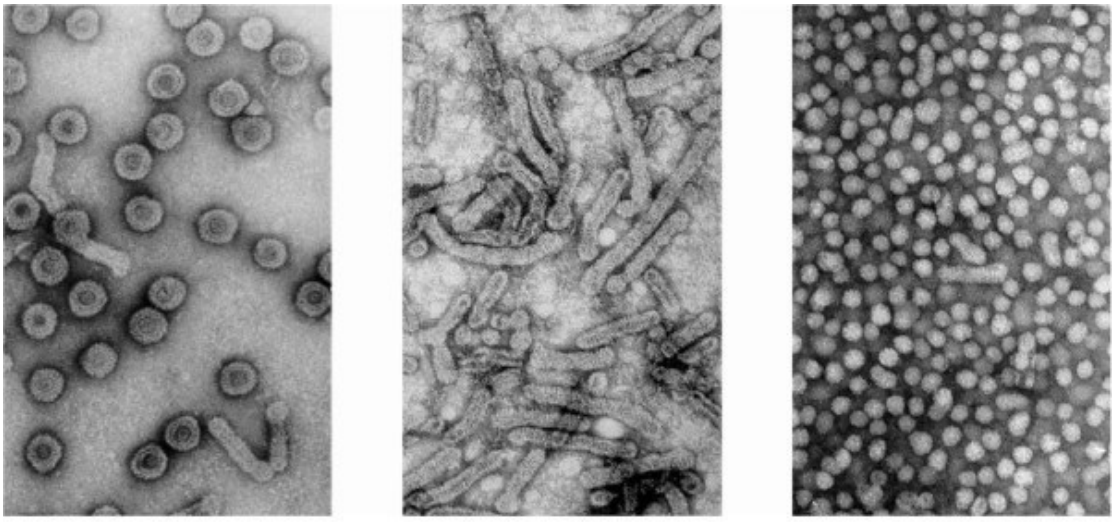


Figure 1.2; HBV-associated viral particles as seen by their negative staining with the use of electron microscopy.

The Dane particles comprise the full HBV infectious virions, while the filaments and spheres are known as the subviral particles as they are only made up of the highly immunogenic HBsAg but lack other important viral features, and hence are non-infectious. *Source:* [16].

1.2.2 Epidemiology of HBV, prevalence of its different serotypes and genotypes, and mode of transmission

HBV, as already mentioned, is one of the most widely spread diseases and as such remains a major public health burden, causing approximately 600 thousand deaths as well as 5 million new infections to arise every year [18-20]. In terms of numbers, approximately 2 billion people have been recorded to have been infected with HBV worldwide, while an estimated 240 million people are currently chronically infected and hence remain at a high risk (nearly a 100 fold higher risk) for progression to other irreversible liver diseases [21-24]. HBV is particularly a major cause of chronic inflammatory liver disease and liver cirrhosis further associated with the development of HCC [25-31]. Nowadays, more than 50% of liver cancers worldwide are attributed to HBV infection [23, 28, 32].

Endemic areas, where the virus has a high prevalence, include North America (including Canada), parts of South America, Sub-Saharan Africa, certain

European areas, and the Middle East, South East Asia and Australasia (mostly areas around Australia). In these places the treatment is possibly scarce or expensive to obtain (*Figure 1.3*) [19, 21, 33, 34].

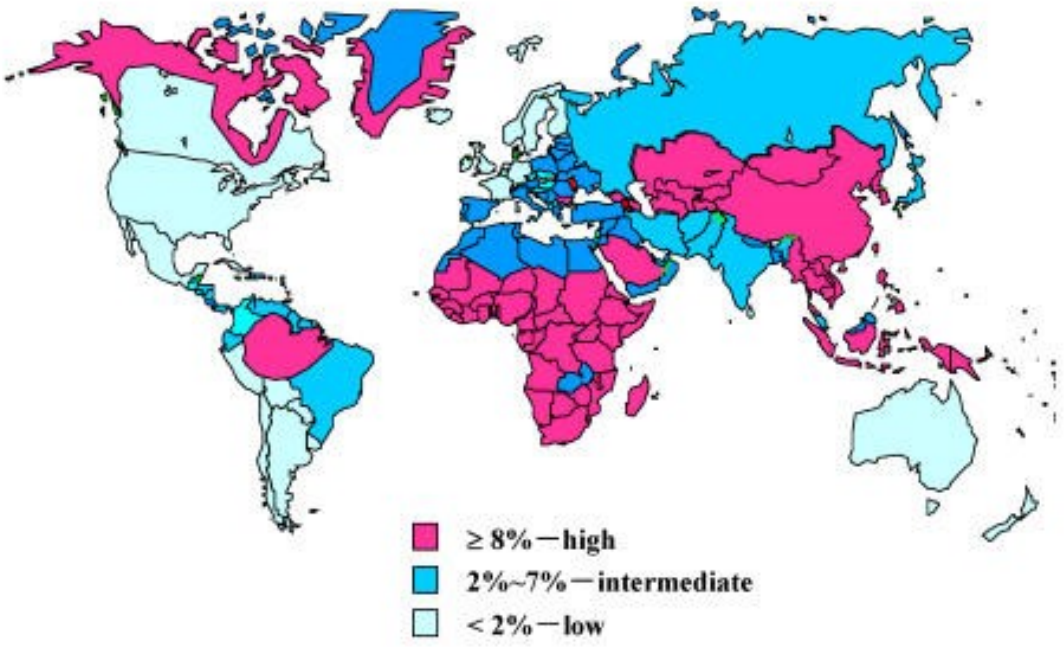


Figure 1.3; Geographic distribution and prevalence of HBV infections.
Highly endemic HBV areas include: North America (including Canada), parts of South America, Sub-Saharan Africa, certain European areas, and the Middle East, South East Asia and Australasia. *Source: World Health Organisation (WHO) and* [21].

As HBV was first identified based on the "Australian antigen", it was originally divided into different serotypes according to the differences in the sequences' antigenicity. In particular, based on the differences in the HBsAg sequence, it was separated into four main subtypes, namely adw, adr, ayw and ayr, and a number of different subtypes. All different HBV serotypes were detected in distinct geographical areas (*Table 1.1*) [35-39]. As technology progressed and it became easier to analyse more extensively genetic information, the HBV serotypes were further divided into different genotypes, and similarly to the HBV serotypes, these genotypes were found in diverse geographic distributions (*Table 1.1; Figure 1.4*). HBV genotypes are currently classified into eight types based on sequence divergence: A, B, C, D, E, F, G and H, and they differ from each other by at least 8%. Recently, two other genotypes

were discovered, affecting distinct populations, but these are still not very well characterised; genotypes I and J. Due to the wide genetic diversity of HBV, these have further been subdivided into numerous subgenotypes (*Table 1.1*) based on phylogenetic relationships, which tend to differ by around 4% [40-43].

Serotypes	Genotypes/ Subgenotypes	Geographical distribution
adw2	A (A1-A7)	North America South America (parts) Sub-Saharan Africa Europe (parts) Australia
adw2, ayw1	B (B1-B9)	Asia and North America
adw2, ayr, adr	C (C1-C16)	Asia and North America, Australia
ayw2, 3, 4	D (D1-D8)	(widespread)
ayw4	E	West and Central Africa
adw4, ayw	F (F1-F4)	Central and South America
adw2	G	(rare)
adw4	H	Central America
adw	I	Vietnam, India, Laos
-	J	Japan

Table 1.1; Summary of the geographical distribution of the different HBV serotypes, genotypes and subgenotypes.
Adapted from [37, 44, 45].

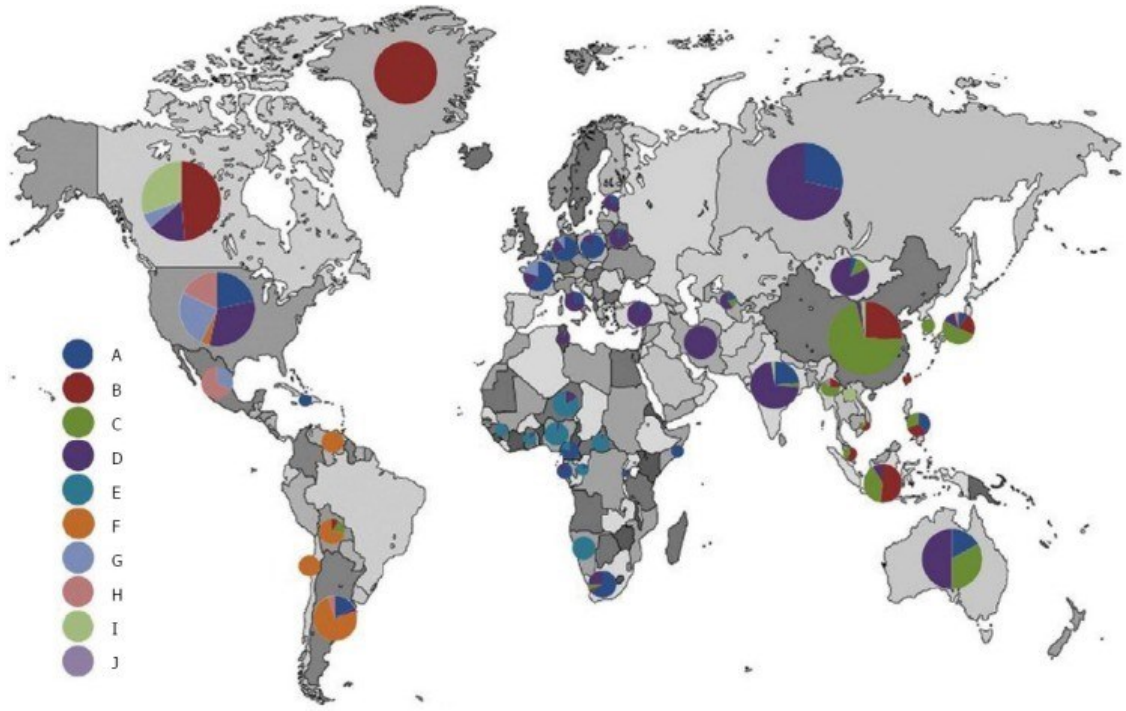


Figure 1.4; Geographical distribution pattern of HBV genotypes.
Illustration of the distinct geographical distribution of the different HBV genotypes, as well as representation of the proportion of each genotype's prevalence in each area. *Source*: *World Gastroenterology Organisation, 2015.*

HBV is known as a bloodborne virus as it is transmitted through person to person contact via primarily virus contaminated blood. However, other bodily fluids have also been implicated, including semen or saliva [21, 46]. There are three accepted routes of transmission: perinatal, sexual and parenteral/ percutaneous, and these are typically separated into two distinct pathways: vertical or horizontal (*Figure 1.5*) [26, 47]. Vertical, or otherwise perinatal, transmission specifically occurs during the perinatal period of a pregnancy when the carrier mother passes on the infection to the baby, and this accounts for a significant number of HBV positive cases [48, 49]. Moreover, HBV is regarded a sexually transmitted disease (STD) with homosexual men being at a higher risk of acquiring the infection via a horizontal route. Nevertheless, heterosexual transmission, due to for example the number of sexual partners or sexual partners of injection drug users, is also associated with high risk of HBV infection. Child-to-child transmission can also occur amongst youngsters, with this being a high risk group for infection [50]. Other ways of horizontal transmission include the parenteral/ percutaneous routes, which particularly refer to injection drug use with needles contaminated with the virus, virus infected blood transfusions, as well as people working in high risk areas such as tattoo studios or healthcare settings [21, 22, 34, 51-53].

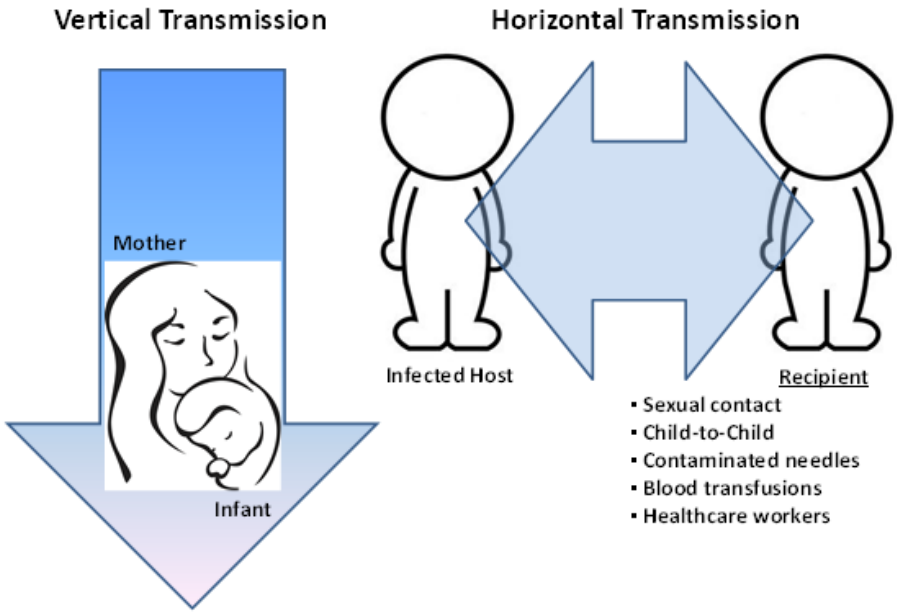


Figure 1.5; Summary of routes of HBV transmission; vertical and horizontal pathways.

1.2.3 HBV pathogenesis, clinical manifestation and persistence

As a general rule, most adulthood HBV infections are acute and self-limiting, meaning that they do not cause disease manifestation particularly in immunocompetent adults. However, approximately 5% infected adults will develop chronic infection, while up to 90% of infected neonates fail to clear infection by mounting an insufficient immune response and as such develop a chronic infection [54-61]. During chronic infection there is constant virus replication occurring in the liver, which will eventually result in the development of other irreversible liver diseases. In chronic infection, generally the time period between HBV infection and manifestation of clinical illness can be decades (*Figure 1.6*), and therefore there is significant opportunity for therapeutic intervention [58, 62].

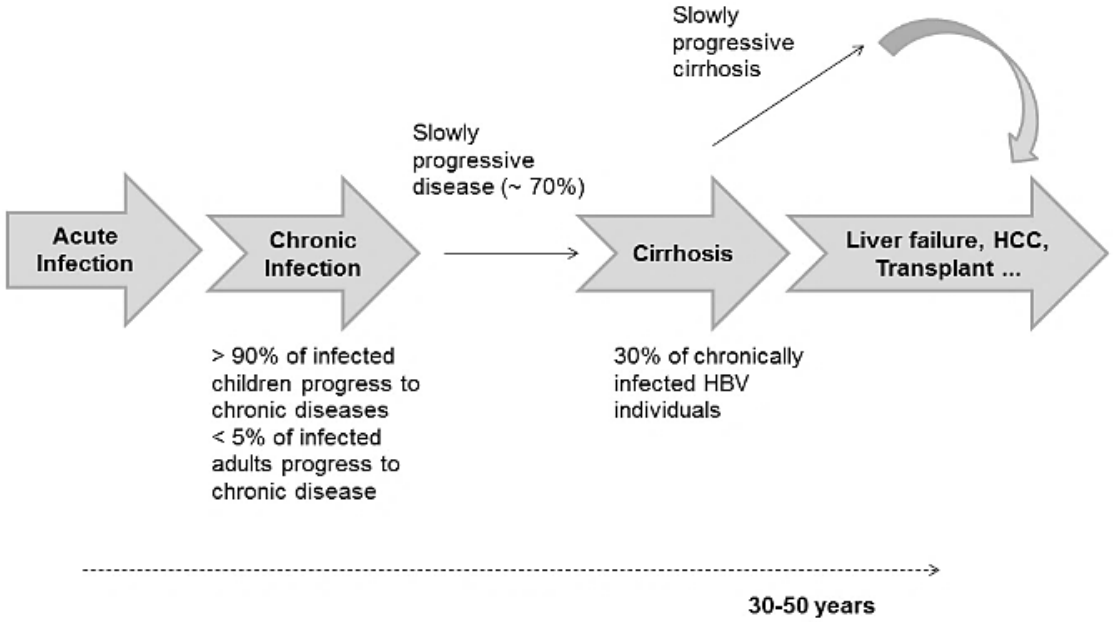


Figure 1.6; Natural history of HBV progression and persistence.

Adapted from [12, 19, 61, 63, 64]

Consequently, HBV causes a broad spectrum of liver disease, which, as previously mentioned, ranges from acute hepatitis (also including fulminant hepatic failure) to chronic hepatitis, followed by progression to irreversible liver failure and eventually HCC [47, 56-58, 61, 65, 66]. The typical clinical courses and serological profiles of both acute and chronic hepatitis are shown

in *Figure 1.7,* and regardless of whether the infection is transient or persistent it may be characterised as either symptomatic or asymptomatic [47, 67].

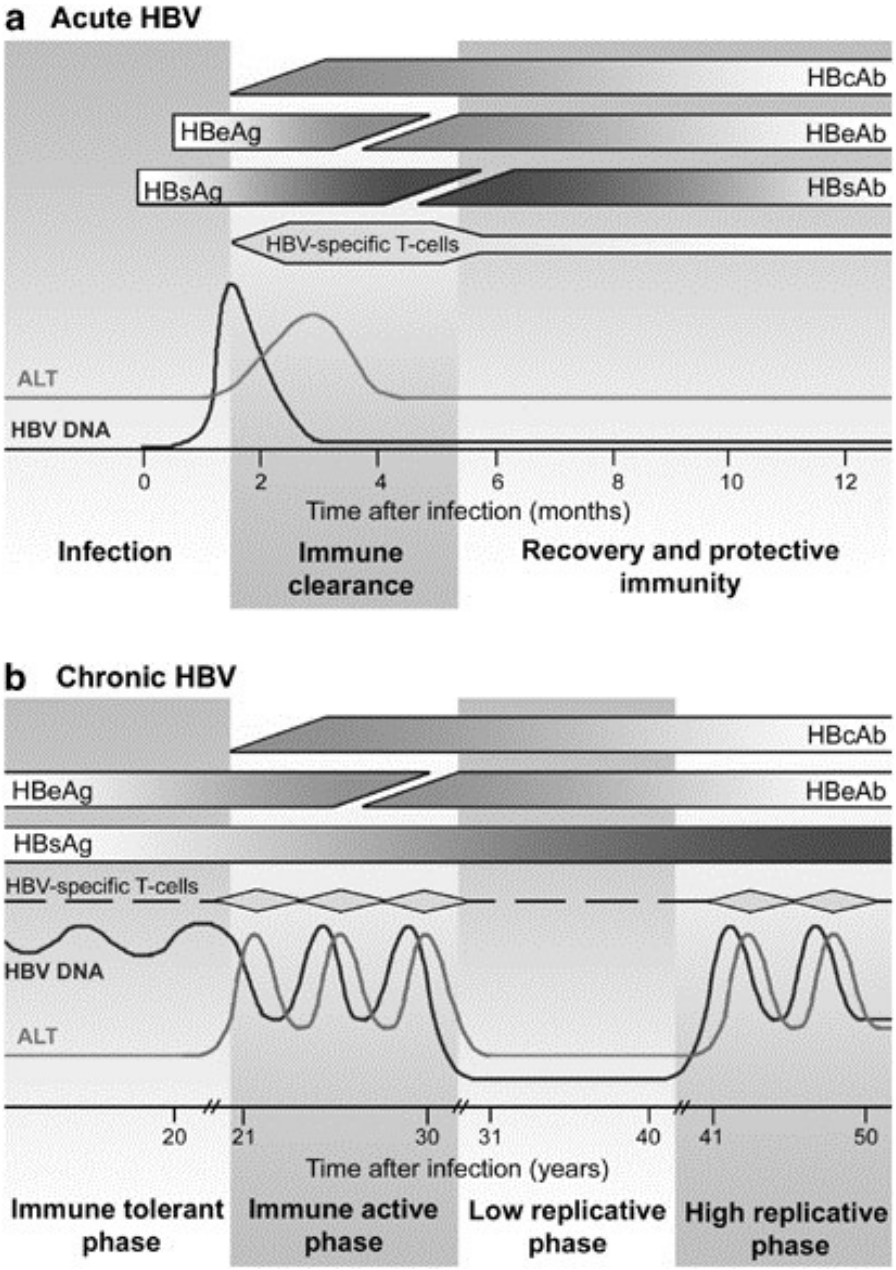


Figure 1.7; The clinical course and serological profiles of acute (a) and chronic (b) HBV infections.

See below for full description. *Source:* [66].

Acute HBV infection

In acute infection (*Figure 1.7*), patients generally are either completely asymptomatic or have very mild symptoms that typically go undetected. Some of these symptoms include weakness and fatigue, nausea and vomiting, anorexia and body aches, and sometimes fever [58]. After acute infection has been established and early during the immune clearance phase, the HBV deoxyribonucleotide (DNA) viral load together with HBsAg and hepatitis e antigen (HBeAg) are the first viral markers that can be detected in the patient's serum. However, only when the levels of the HBV DNA viral load have declined, which is also when the symptoms if any would typically appear, do the levels of alanine transaminase (ALT) rise but they eventually recover. ALT is used in HBV infection as a marker of liver injury. During the recovery and protective immunity phase is when HBsAg and HBeAg are seroconverted and antibodies against these antigens start being produced and detected in the patient's circulating blood. Hepatitis core antigen (HBcAg) antibodies appear during the initial clinical manifestation of the infection. At this point, viral infection has completely been resolved (*Figure 1.7*) [58, 66, 68].

Chronic HBV infection, Stage #1; Immune tolerant phase

As previously mentioned, on occasions acute infections may result in the progression to chronic HBV infections. In chronic infection (*Figure 1.7*), in contrast to acute infections however, there is no initial delay in the increase of the HBV DNA viral load, HBsAg and HBeAg. During this first stage, known as the immune tolerant phase, there is a very high viral load detected but the ALT levels remain normal. Originally this was thought to be the case due to the absence of significant immune response to the virus, while no inflammation and generally no clinical signs of infection could be detected. However, more and more evidence is proving that there is in fact marked disease activity during this early phase of infection, with patients already exhibiting substantial liver inflammation and fibrosis. Patients at this stage of the infection are highly unlikely to lose HBeAg, and hence they are highly

contagious due to the high levels of active vireamia. This phase can persist for decades. Interestingly, children infected perinatally remain in this phase into their adolescence or even early adulthood years. The immune tolerance phase is typically associated with perinatal transmission, and therefore elucidating the exact mechanism of this phase in early infancy is of vital importance. Therapy during this phase of the disease is usually not recommended, but as our knowledge is progressing it is possible that treating the infection earlier will be recommended for patients with higher risk factors for progression to irreversible liver diseases [24, 47, 66, 69-73].

Chronic HBV infection, Stage #2; Immune active phase

In the following, second stage of chronic HBV infection, the immune active phase, that develops quite a few years later and is characterised as the HBeAg-positive chronic hepatitis, there are spontaneous viral flares during which the HBV DNA viral load and the ALT levels characteristically fluctuate. This is typically the stage when HBeAg seroconverts and anti-HBe can be detected in the circulating blood, as well as anti-HBc.

Chronic HBV infection, Stage #3; Inactive carrier phase

The patients then, in the third stage of chronic HBV infection, appear to enter a low replicative stage, or else inactive HBsAg chronic carrier state, during which the HBV DNA load remains reduced and there are normal levels of ALT.

Chronic HBV infection, Stage #4; Virus reactivation phase

Under certain circumstances, however, the virus can flare and enter the fourth stage of chronic HBV hepatitis, which is a higher replicative phase otherwise known as the HBeAg-negative chronic hepatitis, which resembles the immune active phase, with the HBV DNA viral load and the ALT levels being once again elevated and fluctuating. During the course of chronic HBV infection,

patients constantly go between the low and high replicative phases as the viral infection remains unresolved and hence undergoes stages of sporadic reactivation (*Figure 1.7*) [47, 56-58, 61, 66, 69-71].

WHO, as well as the European association for the study of liver disease (EASL), have recently reconsidered the different stages during chronic HBV infection and have decided to specifically separate them into HBeAg-positive and HBeAg-negative chronic HBV infection and chronic HBV hepatitis. The HBeAg-positive phase includes the immune tolerant and immune active stages of chronic HBV infection and chronic HBV hepatitis respectively, while the HBeAg-negative phase includes the inactive carrier (or immune control) stage redefined as chronic HBV infection and the immune escape (or viral reactivation) stage renamed chronic HBV hepatitis (*Table 1.2*) [24, 74].

In terms of symptoms, similarly to patients with acute infections, chronically infected patients can be asymptomatic or have the same general and nonspecific symptoms. However, in cases of severe disease and particularly when the infection has rapidly progressed causing liver failure, patients may develop jaundice (characteristic yellowing of the skin) and the liver will typically have irregular characteristics and slowly begin decomposing [58]. Nevertheless, diagnosis of positive HBV infection is only possible and regarded as accurate when taking into consideration both the clinical manifestation and the serological/ biochemical analysis of the circulating HBV viral antigens and their relevant antibodies in the serum of prospective patients [58, 75, 76]. The current guidelines suggested by EASL for diagnosis of chronic HBV infection are summarised in *Table 1.2.*

	Chronic HBV			
	HBeAg positive		HBeAg negative	
	Infection	Hepatitis	Infection	Hepatitis
Old terminology	**Immune Tolerant**	**Immune Active**	**Inactive carrier**	**Immune Escape***
HBsAg	High	Intermediate/High	Low	Intermediate
HBeAg	+	+	-	-
HBV DNA	$>10^7$ IU/ml	10^4-10^7 IU/ml	<2,000 IU/ml	>2,000 IU/ml
ALT	Normal	Elevated	Normal	Elevated, persistent or sporadic
Liver disease	None/Low	Moderate/Severe	None	Moderate/Severe

Table 1.2; Assessment markers for patients with chronic HBV infection.

***** The virus reactivation phase resembles the immune active phase; in terms of the viral markers being expressed in the patient's circulating blood and the characteristics of liver disease. *Adapted from* [24, 74].

In general, most of our understanding about human HBV pathogenesis has been from studying viral infection in its natural host, the chimpanzee. Chimpanzees, like humans, can develop acute and chronic HBV infections resulting in viral hepatitis, while they also activate a similar immune response [59, 77, 78]. Studies in chimpanzees with acute HBV infections, have specifically demonstrated that the virus early on during infection does not induce any changes to the profile of genes associated with innate immune responses as the infection spreads through the liver of the animal and until the adaptive immune responses activate later on during progression of the infection. This was the first point of evidence that HBV acts like a "stealth virus", thus being very successful at evading the host's innate immune detection mechanisms. In addition, this may possibly explain why 90% of neonates cannot clear acute infection and hence progress to chronic HBV infections. The primary reason for this may actually lie in the way the virus replicates, by specifically incorporating the cccDNA intermediate within the host's nucleus, while at the same time the way the viral capsids are processed (i.e. capped and polyadenylated) makes them resemble normal cellular transcripts. In terms of viral clearance, during acute viral infections in chimpanzees, however, this has been demonstrated to involve direct destruction of infected cells and hepatocellular regeneration. In particular,

antiviral mechanisms have been shown to directly inhibit viral replication by especially targeting the pool of cccDNA templates. Traces of cccDNA have been found to continue to persist in the liver, possibly conferring lifelong immunity to these animals [78-80].

1.2.4 Current HBV treatments

Current available antiviral treatment approved for chronic HBV cases includes the use of nucleoside/nucleotide analogues (e.g. Tenofovir, Entecavir, Lamivudine), which are much better tolerated by patients but lead to the development of drug resistant mutations to varying extents, and the use of pegylated interferon-alpha (IFN-α), which is only partially effective, causes many adverse side effects and is of limited duration [26, 47, 52, 81]. The currently available HBV treatments are summarised in *Table 1.3*; some of these drugs have specifically been developed as targets for the human immunodeficiency virus (HIV) but have been demonstrated to also be active against HBV, hence making treatment of HBV/HIV co-infections slightly easier for clinicians to handle [82].

Antiviral drugs	Potency against HBV	Risk of relapse	Resistance barrier	Activity against HIV
Interferons (PegIFN-α)	Moderate	Low	NA	Moderate
Tenofovir*	High	Moderate/ High**	High	High
Entecavir	High	Moderate/ High**	High	Weak
Lamivudine	Moderate/ High	Moderate/ High**	Low	High
Adefovir	Low	Moderate/ High**	Moderate	-
Telbivudine	High	Moderate/ High**	Low	ND

Table 1.3; Antiviral therapies currently used for treatment of chronic HBV infections.

* Tenofovir disoproxil fumarate and Tenofovir alafenamide.

** Moderate, if “consolidation therapy” is provided after seroconvertion of HBeAg; High, for HBeAg-negative patients.

PegIFN-α can be stopped early if necessary without any concerns regarding the patient's resistance to the drug, however the rest of the treatments should not be stopped before their suggested use as resistance can typically arise. *NA: not applicable, ND: not determined. Adapted from* [24, 74].

Most treated patients, especially ones being treated with nucleoside/nucleotide analogues, relapse shortly after discontinuation of therapy suggesting that the virus persists in the liver during the entire trial period. These treatments are hence life-long and do not cure disease, as they just work by suppressing viral replication, so novel strategies are urgently required [28, 47, 83-85]. Successful HBV therapy may depend on the simultaneous use of multiple compounds with antiviral activity [82, 85]. However, the discovery of additional classes of inhibitors has been hampered by the lack of a suitable cell-based culture system assay or *in vivo* models recapitulating all steps of the virus life cycle so as to study viral entry and infection in greater depth.

1.2.5 HBV taxonomy, genomic structure and organisation

HBV is classified as the prototype member of the *Hepadnaviridae* family and particularly belongs to the mammalian *Orthohepadnavirus* genus. Other HBV species in this genus include the *Woodchuck, Woolly monkey and Ground squirrel hepatitis viruses*. These are not known to infect other primates, while there have definitely not been any reports of them infecting humans, even though they share about 60-70% sequence homology to HBV. HBV, therefore, is the only reported hepadnavirus that infects humans, as well as great apes. The *Hepadnaviridae* family, moreover, also consists of another genus, the *Avihepadnavirus*, specifically consisting of HBV viruses affecting bird species such as the most well known *Duck hepatitis virus*. These viruses are very distant relatives of HBV, with no significant homology existing between the two genera [41, 43, 86-92].

The HBV virion, or else the Dane particle, is a 42 nanometers (nm) enveloped DNA virus containing a partially double-stranded (ds) relaxed circular (rc) genome of 3.2 kilobases (kb). The small HBV genome is encapsidated within an icosahedral nucleocapsid (core) that is surrounded by a lipid bilayer containing the viral glycoproteins (*Figure 1.8 and 1.10; A)*. The extremely compact genome of the virus particularly encodes four overlapping open reading frames (ORFs), which give rise to the core and polymerase proteins, envelope glycoproteins and the X-protein (*Figure 1.8; B*) [26, 32, 47].

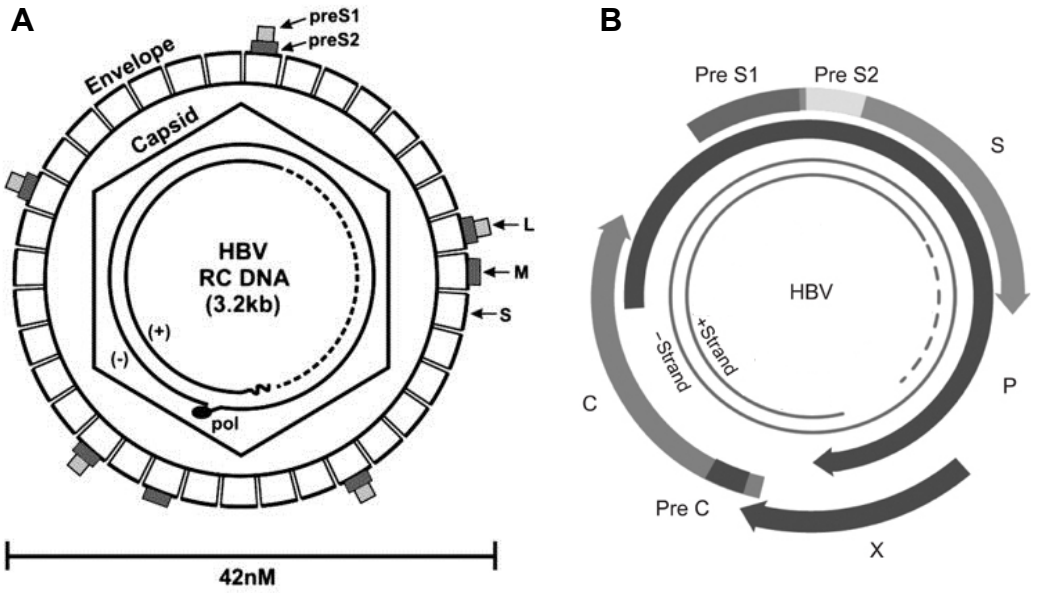


Figure 1.8; The HBV virion and a schematic representation of the virus' compact coding genomic organisation.

Abbreviations: Core (pre-C/C) and Polymerase (P or pol) proteins, and envelope surface glycoproteins (pre-S1/S2/S, or HBsAg). *Adapted from* [26] and [93], respectively.

The HBV genome is unique in the way that the two DNA stands comprising it are actually asymmetric, with the minus strand being complete while the plus strand is incomplete with variable 3' ends, hence resulting in the characteristic partially double-stranded genome – with an overlapping DNA fragment as well as a single-stranded region; a feature exclusive to the *Hepadnaviruses*. In addition, what is unique about the genomic organisation of this virus is the fact that the ends of the full-length minus strand are not covalently bonded but instead the 5' end of the strand is linked to the viral polymerase. On the other

hand, the 5' end of the incomplete plus strand has a capped ribonucleic acid (RNA) oligomer, denoted as the RNA primer (or otherwise the wavy tail) (*Figure 1.8; A* and *Figure 1.9*). The 5' ends of both strands contain direct repeats (DRs), which are short repeat sequences that are crucial for virus replication but also help pair the two strands together which hence obtain this characteristic rc formation (*Figure 1.9*). The rcDNA is eventually converted into the covalently closed circular DNA (cccDNA) in the host nucleus, where infection persists in this form [88, 90, 94-96].

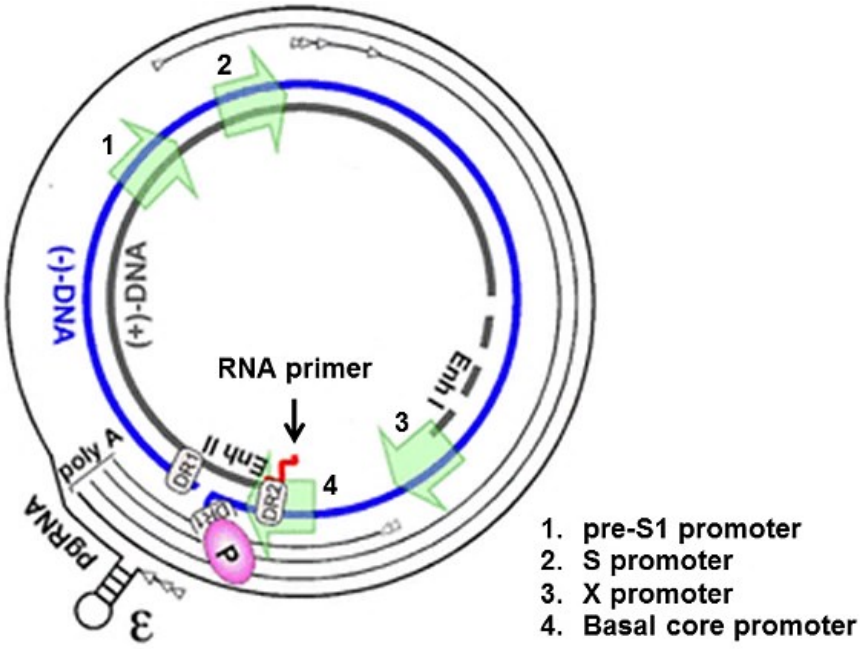


Figure 1.9; Organisation of the HBV rcDNA genome, and representation of the transcriptional regulatory elements.

The four major ORFs are once again indicated (outer lines), as well as the approximate location of the promoter (green arrow heads) and enhancer (Enh I and II) regulatory elements. ε is the RNA encapsidation signal on the pregenomic RNA. The viral polymerase is also denoted and is specifically attached to the 5' end of the minus strand, while the 5' of the plus strand has an RNA primer. DR1/2 sequences indicate the positions of the direct repeats necessary for viral replication. *Adapted from* [95, 96].

The highly regulated coding region of HBV is another unique feature of this virus, where the same nucleotides or codons are utilised by one or more coding regions or ORFs for gene transcription (*Figure 1.8; B* and *Figure 1.9*) [26, 97]. The four viral proteins encoded by the virus, as previously mentioned, are the: core and polymerase proteins, envelope glycoproteins

and the X-protein, whose transcription is tightly regulated by promoter and enhancer elements specifically encoded by the virus. The viral genome, in addition, encodes for various signals used for polyadenylation and encapsidation (*Figure 1.9*) [90, 95, 96]. *For further details on encapsidation, please refer to Section 1.2.6.*

HBV core protein

The core protein is a structural protein that encodes for the nucleocapsid, which encapsidates the HBV genome, as well as the HBeAg, which is the final product generated by post-translational processing of the precore (*see Section 1.2.6 for further details*) [43, 90, 98-100].

HBV polymerase protein

The HBV polymerase, on the other hand, is the central enzyme involved in the virus' genome replication. This has both DNA polymerase, reverse transcriptase and RNase H activity, also allowing for RNA encapsidation (*see Section 1.2.6 for further details*) [43, 90, 98-100].

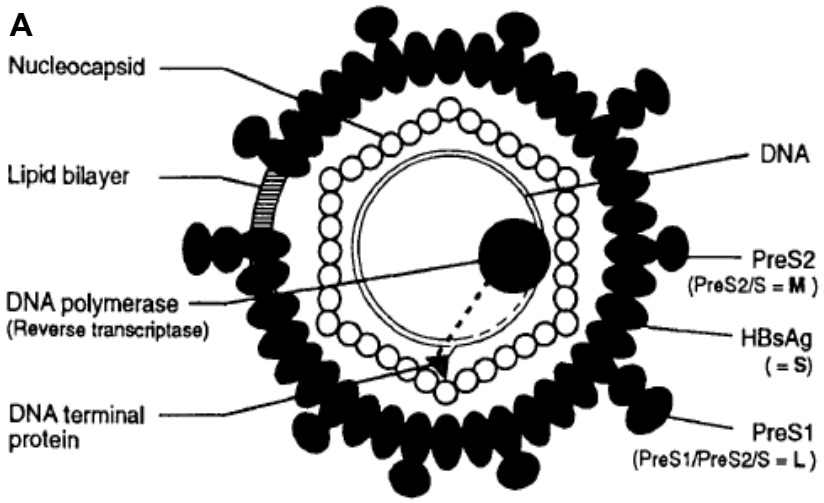
HBV X-protein

The X-protein's function is still enigmatic but it appears to be a non-structural multifunctional regulatory protein and probably is indispensable for virus survival [43, 90, 98-100]. Certain studies have implicated this protein in virus propagation, transcription, cell cycle progression, apoptosis, signal transduction and protein degradation amongst others in the host cells, while it also appears to have oncogenic properties [101, 102].

HBV surface glycoproteins

The surface genomic region particularly encodes for three separate envelope glycoproteins. In more detail, the envelope of HBV specifically consists of three related glycoproteins termed small (S), middle (M) and large (L),

encoded by a single ORF [26, 30, 47]. These are multiple transmembrane spanners (*Figure 1.10; A* and *B*) that share the same carboxyl (C) -terminal domain corresponding to the S-protein, while they differ greatly on their N-terminal domain. Thus, in addition to the shared S-domain, the M-protein contains an extension of a few amino acids termed the pre-S2, while the L-protein has an extra N-terminal myristoylated extension called the pre-S1 in addition to the pre-S2 (*Figure 1.10; C*) [25]. The latter is thought to play a key role in HBV receptor binding and entry into hepatocytes [26], while similarly the integrity of the S-protein domain is also regarded as critical [28, 32, 89, 90]. Studies have shown though that the pre-S2 domain is dispensable for HBV infectivity [103, 104]. However, as knowledge on HBV entry is currently scarce, it may be the case that all three glycoproteins bear candidate attachment sites for the host cells and therefore identification of these sites is of great importance for better understanding of HBV entry and infection, and most importantly, for future research and possible identification of new therapeutic compounds targeted to interfere with and block these stages of the virus' life cycle.



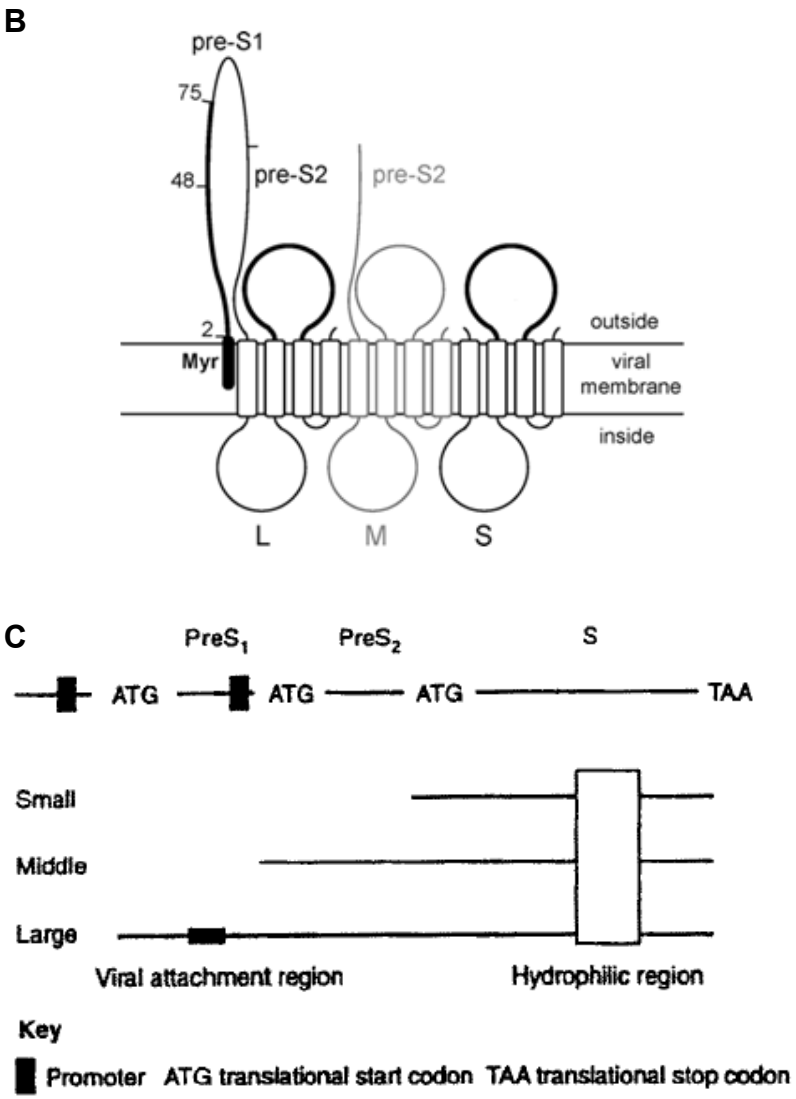


Figure 1.10; Structural organization and expression of the HBV pre-S1/S2/S genes.

(A, B) The HBV glycoproteins are transmembrane spanners that are precisely embedded within the outer envelope of the HBV virion. **(C)** The three proteins, pre-S1, pre-S2 and S, each have their own unique ATG translational start codon but share the same TAA stop codon. Therefore, the surface proteins have a common S-domain to which the pre-S2, or the pre-S1 and pre-S2, domains are optionally added hence forming the respective M- and L- proteins. The whole sequence is known as HBsAg. *Source:* [105], [103] and [106], respectively.

1.2.6 HBV life cycle and replication

The HBV life cycle has been studied extensively over the last decades, allowing for a vast amount of knowledge to be acquired. The virus' life cycle depends specifically on reverse transcription of a longer than full-length RNA copy of its genome termed the pregenomic (pg) RNA, making this virus'

replication similar to that of a retrovirus but with a DNA genome [26, 47, 89, 100].

In brief, HBV as a typical virus follows the same early stages of infection in the target host as other viruses, meaning that there is an initial liquid-phase diffusion followed by a low-affinity attachment to the target cell [107]. Certain signalling events allow the virus to recognise and bind, with the help of its envelope glycoproteins and especially the pre-S1 domain, to its specific surface receptor on the host cell membrane. The virus is then thought to be internalised via endocytosis and membrane fusion, which helps the virus remove its outer lipid layer and expose its nucleocapsid. Following entry, and particularly for the virus to initiate the replication of its genome, the viral core particles migrate to the nucleus of the cell where specific intracellular signals cause them to disintegrate and deposit the virus' genetic material. This then enters through the nuclear pore complexes (NPC) found within the nucleus membrane into the nucleus [33, 38, 47, 70, 98].

Following this stage in the virus' life cycle, is when the HBV replication cycle differs to retroviruses and also other viruses, as HBV replication depends on a not so common process that involves reverse transcription of a RNA pregenome through a cccDNA intermediate [88, 90, 105, 108, 109]. Therefore, the route of reverse transcription and subsequent DNA replication of HBV is fundamentally different from that of retroviruses, which in short enter the host cell after which the reverse transcriptase converts the retroviral RNA genome into a full-length double-stranded DNA genome that will further travel to the cells' nucleus where it will become integrated into the host genome. Subsequently and typically to other viruses, viral genes are transcribed and translated, new particles are assembled and these will exit the infected cell and move on to enter other cells and thus spread the infection [96, 100, 110, 111].

The life cycle of HBV is summarised in Figure 1.16.

1.2.6.1 HBV entry into host cells

HBV, in general, has a very restricted host range infecting only humans, chimpanzees and treeshrews – *Tupaia belangeri*, while the virus also has an extraordinary tropism to infect parenchymal liver cells of its host [26, 112, 113]. Its host and organ specificity may in fact be directly related to its need for hepatocyte-specific receptor(s), as well as additional host factors, for virus entry and infection [28, 30, 32, 114-118]. HBV research has unfortunately been considerably impeded over the years by the lack of understanding HBV entry, and specifically the precise mechanism the virus utilises to infect human liver cells. Previous research has shown that HBV infection is restricted at the host entry level making it probably the sole contributor of the restricted HBV species tropism. These studies particularly showed that HBV infection is indeed supported, for example in transgenic mice, but only when the viral entry stages are completely bypassed [30, 88, 119, 120].

At present, HBV binding and entry into liver hepatocytes is known to be facilitated with the help of its viral envelope glycoproteins, with the most critical site being its N-terminal myristoylated pre-S1 domain of the L-protein [30, 32, 121, 122]. The pre-S1 domain is approximately a 119 amino acid (aa) sequence, depending on the HBV viral subtype, with the unique N-terminal attachment epitope being mapped to aa 21-47. This was later narrowed down, with the use of current and more advanced technologies, to a small epitope sequence, *QLDPAF* (*Figure 1.11*). This sequence is now known to be crucial for cell attachment [121, 123, 124], and therefore, the pre-S1 region has been believed to be the primary determinant of host infectivity [113, 122, 125], while exposure of the myristoylated peptide/ protein is commonly used by other viruses to facilitate entry [89, 126, 127].

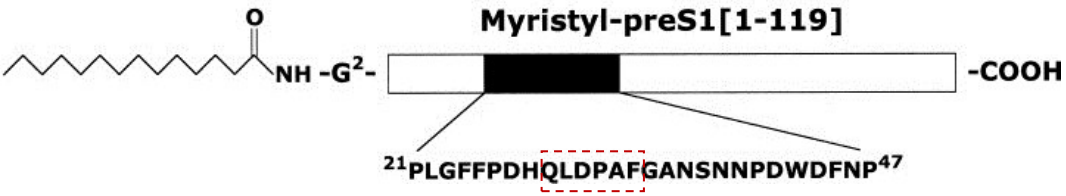


Figure 1.11; Schematic diagram of the myristoylated pre-S1 domain of the HBsAg.
The 21-47 aa protein region is shown in full, and the crucial site for virus attachment to the host cells is indicated by the red, dashed box. *Adapted from* [128].

Interestingly, however, further research eventually revealed that the crucial epitope sequence is in fact not as unique as hoped and hence could not entirely justify the host and organ specificity of HBV. In reality, this sequence remains common amongst viral and bacterial proteins, and in all cases it plays a role in cell adhesion, attachment and fusion. Therefore, it is possible that this sequence has a more general role in viral entry and infection, further suggesting that other crucial epitopes may exist either in the extended region of the pre-S1 domain or even outside this specific region [121, 123, 125]. Past and follow up studies did and continued to reveal that in fact approximately the first 77 aa of the pre-S1 region are indispensable for efficient virus infectivity, so it is not impossible that other important epitopes for virus attachment and entry exist within the pre-S1 domain [28, 129, 130]. For example, from previous research, we know that aa 9 to 15 of the pre-S1 domain are actually believed to be essential for binding of the virus to its receptor, by specifically forming a receptor binding motif [30, 31, 131, 132]. However, other sites outside of the pre-S1 region could additionally facilitate recognition or binding of the virus at the host's cell surface hence resulting in virus entry [89, 130]. In particular, a second epitope outside of the pre-S1 domain has already been suggested to be involved in cell attachment [123]. Therefore, it is possible that, even though the pre-S1 domain contains the major cell attachment epitope(s), there is some synergistic interplay between the HBV envelope glycoproteins for productive attachment and entry of the virus into hepatocytes [124].

The exact mechanism(s) of attachment and productive entrance into the host liver cells, however, are still enigmatic [89, 133]. The general consensus is that attachment and entry of a virus to its host cell typically depends on more than one interaction with cellular receptors, usually starting from lower to higher affinity. For example, in the case of hepatitis C virus (HCV) there are at least nine receptors currently reported to be involved in productive virus entry and infection of hepatocytes [134]. In the case of HBV our current knowledge states that entry into hepatocytes is separated into two distinct steps. There is an initial low-affinity interaction with heparan sulfate proteoglycans (HSPGs), including glypican 5 (GPC5) [135], on the hepatocyte surface mediated by the pre-S1 region of the L-protein and the antigenic loop of the viral S-protein (*Figure 1.10; B*), followed by the attachment of the virus to its recently identified high-affinity sodium taurocholate cotransporting polypeptide (NTCP) receptor, that results in endocytosis of the HBV particles, fusion with the cell membrane and further viral infection (*Figure 1.12*) [32, 116, 131, 132, 136]. Certain drugs can block HBV entry by specifically leading to the degradation of the NTCP receptor [137].

HSPGs are characteristically used by a number of other viruses as primary attachment sites for viral entry, examples include human papillomavirus (HPV) [138] and HIV [139] amongst others. Moreover, even though a number of other HBV host factor/receptor candidates have been reported over the years [89, 115, 121, 133, 136, 140], none of them have been proven to support functional viral infection, as well as overcome the interspecies transmission barrier.

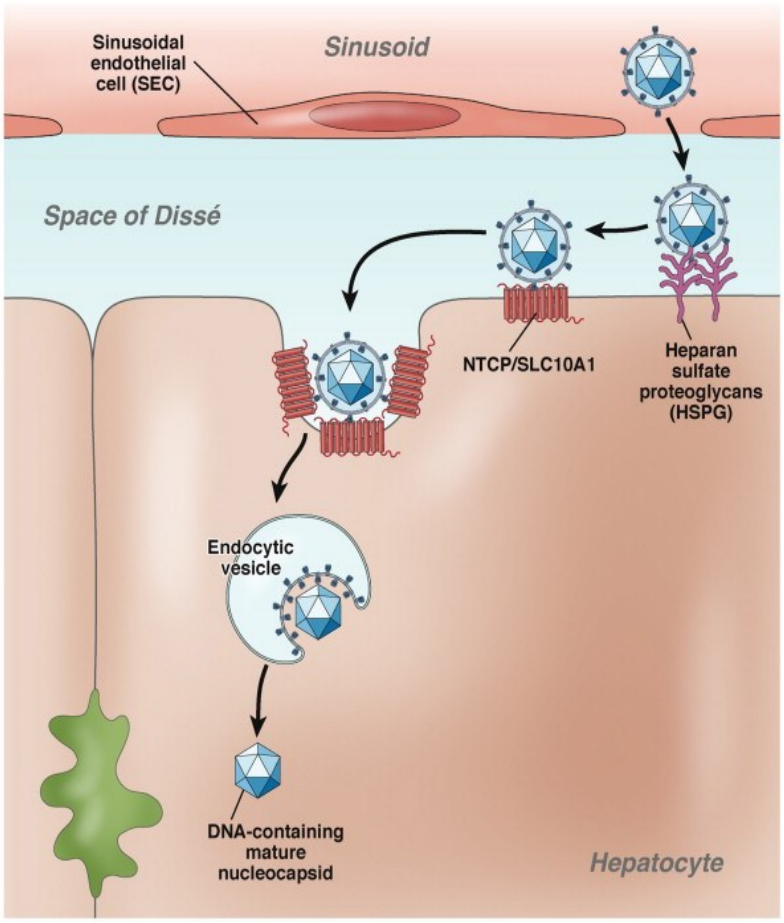


Figure 1.12; Model of HBV entry into hepatocytes.
HBV interacts presumably weakly with its primary binding partner, HSPG, further allowing the virus to slowly move along the surface of the cell until it recognises its high-affinity specific receptor, NTCP, which it then binds to mediating its entry into the cell by endocytosis. *Source:* [141].

Moreover, the virus' internalisation via endocytosis and membrane fusion actually plays an important role in allowing the virus to lose its outer lipid bilayer and hence it is able to release its nucleocapsid into the host cell. It has previously been reported that the presence of low pH within the endosome could be vital during this process, as by inducing conformational changes to the HBV envelope glycoproteins it may actually promote release of the viral nucleocapsid [125]. The nucleocapsid is specifically encoded by the core protein and contains the virus' genome. This viral capsid then migrates to the cell's nucleus where it typically disintegrates and deposits the viral genetic material, which then enters into the host's nucleus through the NPCs embedded within the nuclear membrane [33, 38, 47, 70, 98].

Nevertheless, the steps following HBV internalisation and transport of its nucleocapsids to the host's nucleus are still somewhat elusive, but it is thought to involve active transport and a microtubule-dependent mechanism. Once the capsids arrive to the host's nucleus they interact with importin-α/β receptors that allow them to pass through the NPC into the nuclear basket. These interactions and binding to specific receptor(s) can be direct via recognition of a nuclear localisation signal (NLS) by importin-β, or indirect by binding to importin-α followed by attachment to importin-β, and may also involve certain phosphorylation events. Protein kinase C (PKC) is reported to be involved in these phosphorylation events, as well as two other major cellular kinases, SR protein-specific kinase 1/2 (SRPK1/2), amongst others [142, 143]. Once in the nuclear basket the mature viral capsids, containing the rcDNA, are recognised by particular nucleoporins, such as Nup153, and are allowed to disintegrate and release their viral genome passively through to the nucleus, while the immature viral capsids stay arrested and cannot progress any further (*Figure 1.13*) [144, 145].

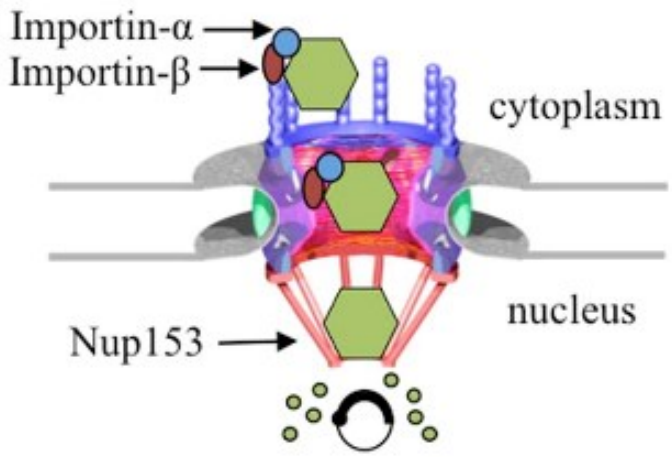
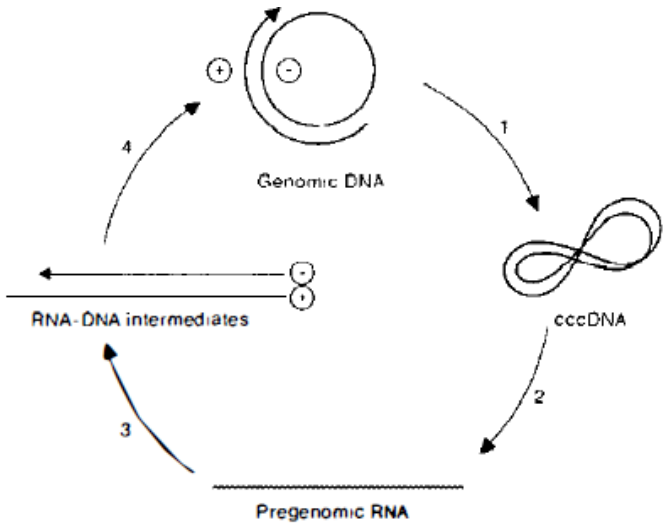


Figure 1.13; HBV genome entry into the nucleus of a host cell via the NPC.
A HBV core capsid is recognised and further interacts with the two importin-α/β receptors, leading to its translocation across the NPC where, with the help of Nup153, it disassembles within the nuclear basket, which is followed by the entry of the virus' genome into the host's nucleus. *Source:* [146]

1.2.6.2 HBV replication cycle

Hepadnaviruses typically use the transcriptional machinery of the host cell for their genomic replication. In particular, once the HBV genome has entered the nucleus it is initially repaired so as to allow formation of the cccDNA. During this repair step, the ds, rc form of the HBV DNA is purposely converted into the cccDNA (*Figure 1.14; A*), a process which primarily relies on the host cellular repair mechanism and principally involves the completion of the plus strand of the dsDNA. This repair mechanism for the conversion to cccDNA is still poorly understood but it is thought to involve the removal of the covalently bound polymerase as well as the RNA primer, allowing for DNA synthesis and ligation to form the closed circular dsDNA [144]. The viral polymerase may also take part in this repair mechanism [88]. The cccDNA, which is formed as a nucleosome-bound minichromosome, is the template for transcription of the viral messenger (m) RNA, a process that takes place on the negative strand of the cccDNA with the help of the host cellular RNA polymerase II [53, 94]. The resulting viral mRNA transcripts specifically refer to the products of the subgenomic (sg) and pg RNA; these are capped and polyadenylated with the help of the unique polyadenylation signal present within the virus' genome (*Figure 1.14; B*) [90, 147].

A



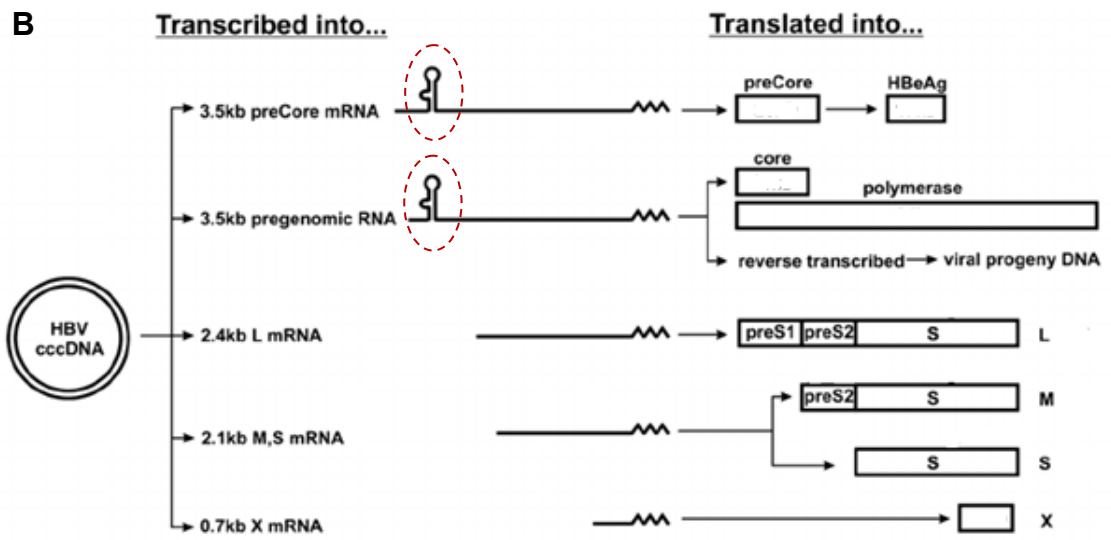


Figure 1.14; (A) HBV replication via reverse transcription of the viral DNA. (B) The products of cccDNA transcription and the respective proteins produced.
(A) The four stages, in summary, are: 1. Repair and conversion of the genomic DNA to the cccDNA minichromosome, 2. cccDNA is then transcribed for the generation of the pgRNA, 3. The pgRNA is used as a template for reverse transcription, when the first strand of viral DNA is synthesised, and 4. The second strand of the viral DNA is synthesised resulting in the production of the mature genomic DNA. *Source:* [108]. **(B)** The cccDNA minichromosome is specifically transcribed into five RNA products, which are either initially encapsidated (precore and pgRNA) or directly translated into the indicated proteins. In the case of the precore product, this is further cleaved for the generation of the HBeAg. The ε encapsidation signal is shown in the red, dashed circles. *Adapted from* [26].

In more detail, the sgRNA transcripts are the 2.4kb, 2.1kb and 0.7kb mRNAs, which respectively translate into: the longest one translates into the full-length HBsAg envelope glycoprotein (L), the middle transcript translates into the other two forms of the envelope glycoproteins (S and M), and the smallest transcript translates into the X-protein. Translation of all viral transcripts is initiated in the cytoplasm of the infected cell. Moreover, one of the two longer transcripts (3.5kb), the precore, translates into the HBeAg, with the help of the unique encapsidation signal. The second longer transcript, the pgRNA, eventually translates into the core and polymerase proteins. This, however, is also utilised as a template for reverse transcription and further synthesis of viral DNA, a process which relies on the encapsidation of the pgRNA by the viral polymerase through recognition of the ε encapsidation signal on the 5'

end of the pgRNA. All transcripts have the same 3' ends but differ significantly on the 5' ends (*Figure 1.14; B*) [109, 147, 148].

Finally, progeny viral capsids assemble and these incorporate the viral pgRNA together with the viral polymerase. The pgRNA within the progeny nucleocapsids then goes through stages of maturation and is eventually reverse-transcribed by the viral polymerase into viral rcDNA (*Figure 1.14*; A) [90, 100, 109]. The stages of pgRNA maturation and eventual reverse transcription for production of rcDNA particularly include:

a. Translation of pgRNA for the production of the viral polymerase. Encapsidation of the pgRNA by the viral polymerase, through recognition of and binding to the ε stem-loop encapsidation signal structure at the 5' end of the pgRNA. Assembly of immature core particles.

b. Initiation of minus strand synthesis, with a complementary four-nucleotide (nt) ε-sequence being initially synthesised. The viral polymerase and the few nucleotides synthesised translocate to a complementary sequence, DR1, near the 3' end of the pregenome, which particularly involves a "first primer shift". Primer shifting is a complex and not well understood mechanism that is particularly facilitated by the DRs. Synthesis continues towards the 5' end of the circular pgRNA template.

c. The minus strand synthesis is completed from 5' to 3' end via reverse transcription using the pgRNA as a template. The pgRNA is finally degraded by the viral polymerase via its RNase H digestion activity, however a small sequence of approximately 15-18 nucleotides,

including the 5’ capped end and the DR1, is not removed during this process.

d. A “second primer and template shift” takes place, during which the remaining capped RNA oligomer primer sequence, including the DR1 sequence and the few remaining nucleotides, is reallocated from the 3’ end of the minus strand to the 5’ end of the DR2 of the plus strand, where the minus strand is finally used as a template for further synthesis of the plus strand. On occasions, when this second priming event fails, double stranded linear HBV DNA is formed instead.

e. Plus strand synthesis initiates using the minus strand as a template, and this is the point when the genome becomes circular. Re-circularisation of the genome specifically aids the plus strand to gain access to the minus strand and utilise as a template for further elongation. Plus strand synthesis, however, remains incomplete and thus the rcDNA genome is formed. The nucleocapsids are now considered as mature particles.

f. rcDNA is then repaired to once again form cccDNA, and so forth.

HBV goes through constant cycles of the same replication stages (*Figure 1.15*). Post-translational modifications of the core particles are thought to possibly occur during this process of genome replication and maturation [90, 100].

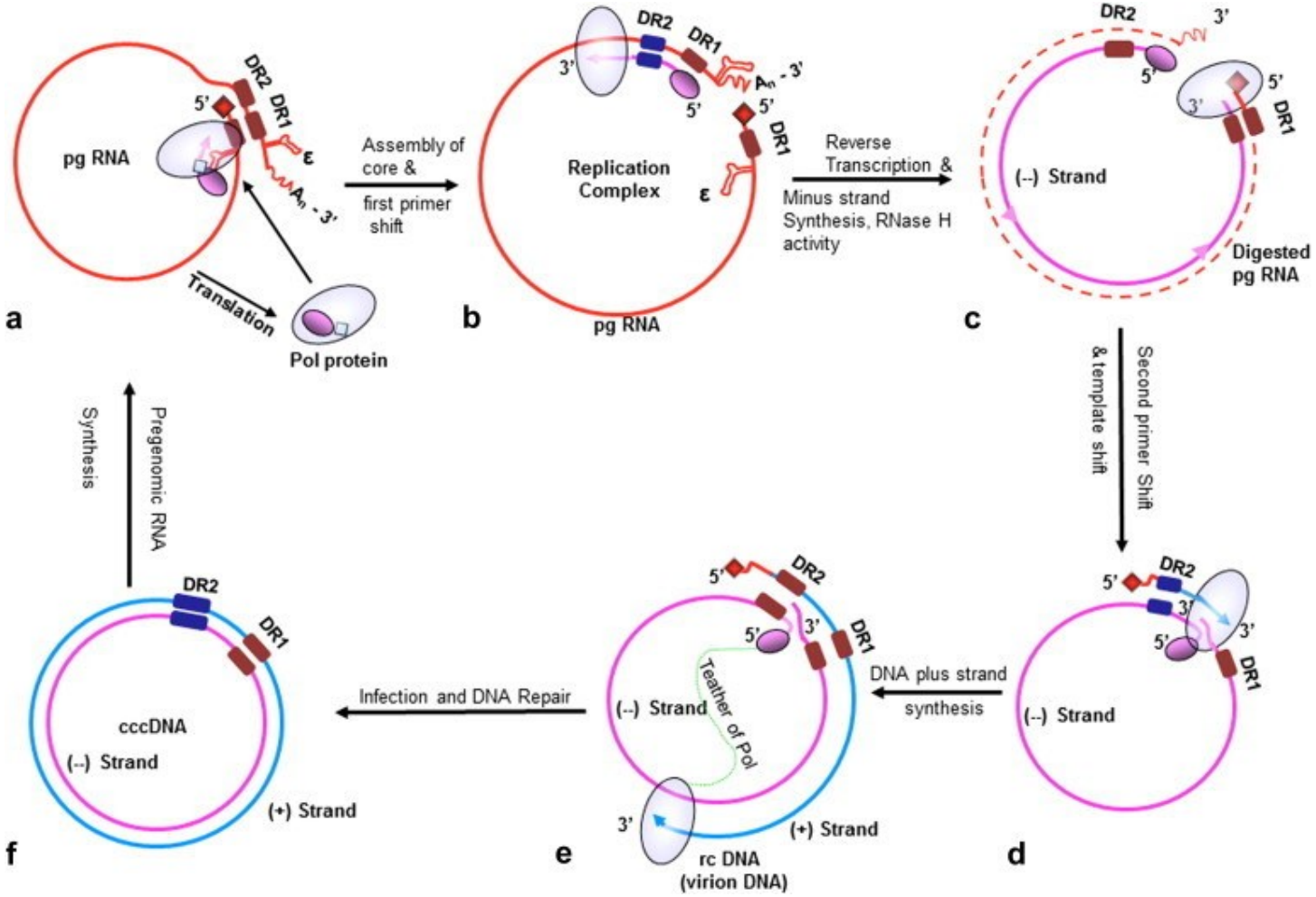


Figure 1.15; The constant cycle of HBV genome replication.
See above for details of each step. *Source:* [90]

The resulting core particles can then follow two distinct pathways; they can either egress by budding into the endoplasmic reticulum (ER) where they are further enveloped and exported from the cell so as to continue infecting other host cells, or they can continue to recycle their genomes into the nucleus of the particular cell for conversion to cccDNA, hence constantly maintaining the virus reservoir within that cell [17, 43, 88, 90, 100, 109, 149-151]. The latter is a critical step of viral persistence and is believed to be one of the main obstacles for curing the disease [96]. The entire genomic replication cycle of HBV is summarised in *Figure 1.16.*

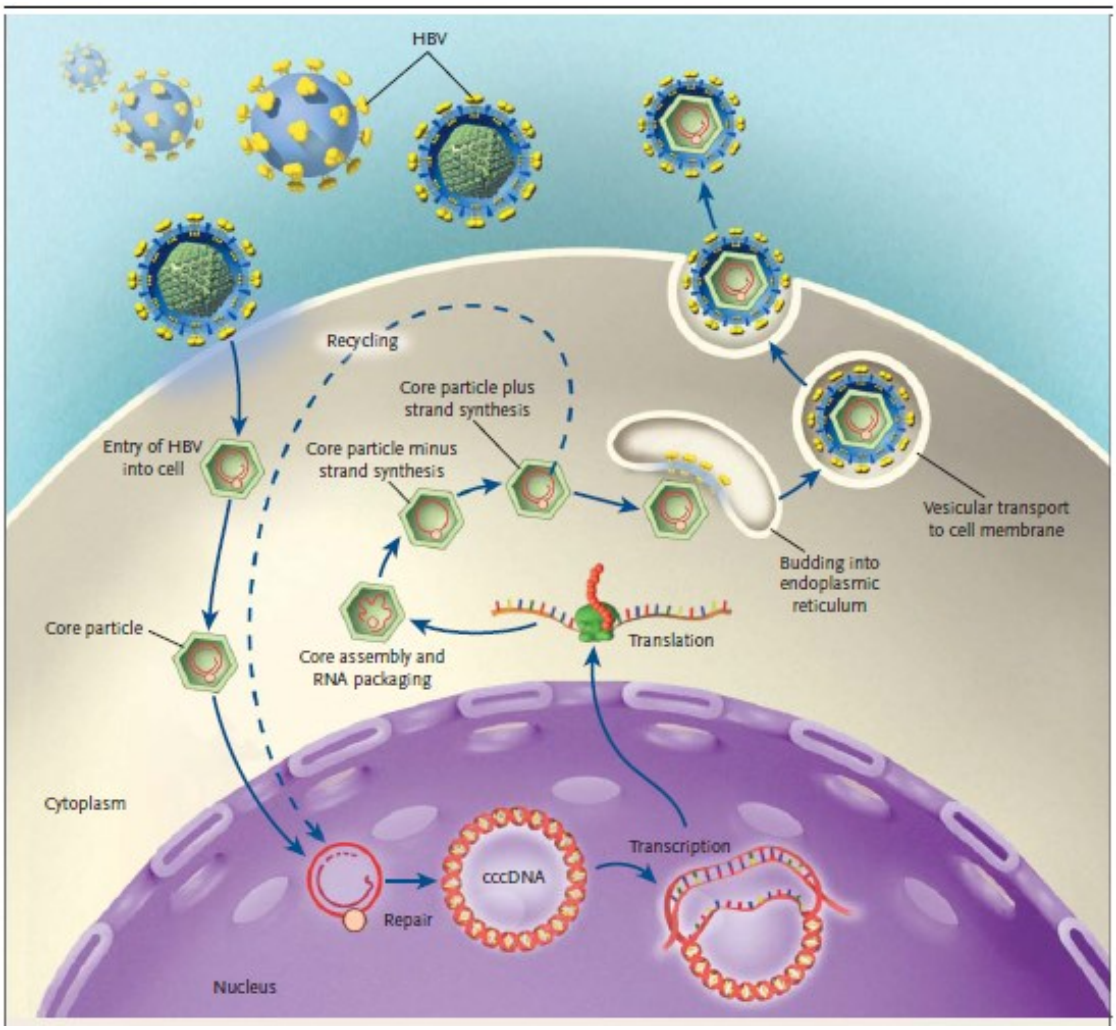


Figure 1.16; HBV life cycle, and the virus' replication strategy.
The HBV life cycle is typical in certain ways to the replication of other viruses and particular retroviruses, but also differs significantly. HBV is characterised as a retrovirus with a DNA genome. *Source:* [47].

Despite the increase of knowledge surrounding the life cycle of this virus, the mechanisms of attachment and productive entrance into the host liver cells are still puzzling, primarily due to the lack of suitable *in vitro* infection systems to allow the in depth study of HBV infection [89, 133]. However, current technologies have allowed the establishment of some long-awaited *in vitro* systems for studying HBV infection and replication, and have most importantly led to the discovery of a HBV-specific receptor. It now seems that there is some light at the end of the tunnel for research on HBV entry, while present discoveries should also optimistically lead to the discovery of additional HBV-specific factors essential for overcoming the HBV interspecies transmission barrier, which are further necessary for the creation of a small animal model that can fully recapitulate the HBV life cycle and hopefully lead to the discovery of a cure for HBV.

1.2.7 The HBV liver-specific receptor, NTCP

In 2012, Yan and his colleagues finally reported NTCP as the long-sought receptor for HBV and hepatitis delta virus (HDV) infection [32, 152, 153]. However, since the early 1990s researches were reporting a candidate with similar characteristics to NTCP, but due to lack of advances in technology in those years the HBV bona fide receptor could not be fully characterised and identified [140, 154]. The NTCP receptor characteristically is a glycosylated multiple transmembrane transporter, with seven to nine predicted transmembrane domains, that is predominantly expressed in the liver (*Figure 1.17; A*). Its polypeptide is exclusively localised to the basolateral membrane of the hepatocytes, and is normally involved in the maintenance and circulation of bile acids in the enterohepatic area of the body (*Figure 1.17; B*) [30, 32, 116, 120, 155-157]. The correct glycosylation of the receptor is believed to be indispensable for its trafficking to the cell surface as well as its stability, and is also essential for productive viral infection [158]. NTCP dimerisation has also been confirmed, with the individual subunits of the protein having been shown to be functional. In the same study performed by Bijsmans and his colleagues they specifically demonstrated that

heterodimerisation of the NTCP receptor may on occasions impair its trafficking to the plasma membrane hence negatively affecting bile salt uptake [159]. However, these aspects of the human NTCP (hNTCP) biology have not been extensively studied yet, and a lot more research is needed to fully understand the characteristics and the regulation of hNTCP expression in the liver and particularly on the hepatocytes surface, including the trafficking, docking, structural conformation and importantly stability of the receptor at its main sites within the cell's membrane.

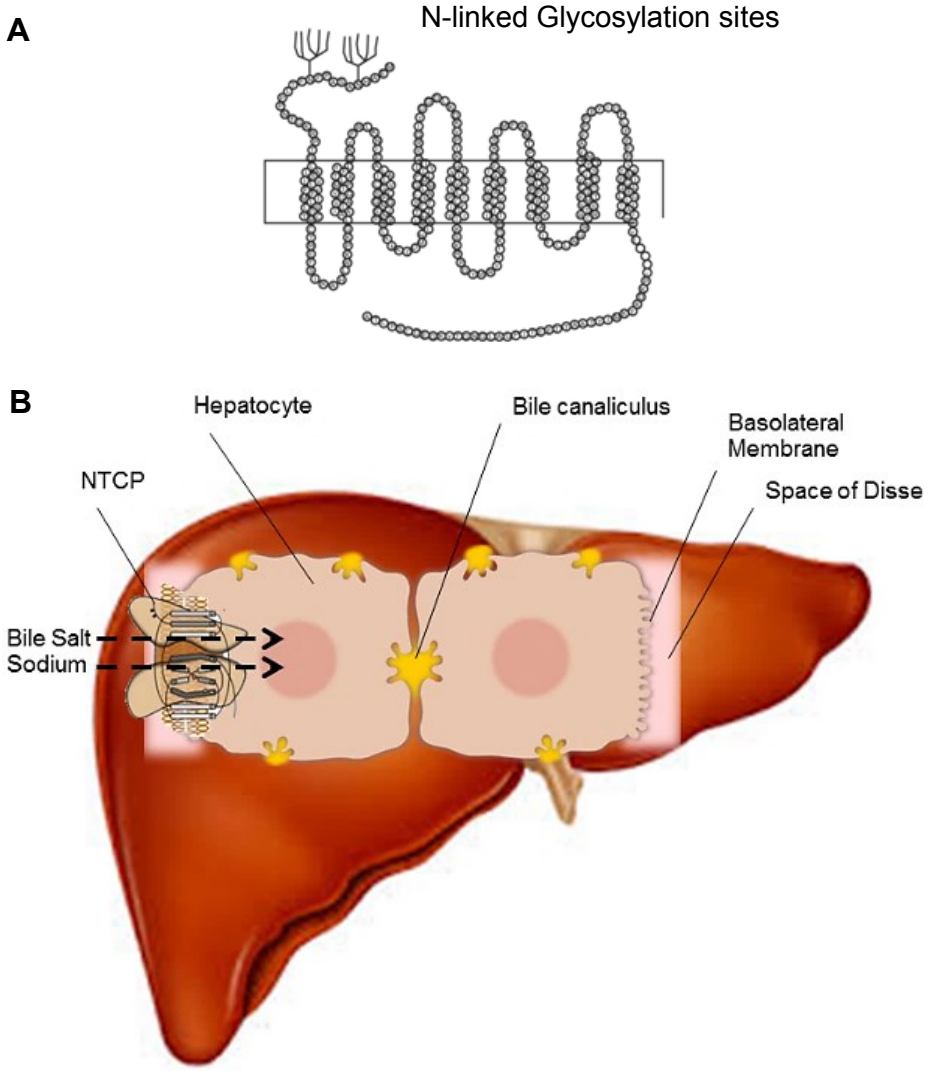


Figure 1.17; (A) Schematic of the multiple transmembrane transporter, NTCP; (B) Representation of the location and function of the NTCP receptor.

(A) The NTCP receptor is predicted to have between seven to nine or even ten transmembrane domains. Its N-terminus has two reported N-linked glycosylation sites and is facing the extracellular space of the cell, towards the space of Disse. The C-terminus is found intracellularly within the hepatocyte. *Source:* [158]. **(B)** The NTCP receptor is specifically found on the basolateral side of hepatocytes, where it has a pivotal role in maintaining the bile acid circulation and homeostasis.

Yan and his colleagues specifically identified NTCP by creating a lipopeptide containing the first 48 aa of the pre-S1 domain, which was then utilised in advanced biochemical techniques like near zero distance photo-cross-linking and tandem affinity purification. However, specific residues from the synthetic peptide were replaced by photo-active non-natural amino acids, such as L-photo-leucines. This was next used as a ligand for recognising and binding to the receptor. In particular, these researchers used ultraviolet (UV) irradiation at 365nm that induced changes to the photo-active peptide ligand further allowing covalent cross-linking at almost zero distance. The cross-linked peptide and the associated partner(s) were then purified through standard streptavidin bead precipitation procedures and separated on a polyacrylamide gel. A monoclonal antibody was specifically designed with a biotin tag at the C-terminus. This antibody recognised a particular epitope adjacent to the critical receptor-binding region. The target protein/ receptor was finally identified through stages of affinity purification followed by mass spectrometry [32, 153].

NTCP in humans is encoded by the gene known as *SLC10A1*, for solute carrier family 10 member 1. NTCP is one of the seminal transporters belonging to this family of solute carriers (*SLC10*), as it is the key player and rate-limiting mediator of bile acid uptake and homeostasis, responsible for over 80% of conjugated bile acid influx into liver hepatocytes, an area where it is typically highly expressed (*Figure 1.18*) [156, 160]. NTCP is particularly located within membrane rafts, where its function is modulated by cholesterol [161]. In general, its expression and function in the liver are very tightly regulated by a number of other factors as well as by its own microenvironment [153, 156, 159]. This could possibly also explain the strict tissue/ organ specificity of HBV infection.



Figure 1.18; RNA and protein expression summary of the *SLC10A1* gene.
Source: The Human Protein Atlas

*(*http://www.proteinatlas.org/ENSG00000100652-SLC10A1/tissue*).*

As previously mentioned, HBV apart from the strict liver tropism, which nicely matches specific liver expression of the NTCP receptor, also has a very narrow species tropism (humans, chimpanzees, and treeshrews). Human NTCP (hNTCP) is 349 aa in length, and this is translated to approximately a 50 kiloDalton (kDa) glycosylated protein (and approximately 35kDa when the

protein is deglycosylated) [162]. *Tupaia* NTCP (tsNTCP) is a bit longer in length compared to hNTCP (379 aa), but shares approximately 84% protein sequence identity with hNTCP (*Figure 1.19*) [32]. Unfortunately, up to date there have been no functional studies regarding the chimpanzee NTCP receptor, probably mostly due to ethical reasons. Therefore, most current studies have focused on trying to understand the rate limiting factor(s) for HBV infection in naturally non-susceptible smaller primates or other hosts [30, 32, 117, 118, 120, 163-165]. These studies have concluded that NTCP largely determines species specificity of HBV entry and infection.

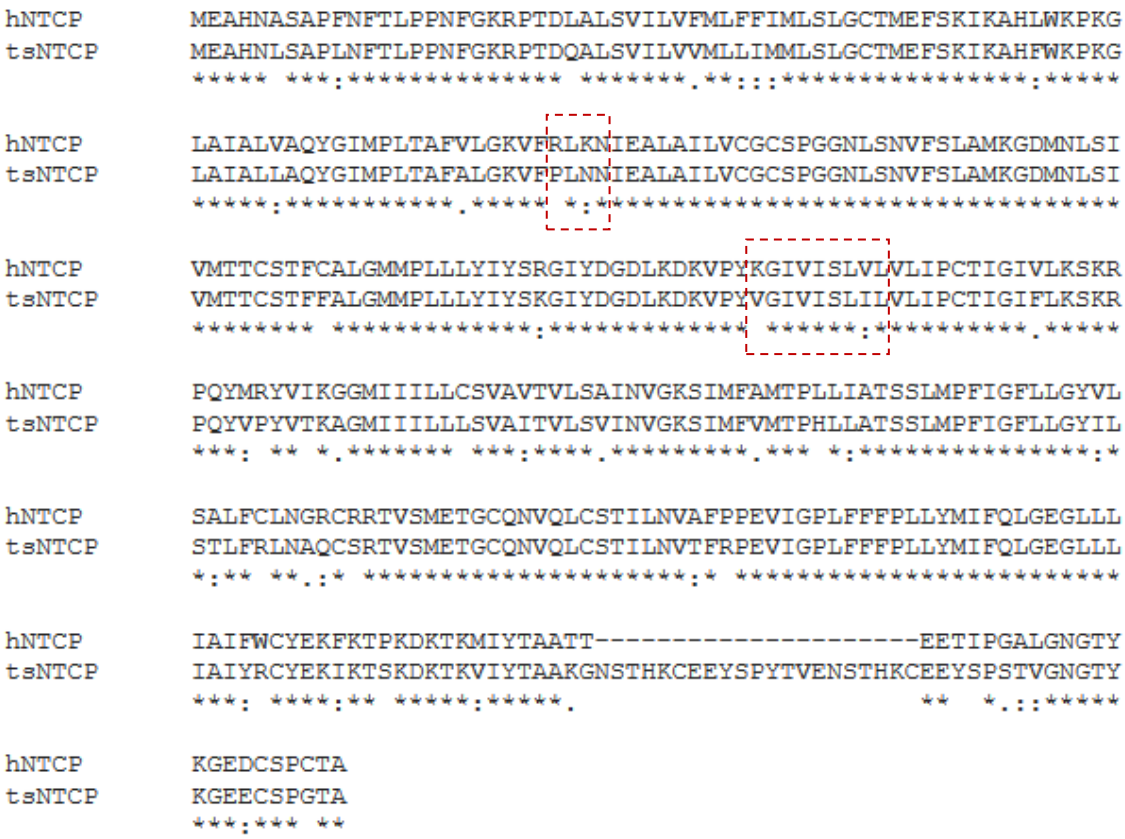


Figure 1.19; Sequence homology analysis between human and *Tupaia* NTCP.
Analysis was performed using the Clustal O (1.2.4) multiple sequence alignment tool. Genebank extension numbers are: hNTCP – NM-003049.3 and tsNTCP – JQ608471.1. The red dashed boxed indicate motifs (residues aa 84-87, and aa 157-165) predicted to be important for recognition and binding to the HBV pre-S1 domain, leading to subsequent viral infection.

NTCP has been shown to bind HBV and HDV viral particles through interaction and attachment to precise pre-S1-related envelope binding sites [30, 32, 166-168], with two specific studies playing a big role in our understanding of virus/host receptor interaction and attachment on the hepatocyte surface. In more detail, Yan and his colleagues, apart from identifying the NTCP receptor as the functional receptor for HBV and HDV, they were also the first to characterise the importance of aa 157 to 165 belonging to hNTCP for virus entry and subsequent infection. These residues were specifically found to be absolutely critical for binding the pre-S1 domain of the virus. The researchers were able to determine this by creating a series of mutated hNTCP variants that spanned the full-length of another NTCP receptor belonging to the crab-eating monkey (*Macaca fascicularis* NTCP – mkNTCP). The researchers investigated the mkNTCP for the reason that it shares more than 96% protein sequence identity with hNTCP, however typically it is non-susceptible and hence cannot support viral infection. During this experiment, they discovered that mutating residues within the 157-165 aa motif completely abolished binding of a synthetic pre-S1 peptide and the ability of hNTCP to support viral infection. On the other hand, replacing that motif in the mkNTCP with its human counterparts conferred susceptibility to viral infection [32].

Nearly a year later, the same group of researchers announced the crucial importance of another hNTCP motif for pre-S1 binding and viral infection; residues 84 to 87 aa. This time they created a series of human and mouse chimeras containing once again different point mutations, and they discovered that replacing the 84-87 aa from the mouse NTCP (mNTCP) with its human counterparts converted that receptor into a fully functional receptor for binding the virus. On the contrary, replacing the same motif from the hNTCP with the corresponding mouse residues, nearly obliterated the ability of hNTCP to support viral infection [30]. All studies mentioned were performed *in vitro*, using either primary isolated hepatocytes or liver hepatoma cell lines (for example HepG2 cells). Furthermore, we now know from follow up studies, that the HBV pre-S1 domain competes with bile acids for binding to the NTCP

receptor (*Figure 1.20*), and specific substrates can prevent this from happening by purposely blocking the virus' entry mechanism. Some of these inhibitors include NTCP substrates like taurocholate, as well as synthetic pre-S1 peptides like Myrcludex B, amongst other drugs [116, 168-170].

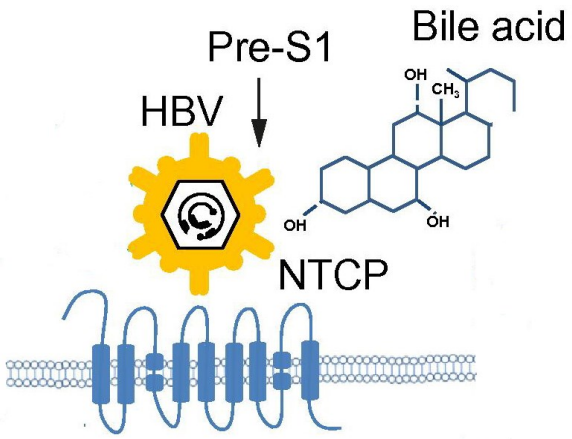


Figure 1.20; The pre-S1 domain of HBV competing with bile acids for entry through the NTCP receptor.
Adapted from [168].

Interestingly, it is noteworthy that humans, mice and rats exhibit marked species-specific differences in NTCP expression and regulation [156]. The mNTCP, as well as the rat NTCP (rNTCP), are 362 aa in length (molecular mass of around 55kDa) and approximately 75% identical to the hNTCP protein sequence (*Figure 1.21*) [30, 156, 162], which could provide an explanation in regards to the species-tropism of HBV. However, the mNTCP and rNTCP have a 91% protein sequence identity between them, as determined by sequence homology analysis using the BLAST® alignment tool. Over the years, rNTCP has been extensively studied.



Figure 1.21; Sequence homology analysis between human, mouse and rat NTCP.

Analysis was performed using the Clustal O (1.2.4) multiple sequence alignment tool. Genebank extension numbers are: hNTCP – NM-003049.3, mNTCP – NM001177561, and rNTCP – NM_017047.1. The red dashed boxed indicate motifs (residues aa 84-87, and aa 157-165) predicted to be important for recognition and binding to the HBV pre-S1 domain and subsequent viral infection.

In summary, it appears that the NTCP receptor is one of the main limiting factors that defines host specificity of HBV and HDV infection at the entry level, more so than the pre-S1 domain of the virus. However, substituting central residue motifs of this receptor from the naturally non-susceptible hosts with their human counterparts, even though it allows those receptors to become functional in regards to binding the virus some of them are still not fully susceptible to viral infection. Therefore, there appears to be a post-entry block in these non-susceptible hosts, suggesting that additional host factors

are necessary to overcome the interspecies transmission barrier [117, 171, 172].

1.2.7.1 The rat NTCP

Receptor functionality depends on its translocation and retention on the plasma membrane of hepatocytes.

Most studies up to date on the molecular mechanisms that govern NTCP translocation to the plasma membrane, followed by the receptors accurate docking and structural conformation that further results in the receptors stable expression, have primarily been performed on rat NTCP. However, as there is some sequence homology between human and rat NTCP maybe there are some lessons to be learned about human NTCP.

Rat NTCP (rNTCP) was the first sodium-dependent bile acid transported to be identified in the early 1990s, so it was the first one to be extensively studied [173]. Rat NTCP similarly to human NTCP localises to the basolateral membrane of the hepatocytes, and also has at least seven predicted transmembrane domains and two reported N-linked glycosylation sites [162, 174, 175]. Translocation of the rNTCP receptor to the plasma membrane and its specific sorting to the basolateral membrane has been shown to be modulated by precise dephosphorylation events triggered by the second messenger cyclic adenosine monophosphate (cAMP) [156, 176]. rNTCP has been reported to be a serine, threonine phosphoprotein and its membrane transport activity is indirectly facilitated by cAMP particularly via its dephosphorylation on residue Ser226 [174, 177, 178]. Originally this was thought to involve the phosphatidylinositol-3-kinase (PI3K)/ protein kinase B (PKB) signalling pathway, while it was suggested that the process required intact actin filaments and the presence of protein kinase C zeta (PKCζ). A second mechanism has also been proposed which involves the activation of a Ca^{2+}/calmodulin-dependent protein phosphatase 2B (PP2B) by cAMP, which then mediates rNTCP dephosphorylation and subsequent cAMP-induced

translocation to the plasma membrane (*Figure 1.22*). In the original study, wortmannin, a PI3K inhibitor, was shown to inhibit the specific cAMP-mediated translocation of rNTCP [179-182].

In addition, another study showed that hyperosmolarity caused the rNTCP to undock from the basolateral membrane of liver cells, an outcome which was demonstrated to be easily reversed by the presence of a NTCP substrate, tauroursodeoxycholate (TUDC), as well as cAMP that further indirectly dephosphorylated rNTCP on the particular serine residue and hence resulted in the re-insertion of the receptor back into the plasma membrane [183]. All-in-all it appears that NTCP requires cAMP for its productive subsequent dephosphorylation and trafficking to the cell surface.

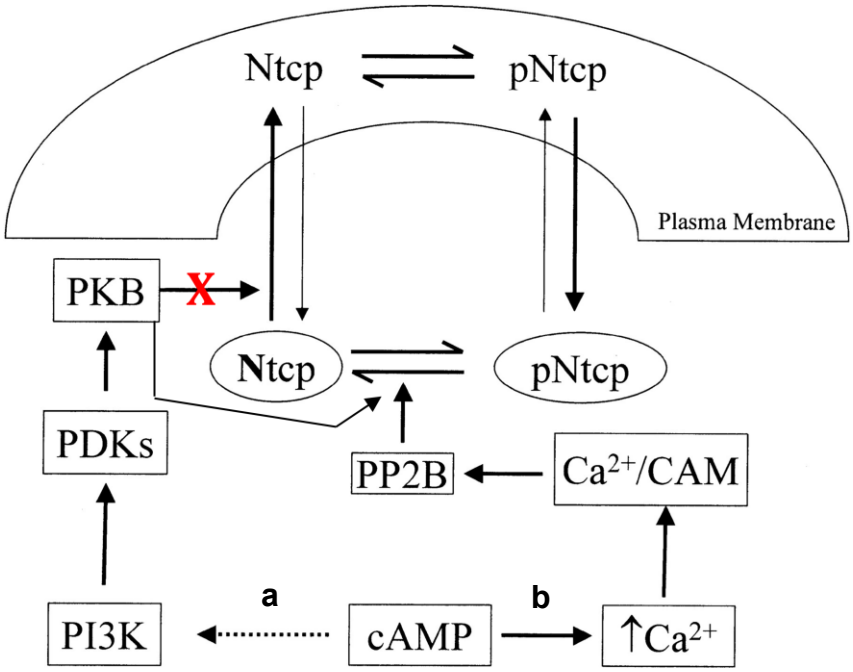


Figure 1.22; Proposed mechanism of cAMP-mediated rNTCP dephosphorylation and translocation to the plasma membrane.

Under normal conditions there is a stable pool of both unphosphorylated and phosphorylated (p) *Ntcp* (rNTCP) in the plasma membrane and within the cell's endosomes. Translocation of the receptor to the cell surface relies on possibly the simultaneous activation of two pathways. cAMP specifically activates the (a) PI3K/PKB and (b) PP2B signalling pathways, resulting in *Ntcp* dephosphorylation and translocation to the plasma membrane. *Adapted from:* [181].

Degradation of the NTCP receptor, for example when there is a toxic overload of bile salts in the hepatocytes, has been shown to involve its clathrin-mediated intracellular endocytosis by activation of PKC, followed by its lysosomal degradation. A different study has also shown that NTCP gets degraded through the ubiquitin-proteasome system [184-186]. NTCP degradation, however, has not been extensively studied over the years.

1.2.8 Previously reported HBV receptors

Asialoglycoprotein receptor (ASGPR), Transferrin receptor (TfR) and Epidermal growth factor receptor (EGFR).

The ASGPR, which was first discovered by Ashwell and Morell as early as 1965 and hence is also known as the Ashwell receptor, is a hepatic C-type (calcium-dependent) lectin [187]. ASGPR is a transmembrane receptor, with a molar mass of approximately 50kDa (38kDa under reducing conditions), that is specifically expressed on the basolateral and sinusoidal hepatic membrane where it primarily mediates the uptake by endocytosis and intracellular lysosomal degradation of desialylated glycoproteins, otherwise known as asialoglycoproteins, in the hepatocytes (*Figure 1.23*). Its highly active function is dependent on the proper assembly of its two subunits, the homologous polypeptides known as HL-1 and HL-2, with the HL-1 being the major subunit [25, 188, 189].

Similarly to NTCP, it has previously been suggested by a few studies that ASGPR may bind to HBV particles through interaction and attachment to specific pre-S1-related envelope binding sites, such as carbohydrate binding sites and N-linked glycosylation sites [25, 188, 190, 191]. However, as with other previously suggested host factors/ receptors there is currently no strong evidence demonstrating that this receptor can support functional virus entry and infection.

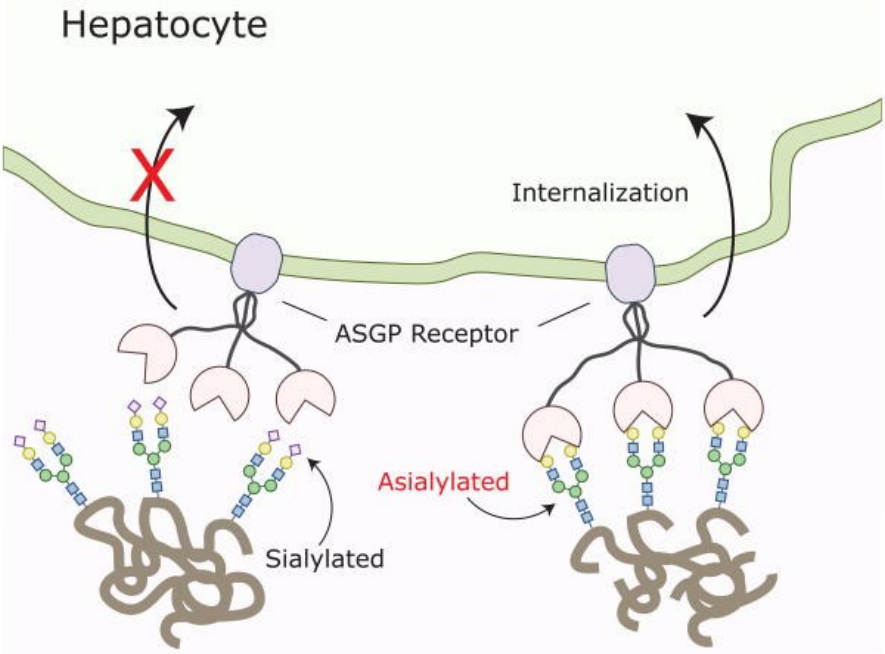


Figure 1.23; The ASGPR receptor functions by degrading asialoglycoproteins via endocytosis.

Sialylated serum proteins are not recognised by the ASGPR receptor and hence cannot be degraded by endocytosis. However, when these become desialylated, or otherwise asialylated, by progressively losing sialic acid moieties then they are recognised by the ASGPR receptor and are further targeted for uptake and degradation via endocytosis. *Source:* [192].

The TfR is a transmembrane glycoprotein with a predicted molecular weight of approximately 80kDa, whose particular role is to mediate cellular uptake of circulating iron from a plasma glycoprotein known as transferrin, which binds iron with a high affinity. This specifically involves the internalisation of transferrin-iron molecules via receptor-mediated endocytosis, which occurs at a neutral pH, and the further release of the iron within the endosome as a result of a decrease in the endosomal pH. The iron after going through certain alterations is then released into the cytosol where it is stored either as haemoglobin or ferritin, while the TfRs are recycled back to the plasma membrane for further use (*Figure 1.24*). TfRs are highly expressed on all cell types, with the exception of highly differentiated cells; however their levels do vary greatly. Two TfRs have been characterised up to date; TfR-1 and TfR-2, with the former being the main receptor for uptake of the transferrin-bound iron molecules as it is ubiquitously expressed in many cell types and binds transferrin with a high affinity [193-195]. TfR is also expressed highly on

hepatocytes where is has been suggested in the past that it may mediate the entry of HBV. This study specifically suggested that T lymphocytes possibly interact with HBV via a recognition site found within the pre-S2 region, further presenting the virus to the TfR which can then enter and further infect cells [196].

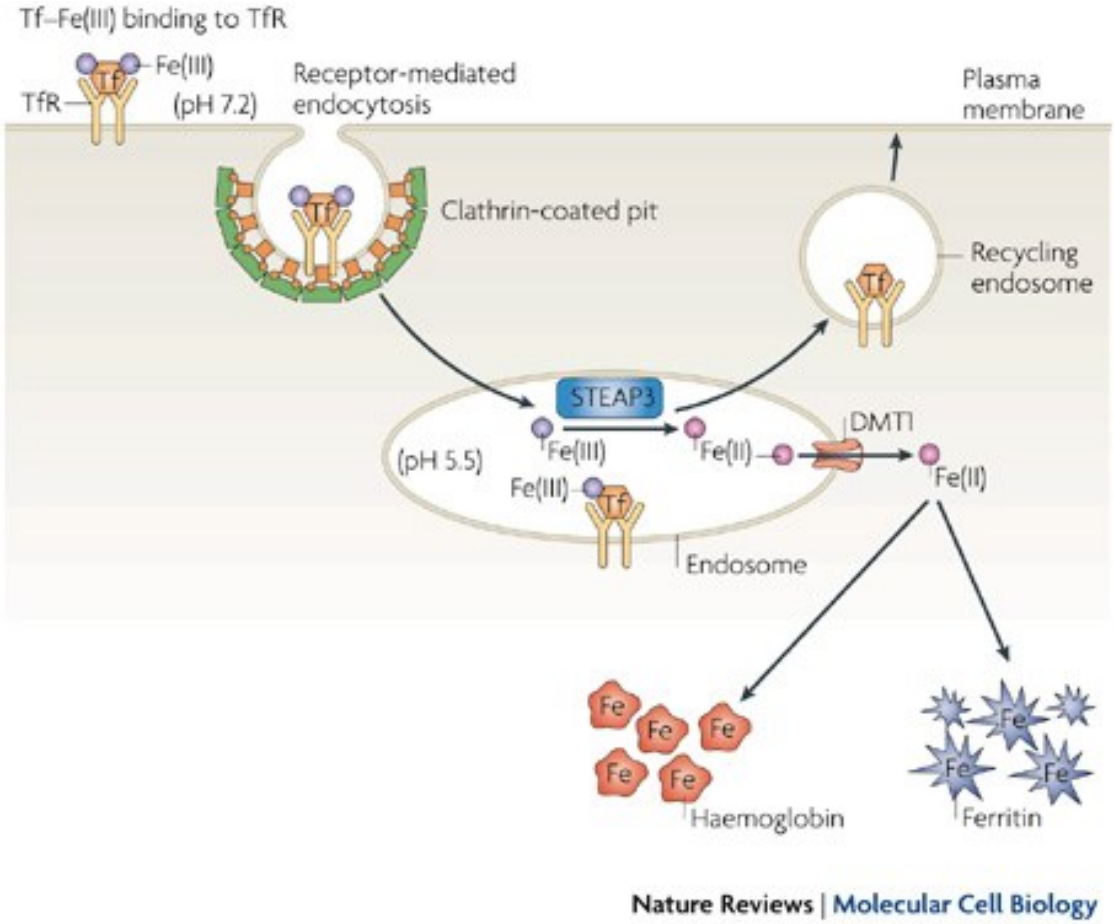


Figure 1.24; Function of the TfR in uptake of transferrin-bound iron molecules. In brief, TfRs bind transferrin-iron molecules that are then internalised via receptor-mediated endocytosis. Within the endosome the iron molecules undergo certain alterations and are eventually released into the cytosol. These processes involve changes in the pH. Once in the cytosol, the iron is stored in either one of two types, while the TfRs are recycled within endosomes back to the plasma membrane. *Source:* [195].

The EGFR is another transmembrane protein, which primarily acts as a receptor for members of the EGF family of extracellular protein ligands. It specifically belongs to a family of four receptor tyrosine kinases (RTKs): EGFR/HER1/ErbB-1, HER2/ErbB-2, HER3/ErbB-3 and HER4/ErbB-4. EGFR

is a 170kDa protein consisting of four conserved domains: an extracellular N-terminal ligand-binding domain, a hydrophobic short transmembrane domain, a cytoplasmic domain with tyrosine kinase activity and a C-terminal regulatory domain. Following ligand binding, EGFR transduces important signals from the extracellular environment into the cells and hence activates vast downstream signalling events that are implicated in various cell functions (*Figure 1.25*). The receptor is widely distributed in many tissues and organs, and deregulation of its tightly controlled signalling network is typically associated with the development of several types of human cancers. A number of different viruses have been demonstrated to use EGFR directly for their endocytosis into host cells, or even rely on EGFR-mediated signalling for their entry, replication and infection of cells [197-199]. For example, it has been suggested that HBV activates EGFR during invasion processes via its *trans*activation by the X-protein, hence possibly implicating the receptor in certain cell phenotypes seen during HCC [199, 200].

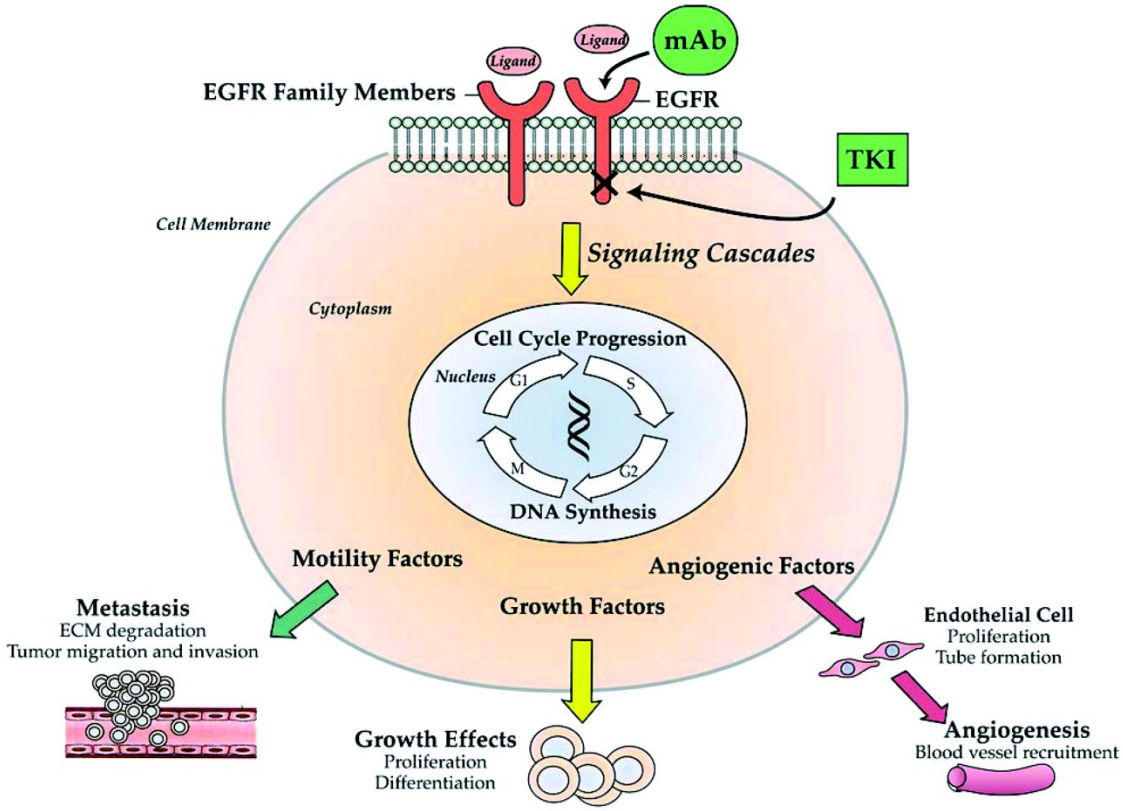
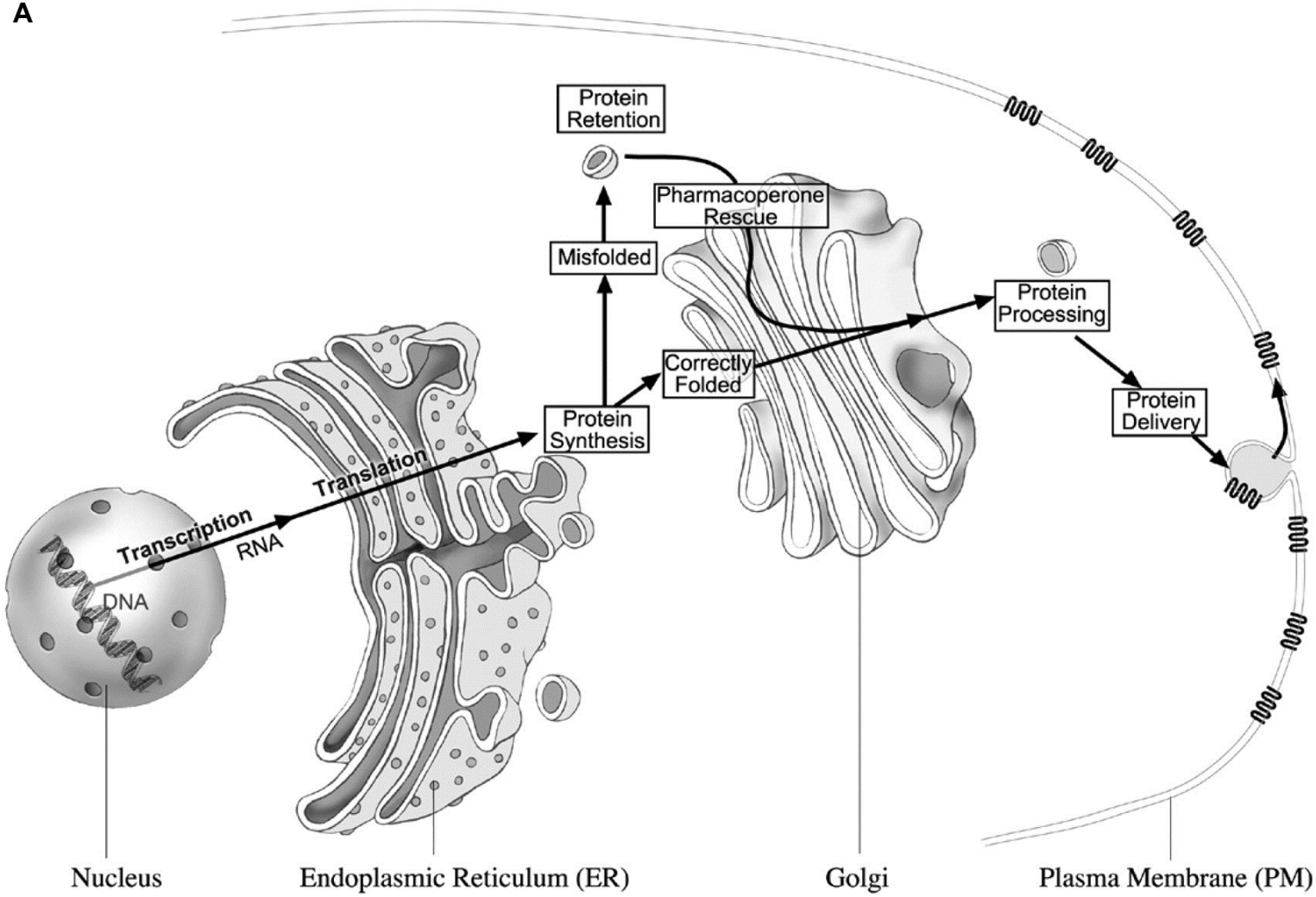


Figure 1.25; Simplified schematic of the EGFR signalling cascade.
EGFRs bind their ligands resulting in activation of vast signalling cascades involved in cell cycle progression and DNA synthesis, and affecting motility, growth and even angiogenic factors. *Source:* [197].

1.2.9 Protein trafficking mechanisms of the predicted HBV receptors

In general, membrane proteins are translated and synthesised in the ER following gene transcription within the nucleus. During the synthesis of the proteins is also when the *N*-linked glycans are appropriately added. Before undergoing any further transportation, the proteins are assessed within the ER for their overall quality before a decision is made as to whether they can continue to translocate to the plasma membrane or not (*Figure 1.26; A*). Specifically, if the proteins are correctly folded, which is typically facilitated by molecular and chemical chaperones, they can be further processed and integrated into the cell surface, however, if for whatever reason the synthesised protein is misfolded then this is retained in the ER and the chaperones attempt to reprocess it for correct folding. If proteins cannot be folded properly they are removed into the cytoplasm after chaperone dissociation where they are targeted for proteasomal degradation. Alternatively, misfolded proteins can also be exported to the Golgi, retrospectively transported back to the ER where correct folding is once again attempted. If the proteins remain defective they are finally degraded within the lysosome (*Figure 1.26; B, and Figure 1.27*) [201-203].

A


Protein Retention
Pharmacoperone Rescue
Misfolded
Protein Processing
Correctly Folded
Protein Delivery
Protein Synthesis
Translation
Transcription
RNA
DNA
Nucleus
Endoplasmic Reticulum (ER)
Golgi
Plasma Membrane (PM)

B

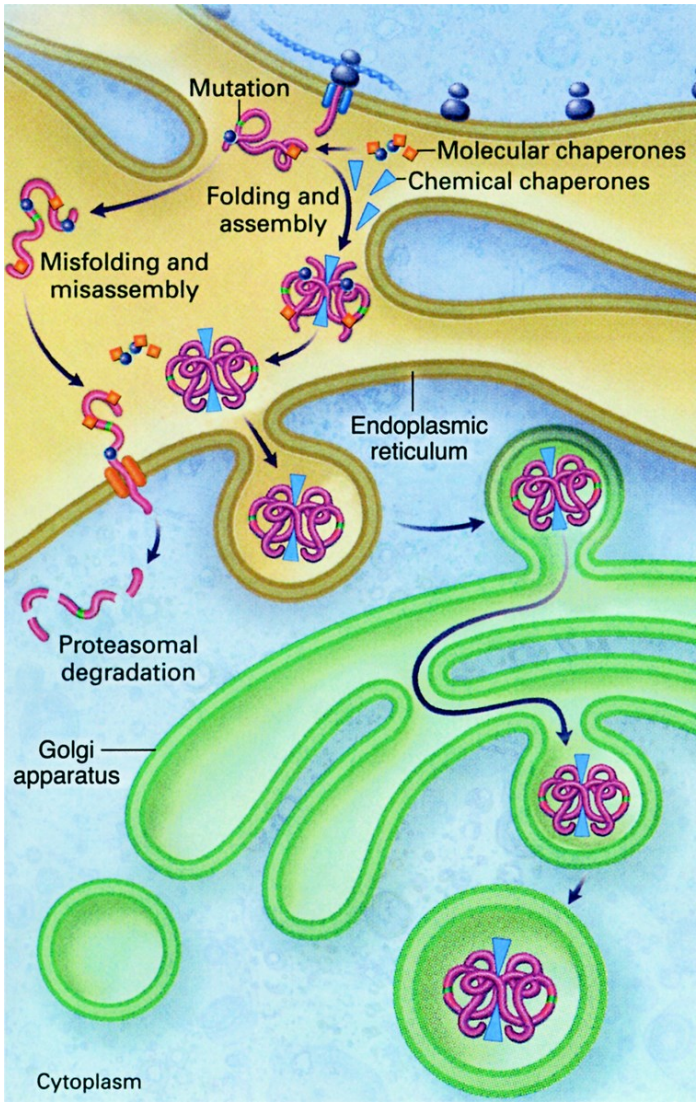


Figure 1.26; (A) Schematic of the overall cellular sites associated with protein synthesis and trafficking; (B) Schematic of the endosomal trafficking/ sorting pathway of newly synthesised proteins.

See text above for full description. Source: [204] and [202], *respectively*.

Proteins at the plasma membrane continuously undergo endocytosis and further transport to the different compartments of the endosome for subsequent processing, that being either degradation or trafficking back to the plasma membrane. From the early endosome, specifically, cell surface proteins are typically sorted either into late endosomes for subsequent degradation or recycling endosomes where they are sorted and migrate back to the plasma membrane (*Figure 1.27*). During these events, subcellular trafficking of the proteins usually takes place on intact microtubules with the help and co-ordinated activity of microtubule motor proteins [205, 206].

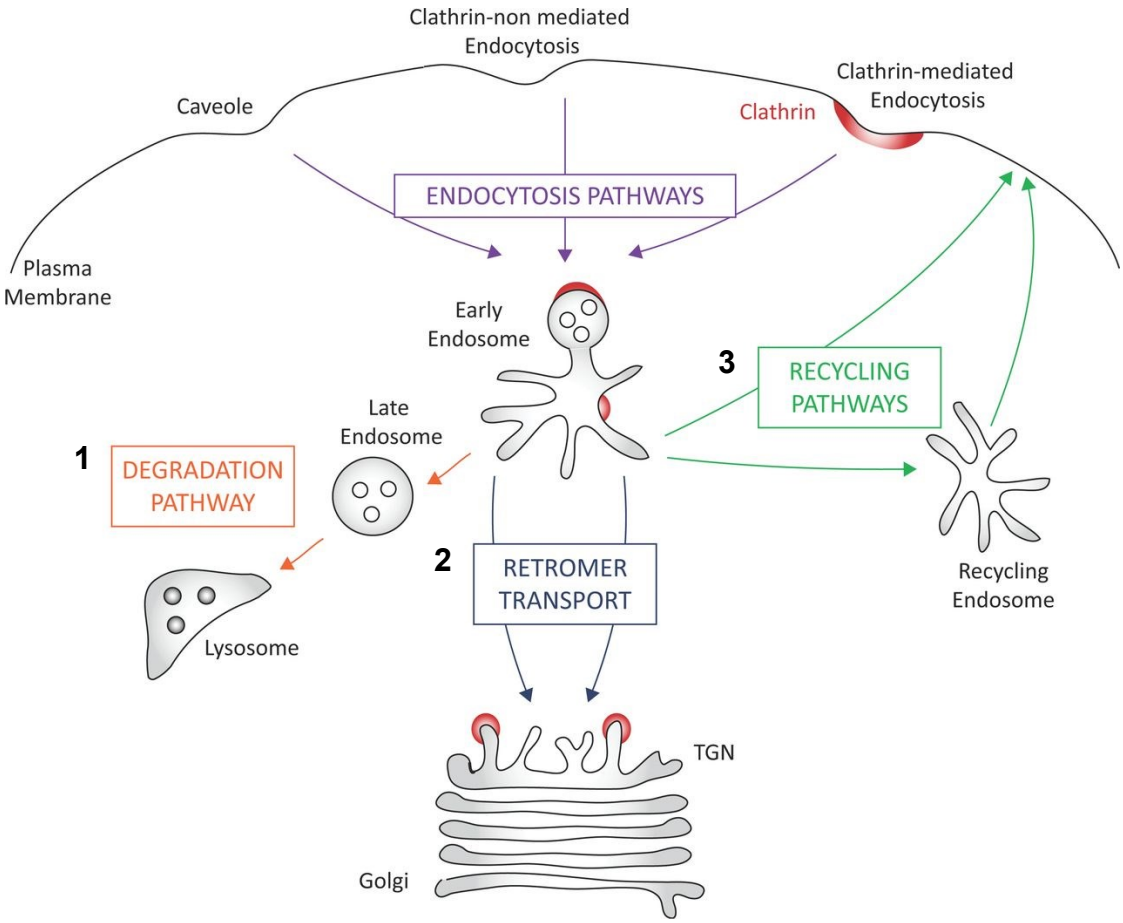


Figure 1.27; Endosomal trafficking/ sorting pathway of plasma membrane proteins after endocytosis.

After endocytosis and delivery to the early endosome, plasma proteins are then sorted and can follow three distinct pathways: 1. Degradation pathway, where they are destined for degradation within the lysosome; 2. Retromer transport between the Golgi and the early endosome; and 3. Recycling pathway, where they are recycled back to the plasma membrane within a recycling endosome. *Source:* [206].

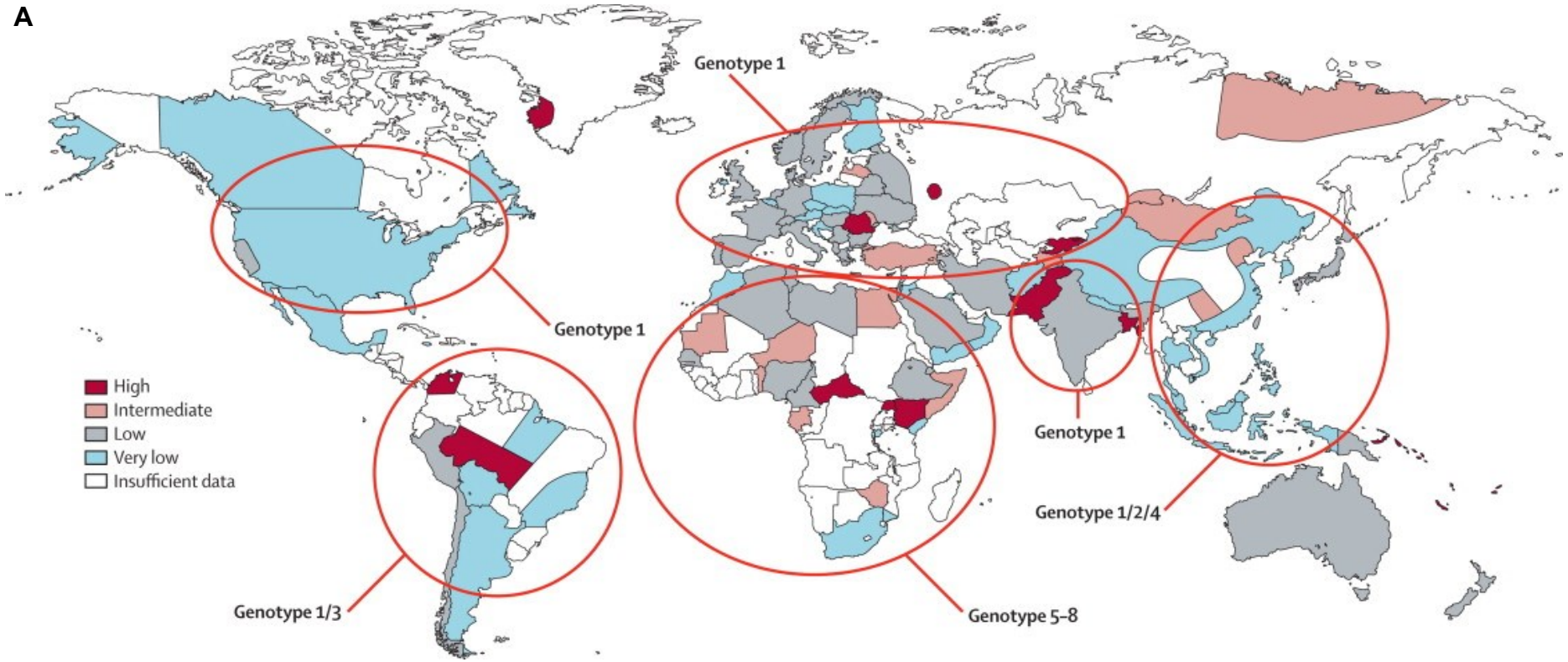
In 2008, Murray and his colleagues looked at endocytic vesicle-based trafficking of the previously described receptors on microtubule-based cytoskeletons using rat hepatocytes *in vitro.* They specifically looked at NTCP, ASGPR, TfR and EGFR and showed that adenosine triphosphate (ATP) was responsible for causing fission of the endocytic contents along the microtubules, further resulting in the segregation of proteins targeted for different cellular compartments. Most interestingly, they showed that NTCP and TfR remained together (did not segregate) during fission events and were recycled back to the plasma membrane in the same endocytic vesicle. From previous studies, we know that ASGPR and TfR once internalised are directly

recycled back to the plasma membrane, while EGFR is directly targeted for degradation within multivesicular bodies. NTCP containing vesicles are typically trafficked between the cell surface and an intracellular vesicular pool, a process that as previously mentioned requires the presence of PKCζ [182, 207].

Interestingly, in 2015, Wang and his colleagues (belonging to the same group of researchers that previously had established that NTCP and TfR recycled back to the plasma membrane in common endocytic vesicles) further determined that NTCP and EGFR also traffic together in the same endocytic vesicles, a process once again regulated by the presence of PKCζ within these vesicles and with inhibition of this kinase resulting in loss of endocytic vesicle motility. Specifically, they observed that upon stimulation of cells with EGF, NTCP and EGFR that were typically found on the plasma membrane now underwent cellular redistribution and became internalised in the same endocytic vesicles, from which they finally segregated, recycling NTCP back to the plasma membrane and targeting the EGF-EGFR complex for lysosomal degradation [207, 208]. Therefore, it would appear that NTCP, TfR and EGFR internalise with similar kinetics, even though they are eventually targeted to different subcellular compartments, while it is probable that ASGPR also undergoes similar internalisation kinetics.

1.3 Hepatitis Delta virus (HDV)

HDV was first discovered in Italy in 1977 [209], and is the smallest human RNA virus currently described. HDV, similarly to HBV, is a blood-borne virus (for transmission details see *Figure 1.5*), which is present in many different countries over the world (*Figure 1.28; A*), and it is estimated that from the approximately 240 million people that are currently chronically infected with HBV roughly 15-20 million of them are additionally infected with HDV. HDV infections are separated into two types: the ones that arise as co-infections together with HBV infections, and ones that arise as superinfections of already infected HBV individuals (*Figure 1.28; B*). In general, acute HDV infections in patients create a much greater risk of progression to fulminant hepatitis and liver failure compared to patients infected with HBV alone, and in addition, chronic HDV infections also appear to progress more rapidly to liver damage and cirrhosis compared to HBV infections alone. Therefore, HBV and HDV co-infections and superinfection result in more severe forms of viral hepatitis. Vaccination against HBV should in theory resolve HDV infections too. Treatment options are currently limited, with only pegulated IFN-α therapy proving to have some antiviral efficacy against the virus. Treatments with other antivirals, as well as combination therapies, have not been well studied as of yet but could possibly be advantageous for virus suppression. In cases where disease has rapidly progressed, liver transplantation is urgently required [210-213]. Appropriate diagnostic markers for disease assessment can be found in *Table 1.4.*


A
Genotype 1
Genotype 1
Genotype 1
Genotype 1/2/4
Genotype 1/3
Genotype 5–8
High
Intermediate
Low
Very low
Insufficient data

B

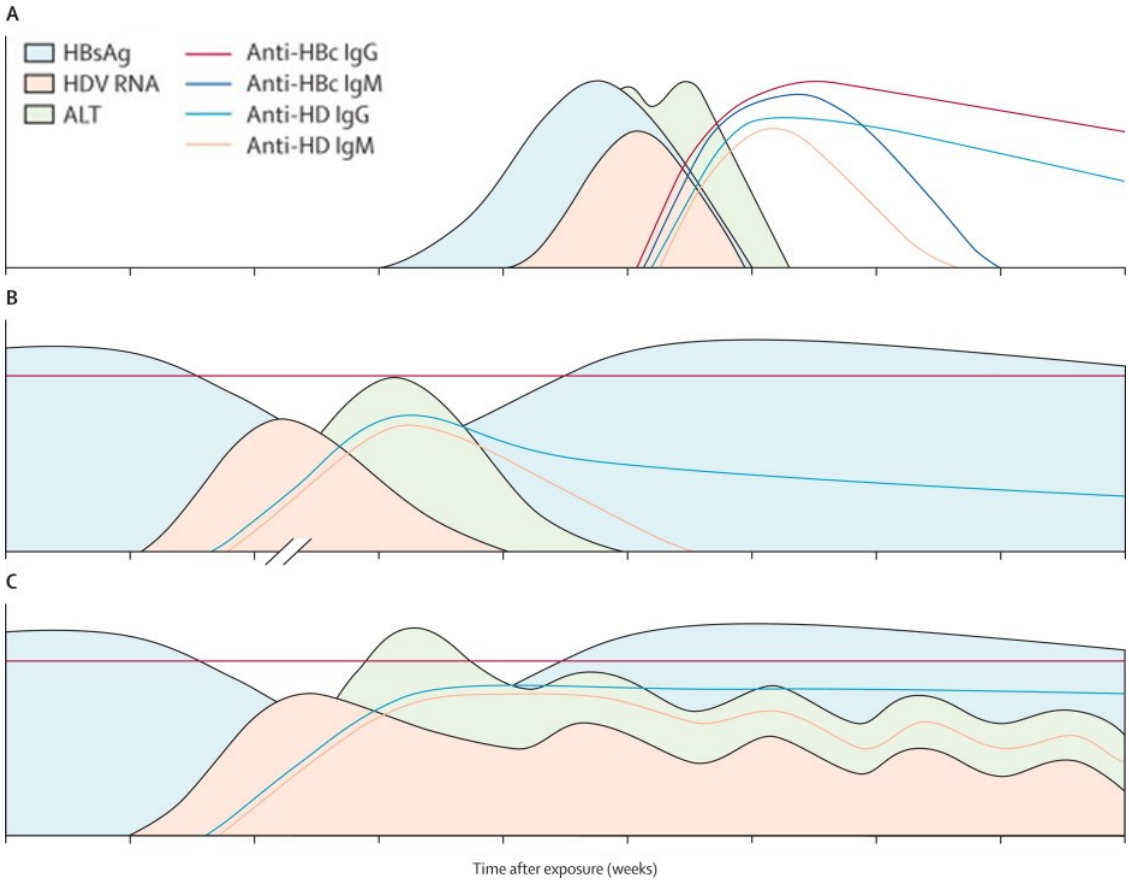


Figure 1.28; (A) Geographic distribution and prevalence of HDV infections and its different viral genotypes. (B) The clinical course and serological profiles of HDV and HBV infections.

(A) Highly endemic HDV areas include: A specific area in Greenland, parts of South America, certain areas in Europe, Sub-Saharan Africa, and the Middle East. **(B)** HDV and HBV simultaneous co-infections that are naturally resolved after a few weeks (a), HDV superinfection of a HBV carrier with self-limited infection, during which spontaneous clearance may take years to occur (as indicated by the break in the x-axis) (b), and HDV superinfection of a HBV carrier with chronic persistent infection. The precise time it takes for a HDV infection to develop, clear or progress to chronicity, is unclear and can be anything from weeks to months to years.
Source: [211].

As illustrated in *Figure 1.28; B*, for the diagnosis of HDV/ HBV co-infections or superinfections the following markers (*Table 1.4*) can be used during the course of the disease:

Test	HDV/ HBV co-infections	HDV superinfection of a HBV carrier with self-limited infection	HDV superinfection of a HBV carrier with chronic persistent infection
Anti-HDV IgG	+	+	+
Anti-HDV IgM	+: in acute and chronic infections -: in past infections		
HDV RNA	-	-: in spontaneous infections (takes years to clear) or where viral clearance has occurred in response to treatment	+
HBsAg	Positive, (associated with HDV RNA)		
HBeAg	Negative: associated with detectable anti-HBe		
HBV DNA	Negative or low: suppressed by HDV (detectable only when HBeAg is also detectable, can reactivate)		
ALT	Elevated/ Normal	Elevated/ Normal	Elevated

Table 1.4; HDV diagnostic markers.
Modified and adapted from [211].

HDV has not been assigned into a family yet but it does belong to the *Deltavirus* genus, and a total of eight genotypes have been classified revealing a complex genetic variability, as well as geographical distribution (*Figure 1.28; A*) [214]. The virus is 36nm in diameter and specifically has a small circular single-stranded, negative-sense RNA genome of approximately 1.7kb that forms a covalently closed circle, while the viral RNA has a rod-like structure that has approximately 70% self-complementation. One unique open reading frame is actively transcribed and this encodes for two isoforms of the hepatitis delta antigen (HDAg), small (S) and large (L), that undergo post-translational modifications, which confer them their specific properties, and together these form the HDV nucleocapsid. Specifically, the S-HDAg (24kDa) is required for initiation of replication, while the L-HDAg (27kda) acts as an inhibitor of replication and is responsible for assembly of new infectious particles. The HDV genome is purposely encapsidated by an outer envelope embedded with the HBV surface glycoproteins (*Figure 1.29*) [210, 211, 215-218].

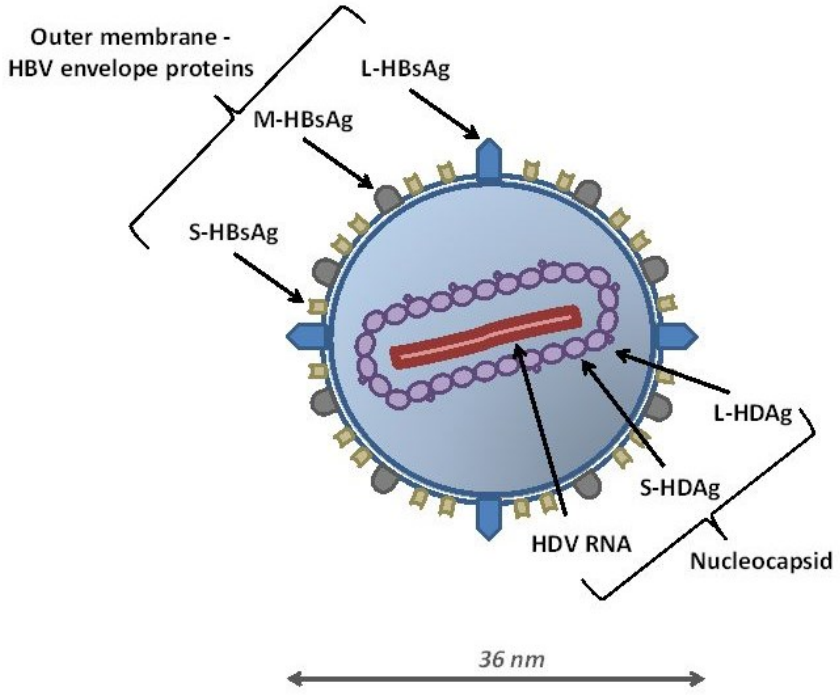


Figure 1.29; Structural organisation of the infectious HDV virion.
Source: [219]

HDV is characterised as a defective satellite virus of HBV, as it requires HBV to complete its own life cycle. In particular, it requires the surface glycoproteins of HBV for cell entry and establishment of infection, as well as the assembly of new virions. Consequently, both viruses are thought to share the same receptor(s), such as NTCP, on liver cells and follow the same entry pathway, including virus uncoating and nucleocapsid transportation (with the help of a HDAg signal) to the host cell's nucleus; however, as already mentioned, these stages of the virus' life cycle require further investigation. HDV, similarly to HBV, has been shown to recognise and bind to its receptor via an interaction with its N-terminal pre-S1 domain, including particularly residues 9-15 aa [122]. HDV replicates its genome via RNA-directed RNA synthesis, in contrast to HBV that requires a DNA intermediate, with three different RNA forms being produced during replication: a circular genomic and a complementary circular antigenomic RNA, as well as a linear mRNA. Moreover, HDV does not have its own RNA polymerase so it makes use of the cellular RNA polymerases I-III [32, 163, 210, 211, 216, 220-222]. For more details on HDV infection and the virus' replication cycle see *Figure 1.30.*

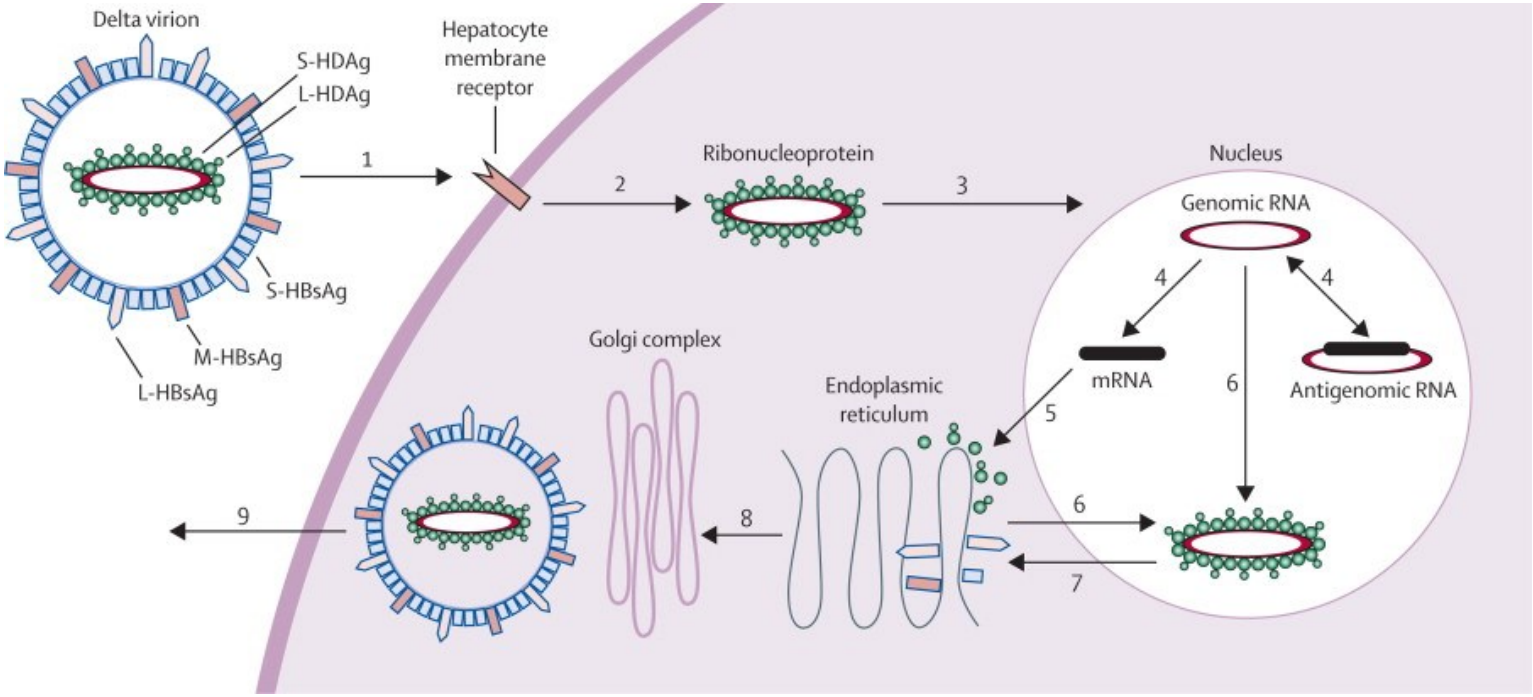


Figure 1.30; Schematic representation of the HDV infection and replication cycle.

1. Virus attachment to the host cell with the use of its HBV-expressing envelope glycoproteins and particularly its pre-S1 N-terminal domain; 2. Virus entry, possibly by endocytosis, and uncoating mediated by membrane fusion; 3. The ribonucleoprotein translocates via a HDAg signal and enters the nucleus; 4. The circular HDV genomic RNA is transcribed with the help of the cellular RNA polymerases to form both the complementary circular antigenomic RNA, which is the template for replication of new transcripts, and the linear mRNA, which contains the open reading frame; 5. The mRNA is transported to the cytoplasm where it is translated in the ER to produce new molecules of HDAg, (6.) which then return to the nucleus where the S-HDAg isoform supports further replication, and where eventually both isoforms, S- and L-HDAg, form new ribonucleoproteins incorporating the new RNA transcripts; 7. These are further exported to the cytoplasm where the L-HDAg promotes the assembly of new viral particles, which are specifically produced in the ER where the HBV envelope glycoproteins are embedded within the HDV virions outer membrane; 8. The newly synthesised infectious particles bud through the ER and the Golgi, and (9.) are finally exported from the infected cell to continue infection of other cell. *Source:* [211].

Interestingly, in regards to HBV and HDV entry and the interspecies transmission barrier, studies to date have revealed that exogenous expression of the human NTCP receptor is sufficient to render *in vitro* human, but not mouse, hepatocytes permissive to HBV and HDV infection, while expressing human NTCP in murine cells only supports HDV infection. Therefore, there appears to be an entry (possibly including differential regulation of human and mouse NTCP, or mouse NTCP simply does not express the appropriate molecular determinants required for entry) or additionally a post-entry block in mouse cells making them non-susceptible to HBV infection [30, 117, 163, 164, 223-226].

1.4 Current systems for studying HBV and HDV infections

The lack of reliable *in vitro* infection systems and convenient small animal models has hindered progress in HBV research over the years, as well as the development of new therapeutic options [89, 225, 227, 228].

Viral infections *in vitro* have been difficult to demonstrate over the years, as they are typically associated with a number of important limitations that need to be overcome. Some of the reasons specifically are that, first of all, the functional receptor for virus entry and infection was only recently discovered and characterised [32], and secondly, cell culture infections currently rely on very high multiplicities of infection (MOIs; for example, the typically used HepG2 cells overexpressing the human NTCP receptor require anything from 100 to 10,000 viral copies for infection of a single cell), as well as addition of certain reagents (such as dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG)) to presumably promote hepatocyte differentiation/ polarisation followed by enhanced virus attachment and entry into the host cell respectively, and even in this case only a very small proportion of the cells actually become infected [136, 226, 229-232]. A summary of *in vitro* models can be found in *Table 1.5.*

On the other hand, *in vivo* models closely related to the natural hosts of HBV (such as chimpanzees) are definitely more efficient in becoming susceptible to and supporting infection, but these as well as others (such as treeshrews and woolly monkeys) are costly, difficult to handle and obtain in bulk, while there is still strong ethical concern in regards to using such animals for this type of research (especially when the animals are so closely related to humans). Regardless, HBV infection, depending on the animal used for its study, can on occasions be mild and self-limiting, making these models generally not as ideal for studying HBV infection. Moreover, other invaluable animal models that have been extensively and successfully studied over the last years include woodchucks and ducks, these however are not naturally susceptibility to human HBV infection and this is probably due to the poor homology of their HBsAg sequence and hence could require different cellular factor(s) or receptor(s) for HBV virus entry and infection [89, 225-227, 231, 233, 234]. A summary of *in vivo* models can be found in *Table 1.6*.

Until the discovery of the NTCP receptor as the long-sought receptor for HBV infection, most models, used both *in vitro* and *in vivo*, bypassed entry by direct delivery of HBV plasmids expressing full-length genomes, however these types of models understandably did not provide information in regards to the virus' full life cycle, which still requires much further investigation and understanding. Nevertheless, as these systems could in fact support HBV infection once entry was circumvented, they did provide useful insight into downstream pathways and the mechanisms activated by the virus, and specifically allowed the collection of the vast amount of knowledge we now have on the virus' replication cycle [43, 225, 226].

1.4.1 *In vitro*, cell culture systems

To date, only a small number of *in vitro* infection systems have been developed and successfully studied for their susceptibility to HBV infection.

One of the first *in vitro* HBV infection systems to be developed were the transfection-based systems. These specifically include cell lines into which overlength HBV genomes have been integrated via transfection. Some of the cell lines that have stably integrated HBV genomes include the HepG2.2.15s, which in particular are HepG2 cells stably expressing a HBV genome, while others have inducible HBV genomes (characteristically tetracycline (Tet)-inducible expression systems) like the HepAD38 and HepDE19, which replicate HBV but under regulated conditions. These have been extensively used for studying HBV viral replication, assemble and secretion, meaning the steps downstream of viral entry, as well as for testing possible HBV inhibitors involved in the particular steps of the virus' life cycle [235-239].

Following on, the infection based systems were developed. These have been proven to be exceptionally suitable *in vitro* models for the study of HBV, as they have exclusively allowed the study of the early events in HBV infection, and they principally include primary human hepatocytes (PHHs) (*Figure 1.31*) and primary tupaia hepatocytes (PTHs). However, limitations to the use of primary hepatocytes include their difficulty to be cultured under normal culturing conditions and their short life span, both of which are associated with the usually abrupt loss of permissiveness to HBV infection primarily due to the loss of their polarity, as well as damages caused to their membranes during cryopreservation. Additionally, their supply is very limited (typically isolated from diseased donors), they display high variability and they are expensive to obtain [89, 225-228, 232, 240-242].

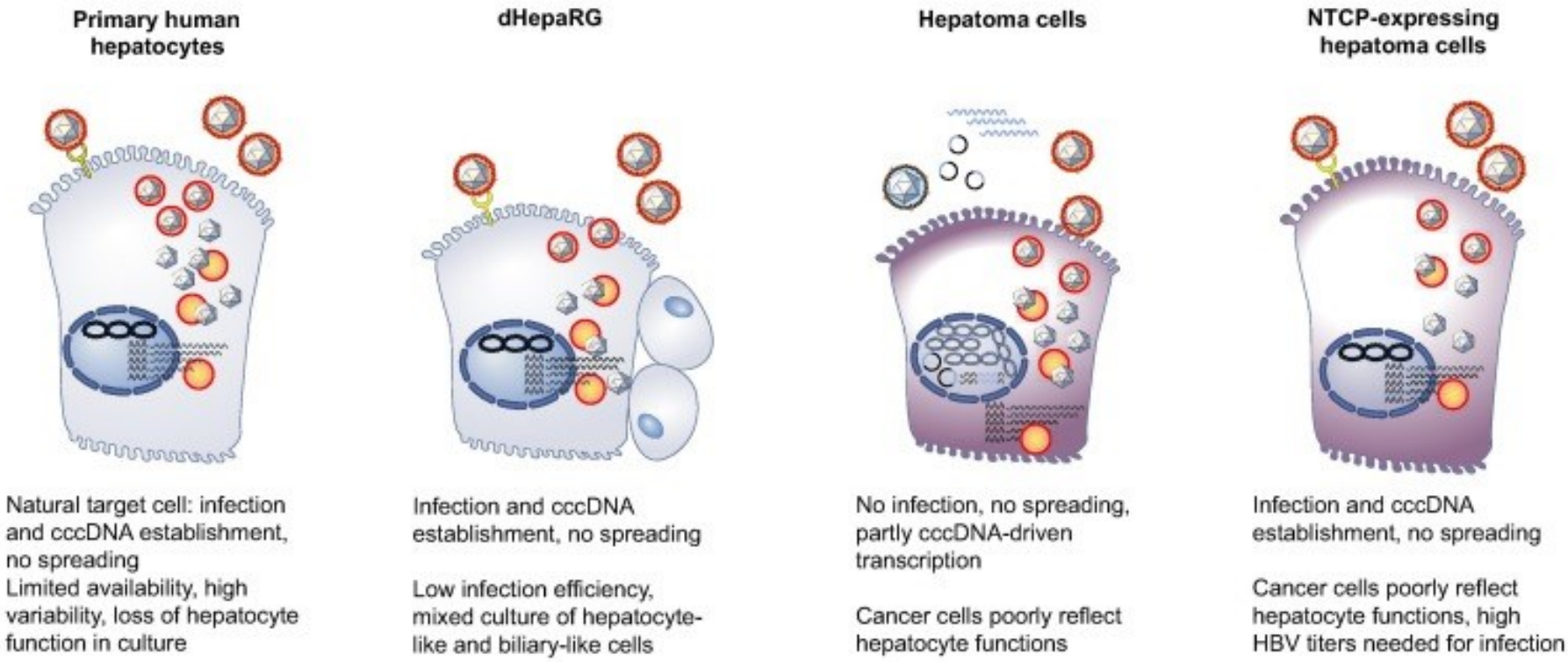


Figure 1.31; Examples of the key human cell culture systems for studying HBV infection.
Source: [225].

Moreover, a human liver progenitor cell line, HepaRG (*Figure 1.31*), was the first cell line to show natural susceptibility to HBV infection without overexpressing any of the known receptors. This cell line was isolated from liver tumour tissue of a female suffering from HCC as a result of a chronic HCV infection and it appears to express key hepatocyte functions and, therefore, has been shown to be efficient at supporting HBV infection, but only after prolonged differentiation with DMSO allowing the cells to differentiate and obtain a more hepatocyte-like morphology. Under diverse differentiation conditions, these cells can become a mix of hepatocyte-like or biliary-like cells, and the presence of hydrocortisone in association with DMSO promotes their phenotypic stability [89, 225-228, 232, 243-246].

Recently, micro-patterned co-culture (MPCC) systems have also been developed and can support HBV infection (*Figure 1.32*). These cell culture systems typically involve the organisation of PHH in micro-patterned colonies that are additionally surrounded by supportive stromal cells, which help to maintain the hepatocellular structure as well as the hepatic function over longer periods of time compared to when PHH are cultured alone on typical tissue culture plates [247, 248]. Human induced pluripotent stem cell (hiPSC) -derived human hepatocytes have also been shown to support HBV infection, allowing for the further study of HBV biology. However, their efficacy at permissiveness to infection does strictly depend on their hepatocyte differentiation state as well as their polarisation that ideally should resemble PHH [225-227, 247, 249-252].

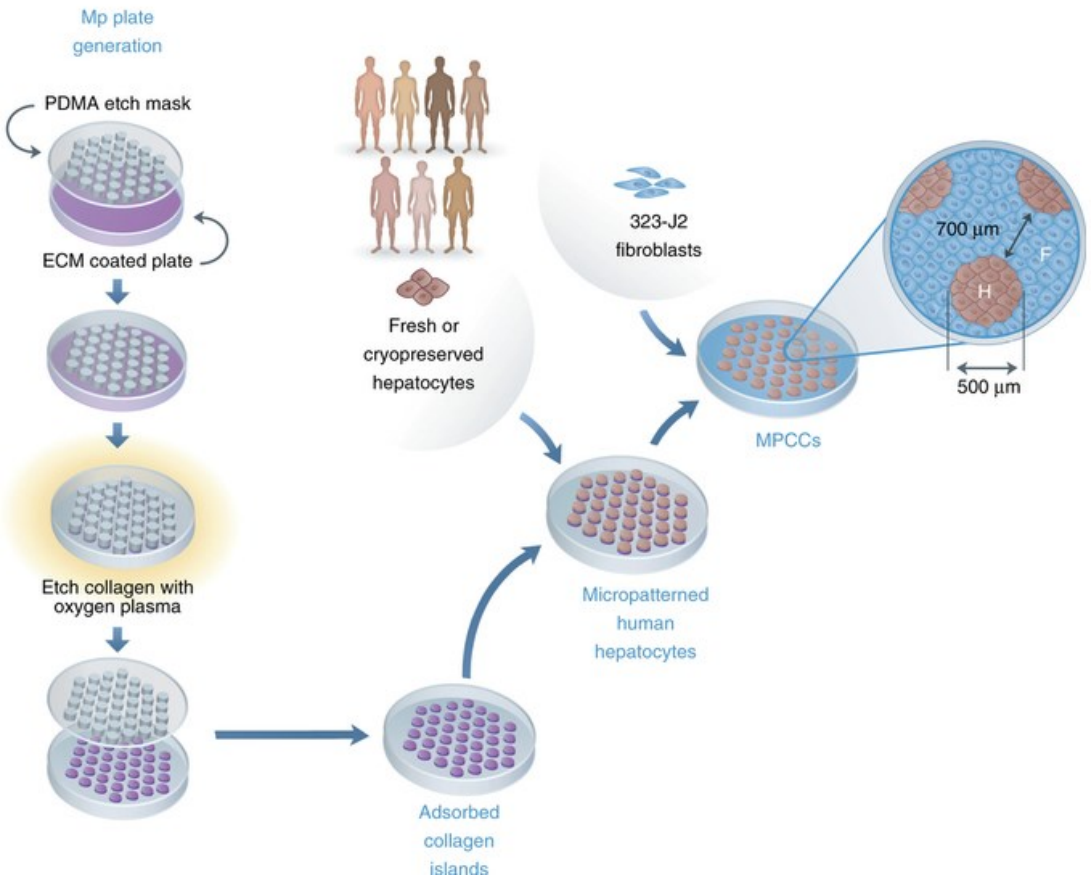


Figure 1.32; Example of a MPCC *in vitro* system model.
Schematic of scaffold construction containing micro-patterned collagen islands. The cells are then seeded and cultured into those islands forming liver tissue-like colonies. *PDMS: polydimethylsiloxane (silicone); ECM: extracellular matrix. Adapted from* [248].

Currently, HepG2 NTCP-overexpressing cell lines have become very popular and these have been shown to be susceptible to HBV infection at low to acceptable levels, as well as a similar Huh7 NTCP-overexpressing cell line (*Figure 1.31*) [116, 225, 227, 253]. These are typically created in two different ways: either by transient transfection of the NTCP receptor, during which the cells will express the foreign gene (i.e. the receptor) but the gene will not actually become integrated into their genome. In this case the gene will not replicate and, therefore, will eventually be lost. The only way to maintain expression of the gene of interest, in this situation, is to apply a constant antibiotic selection pressure [32, 169, 254]; or by creating stable cell lines with the use of lentiviral systems overexpressing the NTCP receptor. In the latter case, the cell culture systems only need to be selected with an appropriate

antibiotic once, after which the NTCP receptor is stably integrated within the host cell's genome [158, 163].

Moreover, to overcome the limitations in regards to the reported moderate HBV infection efficiency levels in these cells, most research groups produce single cell clones of these cell lines and hence select for the ones that have the highest NTCP expression and similarly the highest susceptibility to HBV infection, rather than using polyclonal cells with varying levels of NTCP expression [226, 228, 232, 255, 256]. Mouse Hepa1.6 overexpressing the human NTCP receptor have also been developed but as already mentioned these are not susceptible to HBV infection [223], while another mouse hepatocyte cell line, AML12, overexpressing the human NTCP receptor was in fact recently found to be susceptible to low levels of HBV infection [164, 257], possibly suggesting that the background from where the different cell lines are isolated may play a pivotal role towards understanding aspects of HBV infection and the interspecies transmission barrier. Equivalent HepG2 and Huh7 ASGPR-overexpressing cell lines have additionally been established, but their ability to support HBV infection has not been fully determined [25].

Summary of some of the currently available *in vitro* cell culture systems for the study of HBV:

Cell Culture Systems	Characteristics	Applications	Limitations
Transfection-based systems			
HepG2.2.15	Full HBV genome stably integrated into HepG2 cells.	Study of virus replication cycle and immune responses. Testing drugs that interfere with virus replication.	Do not permit research into the early steps of HBV infection, and do not resemble a natural infection environment.
HepAD38 and HepED19	HBV expression is under the control of a TET-on/off promoter.	*Similar to HepG2.2.15*	*Similar to HepG2.2.15*
Infection-based systems			
PHH	Natural viral host. Exhibit full range of hepatic functions. Freshly isolated cells are typically more susceptible than cryopreserved cells. Approximately 50% infection efficiency.	Allow study of the entire HBV life cycle.	Difficult to be cultured under normal conditions, short life span, easily lose permissiveness to HBV infection, limited availability and high variability. Expensive to obtain. Require the presence of PEG during infection.
PTH	The only other primary cells susceptible to human HBV infection.	*Similar to PHH*	*Similar to PHH*
HepaRG	Isolated from liver tumour of HCC patient chronically infected with HCV. Exhibit hepatic function. Approximately 10% infection efficiency.	Allow study of the entire HBV life cycle, including the early steps during infection, as well as testing drug metabolism and toxicity.	Require the presence of DMSO for long periods to allow differentiation prior to infection, as well as PEG during infection.
MPCC systems	PHH are cultured in what resembles a 3D-like system in small colonies on porous scaffolds that are supported by being co-cultured with other types of cells. Fresh and cryopreserved PHH seem to survive equally as well, and maintain hepatic function for longer.	*Similar to PHH.* This type of system was created to resemble an *in vivo*-like liver tissue; hence it closely resembles the normal cell physiology and provides a more hepatic-like environment.	*Similar to PHH.* Better infection efficiency compared to PHH cultured alone, but still no substantial spread of the virus.

Cell Culture Systems	Characteristics	Applications	Limitations
Infection-based systems			
hiPSC-derived hepatocytes	hiPSC cells become permissive to HBV at around 18-20 days post-differentiation, when their phenotype resembles hepatocyte-like cells.	Study of HBV biology.	Efficacy towards HBV infection is highly dependent on the differentiation status of these cells. Require precise, careful and timely handling.
HepG2- and Huh7- NTCP	Cells lines that transiently or stably overexpress the NTCP receptor. High reproducibility. Easy availability and maintenance. Low to moderate infection efficiencies.	Study of virus/host interaction during the early phases of viral infection, and testing novel inhibitor drugs.	High MOIs in the presence of DMSO and PEG. Limited cccDNA synthesis.

Table 1.5; Examples of current and most widely used cell culture systems for the study of HBV.
Modified and adapted from [226, 227, 232].

1.4.1.1 HBV-mimicking pseudoparticles and recombinant virus

Over the years, as well as trying to generate the best cell culture system for the study of HBV, a lot of interest has also been put into creating different types of hepatitis B viruses, those being either pseudotyped or recombinant viruses, primarily in the hope of enhancing the susceptibility of the available *in vitro* systems but also for studying specific aspects of the HBV biology such as the early events during infection or viral replication, and other cellular processes.

HBV-mimicking lentiviral pseudoparticles (HBVpp) have been used by many scientists hoping to elucidate viral entry into hepatocytes [166]. These particles are specifically created by assembling the HBV envelope glycoproteins into a lentivirus background, following standard techniques for third generation lentivirus production, and therefore are non-HBV/HDV-infectious while as such they also mimic HDV infections. Studies that made

use of these HBV/HDV lentivirus pseudotyped particles additionally shed light on viral assembly [258], as well as the mechanism by which both HBV and HDV attach and enter host cells. One group of researchers, in particular, showed how these pseudoparticles could actually infect PHH, however they could not demonstrate infection of any other cell line such as HepG2 or even primary hepatocytes isolated from other species [259]. Another group showed infection of hepatoma cells overexpressing the HBV-specific NTCP receptor, while they demonstrated that non-hepatic cells overexpressing the receptor were in fact not susceptible to these lentiviral pseudoparticles, further suggesting the need for additional hepatocyte-specific factors for virus entry [260]. Furthermore, more recently another group made use of what they named bio-nanocapsules (BNCs), which specifically expressed a recombinant HBsAg, and they showed that cellular entry of these particles was principally mediated by the HSPGs. Therefore, their conclusion was that NTCP may not be the primary factor involved in virus cellular uptake [261]. Regardless, HBVpp are an invaluable tool for dissecting the pathways and mechanisms involved in virus entry into hepatocytes and they could potentially also be used in the near future for gene deliver and hence gene therapy of the disease.

Recombinant HBV viruses are also an invaluable tool for efficiently monitoring stages of virus infection, further allowing for example identification of host factors involved in the different stages, identifying cells susceptible to viral infection, or even screening inhibitors of virus infection in real-time. Unfortunately, generation of recombinant HBV viruses has also been hampered over the years by specific limitations, which include first and foremost the strict genomic size and extremely compact coding organisation of the virus hence resulting in low yields of infectious recombinant virus and lack of sufficient infectivity [262]. Therefore, so as not to interfere with the coding potential and the replication-competency of the virus, there are believed to be necessary constrictions to the size of the foreign gene that can be inserted. Nishitsuji and his colleagues, to overcome this size limitation, decided to make use of the *NanoLuc* gene which they successfully inserted

into an overlength HBV genome, between the core/precore and the polymerase open reading frames. However, to adhere to the strict size limitation they had to replace part of the core/precore region with the actual *NL* gene, while also deleting part of the polymerase region. They used this newly synthesised recombinant HBV plasmid and transfected it into HepG2 cells for the production of infectious recombinant virus, which was further used to infect PHH and hepatocyte-derived cell lines overexpressing the NTCP receptor, and they reported that they could monitor the early stages of the virus life cycle [263, 264]. Replication-competent recombinant HBV genomes have also been shown to be efficient in delivering other types of proteins as well as small interfering RNAs (siRNAs) into PHH, where their effective packaging additionally occurs [265].

Interestingly, Wang and his colleagues followed a slightly different approach. They specifically decided instead of deleting regions of the HBV genome to duplicate the overlapping regions between the core/precore and polymerase areas, while simultaneously inserting a 22nt artificial internal ribosome entry site (IRES) sequence, RNA-binding motif protein 3 (Rbm3), to control translation of the reporter gene followed by translation of the polymerase (*Figure 1.33*). This specific IRES sequence was chosen due to its limited size. The idea was that the recombinant HBV plasmid when transfected into cells would result in the production of a functional tricistronic pgRNA, while hypothetically expression of the HBV envelope glycoproteins would not be interfered with. The group specifically demonstrated that the smaller the insert of the reporter gene the better its overall replication efficiency, however, no matter the size of the insert they could all form properly enveloped virions. These were further tested for their infectivity and were found to cause infection of HepaRG cells [266].

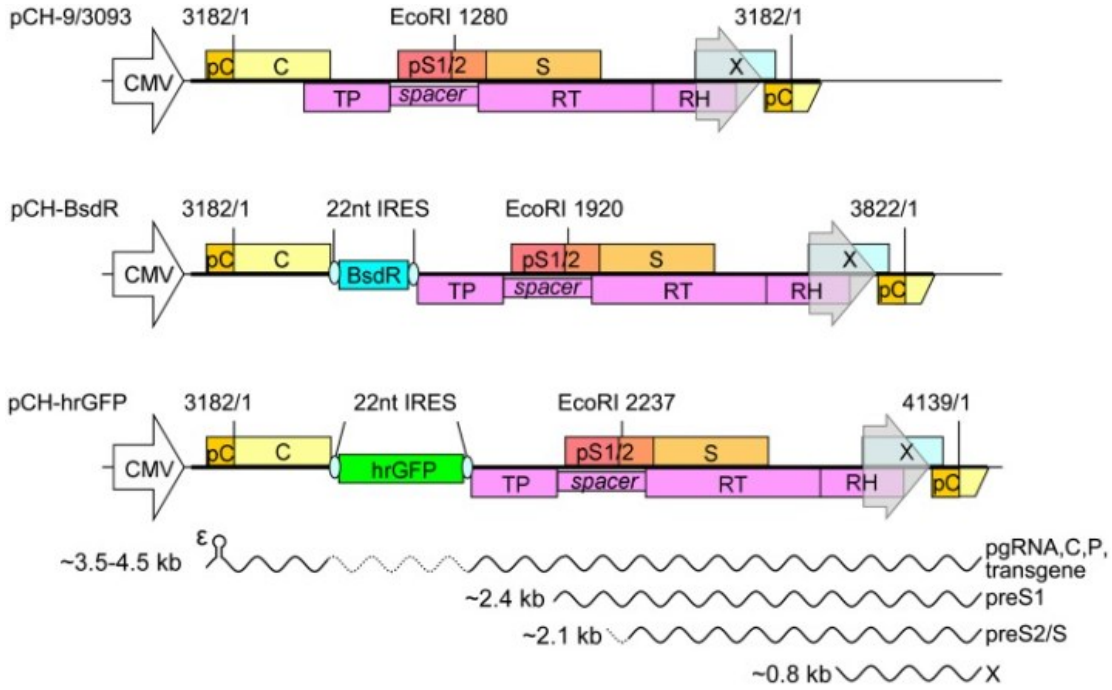


Figure 1.33; Example of genomic organisation of wild-type and recombinant HBV transcripts.

The reporter genes of interest are inserted between the core/precore and polymerase regions, while additionally separated by two 22nt IRES sequences responsible for promoting enhanced expression of the reporter gene as well as the polymerase. *Source:* [266].

In the past, a cyclic lentiviral complementary DNA (cDNA) library repackaging rescue screen approach was used to identify human Occludin as a functional receptor for HCV infection of human and mouse cells (*Figure 1.34*) [267]. This cyclic repackaging rescue screen is possibly an invaluable tool that could theoretically also be used in a similar manner in the case of HBV so as to uncover potential additional host factors or even receptors crucial for susceptibility of naturally non-susceptible cells, such as mouse cells, and there after other non-susceptible hosts to HBV infection. For this, the use of HBV-mimicking pseudoparticles or a recombinant-type viruses encoding for either a fluorescent marker or preferably an antibiotic selection gene would be greatly advantageous.

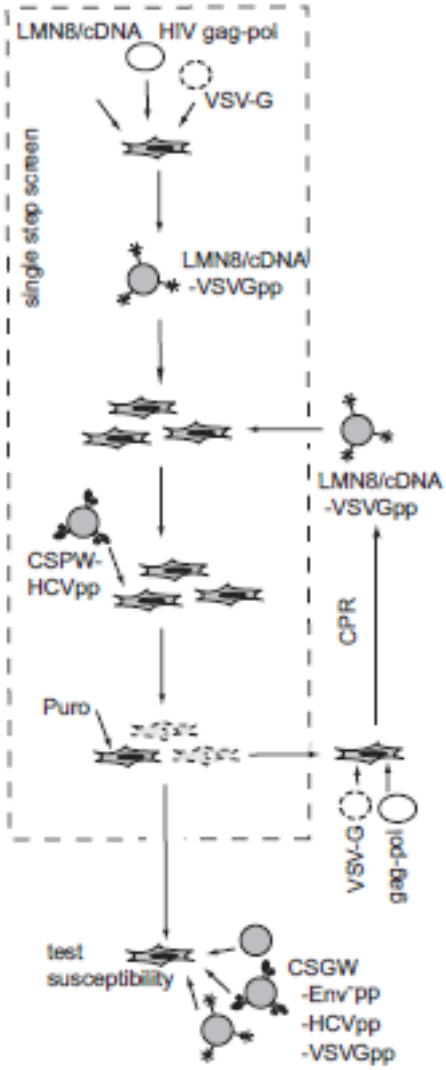


Figure 1.34; Summary of cyclic repackaging rescue screen procedure.

A cDNA library that has previously been derived from Huh7.5 cells, which are highly permissive to HCV, has further been cloned into a retroviral LMN8 vector. This is then packaged into HIV-based pseudoparticles with vesicular stomatitis envelope glycoproteins (VSVg). Cells of interest are then transduced with these particles. Specifically, for the discovery of Occludin as a HCV-specific receptor, Ploss and his colleagues further infected the cells with HCV-mimicking lentiviral pseudoparticles (HCVpp) that additional encoded for a puromycin antibiotic cassette. The cells are then selected for with the specific antibiotic, and surviving cell clones are repackaged into HIV-based pseudoparticles expressing VSVg, resulting in enrichment of the LMN8/cDNA library. This is performed for a number of rounds after which clones are sequenced and any important factors are uncovered. *Source:* [267].

1.4.1.2 Effect of DMSO in the cell culture systems, and particularly on NTCP expression and receptor functionality

Gripon and his colleagues were one of the first research groups to describe that the use of DMSO in cell culture media remarkably enhanced the HBV infection efficiency of human cells [229]. In addition, Gripon and his colleagues were also the first to demonstrate that, besides addition of DMSO, HBV infectivity could also be enhanced with the presence of PEG during infection [230]. Therefore, both of these reagents are nowadays commonly used during the course of HBV infection *in vitro* for induction of cellular differentiation/ polarisation and augmentation of virus binding to its receptor on the hepatocyte-like cell surface.

In 2012, Schulze and his colleagues were particularly interested in characterising the effect of hepatocyte polarisation and how essential it is for productive HBV entry and subsequent infection. They specifically made use of the HepaRG cells, which acquire susceptibility to HBV infection only after prolonged treatment with DMSO resulting in their specific differentiation into hepatic- and biliary-like cells. However, even in the presence of DMSO differentiation, these cells were difficult to infect even when inoculated with very high MOIs, while it was obvious that the small subset of infected cells were in fact islands of hepatocyte-like HepaRG cells, indicating a limited accessibility of the HBV virus to its specific receptor possibly due to the cell's polarity. The group went on to look at the multidrug resistance-associated protein 2 (MRP2), and they determined that in the HepaRG cells this protein exhibits important differences in its localisation pattern. Specifically, it revealed two distinct polarisation phenotypes; infections of HepaRG cells occurring in the subset of cells either exhibiting canalicular structures and hence resembling more PHH, or where cell-to-cell junctions were disrupted. In the latter case, cells could additionally be infected with the virus even if they did not exhibit PHH-like polarisation. Furthermore, the group established that this signalling protein started being expressed as soon as two days after initiation of DMSO treatment, and therefore, they concluded that DMSO is

responsible for hepatocyte-like polarity of these cells. They additionally concluded that HBV infection occurs via the basolateral side of the hepatic membrane, hence partially explaining the difference in infection efficiencies between HepaRG cells and PHH [268]. Below is the proposed model of HBV infection *in vitro*, and the important role of cellular polarisation (*Figure 1.35*).

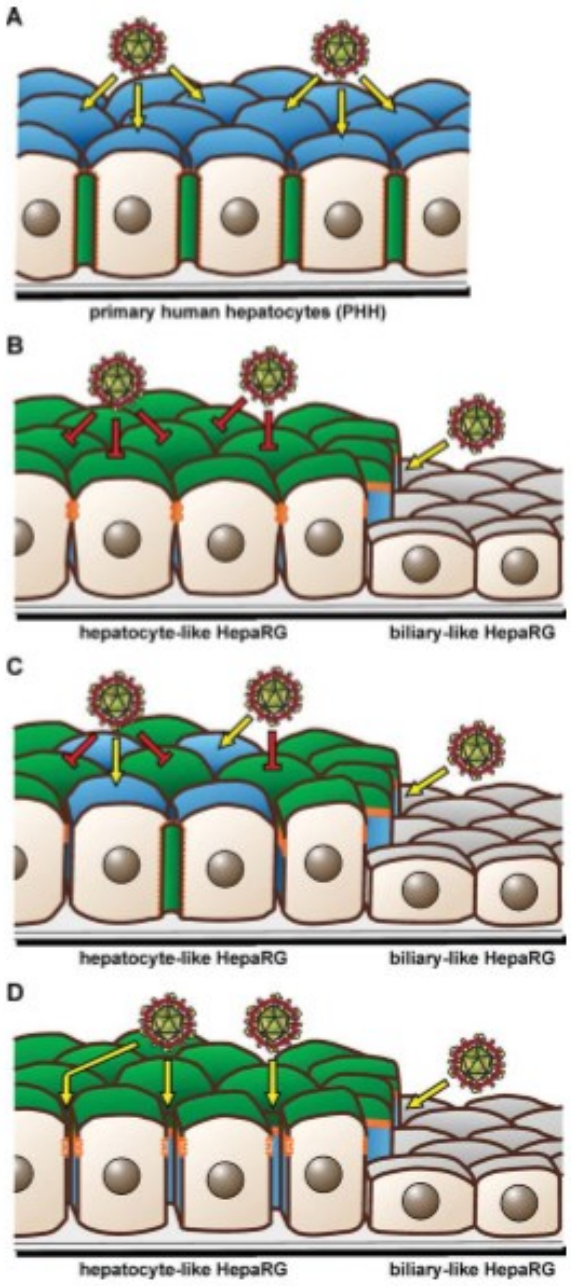


Figure 1.35; Role of cellular polarisation for HBV infection *in vitro*.
Apical membrane (green) and basolateral membrane (blue); Cell junctions (orange). **(A)** Infection of PHH by HBV via the basolateral hepatic membranes. Infection of HepaRG cells via **(B)** epithelial-like cells, **(C)** epithelial-like and PHH-like (containing canaliculi) cells, and **(D)** epithelial-like cells and disrupted cell-to-cell junctions. *Source:* [268].

DMSO throughout history has always been used mainly as a vehicle control and a solvent for water-insoluble reagents, and the only circumstances were DMSO has been used as a differentiation/ polarisation agent is in the case of the previously discussed HepaRG cells, as well as stem cell differentiation [32, 116, 163, 253]. Some examples include differentiation of HL-60 cells to neutrophil-like cells [269], P19 cells into cardial and skeletal muscle cells [270], and generally differentiation of human embryonic stem cells [271]. Differentiation of rat hepatocytes has also been described in the past [272-274], as well as differentiation of an immortalised cell lines derived from human primary hepatocytes, HuS-E/2 [275]. The pharmacological effects of DMSO have also been extensively studied over the years. A minute number of its additional functions include membrane transport and penetration, as well as cell cycle control amongst many others [276].

Recently, it was discovered that NTCP protein expression in HepG2-NTCP cells, despite being under the control of a cytomegalovirus (CMV) promoter, was remarkably increased by the presence of DMSO [254], partially explaining the ability of these cells to become susceptible to HBV infection [163]. Interestingly, DMSO has been reported to act as a PKC activator [276, 277], which may partially associate it with the control of NTCP expression. As discussed previously, rat NTCP is signalled to be degraded in the lysosome via activation of the PKC. PKC, additionally, mediates translocation and retrieval of the rNTCP from the plasma membrane [157]. However, so far no extensive studies have been performed looking at the way by which DMSO is exerting this effect on the human NTCP receptor, and in general there appears to be a lack of complete understanding of the vast effects of DMSO on different biological processes [277]. The effect of DMSO on NTCP appears to be an unusual process and probably much more sophisticated than one may expect. NTCP may possibly be the first reported case of DMSO having such an extensive effect on a receptor protein that lies beyond differentiation and polarisation of cells for correct protein configuration and expression, as will be reported in this current study.

1.4.2 *In vivo*, animal models

Apart from chimpanzees, which have over the years provided as with priceless information on HBV infection, and another example being the *Tupaia* model, mice are still the preferred animal model for the study of human diseases. Mice are one of the most commonly used small animal model, primarily due to the fact they are not costly, they are easy to handle and maintain in bulk, they are easily bred and have a very short reproduction cycle, while their genetic and immune background are well characterised. In addition, due to the progression of technology there are many available genetic modification techniques that can be applied to mice. However, mice, as well as mouse hepatocytes, even though they are able to support HBV replication, are naturally non-susceptible to HBV infection and hence cannot be experimentally infected with the virus. Many research groups have therefore focused on developing alternative mouse models to study HBV, and some of most popular models include HBV transgenic mice, HBV-transfected mice or human-mouse chimera models of HBV infection (*Figure 1.36*) [225, 227, 231].

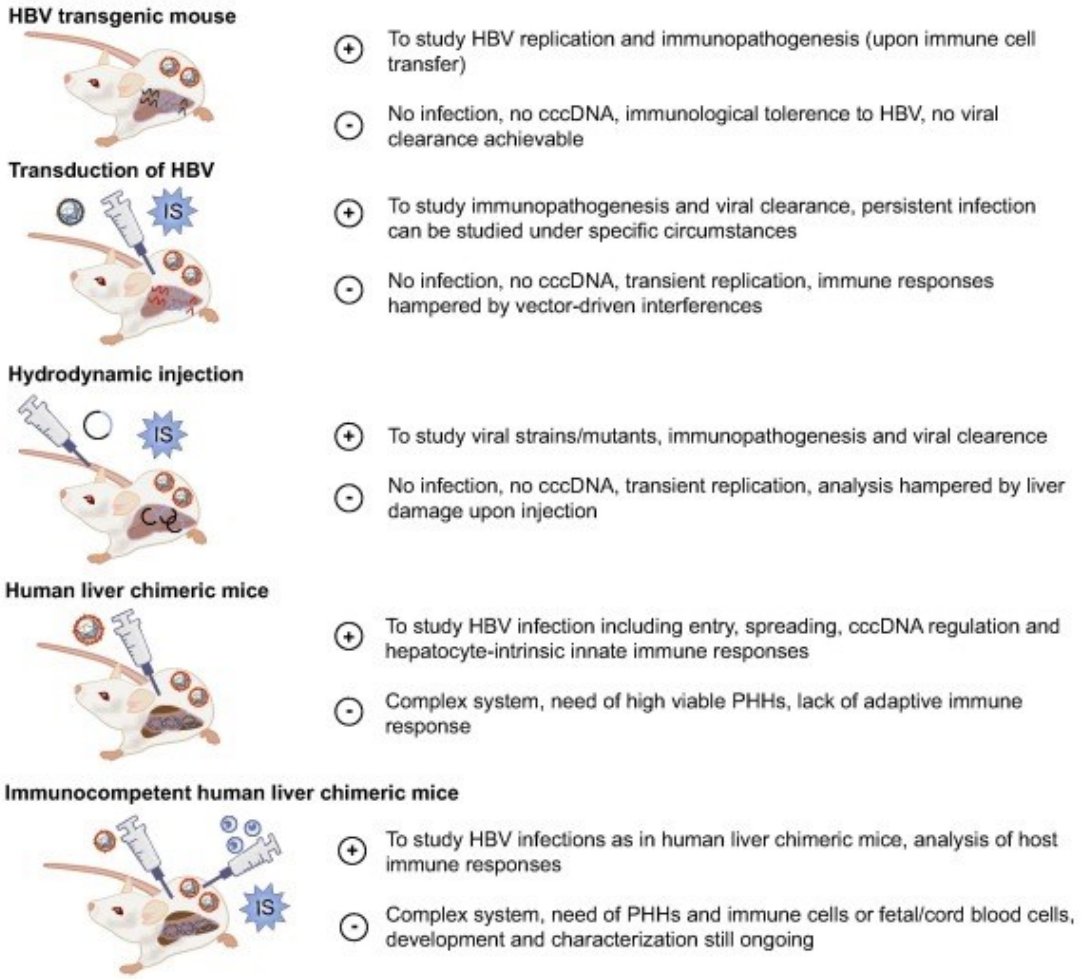


Figure 1.36; Examples of the key mouse models for studying HBV infection. Some of their main characteristics are mentioned. *IS; Immune System.* ***+****; Advantages.* ***-****; Disadvantages. Source:* [225].

HBV transgenic mice (*Figure 1.36*) typically express partial or complete copies of the HBV genome, including particular HBV genes for the study of the individual pathological effects of these viral proteins [278, 279]. This model is created by microinjecting the plasmids of interest into the fertilised eggs of inbred mice, and has allowed the vast study of HBV replication as well as immunopathogenesis of HBV virus in this small animal model. This model, however, does not allow recapitulation of the entire HBV life cycle, and hence knowledge on certain aspects of HBV biology, such as virus entry, cannot be acquired. Additionally, viral clearance in this model cannot be achieved as the HBV genome becomes integrated into the host genome, while no cccDNA can be established hence this aspect of HBV biology also cannot be

investigated [225, 227, 233, 234]. Consequently, a functional HBV receptor-expressing transgenic mouse model would prove to be very useful for the study of the early events during HBV infection [231], as similarly used for the study of HDV infection [224].

The transfected mouse model (*Figure 1.36*) involves vector-mediated transduction of HBV into immunocompetent mice. This is typically performed when high doses of HBV are specifically delivered into the mouse's liver via an adeno-associated virus (AAV) vector by intravenous injection. HBV expression in the liver is under the control of the vector's endogenous promoter. These are typically used to study virus replication, which persists for approximately three months, and thus provide a more physiological environment causing absent to low liver damage compared to the model where HBV gene delivery happens via adenoviruses (AVs). This model also does not allow recapitulation of the entire HBV life cycle, but has allowed the study of viral gene expression, host immune activation and HBV immunotolerance, and the evaluation of immunotherapies [227, 233, 234, 280-282].

Hydrodynamically injected mice, similarly to vector-mediated transduced mice, are mice in which delivery of replication-competent HBV plasmids occurs directly into the animal's liver, but this time by hydrodynamic tail vein injection of a relative large sample volume in a very small amount of time. Characteristic of this model is the efficiency of HBV DNA transfer that results in efficient viral replication [119, 225, 231, 280, 283, 284]. This, as well as the vector-mediated transduction model, could possibly be another model for delivery of the HBV receptor(s) for the creation of a more functional small animal model for the study of the early stages during viral infection.

Human liver chimeric mice (*Figure 1.36*) refer to mice that are xenotransplanted with primary hepatocytes from humans [285, 286], or even

treeshrews [287] amongst others, and are able to recapitulate the entire HBV life cycle and hence are typically used to study the efficacy of HBV drugs including HBV entry inhibitors [288]. However, these models lack an endogenous immune system, which limits their uses as no immunological studies can be performed on them, while at the same time they are extremely fragile and have a high mortality rate. Additionally, it is quite timely and requires great skill to generate them. An example of a chimeric mouse model is the urokinase plasminogen activator (uPA)- severe combined immunodeficiency (SCID) model, others include an uPA model where expression is driven by an albumin promoter (*Figure 1.37*) [227, 289].

Furthermore, the fumaryl acetoacetate hydrolase (Fah) immunodeficient mouse model has also been widely used. In this model the gene encoding for Fah, involved in the catabolic pathway of tyrosine, has been deleted and, therefore, these animals have problematic tyrosine metabolism pathways. Specifically, in these animals there is accumulation of the toxic metabolite fumarylacetoacetate, which eventually results in chronic liver damage. In this model liver injury for the repopulation of the mouse liver with human hepatocytes is under the control of a drug known as Nitisinone or else NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; *typically used for the treatment of hereditary tyrosinemia type I*); the presence of the drug blocks the accumulation of the toxic metabolite, preventing liver damage, and allowing the animals to remain healthy. However, once the drug is removed from the animal's system, selective pressure for the repopulation of the liver is induced as a result of liver injury and hence human hepatocyte transplantation initiates. Transcriptionally active cccDNA can be established in this model [290].

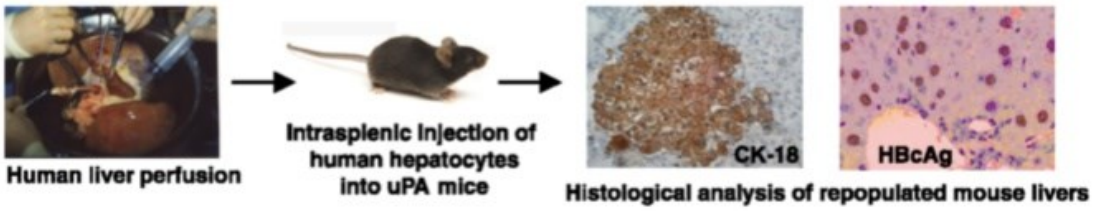


Figure 1.37; The human liver chimeric uPA mouse model.
Human hepatocytes are isolated from a human liver tissue and these are then transplanted into the spleen of the immunodeficient uPA mouse model. The mouse's liver needs time to repopulate with the human hepatocytes. Infection of these mice with HBV leads to establishment of viral replication. Immunostaining with human-specific CK-18 antibody, to determine levels of human-mouse liver chimerism, and HBcAg antibody, to determine establishment of HBV infection. *Source:* [233].

The humanised mouse model (*Figure 1.36*) was generated for the purpose of overcoming the limitations faced with the liver chimeric model, that being the lack of a human immune response system. Even in this model, however, cccDNA is not broadly detectable so certain critical steps in the virus' replication cycle are still difficult to investigate in full. An example of a humanised mouse model is what is referred to as the BLT model for bone marrow/liver/thymus, which is established by surgical transplantation of human fetal liver and thymus in the kidney and intravenous injection of autologous fetal liver hematopoietic stem cells [225, 227, 291-294].

Summary of some of the currently available *in vivo* cell culture systems for the study of HBV:

Mouse models	Characteristics	Applications	Limitations
Transgenic mice	Many advantages for using them: ease to handle due to their size, easy husbandry and short breeding cycle, low cost to purchase and maintain, have been studies for years so well characterised genetic and immune background, numerous manipulation techniques (e.g. genetic) have been fully established.	Expression of partial or complete HBV genomes, to study specific aspects of HBV biology, immune responses and pathogenesis. High replication efficiency. Suitable for evaluation of antiviral drug treatments targeting HBV replication.	Not full HBV cell cycle recapitulation, hence cannot study entry events and further spread of the infection, including HBV pathogenesis and viral clearance. No cccDNA production. HBV tolerance.
Vector-mediated transduced mice	Many advantages for using them. Direct plasmid transfer into the liver.	Acute and self-limiting type of HBV infection hence allows for the study of immune-mediated virus clearance. Secretion of infectious virions.	Vector delivery may induce unwanted immune responses. Cause some damage to liver and may have some cytotoxic effects. Not prolonged establishment of infection (AAV delivery has better results than AV delivery). No cccDNA detectable.
Hydrodynamically injected mice	*Same as vector-mediated transduced mice*	Transient gene expression. Cross species-specific barriers. High replication efficiency. Study of immune responses and antiviral treatments. Use of any mouse genetic background, but different backgrounds respond differently to viral clearance.	The persistence of infection depends entirely on the host immune response system and how quickly it manages to clear infection. Due to the nature of the delivery method, the animals are under strong distress so they require longer periods for recovery during which they need to be under constant observation. Significant liver damage.

Mouse models	Characteristics	Applications	Limitations
Chimeric mice	Human engrafted hepatocytes in the animal's liver. Transplanted hepatocytes are maintained for longer periods of time (with longer-term engraftment in the SCID model).	HBV-permissive human or tupaia hepatocytes (natural virus hosts). Support full HBV infection. Study of virus/host interactions, metabolic pathways, immune responses, virus integration and clearance, and vaccination strategies.	Require skillfulness for generation while they have a slow generation period. Limited period of use. High liver damage induced. Highly immune deficient animals. Regeneration of the mouse liver is timely and levels of human chimerism vary. No functional human immune system hence do not mimic human immune responses to viral infection.
Humanised mice	Human immune system and human liver.	Most physiological model that efficiently supports HBV infection and allows the study of immune responses in response to HBV infection.	Timely and requires skill to produce in bulk.

Table 1.6; Examples of current and most widely used mouse models for the study of HBV.

Modified and adapted from [225, 227, 231, 233, 234].

A small animal model for studying the HBV full life cycle is urgently required, to shed some light into the HBV interspecies transmission barrier as well as facilitate the development of improved therapeutic option, which are also urgently required.

1.5 Project Aims

Due to the recent discovery of NTCP as the functional receptor for HBV, to date, very limited information is available on the early stages during viral infection. This initial step is crucial for primarily gaining a full understanding of how HBV enters cells, spreads infection and persists in the host organism, hence possibly allowing an understanding as to how the virus manages to evade host responses as efficiently as it does. The unavailability of efficient cell culture systems recapitulating the entire virus' life cycle, including the entry phase, has also substantially hampered HBV research over the years. To address these issues, we aimed at dissecting the early events during HBV infection. On completion of this project, we generally hoped to gain a better understanding of the very early events that are necessary for HBV entry into and successive infection of host cells. This would further result in a broader understanding of HBV biology, as well as the possible establishment of novel model systems enabling translational research to investigate novel therapeutics against HBV infection.

We particularly hypothesized that dissecting HBV entry and comparing it in cells, which are either susceptible to infection or those who are refractory to infection, would result in the collection of important data related to the complex virus/host interactions during HBV persistence. Specifically, we aimed at:

1. Dissecting the molecular determinants for HBV entry into human hepatocytes. Since a plethora of receptors have been described throughout the years, we additionally aimed at characterising some of them and defining whether they are in fact essential receptors for HBV infection. To assess entry we particularly generated and made use of a set of tools, including HBV-mimicking lentiviral pseudoparticles and recombinant viruses.

2. Evaluating the signal transduction and regulatory pathways required for HBV receptor functional expression.

3. Comparing the receptors, pathways and their respective activation by HBV in non-human cells, such as mouse cells, in the hope of gaining a better insight into the very narrow species-specific HBV liver tropism and possibly to determine which of these may actually contribute to the definition of the interspecies restriction for HBV infection.

4. Attempting to uncover novel factors crucial for HBV entry.

2. Materials and Methods

2.1 Materials

The majority of consumables and apparatus utilized for this study were purchased from Applied Biosystems (*AB*; UK), Bio-Rad (UK), Eurofins (UK), GE Healthcare (UK), New England Biolabs (*NEB*, UK), Qiagen (UK), VWR International (UK), Abcam (UK), Sigma-Aldrich (UK) and ThermoFisher Scientific (UK).

2.1.1 Reagents

Chemicals and Inhibitors	Suppliers
2-Mercaptoethanol	ThermoFisher Scientific
a- D -Glucose, anhydrous	Sigma-Aldrich
Acetic acid	VWR International
Acrylamide 40% solution	Bio-Rad
Actinomycin D	ThermoFisher Scientific
Agarose	Sigma-Aldrich
Amersham ECL Western Blotting Detection Reagents	GE Healthcare
Ammonium persulfate (APS)	Sigma-Aldrich
Bovine serum albumin (BSA), standard grade powder	Sigma-Aldrich
Bromophenol blue	Bio-Rad
Caffeine	Sigma-Aldrich
cAMP	Sigma-Aldrich
Colchicine	Sigma-Aldrich
Collagen from calf skin	Sigma-Aldrich
Complete mini EDTA-free tablets	Roche (Switzerland)
Coomasie G250	ThermoFisher Scientific
Cycloheximide	Sigma-Aldrich
Cytochalasin D	Sigma-Aldrich
Demecolchine	Sigma-Aldrich
DMSO	VWR International
Dithiothreitol (DTT)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich
Ethanol	VWR International

Ethidium bromide solution	Sigma-Aldrich
Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)	Sigma-Aldrich
Formaldehyde solution, 36.5-38% in H_2O	Sigma-Aldrich
Glucose	Sigma-Aldrich
Glycerol	Sigma-Aldrich
Glycine	Sigma-Aldrich
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich
Hydrochloric acid	VWR International
Hydrocortisone	Sigma-Aldrich
Latrunculin A	Sigma-Aldrich
MDL-12	Sigma-Aldrich
Methanol	VWR International
MG-132	Merck Millipore (US)
Nonidet P40 (NP40)	Sigma-Aldrich
PEG 8000	VWR International
Phenylmethylsulfonyl Fluoride (PMSF)	Sigma-Aldrich
Poly-L-lysine hydrobromide	Sigma-Aldrich
Polybrene	Sigma-Aldrich
2-Propanol (Isopropanol)	Sigma-Aldrich
Roflumilast	SelleckChem (UK)
Skimmed milk powder	Sigma-Aldrich
Sodium bicarbonate	Sigma-Aldrich
Sodium chloride	Sigma-Aldrich
Sodium deoxycholate	Sigma-Aldrich
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Sodium orthovanadate	Sigma-Aldrich
SYBR Safe™ DNA Gel Stain	ThermoFisher Scientific
SuperSignal™ West Pico/ Femto Chemiluminescent substrate	ThermoFisher Scientific
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Thiazolyl Blue Tetrazolium Bromide	Sigma-Aldrich
Triton™ X-100	Sigma-Aldrich
Trypan blue solution (0.4%)	ThermoFisher Scientific
Tunicamycin	Sigma-Aldrich
TWEEN® 20	Sigma-Aldrich
Wortmannin	Sigma-Aldrich

Enzymes and Recombinant Proteins	Suppliers
Alkaline phosphatise, calf intestinal (CIP)	*All enzymes were purchased from* NEB, *unless otherwise stated.*
Beta 1-4 Galactosidase	
Beta-N-Acetylhexosaminidase	
DNaseI	
DpnI	
Endoglycosidase Hf (Endo Hf)	
Gateway™ BP/LR Clonase™ II enzyme mix	
GoTaq® Green Master Mix	Promega (UK)
Lambda phosphatase	
Neuraminidase A	
Peptide *N*-Glycosidase F (PNGaseF)	
Plasmid-Safe™ ATP-dependent DNAse	Epicentre (UK)
Platinum® PCR SuperMix High Fidelity	ThermoFisher Scientific
Platinum® SYBR® Green qPCR SuperMix	ThermoFisher Scientific
Protein Deglycosylation mix	
Quick Ligation™ kit	
SLC10A1 (Human) recombinant protein	Abnova (Taiwan)
T4 DNA ligase	
TaqMan® Universal Master Mix	ThermoFisher Scientific
DNA and Protein ladders	**Suppliers**
HyperLadder™ 1kb DNA ladder	Bioline (UK)
Quick-Load® 100 base pair (bp) or 1kb DNA ladders	NEB
SeeBlue® Plus2 Pre-Stained Protein Standard (4-250kDa)	ThermoFisher Scientific
Color Prestained Protein Standard, Broad Range (11-245 kDa)	NEB
Antibiotics, for Bacteria (BC) or Cell Culture (CC)	**Suppliers**
Ampicillin (1:1000) - *BC*	*All antibiotics were purchased from* Sigma-Aldrich. *They were all prepared and filtered as per the manufacturer's instructions.*
Blasticidin (5ug/ml; 10mg/ml stock) - *CC*	
Chloramphenicol (35ug/ml; 35mg/ml stock) - *BC*	
Geneticin (G418) – Neomycin (400ug/ml; 100mg/ml stock) - *CC*	
Hygromycin B (500ug/ml; 50mg/ml stock) - *CC*	
Kanamycin (1:1000) - *BC*	
Penicillin/Streptomycin (1%) - *CC*	
Puromycin (5ug/ml; 10mg/ml stock) - *CC*	

Bacteria Chemically Competent Cells	Suppliers
Dam- Dcm- (E.coli) DH5alpha™ (E.coli) Library Efficiency® DH5alpha™ One Shot® ccdB survival 2 T1R One Shot® Mach1 T1 (E.coli) One Shot® TOP10 (E.coli)	*All competent cells were initially purchased from* ThermoFisher Scientific, *and these were then used to make follow up stocks.*
Cell Culture Media and Supplements	**Suppliers**
Dulbecco's Modified Eagle's Medium (DMEM) Fetal Bovine Serum (FBS, 10%) Hank's Balanced Salt Solution (HBSS) HEPES (1M) Lipofectamine™ 3000 Transfection Reagent Lipofectamine® RNAiMAX Reagent OptiMEM® (minimum essential medium) OptiPrep™ Density Gradient Medium Phosphate Buffered Saline (PBS) Primary Hepatocyte Maintenance Media (PHMM), including Cocktail B Trypsin/0.05% EDTA William's E (with or without phenol red)	*All cell culture media and supplements were purchased from* ThermoFisher Scientific, *unless otherwise stated.* Sigma-Aldrich
TaqMan® Gene Expression Assays	**Suppliers**
ASGPR1 (Hs01005019_m1) EGFR (Hs01076090_m1) GAPDH (Hs02786624_g1) PDE1A (Hs00897273_m1) PDE1C (Hs01095682_m1) PDE4D (Hs01579625_m1) PDE12 (Hs00698272_m1) Slc10a1 (mouse - Mm00441421_m1) SLC10A1 (human - Hs00161820_m1) TfR (Hs00951083_m1)	*All gene expression assays were purchased from* ThermoFisher Scientific
Small Interference RNAs (siRNAs)	**Suppliers**
Silencer® Select Pre-designed siRNAs [PDE1A, PDE1C, PDE4D, PDE12] *For product information sheets see Appendix A.2.XIV.*	*All siRNAs were ready purchased from* ThermoFisher Scientific

Primary Antibodies	Optimum Concentrations/ Dilutions	Suppliers
anti-ASGPR [ab42488]	*0.5 µg/ml*	Abcam
anti-beta-Actin- Horseradish peroxidise (HRP) [A3854]	*1:35,000*	Sigma-Aldrich
anti-EGFR [ab2430]	*1:1,000*	ThermoFisher Scientific
anti-Flag antibody (DYKDDDDK Epitope Tag antibody) [PA1-984B]	*1:1,000 (WB), 1 µg/ml (IF; immunofluorescence)*	ThermoFisher Scientific
anti-Flag antibody (DYKDDDDK Epitope Tag antibody) – Phycoerythrin (PE), L5	*1:500*	Biolegend (UK)
anti-green fluorescent protein (GFP) [20R-GR011]	*1:1,000*	Fitzgerald (US)
anti-HBcAg [B0586]	*1:200*	Dako, Agilent Technologies (France)
anti-HBsAg Ad/Ay – Fluorescein isothiocyanate (FITC) [PA173086]	*1:1,000*	ThermoFisher Scientific
anti-p53, Clone D01	*1:1,000*	Dako
anti-Proliferating Cell Nuclear Antigen (PCNA) – HRP [EPR3821, ab193965]	*1:5,000*	Abcam
anti-PDE4D [ab59249]	*1:500*	Abcam
anti-SLC10A1 antibody [EPR8606, ab133670]	*1:1,000*	Abcam
anti-SLC10A1 antibody [ab131084]	*1:1,000*	Abcam
anti-SLC10A1 antibody (FITC) [orb14188]	*1:50*	Biorbyt (UK)
anti-SLC10A1 antibody [GTX37362]	*1:1,000*	GeneTex (US)
anti-SLC10A1 antibody [SAB2104761]	*1:1,000*	Sigma-Aldrich
anti-SLC10A1 Human Antibody [LS-C161120]	*1:1,000*	LSBio (US)
anti-SLC10A1 Mouse Antibody [LS-C312688]	*1:500*	LSBio
anti-TfR [ab84036]	*1 µg/ml*	Abcam
anti-Tubulin [T7816]	*1:20,000*	Sigma-Aldrich

Secondary Antibodies	Optimum Concentrations/ Dilutions	Suppliers
anti-mouse Alexa Fluor® 594	*1:1,000*	ThermoFisher Scientific
anti-rabbit Alexa Fluor® 488	*1:1,000*	ThermoFisher Scientific
Donkey anti-rabbit IgG HRP-linked	*1:50,000*	GE Healthcare
Sheep anti-mouse IgG HRP-linked	*1:50,000*	GE Healthcare
Labelled Cell Trackers	**Optimum Concentrations/ Dilutions**	**Suppliers**
4',6-Diamidino-2-Phenylindole, Dilactate (DAPI)	*1:50,000*	*All labelled cell trackers were purchased from* ThermoFisher Scientific
Concanavalin A, Alexa Fluor® 594 Conjugate	*200 µg/ml*	
Lectin Helix pomatia agglutinin (HPA), Alexa Fluor® 647 Conjugate	*10 µg/ml*	
Phalloidin, Alexa Fluor® 647 Conjugate	*1:20*	
Wheat germ agglutinin (WGA), Alexa Fluor® 350	*5 µg/ml*	

Kits, and other Consumables	Suppliers
Amicon® Ultra 15ml Centrifugal Filters	Merck Millipore
cAMP direct immunoassay kit [ab65355]	Abcam
DNeasy® Blood and Tissue kit	QIAGEN
EndoFree® Plasmid Maxi kit	QIAGEN
Hepatitis B E and Surface Antigen (HBe/sAg) chemiluminescent immunoassay kits	Autobio (China)
High-Capacity cDNA Reverse Transcription kit	ThermoFisher Scientific
High-Capacity RNA-to-cDNA kit	ThermoFisher Scientific
HiSpeed® Plasmid Maxiprep kit	QIAGEN
Human Phospho-Kinase Antibody Array kit	R&D Systems (UK)
Pierce BCA kit	ThermoFisher Scientific
QIAprep® Spin Miniprep kit	QIAGEN
QIAquick® Gel Extraction/PCR Purification kit	QIAGEN
RNeasy® Mini kit	QIAGEN
SilverQuest™ Silver Staining kit	ThermoFisher Scientific
TOPO® TA Cloning® kit	ThermoFisher Scientific

Table 2.1; Consumables used during the course of the project.

2.1.2 Solutions and Buffers

	Composition:
10% APS	1g APS in 10ml ddH_2O
5% FBS/PBS **10% FBS/PBS**	5ml FBS and 95ml 1xPBS 10ml FBS and 90ml 1xPBS
4% paraformaldehyde (PFA)	Diluted the original stock of Formaldehyde solution, 36.5-38%, in PBS (approximately 1:10).
0.1% PBS/TWEEN (PBST)	100ml 10x PBS 1ml TWEEN® 20 ~ 900ml ddH_2O
4% PEG 8000	Prepared a 40% stock solution by dissolving 40g of the PEG 8000 into a final 100ml of H_2O. Once filter sterilised this was then diluted accordingly (1:10) directly in cell culture media.
0.1% Triton™-X 100/PBS	100µl Triton™ X-100 10ml 10x PBS ~ 90ml ddH_2O
Agarose Running Buffer; **Tris-Acetate EDTA buffer** **(50x TAE)** **1xTAE**	121g Tris-HCl 28.6ml glacial acetic acid 50ml 0.5M EDTA (pH 8.0) Made to a final volume of 500ml with ddH_2O 20ml 50x buffer diluted in 980ml H_2O.
Blocking Solution	5% Non-fat, dry Milk Powder in PBS-0.1% Tween
$CaCl_2$ stock solution **(for transfections - 2.5M)** **0.25M $CaCl_2$**	36.75g $CaCl_2$ 70ml ddH_2O Made to a final volume of 100ml. Filtered. 10ml of 2.5M $Cacl_2$ diluted to 100ml of ddH_2O.
HEPES buffered saline (HBS; 2x)	8g NaCl 0.2g Na_2HPO_4-$7H_2O$ (0.14g if using dehydrate; [phosphate] must be 1.5mM) 6.5g HEPES pH 7.0 Made up to 500ml with ddH_2O (pH 7.0). Filtered.

Laemmli Buffer (2x)	260mM Tris- Hydrogen Chloride (HCl), pH 6.8 5% SDS 10% B-Mercaptoethanol 14.8% Glycerol 0.001% Bromophenol Blue	
OptiPrep™ diluent	100mM NaCl 30mM $CaCl_2$ 120mM Tris-HCl, pH 7.2	
OptiPrep™ working solutions: **50% iodixanol** **10% iodixanol**	 Mix 5 volumes of the purchased 60% OptiPrep™ solution with one volume of the OptiPrep™ diluents. Mix one volume of the 50% OptiPrep™ working stock solution with 4 volumes of the OptiPrep™ diluents.	
Polybrene	(4mg/ml) - 40 mg polybrene (hexadimethrine bromide) dissolved in 10 ml ddH_2O. Sterilized by passage through a 0.2-µm filter.	
Poly-L-Lysine	(100µg/ml) – 25mg stock powder was dissolved in 250ml ddH_2O. Filtered.	
Radioimmunoprecipitation assay (RIPA) Lysis Buffer (5x) **1x RIPA**	0.75M NaCl 5% (v/v) NP40 2.5% (w/v) Deoxycholate 0.5% (w/v) SDS <u>0.25M Tris-HCl, pH8.0</u> 2ml of the 5x RIPA were diluted to a final volume of 10ml with ddH_2O.	
SDS- PAGE (polyacrylamide gel electrophoresis); Gels	<u>10% Resolving Gel</u> (15ml) 5ml 30% acrylamide 3.8ml 1.5M Tris (pH8.8) 150µl 10% SDS 150µl 10% APS 6µl TEMED 5.9ml H_2O	<u>4% Stacking Gel</u> (10ml) 1.7ml 30% acrylamide 1.25ml 1.5M Tris (pH6.8) 100µl 10% SDS 100µl 10% APS 10µl TEMED 6.8ml H_2O

Sodium Chloride (10mM, 80mM and 140 mM) - Ringer's Solution.	10mM (0.58g/L), 80mM (4.68g/L) and 140mM (8.18g/L). Filtered prior to use.
Terrific Broth (TB) medium	12g Tryptone 24g Yeast extract 4ml Glycerol To a final volume of 900ml with ddH_2O The medium was autoclaved and, once slightly cooled, 100ml of the following sterile solution were added: 0.17M KH_2PO_4 0.72M K_2HPO_4
Tris (1M)	12.1g in 900ml ddH_2O Made up to 1L with ddH_2O, and the pH adjusted accordingly.
Western Blot **Running Buffer (10x, 1L)** **1x Running Buffer (1L)**	250mM Tris = 30.3 gr 1.92M Glycine = 144 gr 100ml 10x buffer 890ml H_2O 10ml 10% SDS
Western Blot **Transfer Buffer (10x, 1L)** **1x Transfer Buffer (1L)**	58gr Tris 29gr Glycine 100ml 10x buffer 700ml ddH_2O 200ml Ethanol
William's E (WE) 10% media with Primary Hepatocyte Maintenance Supplements	[450ml WE] + 50ml FBS 20ml Cocktail B: [2.5ml Penicillin/ Streptomycin; 5ml ITS+ human recombinant insulin - human transferrin (0.625 mg/ml each), selenous acid (0.625 µg/ml), BSA (0.125 g/ml), linoleic acid (0.535 mg/ml); 5ml GlutaMAX™ (200mM/100x); 7.5ml HEPES pH7.4 (1M)] 5µl Dexamethasone (in DMSO, 10mM) Further supplemented with: 18µg/ml Hydrocortisone 2% DMSO *As suggested by* [32, 295].

Table 2.2; Solutions and Buffers used during the experimental procedures.

2.1.3 Sequencing and Cloning Primers

Name of Oligonucleotides	Sequence (5'-3')
Forward Primers for sequencing the full-length pCMV_HBV construct	
HBV_189	TGCTCGTGTTACAGGCG
HBV_612	CATCATCTTGGGCTTTCG
HBV_1178	AAGTGTTTGCTGACGC
HBV_1827	ACCTCTGCCTAATCATCTC
HBV_2440	TTACCACTCCCTATCAGT
HBV_3040	TAATGACTCTAGCTACCTG
HBV_3690	TTACACACTCTATGGAAGG
HBV_4240	GTCAATCTTCTCGAGGA
HBV_4660	AAGGAACCTCTATGTATCC
HBV_5260	ATACCTGAACCTTTACCC
HBV_5830	GGCATACTTCAAAGACTG
Primers used for Gateway cloning of the HBV genomes encoding transgenes	
HBV core Forward (Fw)	GTCGAGCTCggtacc (*KpnI*) CGGGTCGAGTAGGCG
HBV core Reverse (Rev)	TAAGCAacgcgt (*MluI*) CTAACATTGAGGTTCCCG
HBV polymerase Fw	TAAGCAacgcgt (*MluI*) ATGCTTACAGACCACC
HBV polymerase Rev	CAATCctcgag (*XhoI*) AAGATTG
Dest-*MluI* Fw	TAAGCAacgcgtTTTGTACAAAAAAGCTGAAC
Dest-*MluI* Rev	TAAGCAacgcgtAACCACTTTGTACAAGAAAG
attB.IRES.BFP Fw (*blue fluorescent protein*)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTATAATTTCTTCTTCCAGAAAGCCACCATGAGCGAGCTGATTAAG
attB.IRES.BFP Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTGGAAGAAGAAATTATAAAGGTGGCTTTATCTAGATCCGGTGGA
attB.IRES.Bsd Fw (*blasticidin resistance)*	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTATAATTTCTTCTTCCAGAAAGCCACCATGGCCAAGCCTTTGTCT
attB.IRES.Bsd Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTGGAAGAAGAAATTATAAAGGTGGCTTTATATGTGTGGGAGGGC
attB.IRES.Fluc Fw (*Firefly luciferase*)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTATAATTTCTTCTTCCAGAAAGCCACCATGGAAGATGCCAAAAAC
attB.IRES.Fluc Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTGGAAGAAGAAATTATAAAGGTGGCTTTAGACGTTGATCCTGGC

attB.IRES.Gluc Fw (*Gaussia luciferase*)	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTATAATTTCTTCTTCCAGAAAGCCACCATGGAAGATGCCAAAAAC
attB.IRES.Gluc Rev	GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTGGAAGAAGAAATTATAAAGGTGGCTTTAGACGTTGATCCTGGC
Sequencing Fw	CGGGAACCTCAATGTTAG
Sequencing Rev	TTGATAGGATAGGGGCAT
Primers used for cloning of the individual fragments that make up the HBV genome	
HBV Surface Fw	GCTAGC (*NheI*) – AAACAACACATAGCG
HBV Surface Rev	AAGCTT (*HindIII*) – GCAAGGACCCATAAC
HBV Polymerase Fw	GCTAGC (*NheI*) – AGCTTATAGACCACC
HBV Polymerase Rev	AAGCTT (*HindIII*) – GCAACATTCGGTGGG
	The HBV core and EpsilonCre constructs were purchased as GeneArt constructs and utilised in a typical gateway cloning procedure.
Additional HBV primers	
HBV full-length Fw	(*SpeI*) - GATACCACTAGTCCTTTACCACTCCCTATCAG
HBV full-length Rev	(*XbaI*) - GTGCCAAGGTCTGAAGGTCATCTAGAGATACC
HBV core Fw	GCACGAGTGGGTTACA
HBV core – *MluI* Rev	GGTGGTacgcgtCTAACATTGAGGTTCCCGAG
HBV core – *KpnI* Fw	ggtaccGAGCTCGGATCCACTAGTAA
HBV core – *MluI* Rev	TAAGCAacgcgtGAGGGCCCACATATTGTTG
HBV pol. – *MluI* Fw	TAAGCAacgcgtATGCCCCTATCTTATCAACA
HBV pol. – *SacII* Rev	CAATCccgcgcGATTCAGCGCCGACGG
HBV cccDNA primers	
cccDNA Fw	CTCCCCGTCTGTGCCTTCT
cccDNA Rev	GCCCCAAAGCCACCCAAG
Primers used for quantification of HBV DNA copies	
HBV Fw	GTGTCTGCGGCGTTTTATCA
HBV Rev	GACAAACGGGCAACATACCTT
HBV DNA probe	FAM-CCTCTKCATCCTGCTGCTATGCCTCATC-TAMRA
	As previously described by Murray JL et al. [296].

Primers used for the quantification of the HBV replication intermediates	
Pregenomic Fw	GAGTGTGGATTCGCACTCC
Pregenomic Rev	GAGGCGAGGGAGTTCTTCT
Subgenomic Fw	TCACCAGCACCATGCAAC
Subgenomic Rev	AAGCCACCCAAGGCACAG
RPS11 Fw	GCCGAGACTATCTGCACTAC
RPS11 Rev	ATGTCCAGCCTCAGAACTTC
*(*Ribosomal Protein S11 was used as the housekeeping gene)*	*As previously described by Yan H et al. [32].*
Sequencing primers for lentiviral cyclic repackaging screen	
cDNA Fw	ACCGCCCTCAAAGTAGAC
cDNA Rev	GCTTGCCAAACCTACAGG

Table 2.3; List of primers used for sequencing or cloning of various DNA plasmids.

All oligonucleotides were purchased from Eurofins.

2.1.4 DNA constructs

Abbreviations of Plasmids	Notes	Appendix
Vector plasmids		
HIV gag/pol	Construct expressing the HIV type 1 virus. ***	A.2.VIII.i
pCDNA3.1(+)	Mammalian expression vector with the CMV promoter. The multiple cloning sequence (MCS) is in the forward (+) orientation. *Kindly provided by Peter O'Hare (Imperial College London).*	A.2.I.i
pCDNA3.1(-)	Mammalian expression vector with the CMV promoter. The MCS is in the reverse (-) orientation. ***	A.2.I.ii
phCMV_VSVg	Mammalian expression vector encoding for the VSVg under the control of a CMV promoter. ***	A.2.VIII.ii
pLenti_CMV_Dest_ Hygro	3rd generation lentiviral Gateway destination empty vector. Expresses a CMV promoter and a Hygromycin antibiotic selection cassette (Addgene, US).	A.2.I.iii
pTRIP_CMV_IRES_ RFP	Lentiviral vector expressing a red fluorescent protein (RFP) under the control of an IRES element. ***	A.2.I.iv
pTRIP-Fluc_RFP	Lentiviral vector expressing a Fluc sequence. ***	A.2XIII.i
pTRIP_Venus	Lentiviral vector expressing a Venus fragment as an alternative to a GFP sequence. ***	A.2.VIII.iii
HBV-related plasmids		
Jc1-5AB-DEST	This construct contains the full length monocistronic Jc1 plus-strand genome with a Gateway® destination cassette inserted between duplicate NS5A/NS5B cleavage motifs. This vector does not encode a functional genome.	A.2.II
pTagBFP-C	Cloning vector containing the BFP. ***	A.2.III.i
pGL4.12(luc2CP)	Basic vector, with no promoter, encoding the luciferase reporter gene *luc2CP (Photinus pyralis).* ***	A.2.III.ii
pCMV_Gluc	Mammalian expression vector with a CMV promoter encoding for Gluc. ***	A.2.III. iii

pCMV_HBV	Mammalian expression vector with a CMV promoter. Contains the wild-type HBV 1.3kb overlength genomic sequence. *Kindly provided by Christoph Seeger (Fox Chase Cancer Center).*	A.2.IV
pCMV_HBV_ BFP	*Same as above,* but in this plasmid a BFP sequence has additionally been inserted between the core and polymerase HBV sequence.	A.2.V.i
pCMV_HBV_ BSD	*Same as above,* but in this plasmid a BSD has additionally been inserted between the core and polymerase HBV sequence.	A.2.V.ii
pCMV_HBV_ Fluc	*Same as above,* but in this plasmid a Fluc sequence has additionally been inserted between the core and polymerase HBV sequence.	A.2.V.iii
pCMV_HBV_ Gluc	*Same as above,* but in this plasmid a Gluc sequence has additionally been inserted between the core and polymerase HBV sequence.	A.2.V.iv
pCDNA3.1(-)_HBV	Mammalian expression vector with a CMV promoter. The MCS is in the reverse (–) orientation. Contains the wild-type HBV 1.3kb overlength genomic sequence.	A.2.VI
pCDNA3.1(-)_HBV_ BFP	*Same as above, and similar to pCMV_HBV_BFP.*	A.2.VII.i
pCDNA3.1(-)_HBV_ BSD	*Same as above, and similar to pCMV_HBV_BSD.*	A.2.VII.ii
pCDNA3.1(-)_HBV_ Fluc	*Same as above, and similar to pCMV_HBV_Fluc.*	A.2.VII.iii
pCDNA3.1(-)_HBV_ Gluc	*Same as above, and similar to pCMV_HBV_Gluc.*	A.2.VII.iv
pCDNA3.1(+)_HBs Ag	Mammalian expression vector with the CMV promoter. The MCS is in the (+) orientation. Contains the full HBsAg sequence from the HBV genome.	A.2.IX.i
pCDNA3.1(+)_Core	*Same as above,* but this construct contains the core fragment from the HBV genome.	A.2.IX.ii
pCDNA3.1(+)_ Polymerase	*Same as above,* but this construct contains the polymerase fragment from the HBV genome.	A.2.IX.iii
pCDNA3.1(+)_ EpsilonCre	*Same as above,* but this construct contains the HBV RNA encapsidation signal Epsilon and additionally encodes for a Cre-recombinase.	A.2.IX.iv

Relevant GeneArt constructs		
BsdR	*Synthesised by* ThermoFisher Scientific, *and contained the equivalent fragments as suggested by their names.*	A.2.X.i
HBV Core		A.2.X.iii
HBV Core/Polymerase		A.2.X.iv
HBVepsilonCre		A.2.X.v
HBV receptor-related plasmids		
EX-C0391-LV105	Human-SLC10A1, Puromycin selection cassette (GeneCopoeia, US)	A.2.XI.i
EX-C0391-LV158	Human-SLC10A1 with a C-terminal Flag-tag, Neomycin selection cassette (GeneCopoeia)	A.2.XI.ii
EX-A0379-Lv105	Human-ASGPR, Puromycin selection cassette (GeneCopoeia)	*Similar to A.2.XI.i.*
chimNTCP ***(GeneArt)***	*Sequence synthesised by* ThermoFisher Scientific. This sequence has been designed to contain attB sites, on either side, for subsequent gateway cloning.	A.2.X.ii
pLenti_CMV_ chimNTCP_ Hygro	3rd generation lentiviral plasmid expressing a chimeric form of the NTCP receptor (i.e. the amino acids mapping to the important HBV pre-S1 binding domains of the mouse NTCP have been replaced with their human counterparts) under the control of a CMV promoter. It also expresses a Hygromycin selection cassette.	A.2XII.i
pTRIP_chimNTCP_ RFP	*Similar to above,* but this plasmid encodes for RFP rather than an antibiotic selection cassette.	A.2XII.ii
pTRIP_hNTCP_ RFP	*Similar to above,* but this plasmid encodes for the human form of the NTCP receptor instead of the chimeric form.	A.2XIII.ii

Table 2.4; List of DNA plasmids utilised during the experimental procedures.

**** These plasmids were all kindly provided by Charles Rice (Rockefeller University). The remaining plasmids, unless otherwise stated, were specially created during the course of this project.*

2.1.5 Cell lines

Cells	Description	Provided by
HepG2	Human liver hepatocellular carcinoma, or otherwise hepatoma, cell line	Paul Farrell (*Imperial College London, UK*)
Huh7	Human hepatocyte-derived cellular carcinoma cell line	Charles Rice (*Rockefeller University, US*)
Huh7.5	Clone of Huh7 cells with a mutation in the retinoic acid-inducible gene 1 (RIG-1), allowing for improved replication of some viruses as shown by previous studies (i.e. HCV; [297]).	
Hepa1.6	Mouse liver hepatoma cell line	
H2.35	Mouse liver hepatocyte cell line	
NIH3T3	Mouse embryo fibroblast cell line	Martin Allday (Imperial College London, UK)
HEK293T	Human embryonic kidney cells, derived from the HEK293 cell line with the addition of the SV40 large T-antigen that has been shown to increase vector production of some viral vectors (*National Gene Vector Biorepository*)	Mike Malim (*King's College London, UK*) *- Hek293 were kindly provided by Charles Rice (Rockefeller University, US)*
HepG2.2.15	Human hepatoma cell line derived from HepG2 cells, which stably expresses and replicates HBV in culture.	Peter Karayiannis (*University of Nicosia, Cyprus*)
HepDE19	Human hepatoma cell line derived from HepG2 cells, which stably expresses and replicates HBV in culture, but under the control of a Tet-on/off system [298].	Haitao Guo (*Indiana University*)

Table 2.5; Various cell lines utilised during the course of the project.

**All cell lines were a kind gift from the persons mentioned.*

2.1.6 Equipment

Types of Equipment	Instruments and Suppliers
Bacteria Cell Culture Shaking Incubators	MaxQ™ 4450, ThermoFisher Scientific Innova™ 4300, Eppendorf (UK)
Cell Counter	Countess II FL Automated Cell Counter, ThermoFisher Scientific
Centrifuges ***Cont.* Centrifuges**	Eppendorf Centrifuge 5424, Eppendorf Sorvall® Legend® RT+ and Sorvall® WX Ultra 80 Series Centrifuges, ThermoFisher Scientific
Confocal Microscope	LSM 5 Pascal, Zeiss (Germany)
Electroporator	ECM 830, ElectroSquarePorator™, BTX (US)
Enzyme-linked Immunosorbent Assay (ELISA) Microplate Reader	Spectramax® Plus 384, Molecular Devices (US) FLUOstar® Omega, BMG LabTech (Germany)
Flow Cytometry System	BD™ LSR II, BD Biosciences (UK)
Fluorescent and other Microscopes	Olympus 1X70 connected to an Olympus U-RFL-T, Olympus (UK) Axiovert 135 TV, Zeiss Wilovert, WILL Wetzlar (Germany) EVOS® FL Auto Cell Imaging System, ThermoFisher Scientific
Gel Imaging; e.g. Agarose gels	Molecular Imager Gel Doc™ XR+ Imagine System, Bio-Rad
Gel Extraction Imager	Safe Imager™, ThermoFisher Scientific
Heating Block	Analog Heatblock, VWR International
Luria-Bertani (LB) Plate Incubator	Heraeus (Germany)
Polymerase Chain Reaction (PCR) Thermal Cycler	DNA Engine DYAD™, Bio-Rad GeneAmp® PCR System 9700, AB ProFlex™ PCR System, ThermoFisher Scientific
pH Meter	Beckman pHI 40, Beckman (US)
Quantitative Real-Time or Reverse-Transcription (qRT) PCR Cycler	StepOne Plus™, AB QuantStudio™ 7 Flex Real-Time PCR, AB
Shaker	Mini Blot Mixer, VWR International
Spectrophotometer	Nanodrop Lite™, ThermoFisher Scientific

Tissue Culture (TC) Incubator	In-VitroCell™, NUAIRE (US)
UV Transilluminator	High Performance Ultraviolet Transilluminator, UVP (UK)
Vortex	WhirlIMixer™, FISONS (UK)
Western Blot Developer	ChemoDoc-IT®, UVP JP-33, JPI Healthcare (US)

Table 2.6; The apparatus used during the experimental procedures.

2.1.7 Computer Programs

Software	Suppliers
Confocal Microscopy Imager	LSM Image Browser, Zeiss
Data Analysis and Statistics	GraphPad Prism (US)
ELISA	MARS data analysis, BMG LabTech www.elisaanalysis.com (web-based analysis tool sponsored by ELISAkit.com; Australia)
Flow cytometry	BD FACS Diva™ Software, BD Biosciences FlowJo v7.61 software, Tree Star Inc. (US)
Fluorescent Microscopy Imager	Image Pro Plus MDA, MediaCybernetics (US)
Gel Imaging; e.g. Agarose gels	ImageLab™ 4.1, Bio-Rad
Graphics Design	Adobe Illustrator and Photoshop
Image Analysis, e.g. Western Blot band quantification	ImageJ, RSB GIMP
Plasmid Viewer	MacVector, Inc. (US) SnapGene® Viewer
qRT-PCR	StepOne™ Software and QuantStudio™ Real-Time PCR Software, ThermoFisher Scientific

Table 2.7; Computer software programs used for experimental analysis of the data.

2.2 Methods

2.2.1 Molecular techniques

2.2.1.1 General Cloning Strategy

For the purpose of this project we employed two distinct ways of cloning; we either went down the traditional cloning route by performing restriction enzyme digests as appropriate or we used Gateway® recombination technology, *as described by ThermoFisher Scientific (https://www.thermofisher.com/uk/en/home/life-science/cloning/gateway-cloning.html)*. In the case of the traditional cloning, the fragments of interest were either directly digested out of a plasmid and re-inserted into an appropriate vector backbone, or on different occasions the fragments of interest were PCR amplified from the plasmid with purposely designed primers that expressed specific restriction enzyme sites on either end so that the isolated PCR fragment could then be digested and reinserted into the vector backbone of interest. The traditional cloning strategy, for example, was carried out for the creation of the different plasmids expressing the individual HBV fragments (HBsAg, Core, Polymerase and EpsilonCre; *Table 2.4*) in a pCDNA3.1(+) vector background. Gateway® cloning, on the other hand, generally allows for the efficient transfer of DNA fragments between plasmids with the use of specific recombination sequences, known as the "Gateway® att" sites (*Figure 2.1*). This technology, for example, was employed for the transfer of the chimNTCP sequence into eventually the two lentiviral vectors expressing either the Hygromycin antibiotic resistance or the RFP reporter for subsequent creation of lentiviral pseudoparticles.

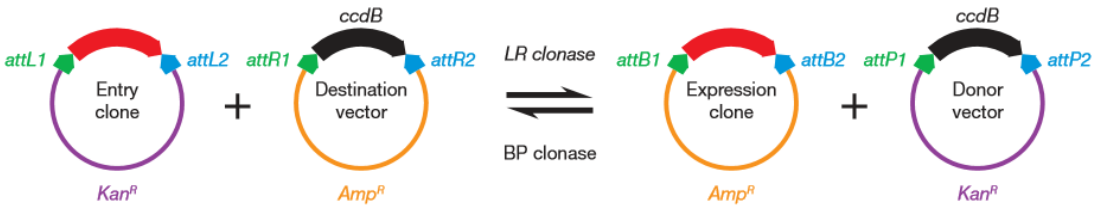


Figure 2.1; The Gateway® reaction.
Source ThermoFisher Scientific.

2.2.1.1.1 Summary of Gateway® Cloning

As suggested by *ThermoFisher Scientific* (*Figure 2.1*), the steps for Gateway® cloning are as follows:

1) A typical BP Gateway® reaction protocol for the creation of entry clones:

BP Gateway®; Creation of entry clone.	
Ingredients	**Concentration/ Quantity**
pDONR221 Entry Vector (~75ng/µl)	1µl
Purified PCR product (~100ng total)	1µl
BP Clonase II Enzyme Mix	1µl
TE Buffer (pH8.0)	2µl
Final volume	5µl

Table 2.8; Example of a standard BP Gateway® reaction.

The reactions were incubated for one to three hours at room temperature (25^{o}C). After bacteria transformation, isolation of the appropriate DNA plasmids and confirmation of the accuracy of the DNA sequences, following standard procedures as will be discussed below, these entry clones were utilised in an LR Gateway® reaction.

2) A typical LR Gateway® reaction protocol for the creation of recombinant destination constructs:

LR Gateway®; Creation of recombinant destination clone.	
Ingredients	**Concentration/ Quantity**
Destination Vector (~75ng/µl)	1µl
Entry Clone (~100ng total)	1µl
LR Clonase II Enzyme Mix	1µl
TE Buffer (pH8.0)	2µl
Final volume	5µl

Table 2.9; Example of a standard LR Gateway® reaction.

These reactions were treated similarly to the BP Gateway® reactions.

2.2.1.1.2 Example of a cloning strategy designed for the construction of replication-competent HBV genomes encoding reporter genes

In 2013, *Wang Z et al.* described that HBV can maintain stable transgene expression if these sequences were specifically inserted after the core gene and separated by two minimal 22nt IRES sequences (*Figure 1.33*) [266]. IRES-containing bicistronic vectors generally allow the simultaneous expression of two proteins separately but from the same RNA transcript. The aim of our cloning technique was to create recombinant HBV particularly expressing BFP, BSD, Fluc and Gluc, in the exact same manner as described in the reference publication. For this reason, we performed the following cloning strategy:

Step 1: Creation of pCMV_HBV-*MluI*.

We utilised the original pCMV_HBV construct (*Table 2.4*), and in order to achieve the individual insertion of the selected transgenes into this we initially had to isolate the overlapping HBV core and polymerase fragments. This would then be followed by their re-insertion back into the original HBV construct but in a linear manner, while they would further be separated by a *MluI* restriction site. Addition of the *MluI* restriction site would allow for easy exchange of initially a destination cassette followed by the transgenes while maintaining full in frame translation of the core and polymerase sequences.

To begin with, we generated partial HBV core and polymerase fragments by PCR amplification, using specifically designed primers that contained a *MluI* restriction site on either the reverse or the forward primer respectively for either the core or the polymerase sequence (*Table 2.3; Primers used for Gateway cloning of the HBV genomes encoding transgenes*). Accordingly, the

remaining primers included different enzymatic restriction sites, hence allowing digestion of the PCR products on either side. Both restriction sites would eventually be added to the PCR products during the primer annealing and extension steps. Correct size products were determined by visualisation on a standard agarose gel (*Figure 2.2*), and these were cut out from the gel and purified for further use.

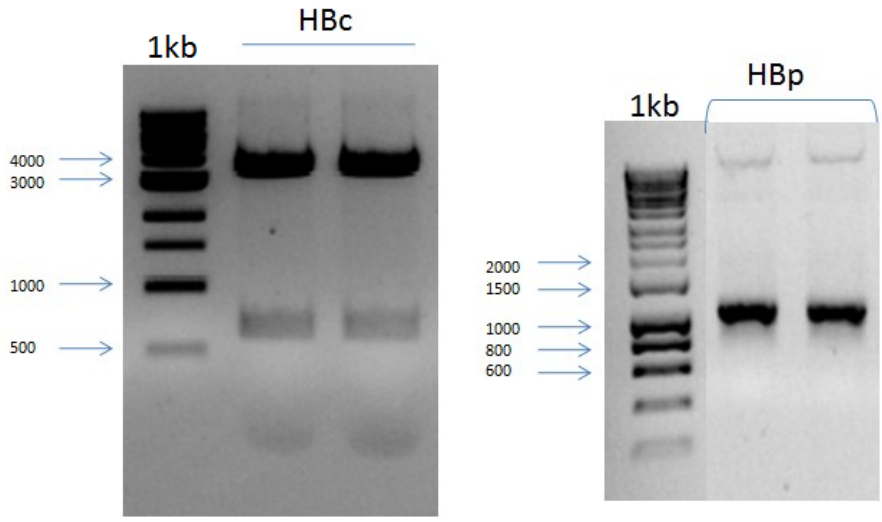


Figure 2.2; Visualisation of the HBV core and polymerase PCR products on a typical agarose gel.

The DNA band of interest for the HBV core was the 700bp fragment, while the expected size for the polymerase fragment was around 1kb. The additional band seen for core is a longer PCR-amplified sequence produced due to the fact that one of the primers bound twice to the original HBV construct, which is a 1.3kb overlength plasmid expressing part of the core fragment also at the end of its one copy sequence. *1kb DNA ladders were used for size comparison (Table 2.1; NEB and Bioline, respectively.)*

Following on, the PCR purified products as well as the original HBV construct were digested with the appropriate restriction enzymes. The digested PCR fragments were column purified, while the digested HBV plasmid was further run on an agarose gel to confirm correct restriction digest and to isolate and gel purify only the vector backbone. To re-insert the HBV core and polymerase PCR purified fragments back into the original pCMV_HBV construct we performed a three-fragment ligation, followed by a typical transformation procedure into DH5α bacteria cells and DNA extraction.

Once correct DNA plasmids had been validated both by restriction digest and by sequencing we continued our cloning strategy.

Step 2: Creation of pCMV_HBV_Dest.

The aim of this step of the cloning procedure was to insert a Gateway® destination cassette into the *MluI* site of the newly synthesised pCMV_HBV_*MluI* construct. Therefore, we isolated the cassette encoding the Gateway® region from an appropriate construct (*Jc1-5AB-DEST; Table 2.4*) by PCR amplification, while additionally adding *MluI* restriction sites on both ends of the sequence by PCR primer amplification. The PCR product was run on an agarose gel for visualisation (*Figure 2.3*) and further purification. This purified product, as well as the newly created pCMV_HBV_*MluI* construct, were both then digested with the *MluI* enzyme. After they were validated and gel purified, they were used in a two-fragment ligation and transformed into ccdB bacteria cells. Correct DNA plasmids were once again validated by both restriction digest and sequencing.

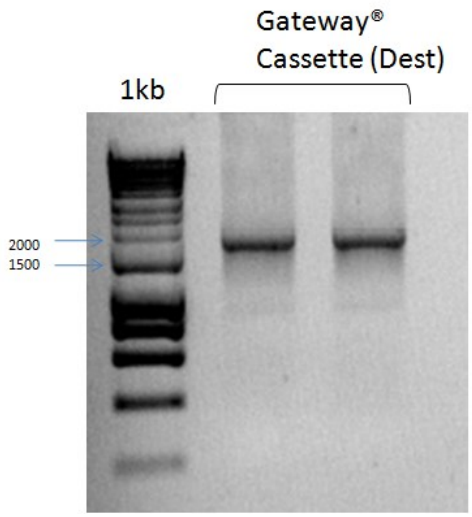


Figure 2.3; Visualisation of the Gateway® destination cassette PCR product on a typical agarose gel.
The anticipated size of the produced sequence was 1.6kb. *1kb DNA ladder (Bioline).*

Step 3: Final assembly of recombinant HBV genomes.

The final step in the creation of reporter HBV constructs was PCR-based amplification of the particular reporter constructs from appropriate constructs (*Table 2.4*), followed by Gateway®-guided recombination with the newly created pCMV-HBV-DEST.

During the initial PCR amplification, especially designed primers containing both the "Gateway® att" sites and the 22nt IRES sequence were utilised (*Table 2.3; Primers used for Gateway cloning of the HBV genomes encoding transgenes*). Accurate size validation of the PCR products was confirmed by agarose gel electrophoresis (*Figure 2.4*). The DNA plasmids of interest were isolated and purified for further use in a BP Gateway® reaction, following standard protocols. Once correct DNA clones had been confirmed, as typical, these were finally utilised in an LR Gateway® reaction and further confirmed for their accuracy.

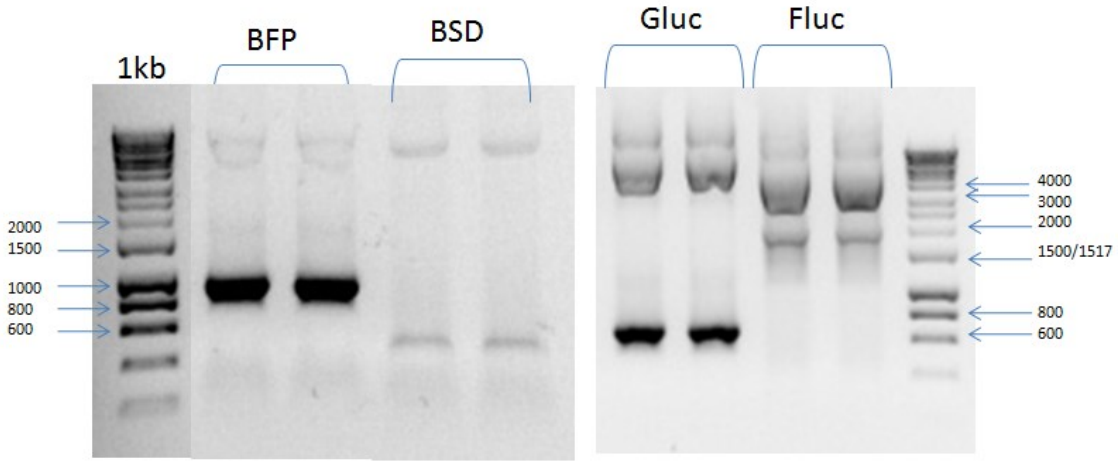


Figure 2.4; Visualisation of the reporter PCR products on a typical agarose gel. The expected product sizes, as well as the plasmids from which the fragments were PCR amplified from (*Table 2.4*), were: 900bp for BFP (*pTagBFP-C*), 500bp for BSD (*GeneArt construct*), 650bp for Gluc (*pCMV_Gluc*) and nearly 2kb for Fluc (*pGL4.12(luc2CP)*). *1kb DNA ladder (Bioline).*

Figure 2.5 shows the schematic summary of the cloning strategy described.

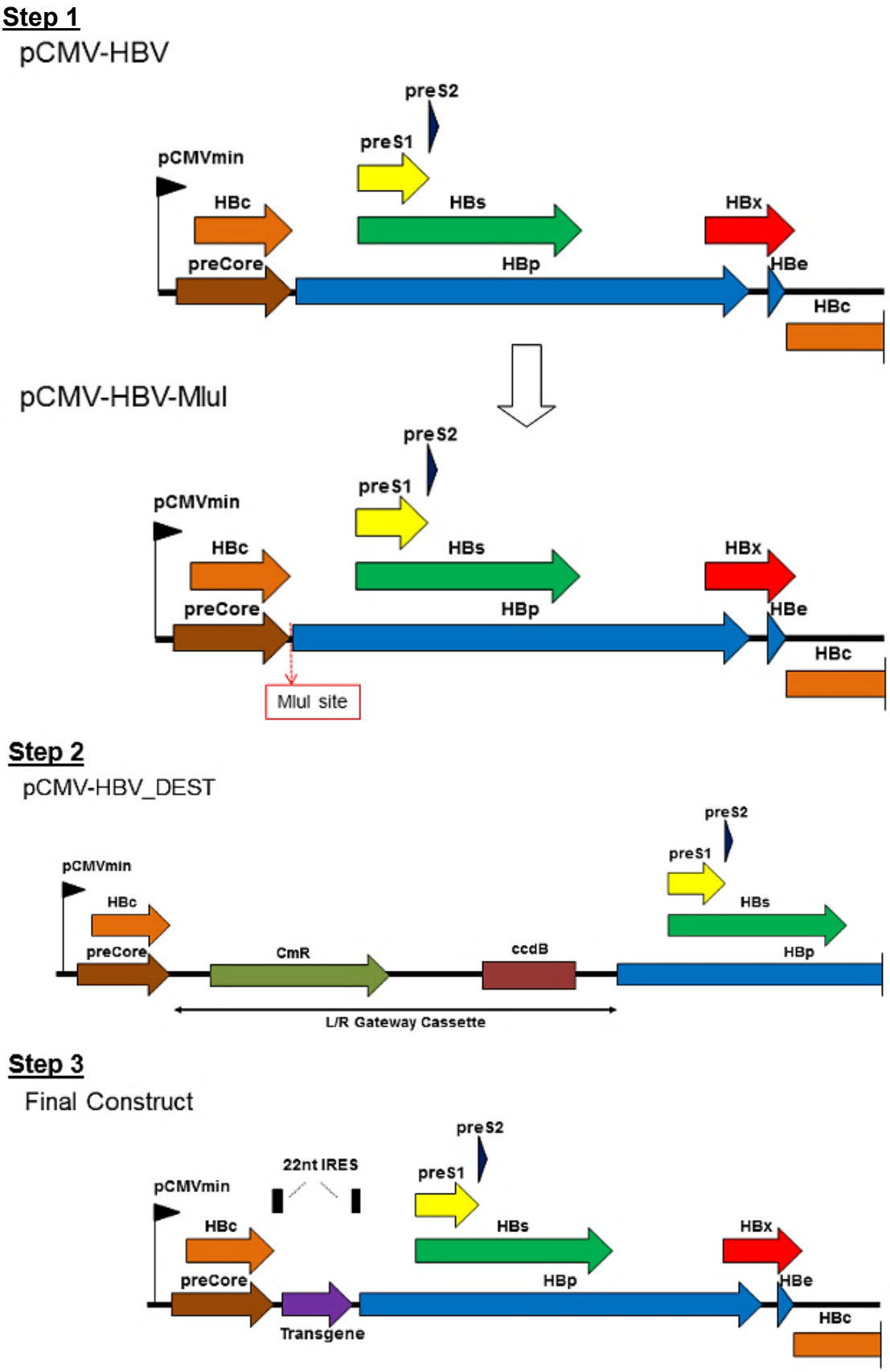


Figure 2.5; Diagrammatic overview of the cloning strategy for the construction of HBV genomes encoding transgenes.

Figure 2.5 description: "In the initial step of this cloning procedure, a portion of the overlapping HBV core and polymerase fragments were isolated by PCR and re-inserted into the original HBV construct in a linear manner, further being separated by a *MluI* restriction site. Next, a destination cassette was added at the created *MluI* restriction site. The final step in the creation of reporter HBV constructs was PCR-based amplification of the reporter constructs of interest (*Transgenes*: BFP, BSD, Fluc, Gluc) and Gateway®-guided recombination of those between the core and the polymerase sequences of the pCMV_HBV construct by specific exchange with the destination cassette. The 22nt IRES sequences on either side were added during the final PCR amplification procedure by PCR primer extension."

These HBV genomes encoding the different reporter genes, as well as the original full-length HBV genome, were finally cloned into a pCDNA3.1(-) vector background following traditional cloning procedures.

All procedures utilised during the various cloning strategies are mentioned below in detail.

2.2.1.2 Restriction digest of DNA fragments

A typical restriction digest procedure was as follows:

Restriction Digest Protocol	
Ingredients	**Concentration/ Quantity**
DNA template - DNA fragment/plasmid, or - PCR purified product (*Section 2.2.1.8*)	 ~2µg 23.5µl
Restriction Enzymes (NEB)	0.25µl/ each
Buffer	2.5µl
BSA	0.5µl
Final volume with ddH_2O	25µl

Table 2.10; Example of a standard restriction digest reaction.

The reaction was usually incubated at 37^{o}C (depending on the enzyme used) for approximately 3 hours, after which all the digested product was loaded

onto an agarose gel. The fragments of interest were gel excised, after they had separated appropriately, and purified for further use (*see below for protocol details*).

2.2.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis was generally performed for the visualisation of DNA, for example digested DNA constructs or particular fragments generated by PCR. Principally, 1g of agarose powder was dissolved in 100ml of a 1x preparation of TAE buffer (*Table 2.2*) by heating, thus resulting in a 1% agarose gel preparation. Once the solution had slightly cooled down, 10µl of SYBR® safe DNA gel stain were added. The mixture was then poured into an electrophoresis tank, containing a comb with an appropriate number of wells, and left to solidify. Once the gel had cast, the DNA samples mixed together with a 1x sample loading dye were loaded into the wells of the gel alongside with either a 1kb or 100bp DNA ladder (*Table 2.1*) to allow for the determination of the exact size of the DNA fragment of interest. The gel was run at approximately 80-120 Volts for 30 minutes to one hour and the bands were finally visualised with the use of a particular piece of equipment (Molecular Imager Gel Doc^{TM} XR+ Imagine System; *Table 2.6*).

2.2.1.4 Gel extraction of DNA fragments from agarose gels

Once the DNA products, digested or PCR amplified, had separated accordingly on the appropriate percentage agarose gel (usually 1%) they were visualised under a safe imager (Safe Imager^{TM}; *Table 2.6*). The appropriate size bands were then cut out from the gel using always a clean scalpel. The fragments of interest were gel purified, as discussed above, while the concentration and the purity of the DNA were both assessed with the use of a nanodrop system (Nanodrop Lite^{TM}; *Table 2.6*).

2.2.1.5 Ligation of the DNA fragments of interest into the appropriate vectors and Transformation into bacteria cells

A typical DNA ligation procedure was as follows:

Ligation Protocol	
Ingredients	**Concentration/ Quantity**
DNA	
- Vector	50ng
- Insert	(1:3 molar ratio of vector to insert)
DNA ligase (T4 or Quick Ligase™)	0.2µl
DNA ligase reaction buffer (10x-2x, *respectively*)	1-5µl (depending on the concentration)
Final volume with ddH_2O	10µl

Table 2.11; Example of a standard DNA ligation reaction.

The reaction was incubated for approximately one hour at room temperature, after which it was directly transformed into bacteria cells (usually E.coli chemically competent cells; *Table 2.1*). Specifically, 50ul of the competent bacteria cells were removed from the -80^{o}C and left for a few minutes to thaw on ice. Once thawed, 5ul of the ligation reaction was carefully added to the cells and gently mixed by flicking the tube. We performed a quick transformation procedure; therefore, the competent cells containing the ligation reaction were only incubated on ice for two minutes before they were directly heat shocked at 42^{o}C for 45 seconds. They were immediately placed back on ice for a further two minutes, after which 1ml of LB medium was added to the bacteria sample and this was left to incubate for 20-30 minutes on a shaker set at 37^{o}C. The sample was then quickly/gently centrifuged so as to collect all cells in a smaller volume of sample. All cells were finally spread onto LB plates containing the appropriate antibiotic resistance, and were incubated overnight at 37^{o}C. For bacteria transformation of full-length DNA plasmids, and not DNA ligations, only a small number of cells were spread onto the LB plates.

2.2.1.6 DNA plasmid preparation

Transformed bacteria colonies were grown initially on a small scale preparation so as to determine the correct insertion of the DNA fragment of interest into the chosen vector. For this purpose, a number of colonies were picked and each one was directly inoculated into 5ml of LB medium containing the appropriate antibiotic and these were further grown at 37^{o}C overnight in a shaking incubator. The next day, we isolated 4ml of each sample and the DNA plasmids were purified from each using the QIAprep® Spin Miniprep kit (*Table 2.1*) according to the product manual. To validate the accuracy of the DNA plasmid mini-preparations we performed typical restriction digest reactions and visualised the expected size fragments by agarose gel electrophoresis. Once we were certain of the correct insertion of the DNA fragments of interest, we went on to produce bigger scale preparations. Specifically, the remaining 1ml of each validated DNA miniprep was further inoculated in a final 250ml LB medium containing the appropriate antibiotic and incubated similarly overnight. These DNA preparations were purified the following day using either the HiSpeed® or the EndoFree® Plasmid Maxiprep kits, depending on the future use of these plasmids.

2.2.1.7 DNA sequencing

All individual PCR products, as well as the final cloned DNA constructs, generated were confirmed for accuracy of their sequence by DNA sequencing. This procedure was outsourced to Eurofins.

2.2.1.8 PCR amplification

A typical PCR protocol was as follows:

PCR Protocol	
Ingredients	**Concentration/ Quantity**
Template DNA	100ng
Platinum® PCR SuperMix High Fidelity	22.5µl
Forward/ Reverse Primers (100nM)	0.25µl/each
Final volume with ddH_2O	50µl

Table 2.12; Example of a standard PCR method.

The samples were run on a PCR thermal cycler (*Table 2.6*) following the protocol below:

Cycling Conditions for PCR			
Cycle		**Temperature**	**Time**
Initial Denaturation		94°C	1 minute
34 cycles	Denaturation	94°C	30 seconds
	Annealing	48 °C (depending on the primers)	30 seconds
	Extension	68°C	60-90 seconds (depending on the product size)

Table 2.13; Example of the usual cycling conditions followed for a standard PCR.

The resulting PCR products were either directly purified through specific columns using the QIAquick® PCR Purification kit (*Table 2.1*) or were run on an agarose gel electrophoresis before being excised and gel purified using the equivalent QIAquick® Gel Extraction kit, and always following the manufacturer's instructions.

2.2.1.8.1 Bacteria Colony PCR

On occasions, before setting up DNA plasmid preparations, we would perform bacteria colony PCR. This technique allows for quick and easy screening of a much greater number of bacteria colonies, thus permitting the precise

selection of only those colonies that have been confirmed to be accurate. These would then be incubated overnight in LB medium, followed by extraction of the DNA plasmids. In particular, a number of colonies were picked and each one was resuspended in 50µl of ddH_2O; 5µl of each diluted bacteria sample was then used in a typical PCR reaction, but instead of using a high-fidelity polymerase enzyme we used a non-proof reading GoTaq® Green polymerase (*Table 2.1*). Correct bacteria colonies would be verified by visualisation of the PCR products on an agarose gel.

2.2.1.8.2 Detection of HBV cccDNA formation

On particular occasion, the DNA extracted from the cell lysates transfected with various HBV-expressing plasmids was used for detection of HBV cccDNA formation by a traditional PCR technique while using specific HBV cccDNA primers (*Table 2.3*), targeted to opposite sides of the single-stranded gap region within the relaxed circular HBV DNA [299]. However, before subjecting the samples to PCR analysis, they had to be treated first with Plasmid-Safe™ ATP-dependent DNAse (*Table 2.1*), following the manufacturer's instructions, so as to break down any traces of relaxed circular and single-stranded HBV DNA. The samples were then utilised in a typical PCR procedure, using a non-high-fidelity polymerase, and analysed by agarose gel electrophoresis.

2.2.1.9 Quantitative PCR amplification

Note*:* This protocol specifically describes the quantification of viral HBV DNA copies within samples extracted from cellular supernatants or lysates. As these samples are considered infectious, all handling up till the point of their protein inactivation by Proteinase K (following the manufacturer's instructions) was performed under containment level 3. Total DNA was specifically extracted using the DNeasy® Blood and Tissue kit (*Table 2.1*) and following the products manual. The procedure is the same for extracting DNA either from the supernatants or the cell lysates. The DNA yield was measured with

the use of a nanodrop and eventually used to calculate the HBV DNA copies per ng of DNA sample.

The samples were quantified for HBV DNA copies with a typical Taqman® qPCR procedure.

Taqman® qPCR Protocol	
Ingredients	**Concentration/ Quantity**
DNA	5µl
Taqman® Universal Master Mix	12.5µl
Forward/ Reverse Primers (100uM) *	0.2µl/each
Probe *	0.1µl
Final volume with ddH_2O	25µl per reaction

Table 2.14; Example of a standard Taqman® qPCR method.

***** *Table 2.3; Primers/Probes used for quantification of HBV DNA copies.*

Usually a master mix would be prepared according to the amount of samples needing to be tested, so as to avoid pipetting errors. The DNA template would be added at the end, directly into each well of a 96-well plate. However, apart from the DNA samples in question, HBV standards would also be included onto the same plates so as to generate a standard curve for the purpose of data analysis. The standards were specifically prepared by performing 1:10 serial dilutions of the pCMV_HBV plasmid, starting from $2e^9$ all the way down to $2e^1$ HBV copies/reaction. A non-template negative control (NTC) sample, containing water instead of DNA, was also included. The HBV standards, NTC, as well as the unknown DNA samples were all pipetted onto the plate in duplicate, under sterile conditions. If the DNA samples to be tested would not fit in a 96-well plate, then a 384-well plate would be used instead and the quantities of all reagents would be adjusted accordingly to make a final volume of 10ul per reaction.

The plates were then run on a qPCR thermal cycler (*Table 2.6*), following the systems internal cycling conditions.

Cycling Conditions for Taqman® qPCR			
Cycle		**Temperature**	**Time**
Hold		50°C	2 minutes
Hold		95°C	10 minutes
40 cycles	Denaturation	95°C	15 seconds
	Annealing/ Extension	60 °C	1 minute

Table 2.15; Example of the usual cycling conditions for a standard Taqman® qPCR reaction.

2.2.1.10 Analysis of mRNA expression by qRT-PCR

Total RNA was extracted from collected cells using the RNeasy® Mini kit (*Table 2.1*) and was quantified by a nanodrop. Equal concentrations of all unknown RNA samples were then used and converted into cDNA. Specifically, cDNA was synthesised using the High-Capacity cDNA Reverse Transcription kit, according to the product manual.

During the course of this project, this procedure was carried out for two separate types of qRT-PCR analysis:

1) Quantification of the HBV replication intermediates using the SYBR® technology (*ThermoFisher Scientific*).

The HBV-specific 3.5kb pre-C and pregenomic RNA, as well as the total HBV subgenomic RNA, were specifically quantified using the following SYBR® qPCR procedure.

SYBR® qPCR Protocol	
Ingredients	**Concentration/ Quantity**
cDNA	2µl
Platinum® SYBR® Green qPCR SuperMix	12.5µl
Forward/ Reverse Primers (10µM) *	0.5µl/each
Final volume with ddH_2O	25µl per reaction

Table 2.16; Example of a standard SYBR® qPCR method.

***** *Table 2.3; Primers used for the quantification of the HBV replication intermediates.*

And again a master mix would be prepared according to the number of unknown samples needing to be tested, in duplicate.

The plates were then run on a qPCR thermal cycler, following a very similar protocol to the one performed for the Taqman® qPCR procedure.

Cycling Conditions for SYBR® qPCR			
Cycle		**Temperature**	**Time**
Hold		50°C	2 minutes
Hold		95°C	2 minutes
40 cycles	Denaturation	95°C	15 seconds
	Annealing/ Extension	60 °C	*30 seconds*

Table 2.17; Example of the usual cycling conditions for a standard SYBR® qPCR reaction.

Finally, all values were normalized to expression of the ribosomal protein S11 (RSP11) housekeeping gene using the $2\Delta\Delta C_T$ formula:

= 2^ - ddC_T (gene in questions)/ 2^ - ddC_T (housekeeping gene).

2) Taqman® Gene expression assays

For this type of analysis we principally followed the instructions supplied by *ThermoFisher Scientific* (https://tools.thermofisher.com/content/sfs/manuals/cms_041280.pdf), but the procedure was in general very similar to the previously described Taqman® qPCR protocol (*Section 2.2.1.9*). Taqman® Gene expression assays were purchased according to the gene of interest (*Table 2.1*) and these were provided by the company as a mix of both primers and probe. All expression levels were normalised to the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference gene, and were once again analysed using the $2\Delta\Delta C_T$ formula. Graphs and statistical analysis were performed using GraphPad, Prism (*see Section 2.2.3*).

2.2.1.11 Analysis of protein expression by Immunoblotting

For protein analysis by immunoblotting, whole-cell lysates were prepared by directly lysing cells in an appropriate volume of 1x RIPA buffer (*Table 2.2*). After vortexing or pipetting the samples considerably, to assure complete resuspension of the cells in the lysis buffer, these were kept on ice for approximately 30 minutes for complete cell lysis. Samples were then centrifuged for 15 minutes at full speed so as to remove any insoluble material. The supernatant from the lysed cells was collected into new eppendorf tubes, the protein yield was quantified by a nanodrop, and the samples were kept on ice until further use. On occasions, if certain protein samples were too viscous, these would be briefly sonicated.

The protein samples were typically separated on a 10% polyacrylamide gel (SDS-PAGE; *Table 2.2*), as the majority of the proteins of interest had a molecular weight between 35-55 kDa. They were mixed with the appropriate volume of Laemmli sample buffer (*Table 2.2*), however, they were not denaturated by heating at 95^{o}C prior to loading them on the gel as this appeared to completely degrade some of the proteins. An appropriate protein

ladder (*Table 2.1*) would always be run alongside the samples, as a size standard for comparison purposes. The SDS-PAGE was run at 80-120 Volts for approximately one hour and a half.

After gel electrophoresis, the proteins were transferred onto nitrocellulose membranes (*Bio-Rad*) using a wet tank electrotransfer chamber. The gel and the membrane were sandwiched together, following standard procedures, allowing for the proteins to migrate from the gel onto the membrane when an electric current of 100 Volts was applied to the chamber for one hour.

After completion of the proteins' transfer, the membranes were blocked for unspecific binding of antibodies with 5% milk powder resuspended in 0.1% PBST (*Table 2.2*) for one hour at room temperature. Primary antibodies *(Table 2.1)* were also resuspended in 5% milk/ 0.1% PBST and the membranes were further incubated overnight on a shaker in a cold room. The concentrations of the antibodies used were as per the manufacturer's suggestion and specific for this type of analysis. The following day, after five 10 minute washes with the 0.1% PBST buffer, the membranes were incubated with an appropriate concentration of a secondary ECL (enhanced chemiluminescence)-conjugated antibody (*Table 2.1*) resuspended in 5% milk/ 0.1% PBST for one hour at room temperature, which was also followed by a final five 10 minute washes with the same buffer (i.e. 0.1% PBST).

Finally, the proteins were visualised with the use of an ECL substrate, following the manufacturer's instructions.

Note: A comparison between ECL and LI-COR® technology for two particular antibodies (a-NTCP and a-ASGPR) revealed that, even though LI-COR® had a greater linear range for the protein concentrations tested, ECL was still optimum for protein concentrations between 5-25µg (*Appendix A.2.XV*).

2.2.1.11.1 Deglycosylation/ Dephosphorylation of protein samples prior to Immunoblotting

Deglycosylation/ Dephosphorylation was performed on the whole-cell lysates, according to the enzyme's protocol manual (PNGaseF or EndoH, for deglycosylation; and Lambda phosphatase, for dephosphorylation). The enzymatic reactions were typically incubated at room temperature overnight to assure complete digestion, before being run on a SDS-PAGE the next day.

2.2.1.12 Analysis of protein expression by Immunofluorescence

For protein analysis by immunofluorescence, cells were typically grown to approximately 80% confluency on appropriate Poly-L-Lysine-treated (*Table 2.2*) coverslips so as to obtain a nicely even cell monolayer. Once the cells were ready, the following procedure was performed:

Cells were briefly washed with PBS, followed by them being fixed with 4% PFA (*Table 2.2*) for 20-30 minutes at room temperature, after which they were washed again three times with PBS. They were then permeabilised primarily with the use of 0.1% Triton^{TM}-X 100/PBS (*Table 2.2*) for 5-10 minutes at room temperature. On a different occasion, however, simultaneous methanol (100%) fixation and permeabilisation of the cells was performed for 10 minutes at room temperature, so as to assess any differences in the staining of the cells when following this protocol compared to the PFA fixation and Triton^{TM}-X permeabilisation procedure.

After removal of the detergent or chemical respectively by washing a further three times with PBS, non-specific binding of the antibodies was blocked with 10% FBS/PBS (*Table 2.2*) for approximately 30 minutes at room temperature. Appropriate concentrations of the primary antibodies were diluted in 5% FBS/PBS and were directly used to stain the cells for one hour at room

temperature. The cells were then washed, followed by their incubation with a suitable fluorescently-labelled secondary antibody for a further one hour at room temperature but in the dark (i.e. wrapped in aluminium foil). On occasions, labelled cell trackers were utilised to additionally stain specific compartments or characteristics of the cells (*Table 2.1*). The cells were incubated with these antibodies for an extra hour at room temperature in the dark. The concentrations of all antibodies used were once again as per the manufacturer's suggestion. Moreover, during the course of this procedure, the coverslips with the cells were gently rocked on a shaker.

Below is a list of the antibodies used during the immunofluorescence procedures (*Table 2.18*):

Antibody	Used for the detection of:
Phalloidin *(Alexa Fluor ® 594- Texas Red)*	Actin
Concanavalin A (*Alexa Fluor® 594 - Texas Red*)	ER
Lectin HPA (*Alexa Fluor® 647 – Cy5*)	Golgi
Anti-HBcAg and Alexa Fluor® 350 (UV)	HBcAg
anti-FLAG and Alexa Fluor® 488 (FITC) (or Alexa Fluor® 594 - Texas Red*)*	hNTCP_FLAG-tag
anti-beta-Tubulin and Alexa Fluor ® 647 (Cy5)	Tubulin

Table 2.18; Commonly used antibodies for immunofluorescence detection of the expression of particular proteins.

Finally, the coverslips with the cells were washed with PBS and moulded with Mowiol® (*Sigma-Aldrich*) onto microscope slides. These were left to set for approximately an hour at 37^{o}C in a humidified incubator. Images were taken, for each experiment under identical conditions, either on a traditional fluorescent microscope or on a confocal microscope for better definition (*Table 2.6*).

Note*:* Direct immunofluorescence of cells expressing a fluorescent protein (e.g. BFP, GFP, RFP), either due to transfection with a plasmid encoding a fluorescent protein or due to transduction with lentiviral pseudoparticles expressing a fluorescent protein, was also performed. In this case, cells were directly visualised under a fluorescent microscope while being maintained in culture, so as to assess the efficiency of the transfection/ transduction procedure before continuing with the experimental plan.

2.2.1.13 Analysis of protein expression by Flow Cytometry

Similarly to the procedure performed for protein immunofluorescence analysis; cells were washed with PBS, trypsinised, washed further to remove any residual media, and then fixed with 4% PFA in suspension. After removal of the fixative solution, the cells were either permeabilised with 0.1% Triton™ X-100/PBS or they were not permeabilised at all, depending on the known location of the protein of interest. After removal of the detergent, unspecific binding of the antibody was blocked as previously described, followed by the staining of the cells with a directly fluorescently-conjugated antibody (*Table 2.1; e.g. a-Flag-PE or HBsAg-FITC*) for one hour at room temperature in the dark. The residual antibody was then washed away from the cells and the cells were immediately run on a suitable flow cytometry system (*Table 2.6*). Data analysis was performed using FlowJo.

On different occasions, when measuring cells expressing an internal fluorescent marker, the same procedure as described above was performed up till the point of the cells' permeabilisation, after which these samples were directly run on a flow cytometry system without further being stained with any antibodies.

2.2.1.14 ELISA analysis

Note*:* This protocol primarily describes the quantification of two viral HBV antigens, HBeAg and HBsAg, secreted by cells into the supernatant after they had been transfected or infected with a HBV genome. As these samples are considered infectious, the entire procedure was performed under containment level 3. Supernatants were specifically collected and directly used in two individual commercial chemiluminescence immunoassays (*Table 2.1*), following the manufacturer's instructions. All samples were run in duplicate for determination of statistical significance. At the end of the procedure, the values of each sample were read on a microplate reader (*Table 2.6*), and data analysis was performed using an open-source online program (*Table 2.7*).

Moreover, a precisely similar procedure was followed when quantifying the cAMP released from the cells under different conditions, using a commercial kit (*Table 2.1*) and following the manufacturer's product manual.

2.2.2 Cell-based techniques

2.2.2.1 General Cell Culture

Unless otherwise stated, all adherent cells were maintained in DMEM supplemented with 10% FBS, referred to from now on as complete medium. They were passaged by trypsinisation as necessary according to their confluency and typically split at a ratio of 1:20. Adherent cells were grown in a 5% CO_2 atmosphere in a humidified incubator set at 37^{o}C.

2.2.2.2 Optimised cell culture for cellular differentiation and polarisation

Originally, the optimised cell culture medium referred to the use of WE media supplemented with 10% FBS. Moreover, this medium contained primary hepatocyte maintenance supplements, as provided by the manufacturer, and was further complemented with dexamethasone, hydrocortisone and 2% DMSO (*see Table 2.2*).

During the course of the project, however, we established that the only essential reagent for cellular differentiation and polarisation was the 2% DMSO, and since then and according to the experimental procedure we would supplement the typical complete medium with 2% DMSO and refer to this as the optimised cell culturing medium, or else PHMM for primary human maintenance media.

The cells, however, typically continued to be cultured and maintained under normal media conditions, as previously described, and only when the experimental protocol required it we would switch the complete media to the optimised media.

2.2.2.3 Electroporation of cells with plasmids of interest

On occasions, instead of using typical transfection techniques for delivery of DNA constructs directly into cells, as will be discussed below, we preferred to electroporate the DNA into the cells. The elecroporation technique particularly relies on the use of a sort pulse of electricity on the cell sample that results in the opening of the pores within the cell membrane, hence permitting entry of the DNA construct of interest.

For this procedure, we specifically utilised an ECM 830 ElectroSquarePorator™ (*Table 2.6*), and precisely followed the manufacturer's suggested protocols for electroporation of hepatic cell lines, such as HepG2 and Hepa1.6 cells (http://www.btxonline.com/technical-resources/protocol-database.html).

2.2.2.4 Transfection of cells with plasmids of interest

Confluent cells were trypsinised and counted using an automated cell counter. A specific number of them were then seeded in appropriate cell culture containers (*Table 2.19*), according to the experimental plan.

	Seeding Densities
Dishes	
10cm	1×10^6
15cm	$2.5 - \times 10^6$
Plates	
6-well	0.2×10^6
12-well	80×10^5
24-well	$30 - 50 \times 10^4$
96-well	$10 - 15 \times 10^4$

Table 2.19; Typical number of cells seeded per cell culture dish or plate.

Once seeded, the cells were left to settle either during the course of the day or overnight, depending on how quickly they adhered to the surface of the container. When adherent, the cells were directly transfected with DNA plasmids by means of Lipofectamine™ 3000, while in the case of siRNA plasmids (*Table 2.1*) the cells were transfected by means of the Lipofectamine® RNAiMAX reagent. The process of lipofection specifically relies on the use of liposomes for insertion of genetic material into cells; liposome vesicles can typically merge very easily with the cell's membrane, since they are both made of a phospholipid bilayer. In particular, a transfection mixture was prepared, according to the two different product protocol manuals, containing the plasmid of interest with all the necessary transfection reagents, and this was incubated for 15-30 minutes prior to being pipetted drop-by-drop onto the media covering the cells.

After overnight incubation of the cells with the transfection mixture, the cells were then washed approximately six times with PBS. A final wash was performed with complete medium, which was then replaced and the cells were further incubated according to the experimental plan.

2.2.2.4.1 Production of lentiviral pseudoparticles

In general, we followed the protocol described by Tiscornia G et al. [300].

For the production of 3rd generation lentiviral pseudoparticles, Human embryonic kidney (HEK293T) cells (*Table 2.5*) were specifically seeded on a number of poly-L-lysine coated 15cm^2 plates, according to the final quantity of lentiviruses we wanted to produce. When they had reached 80-90% confluency, they were transfected with three plasmids, following standard procedures: a packaging plasmid expressing the HIV gag/pol polyprotein, an envelope plasmid expressing the VSVg and a transfer plasmid expressing for example a GFP reporter (*Table 2.4; Figure 2.6*). On occasions and depending

on what kind of lentiviral pseudoparticles we wanted to produce for the need of this project, we would separately either replace the GFP-expressing plasmid with a plasmid encoding for one of the HBV-related receptors (i.e. human or chimeric NTCP, or human ASGPR; *Table 2.4*) or we would replace the VSVg expressing plasmid with another plasmid encoding the full-length HBV surface antigen glycoprotein (*Table 2.4*). The former would result in the production of lentiviruses expressing the specific receptors of interest, which would get integrated into the host genome of subsequently transduced cells (*Figure 2.8*) hence creating a receptor overexpression cell culture system allowing for the study of HBV entry. On the other hand, in the second case we would ideally produce HBV- (or HDV-) mimicking lentiviral pseudoparticles that were named HBSpp, which were of interest for studying once again virus entry into cells.

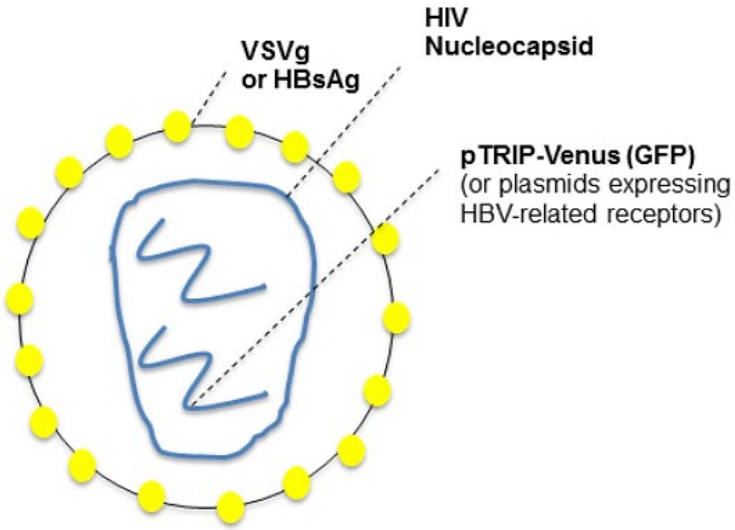


Figure 2.6; Schematic of typical lentiviral pseudoparticles produced.

The standard procedure followed was specifically a calcium phosphate precipitation protocol, during which the plasmid DNAs were mixed with an appropriate volume of a 0.25M $CaCl_2$ solution. This mix was then added in a controlled manner (i.e. gently while vortexing) to a buffered saline/phosphate solution of 2x HBS.

A typical mix would be as follows:

DNA plasmid Concentrations	**$CaCl_2$ (0.25M) ***	**2x HBS ***
20µg Transfer vector (i.e. pTRIP-Venus, HBsAg or HBV-related receptors; *Table 2.4*) 13µg HIV gag/pol 7µg VSVg	1ml	1ml

Table 2.20; Typical preparation of a transfection mixture for a $CaPO_4$ precipitation protocol.
Concentrations and values are shown as per reaction sample.
***** *Preparation of buffers can be found in Table 2.2.*

The samples were incubated at room temperature for approximately 15 to 30 minutes, after which the transfection mixture was added drop by drop to the appropriate plates. The plates were gently swirled to assure even distribution of the mixture and incubated overnight in a humidified incubator at 37^{o}C.

After overnight transfection of the HEK293T cells with the mixture of the three plasmids, the following day the cells were briefly washed and the media was replaced. Supernatants containing the lentiviruses of interest were collected at 48 hours and 72 hours post-transfection, they were then filtered through a 0.45µm filter and supplemented with 20mM HEPES and 4µg/ml Polybrene (*Table 2.2*) before further use or before being stored for future use.

The transfection efficiency was verified by visualisation of the GFP signal post-transfection of the HEK293T cells with the pTRIP-Venus (*Table 2.4*) construct by immunofluorescence (*Figure 2.7, A*), while the infection efficiency of the produced lentiviruses was confirmed by infecting another cell line (different to the HEK293T cells) with varying dilutions of the VSVg pseudoparticles and quantifying the GFP signal by flow cytometry analysis (*Figure 2.7, B*).

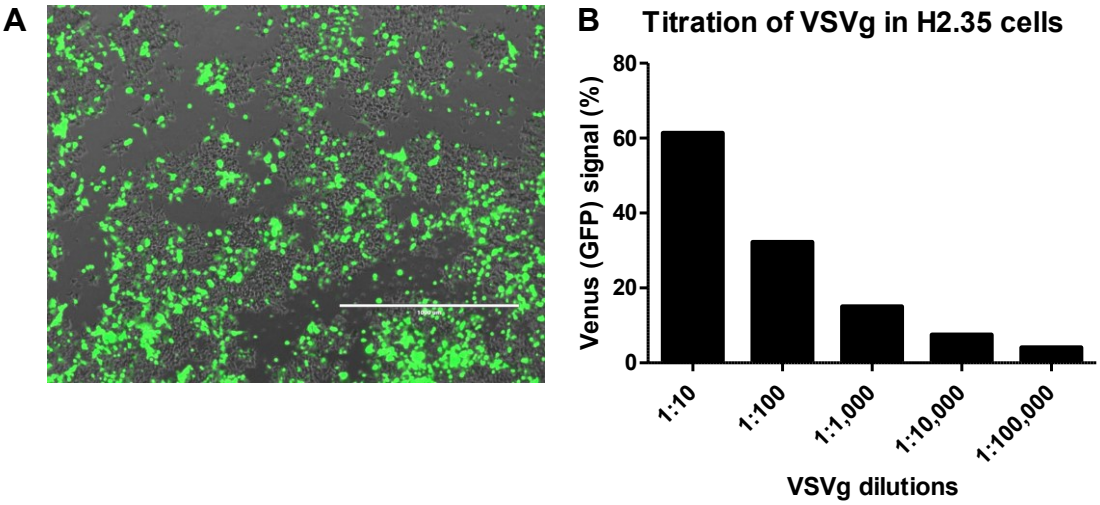


Figure 2.7; Production of lentiviral pseudoparticles by performing a $CaPO_4$ precipitation technique.

(A) Transfection efficiency of HEK293T cells with the pTRIP-Venus plasmid, analysed by immunofluorescence. *Merged image of bright field and GFP channels. The image bar is 1000µm.* **(B)** Example of the transduction efficiency of varying dilutions of the VSVg lentiviral pseudoparticles used to specifically transduce H2.35 cells (*Table 2.5*). The data was analysed by flow cytometry.

Below is a schematic summary of the production of lentiviral pseudoparticles, and how these are further used to transduce other cells (*Figure 2.8*).

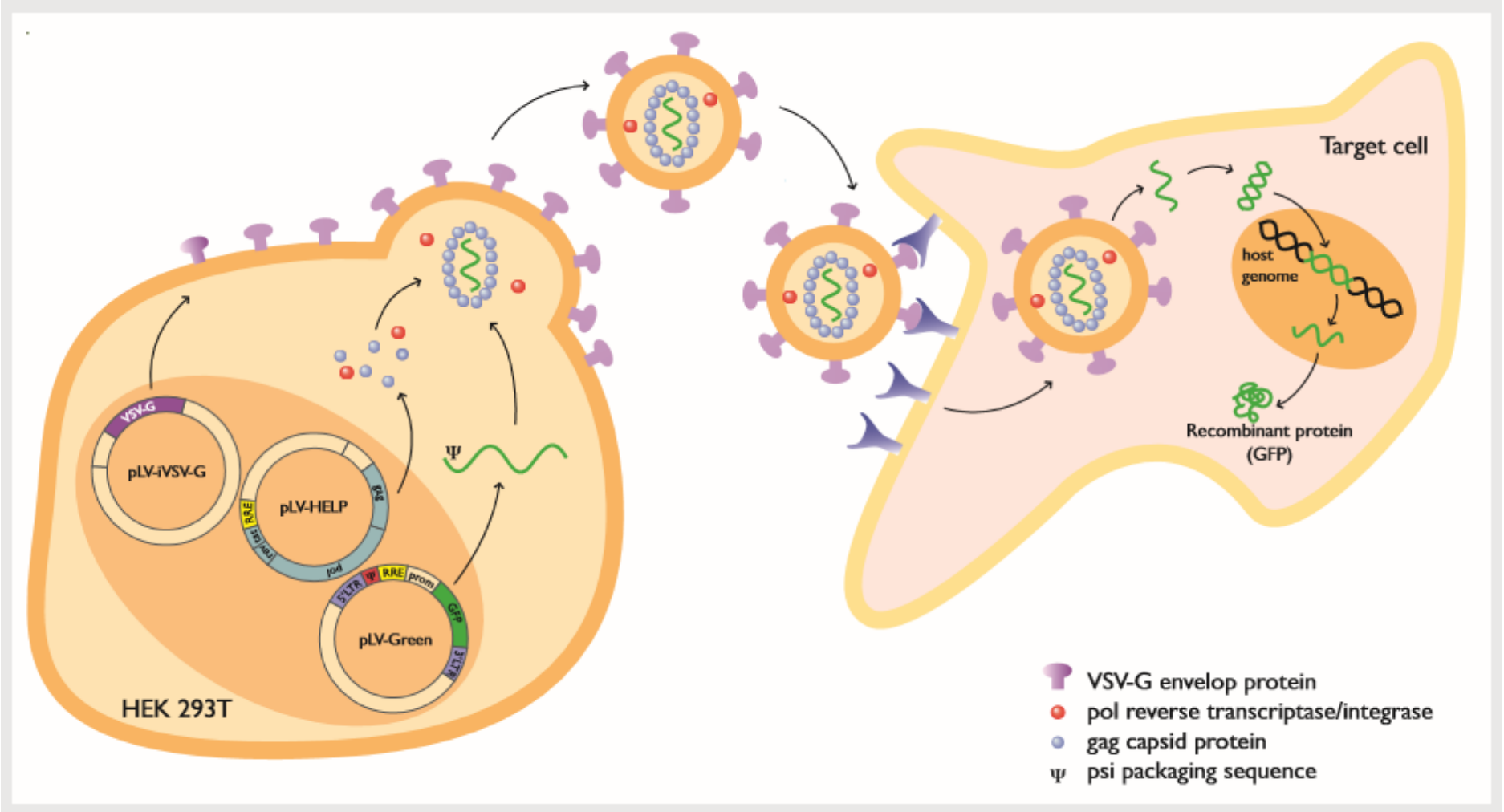


Figure 2.8; Lentiviral vector production and Cell transduction.
Source InvivoGen (http://www.invivogen.com/docs/Insight_201004.pdf).

2.2.2.4.1.1 Production of lentiviral pseudoparticles expressing HBV-related receptors

For the production of lentiviral pseudoparticles expressing the human NTCP receptor we purchased two individual lentiviral plasmids from GeneCopoeia (EX-C0391-Lv105/Lv158; *Table 2.4*). They both expressed the full NTCP ORF under the control of a CMV promoter. However, the former expressed a puromycin selection cassette for the subsequent antibiotic selection of cell clones that had stably integrated one-copy of the NTCP gene into their genome, while the latter expressed a neomycin selection cassette and most importantly a C-terminal FLAG-tag to assist protein detection.

The lentiviral transfer plasmid encoding the hNTCP, as purchased from the company, was then transfected into HEK293T cells together with the HIV gag-pol polyprotein and VSVg plasmids. Lentiviral pseudoparticles were therefore produced following standard techniques (*Figure 2.9*), as described above. Fresh supernatant collected at 48 hours and 72 hours post-transfection was used to further transduce the cell lines of interest for the production of stable cells overexpressing the hNTCP. Only in the case of the lentiviruses expressing the hNTCP_FLAG-tag, prior to them being used for the transduction of other cells, their efficacy was analysed by titrating varying concentrations back in HEK293T cells and thus determining what concentration was the most optimal for further use (*Appendix A.2.XVI*).

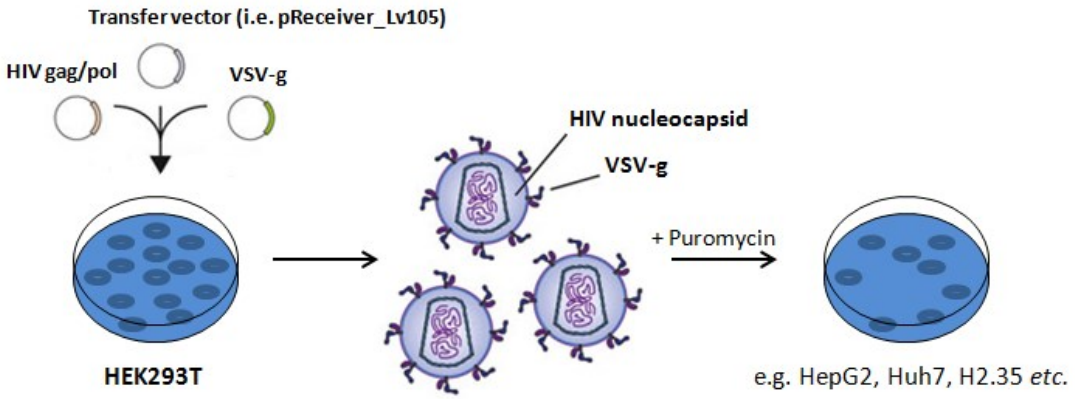


Figure 2.9; Production of lentiviral pseudoparticles expressing the hNTCP.
HEK293T cells were transfected with three plasmids; a lentiviral transfer plasmid encoding the hNTCP, a packaging plasmid encoding for the HIV gag/pol polyprotein and an envelope plasmid encoding for the VSVg. These plasmids will be packaged together to form pseudotyped viral particles; specifically, the hNTCP will get encapsidated into HIV particles that will further assemble into viral particles expressing the VSVg on their cell surface. The media was changed after overnight transfection and supernatants containing the infectious lentivirus were collected and used to further transduce the cells of interest. *Adapted from:* [301].

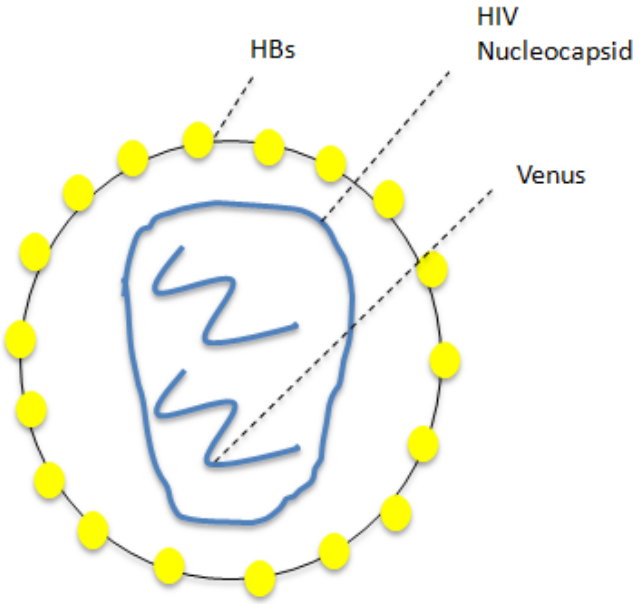
2.2.2.4.1.2 Production of lentiviral pseudoparticles expressing the HBV-mimicking lentiviral particles

In parallel to using infectious supernatants derived from the HepG2.2.15 cells for infection experiments that had to be performed in a Category 3 lab, we also wanted to develop a non-infectious system for performing HBV infections in a low security Category 2 tissue culture lab. Therefore, we produced HBV-mimicking particles making use of the currently available lentiviral technology systems. We decided to name these “HBSpp” due to the fact they are pseudoparticles expressing the HBV glycoproteins on their cell surface, hence somewhat mimicking both HBV and HDV infection.

We specifically designed and produced two types of HBV-mimicking lentiviral pseudoparticles; the first type only expressed the full-length HBsAg together with GFP for subsequent visualisation (Type I; *Figure 2.10*), while the second type together with the full-length HBsAg additionally expressed the HBV core, polymerase and an epsilon-Cre (Type II; *Figure 2.10*). For type I, the GFP (a Venus sequence) specifically was encapsidated into HIV particles that were

assembled into viral particles expressing the full-length HBsAg on their cell surface. For type II, the theory behind the design was that the epsilon-Cre would interact with the polymerase, and together they would be encapsidated into core particles, which would then further get assembled into viral particles expressing the full-length HBsAg. Therefore, Type II HBSpp were considered to be more physiological compared to Type I. GFP was not expressed in this latter case.

Type I



Type II

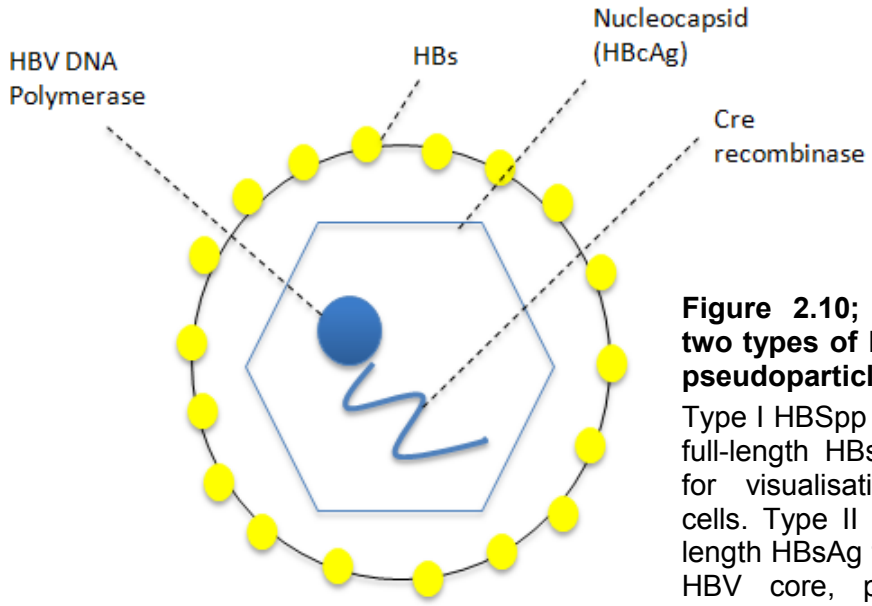


Figure 2.10; Production of two types of HBV-mimicking pseudoparticles.

Type I HBSpp only express the full-length HBsAg with a GFP for visualisation of infected cells. Type II express the full-length HBsAg together with the HBV core, polymerase and epsilon-Cre.

Type I were specifically produced by transfecting HEK293T cells with three plasmids, as previously described in *Section 2.2.2.4.1*: a transfer vector expressing the GFP (Venus), a packaging plasmid expressing the HIV gag-pol polyprotein and an envelope plasmid encoding the full-length HBsAg (*Figure 2.11*). Type II were produced simply by transfecting simultaneously all four plasmids; HBsAg together with HBV core, polymerase and epsilon-Cre (*Table 2.4*). However, as the latter HBSpp were not produced to express any fluorescent protein for easy visualisation of the infected cells, we only went on to characterise Type I.

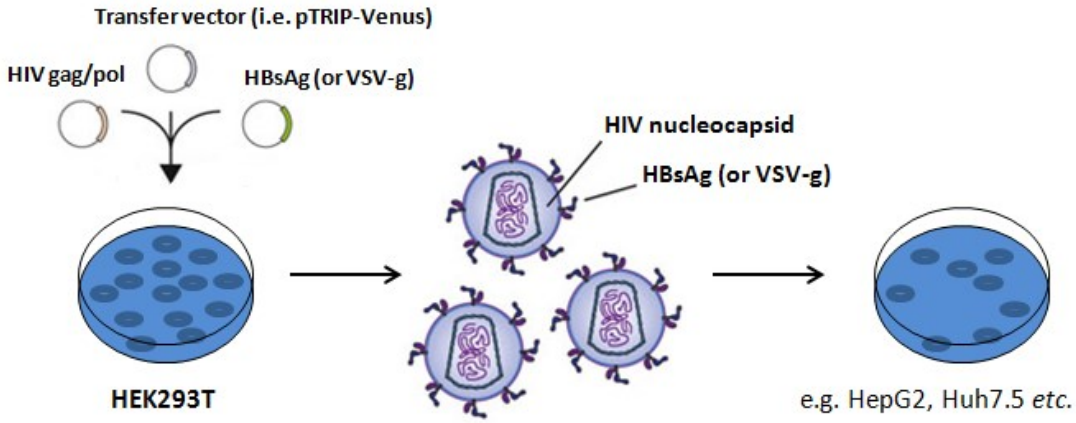


Figure 2.12; Production of non-infectious HBV-mimicking lentiviral particles.
HEK293T cells were transfected with three plasmids; a lentiviral transfer plasmid encoding the GFP (Venus) sequence, a packaging plasmid encoding for the HIV gag/pol polyprotein and an envelope plasmid encoding either for HBsAg or VSVg (positive control). These plasmids will be packaged together to form pseudotyped viral particles; specifically, the GFP will get encapsidated into HIV particles that will further assemble into viral particles expressing the HBsAg or VSVg on their cell surface. *Adapted from:* [301].

In the case of the HBSpp Type I, we additionally produced two other types of lentiviral pseudoparticles. As a negative control, we produced lentiviruses expressing no envelope glycoproteins that would not be able to enter any cells hence no GFP would be detectable, and these were named non-envelope pseudoparticles (NEs), while for a positive control we produced the VSVg pseudoparticles (*Figure 2.11*). VSVg lentiviral pseudoparticles typically have a high infection efficiency, thus the majority of cells should theoretically be GFP-positive.

2.2.2.4.1.3 Production of LMN8-Huh7.5 lentiviral cDNA library, for use during the cyclic repackaging rescue screen

The procedure undertaken was based on a previous study performed by *Ploss A et al*. [267], while also adjusting the protocol described by *Tiscornia G et al.* [300].

In particular, and before commencing the production of lentiviruses expressing the entire Huh7.5 cDNA library, initially the LMN8 retroviral vector containing the Huh7.5-derived cDNA library was transformed into Library Efficiency® DH5α^{TM} E.coli competent cells (*Table 2.1*), following the manufacturer's instructions. Principally, 1μl of the lentiviral cDNA library construct was transformed into 100μl of the DH5α bacteria. To assess the transformation efficiency we additional made use of the pUC19 control vector, as well as X-gal (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) for blue/white colony screening. The two bacteria transformation samples were spread onto LB plates, containing an appropriate antibiotic and the X-gal, and incubated at 37^{o}C overnight. At the same time, a maxi-preparation for the pooled lentiviral cDNA library was directly set up overnight; ~900μl of the remaining LMN8 bacteria transformation sample were directly inoculated into 100ml of TB broth (*Table 2.2*). The maxi-preparation was left to shake overnight in a shaker set at 37^{o}C, so as to allow bacteria growth.

The following day, after assessing the transformation efficiency, the cDNA library was purified from the LMN8 maxi-preparation that had previously been left to grow overnight, following standard techniques. Subsequently, production of lentiviruses expressing the Huh7.5-derived cDNA library was performed in the HEK293T cells, following once again standard procedures as described above. The cells were seeded onto poly-L-lysine coated plates, left to attach onto the plates overnight and the next day they were transfected by a typical calcium-phosphate precipitation method. The transfection mix

contained the LMN8-Huh7.5 cDNA library, VSVg and HIV gag/pol plasmids. Supernatants were collected from 24 hours up to 96 hours post-transfection and purified by filtration, before being stored in -80oC for future use.

Note: The LMN8-Huh7.5 cDNA library-expressing construct was kindly provided by Charles Rice (Rockefeller University, US).

2.2.2.4.1.3.1 Lentiviral cyclic repackaging screening for the identification of novel host factors required for HBV entry

Despite the recent description of NTCP as the functional receptor for HBV, as well as the previously characterised ASGPR receptor amongst others, it is possible that other currently unknown receptors may also be essential for HBV entry. Moreover, apart from additional receptors, HBV entry *in vitro* may generally rely on supplementary host factors which could be responsible for limiting infection of non-human cells. Even the fact that we still require very high MOIs to infect human hepatoma cells overexpressing the NTCP receptor, ranging from 100-10,000 DNA copies/cell, could be justified with the need for an indispensable host factor. To uncover any such factors necessary for efficient HBV entry into cells, a cDNA library screening approach could be performed. This assay, which is summarised below (*Figure 2.12*) and has already been applied successfully in the case of HCV [267], could possibly hold the answer towards the long-awaited creation of a small animal model for the study of HBV.

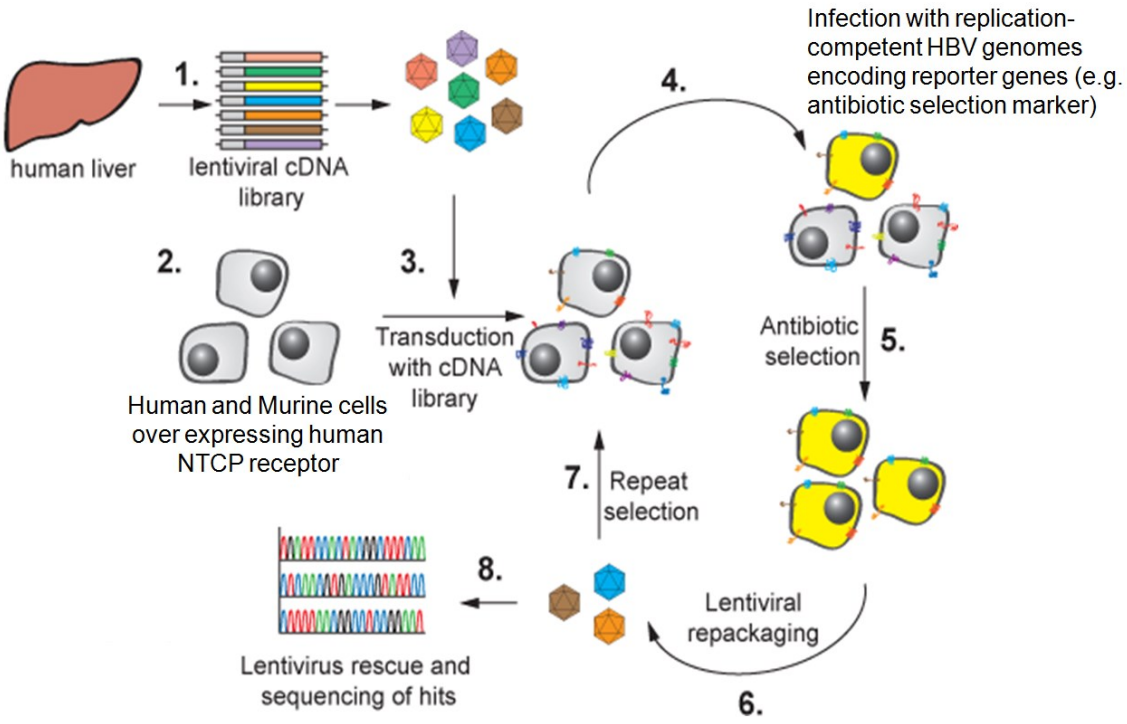


Figure 2.13; Diagrammatic summary of the "cyclic repackaging rescue screen" (CRRS).

Briefly, a lentiviral cDNA library obtained from human liver, which is permissive to HBV, is used to transduce human or mouse cell lines overexpressing the human NTCP receptor. Upon infection of the human or mouse cells, now overexpressing both hNTCP and the cDNA library, with a replication-competent drug-selectable HBV genome and subsequent antibiotic selection, only those cells expressing factors restricting infection of the human or mouse cells will survive. Supplying these cells with HIV gag/pol and VSVg then leads to re-amplification of an enriched lentiviral library. Repeated selection followed by RNA sequencing will theoretically uncover previously unknown HBV entry factors limiting infection of the original cells.

However, we attempted to perform an altered version of the CRRS, summarised below (*Figure 2.13*). In particular, we chose to use only human cells, rather than both human and mouse cells, believing it could still be possible to uncover essential host factors limiting HBV infection in these cells. In this altered version of the CRRS, the generation of the HBV- & HDV-mimicking lentiviral pseudoparticles (*Sections 2.2.2.4.1.2* and *3.1.5.3.1*), as well as the generation of recombinant HBV viruses (*Section 2.2.1.1.2* and *3.3.1*), played a very important role.

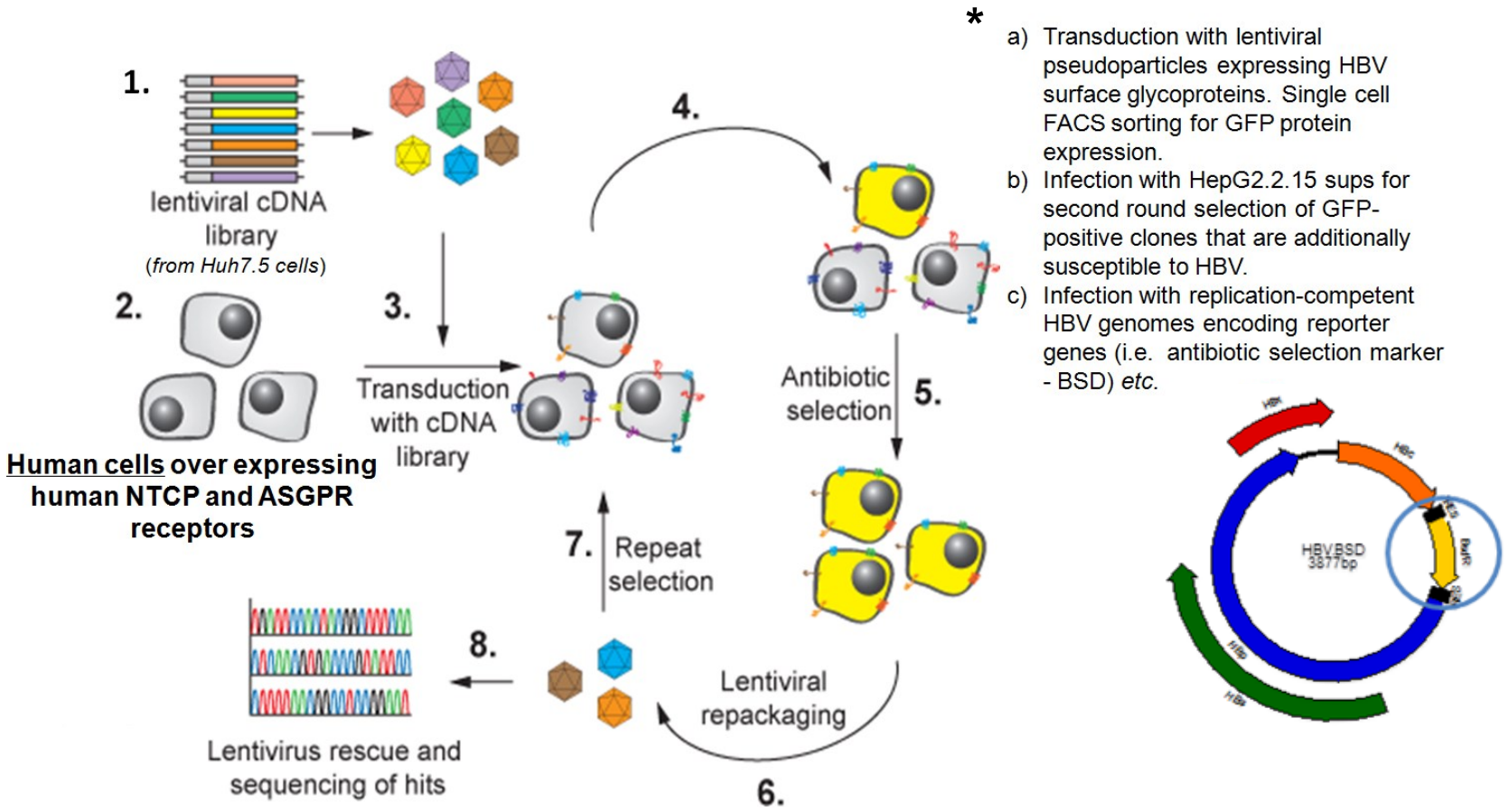


Figure 2.14; Diagrammatic summary of an altered version of the CRRS.

Briefly, a lentiviral cDNA library obtained from human liver cells (e.g. Huh7.5) was used to transduce a human cell line overexpressing the human NTCP and ASGPR receptors. The human cells, now overexpressing both hNTCP/hASGPR and the cDNA library, underwent three stages of "HBV infection" selection (*). After final antibiotic selection of the cells, the experimental plan remains the same as described above (*Figure 2.12*).

2.2.2.4.2 Concentration of lentiviral pseudoparticles

Two types of concentration procedures were performed for the lentiviral pseudoparticle preparations:

1) Amicon® filter centrifugation.

Simply, up to 15ml of supernatants were directly added to these filter units which were then centrifuged at speeds of approximately 1000xg for 30 minutes at 4^{o}C. After rounds of centrifugation with more supernatant being added to the filter unit, the lentiviral preparation would eventually get concentrated to a suitable final volume. The concentrate was then collected from within the filter device sample reservoir and used or stored accordingly, while the flow-through collected in the actual centrifuge tubes was discarded.

2) Ultracentrifugation.

The lentiviral preparations were also concentrated by ultracentrifugation, following once again the protocol described by *Tiscornia G et al.* In particular, supernatants were carefully loaded into conical 30ml tubes and centrifuged on a Beckman swinging bucket SW 28Ti rotor at approximately 20.000xg for 2 hours at room temperature in an ultracentrifuge. Additional supernatants were loaded as appropriate and extra spins were carried out where necessary. Following ultracentrifugation, the supernatant was discarded carefully so as not to disrupt the barely visible pellet. Any remaining liquid was removed and the pellet was finally resuspended in a very minute volume (~100μl) of 1x HBSS (T*able 2.1*) and stored for future use.

2.2.2.5 Transduction of cells with lentiviral pseudoparticles

A summary of the cell transduction procedure is depicted in Figure 2.8.

2.2.2.5.1 Creation of cell lines stably overexpressing the HBV-related receptors

Cells of interest were maintained in complete medium. Once they were 80-90% confluent, they were trypsinised, counted and seeded in appropriate cell culture plates (*Table 2.19*), following standard techniques. Specifically, 200.000 cells were seeded into each well of a 6-well plate and left to settle usually overnight, after when they were separately transduced with the lentiviral pseudoparticles expressing either of the different HBV-related receptors, i.e. human or chimeric NTCP, or human ASGPR. On occasions, however, cells were transduced simultaneously with lentiviruses expressing both the human NTCP and ASGPR receptors so as to create double-receptor stably overexpressing cell lines.

A transduction mixture was particularly prepared containing one volume of lentiviral pseudoparticles mixed in one volume of complete media, while it was further supplemented with 20mM HEPES and 4μg/ml Polybrene so as to enhance the infection efficiency. The complete media on the cells was removed and replaced with the transduction mixture. The cells were then allowed to incubate with the mixture overnight in a humidified incubator set at 37^{o}C. The following day the medium was changed to complete media and 48 hours post-transduction of the cells antibiotic selection commenced for the production of stable polyclonal cell populations overexpressing the HBV-related receptors of interest.

Cell lines transduced with the human NTCP receptors were selected for with G418 (400μg/ml; *Figure 2.14*) antibiotic, cell lines transduced with the chimeric NTCP receptors were selected for with Hygromycin (500μg/ml)

antibiotic, and finally cell lines transduced with the human ASGPR receptors were selected for with Puromycin (5µg/ml) antibiotic. The suitable concentration of antibiotics utilised was determined by performing dose response curves for each antibiotic on one representative cell line. The complete media containing the appropriate antibiotic selection was replaced every other day. All cells were ultimately selected as a mixed population for at least two weeks, and usually up to a full month, before being used for further experiments or stored for future use.

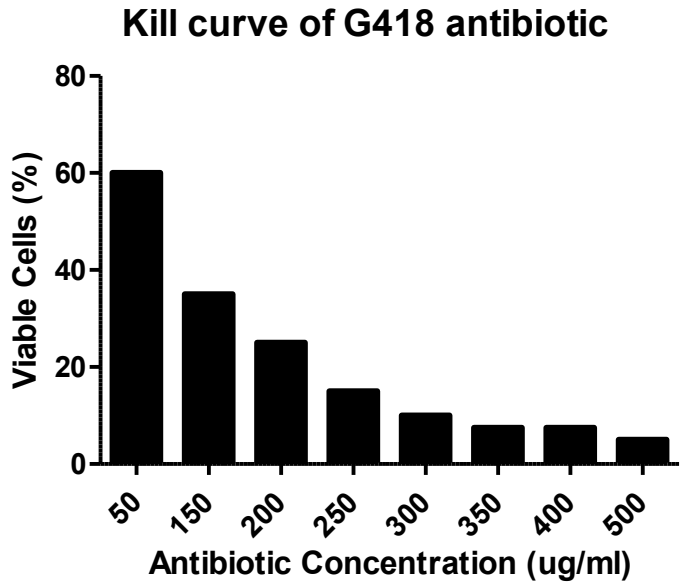


Figure 2.15; Example of a titration kill curve of HepG2 cells treated with G418 antibiotic.

For the production of monoclonal populations of cell lines stably overexpressing the human NTCP receptor, the equivalent mixed population of cells were appropriately diluted in complete media and single cells were seeded individually each in one 96-well. These cells were then allowed to grow to confluency, a process that took approximately two to three months, before being further expanded and used in future experiments.

2.2.2.5.2 Transduction of cells overexpressing the HBV-related receptors with HBSpp

Similarly as described above, either 50.000 cells were seeded into each well of a 24-well plate or 15.000 cells were seeded into each well of a 96-well plate, and left to adhere overnight. The next day, the medium was removed and the cells overexpressing the HBV-related receptors were particularly transduced with either of the following lentiviral preparations: NE (negative control), VSVg (positive control) and HBSpp (HBV glycoprotein-expressing lentiviral pseudoparticles), following standard procedures as described previously. The parental cells were also included as further negative control samples.

Additionally, on one occasion, varying sodium concentrations (i.e. 10mM, 80mM and 140mM; *Table 2.2*) were tested for their efficacy in enhancing the infection process. In this case, the samples were prepared in the following manner: a lentiviral pseudoparticle-NaCl-complete media solution was prepared in a 2:1:2 ratio, and the mixture was added directly to the cells.

After overnight transduction of the cells with the lentiviruses, the media was replaced. The cells were maintained up to 96 hours, checking daily for cells with positive fluorescent signal with the use of a fluorescent microscope (*Table 2.6*). Once a maximum number of fluorescent cells had been determined then, the ones in the 24-well plates were typically trypsinised and fixed in suspension with 4% PFA for flow cytometry analysis of the GFP signal, while the cells in the 96-well plates were fixed and further stained with a DAPI solution (1:10,000 dilution of a 10mg/ml stock) for further immunofluorescence analysis.

2.2.2.6 Infection of cells with infectious HBV supernatants

2.2.2.6.1 Production of infectious HBV supernatants

Infectious HBV was principally generated from the HepG2.2.15 cells (*Table 2.5*). These cells have been derived from HepG2 cells that at some point in time were transfected with the complete HBV genome that became stably integrated. Therefore, HepG2.2.15 cells support stable HBV expression and replication and hence constantly produce infectious virus. The cells were seeded in appropriate flasks and once confluent were split accordingly. They continued to be grown in complete media and once confluent again then the supernatants were collected every other day. This was carried out for a maximum of seven days or until a sufficient quantity of infectious supernatants had been collected, after which the cells were discarded. The supernatants which were maintained in a fridge were then filter sterilised and used fresh to directly inoculate cells.

On different occasions, we produced recombinant HBV virus by transfecting typically HepG2(.2.15) or Huh7(.5) cells (*Table 2.5*) with the replication-competent HBV genomes encoding reporter genes. In this case, the cells were transfected with the HBV plasmid of interest, following standard procedures as described above (*Section 2.2.2.4*). Supernatants were collected and filtered every day from 48 hours up till 7 days post-transfection, after which they were either concentrated or used fresh to directly re-infect other cells of interest.

2.2.2.6.2 Concentration of recombinant HBV virus through an Iodixanol Gradient

We followed the OptiPrep™ Application Sheet V01 and V36 manuals (http://www.axis-shield-density-gradient-media.com/V01.pdf and /V36.pdf; *Axis-Shield*) and adjusted them accordingly to our protocol. OptiPrep™ is generally purchased as a 60% (w/v) stock solution of iodixanol diluted in water, with a density of 1.32 g/ml.

Characteristically, 1ml to maximum 2ml of infectious cell culture supernatant(s), previously concentrated with the use of the Amicon® filter units, were loaded onto an iodixanol gradient ranging from 10% to 50% (*Table 2.2*). A linear 10-50% iodixanol continuous gradient was specifically prepared in 14ml Beckman tubes by allowing a discontinuous gradient to diffuse, meaning that the 50% iodixanol solution was loaded approximately up to the middle of the tube and then the 10% iodixanol solution was very slowly and carefully loaded on top of the 50% solution, leaving enough room for the infectious virus to be loaded on top, and hence forming a linear gradient. The layered tubes were left to settle at room temperature for approximately 4-6 hours before the infectious supernatant was loaded. The mixtures were then ultracentrifuged on a swinging-bucket Beckman SW 40Ti rotor set at 4^{o}C at 200.000xg overnight.

The following day a number of 0.5ml fractions were collected from top to bottom of the density gradient, and 10μl of each fraction were used for further analysis so as to determine the precise infectiousness of the collected fraction samples for future experimental use.

2.2.2.6.3 HBV infection of cell lines stably overexpressing the HBV-related receptor, NTCP

For infection experiments, in general, the cells of interest were seeded at 80% confluence (approximately 15.000 cells per well) on previously collagen-coated 96-well plates. These were typically maintained in complete medium, in the presence or absence of 2% DMSO, for 24 hours prior to infection. This was performed so as to allow for DMSO-mediated differentiation of the cells. On the day of the actual infection, HepG2.2.15 supernatants were collected fresh from confluent cells that had been maintained in culture for approximately 5 days prior to collection. The infectious supernatant was then filtered, just before use; the appropriate quantity, according to our experimental plan, was used and additionally mixed with 2% DMSO and 4%

PEG 8.000 (*Table 2.2*). This infectious mixture was then directly added onto the cells. HBV infections were carried out overnight in a humidified incubator set at 37ºC. The next day the infectious media was removed, the cells were washed six times with PBS followed by a final wash with complete medium, and then the complete media was replaced. Supernatants and cells were finally collected at 5 to 7 days post-infection for further analysis (*Figure 2.15*), while the MOI of the inoculum was also calculated. The parental cells were always included in these experiments as negative control samples.

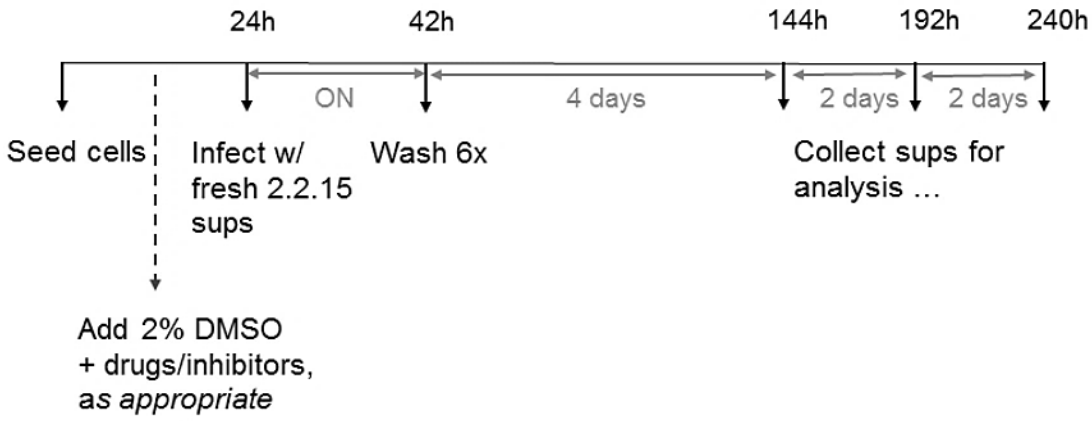


Figure 2.16; Timeline of HBV infection experiments.
The experimental set up was as follows: Cells were seeded on collagen-treated plates and after overnight (ON) incubation were either cultured in the presence or absence of 2% DMSO accordingly. At this point, any other drugs/ inhibitors necessary for the specific experiment(s) were also added to the cell culturing media, together or not with DMSO. 24 hours to 48 hours post-DMSO and/or other drug treatment(s), all cells were infected with freshly collected HepG2.2.15 infectious supernatants. The HepG2.2.15 cells had to be at 90% confluency for the infectious virus to be collected for further infections. 4% PEG was always added during the infection procedure. After overnight infection, the cells were washed with complete medium approximately six times and were incubated for a further five to seven days during which time samples were collected for analysis. Typically analysis involved quantitative PCR analysis for the determination of HBV DNA copies, as well as evaluation of the levels of secreted antigens (HBs/eAg) by ELISA analysis.

2.2.2.6.3.1 Treatment of cells with various drugs, and their optimum concentrations

During the course of this project and depending on the specific experiment, cells were treated with a number of reagents/ drugs/ inhibitors. This was typically performed for analysis of human NTCP receptor expression and

subsequent susceptibility of these cells to HBV infection. Below is a list of all these agents, while the final concentrations used during the experimental procedures are additionally mentioned (*Table 2.21*).

Reagents/Drugs/Inhibitors	Optimum Concentrations
Actinomycin D	5µg/ml
BIX-01294	10µM
C646	1µM
Caffeine	2mM
cAMP	10µM
Colchicine	1µg/ml
Cycloheximide	50µg/ml
Cytochalasin D	4µg/ml
Demecolchine	1µg/ml
Latrunculin A	0.1µg/ml
MB-3	10µM
MDL12	10µM
MG132	1µM
Roflumilast	20nM
Sodium Butyrate	1µM
Tunicamycin	5µg/ml
Valproic acid	10µM
Wortmannin	3mM

Table 2.21; Different reagents/ drugs/ inhibitors utilised during various experimental procedures and their optimum concentrations.

2.2.2.7 Cell viability assay

To determine viable cells during the course of certain experiments we specifically performed an MTT assay. This is a colorimetric assay that allows assessment of the cell's metabolic activity, and hence permits analysis of any cytotoxic effect(s) that certain cell culture reagents (i.e. DMSO) or drug/ inhibitors may have on the cultured cells.

Therefore, for assessing specifically the effect of DMSO on the cell's viability, approximately 8.000 cells were seeded per well of a 96-well plate and maintained in complete medium. Once the cells reached 90% confluency, they were exposed to the different concentrations of DMSO, ranging from 0.5% to 4%, for a total of 24 hours. At that point the MTT reagent (Thiazolyl Blue Tetrazolium Bromide) was added to each well and the cells were further incubated for 4 hours in a humidified incubator at 37^{o}C. The supernatant was then removed and the cells were resuspended in an appropriate volume of the DMSO stock solution (100%). After allowing the colorimetric reaction to take place for at least 10 minutes, finally the absorbance was measured at 562nm on a plate reader (*Table 2.6*).

2.2.3 Statistics

The experiments were performed in triplicates. Graphs and statistical analysis were specifically performed using the GraphPad, Prism software. Error bars depict the standard error of the mean (SEM). For analysing two variables, an unpaired Student's t-Test was performed, while when analysing more than two variables, a one-way ANOVA was performed for one-way analysis of variance after which the Bonferroni correction was applied to compare all pairs of columns. Two-tailed *p*-values were calculated and any *p*-value lower than 0.05 (95% confidence intervals) was considered statistically significant.

3. Results

3.1 HBV entry into human cells

3.1.1 Lentiviral overexpression of human NTCP does not readily result in functional protein expression of the receptor, further impacting on cells susceptibility to HBV infection

3.1.1.1 Effect of lentiviral overexpression of human NTCP on the mRNA and protein levels

Since HBV entry relies on the expression of the human NTCP receptor, we utilised lentiviral overexpression of hNTCP driven by a CMV promoter. Following selection of stable human hepatic (HepG2, Huh7 and Huh7.5) cell lines via antibiotic selection, mRNA expression of hNTCP was determined by quantitative PCR (*Figure 3.1*). All tested cell lines exhibited minimal expression of hNTCP mRNA at baseline, whereas hNTCP mRNA in stably selected cell lines was increased by approximately 1,000-100,000 fold.

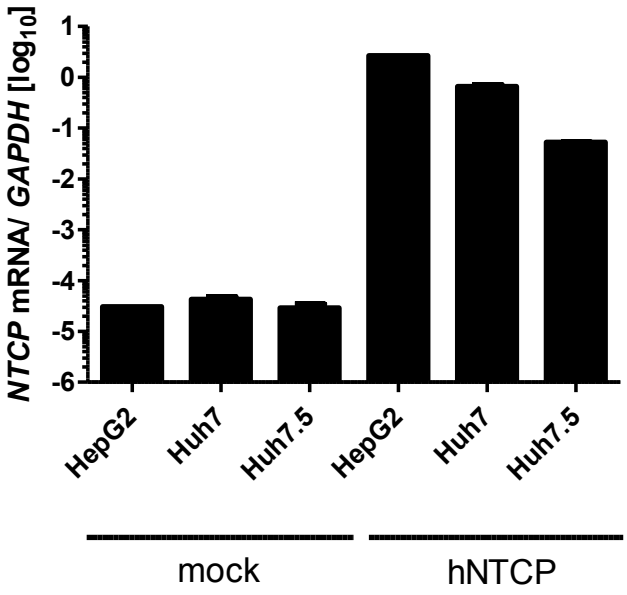


Figure 3.1; Human NTCP mRNA overexpression in stable human hepatic cells. Comparison between the untransduced (mock) and the transduced cell lines (hNTCP) reveals that the lentiviral transduced cells had between three to five log higher hNTCP expression, at the mRNA level, compared to the corresponding untransduced parental cell lines. *Analysis was performed after approximately a month of the cells being cultured under antibiotic selection for the creation of stable cell lines.*

Strikingly, even though all stable cell lines expressed significant amounts of hNTCP at the mRNA level, no expression of the receptor could be detected at the protein level (*Figure 3.2; A)*. To rule out antibody specificity, we performed Western blot analysis of a recombinant human NTCP protein, which could easily be detected by all four evaluated antibodies against hNTCP (*Figure 3.2; B* and *C*). Irrespective of this, none of the antibodies was able to detect endogenous or overexpressed hNTCP in any of the cell lines tested.

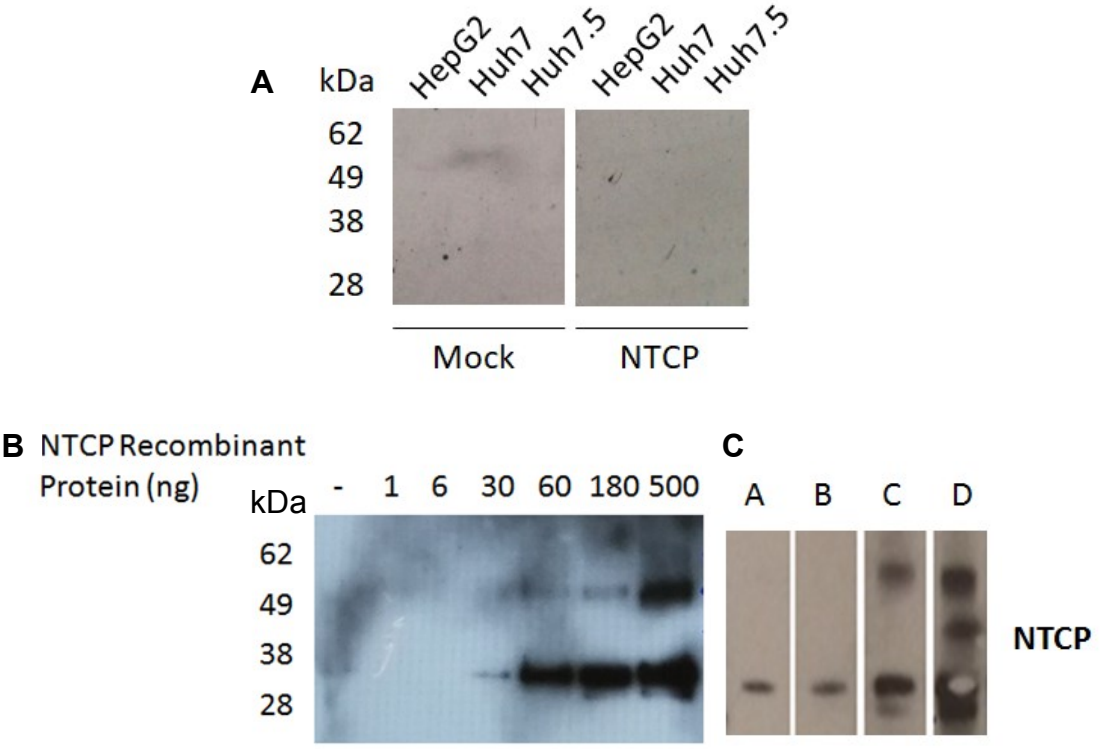
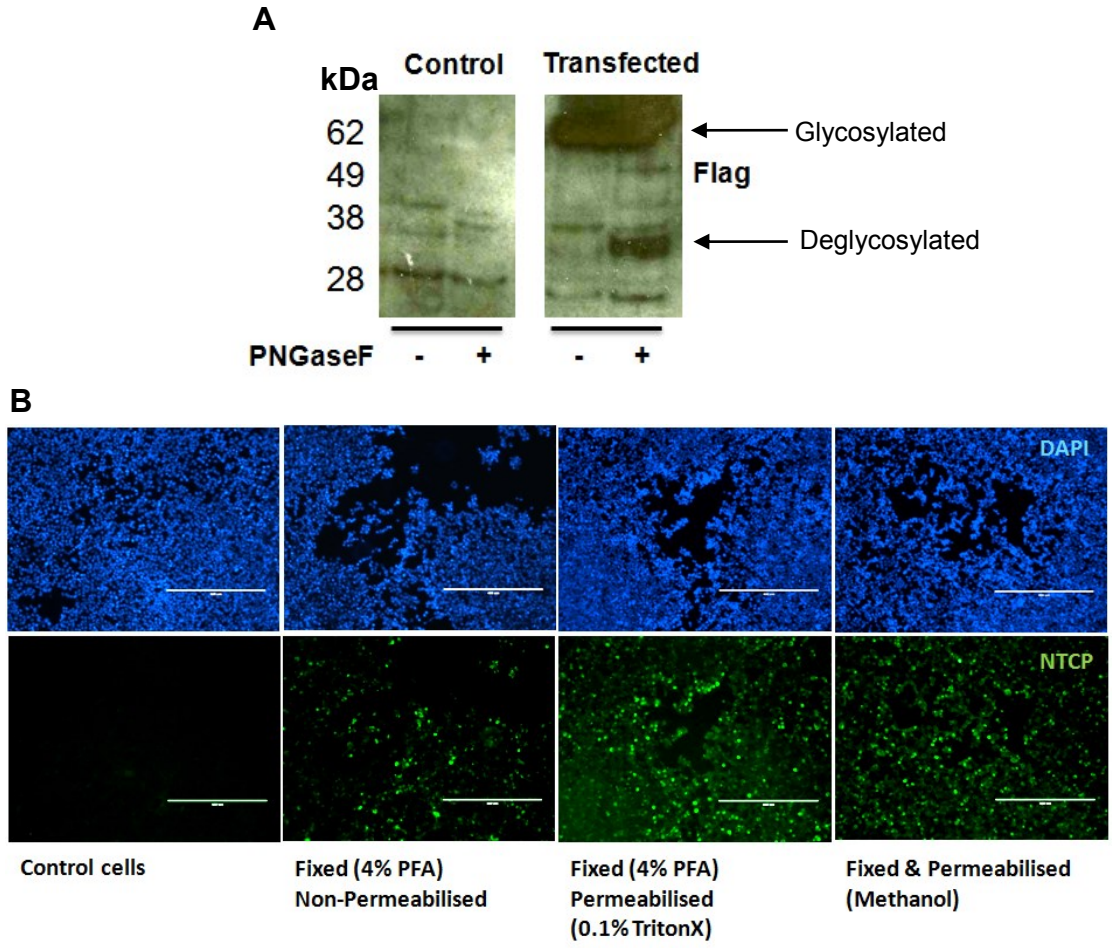


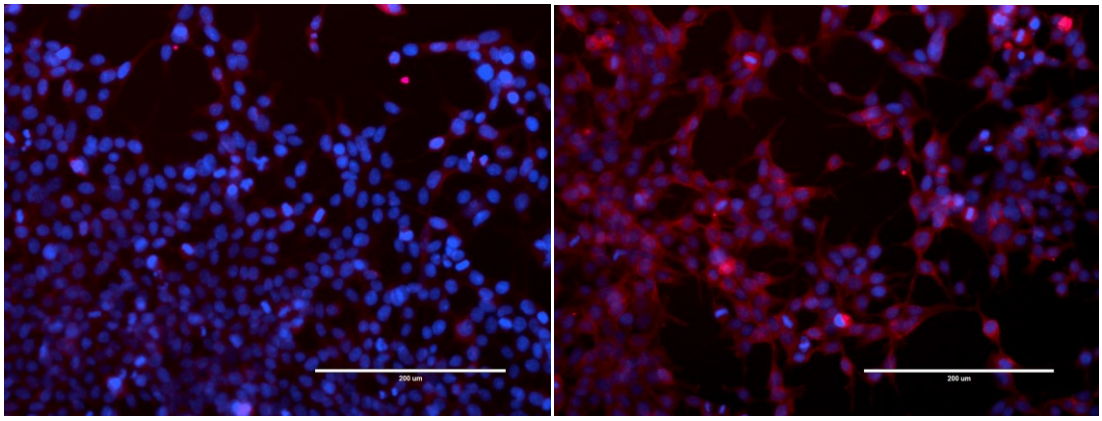
Figure 3.2; (A) Absence of hNTCP protein detection on a western blot, using samples isolated from stable, receptor overexpressing cell lines; (B, C) Detection of a recombinant hNTCP protein by western blot when using anti-NTCP-specific antibodies.

(B) Various concentrations of the recombinant hNTCP protein were tested and detected with the *LSBio* anti-SLC10A1 antibody. The limit of detection for this antibody by western blot was determined to be at 60ng. **(C)** A total of four anti-SLC10A1 polyclonal antibodies were further tested (A. *Abcam*; B. *GeneTex*; C. *LSBio*; D. *Sigma*) for detection of 500ng of the recombinant hNTCP protein. In both sets of blots, two forms of the hNTCP protein were observed; the glycosylated form at approximately 50kDa and the deglycosylated form just below 38kDa. However, only the *LSBio* and *Sigma* antibodies could detect both forms of the protein (at 500ng), while the *Abcam* and *GeneTex* antibodies only detected the deglycosylated form. This possibly suggested that higher concentrations of hNTCP protein were probably required for the detection of both forms by these two antibodies. Otherwise, it is also possible that the antibody recognition site within the glycosylated form of the protein was somehow being masked.

To address as to whether the lack of hNTCP protein expression was due to antibody recognition or a general lack of the receptor's protein expression, we next created stable cell lines overexpressing a C-terminally FLAG-tagged hNTCP, which has previously been shown to be functional in cells [223]. Using transient transfection of HEK293 cells, we were able to detect hNTCP overexpression by western blot and immunofluorescence when using an anti-FLAG-tag antibody (*Figure 3.3; A* and *B*), whereas the human NTCP-specific antibodies still were not able to detect any considerable overexpression, possibly suggesting that the glycosylation patterns of human NTCP produced in mammalian cells may be interfering with antibody recognition. hNTCP protein detection, with the use of the a-FLAG antibody, was determined to be specific (*Figure 3.3; C*).



C



Mouse IgG1, kappa **Mouse a-FLAG**

Figure 3.3; Human NTCP (FLAG-tag) protein overexpression in a transiently-transfected non-hepatic cell line.

(A) *Analysis for performed 72 hours post-transfection.* Detection of the hNTCP_FLAG-tag by western blot, and comparison between the control (untransfected) cells and the cells transfected with the hNTCP_FLAG-tag construct. Analysis was performed 48 hours post-transfection. Both forms of the hNTCP protein were detected only in the transfected cells; the glycosylated form at 50kDa and the deglycosylated form just below 38kDa (the latter resulted from digestion of the whole protein lysate with a PNGaseF enzyme). *For western blot analysis, always 5µg of each protein sample were loaded onto the gel.* **(B)** Detection of the hNTCP_FLAG-tag by immunofluorescence, while testing different cell fixation and permeabilisation conditions. Control cells refer to untransfected cells and these were negative for detection of the FLAG-tag. Transfected cells positive for detection of the FLAG-tag were seen in all other conditions, regardless of the fixation protocols and whether the cells were permeabilised or not. Thus, indicating that the hNTCP C-terminus may possibly be located extracellularly. The image scale bar is 400µm. **(C) Determining specificity of a-FLAG antibody, towards the detection of hNTCP receptor, by performing immunofluorescent staining for immunoglobulin (IgG) as a negative control.** *An A647 anti-mouse secondary antibody was used for immunofluorescence detection.* The image scale bar is 200µm.

Thus, for all further experiments, the FLAG-tagged hNTCP-expressing stable hepatic (HepG2 and Huh7.5) cell lines were utilised, while a stable non-hepatic (HEK293) cell line was additionally created for comparison purposes, and the expression of hNTCP was once again confirmed at the mRNA level by quantitative PCR (*Figure 3.4*). All tested cell lines exhibited minimal to no expression of hNTCP mRNA at baseline, whereas hNTCP mRNA in stably selected cell lines was once again increased by approximately 1,000-1,000,000 fold.

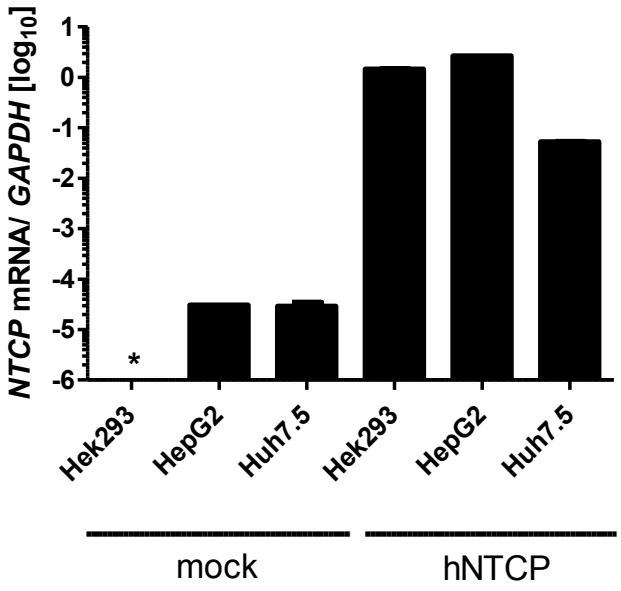


Figure 3.4; Human NTCP (FLAG-tag) mRNA overexpression in stable human hepatic and non-hepatic cells.

Comparison between the untransduced (mock) and the transduced cell lines (hNTCP) revealed that the lentiviral transduced cells had once again between three to six log higher hNTCP expression, at the mRNA level, compared to the corresponding untransduced parental cell lines. * *undetermined.*

In summary; Using a plasmid expressing hNTCP under the control of a CMV promoter we were able to produce various stable cells lines all overexpressing the human NTCP receptor at the mRNA level. However, no data could be originally collected about the hNTCP expression at the protein level. This was thought to be due to the anti-SLC10A1 antibody specificity. Subsequently, we went on to use a plasmid expressing the same hNTCP but with a C-terminus FLAG-tag to assist protein expression. This was determined to be functional, as demonstrated by positive protein overexpression post-transient transfection of a non-hepatic cell line. Nevertheless, anti-SLC10A1 antibody specificity appeared to still be an issue. Therefore, we continued to make use of the hNTCP (FLAG-tag) construct and specifically created a series of new stable cell lines, which were also determined to overexpress the hNTCP receptor at the mRNA level, while with the assistance of the FLAG-tag we hoped we would now be able to determine hNTCP overexpression additionally at the protein level.

3.1.1.1.1 Effect of antibiotic selection on functional human NTCP (FLAG-tag) protein expression during the production of stable cell lines

Interestingly, upon antibiotic selection of the stable cell lines overexpressing the FLAG-tagged hNTCP, it became obvious that forced overexpression of hNTCP via antibiotic selection resulted in a gradual loss of hNTCP protein expression (*Figure 3.5*), despite the fact that considerable levels of hNTCP mRNA expression could still be detected. This effect was however restricted to hepatic HepG2 cells, whereas non-hepatic HEK293 cells retained stably high hNTCP protein levels throughout the course of antibiotic selection. In particular, both cell lines, HEK293 and HepG2, three days post-transduction with the hNTCP_FLAG-tag-expressing lentiviral pseudoparticles but prior-antibiotic selection were positive for detection of the FLAG-tag. As already mentioned, the majority of the HEK293_hNTCP cells even post-antibiotic selection and throughout the course of the treatment remained positive for detection of the FLAG-tag. However, in the case of the HepG2_hNTCP cells, surprisingly these started losing expression of the receptor fifteen days post-antibiotic selection with only very few cells still being detected as positive for FLAG-tag hNTCP protein expression at eighteen days post-selection, as determined by immunofluorescence analysis.

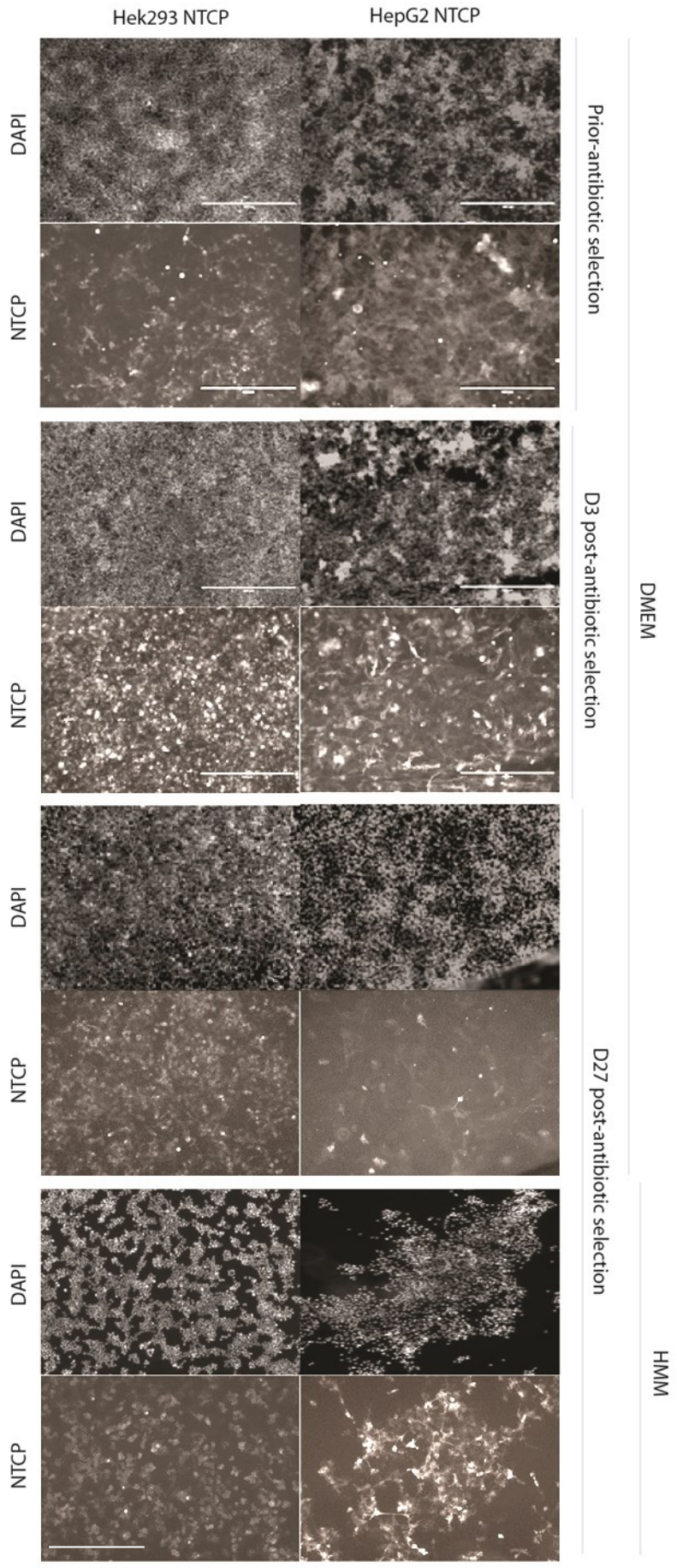

Hek293 NTCP
HepG2 NTCP
DAPI
NTCP
Prior-antibiotic selection
D3 post-antibiotic selection
D27 post-antibiotic selection
DMEM
HMM

Figure 3.5; Effect of antibiotic selection on hNTCP (FLAG-tag) protein expression during the generation of stable cell lines.

Detection of hNTCP_FLAG-tag by immunofluorescence during the course of antibiotic selection for the production of cells that have stably integrated the hNTCP_FLAG-tag receptor. HEK293_hNTCP cells remained unaffected by the presence of antibiotic selection, while HepG2_hNTCP cells gradually lost or suppressed hNTCP_FLAG-tag expression over the course of the treatment. However, the protein expression in these cell lines was fully restored when they were cultured for 48 hours in the presence of optimised media (HMM). The image scale bar is 400µm.

Since hepatic cells often require specifically formulated media containing hepatocyte-specific growth factors and nutrients, we decided to compare hNTCP protein expression in conventionally used media (DMEM/10%FBS; complete medium) with specifically hepatocyte-defined media supplements (William's E/10% FBS/ITS/DMSO/Dexamethasone/Hydrocortisone; HMM or optimised medium) and found that hepatocyte-defined medium resulted in a rapid gain of the hNTCP protein expression that had been lost or suppressed during antibiotic selection in the hepatic cell lines overexpressing hNTCP. However, the same supplements did not result in obviously increased hNTCP expression in the non-hepatic cells, which particularly appeared to be unaffected by the change of the culturing conditions (*Figure 3.5*).

In summary; The presence of antibiotic selection during the production of cell lines stably overexpressing hNTCP (FLAG-tag) appeared to have a negative impact on the hNTCP protein expression in the human hepatic HepG2 cells, while it did not obviously affect the non-hepatic HEK293 cells. A primary hypothesis for this being that the human NTCP receptor, in the specific hepatic cell line, may have a very complex transcriptional and translational processing pathway that it requires to undertake before being fully transported and expressed on the cell surface; a process that may be affected by antibiotic selection. Another explanation for this outcome observed in the stable human hepatic cell line, apart from the possible negative impact of antibiotic selection directly on the expression of the receptor, could be simply the passage of time during which the cells are maintained in culture; meaning

once again that appropriate expression and integration of this receptor within the plasma membrane of the particular cell line requires more complex processing over a longer period of time (a process that occurs in a more straight-forward manner in the stable non-hepatic cell line). This, however, appeared to be directly regulated by appropriate cell culturing conditions, during which the HepG2_hNTCP cells were particularly observed to rapidly restore expression of the hNTCP protein.

3.1.1.1.2 Culturing single cell clones from the hepatic cell line overexpressing human NTCP, so as to select a stable monoclonal cell population positive for functional hNTCP protein expression

To overcome the problem in regards to the loss of hNTCP (FLAG-tag) protein expression in the HepG2_hNTCP stable cell lines post-antibiotic selection, while also wanting to eliminate the need for a highly specialised and expensive culturing media like HMM, we decided to additionally grow individual cell clones of this cell line. The idea was that by single cell cloning the HepG2_hNTCP we would be able to isolate and rescue those very few cells that had sustained expression of the hNTCP protein throughout the course of the antibiotic treatment.

After some considerable amount of time of culturing the single cell clones to confluency in complete media, we performed immunofluorescence for FLAG-tag detection (*Figure 3.6*). However, as previously, it was impossible to detect any single cell clones positively expressing the hNTCP protein. Thus, we either had not managed to select for any of the cells that were originally thought to be positive for hNTCP protein expression or all cells had once again managed to lose or suppress protein expression. As soon as these single cell clones were cultured for 48 hours in the optimised media (HMM), they all restored hNTCP protein expression but to somewhat different degrees.

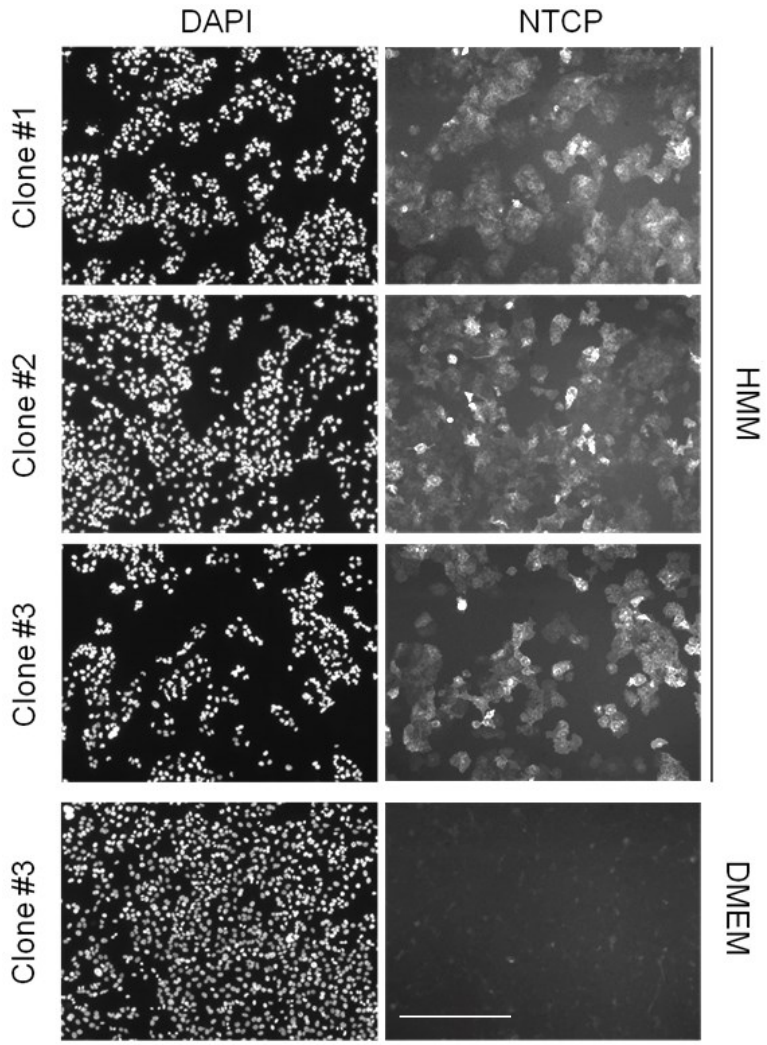


Figure 3.6; Examples of stable HepG2_hNTCP monoclonal cell populations cultured in optimised media (HMM) versus complete media (DMEM).

Detection of the hNTCP_FLAG-tag by immunofluorescence in different HepG2_hNTCP single cell clones cultured in optimised media, and an example of a single clone cultured in normal complete media. hNTCP protein expression was lost or suppressed when cells were cultured under complete media conditions, but was rapidly restored when the cells were cultured for 48 hours in the presence of the optimised culturing media. The image scale bar is 400μm.

Consequently, regardless whether the stable HepG2_hNTCP cells were a polyclonal or monoclonal cell population, they appeared to behave in a similar manner; they required some active ingredient(s) within the optimised media, obviously missing from the complete media, to support proper hNTCP protein expression on the cell surface. The similarity of the polyclonal and monoclonal

cell populations & clones, respectively, could also be seen at the mRNA level (*Figure 3.7*), where both polyclonal and monoclonal HepG2_hNTCP cell populations expressed comparably high hNTCP expression levels.

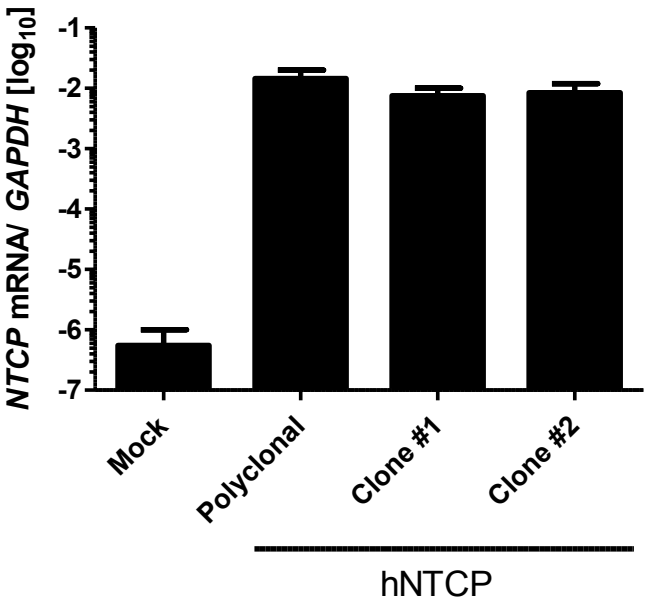


Figure 3.7; Human NTCP (FLAG-tag) mRNA overexpression in a selection of stable HepG2_hNTCP polyclonal and monoclonal cell populations.
Examples of a polyclonal and two monoclonal cell populations that were determined to express roughly equal levels of hNTCP mRNA. Mock cells refer to the HepG2 untransduced, parental cells.

In summary; Polyclonal populations and monoclonal stable HepG2_hNTCP cells were determined to require the presence of one or more ingredients found within the optimised culturing media for expression of hNTCP at the protein level. It is possible that whatever the ingredient(s), they may play a role on proper translation of the protein, while they may also have a part in post-translational events, such as assuring accurate transportation of the hNTCP protein to the cell surface. One observation that was clearly obvious when using the optimised media, compared to the complete media, was that the HepG2_hNTCP cells seemed to differentiate and hence acquired a vastly different morphology resembling more hepatocyte-like structures (*Figure 3.8*).

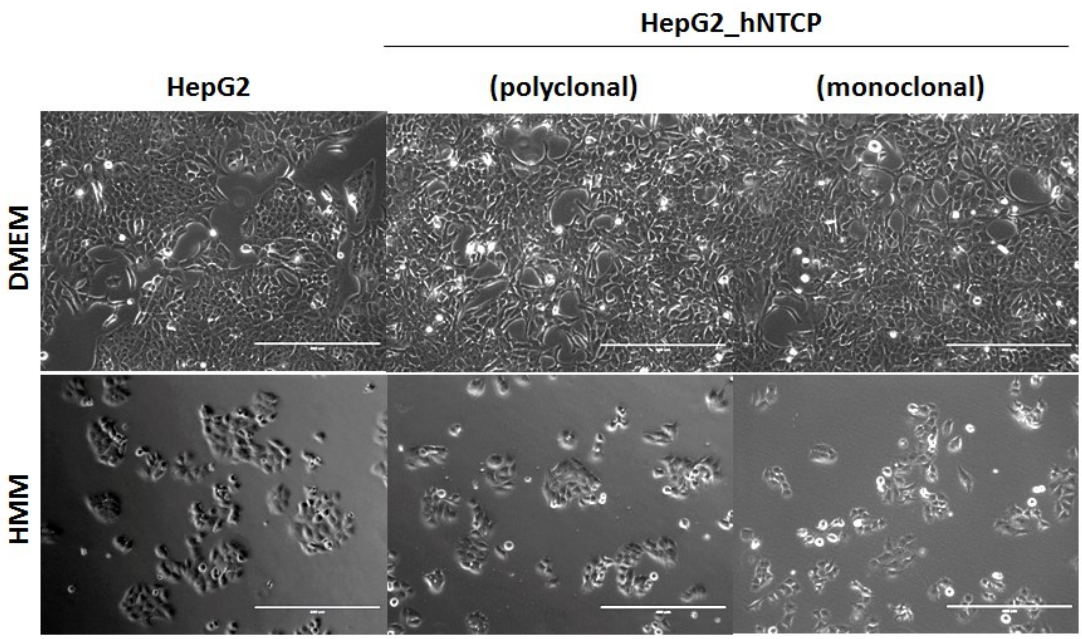


Figure 3.8; Differential morphology of stable HepG2_hNTCP cell populations when cultured in complete (DMEM) versus optimised (HMM) media conditions.

Bright field images of various HepG2 cells. Generally, the effect of the optimised media, in regards to cell differentiation, was identical on all HepG2 cells, parental as well as cells overexpressing hNTCP. Particularly, the cells transformed from more elongated structures, when cultured in DMEM, to structures resembling more defined cuboidal shapes, when cultured for 48 hours in the presence of the optimised media. The image scale bar is 400μm.

3.1.1.2 Effect of human NTCP overexpression on susceptibility of cells to HBV infection

The gradual loss or suppression of the hNTCP protein observed in the stable hepatic cells during antibiotic selection might actually explain the overall poor susceptibility of these hNTCP-overexpressing HepG2 cells to HBV infection, which we were typically observing (*Figure 3.9*).

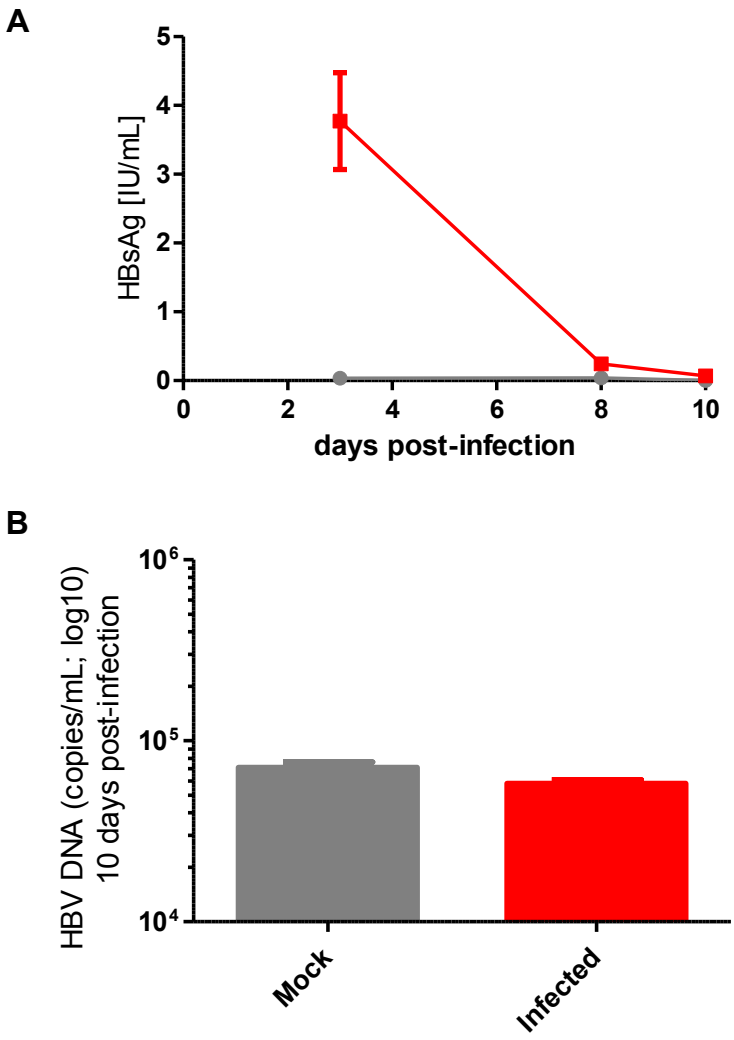


Figure 3.9; Representative example of an infection experiment of HepG2_hNTCP cells with infectious HBV.

No infection of stable hepatic HepG2 cells overexpressing hNTCP could be established when infecting the cells with HepG2.2.15 infectious supernatants under non-optimised culturing conditions, as determined by declining HBsAg secretion levels over time **(A)**, as well as slightly lower levels of HBV DNA copies being detected in the infected versus the mock (uninfected) cells **(B)**.

In summary; When the stable HepG2_hNTCP cell lines were systematically infected with HBV infectious supernatants in the presence of conventional media, these cells were determined to be non-susceptible to viral entry and infection/ replication.

3.1.2 DMSO differentiation of certain human cells is required for functional human NTCP protein expression and subsequent susceptibility of cells to HBV infection

To determine, which of the added supplements within the optimised media was responsible for this dramatic effect, in regards to rapidly restoring the protein expression of the receptor, on the stable hepatic cell lines overexpressing hNTCP, we performed experiments adding individually each of the supplements to the cultured HepG2_hNTCP cells. Therefore, by process of elimination, we finally determined that none of the hepatocyte-specific growth factors, or even the base of the culturing media, had a significant impact on hNTCP protein expression. Instead, we discovered that addition of 2% DMSO by itself significantly enhanced hNTCP expression.

3.1.2.1 Effect of DMEM versus William's E base media on human NTCP mRNA expression

Optimised HMM media, which is typically used for culturing primary human hepatocytes, is as a rule made up in Williams' E culturing medium, rather than DMEM, which we principally used for our experiments. Therefore, the first step towards analysing the optimised media was to determine whether Williams' E itself was having an enhanced effect on the expression of hNTCP in the cells overexpressing the receptor. Our initial observation, at the mRNA level, was that the Williams' E optimised media was in fact not responsible for the pronounced effect on restoring hNTCP expression in the stable hepatic cells previously seen, as the use of either DMEM or William's E as a base for the optimised media resulted in highly comparably hNTCP mRNA levels (*Figure 3.10*). As a result, for subsequent experiments we continued using our standard and more readily available DMEM base media and optimised it accordingly.

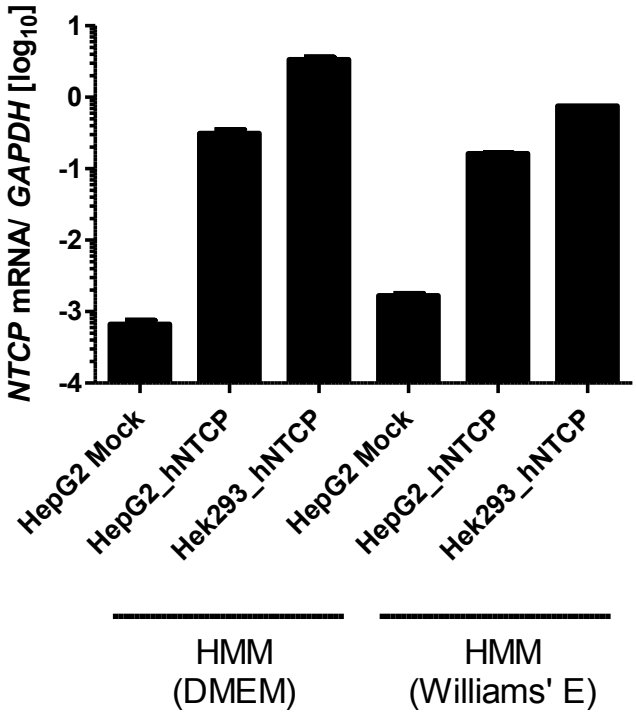


Figure 3.10; Effect of DMEM versus William's E optimised media on hNTCP (FLAG-tag) in stable hepatic and non-hepatic cell lines.

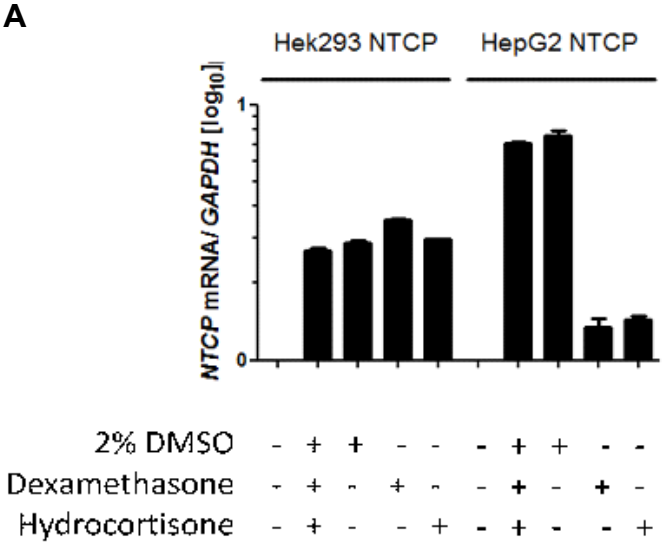
Comparison between DMEM and Williams' E optimised media and their effect on hNTCP mRNA overexpression. Both media resulted in comparable hNTCP mRNA expression levels in the cell lines tested. HepG2 parental cells (Mock) were used as negative controls.

In summary; The base media used for further supplementation with the HMM components does not have a significant effect on hNTCP protein overexpression.

3.1.2.2 Effect of DMSO differentiation on human NTCP mRNA and protein expression

Subsequently, the second step of the analysis was to isolate as many of the ingredients as possible from the HMM optimised media and determine their individual effect on hNTCP (FLAG-tag) expression. To begin with we isolated DMSO, Dexamethasone and Hydrocortisone, while also including a mix of all three of the reagents together with “cocktail B”. Cocktail B, as supplemented by the manufacturer, refers to the mix of all important nutrients and growth factors necessary for culturing primary hepatocytes (*Table 2.2*). We primarily analysed the mRNA and protein expression levels (*Figure 3.11; A* and *B,* respectively), and that was when we determined that addition of DMSO was in fact responsible for the pronounced hNTCP protein overexpression observed in the stable hepatic cell line post-antibiotic selection. Interestingly, the effect DMSO was having on the ectopically expressed hNTCP in the stable human hepatic cell line was also obvious on the endogenous hNTCP expression in the parental cells (*Figure 3.11; C*).

A



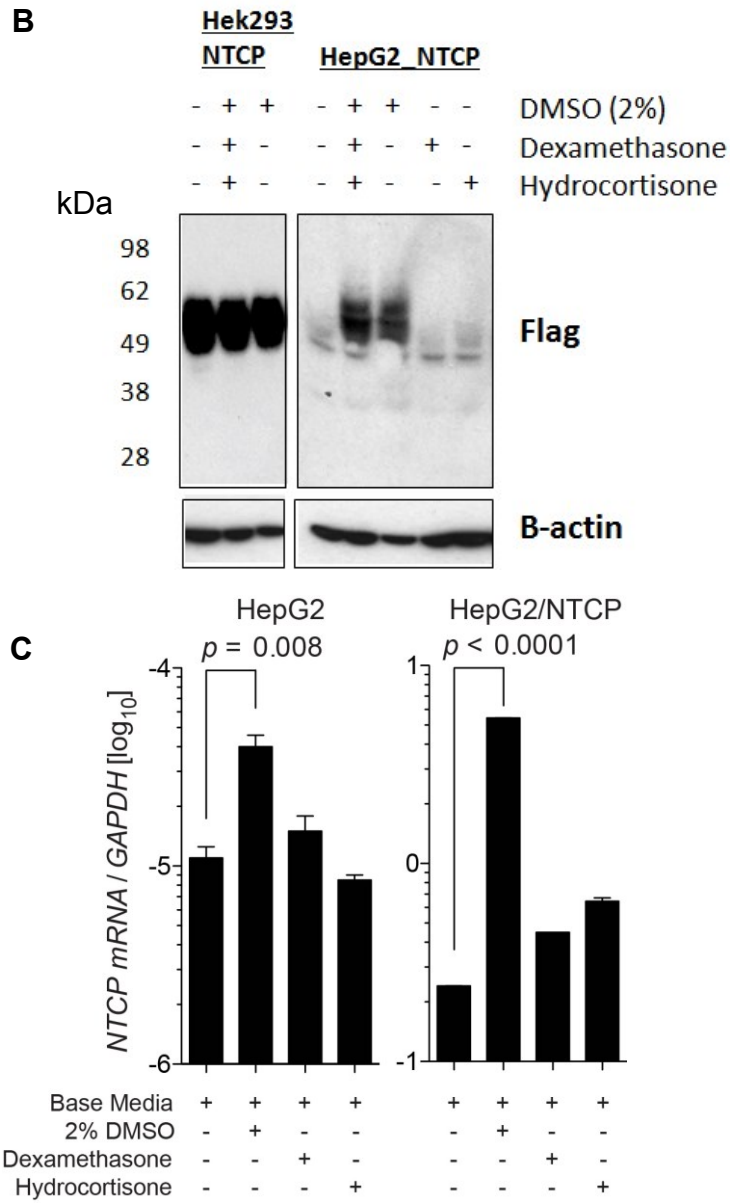


Figure 3.11; Effect of DMSO on hNTCP (FLAG-tag) mRNA (A) and protein (B) overexpression in the stable cell lines created, as well as on the endogenous levels of the receptor expressed in the parental human hepatic cells (C).

The cells were treated with the different reagents for a total of 48 hours. **(A, B)** HEK293_hNTCP cells expressed relatively constant levels of hNTCP, on both the mRNA and protein level, regardless of the culturing conditions tested. On the contrary, HepG2_hNTCP cells required the presence of DMSO (2%) for noticeable hNTCP protein overexpression, while the presence of DMSO in these cells also seemed to cause an increase on the expression of hNTCP mRNA. In the case were all three ingredients were mixed together with cocktail B, the same result was obtained as when DMSO alone was added. This confirmed the fact that none of the supplementary nutrients and growth factors contained within the cocktail B were responsible for the pronounced effect seen on the hNTCP expression in the stable hepatic cells. DMSO was definitely the ingredient necessary for restoring functional hNTCP protein expression in the stable HepG2 cells. *For the qRT-PCR analysis the values are depicted as fold over the untreated samples.* **(C)** A similar effect of DMSO on hNTCP expression was also obvious on the endogenous levels in the parental HepG2 cells.

DMSO was, therefore, principally responsible for restoring the functional protein expression of hNTCP in the stable HepG2 cells that had been suppressed during antibiotic selection. On the other hand, the non-hepatic HEK293_hNTCP cells remained unaffected by the different culturing reagents tested and hence expressed similar levels of hNTCP under all conditions. This result was fairly anticipated considering HEK293_hNTCP cells maintained hNTCP protein expression throughout the course of the antibiotic treatment. These conclusions were also verified by immunofluorescence analysis (*Figure 3.12*).

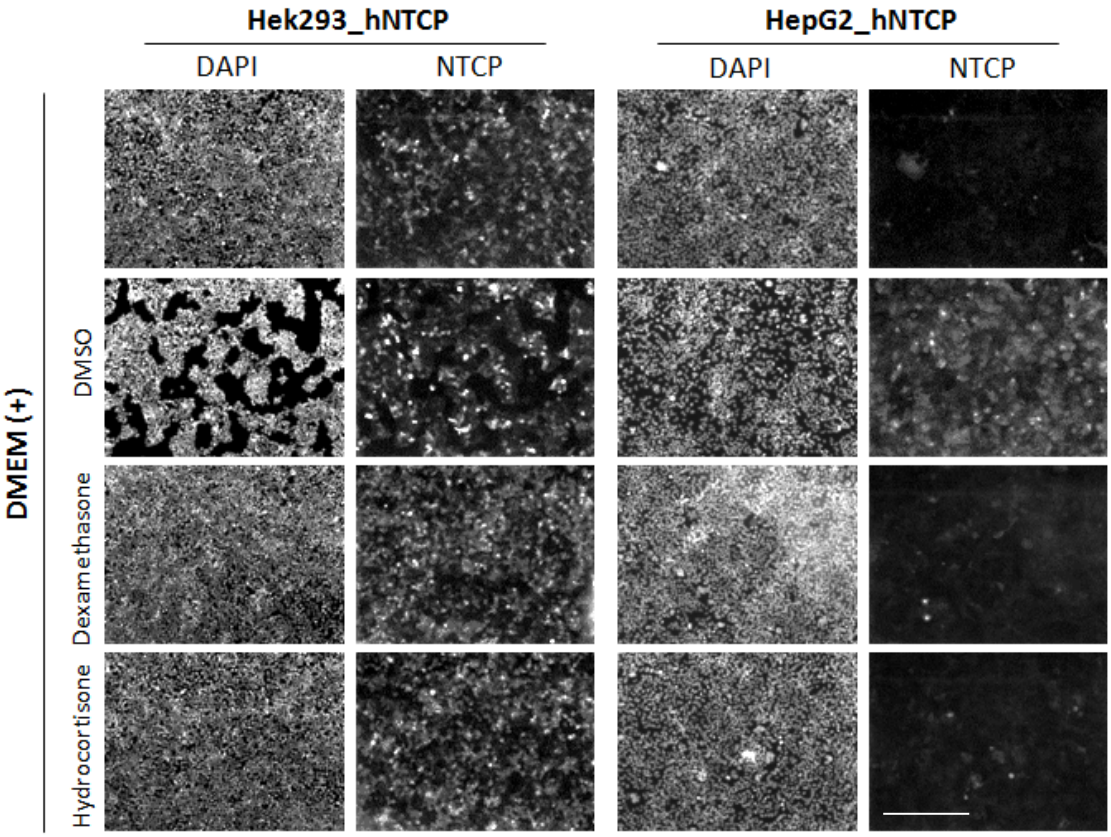


Figure 3.12; Effect of different culturing conditions on the expression of hNTCP (FLAG-tag) in stable cell lines, as seen by immunofluorescence.

Detection of the hNTCP_FLAG-tag by immunofluorescence in stable HEK293 and HepG2 cells cultured in the presence of different reagents. Isolating some of the main ingredient found within the HMM optimised media, it was obvious that particularly the human hepatic cell line, HepG2_hNTCP, required the presence of DMSO (2%) for protein expression of hNTCP on the surface of the cells. The specific reagent appeared to play a role in the recovery of hNTCP protein expression after it was suppressed during antibiotic selection when creating the stable cell lines. In contrast, HEK293_hNTCP cells had a comparable expression of hNTCP on the cell surface under all culturing conditioned tested. The image scale bar is 400µm.

In summary; HepG2_hNTCP, unlike HEK293_hNTCP, cells required the presence of 2% DMSO in the cell culturing medium for the vast recovery of hNTCP expression, as additionally confirmed by flow cytometry analysis (*Appendix A.3.I*). The fact that this small molecule appeared to have an effect on the hNTCP mRNA and protein expression levels, would suggest that it was involved in the transcriptional, translation and possibly trafficking processes that are necessary for hNTCP to undertake for accurate expression specifically in this hepatic cell type.

3.1.2.2.1 Characteristics of DMSO-induced human NTCP expression and the reversibility of this effect

We then went on to further characterise this effect of DMSO on the human NTCP receptor in the stable human hepatic cell line. To begin with, we wanted to determine at what time point DMSO exerted its effect on hNTCP expression. For this reason, we performed a time course experiment where we treated the HepG2_hNTCP cells with 2% DMSO for up to 30 hours, and we looked at both mRNA and protein expression of the hNTCP (*Figure 3.13; A* and *B,* respectively). The outcome of this experiment was that DMSO had to be present in the cell culturing media for at least 15 hours to assist and have a positive effect on hNTCP overexpression. In addition, at the mRNA level, it was determined that hNTCP overexpression remained constantly upregulated for at least up to 14 days in the presence of DMSO.

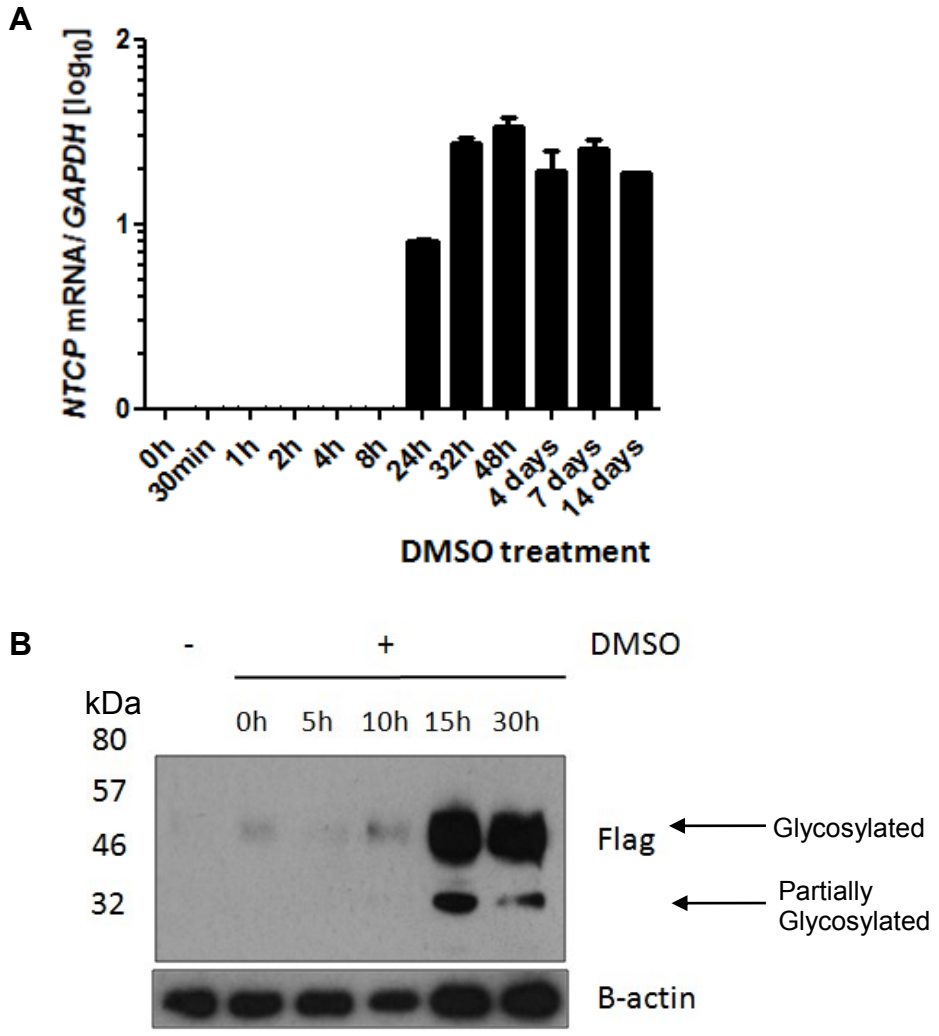
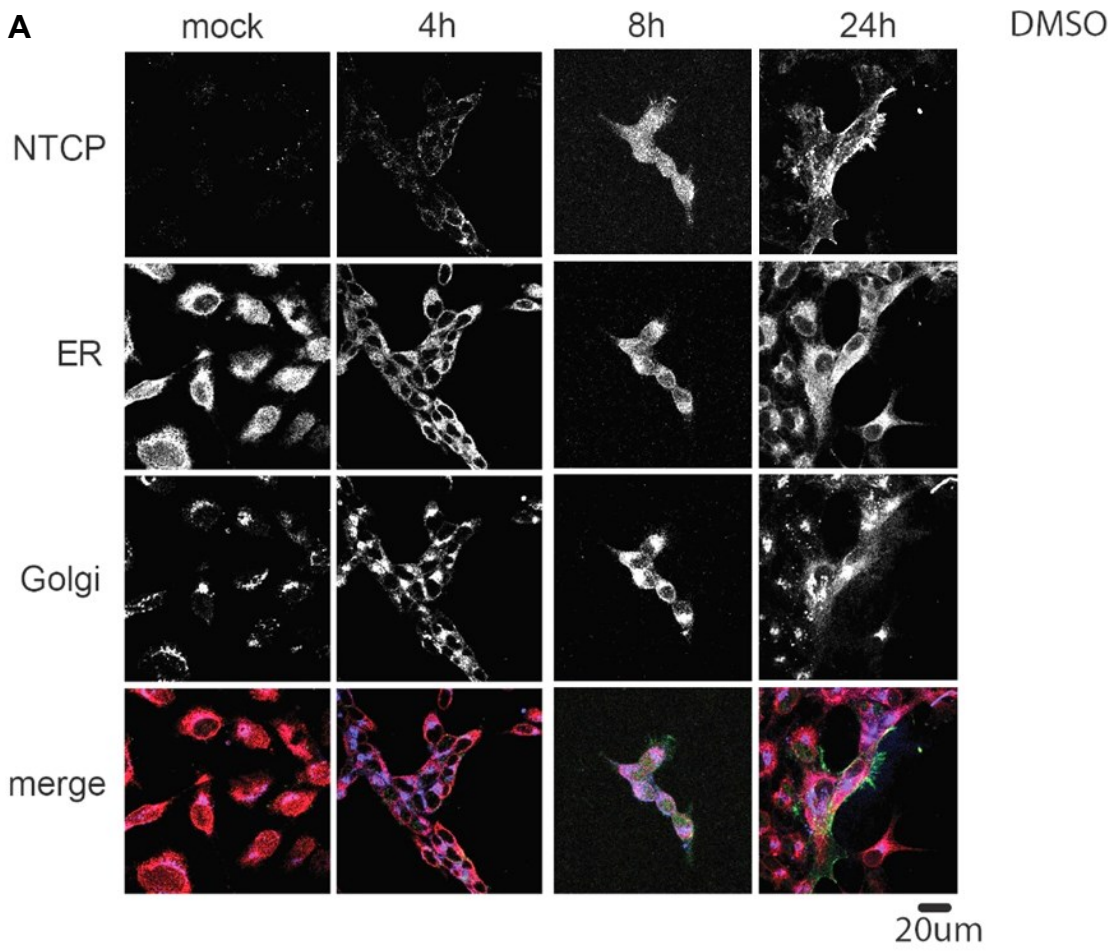


Figure 3.13; Kinetic analysis of the DMSO-induced effect on hNTCP (FLAG-tag) expression in the stable human hepatic cell line.

On both the mRNA **(A)** and protein **(B)** levels, the effect of DMSO on hNTCP expression was obvious after overnight incubation, or more precisely between 10 hours to 15 hours. Specifically, hNTCP mRNA expression was determined to have increased 24 hours post-incubation with DMSO, while hNTCP protein overexpression was observed after 15 hours post-DMSO addition to the culture medium. *For the qRT-PCR analysis the values are once again depicted as fold over the untreated (0h) samples.*

This outcome was also confirmed by immunofluorescence analysis with the use of confocal microscope (*Figure 3.14; A*), during which we were able to observe the shuttling of the hNTCP to the cell surface over time in the presence of DMSO. At a total of 24 hours hNTCP had reached the cell surface, after *de novo* translation seemingly taken place with the protein gradually passing through the ER (4 hours post-DMSO treatment) and the

Golgi (8 hours post-DMSO treatment). An attempt at fractionating different subcellular compartments confirmed that the majority of the protein was found in the membrane fraction, after culturing the cells for 24 hours with DMSO. Some of it was retained within the nucleus, while no protein could be detected in the cytoplasmic fraction (*Figure 3.14; B*).



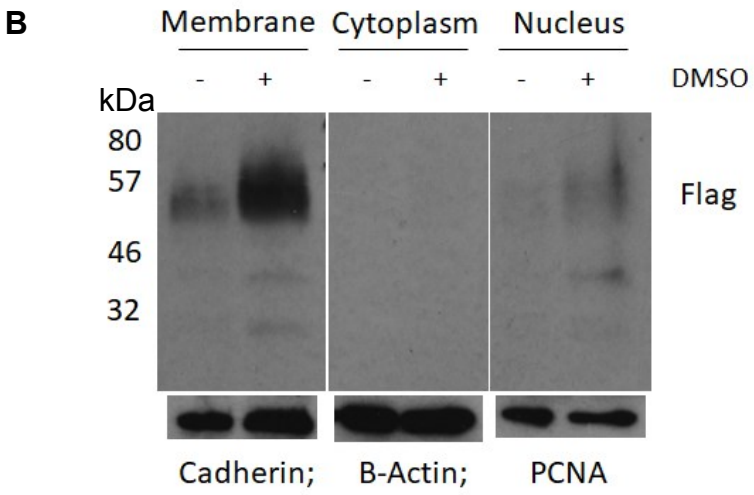
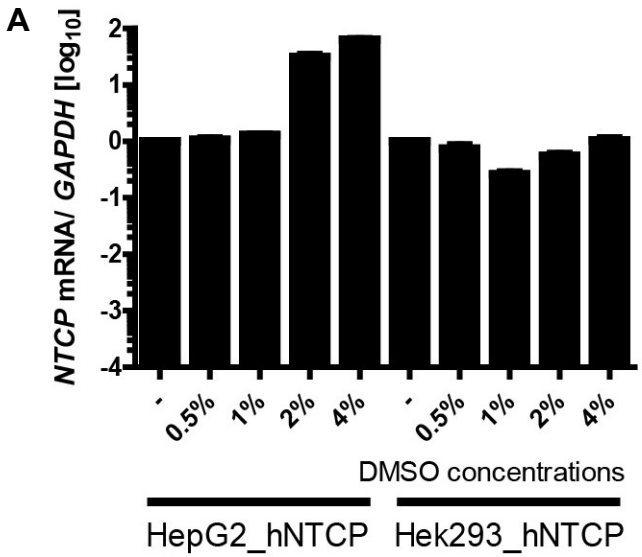
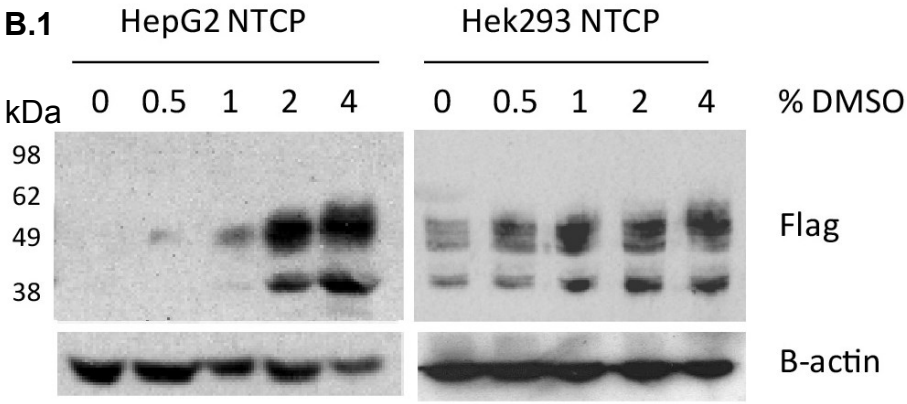


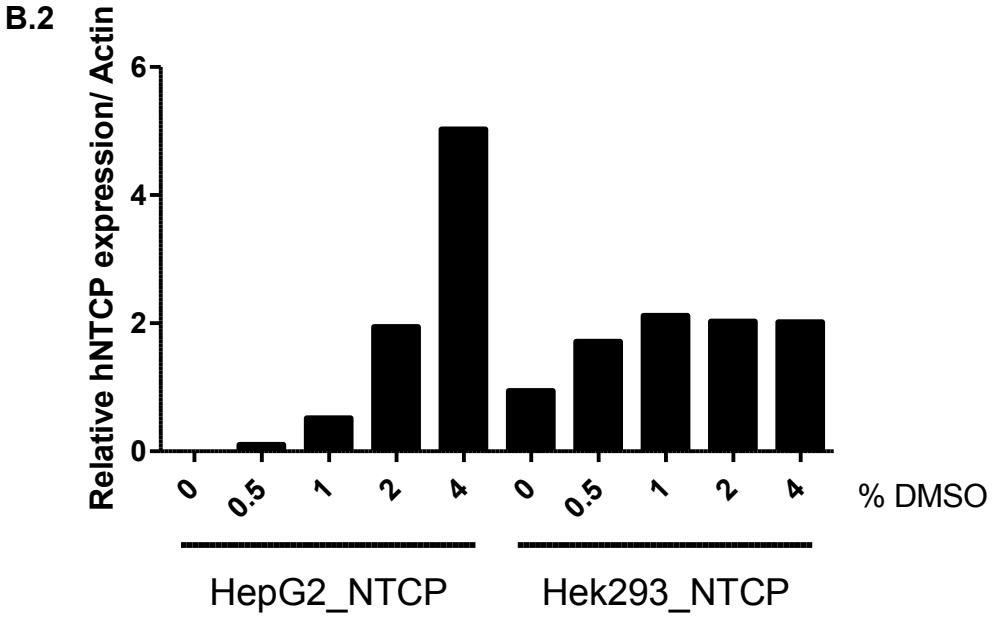
Figure 3.14; (A) Shuttling of hNTCP towards the cell surface over time and in the presence of DMSO; (B) Subcellular fractionation experiment for the expression of the hNTCP receptor.

(A) Detection of the hNTCP_FLAG-tag by immunofluorescence in stable HepG2 cells that were cultured in the presence of DMSO over time. hNTCP at 4 hours was determined to pass through the ER, moving to the Golgi at 8 hours, and finally reaching the cell surface at around 24 hours. **(B)** Expression of the hNTCP receptor, after culturing for 24 hours in the presence of DMSO, is primarily found on the plasma membrane. Some of the protein is also found in the nucleus, while none (or extremely low levels) is obvious in the cytoplasm.

Following on, we wanted to confirm whether or not 2% DMSO was sufficient for higher levels of hNTCP expression seen in the hepatic cells. For this experiment, HEK293_hNTCP cells were also included for comparison purposes. We tested a series of different DMSO concentrations ranging from 0.5% to 4%, and analysed their effect on hNTCP expression on both the mRNA and protein levels (*Figure 3.15; A* and *B,* respectively). We determined that, in the stable human hepatic cell line, 2% DMSO was indeed sufficient as higher concentrations of DMSO, albeit responsible for somewhat higher expression levels of hNTCP, resulted in approximately 50% cell death (*Figure 3.15; C*), while much lower concentrations of DMSO did not support hNTCP overexpression particularly at the protein level (when lower concentrations of DMSO were tested, however, they resulted in typical levels of hNTCP overexpression on mRNA level). In the case of HEK293_hNTCP cells, as expected these were not affected significantly, or to the same extent as the HepG2_hNTCP cells, by the different concentrations of DMSO tested and instead expressed consistent levels of hNTCP expression.


A
NTCP mRNA/ GAPDH [log10]
2
1
0
-1
-2
-3
-4
-
0.5%
1%
2%
4%
-
0.5%
1%
2%
4%
DMSO concentrations
HepG2_hNTCP
Hek293_hNTCP


B.1
HepG2 NTCP
Hek293 NTCP
kDa
0 0.5 1 2 4
0 0.5 1 2 4
% DMSO
98
62
49
38
Flag
B-actin


B.2
Relative hNTCP expression/ Actin
6
4
2
0
0
0.5
1
2
4
0
0.5
1
2
4
% DMSO
HepG2_NTCP
Hek293_NTCP

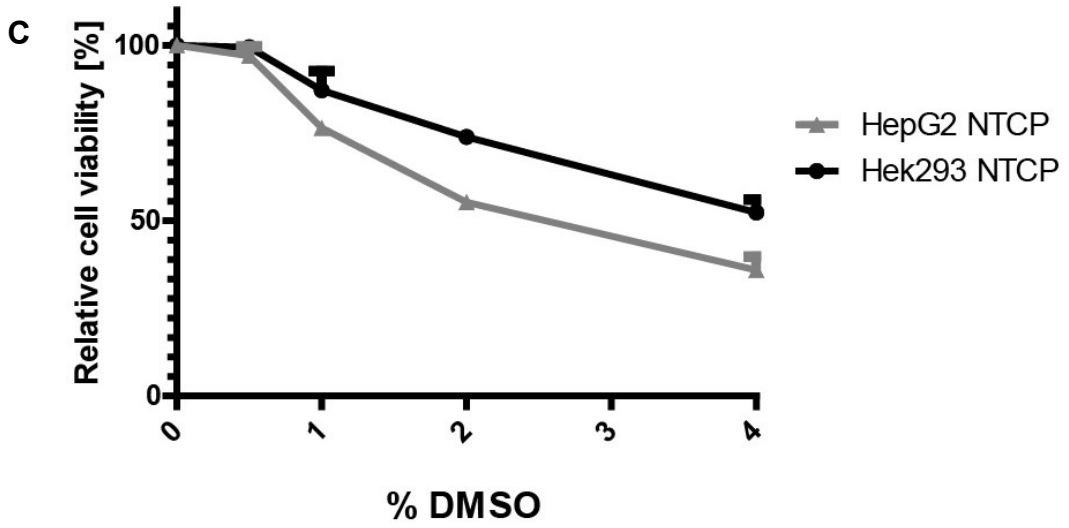


Figure 3.15; Testing varying concentrations of DMSO and their effect on hNTCP (FLAG-tag) overexpression on the mRNA (A) and protein (B) level, as well as on cell viability (C).

The cells were treated with the different concentrations of DMSO for a total of 24 hours. Only in the presence of 2% or 4% DMSO did HepG2_hNTCP cells demonstrate an overexpression of hNTCP on both the mRNA **(A)** and protein **(B)** level, while concentrations lower than 2% were certainly not sufficient for supporting hNTCP protein overexpression. HEK293_hNTCP cells, as previously shown, were not significantly affected by the presence of varying concentrations of DMSO and instead expressed relatively comparable levels of hNTCP regardless of the DMSO conditions tested, as additionally confirmed by densitometry analysis (*Figure B.2*). **(C)** Both non-hepatic and hepatic cell lines, respectively, showed a reduction in their cell numbers when higher concentrations of DMSO were tested, losing nearly 50% of their cell population at the highest DMSO concentration (4%).

Subsequently, we were curious to uncover whether this effect we were observing of the presence of DMSO on hNTCP expression could actually be reversed after withdrawal of the treatment. Thus, we decided to treat both the HEK293 and HepG2 cells overexpressing hNTCP (FLAG-tag) for 48 hours with 2% DMSO, after which we withdrew the reagent and cultured the cells in conventional complete media. We then performed a time course analysis from zero hours post-DMSO removal up till 48 hours later, and once more analysed the expression of hNTCP on both the mRNA and protein level (*Figure 3.16; A* and *B,* respectively).

Interestingly, what we determined was that, unlike HEK293_hNTCP cells where hNTCP expression generally remained more or less stable regardless the conditions tested, in HepG2_hNTCP cells expression of hNTCP declined post-DMSO removal. In particular, in the case of hNTCP mRNA expression levels (*Figure 3.16, A*), these decreased nearly by 100 fold immediately after DMSO was removed (4 hours post-DMSO removal the mRNA levels had returned back down to normal – as previously seen in the complete absence of DMSO), nevertheless hNTCP protein expression (*Figure 3.16, B*) was relatively stable up till 6 hours post-DMSO removal after which there was a gradual decline with the majority of hNTCP protein expression being lost or suppressed by 48 hours post-DMSO removal. Moreover, in the case of HEK293_hNTCP mRNA expression levels, what was interesting was that even though there was an initial drop in the expression levels also immediately after DMSO removal, these cells managed to recover the levels of hNTCP mRNA in a total of 48 hours.

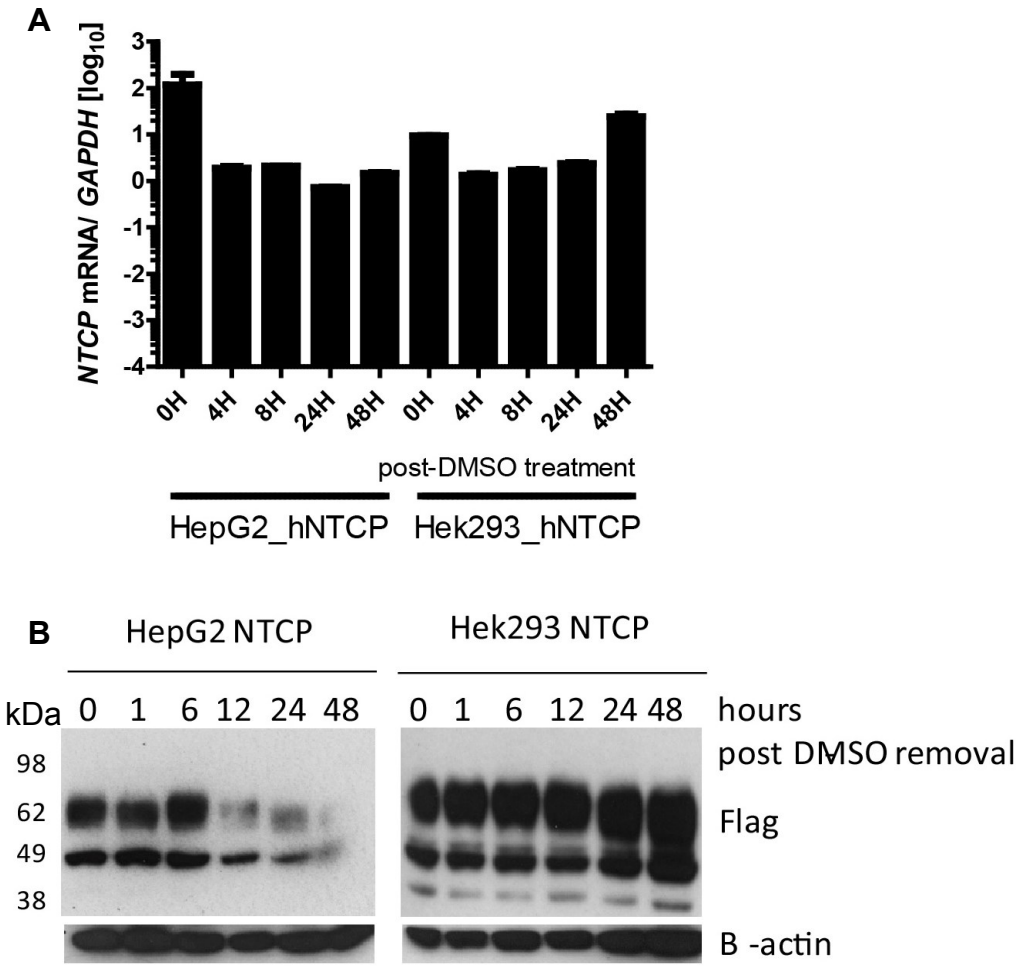


Figure 3.16; Reversibility of the DMSO-induced effect on hNTCP (FLAG-tag) mRNA (A) and protein (B) expression levels in the stable cell lines.

The cells were initially cultured in the presence of DMSO for a total 24 hours. Post-DMSO removal, the HepG2_hNTCP cells showed an immediate decline in hNTCP expression at the mRNA level, while at the protein level the decrease was more gradual with a stable protein level being maintained up till 6 hours. Finally, the majority of the hNTCP protein was lost or suppressed by 48 hours after DMSO treatment was removed. On the other hand, HEK293_hNTCP showed stable levels of hNTCP protein expression throughout the experiment; however at the mRNA level there was a similar immediate decline of hNTCP expression but the cells managed to recover this expression by the end of the experiment at 48 hours post-DMSO removal.

In summary; In stable human hepatic HepG2 cells, hNTCP expression on the cell surface was found to be highly dependent on the cell culturing conditions and specifically the presence of DMSO. The effect of DMSO on hNTCP expression was regulated in a time and concentration dependent manner. However, this effect could be rapidly reversed by removing the treatment from the cells.

3.1.2.2.2 Effect of DMSO on the CMV promoter driving expression of genes

Furthermore, we wanted to confirm that the effect we were observing when using DMSO to culture the HepG2_hNTCP cells was directly influencing the expression of the hNTCP receptor rather than acting on the CMV promoter that was driving expression of the hNTCP in these cell lines; recently, another group demonstrated that this was the case [254]. For this reason, we used HepG2 parental cells and transiently transfected these with a plasmid containing a GFP sequence under the control of a CMV promoter (pTRIP_Venus; *Table 2.4*). Post-transfection and after allowing the cells to incubate for a couple of days, we further cultured these in the absence and presence of 2% DMSO for a total of 24 hours. We then analysed expression of the GFP protein, by western blot and immunofluorescence (*Figure 3.17; A* and *B,* respectively), to determine whether DMSO also exerted an effect on this protein. We discovered that the addition of DMSO to the cell culture medium of cells transfected with this CMV_GFP plasmid in fact did not affect the expression levels of GFP, with equal levels of expression particularly being seen in the minus and plus DMSO samples in both types of analysis undertaken. Consequently, we also concluded that DMSO did not work on the CMV promoter but rather it directly affected the expression of hNTCP receptor.

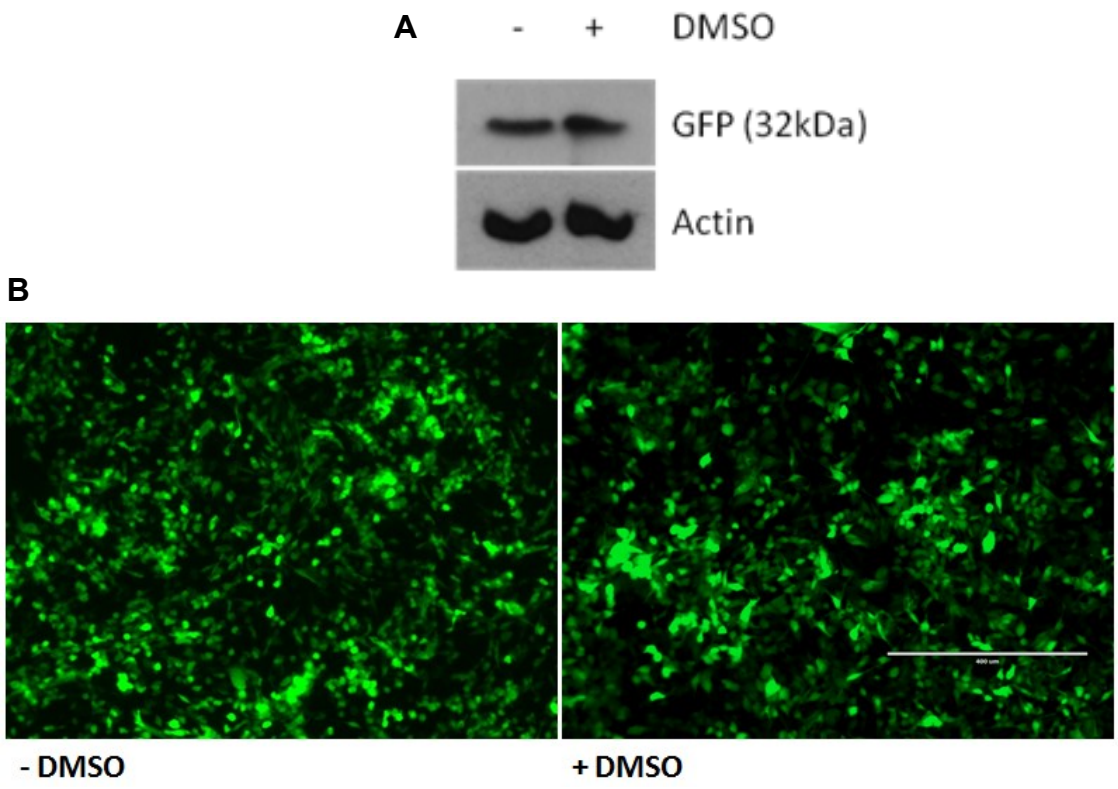


Figure 3.17; Effect of DMSO on the CMV promoter driving expression of a GFP sequence.

The HepG2 parental cells were analysed for GFP expression at 72 hours post-transfection with the CMV_GFP-expressing plasmid. DMSO treatment was initiated at 48 hours post-transfection of these cells, and hence the cells were maintained in the presence of the reagent in total for 24 hours. Equal levels of GFP expression were determined, by western blot **(A)** and immunofluorescence **(B)** analysis, in samples either untreated or treated with DMSO, indicating that the presence of DMSO in the cell culturing media did not affect the CMV promoter driving expression of the GFP sequence. The image scale bar is 400µm.

In summary; DMSO appears to have a direct effect on hNTCP overexpression, and does not seem to influence the CMV promoter driving expression of the gene.

3.1.2.2.3 Effect of DMSO on human NTCP expression in other stable human hepatic cell lines

Having created in total three stable human non-hepatic and hepatic cell lines, Hek293, HepG2, and Huh7.5 (*refer to Figure 3.4, for equivalent hNTCP mRNA expression levels*), we were particularly interested in determining whether DMSO had the same effect on hNTCP expression in the remaining stable human hepatic cell line (Huh7.5) as it had in the HepG2_hNTCP cells.

For this purpose, we cultured the cells in the presence or absence of 2% DMSO, for a short (4 hours) and a longer (24 hours) duration, and then we analysed the samples for hNTCP mRNA and protein expression (*Figure 3.18; A* and *B,* respectively). Surprisingly, we observed that, apart from the HepG2_hNTCP cells that we already knew were greatly affected by the addition of DMSO, the other human hepatic cell line (Huh7.5_hNTCP), similarly to HEK293_hNTCP, was not affected by the presence of DMSO.

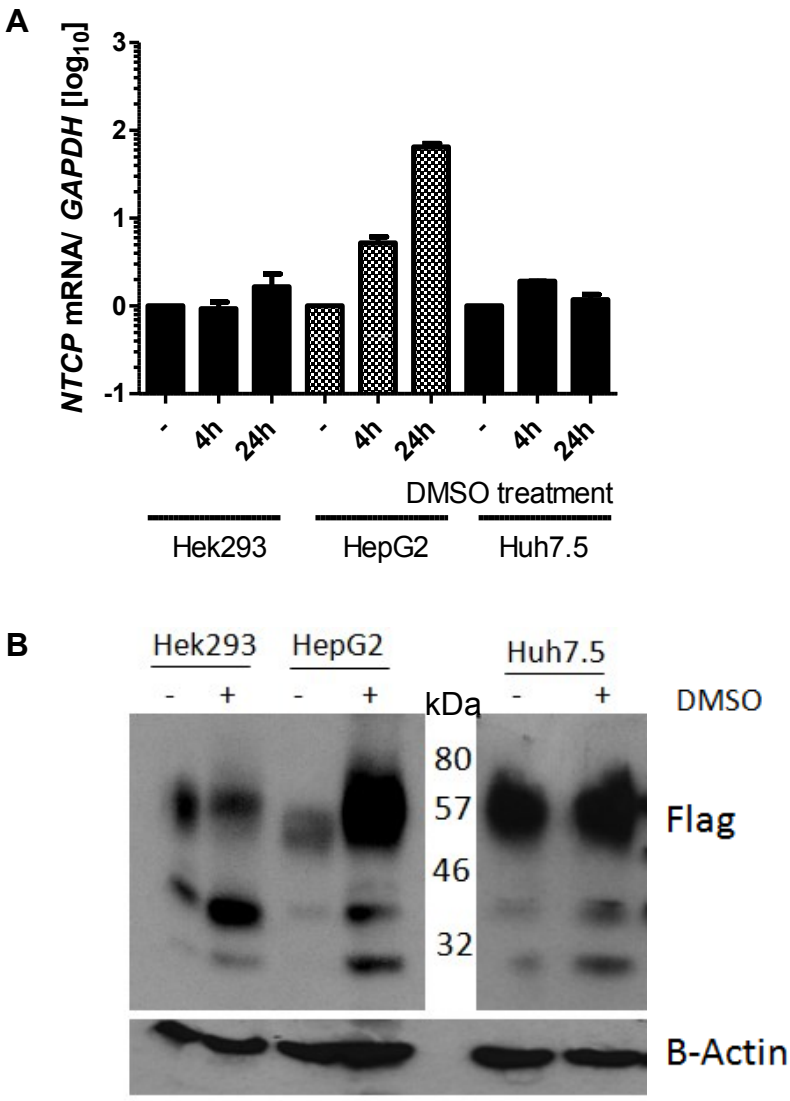


Figure 3.18; The effect of DMSO on hNTCP (FLAG-tag) expression in another stable human hepatic cell line.

Comparison between two stable human hepatic cell lines overexpressing hNTCP, and a non-hepatic cell line. Addition of DMSO to the cell culture medium only affected stable HepG2_hNTCP overexpressing cell lines, while it did not affect whatsoever hNTCP expression in HEK293 and Huh7.5 cells. These outcomes were determined on both the mRNA **(A)** and protein **(B)** levels. *For western blot analysis, the cells were treated for a total of 24 hours in the presence of DMSO.*

In summary; It is possibly that the mechanism DMSO regulates in the HepG2_hNTCP cells, may in fact be fully functioning in the Huh7.5_hNTCP cells allowing for functional expression of the receptor, and thus explaining why the latter do not obviously require DMSO for induction of hNTCP expression. Nevertheless, it should be mentioned that, over time and in the absence of DMSO, the baseline hNTCP protein expression in the stable

human hepatic HepG2 cell line was in fact established to naturally increase (*Appendix A.3.II*), possibly once again verifying that the process which regulates functional hNTCP expression in this cell lines is somewhat problematic and not as efficient as it possibly is in other cell lines. Regardless, the presence of DMSO was verified to always have an enhanced effect on hNTCP protein expression in these stable human hepatic cells.

3.1.2.3 Glycosylation of the human NTCP receptor following induction with DMSO

It is well known that human NTCP is highly dependent on its accurate *N*-glycosylation for the receptor's stability and trafficking to the cell surface [158]. Therefore, we wanted to ensure that the hNTCP being overexpressed in the stable cell lines was for certain being properly glycosylated. We used initially a PNGaseF enzyme that commonly cleaves complex sugars from *N*-linked glycoproteins. The experimental set up was that cells were treated in the presence of 2% DMSO, for induction of hNTCP overexpression, for either 8 hours or 24 hours after which they were collected and incubated in the presence or the absence of the enzyme and further analysed by western blot (*Figure 3.19*). As a result of this treatment, the hNTCP protein was completely deglycosylated at both time points, while interestingly the same deglycosylated protein bands were seen for both, hepatic and non-hepatic, cell lines, thus indicating that hNTCP glycosylation was being processed as anticipated and in a similar manner in both cell lines. Therefore, all further investigations were performed only on the stable human hepatic cell line overexpressing hNTCP.

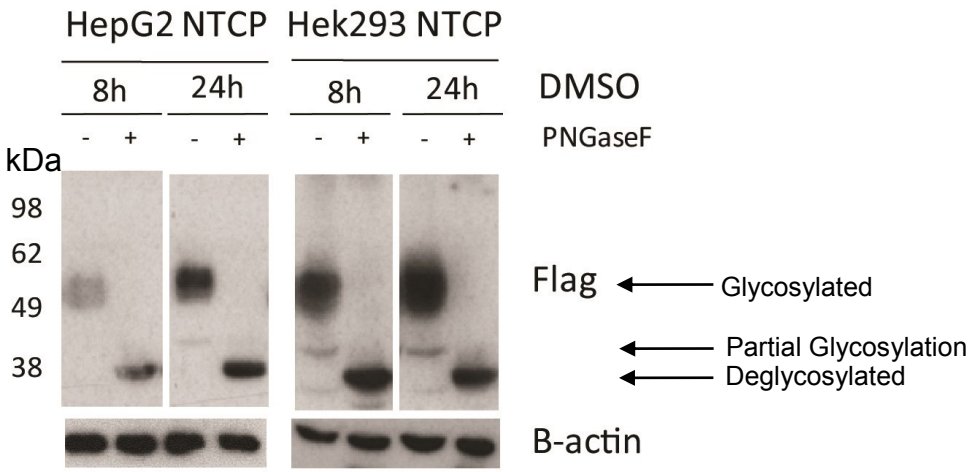


Figure 3.19; Analysis of the hNTCP glycosylation status upon induction with DMSO and treatment with a PNGaseF glycosidase enzyme.

Digestion of the protein samples with PNGaseF enzyme resulted in the complete deglycosylation of hNTCP, as seen by western blot analysis. The glycosylated protein band runs at 50kDa, while the deglycosylated protein band runs at 38kDa. A partially glycosylated band (at approximately 40-45kDa) is shown between the deglycosylated and the fully glycosylated bands.

Subsequently, we thought of checking what would happen when simple sugars were digested. We specifically utilised the HepG2_hNTCP protein samples, which were treated similarly to the previous experiment, and incubated these with an Endo H enzyme prior to analysing them by western blot (*Figure 3.20*). As expected, Endo H cleaved a lower molecular weight protein band found just below the fully glycosylated form of hNTCP, and present only in the samples treated with DMSO for the longest period of time, while it did not have any obvious effect on the fully glycosylated, higher molecular weight band. The sugars cleaved by Endo H were probably sugars that were either on route to being processed into the fully glycosylated form before the samples were collected or could not be processed any further due to protein glycosylation overload.

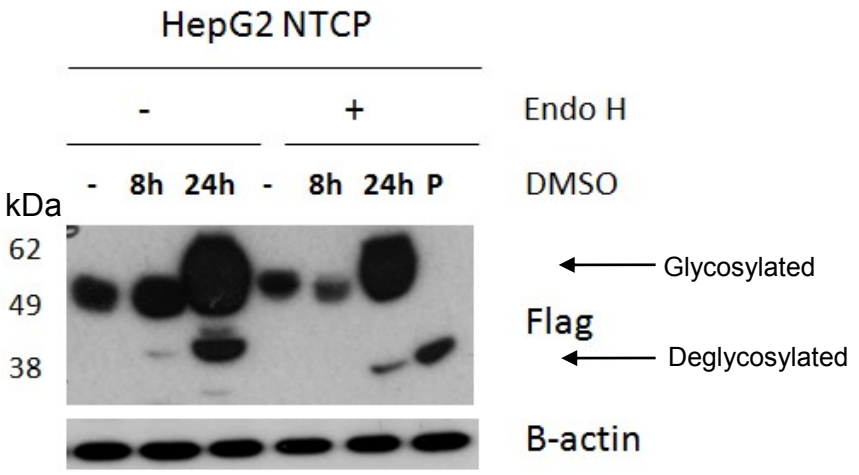


Figure 3.20; Analysis of the hNTCP glycosylation status upon induction with DMSO and treatment with a Endo H glycosidase enzyme.

Digestion of the protein samples with Endo H enzyme resulted in the deglycosylation of a lower molecular weight band (~40kDa) to the fully deglycosylated protein band form (38kDa), as determined by western blot analysis. This band was only seen in the samples treated with DMSO for 24 hours, possibly indicating glycosylation overload. *P indicates the PNGaseF enzyme*, and this was used as a comparison control for determination of the deglycosylated protein band size.

Finally, we were interested in characterising the glycosylation kinetic status of HepG2_hNTCP cells in the presence of DMSO. Accordingly, we made use of an inhibitor of *de novo* protein glycosylation, Tunicamycin. We specifically cultured cells either in the presence or the absence of 2% DMSO while at the same time treating with Tunicamycin for varying amounts of time. The samples were analysed by western blot (*Figure 3.21*). Our data suggested that, as very low levels of glycosylated hNTCP were being processed in the cells when Tunicamycin was present for 8 hours or more, hence the hNTCP protein had to be translated and glycosylated at around 8 hours post-addition of DMSO to the cell culture.

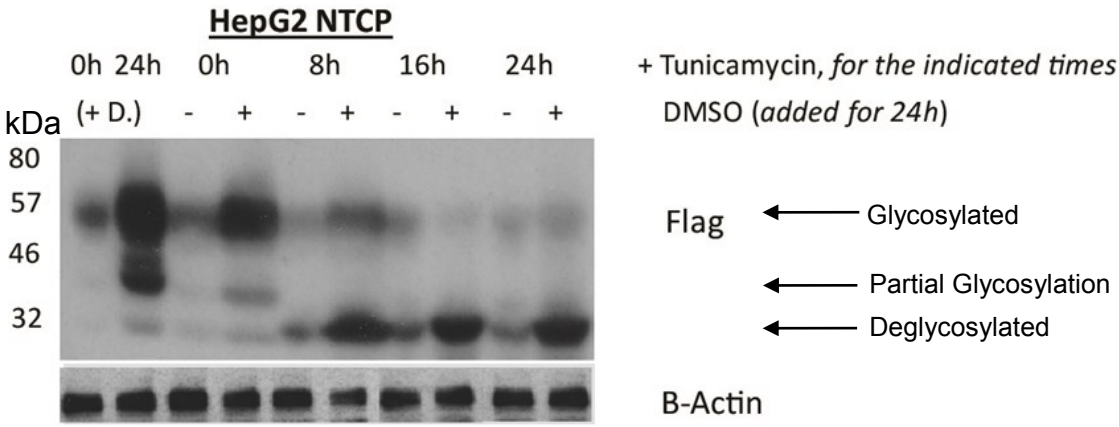


Figure 3.21; Kinetic analysis of the hNTCP glycosylation status upon induction with DMSO and treatment with Tunicamycin.

DMSO treatment was added for a total of 24 hours, while concurrently Tunicamycin treatment took place for the indicated amounts of time. Blocking *de novo* protein glycosylation with Tunicamycin revealed that in the presence of DMSO hNTCP overexpressed in the hepatic HepG2 cells required approximately 8 hours before it was properly translated and glycosylated. *D. stands for DMSO*.

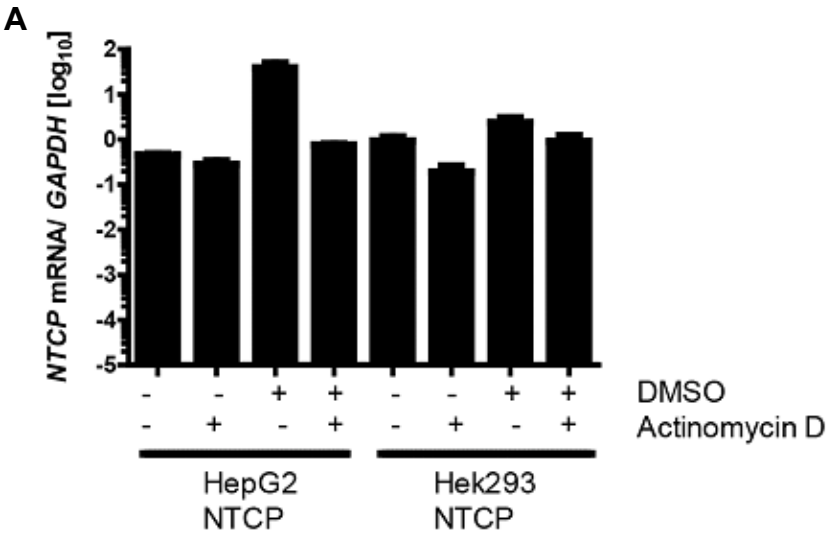
In summary; hNTCP glycosylation was processed post-translationally, as anticipated, and in a similar way in both cell lines tested, hepatic and non-hepatic. In the cases were DMSO was added to the cell culture media, this process seemed to take place around 8 hours post-addition of the reagent.

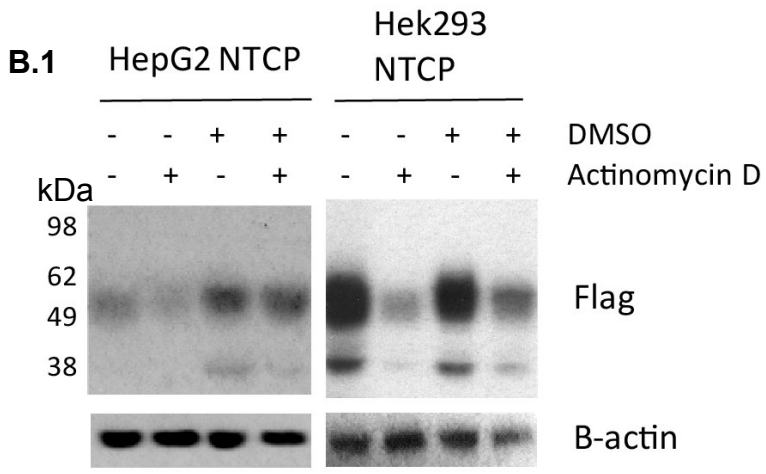
3.1.2.4 mRNA and protein stability of the human NTCP receptor

To gain an understanding about hNTCP stability and particularly the reason why hNTCP expression may be lost or suppressed under certain instances, such as during the course of antibiotic selection, we performed a set of experiments looking separately at the hNTCP stability on the mRNA or the protein level.

Initially we took a look at hNTCP expression stability on the transcriptional level. We were particularly interested in investigating whether there were any stability problems at this stage of the hNTCP expression pathway and if so was DMSO regulating transcription of hNTCP mRNA. For this reason, we

used a drug that inhibited *de novo* transcription, Actinomycin D. The experimental set up was that the stably overexpressing cells were cultured either in the presence or absence of 2% DMSO while at the same time they were either treated or not treated with Actinomycin D for a total of 24 hours. We analysed the samples for hNTCP expression both on the mRNA and the protein level (*Figure 3.22; A* and *B,* respectively).





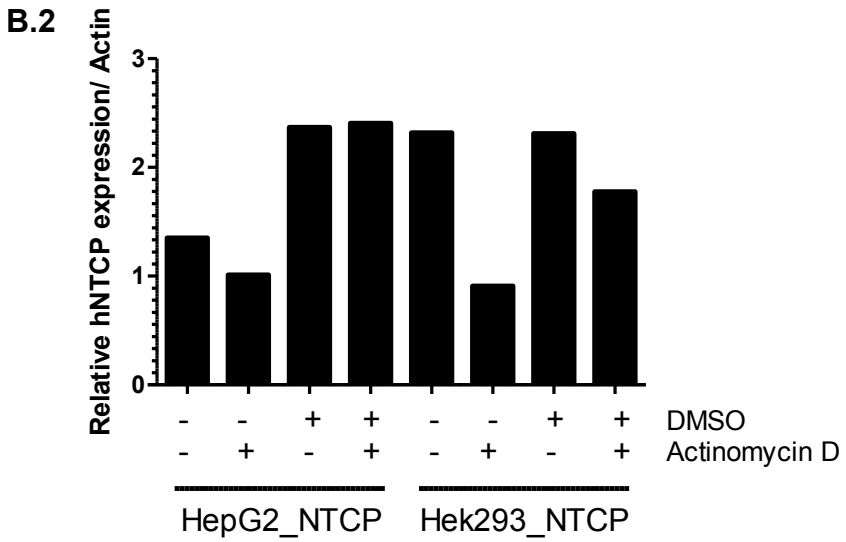


Figure 3.22; Effect of Actinomycin D on *de novo* transcription of hNTCP after induction with DMSO.

The cells were treated in the absence or simultaneous presence of the different reagents (i.e. DMSO and/or Actinomycin D) for a total of 24 hours. **(A)** The presence of Actinomycin D, irrespective of whether DMSO was present or not, resulted in most cases in a low but detectable decline in hNTCP mRNA expression. **(B)** Nonetheless, even though this outcome was also true for hNTCP protein expression in the non-hepatic HEK293_hNTCP cells, it appeared as if the presence of DMSO in the hepatic HepG2_hNTCP cells efficiently blocked suppression of hNTCP protein expression, as demonstrated by western blot and subsequent densitometry analysis. This possibly indicates that DMSO may have been affecting the expression of hNTCP primarily at a translational level, rather than at a transcriptional level.

At the mRNA level (*Figure 3.22, A*), and in both hNTCP overexpressing cell lines tested, as anticipated we detected that the addition of Actinomycin D resulted in a detectable decrease in the levels of hNTCP. However, this decrease was nearly 100 fold in the HepG2_hNTCP cells, when DMSO had been added for induction of hNTCP overexpression. Interestingly, however, at the protein level (*Figure 3.22, B*), unlike HEK293_hNTCP cells where addition of Actinomycin D once again resulted in evidently decreased levels of hNTCP protein expression irrespectively of whether DMSO was present or not, in the case of HepG2_hNTCP cells, and particularly in the samples where DMSO had been added, the presence of Actinomycin D did not result in a noticeable decline in the expression of hNTCP; comparable levels of hNTCP expression could be observed in both samples (i.e. Actinomycin D treated and untreated, in the presence of DMSO). In the HepG2_hNTCP samples where DMSO had

not been added there was a slightly detectable decrease in hNTCP protein expression. Our data most likely demonstrates that hNTCP expression was being regulated via translational, or possibly post-translational, mechanism(s) by DMSO.

Having established that DMSO possibly does not affect transcription of hNTCP, at least not to the same extent as it affects translation of the receptor, we turned our attention to its protein stability. This time we used Cycloheximide, a *de novo* protein synthesis inhibitor, to determine how stable the hNTCP protein actually was. The experimental set up was that HepG2_hNTCP cells were treated for 24 hours with 2% DMSO after when DMSO was removed and the cells were treated with Cycloheximide for various lengths of time. We specifically collected samples from zero hours to 24 hours post-DMSO removal of cells additionally treated in the presence of the *de novo* protein synthesis inhibitor. Samples were analysed for hNTCP protein expression (*Figure 3.23*). The results showed that, when inhibiting *de novo* protein synthesis with the use of Cycloheximide, the half-life of hNTCP protein after 24 hours induction with DMSO was stable for at least a further 24 hours after DMSO had been removed; so in the presence of cycloheximide the hNTCP receptor is possibly degraded very slowly. On the contrary, after 24 hour DMSO induction of hNTCP expression and in the absence of the *de novo* protein synthesis inhibitor, the receptor was completely stable for at least a further 6 hours after which the protein slowly started to degrade (at 24 hours there was obviously less protein present on the western blot compared to the amount seen at 6 hours). The latter outcome matched nicely with the results demonstrated in *Figure 3.16*, where after hNTCP DMSO induction for 24 hours, and subsequent removal of the reagent, hNTCP protein expression was once again stable for up to 6 hours after which point it slowly started to decline.

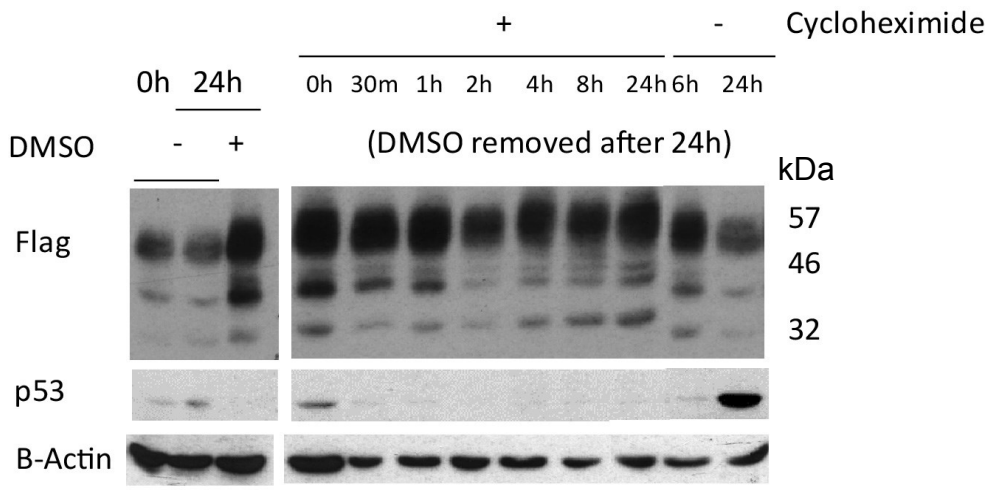


Figure 3.23; Effect of Cycloheximide on *de novo* protein synthesis of hNTCP after induction with DMSO and subsequent withdrawal of the agent.

After induction of hNTCP protein expression for 24 hours with DMSO (*at which point DMSO was removed*), hNTCP protein expression remained stable for at least another 24 hours in the presence of Cycloheximide. However, in the absence of Cycloheximide the hNTCP protein expression became fairly unstable and was gradually suppressed or degraded, similarly to what was seen in previous experiments (*refer to Figure 3.16, B*). The p53 protein was used as an experimental control to confirm inhibition of *de novo* protein synthesis.

Consequently, it would appear that in the presence of Cycloheximide the levels of the receptor remain stable within the cell, suggesting that the presence of the *de novo* protein synthesis inhibitor may in fact result in the suppression or degradation of another protein, whose presence typically impacts negatively the hNTCP protein expression. This other protein may similarly also become suppressed or degraded in the presence of DMSO, hence resulting in the over-induction of hNTCP expression. *Refer to the Discussion chapter; Section 4.2.2. and Figure 4.1.*

Finally, we decided to investigate whether the receptor was being degraded in the proteasome, due to protein misfolding during protein synthesis. Therefore, we used a proteasome inhibitor, MG132, and we specifically performed two sets of experiments on HepG2_hNTCP cells that were cultured in complete medium in the absence of DMSO, so as to determine possible recovery of hNTCP expression. In the first experiment we tested varying concentrations of the drug at 6 hours post-treatment, and in the second, we carried out a time

course experiment from zero to 24 hours post-drug treatment, using what we believed to be the optimum concentration of the inhibitor. However, when we analysed the cells by immunofluorescence we could not observe any hNTCP protein expression. We concluded that the hNTCP protein was most likely not being degraded in the proteasome, however this requires further investigation.

In summary; DMSO appears to exert its mechanism of action on hNTCP mainly via a translational and possibly post-translational (e.g. trafficking) mechanism. However, the effect of DMSO on hNTCP at a transcriptional level cannot be completely excluded.

3.1.2.5 Susceptibility of cells to HBV infection upon DMSO differentiation

To evaluate the effect of DMSO on the susceptibility of liver cells to HBV infection, we utilised the stable human HepG2_hNTCP cells. We infected this hNTCP stably overexpressing cell line with infectious sups at a MOI of 5000 and analysed the samples at seven days post-infection for HBV DNA copies within the actual cells (*Figure 3.24*). Surprisingly, the susceptibility of the HepG2_hNTCP cells to HBV infection had considerably increased in the presence of DMSO. Consequently, DMSO is actively responsible for enhancing, not only the hNTCP protein expression on the plasma membrane, but also the susceptibility of cells to HBV infection. However, the enhanced effect of DMSO on the susceptibility of cells to HBV infection has been acknowledged since the late 1980s [229].

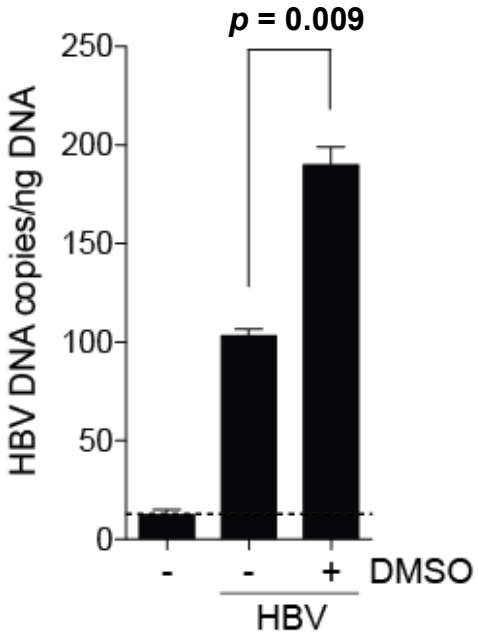


Figure 3.24; Enhanced susceptibility of human liver cells overexpressing hNTCP to HBV infection in the presence of DMSO.

The general experimental set-up for HBV infections is summarised in Figure 2.16. The presence of DMSO during HBV infection of human liver cells overexpressing hNTCP resulted in a considerable increase in the amount of cells becoming susceptible to the virus. *HBV DNA copies/ng DNA refers to the total count of HBV DNA copies within the total number of cells analysed. An infected cell may contain more than one HBV DNA copies.*

In summary; In the presence of DMSO, certain cell lines become more readily susceptible to HBV infection, confidently revealing that there is a direct correlation between the levels of hNTCP protein expression on the cell surface and the number of cells that become permissive to HBV.

3.1.3 DMSO results in human NTCP expression via cAMP-mediated restructuring of actin filaments, which further enhances susceptibility of cells to HBV

3.1.3.1 DMSO-induced changes in DMSO-treated cells suggest epigenetic and cytoskeletal modifications could be affecting human NTCP expression

Proteomic analysis of the stable human hepatic cell lines overexpressing hNTCP and cultured in the presence or the absence of 2% DMSO, revealed a significant upregulation of certain epigenetic and cytoskeletal factors particularly induced in the presence of DMSO (*Table 3.1*).

	HepG2_hNTCP (- DMSO)	HepG2_hNTCP (+ DMSO)
Epigenetic factors	-	Histones H2B types: 1-B 1-C/E/F/G/I 1-D 1-J, K, L, M, N, O 2-F 3-B
Cytoskeletal factors	-	Tubulin alpha chains: 1A-B-C, 3C/D-E, 4A, 8 Tubulin beta chain and Tubulin beta chains: 1, 2A-B, 3, 4A-B, 6 Cofilin 1/2

Table 3.1; Proteomics analysis of DMSO-treated cells revealed upregulation of a number of epigenetic and cytoskeletal factors.

Histone H2B is one the main histone proteins involved in the regulation of eukaryotic chromatin structures. In terms of the H2B isoforms, there are a total of 16 human variants (15 of which are shown to be upregulated in our experiment, in the presence of DMSO). These are structurally very similar to the original H2B histone protein, and only have slight sequence differences with the number of amino acids remaining unaffected. However, they have been demonstrated to undergo different post-translational modifications, they are found in different chromatin areas and interact differently with proteins. Therefore, they all have unique functions [302]. *Proteomic analysis of these samples was performed by Professor Anne Dell's group, in South Kensington (Imperial College London).*

Therefore, we were particularly interested in analysing the effect of epigenetic modifications and cytoskeletal reorganisation on hNTCP protein expression and subsequent susceptibility of cells to HBV infection.

3.1.3.1.1 Impact of epigenetic regulation on human NTCP expression

First of all, we looked at epigenetic modifications. To specifically investigate the effect of epigenetic regulation on hNTCP protein expression in the stable human hepatic cells, HepG2, we decided to treat these cells with a number of histone modification drugs, in the presence of absence of 2% DMSO. The reason being that we wanted to establish whether interfering with certain epigenetic regulation processes, in the absence of DMSO, would actually allow for some recovery of hNTCP protein expression, while we were additionally interested to determine whether the presence of DMSO would enhance this effect.

The drugs we chose to use, in particular, were as follows (*Table 3.2*):

Epigenetic modification drugs	Function
BIX-01294	Histone-lysine methyltransferase inhibitor
C646	Histone acetyltransferase p300 inhibitor
Sodium Butyrate	Histone deacetylase inhibitor
Valproic acid	Histone deacetylase inhibitor
MB-3	Histone acetyltransferase inhibitor

Table 3.2; Epigenetic modification drugs and their particular functions.

However, all drugs tested had no significant effect on enhancing hNTCP protein expression in the absence of DMSO (*Figure 3.25*), and thus, we did not go any further into examining the effect of epigenetic regulation on hNTCP expression but instead we went on to investigate the effect of cytoskeletal reorganisation on hNTCP protein expression and trafficking to the cell surface.

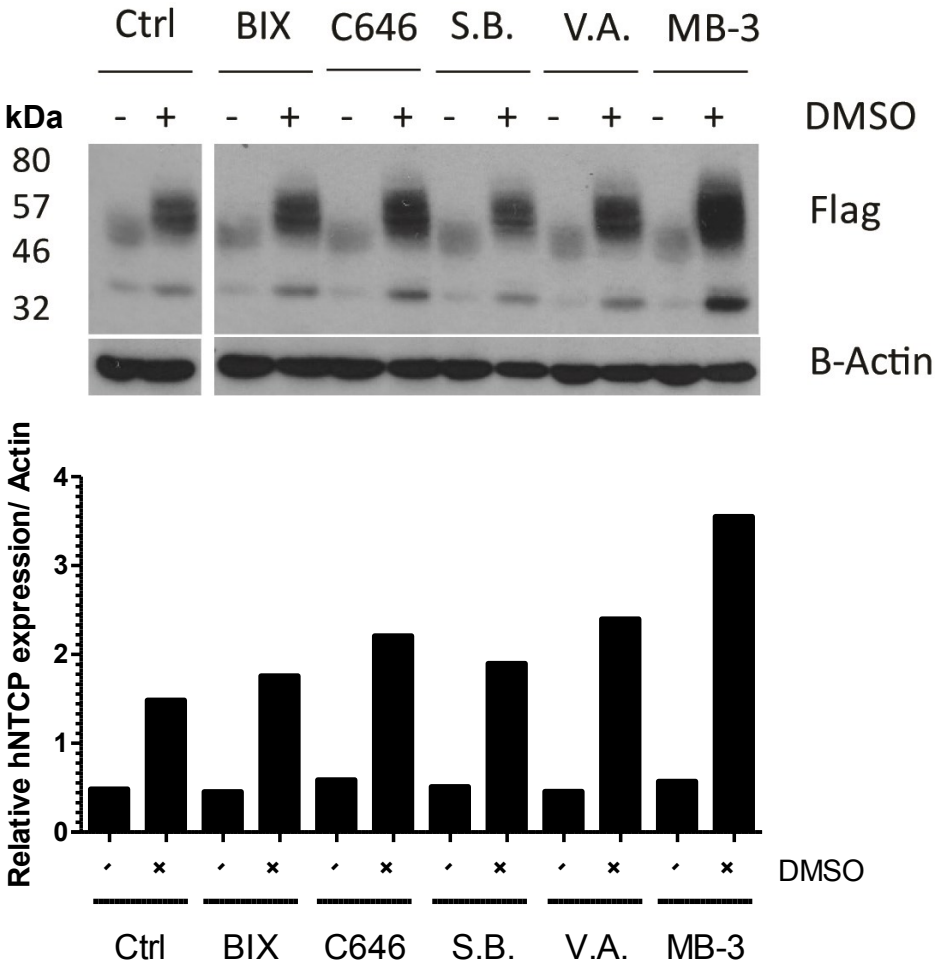


Figure 3.25; The effect of epigenetic modification drugs on hNTCP (FLAG-tag) protein expression.

Treating the cells with the various epigenetic modification drugs, in general, did not have a significant effect on hNTCP protein overexpression in the absence of DMSO, as observed on the western blot and confirmed by densitometry analysis. Interestingly however, the combination of DMSO and MB-3 did in fact have an obvious effect on enhancing hNTCP protein expression; thus, it is possible that these two reagents together are involved in some common mechanistic pathway indirectly or directly affecting the receptors expression. *The cells were treated for a total of 24 hours in the presence of the various histone modifying drugs, as well as in the absence or simultaneous presence of DMSO, prior to analysis. Ctrl (control), refers to the sample that was not treated with any of the histone inhibitors.* ***BIX (-01294)****: 2-(Hexahydro-4-methyl-1H-1,4-diazepin-1-yl)-6,7-dimethoxy-N-[1-(phenylmethyl)-4-piperidinyl]-4-quinazolinamine trihydrochloride hydrate;* ***C646****: 4-[4-[[5-(4,5-Dimethyl-2-nitrophenyl)-2-furanyl]methylene]-4,5-dihydro-3-methyl-5-oxo-1H-pyrazol-1-yl]benzoic acid;* ***S.B.****: Sodium Butyrate;* ***V.A.****: Valproic acid.* ***MB-3****: (2R,3S)-rel-4-Methyl-5-oxo-2-propyl tetrahydrofuran-3-carboxylic acid.*

In summary; Even though the presence of DMSO induced expression of different epigenetic markers within the cells tested, this did not appear to have a considerable effect on the protein expression of hNTCP in those cells in the absence of DMSO. Only when a combination of DMSO and MB-3 was used to

treat the cells, a somewhat greater effect on hNTCP protein expression was observed; an outcome that would be of interest to investigate further in the future.

3.1.3.1.2 Impact of drugs targeting the cell's cytoskeleton on human NTCP expression

Interestingly, one of our original observations when adding DMSO to the cell culture media was how the HepG2_hNTCP cells to some extent obtained an apparently different morphology compared to when DMSO was absent from the media (*refer to Figure 3.8*). And this became even more obvious when we started staining these cells for Actin (Phalloidin; *Figure 3.26*).

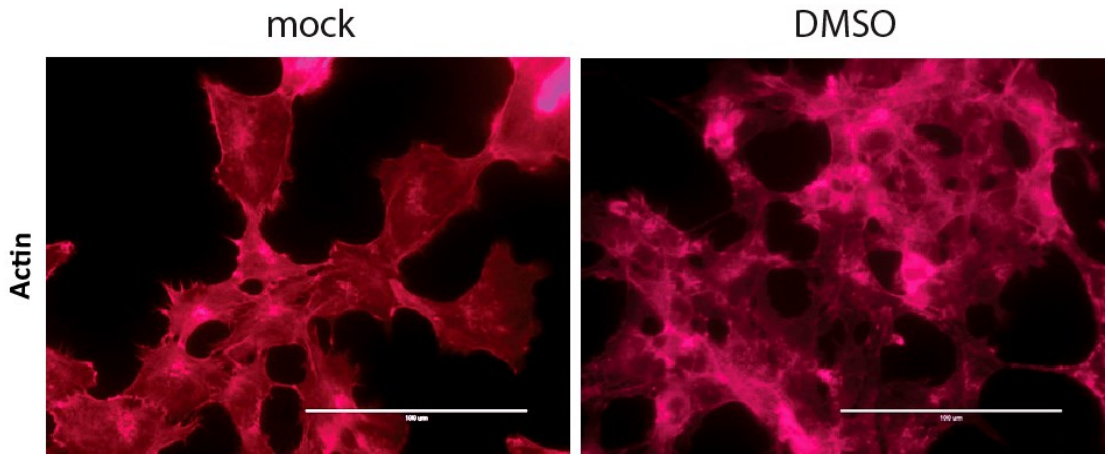


Figure 3.26; Actin (Phalloidin) stain of HepG2_hNTCP cells in the presence or absence of DMSO.
Addition of DMSO to the cultured cells seemed to interfere with and induce changes to the cell's cytoskeleton. The image scale bar is 100µm.

Wondering whether DMSO's interference with the cell's cytoskeleton was directly responsible for regulating the transport of the hNTCP receptor to the plasma membrane, we decided to interfere even further with the actin and tubulin polymerisation processes within the stable human hepatic HepG2_hNTCP cells and examine any possible outcomes on hNTCP protein expression. To this end, we utilised a series of drug inhibitors selective against

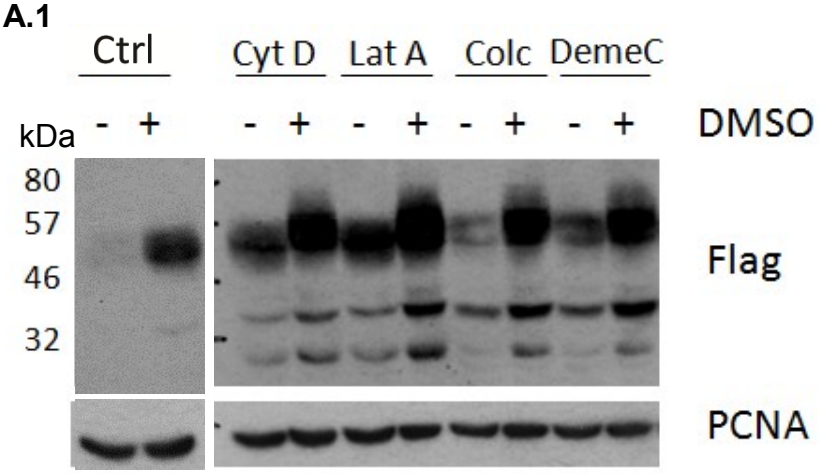
either actin or tubulin polymerisation (*Table 3.3*), some being actual polymerisation inhibitors while other were direct depolymerisers.

Polymerisation interfering drugs/ inhibitors			
Actin		**Tubulin**	
Cytochalasin D (polymerisation inhibitor)	**Latrunculin A** (depolymerisation drug)	**Colchicine** (polymerisation inhibitor)	**Demecolchine** (depolymerisation drug)

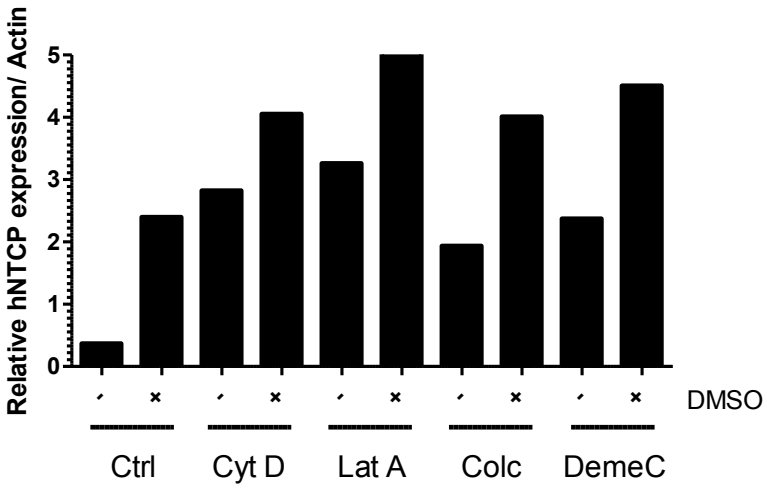
Table 3.3; Actin and Tubulin polymerisation inhibitors and active depolymerisers.

We treated the HepG2_hNTCP cells in the presence or absence of these drugs, as well as 2% DMSO, and analysed the samples at the protein level by western blot and confocal microscopy (*Figure 3.27; A* and *B,* respectively). We confirmed that disruption of the cell's cytoskeleton with the drugs targeting either actin or tubulin polymerisation resulted in the same, and on occasions even higher, levels of hNTCP protein expression compared to when DMSO alone was used in the cell culture medium. In addition, when the individual drugs were used in combination with DMSO they definitely produced a cumulative effect. Interestingly, disruption of the actin filaments appeared to result in a slightly more pronounced effect compared to when tubulin filaments were targeted, particularly in the absence of DMSO. However, the precise specificity of these inhibitors cannot be guaranteed and hence it is possible that changes caused to actin may also be affecting tubulin, and vice versa.

A.1

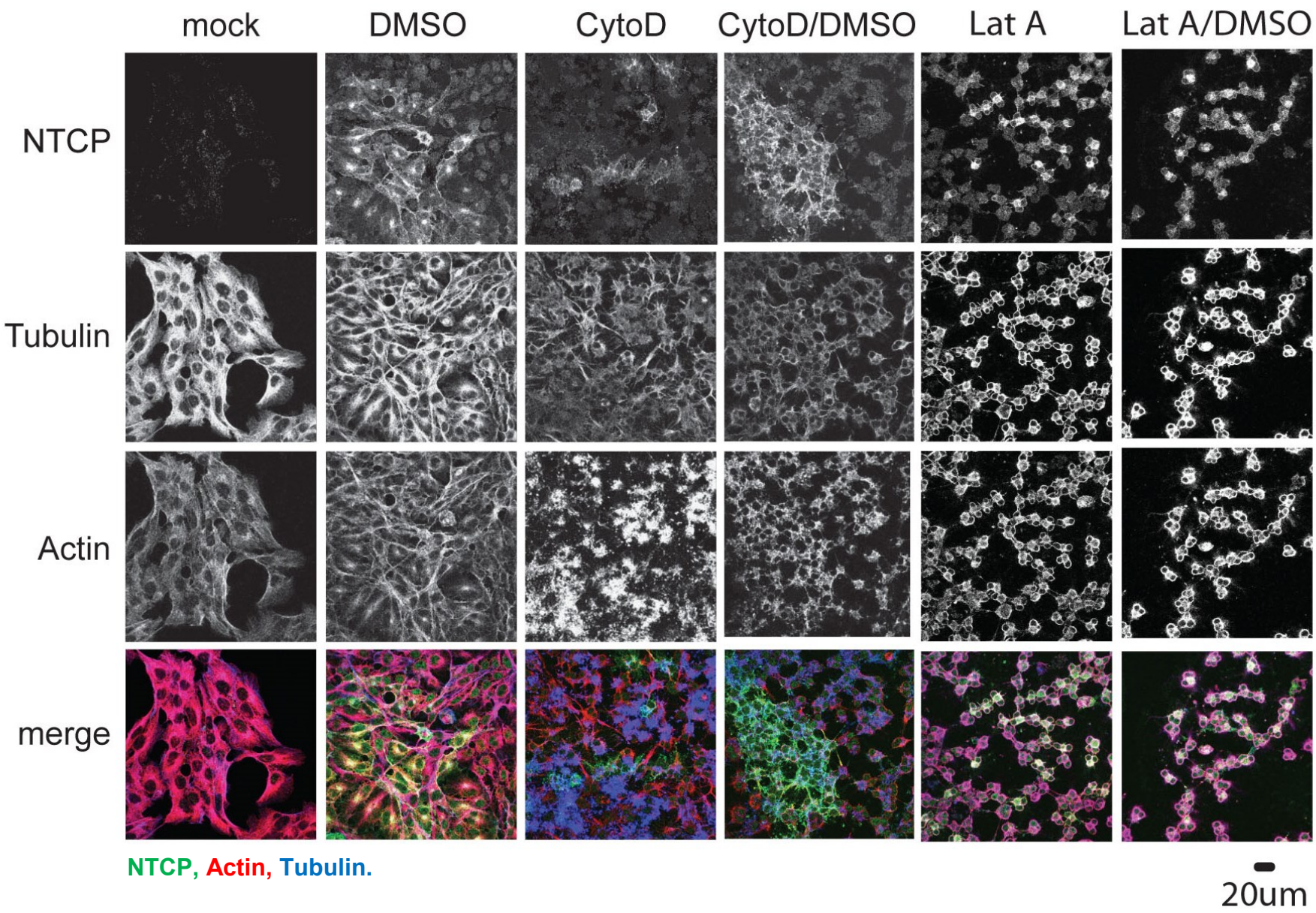


A.2



B

(*See next pages.*)



NTCP, Actin, Tubulin.

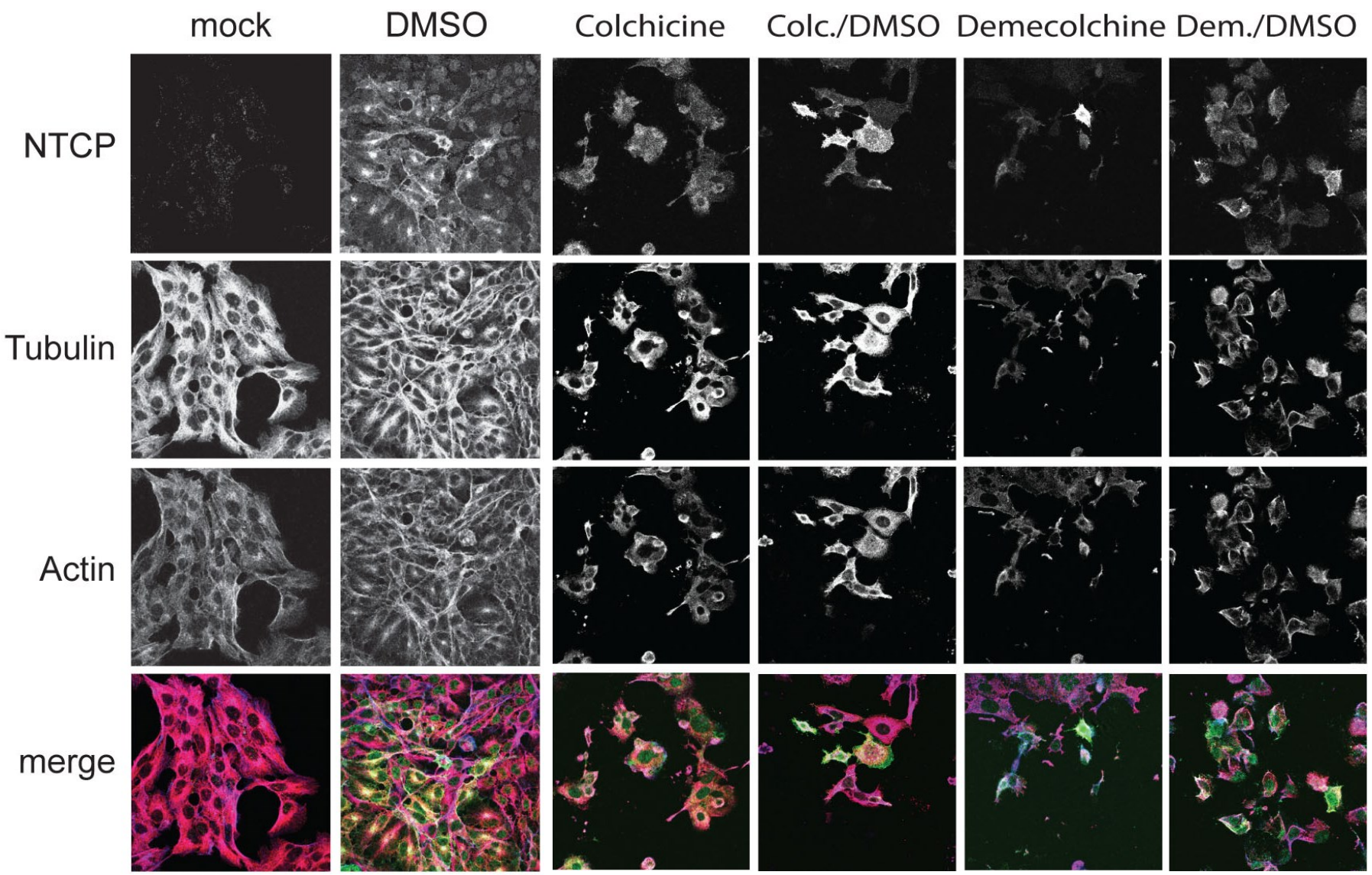

mock
DMSO
Colchicine
Colc./DMSO
Demecolchine
Dem./DMSO
NTCP
Tubulin
Actin
merge
NTCP, Actin, Tubulin.
20um

Figure 3.27; The effect of actin and tubulin polymerisation inhibitors or active depolymerisers on the expression of hNTCP (FLAG-tag) protein, and comparison with the effect of DMSO.

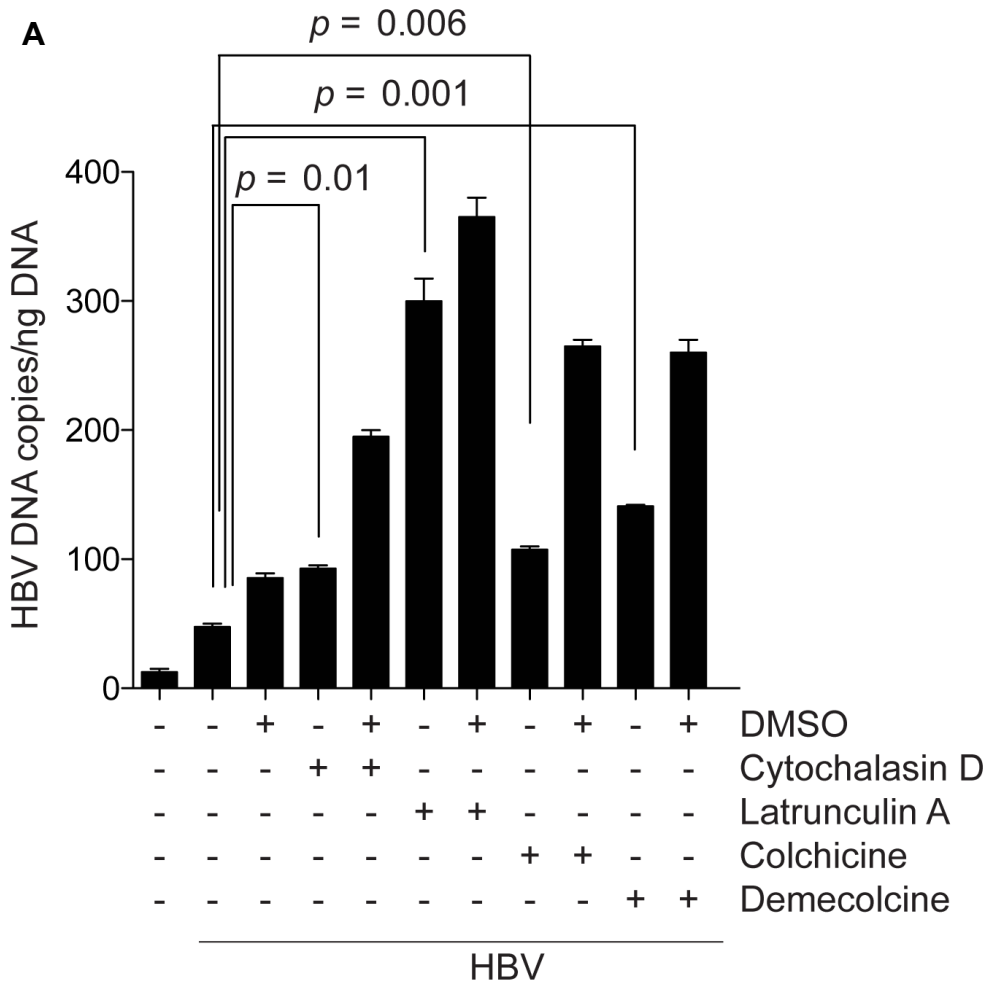
The cells were treated for a total of 24 hours in the presence of the various cytoskeleton modifying drugs, as well as in the absence or simultaneous presence of DMSO, prior to analysis. Analysing the addition of the various drugs inhibiting actin or tubulin polymerisation to cultured HepG2_hNTCP cells by both western blot (followed by densitometry analysis) **(A)** and immunofluorescence **(B)**, we verified that targeting and disrupting the cell's cytoskeleton resulted in enhanced hNTCP protein expression on the plasma membrane with the addition of DMSO augmenting this effect even further. PCNA was used in this experiment as a loading control for western blot analysis, instead of B-actin, so as to rule out any differences in the detection of the whole protein that could have arisen from the use of the drugs interfering with actin polymerisation. *Ctrl (control), refers to the sample that was not treated with any of the actin or tubulin inhibitors. Cyt D: Cytochalasin D, Lat A: Latrunculin; Colc: Colchicine; DemeC: Demecolchine.*

In summary; One of DMSO's mechanism of action in regards to hNTCP protein overexpression appears to be the direct induction of cytoskeletal changes within the cell that may in fact aid the translocation, and possibly the stabilisation, of the hNTCP receptor to the cell surface.

3.1.3.1.3 Disrupting the actin and tubulin cytoskeleton and its subsequent effect on susceptibility of cells to HBV infection

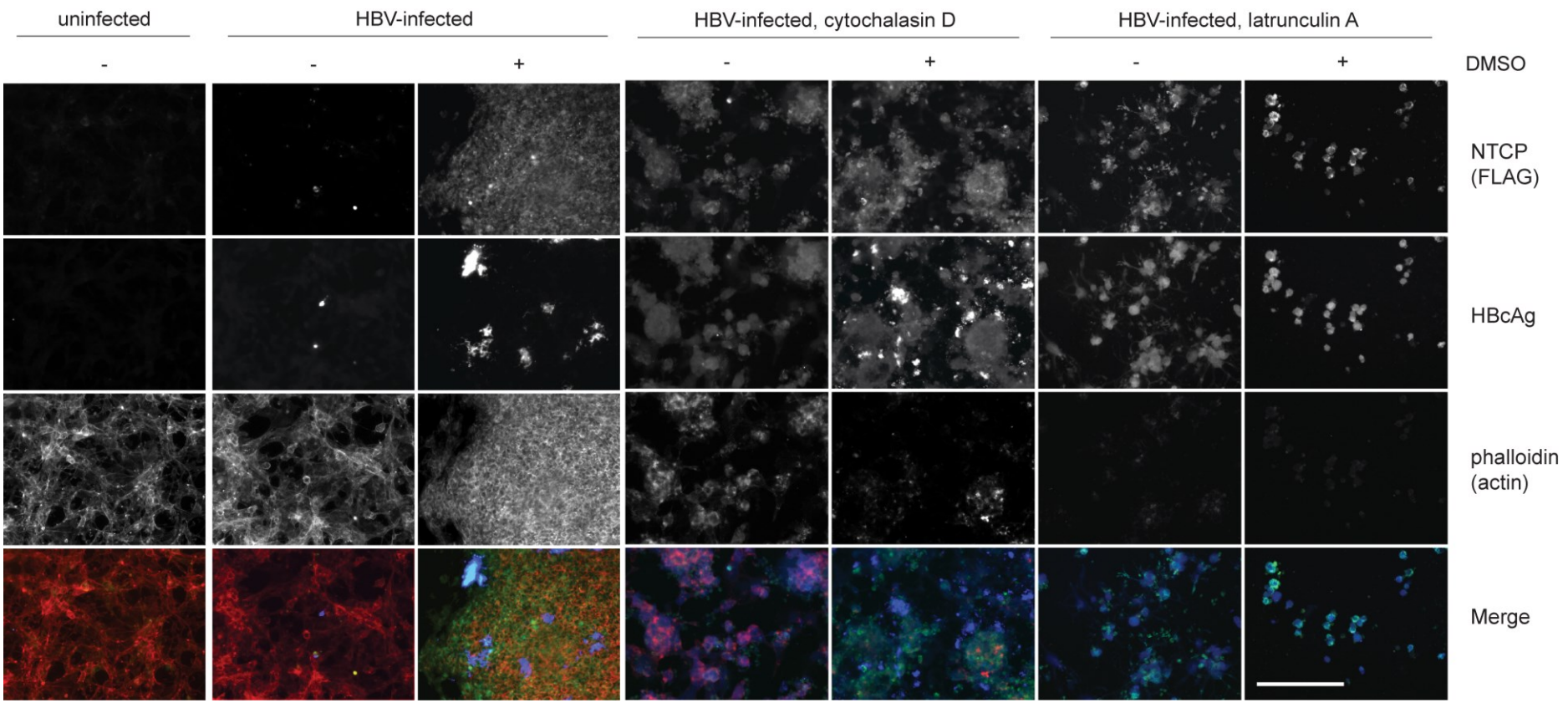
Following on, we investigated the effect of the actin and tubulin polymerisation inhibitors on susceptibility of the stable human hepatic cell line overexpressing hNTCP to HBV infection. For this purpose, the HepG2_hNTCP cells were treated in a similar manner as described previously for analysis of hNTCP overexpression, after which they were particularly infected with HepG2.2.15 infectious sups at a MOI of 2500. The samples were analysed at seven days post-infection for total HBV DNA copies/ng of DNA cell sample, while they were additionally stained for HBcAg to visualise possible infected cells by immunofluorescence (*Figure 3.28; A* and *B,* respectively).

A

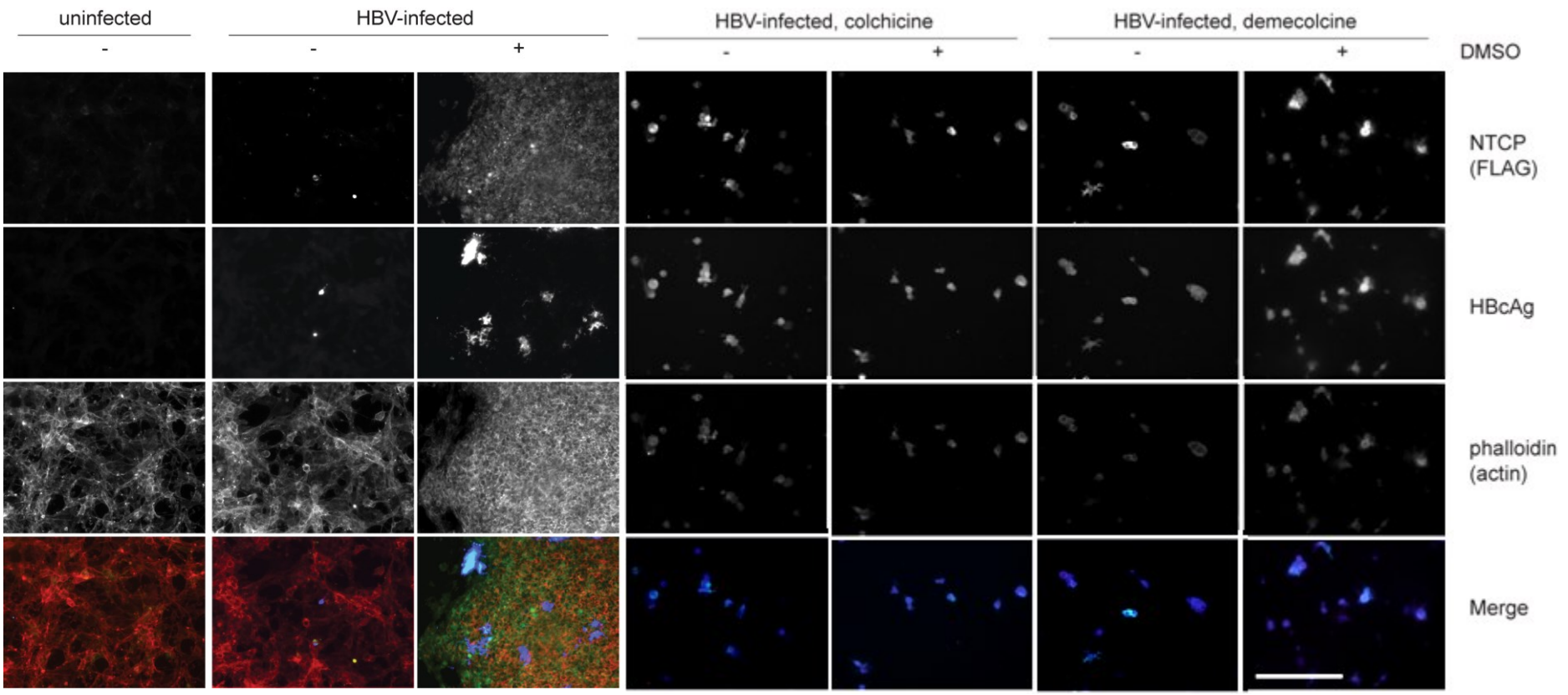


B

(See next pages.)



NTCP, HBcAg, Actin.



NTCP, HBcAg, Actin.

Figure 3.28; Analysing the susceptibility of HepG2_hNTCP cells to HBV infection in the presence of actin and tubulin polymerisation inhibitors, as well as DMSO.

The cells were treated as mentioned in Figure 3.27. A significant number of cells were strikingly infected when the cells had been cultured and infected in the presence of the actin and tubulin polymerisation inhibitors, some of them infecting a comparable number of cells to what was established only in the presence of DMSO, while others permitting the infection of a significantly higher number of cells. However, when DMSO was added simultaneously with the inhibitors during HBV infection there was a cumulative effect on the susceptibility of cells to the virus. These outcomes can be seen both by quantification of the HBV DNA copies/ng of DNA sample **(A)** and by the HBcAg staining of the infected cells visualised by immunofluorescence **(B)**. The image scale bar is 200μm.

In summary; Addition of the actin or tubulin polymerisation inhibitor drugs to cultured HepG2_hNTCP cells resulted either in an equivalent or a significantly higher number of cells becoming susceptible to HBV infection, compared to when DMSO alone was used to culture these cells before and during viral infection. However, the effect of these drugs is relatively dependent on the presence of DMSO, as simultaneous addition of DMSO to the cells during infection certainly resulted in an augmented effect and hence an even greater number of cells became susceptible to viral infection, suggesting that DMSO and these polymerisation drugs possibly function in a very similar manner. Moreover, and as we showed previously, addition of these inhibitor drugs to cultured HepG2_hNTCP cells also greatly induced hNTCP protein expression on the plasma membrane. Consequently, there is definitely a connection between the level of hNTCP protein expression on the cell surface and the number of HBV permissive cells.

3.1.3.2 DMSO results in accumulation of cAMP, which in turn may drive remodelling of actin filaments

NTCP is a known glycoprotein, but it is also a serine, threonine phosphoprotein, and its phosphorylation status is affected by the presence of cAMP within cells. In particular, cAMP has been shown to indirectly dephosphorylate rat NTCP and hence result in the retention of the receptor within the plasma membrane [174, 178].

To determine whether DMSO and cAMP were possibly involved in the same mechanism in regards to induction of human NTCP protein expression, we initially assessed whether addition of DMSO to the culture media resulted in an increase of the endogenous cAMP levels. We treated the samples as usual in the presence and absence of 2% DMSO and analysed the endogenous cAMP levels using a commercial direct immunoassay (*Figure 3.29*). Interestingly, we noticed that the presence of DMSO in the cells caused nearly a doubling of the endogenous cAMP levels. Thus, we hypothesised that DMSO and cAMP most likely are involved in some common pathway that results in hNTCP protein overexpression, with DMSO presumably working upstream of cAMP.

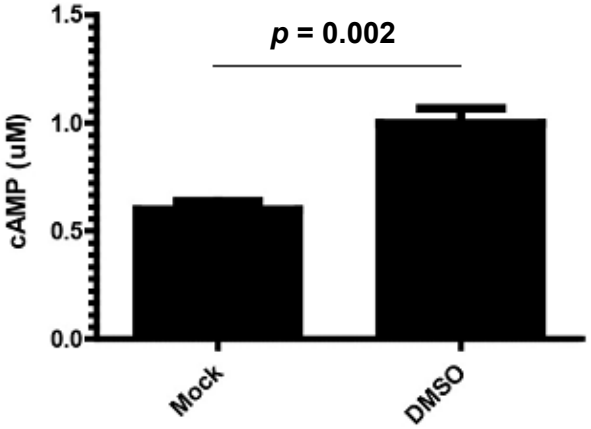


Figure 3.29; Analysis of the endogenous cAMP levels in HepG2_hNTCP cells treated with DMSO.

Addition of DMSO for 24 hours to the cell culturing media of HepG2_hNTCP cells resulted in an significant increase of the endogenous levels of cAMP, hence it is likely that DMSO and cAMP result in the induction of hNTCP protein expression by being involved in the same mechanistic pathway. *'Mock' refers to the sample that was not treated with DMSO.*

Interestingly, after discovering that disturbing the cell's cytoskeleton by targeting actin and tubulin polymerisation led to augmented hNTCP expression on the plasma membrane and that possibly this may be a pathway that DMSO participates in to enhance hNTCP protein expression, a literature review also revealed the involvement of cAMP in this mechanism. In particular, previous research has illustrated that cAMP indirectly induces

remodelling to the cell's cytoskeleton by specifically participating in the phosphorylation of actin and hence causing changes to its polymerisation status [303-305]. Therefore, cAMP, as well as indirectly dephosphorylating hNTCP and hence allowing its translocation to the cell surface, similarly to DMSO may be actively inducing changes to the cell's cytoskeleton by participating in the phosphorylation of actin, causing an indirect disruption of its filaments and hence providing easier access for hNTCP to be transported to the plasma membrane.

In summary; Addition of DMSO to the cell culture media resulted in an increase of the endogenous levels of cAMP released by the cells, which may partially explain the further induction of hNTCP protein overexpression. Therefore, DMSO and cAMP may possibly be involved in the same mechanism(s) in regards to inducing hNTCP expression, which may specifically involve cytoskeletal reorganisation and restructuring of the cell, further promoting the translocation of hNTCP to the cell surface.

3.1.3.3 The second messenger cAMP increases human NTCP protein levels on the plasma membrane and thus renders cells more susceptible to HBV infection

Following on, we wanted to assess the effect addition of exogenous cAMP would have on hNTCP protein expression in the human hepatic cell line, in the presence or absence of 2% DMSO. We analysed the samples by immunofluorescence (*Figure 3.30*) and were able to determine that even in the absence of DMSO the addition of cAMP allowed to some extent for induction of hNTCP protein expression on the cell surface. When both reagents were added together to the cell culture media they had an additive effect.

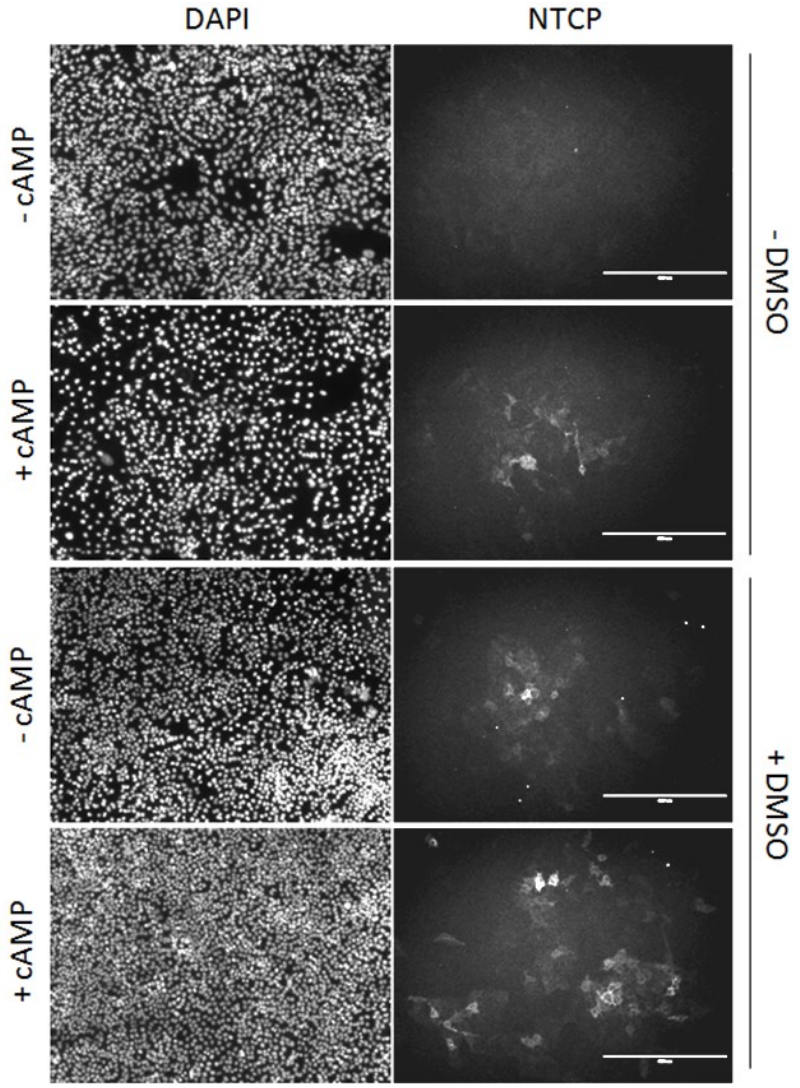


Figure 3.30; The effect of exogenous cAMP on the expression of hNTCP (FLAG-tag) protein.

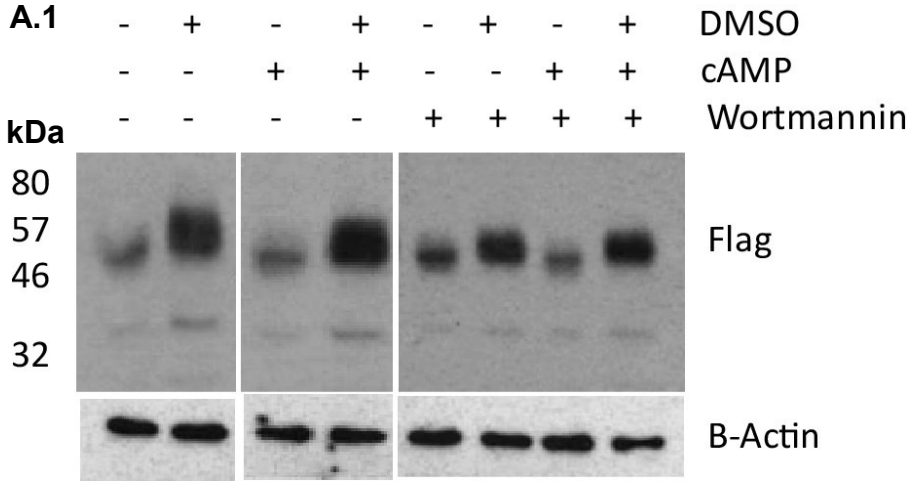
The cells were treated in the absence or presence of DMSO and/or cAMP for a total of 24 hours. Addition of exogenous cAMP when culturing HepG2_hNTCP cells resulted in approximately equivalent levels of hNTCP protein induction compared to when only DMSO was added to the cell culturing media. And when both reagents were added simultaneously they generated a cumulative effect, as determined by immunofluorescence. The image scale bar is 400μm.

To confirm that the effect we were observing on hNTCP protein expression when adding exogenous cAMP to the cell culture media was specific, we treated the cells with Wortmannin. This is a drug that inhibits the PI3K pathway and hence targets cAMP-downstream signalling events. If cAMP was involved in a signalling pathway that resulted in the overexpression of the hNTCP protein on the cell surface, then addition of Wortmannin to the

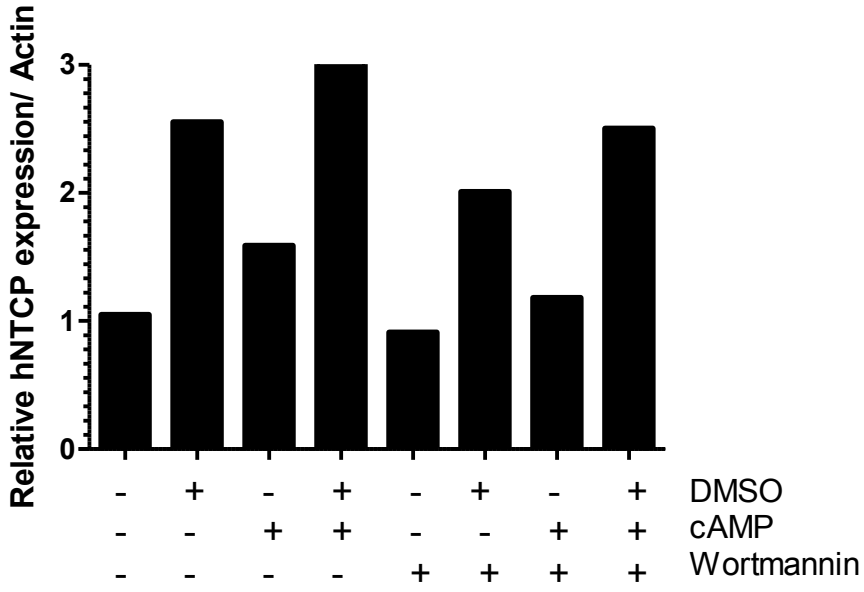
culturing media would block all downstream cAMP-signalling and hence prevent hNTCP protein overexpression. Moreover, if DMSO and cAMP were working together in a common pathway then theoretically addition of Wortmannin should inhibit hNTCP overexpression induced also by DMSO, in the absence of exogenous cAMP.

To this end, we treated the cells in the presence or absence of either cAMP or DMSO, accordingly, while in the meantime also treating them or not with Wortmannin. We analysed the cell samples by western blot and confocal microscopy (*Figure 3.31; A* and *B,* respectively), and as expected we found that addition of Wortmannin to cultured HepG2_hNTCP cells blocked the overexpression of hNTCP induced independently by both DMSO and cAMP. The way the inhibitor seemed to act was by generally suppressing hNTCP within the ER/Golgi complex and hence blocking the receptors efficient transport to the cell surface (*Figure 3.31, B*). As a result, the total hNTCP protein within the cells was not affected to a great extent, with addition of Wortmannin resulting in a slight decline in the total protein levels (*Figure 3.31, A*).

A.1

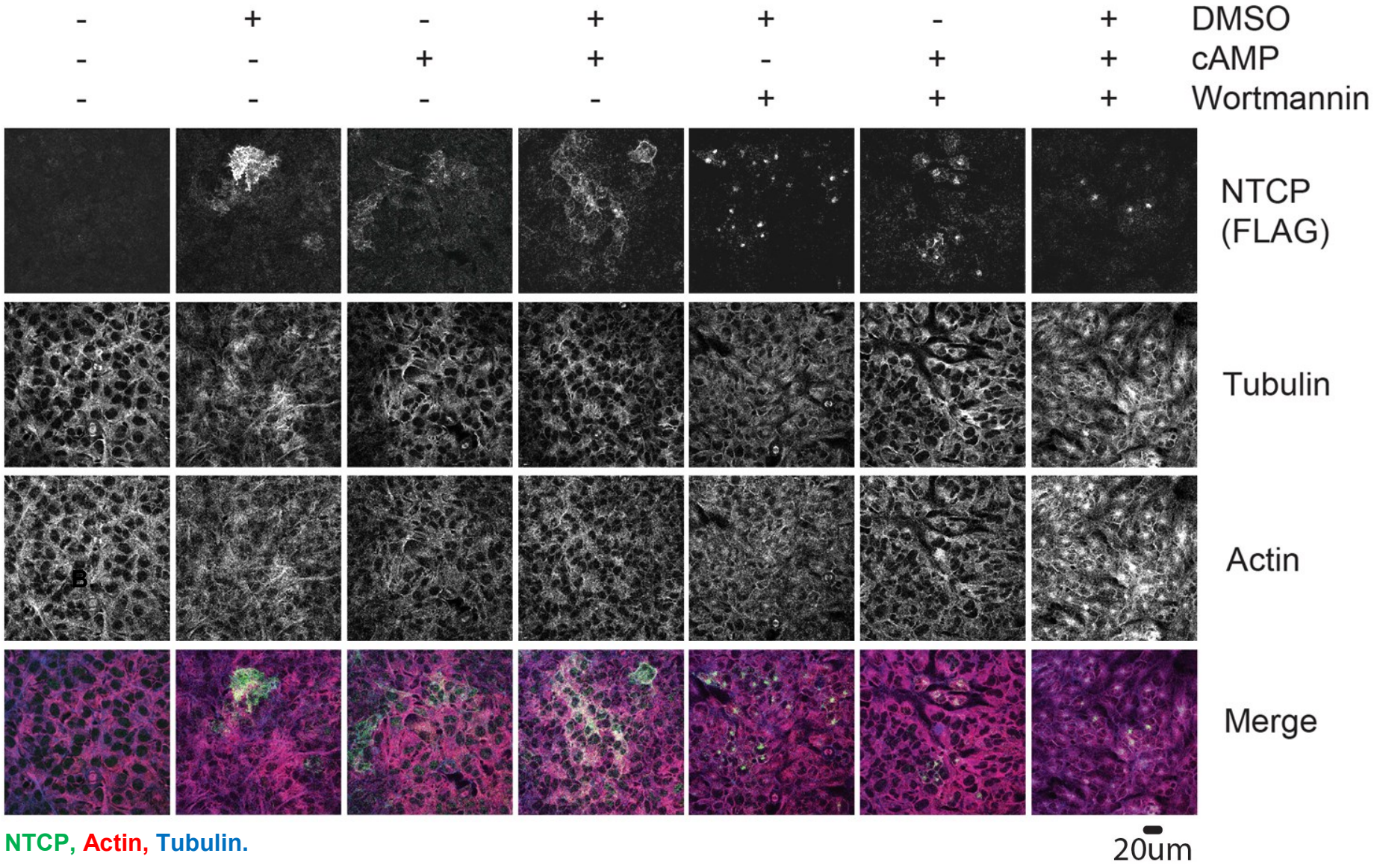


A.2



B

(*See next page.*)



NTCP, Actin, Tubulin.

Figure 3.31; The effect of Wortmannin on the protein expression of hNTCP (FLAG-tag), after induction by DMSO and/or exogenous cAMP.

The cells were treated in the absence or presence of DMSO and/or cAMP and/or Wortmannin for a total of 24 hours. **(A)** Analysing the addition of Wortmannin to cultured HepG2_hNTCP cells after treatment with DMSO and/or cAMP by western blot, followed by densitometry analysis, we detected lower levels of hNTCP expression, but there was still a considerable amount of total protein present. It should be noted that treating the cells with cAMP, due to the reagents function as a indirect hNTCP dephosphorylase, generally resulted in the production of a protein band that was not as smeary as typically seen when the hNTCP protein was fully glycosylated/phosphorylated. This observation was further confirmed with the use of a lambda phosphatase enzyme, which typically dephosphorylates serine and threonine residues, revealing a less smeary than typical hNTCP protein band on a western blot (*Appendix A.3.III*). **(B)** Analysing the samples by immunofluorescence, it was obvious that addition of Wortmannin inhibited the transport of the hNTCP receptor to the cell surface, regardless of the presence of DMSO and/or cAMP.

In a similar manner, we studied the effect of the exogenous cAMP and wortmannin on the susceptibility of stable human HepG2_hNTCP cells to HBV infection. We particularly infected these cells with infectious sups at a MOI of 2500 and analysed the samples at seven days post-infection for HBV DNA copies within the actual cells (*Figure 3.32*). Our conclusions were:

1) Addition of cAMP enhanced the susceptibility of cells even more so than what treatment with DMSO alone did (nearly a third of extra cells became susceptible to viral infection), while simultaneous addition of both cAMP and DMSO had a cumulative effect and resulted in a even greater number of cells getting infected with HBV.

2) Addition of Wortmannin resulted in an obvious reduction in the number of cells infected by the virus.

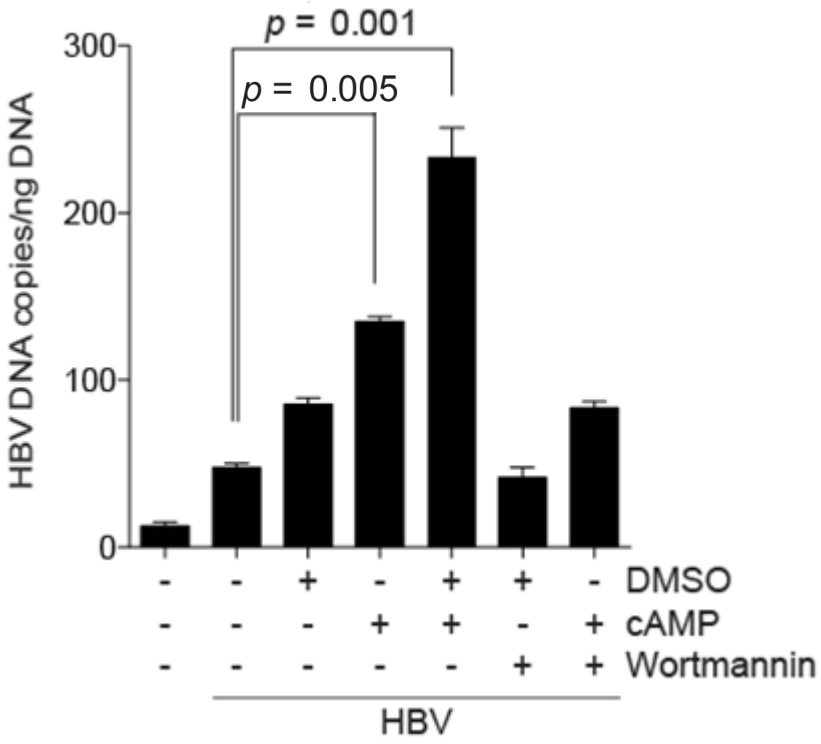
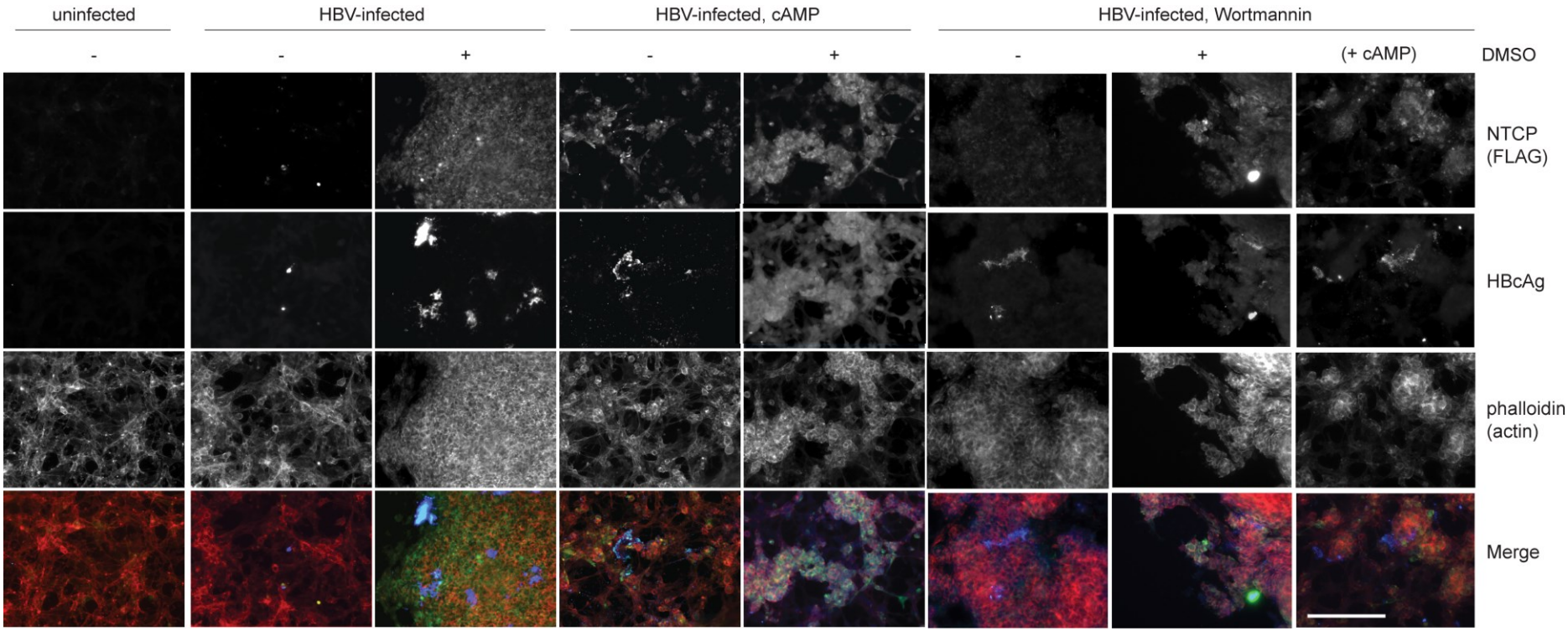


Figure 3.32; The effect of exogenous cAMP and wortmannin, as well as DMSO, on the susceptibility of HepG2_hNTCP cells to HBV infection.

The cells were treated as mentioned in Figure 3.31. The presence of exogenous cAMP enhanced susceptibility of cells to HBV infection, more so than what treatment with DMSO alone did. However, the two reagents together had a cumulative effect. On the contrary, the presence of wortmannin resulted in a decreased number of cells becoming susceptible to infection.

These outcomes were also confirmed by performing HBcAg staining of the infected cells and analysing them by immunofluorescence (*Figure 3.33*).



NTCP, HBcAg, Actin.

Figure 3.33; Analysing the susceptibility of HepG2_hNTCP cells to HBV infection, in the presence of exogenous cAMP, Wortmannin, and DMSO, by staining for HBcAg and visualising the cells by immunofluorescence.

The cells were treated as mentioned in Figure 3.31. A significant number of cells were markedly infected when the cells had been cultured and infected in the presence of both exogenous cAMP and DMSO, while a somewhat fewer number of cells became infected in the presence of either DMSO or cAMP alone. In the presence of wortmannin no cells became infected with HBV, while the additional presence of DMSO or cAMP seemed to slightly rescue the phenotype. The scale bar is 200μm.

In summary; Addition of cAMP to the cell culture media increased hNTCP cell surface expression but not to the same extent as DMSO. This suggests that DMSO and cAMP function in a common mechanistic pathway that induces hNTCP expression, and most likely DMSO works upstream of cAMP. However, the positive effect of both DMSO and cAMP on hNTCP protein overexpression can be blocked by Wortmannin, with the drug efficiently suppressing transport of the hNTCP receptor to the cell surface. As a result, DMSO and cAMP must be specifically involved in a pathway that induces translocation of the receptor to the plasma membrane, and specifically in the case of cAMP, while also according to the literature, this must involve the dephosphorylation of the hNTCP protein. The effect of these reagents/drugs was also obvious on the susceptibility of cells to HBV infection, which was comparable to the effect the reagents had on hNTCP expression on the plasma membrane; meaning that DMSO and cAMP increased susceptibility of cells to HBV infection, while wortmannin blocked this process. However, the additional presence of DMSO and cAMP slightly rescued this phenotype.

3.1.4 Intracellular cAMP levels regulate the expression of human NTCP on the plasma membrane, as well as the resulting susceptibility of cells to HBV infection

3.1.4.1 Modulating intracellular cAMP levels by targeting PDEs or adenylyl cyclase greatly affects human NTCP expression on the plasma membrane and subsequent HBV susceptibility of cells

Having established that one way by which DMSO induces hNTCP protein expression on the cell surface is by interfering with the cell's cytoskeleton, while also having determined that DMSO and cAMP may function in a common mechanistic pathway that results in the same outcome (i.e. hNTCP overexpression on the plasma membrane), we were now particularly interested in understanding how DMSO and cAMP were possibly connected within this common regulatory pathway.

Another hypothesis we had in regards to DMSO's mechanism of action was that, DMSO may be indirectly acting on cAMP by specifically targeting a PDE and hence enhancing hNTCP receptor translocation to the cell surface. PDEs are a diverse group of enzymes that degrade phosphodiester bonds within cAMP molecules. Consequently, by DMSO directly targeting one or more of these enzymes, it may be indirectly targeting the breakdown of cAMP by a particular PDE. By doing so, DMSO participates in maintaining the cAMP levels within the cells, and hence the subsequent translocation of the hNTCP receptor to the plasma membrane occurs uninterrupted.

To test our hypothesis we initially used two drugs; MDL12, an adenylyl cyclase inhibitor blocking the synthesis of *de novo* cAMP in the cells, and Caffeine, a pan-PDE inhibitor. The purpose for using the former drug was to confirm that by completely blocking *de novo* synthesis of endogenous cAMP, even in the presence of DMSO, would result in no hNTCP protein expression, hence also confirming our previous hypothesis that DMSO was partially responsible for indirectly increasing the levels of cAMP in the cell, further resulting in higher hNTCP protein expression. Moreover, this phenotype theoretically should be overcome to some degree when exogenous cAMP gets added to the cultured cells. So if our current hypotheses stand true, we would expect that MDL12 would function by inhibiting the cAMP-specific hNTCP receptor translocation to the cell surface, while on the contrary Caffeine in addition with DMSO should enhance the transport of the hNTCP receptor to the cell surface by targeting PDEs. Caffeine and DMSO, if our theory is correct, should function in a similar manner and result in a comparable outcome.

We treated the HepG2_hNTCP cells with all different combinations of the drugs, and analysed the samples by western blot and confocal microscopy (*Figure 3.34; A* and *B,* respectively). As previously, the results were easier interpreted from the immunofluorescent data (*Figure 3.34, B*), as the drugs could possibly be having other various effects on the hNTCP protein status, hence affecting the band patterns on the western blot (*Figure 3.34; A*).

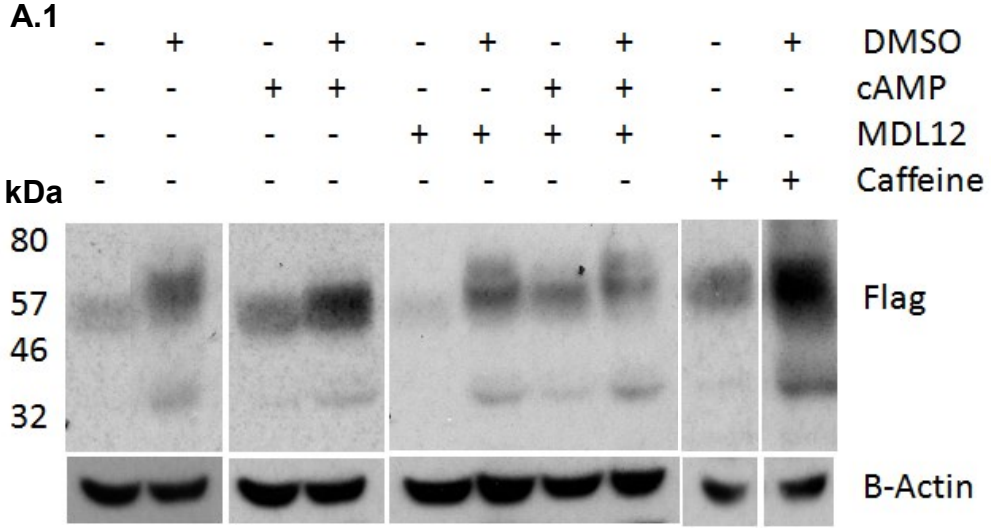
The important outcomes from this experimental procedure were as follow:

1) Addition of MDL12, similarly to Wortmannin, but more profoundly suppressed hNTCP protein overexpression even when DMSO was additionally added to the cell culture. Some protein was detected in the ER/Golgi complex but in only very few of the cells analysed. MDL12 and Wortmannin, even though they both act indirectly on cAMP, are two very different inhibitors and hence will function at different stages to block hNTCP expression, but the final outcome should be relatively the same.

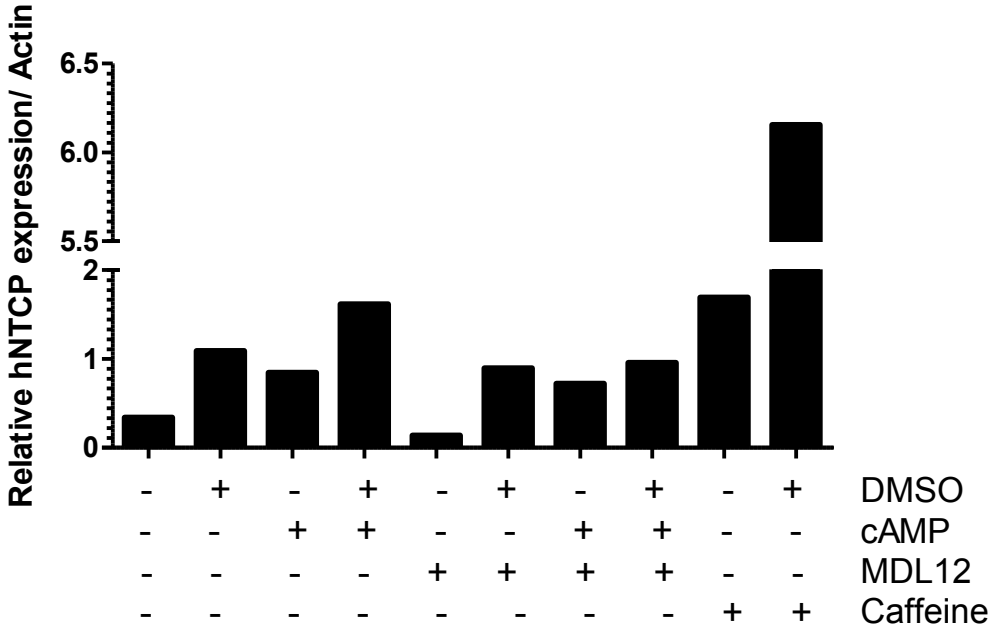
2) Addition of MDL12 with simultaneous addition of exogenous cAMP, as expected, rescued the phenotype to some extent, with further addition of DMSO playing an important role for hNTCP protein expression on the cell surface.

3) Addition of Caffeine to the cell culture media, similarly to addition of exogenous cAMP, increased hNTCP cell surface expression but not to the same extent as DMSO did when added to the cell culture media alone. However, when Caffeine and DMSO were added simultaneously they had a very pronounced cumulative effect. This could be due to the fact that DMSO is targeting a specific PDE responsible for degrading cAMP and hence suppressing hNTCP translocation to the plasma membrane, while Caffeine has a broader action not necessarily targeting the PDE in question to the same extent as DMSO may be targeting it.

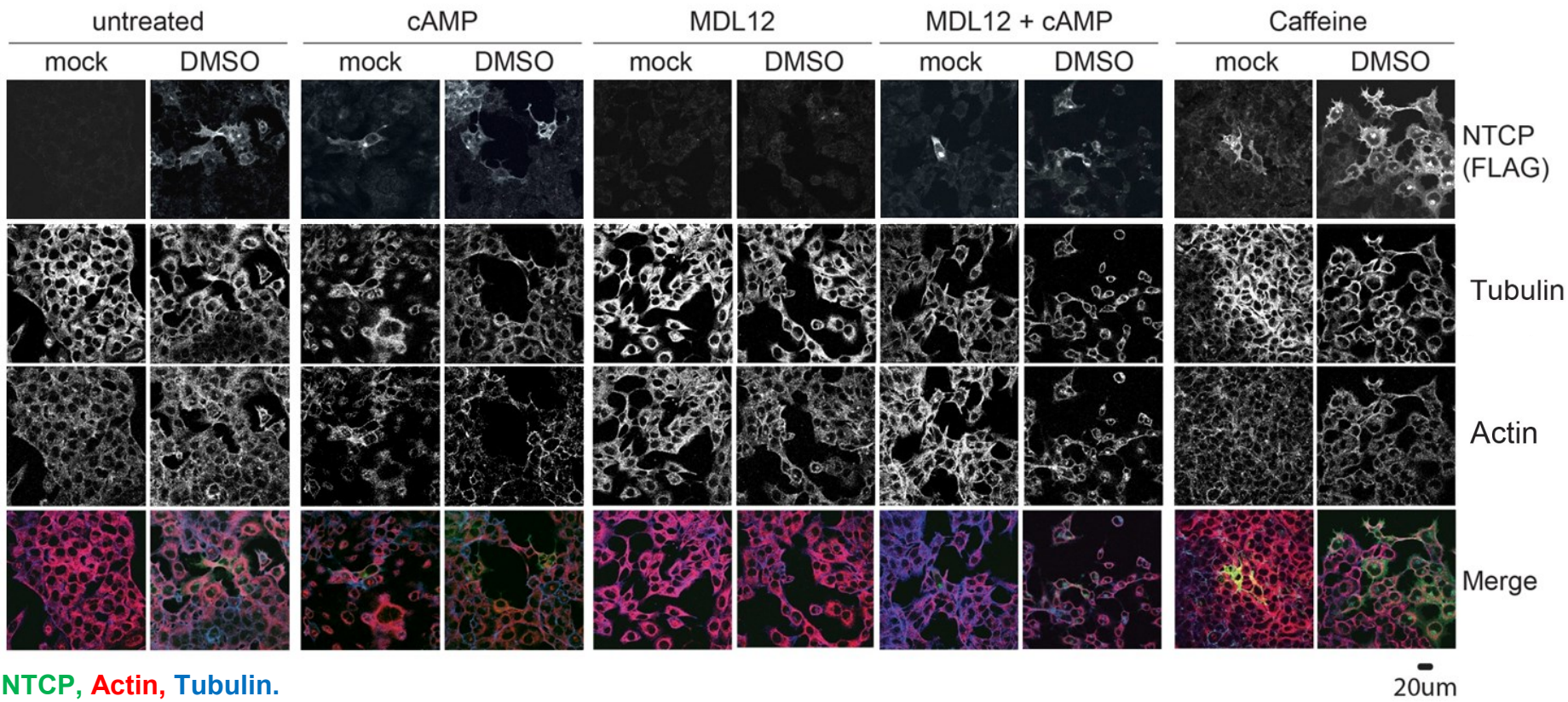
A.1



A.2



B (*See next page.*)



NTCP, Actin, Tubulin.

Figure 3.34; The effect of MDL12 or Caffeine on the protein expression of hNTCP (FLAG-tag), after induction by DMSO and/or exogenous cAMP.

The cells were treated in the absence or presence of DMSO, cAMP, MDL12 and/or Caffeine for a total of 24 hours. **(A)** The analysis of the results from the western blot was a bit ambiguous (with the densitometry analysis being somewhat more informative), **(B)** nevertheless from the immunofluorescence images it can be clearly deduced that addition of MDL12 to cultured HepG2_hNTCP cells even after treatment with DMSO suppressed expression of the hNTCP protein. However, when additional exogenous cAMP was added to the cell culture the phenotype was rescued to some extent and further addition of DMSO augmented this effect. In the case when Caffeine was added to the cultured HepG2_hNTCP cells, cell surface hNTCP protein expression increased but not to the same extent as when DMSO alone was added to the media. Together, however, both reagents caused a significant increase in hNTCP protein expression on the plasma membrane. Comparable effects of these drugs were observed also on the levels of cAMP within the cells (*Appendix A.3.IV*).

In a similar manner, we studied the effect of MDL12 and Caffeine on the susceptibility of stable human hepatic HepG2_hNTCP cells to HBV infection. We particularly infected these cells with infectious sups at a MOI of 2500 and analysed the samples at seven days post-infection for HBV DNA copies within the actual cells (*Figure 3.35*). Our conclusions were:

1) Addition of MDL12 resulted in an obvious reduction in the number of cells infected by the virus, compared to the number of cells that were infected when treated with DMSO alone, while the extra addition of DMSO did not enhance the susceptibility of cells to viral infection in the presence of MDL12.

2) Addition of Caffeine, in the absence of DMSO, caused the infection of approximately an equivalent number of cells as treatment with DMSO alone did. However, caffeine appeared to be very slightly more effective compared to DMSO. When the two reagents were added together to the culture medium, they produced a cumulative effect that resulted once again in a considerable number of cells becoming infected with the virus.

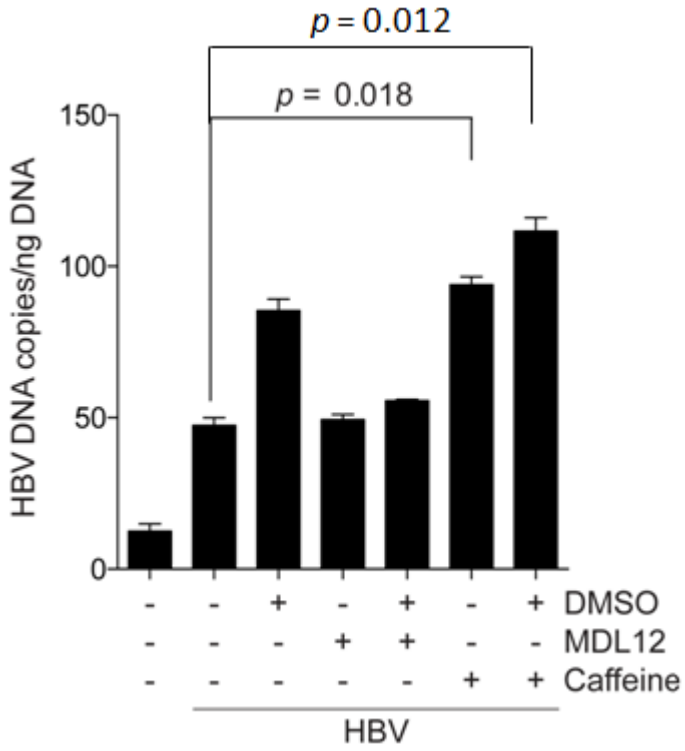
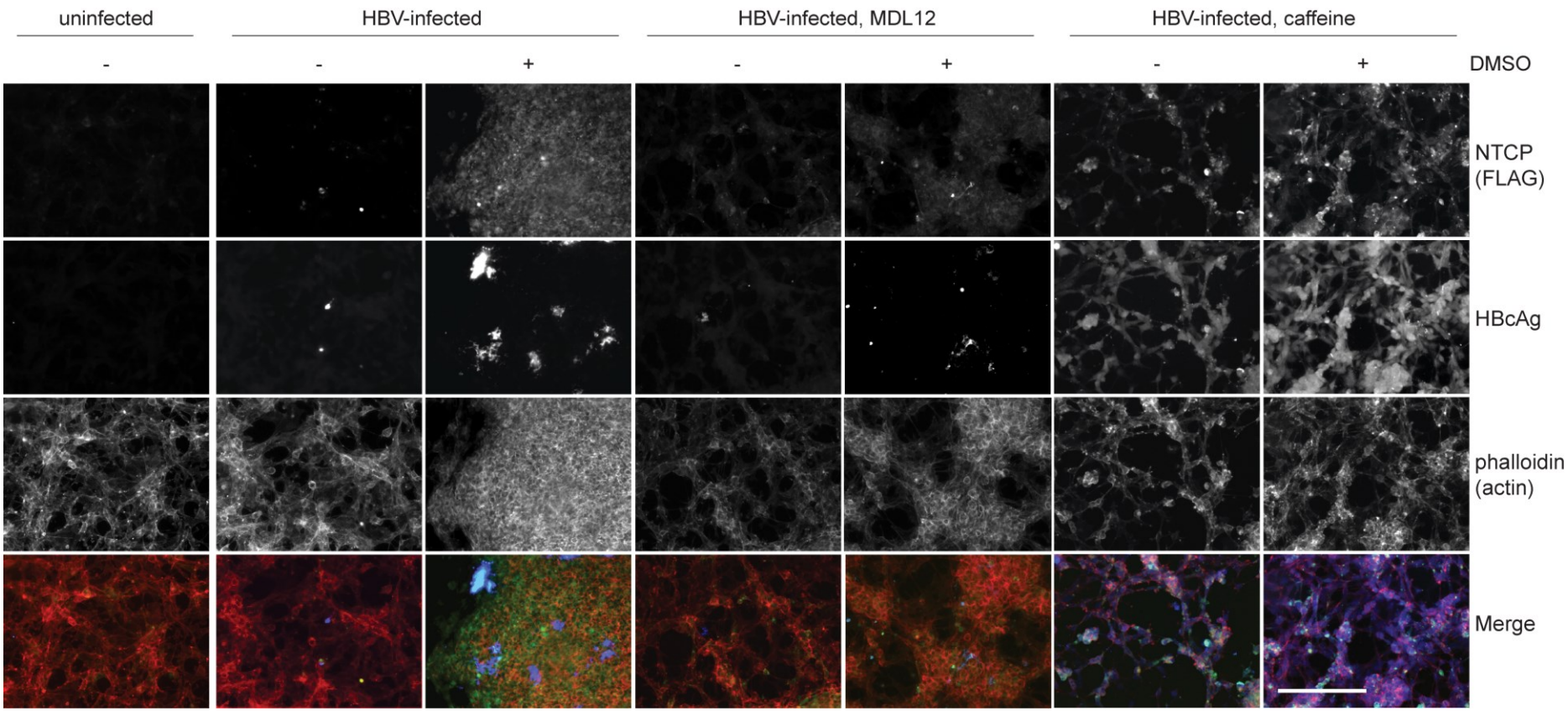


Figure 3.35; The effect of MDL12 and caffeine, as well as DMSO, on the susceptibility of HepG2_hNTCP cells to HBV infection.

The cells were treated similarly as in Figure 3.34. The presence of MDL12 resulted in a decreased number of cells becoming susceptible to infection, compared to the number of cells that become susceptible post-DMSO treatment. On the contrary, the presence of caffeine enhanced susceptibility of cells to HBV infection, comparably to what treatment with DMSO alone did, while the two reagents together had a somewhat cumulative effect.

These outcomes were also confirmed by performing HBcAg staining on the infected cells and analysing them by immunofluorescence (*Figure 3.36*).



NTCP, HBcAg, Actin.

Figure 3.36; Analysing the susceptibility of HepG2_hNTCP cells to HBV infection, in the presence of MDL12, Caffeine, and DMSO, by staining for HBcAg and visualising the cells by immunofluorescence.

The cells were treated similarly as in Figure 3.34. A significant number of cells were markedly infected when the cells had been cultured and infected in the presence of both caffeine and DMSO, individually or even simultaneously. In the presence of MDL12 a considerable lower number of cells became infected with HBV. The scale bar is 200µm.

In summary; Another of our hypotheses as to how DMSO exerts its function on hNTCP expression is that it indirectly acts on cAMP by specifically targeting its breakdown by a particular PDE. So DMSO directly targets and blocks one or more PDE(s) within the cell. Therefore, the levels of cAMP within the cell remain stable further allowing for indirect hNTCP protein dephosphorylation and subsequent trafficking to the plasma membrane (always presuming that cAMP acts in a similar manner in regards to enhancing hNTCP expression on the cell surface as it does for rNTCP expression).

Towards verifying our hypothesis we treated the cells with MDL12 that inhibits adenylyl cyclase from catalysing the synthesis of cAMP from AMP. This resulted in no *de novo* cAMP being produced, while any endogenous cAMP that was already present in the cells would in this instance get fully degraded by the PDEs present within the cell. And even though with the further addition of DMSO, which is thought to be indirectly acting on cAMP, any PDE(s) would in theory be inhibited this still wouldn't help increase the cAMP levels necessary for subsequent hNTCP dephosphorylation and subsequent trafficking to the plasma membrane. As a result, no hNTCP protein expression should have been or was detected in these samples. To continue, we treated the cells with Caffeine that inhibits unspecifically all PDEs within the cell, so should behave similarly to DMSO if our hypothesis is correct. By blocking the action of PDEs this would allow for constant synthesis of cAMP, hence hNTCP would be dephosphorylated, through the particular cAMP-mediated processes/pathways, and subsequently trafficked to the plasma membrane. Even though Caffeine is a broad range inhibitor, while DMSO is believed to be a more specific PDE inhibitor, it did enhance hNTCP protein expression on the cell surface but not too the same extent as DMSO. Finally, the effect of these drugs was also obvious on the susceptibility of cells to HBV infection, and this effect was once again comparable to the effect the drugs had on hNTCP expression on the plasma membrane; meaning that DMSO and caffeine increased susceptibility of cells to HBV infection, while MDL12 inhibited this process.

3.1.4.2 PDE 4 and 12 are important players in suppressing baseline human NTCP expression and subsequent susceptibility of cells to HBV

To determine which PDE(s) could be possibly inhibited by DMSO we chose to study ones that were determined to be highly expressed either in liver tissue or in HepG2 cells. We verified these by performing an analytical search of all accounted PDEs on the Human Protein Atlas website (http://www.proteinatlas.org/), and specifically evaluated their expression in liver tissue as well as in HepG2 parental cells. We concluded that the PDEs of interest were PDE1A, PDE1C, PDE4D and PDE12. To this end, we purchased the particular siRNAs, so as to silence the expression of these PDEs within the HepG2_hNTCP cells and establish whether by doing so we could already detect a recovery or an increase in the expression of hNTCP protein in the absence of DMSO, hence demonstrating our hypothesis that another of DMSO's mechanisms of action, in regards to enhancing hNTCP protein expression, is by interfering with and blocking the function of a certain PDE within these cells, further preventing cAMP from getting degraded and thus resulting in hNTCP protein dephosphorylation and subsequent trafficking to the plasma membrane.

We transfected the different PDE siRNAs into the cells. We incubated these cells for 72 hours to ensure complete silencing, at which point 2% DMSO was added for a further 24 hours. We initially analysed the mRNA levels of the PDEs of interest within the HepG2_hNTCP cells to confirm the degree of silencing we had achieved (*Figure 3.37*).

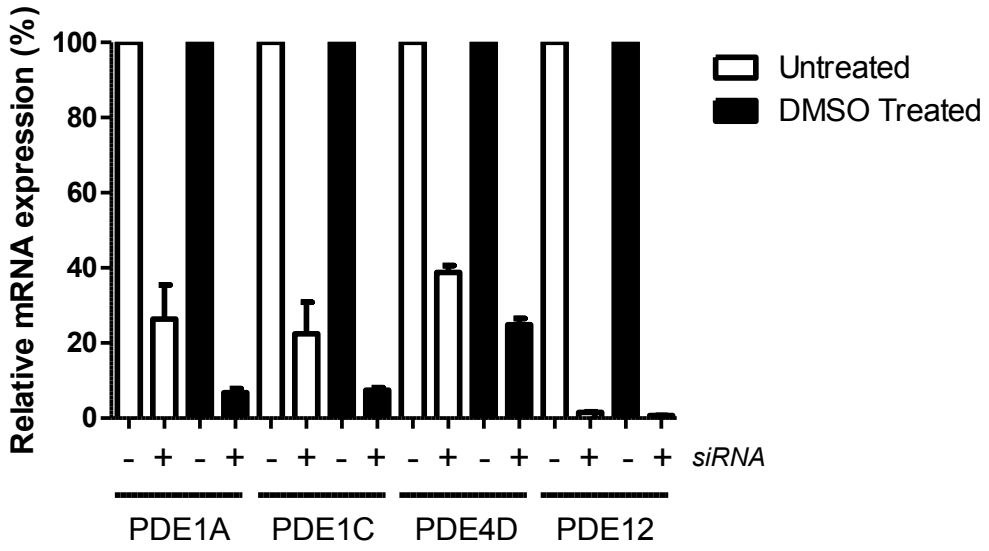


Figure 3.37; Degree of silencing of the different PDEs via siRNAs within the stable hepatic cells overexpressing hNTCP.

The cells were transfected with the various siRNAs for a total of 72 hours, at which point DMSO was added for a further 24 hours. Comparison between the HepG2_hNTCP cells where the different PDEs were silenced (+ siRNA) and the "non-silenced" parental HepG2_hNTCP cells (- siRNA), while additionally comparing the cells cultured in the presence or absence of DMSO. The presence of DMSO appeared to enhance the silencing of the various PDEs tested, which would be anticipated if our hypothesis was correct. *A scramble siRNA control was not included in this experiment.*

The levels of silencing we attained varied from 90-100% in the best cases to 60-70% particularly for PDE4D, while the presence of DMSO improved the silencing efficiency. We next went on to evaluate the protein levels of hNTCP expression in the stable hepatic HepG2 cells where the particular PDEs had been silenced (*Figure 3.38; A*), and we determined that interfering explicitly with PDE4D and PDE12 seemed to have a slightly positive effect on hNTCP protein expression in the absence of DMSO, while addition of DMSO seemed to augment this effect even further. No obvious effect could be observed for PDE1A and C. Therefore, between PDE4D and PDE12, and especially as PDE12 is not that well characterised up to date and has no established functional inhibitor, we decided to continue analysing only PDE4D and its effect on interfering with hNTCP expression. Before moving on to any further inhibition experiments, however, we validated the degree of PDE4D silencing additionally at the protein level (*Figure 3.38; B*).

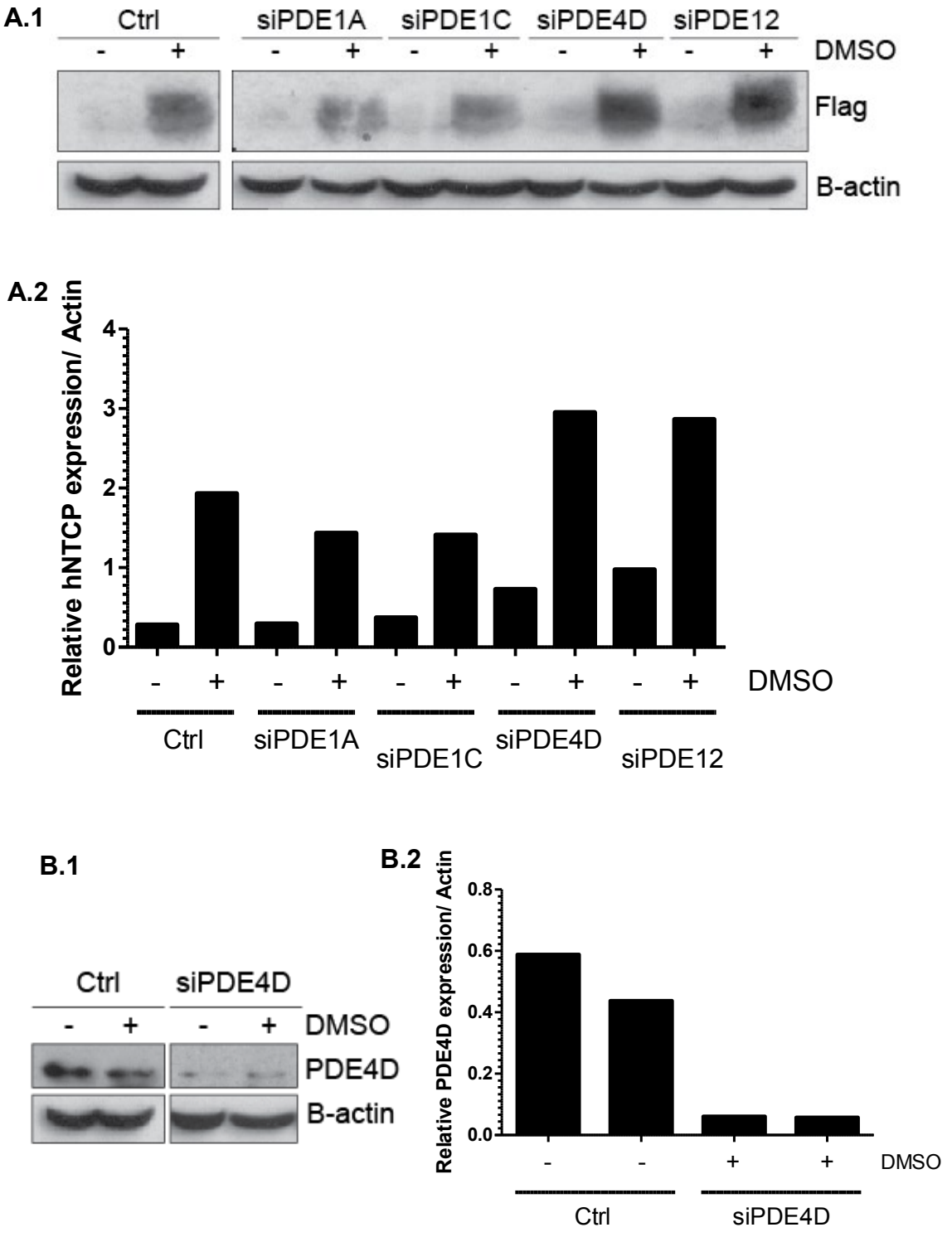


Figure 3.38; Effect of silencing of the various PDE(s) on hNTCP (FLAG-tag) protein expression in the stable human hepatic cell line.

The cells were treated as described in Figure 3.3.7. **(A)** Higher protein expression levels of hNTCP were detected in the stable HepG2 cells, specifically, when PDE4D and PDE12 had been silenced. **(B)** Determination of the precise degree of PDE4D protein silencing within the same cell samples revealed considerable silencing of the enzyme, as also demonstrated by densitometry analysis. *Ctrl, refers to the siRNA untransfected cells.*

In summary; Targeting PDE4D and PDE12 specifically enhanced hNTCP protein expression. Consequently, DMSO's mechanism of action could possibly be to specifically target PDE4D and PDE12, and hence partially exert its effect on the induction of hNTCP protein expression.

3.1.4.3 Targeting PDE4 greatly enhances human NTCP expression and subsequent susceptibility of cells to HBV infection

To verify the data obtained for PDE4D by siRNA technology (i.e. silencing of the specific enzyme resulted in vaguely increased levels of hNTCP expression), we decided to make use of a specific PDE4D inhibitor in the hope that it would allow us to reach a more confirmatory outcome. We therefore used a selective and long-lasting inhibitor drug called Roflumilast that has a very high affinity towards targeting PDE4 within cells. We tested varying concentrations of the drug for their effect on hNTCP protein expression in the stable human hepatic cells, while further incubating the cells in the presence or absence of 2% DMSO. We analysed hNTCP protein expression under the diverse conditions by immunofluorescence (*Figure 3.39*). Interestingly, we were able to clearly verify that inhibition of PDE4 by Roflumilast in the HepG2_hNTCP cells resulted in enhanced expression of the hNTCP protein on the cell surface, and this effect was drug dose dependent (i.e. higher drug doses resulted in higher hNTCP expression on the cell surface) and interestingly was independent of the presence or absence of DMSO. This outcome validated that Roflumilast and DMSO perform the same action and to the same degree in regards to hNTCP protein overexpression.

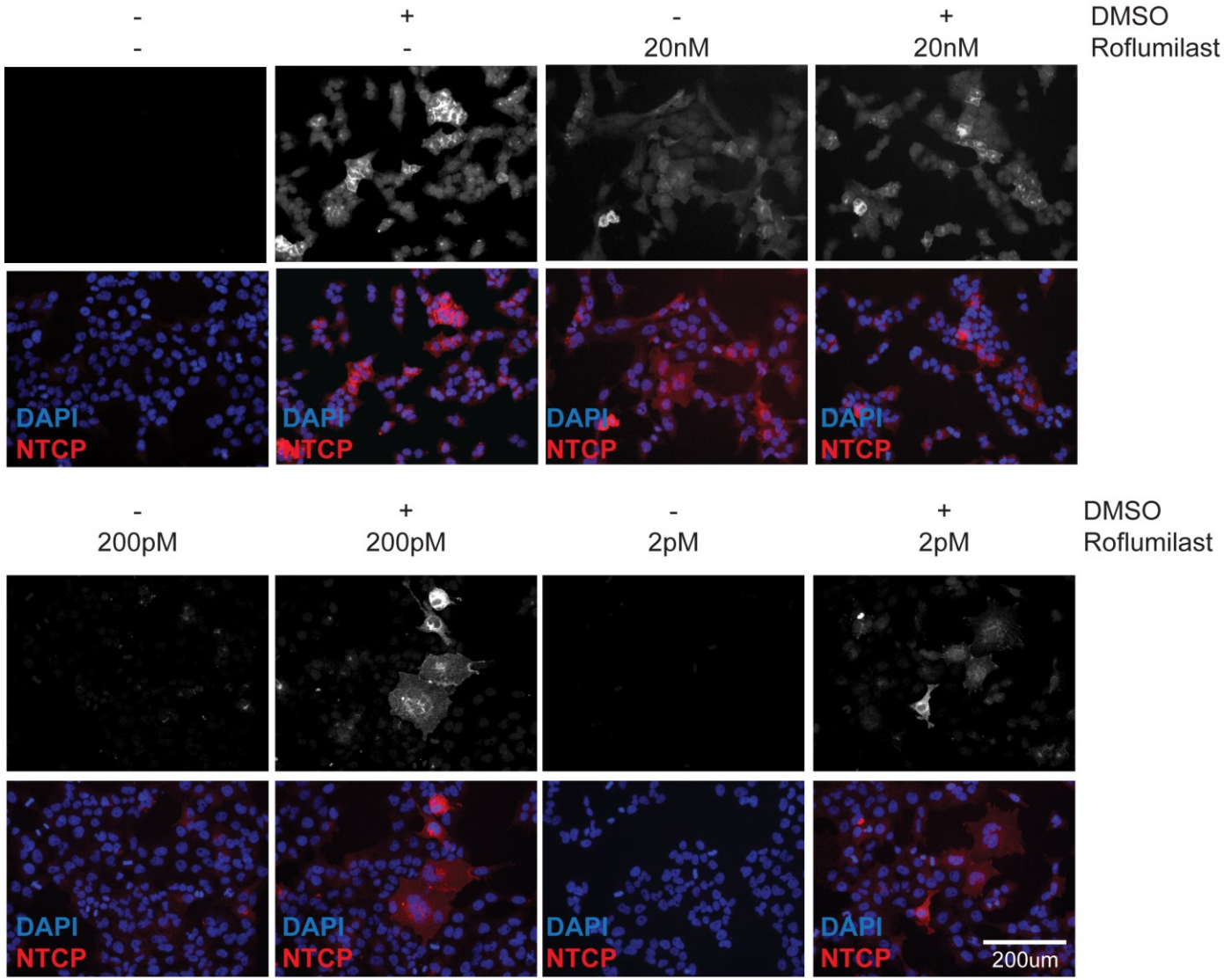
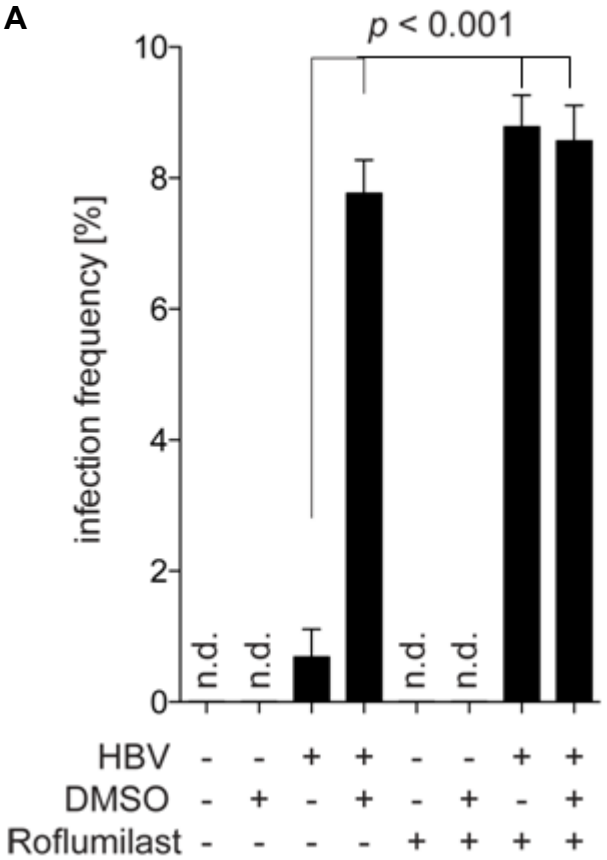


Figure 3.39; The effect of Roflumilast on inhibition of PDE4, resulting in indirect stimulation of hNTCP protein expression on the plasma membrane.

The cells were treated in the absence or presence of DMSO and Roflumilast for a total of 24 hours. Addition of Roflumilast when culturing the HepG2_hNTCP cells resulted in evident expression of hNTCP on the cell surface, and this was obvious in a dose dependent manner and was independent of the presence of DMSO. The image scale bar is 200µm.

[This experiment was performed and analysed by Dr Marcus Dorner].

Following on, we investigated the effect of Roflumilast on susceptibility of the stable human hepatic cells overexpressing hNTCP to HBV infection. The HepG2_hNTCP cells were treated and infected as previously described. The cells were then stained for HBcAg and analysed by flow cytometry, as well as immunofluorescence (*Figure 3.40; A* and *B, respectively*). Our data revealed that addition of Roflumilast to the cultured HepG2_hNTCP cells enhanced not only the hNTCP protein expression on the plasma membrane, as previously demonstrated, but also the susceptibility of cells to HBV infection. This result was independent of the presence of DMSO once again, meaning that further addition of DMSO together with Roflumilast did not really produce a cumulative effect on the number of cells that became susceptible to HBV infection, hence providing further proof that Roflumilast and DMSO have a very similar, if not exactly the same, mechanism of action in regards to hNTCP overexpression on the plasma membrane and subsequent susceptibility of cells to HBV infection.



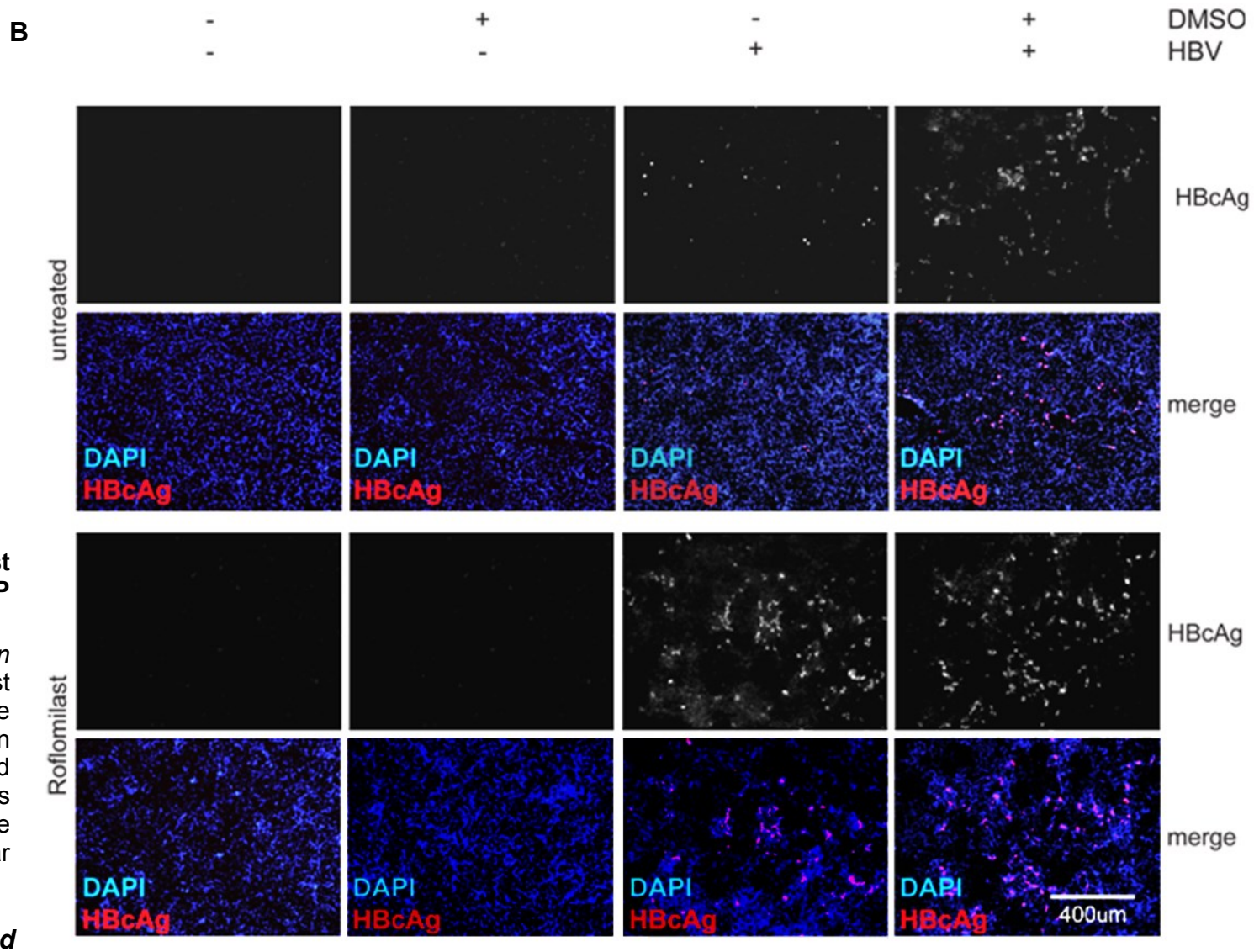


Figure 3.40; The effect of Roflumilast on susceptibility of HepG2_hNTCP cells to HBV infection.

The cells were treated as mentioned in Figure 3.39. Addition of Roflumilast (20nM) resulted in evident infection of the HepG2_hNTCP cells with HBV, as seen by both flow cytometry **(A)** and immunofluorescence **(B)** analysis. This outcome was independent of the presence of DMSO. The image scale bar is 200µm. *n.d.; nondetermined.*

[This experiment was performed and analysed by Dr Marcus Dorner].

In summary; We have demonstrated that Roflumilast, a selective PDE4 inhibitor, and DMSO have a similar mechanism of action and hence the same positive outcome on expression of hNTCP. Therefore, we can conclude that our second hypothesis, in regards to DMSO's mechanism of action, stands true; DMSO's role, in regards to induction of hNTCP protein expression, is to inhibit a certain PDE within the cells. And now we know that this PDE is PDE4D, and by doing so DMSO indirectly blocks the degradation of cAMP that can continue to indirectly dephosphorylate hNTCP and hence allow its constant and stable transportation to the plasma membrane. However, it is still possible that other PDEs may still get inhibited by the addition of DMSO and this is worth looking into further in the near future. Moreover, in the presence of Roflumilast, a comparable number of cells became susceptible to HBV infection to when DMSO alone was used to culture these cells during HBV infection. So once again the effect of Roflumilast was independent of the presence of DMSO, providing further proof that both ingredients work in a very, if not exactly, similar fashion and are part of the same pathway concerning hNTCP expression on cell surface and subsequent susceptibility of the cells to HBV infection.

3.1.5 Characterising another HBV-related receptor; Lentiviral overexpression of human ASGPR and subsequent susceptibility of cells to HBV infection

3.1.5.1 Effect of lentiviral overexpression of human ASGPR on the mRNA and protein levels

ASGPR has in the past been characterised as a possible receptor that HBV utilises for entry into hepatocytes and establishment of infection [25, 190]. Similarly to the production of cell lines overexpressing the hNTCP receptor, we utilised lentiviral overexpression of hASGPR driven by a CMV promoter. Following selection of stable human hepatic (HepG2 and Huh7.5) cell lines via antibiotic selection, mRNA and protein expression of hASGPR was determined by quantitative PCR and western blotting, respectively (*Figure 3.41; A* and *B, respectively*). All tested cell lines exhibited minimal expression of ASGPR mRNA at baseline, whereas ASGPR mRNA in stably selected cell lines was increased by approximately 1,000-100,000 fold (*Figure 3.41, A*). Interestingly, stable HepG2 cells had a significantly higher protein overexpression of hASGPR compared to the stable Huh7.5 cells (*Figure 3.41, B*), indicating once again differences in the protein processing mechanisms taking place in the various cell lines.

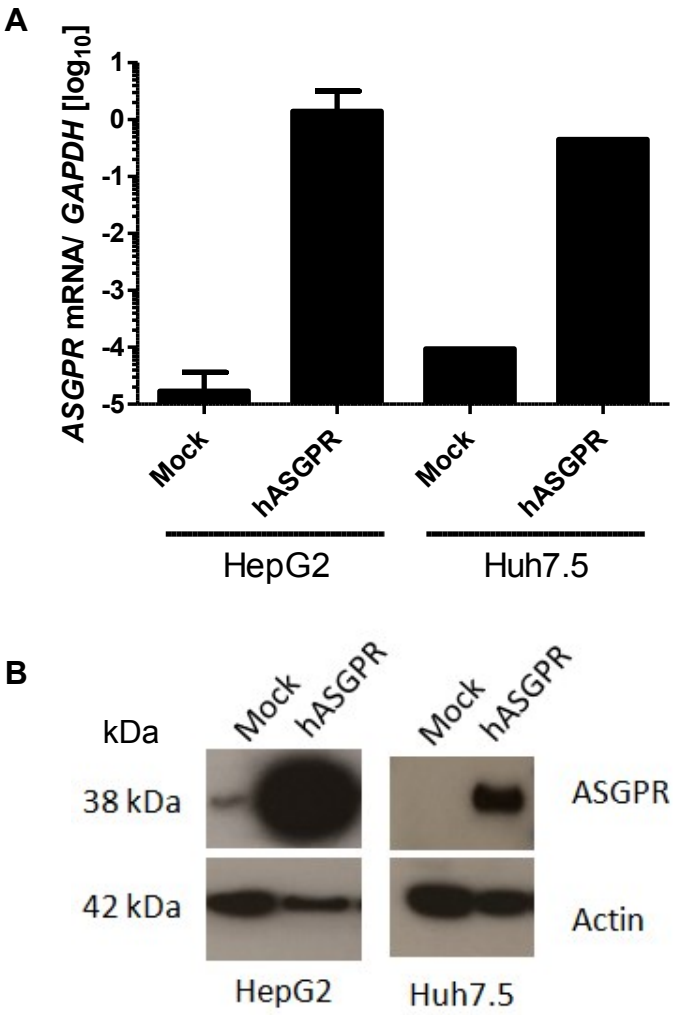


Figure 3.41; Human ASGPR mRNA overexpression in stable human hepatic cell lines.

(A) Comparison between the untransduced (mock) and the transduced cell lines (hASGPR) revealed that the latter had between three to five log higher hASGPR expression, at the mRNA level, compared to the corresponding former parental cell lines. **(B)** Detection of the hASGPR by western blot in the two stable hepatic cell lines, and comparison between the mock (untransduced) cells and the cells transduced with the hASGPR lentiviruses, with the latter visibly demonstrating hASGPR overexpression.

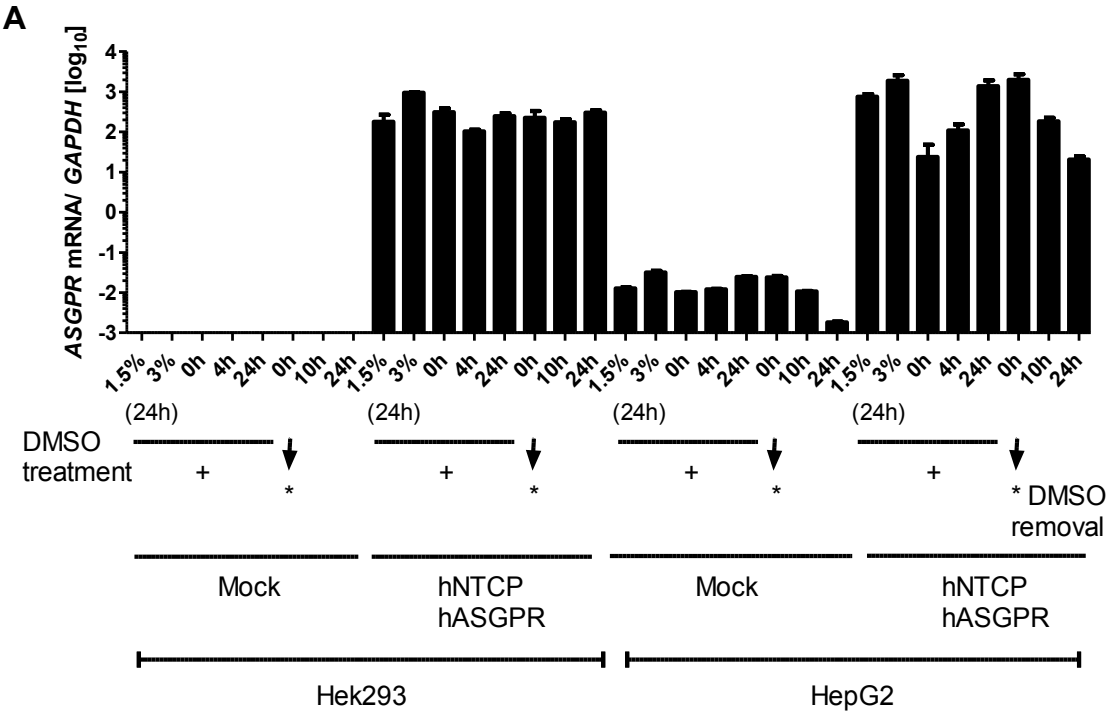
In summary; Using a plasmid expressing hASGPR under the control of a CMV promoter we were able to produce different stable cells lines overexpressing the human ASGPR receptor at the mRNA and protein levels.

3.1.5.2 The expression of the human ASGPR receptor may be regulated in a similar manner to the expression of the human NTCP receptor during DMSO differentiation

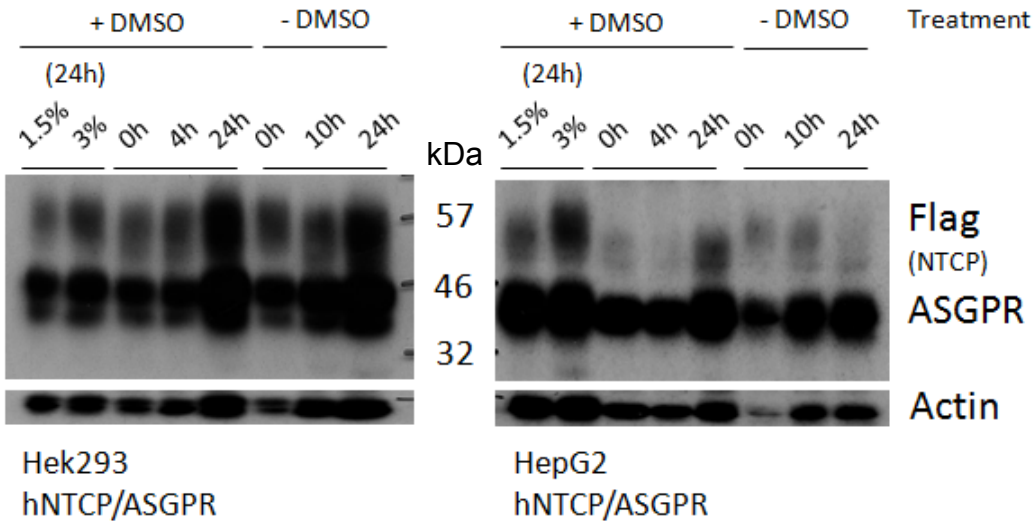
To characterise the effect of DMSO on hASGPR, apart from the single transduced hASGPR cell lines we had already created, we decided to also produce double transduced cell lines; meaning cell lines that simultaneously overexpressed the hNTCP and hASGPR receptors. For this purpose, we made use of the formerly created hNTCP (FLAG-tag) stably overexpressing cell lines and further infected them with the lentiviruses expressing hASGPR. The exact same process was followed, as if we were producing single transduced hNTCP or hASGPR cells.

Initially, we made use of the hepatic HepG2_hNTCP/hASGPR cell line, as well as the non-hepatic HEK293_hNTCP/hASGPR cells for comparison purposes, and similarly to what was previously described for the characterisation of the effect of DMSO on the hNTCP stably overexpressing cell lines, we were interested in characterising the effect of DMSO on these double-receptor-expressing cell lines. We were particularly interested in understanding whether the effect of DMSO was unique to the hNTCP receptor, especially in the HepG2 stably overexpressing cells, or whether DMSO had a broader effect on other receptors such as hASGPR. Moreover, we were curious to learn whether or not any possible effect of DMSO on hASGPR would be lacking in the HEK293 stable cell lines, as was shown to be the case for hNTCP. To this end, we treated the cells in the presence of varying concentrations of DMSO for selected periods of time, while also eventually removing the DMSO to establish whether the effect could be reversed. We analysed the samples on both the mRNA and protein levels (*Figure 3.42; A* and *B,* respectively), while we also quantified the protein bands by densitometry (*Figure 3.42, C*).

A


ASGPR mRNA/ GAPDH [log10]
4
3
2
1
0
-1
-2
-3
1.5% 3% 0h 4h 24h 0h 10h 24h 1.5% 3% 0h 4h 24h 0h 10h 24h 1.5% 3% 0h 4h 24h 0h 10h 24h 1.5% 3% 0h 4h 24h 0h 10h 24h
(24h)
(24h)
(24h)
(24h)
DMSO treatment
+
*
+
*
+
*
+
* DMSO removal
Mock
hNTCP hASGPR
Mock
hNTCP hASGPR
Hek293
HepG2

B


+ DMSO
- DMSO
+ DMSO
- DMSO
Treatment
(24h)
(24h)
1.5% 3% 0h 4h 24h 0h 10h 24h
kDa
1.5% 3% 0h 4h 24h 0h 10h 24h
57
46
32
Flag
(NTCP)
ASGPR
Actin
Hek293
hNTCP/ASGPR
HepG2
hNTCP/ASGPR

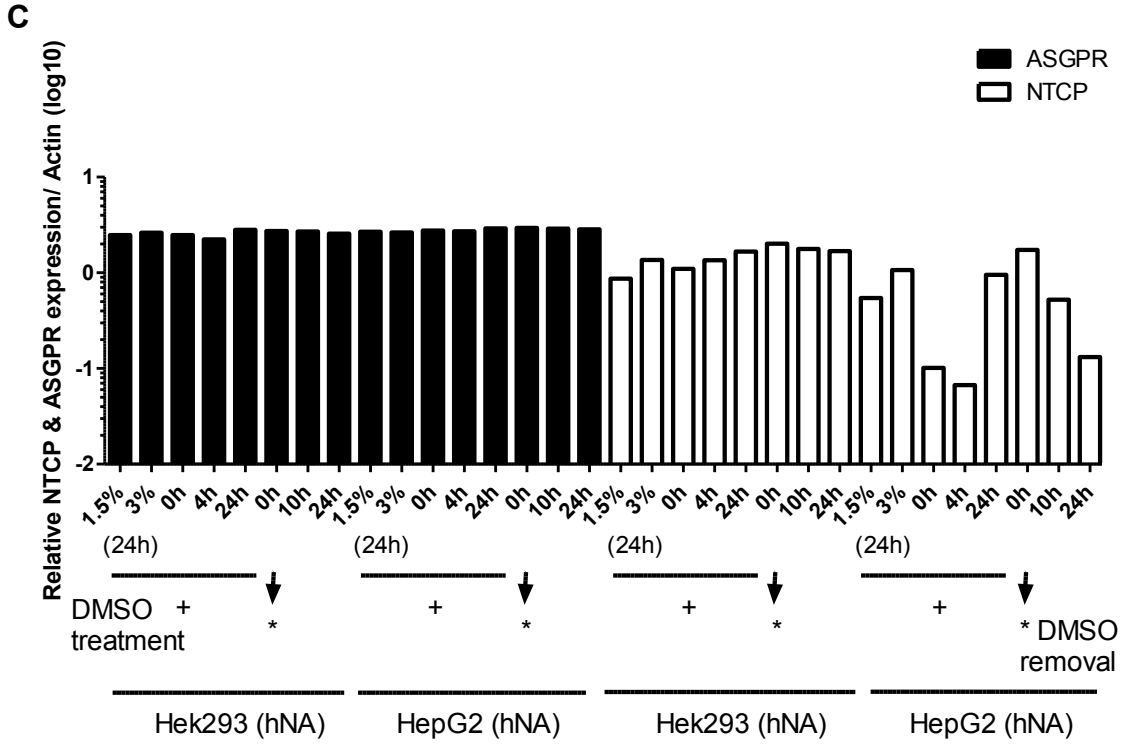


Figure 3.42; The effect of DMSO on hASGPR expression in a stable human hepatic and non-hepatic cell line.

The cells were treated in the absence or presence of DMSO, as indicated on the graphs. **(A)** At the mRNA level, DMSO exhibited some effect on hASGPR in the stable HepG2 double overexpressing cell lines, while no major effect was seen in the equivalent HEK293 cells. **(B)** On the western blot, it was difficult to distinguish any significant difference between the stable HEK293 and HepG2 double overexpressing cell lines, in regards to the effect of DMSO on hASGPR. However, for comparison reasons we concurrently stained the same blot for hNTCP (FLAG-tag) detection, and in this case, the effect of DMSO on hNTCP was once again clearly obvious, primarily in the HepG2 cells. As it was difficult to differentiate the effect of DMSO between the hASGPR samples, we performed densitometry of the protein bands **(C)**, which revealed no major effect of DMSO in either the HEK293 or the HepG2 hNTCP/hASGPR overexpressing cell lines specifically in regards to hASGPR protein expression. However, once again the effect of DMSO on hNTCP was clearly obvious principally in the HepG2 cells. *Mock, refer to the parental cell lines. hNA; hNTCP/hASGPR. Downward-pointing arrows and asterisks refer to the time-point(s) when DMSO was removed from the cell culture media.*

The conclusions we could draw from this experiment were as follows:

1) hASGPR, at the mRNA level and specifically in HepG2_hNTCP/hASGPR cells, appeared to be affected by the addition of DMSO, with the levels of expression subtly changing according to the DMSO concentrations tested, the periods of time they were tested for and whether DMSO was eventually removed from the cell culture.

   Interestingly, the addition of DMSO seemed to have the exact same effect on the endogenous hASGPR mRNA expression levels, as it did on the exogenous mRNA levels, further indicating that the effect produced by DMSO was independent of the CMV promoter driving expression of the receptor.

   However, the same did not seem to be the case at the protein level, where no significant differences could be distinguished between the different samples. *The data on the hASGPR protein expression in the HepG2_hNTCP/hASGPR cells was, however, considered inconclusive.*

2) No considerable differences were observed in the HEK293_hNTCP/hASGPR cells, on the mRNA or protein levels, for either hASGPR or hNTCP expression.

3) These outcomes were also confirmed in the equivalent single hASGPR overexpressing cell lines.

4) The effect of DMSO on hNTCP expression was once again confirmed in the stable human hepatic cell line, and was demonstrated to be independent of the presence of other receptors.

Following on, as the effect of DMSO on the hASGPR expression in the stable HepG2 double overexpressing cell lines was only really obvious at the mRNA level, we wanted to compare the precise extent of the effect of DMSO on both hNTCP and hASGPR in the HepG2_hNTCP/hASGPR cell lines at the mRNA level. As we had already quantified the hASGPR mRNA expression levels in the various samples (*Figure 3.42, A*), we just used the exact same samples and reanalysed them for the mRNA expression levels of hNTCP (*Figure 3.43*).

Accordingly, we discovered that the addition of DMSO to the cell culture media affected the expression of hNTCP on occasions nearly more than 10 fold greater when compared to the corresponding effect it had on hASGPR expression.

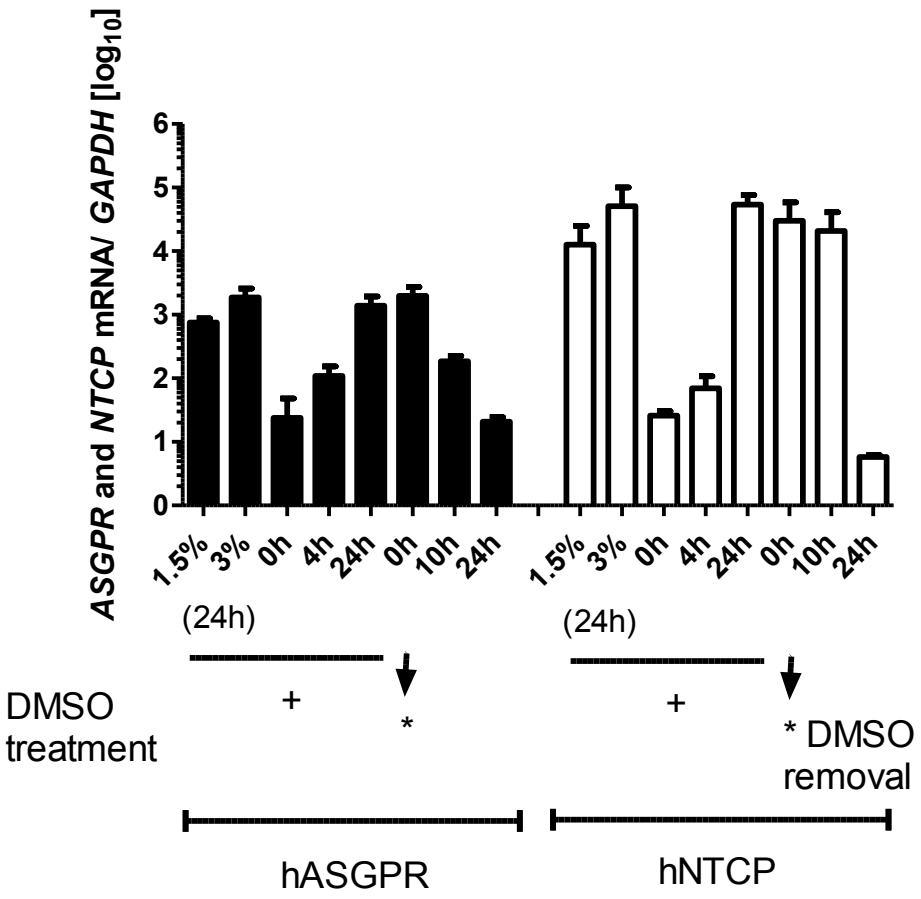


Figure 3.43; Comparison of the effect of DMSO on hASGPR and hNTCP expression in stable human hepatic (HepG2) cells, when cultured under varying DMSO conditions.

DMSO had a similar effect on the mRNA expression of both hASGPR and hNTCP, nevertheless it was evident that DMSO affected hNTCP expression more so than what it affected hASGPR expression. *Downward-pointing arrows and asterisks refer to the time-point(s) when DMSO was removed from the cell culture media.*

Furthermore, we decided to create some double transduced Huh7.5_hNTCP/hASGPR cell lines and we evaluated these, on the mRNA and protein level, in regards to the effect of DMSO only on expression of hASGPR (*Figure 3.44; A* and *B,* respectively). Analysis of the expression of hNTCP in Huh7.5_hNTCP cells, in the presence or absence of DMSO, previously revealed no differences in the receptor's expression between the conditions

tested (*Figure 3.18*). For confirmation purposes, we re-included in this experiment the HepG2_hNTCP/hASGPR cell lines and similarly repeated the analysis of hASGPR expression but only at the protein level. We were hoping to validate whether DMSO, besides having an effect at the mRNA level (*Figure 3.42, A*), actually also had a subtle effect at the protein level that we were unable for one reason or another to accurately detect previously. This could be primarily due to the fact that the effect of DMSO on hASGPR is more obvious at the mRNA level rather than at the protein level. We therefore concluded, first of all, that the presence of DMSO in the stable Huh7.5 double overexpressing cell lines did not have an effect on the expression of hASGPR, both on the mRNA and protein level, which compares to our previous data on the effect of DMSO on hNTCP expression in single Huh7.5 hNTCP overexpressing cell lines, and demonstrates that the two cell lines have differences in their regulatory pathways/ processes influencing functional expression of these receptors. Secondly, the presence of DMSO did in fact have a subtle effect on hASGPR protein expression in the HepG2_hNTCP cells.

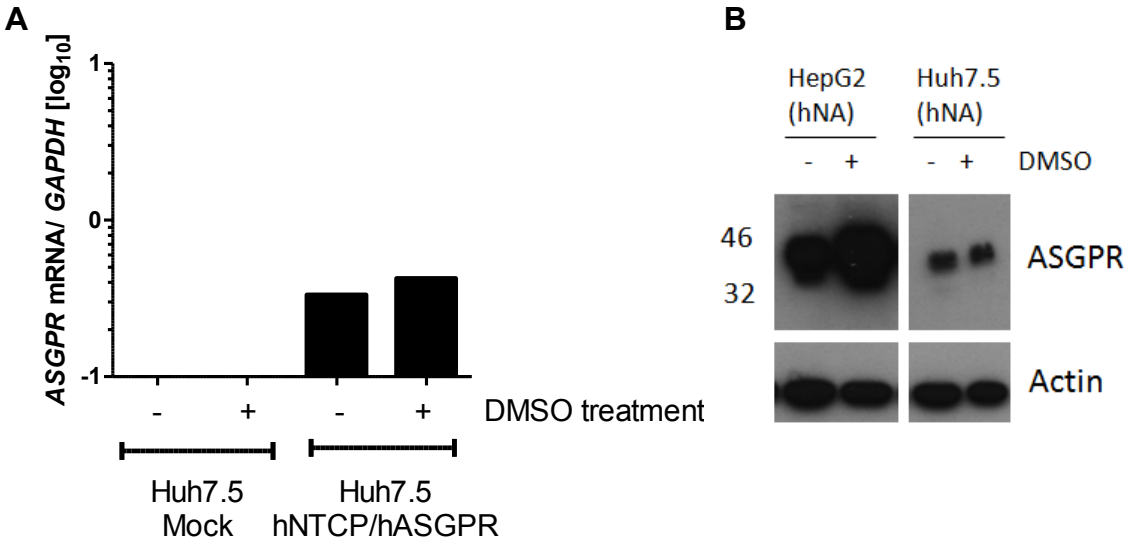


Figure 3.44; Effect of DMSO on hASGPR expression in stable human hepatic cell lines.

The cells were treated with DMSO as usual for a total of 24 hours. DMSO, similarly to what was previously seen for hNTCP expression, did not affect hASGPR mRNA **(A)** or protein **(B)** expression in Huh7.5 overexpressing cell lines. However, DMSO appeared to affect to some extent hASGPR protein expression **(B)** in HepG2 overexpressing cell lines.

In summary; The presence of DMSO in cell culture appears to induce both hNTCP and hASGPR expression in a similar manner. This effect was particularly established in HepG2 stably overexpressing cell lines, with no effect of DMSO being exhibited in equivalent HEK293 and Huh7.5 cell lines. However, the extent by which DMSO affected the two receptors varied, with hNTCP possibly being affected to a greater and more obvious extent compared to hASGPR. However, our data generally illustrates that DMSO may have a broader effect on a number of other receptors, with its impact possibly being exerted to varying degrees.

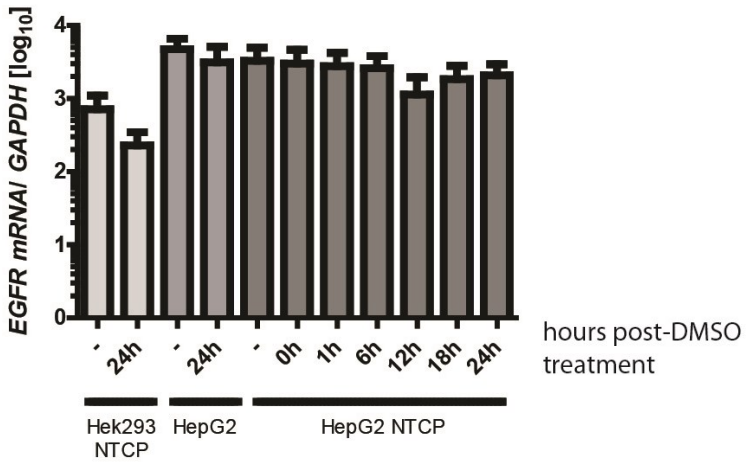
3.1.5.2.1 DMSO has a broad, but variable, effect on the expression of different receptor proteins

Moving on, we specifically decided to further investigate the human EGFR and TfR receptors primarily due to the fact that they have previously been shown to be somewhat involved with NTCP receptor trafficking within the cell [207, 208]. A particular study made mention of NTCP trafficking between the cell surface and an intracellular pool within endocytic vesicles on microtubules, and that within the same endocytic vesicles EGFR was also present as it is thought to be essential for regulating this process [208]. Furthermore, NTCP and TfR have been determined to get recycled back to the plasma membrane in the same endocytic vesicle [207], while, once internalised, ASGPR and TfR have also been shown to traffic together in the same endocytic vesicles [182]. As a result, we were interested in investigating the effect of DMSO on these two additional receptors.

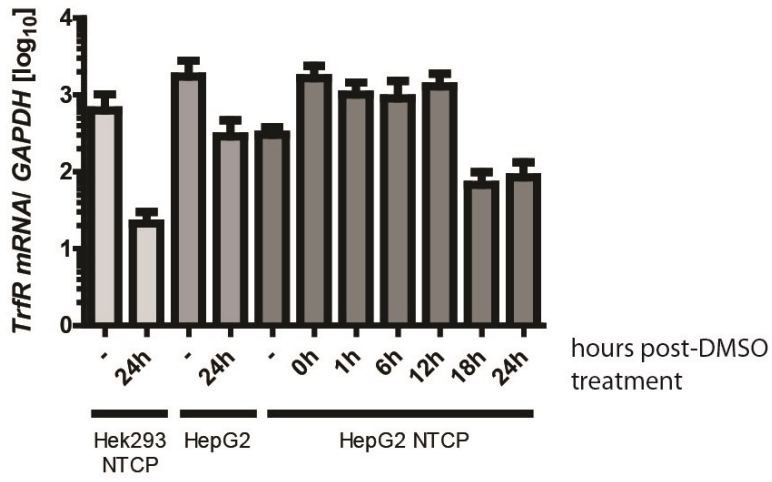
For this purpose, we made use of the stable human non-hepatic HEK293 and hepatic HepG2 hNTCP overexpressing cell lines, as well as the HepG2 parental cells as controls, and we assessed whether endogenous expression of human EGFR (hEGFR) and TfR (hTfR) was affected similarly in all three cell lines by the presence of DMSO over time. We analysed the samples on the mRNA and protein level (*Figure 3.45; A* and *B,* respectively), and determined

that, in general, expression of both hEGFR and hTfR decreased in the presence of DMSO over time, with hTfR being more obviously affected compared to hEGFR. Moreover, this negative impact of DMSO on the hEGFR and hTfR expression was the same regardless of the cell line tested or the additional presence of hNTCP.

A.1



A.2



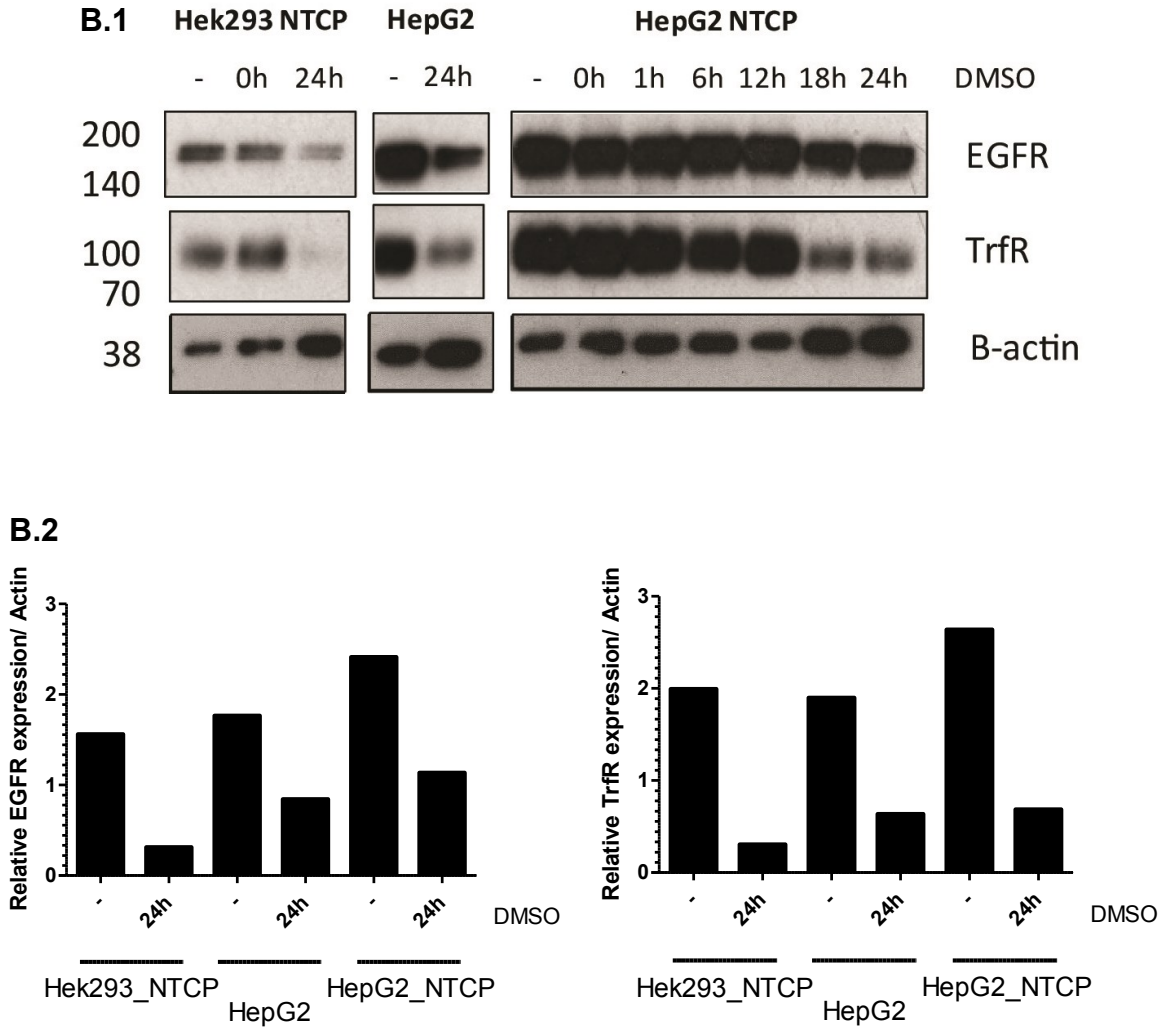


Figure 3.45; The effect of DMSO on expression of human EGFR and TfR in stable human hepatic and non-hepatic cell lines.

Expression of hEGFR and hTfR declined in the presence of DMSO over time, on both the mRNA **(A)** and protein **(B)** level. The effect was more obvious on hTfR expression rather than hEGFR, but was fairly consistent in all cell lines tested. HepG2 parental cells were used as controls for comparison reasons.

In summary; In the presence of DMSO, the expression of human EGFR and TfR appeared to be regulated in the completely opposite way compared to human NTCP and ASGPR expression. While the addition of DMSO to the cell culture media had a positive effect on the expression of NTCP and ASGPR, yet to varying degrees, in the case of EGFR and TfR the presence of DMSO had an apparently negative effect on their expression, again to varying extents. The presence of DMSO for longer periods of time or at higher concentrations

resulted in enhanced downregulation of these two receptors. Nevertheless, the effect of DMSO could be reversed upon its removal. Consequently, DMSO has been shown to regulate a number of receptors, possibly with more to be discovered, but sometimes it has an upregulating effect on their expression while other times it causes their downregulation.

3.1.5.3 Infection of cells with HBV- & HDV- mimicking particles appears to require expression of both the human NTCP and ASGPR receptors

3.1.5.3.1 Generation and characterisation of HBV- & HDV- pseudotyped lentiviral particles

Specifically following the production of the Type I HBSpp (*refer to Section 2.2.2.4.1.2*), we collected the infectious lentiviral supernatants and analysed them for expression of HBsAg (*Figure 3.46, A*). However, as we had collected a big quantity of supernatants, we also decided to concentrate some of these particles either by concentration through an Amicon® filter unit or by ultracentrifugation, and then we re-analysed the concentrated samples for HBsAg expression levels (*Figure 3.46, A*). Moreover, we also used the two types of concentrated samples to re-infect stable HepG2_hNTCP overexpressing cells, so as to determine their actual infection efficiency (*Figure 3.46, B*).

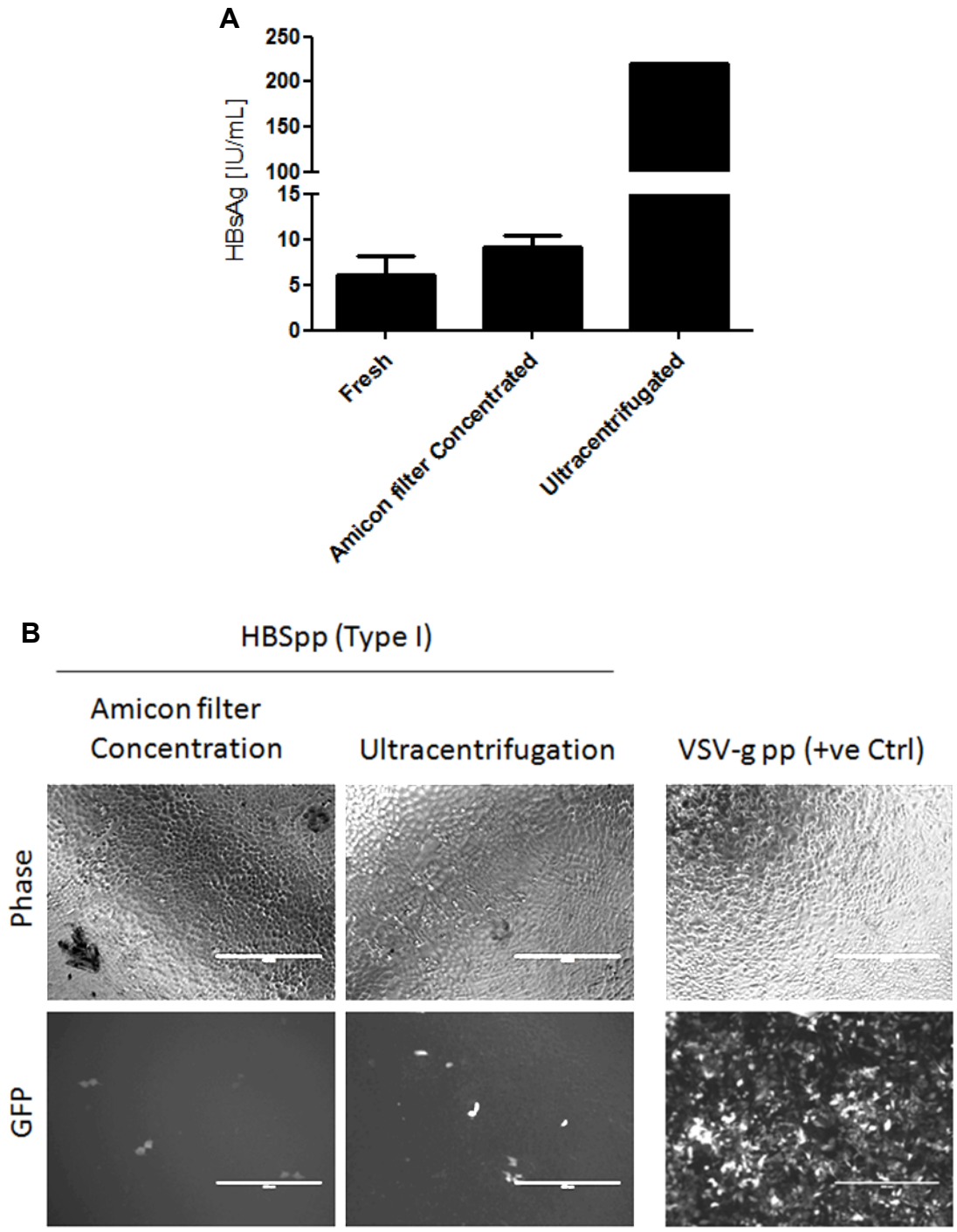


Figure 3.46; Characterisation of Type I HBSpp.

(A) Analysis of the HBsAg expressed on surface of these lentiviral pseudoparticles. Ultracentrifugation clearly resulted in a much higher level of HBSpp concentration, while it would appear as if some lentiviral particles were possibly lost when concentrated through the filter units. **(B) Immunofluorescence of HepG2_hNTCP cells infected with the concentrated Type I HBSpp, as well as the positive control VSVg pseudoparticles (VSVg pp).** In general, the infection efficiency of the cells is very low compared to the positive control sample. However, the ultracentrifuged particles are still more effective compared to the filter concentrated particles. The images were taken 72 hours post-infection of the cells. The image scale bar is 400µm.

From the results of the HBsAg ELISA and immunofluorescence of GFP-positive cells (i.e. HBSpp infected cells) we decided to ultracentrifuge all remaining supernatants and only use those for follow up experiments, as undoubtedly ultracentrifugation resulted in a greater pseudoparticle concentration efficiency hence theoretically increasing the chances of more cells becoming infected.

In summary; We generated "Type I" HBSpp and characterised them for their expression of HBsAg on their cell surface, as well as their ability to infect stable human hepatic cell lines overexpressing the hNTCP receptor. Unfortunately, the infection efficiencies we obtained were rather low, but nevertheless, we utilised these pseudoparticles for further infection experiments.

3.1.5.3.2 Effect of ASGPR overexpression on susceptibility of cells to HBV- & HDV- mimicking particles

Following on, we used the ultracentrifuged Type I HBSpp and further infected HepG2 cells. This time, apart from the HepG2_hNTCP, we also included the stable overexpressing HepG2_hASGPR and the double-expressing HepG2_hNTCP/hASGPR. The HepG2 parental cells (mock) were also included as negative controls. We infected all cells with the Type I HBSpp, as well as the NE pp (negative controls) and the VSVg pp (positive controls), and analysed the cells by immunofluorescence at 72 hours post-HBSpp infection of the cells (*Figure 3.47, A*). However, the infection efficiency of the HBSpp on this occasion was unexplainably even worse compared to the original test in the HepG2_hNTCP cells (*Figure 3.46, B*), with only the double-expressing cells showing very few signs of GFP-positive cells. NE pp caused no infection, while VSVg pp entered a considerable number of cells, as expected.

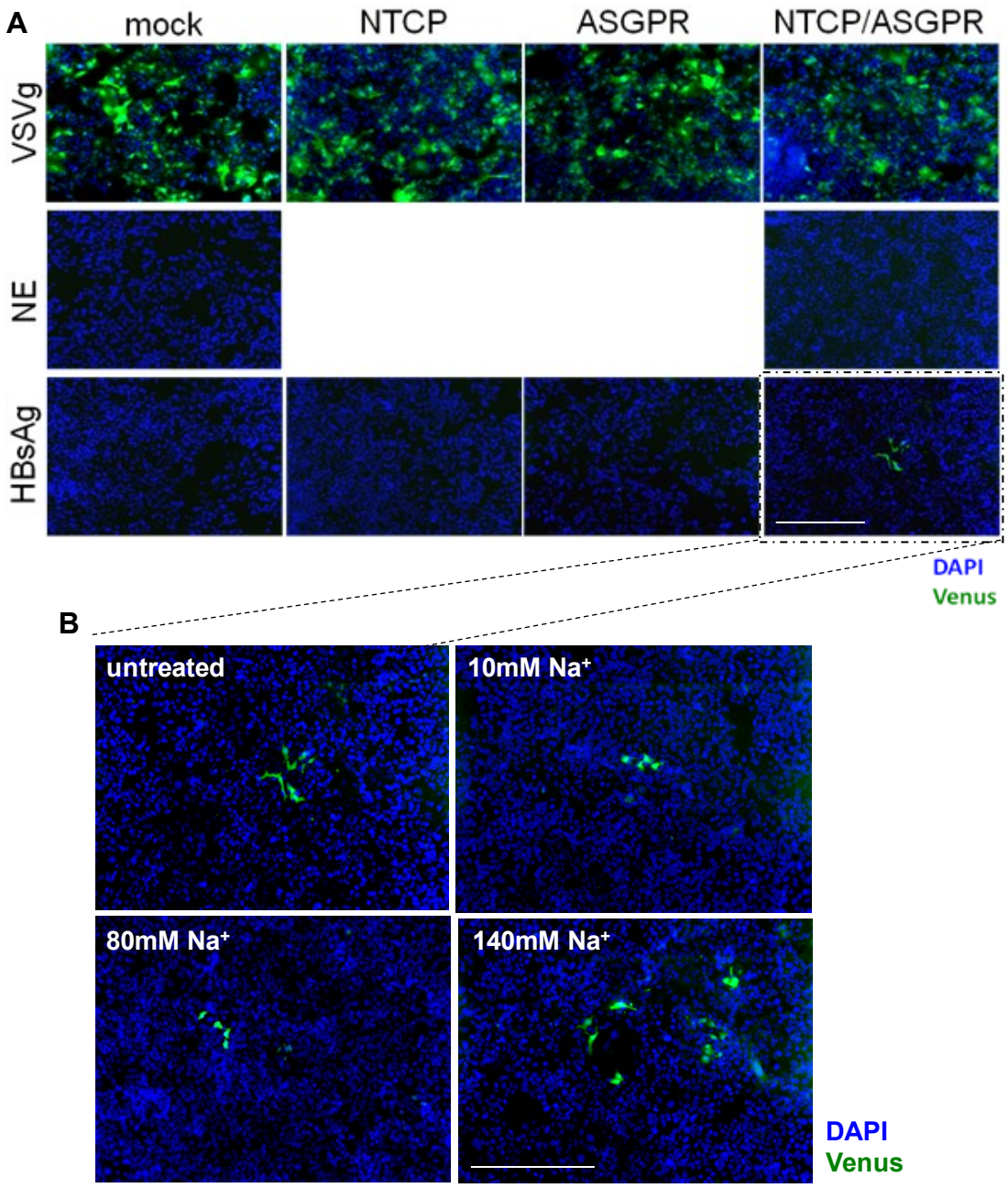


Figure 3.47; Infection of human hepatic cell lines, overexpressing two HBV-related receptors, with HBSpp.

Analysis was performed 72 hours post-infection of these cells with the lentiviral pseudoparticles. **(A) Immunofluorescence of varying HepG2 cells infected with the ultracentrifuged Type I HBSpp, as well as the negative control NE pseudoparticles (NE pp) and positive control VSVg pp.** Only the double-expressing stable HepG2_hNTCP/hASGPR cells showed signs of HBSpp entry and hence were GFP-positive. (**B) Immunofluorescence of the HepG2_hNTCP/hASGPR cells infected with the ultracentrifuged Type I HBSpp, in the presence of varying sodium concentrations.** Higher sodium concentrations resulted in more cells becoming infected. *The sodium solutions were added during the infection procedure.* The image scale bar is 400µm.

Thinking of possible ways to improve entry of the HBSpp into the cells we decided to test the presence of varying concentrations of sodium in the cell media during infection only of the hNTCP/hASGPR double-expressing cells (*Figure 3.47, B*). It appeared that when higher concentrations of sodium were present during the infection procedure this resulted in a higher number of cells becoming infected and hence GFP-positive. Presumably influx of sodium into the cells may have simultaneously allowed the particles' entry. However, the number of infected cells was still on the very low side. In a different experiment, we additionally tested the presence of 4% PEG, widely used during HBV infections, but this had no enhanced effect on entry of the HBSpp.

We next decided to test whether the presence of DMSO (2%) would enhance entry of the HBSpp. Apart from the HepG2_hNTCP/hASGPR cell lines, we also used the HEK293_hNTCP/hASGPR for comparison purposes, while we also included the equivalent single-expressing hNTCP cell lines. The parental cells of both cell lines were also included as controls for determining background infection levels. On this occasion, and so as to determine the exact number of cells that had been infected with the Type I HBSpp, we decided to actually count the GFP-positive cells. We counted all cell samples twice at both 72 and 96 hours post-infection and plotted the means (*Figure 3.48*). The addition of DMSO was verified to have a positive outcome on the number of cells infected with the HBSpp, however in most cases the induction of HBSpp entry in the presence of the solvent was not considered to be especially significant, especially in the human hepatic cell lines. Additionally, and interestingly, it would appear that entry of HBSpp required the simultaneous presence of both the hNTCP and hASGPR receptors.

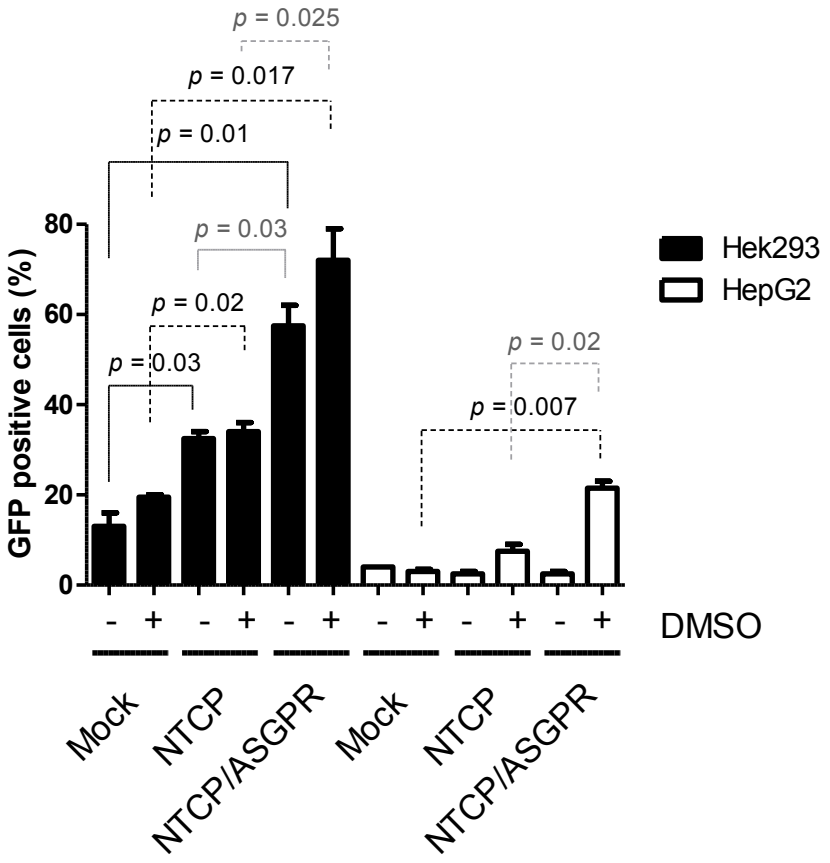


Figure 3.48; Infection of non-hepatic and hepatic cell lines overexpressing the HBV-related receptors with HBSpp.

Addition of DMSO to the cell culture during HBSpp infection definitely enhanced the number of GFP-positive cells, but in most samples not significantly. Interestingly, the presence of the human ASGPR receptor appeared to be important during these HBV entry events. *Mock, refers to the parental/untransduced cells. Statistical analysis: Solid lines indicate comparison between Mock and NTCP/ASGPR samples, while dashed lines indicate comparison between NTCP and NTCP/ASGPR samples.*

In summary; Entry of the Type I HBSpp in all cell types tested was low and only slightly improved in the presence of high sodium concentrations or DMSO. Interestingly, though it appeared that the particles entered cells more freely when both the hNTCP and hASGPR receptors were present, compared to when the NTCP receptor alone was present. However, it is possible that even effective entry of HBSpp into cells requires more than just expression of these two receptors, such as another possible receptor or additional factors.

3.2 HBV entry into mouse cells, and the interspecies restriction of HBV infection

3.2.1 Human NTCP protein expression in some mouse cell lines is regulated similarly to human cells

Since HBV entry relies on the expression of the human NTCP receptor, we once again utilised lentiviral overexpression of hNTCP (C-terminal FLAG-tag) driven by a CMV promoter. Following selection of stable mouse hepatic (Hepa1.6 and H2.35) cell lines via antibiotic selection, mRNA expression of hNTCP was determined by quantitative PCR (*Figure 3.49*). All tested cell lines exhibited no expression of hNTCP mRNA at baseline, whereas hNTCP mRNA in stably selected cell lines was increased by approximately 1,000-100,000 fold.

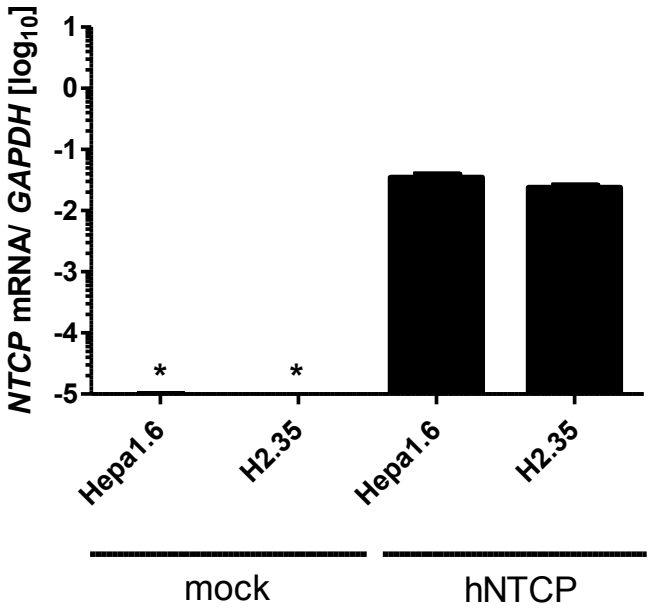


Figure 3.49; Human NTCP mRNA overexpression in stable mouse hepatic cells. Comparison between the untransduced (mock) and the transduced cell lines (hNTCP) reveals that the lentiviral transduced cells had between three to five log higher hNTCP expression, at the mRNA level, compared to the corresponding untransduced parental cell lines. * *undetermined.*

Following on, we were interested in characterising the effect of DMSO on hNTCP expression in these stable mouse cell lines, and perform a comparison to the effect seen in the stable human cell lines. After culturing the cell lines in

the presence or absence of 2% DMSO, for a short (4 hours) and a longer (24 hours) duration, we analysed the samples for hNTCP mRNA and protein expression (*Figure 3.50; A* and *B,* respectively). To our surprise, we observed that, apart from the HepG2_hNTCP cells that we already knew were greatly affected by the addition of DMSO, the only other cell line that appeared to respond in a very similar manner was the mouse hepatocyte cell line H2.35_hNTCP. The mouse hepatoma cell line (Hepa1.6_hNTCP), similarly to the second human hepatoma cell line (Huh7.5_hNTCP) and to the non-hepatic cell line (HEK293_hNTCP), was not affected by the addition of DMSO.

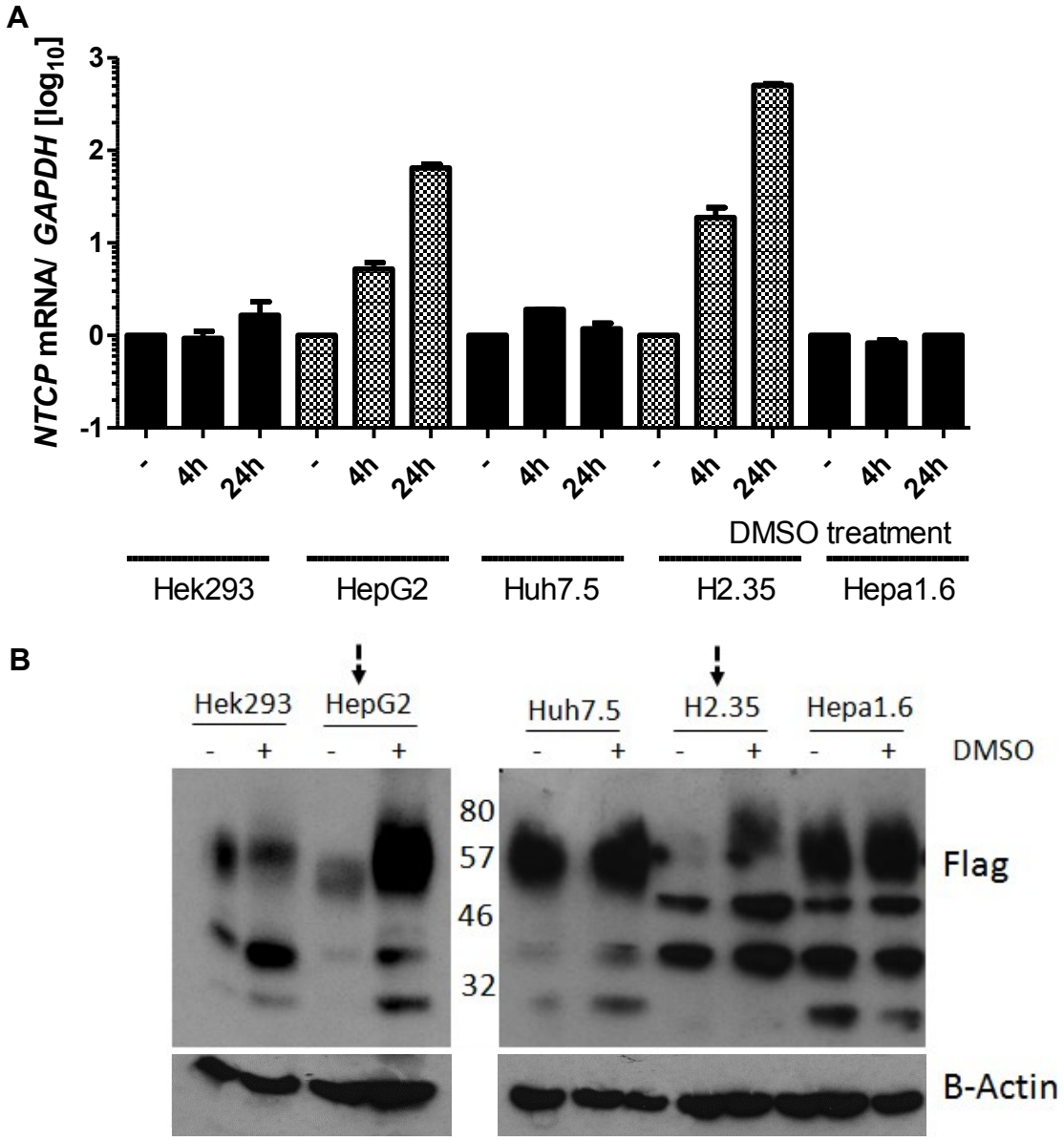


Figure 3.50; The effect of DMSO on hNTCP (FLAG-tag) expression in stable mouse hepatic cell lines.

The cells were treated with DMSO as usual for a total of 24 hours. Comparison between stable human and mouse cell lines overexpressing hNTCP. Addition of DMSO to the cell culture medium affected stable HepG2 and H2.35 hNTCP overexpressing cell lines, while it did not affect whatsoever hNTCP expression in HEK293, Huh7.5 and Hepa1.6 cells. These outcomes were determined on both the mRNA **(A)** and protein **(B)** levels. In addition, hNTCP in mouse cells appeared to run at a slightly higher molecular weight compared to hNTCP in human cells, possibly due to natural differences in glycosylation processing and final status. However, PNGaseF digestion resulted in full deglycosylation of all protein bands in all cell lines (*Appendix A.3.V*). *Downward-pointing arrows depict the cell lines where hNTCP expression was demonstrated to be affected by the presence of DMSO.*

In summary; Using a plasmid expressing hNTCP (FLAG-tag) under the control of a CMV promoter we were able to produce various stable mouse cells lines. These overexpressed the human NTCP receptor on the mRNA and protein level. Interestingly, the addition of DMSO for various time points had an enhanced effect but only in two particular stable cell lines; HepG2, as previously determined, and H2.35. The rest of the stable cells, regardless of whether they were human or mouse, were unaffected by the presence of DMSO. This was obvious on both the mRNA and protein level. Therefore, DMSO probably affects hNTCP overexpression by a similar mechanism in both HepG2 and H2.35 cells, while possibly this mechanism DMSO regulates in those specific cell lines may in fact be fully functioning in the remaining cells, explaining why they do not require DMSO for induction of hNTCP expression.

3.2.2 A mouse/human chimeric NTCP receptor renders murine cells susceptible to HBV infection

Human and mouse hepatocytes, as we know, are different in regards to their ability to become susceptible and hence infected with HBV. In part this is believed to be due to differences in the sequence of the NTCP receptor they express. NTCP has been shown to be sufficient to render human but not mouse cells permissive to HBV and HDV infection. Expression of the human NTCP, however, in mouse cells only supports HDV infection. As the HDV virus is a satellite virus of HBV and specifically requires the HBV surface glycoproteins for the envelopment of its genome, therefore it is also plausible that mice either encode for a restriction factor or fail to encode a crucial host factor necessary for their subsequent infection by HBV [30, 117, 163, 164, 223-226].

To examine this scenario further (i.e. differences between the sequences of the human and mouse NTCP receptor are responsible for their differences in susceptibility to HBV infection) we designed a chimeric NTCP receptor which predominantly expressed the mouse NTCP receptor sequence but had very

few amino acid substitutions from the human NTCP receptor sequence (*Figure 3.51*). We particularly replaced amino acids 157-165 of the non-functional mouse NTCP with the human counterparts. This region within the human NTCP receptor has previously been reported as being one of the crucial areas for recognition and binding to the HBV pre-S1 domain hence permitting human cells to effectively support viral infection [30, 32, 170].



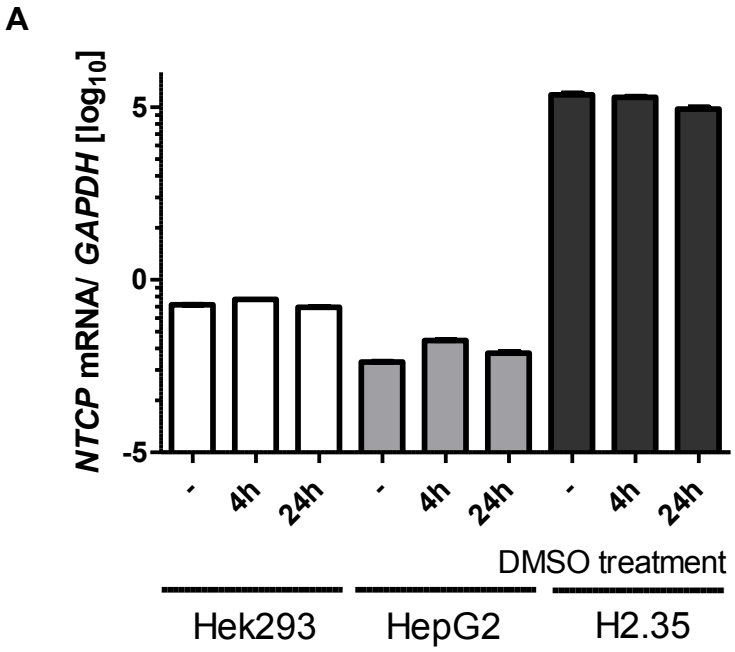
Figure 3.51; Sequence alignment analysis of the human, mouse and newly synthesised chimeric form of the NTCP receptor.

The DNA sequence alignment was performed using the Clustal Omega web program (http://www.ebi.ac.uk/Tools/msa/clustalo/). The red box specifies the mouse 157-165 amino acids substituted with their human counterpart, and also illustrates the final chimNTCP product sequence.

Abbreviations: mNTCP – mouse, chimNTCP – chimeric, and hNTCP – human.

The commercially synthesised construct (entry clone) was used to perform gateway recombination cloning into particular destination constructs, which were then utilised for the production of lentiviral pseudoparticles expressing the chimNTCP receptor. chimNTCP expression was also under the control of a CMV promoter. Stable human and mouse hepatic (HepG2 and H2.35) cell lines, as well as stable non-hepatic (Hek293) cell lines, were finally created.

Initially, we were interested in once again characterising the effect of DMSO on chimNTCP expression in these cell lines. Due to the differences in human and mouse NTCP sequences, we were particularly interested to understand whether the chimNTCP would respond similarly to the hNTCP in the HepG2 and H2.35 stably overexpressing cells lines in regard to the presence of DMSO, and whether this effect once again would not be apparent in the stable non-hepatic HEK293 cell lines. For this purpose, we treated the cells in the presence of varying concentrations of DMSO for selected periods of time, while also eventually removing the treatment to establish whether the effect of the agent could be reversed. We analysed the samples on both the mRNA and protein levels (*Figure 3.52; A* and *B,* respectively).



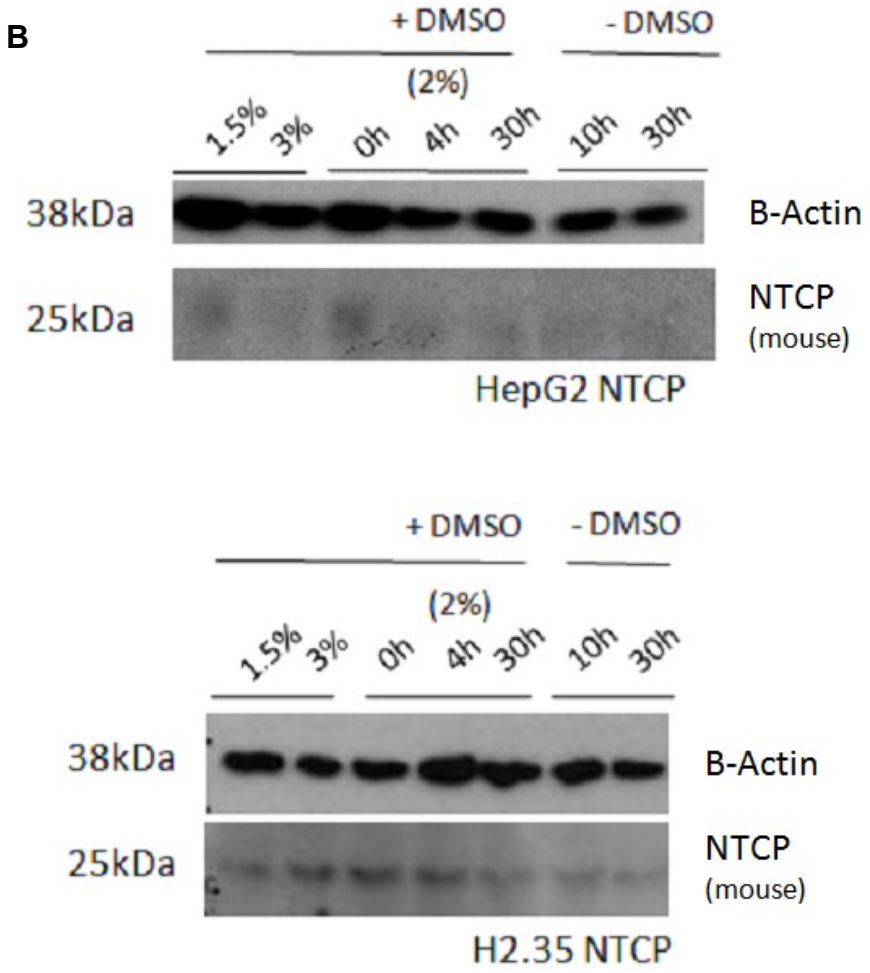


Figure 3.52; Effect of DMSO on the expression of a chimNTCP in stable non-hepatic and hepatic cell lines.

Treatment with DMSO was performed as indicated on the graphs. **(A)** Representative analysis of the mouse NTCP (chimNTCP) mRNA levels under the different conditions tested. *The values are depicted as fold over the untreated samples.* **(B)** As no difference was identified between the samples during the mRNA analysis, only the stable HepG2 and H2.35 chimNTCP cell lines were used for protein analysis, as these were of primary interest. A protein band of approximately 25kDa was detected for the mouse NTCP (chimNTCP), in both cell lines and in all samples tested.

At the mRNA level (*Figure 3.52, A*), no difference whatsoever could be detected between all the samples collected and tested from the same stably overexpressing cell lines. Specifically, regardless of the DMSO concentrations tested and the periods of time they were tested for or whether DMSO was finally removed from the cell culture media, the mouse NTCP mRNA levels were constant in all stable cell lines overexpressing the chimNTCP under all culturing conditions. In general, the H2.35 stable cell lines revealed the highest levels of chimNTCP overexpression compared to the other two cell lines that

demonstrated lower but comparable levels of receptor expression. For this reason, when we came to analysing the protein samples (*Figure 3.52, B*), we decided to continue only with the samples collected from the stable hepatic HepG2 and H2.35 chimNTCP cell lines, which were of primary interest. Similarly to the mRNA analysis, no differences could be seen either at the protein level. It should be noted that there is still some uncertainty about the a-mouse NTCP antibody's specificity (even though the signal detected in the chimNTCP stably overexpressing cell lines was non-existent in the parental cells). Nevertheless, the mRNA data clearly revealed that DMSO did not affect the chimNTCP in the same manner it did the hNTCP, meaning that possibly the chimNTCP was being regulated differently to hNTCP and as such was possibly not dependent on DMSO for its functional expression on the plasma membrane in HepG2 and H2.35 stably overexpressing cell lines. This requires further investigation though.

Subsequently, we went on to investigate the susceptibility of the stable hepatic cells expressing the chimNTCP receptor to HBV infection, while we additionally included the human hepatic cell lines stably overexpressing the hNTCP receptor. We particularly infected the cells with the infectious sups at an MOI of 1000 and analysed the samples at seven days post-infection for HBV DNA copies within the actual cells, specifically measuring the total HBV DNA copies/ng of sample DNA (*Figure 3.53*). To our surprise, and to our current knowledge, this is the first reported case of a mouse liver cell line becoming susceptible to HBV infection *in vitro*, under these particular circumstances (i.e. by overexpressing a particular chimeric NTCP receptor).

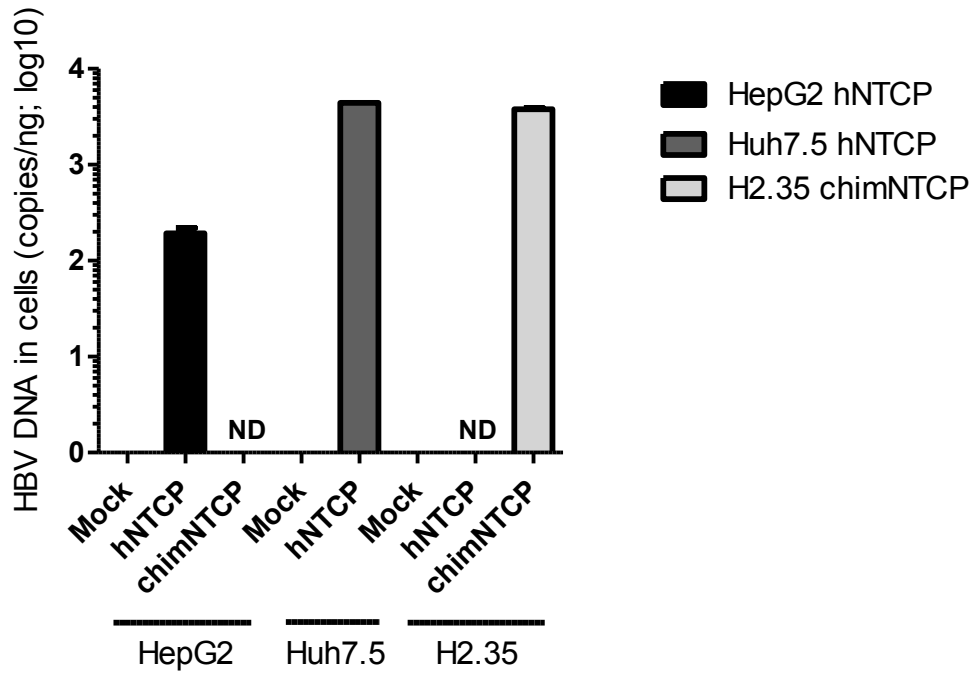


Figure 3.53; Susceptibility of human and mouse hepatic cells expressing two different forms of the NTCP receptor to HBV infection, *in vitro*.

Mouse H2.35 liver cells, upon overexpression of a synthetic chimNTCP receptor, became readily susceptible to HBV infection. In addition, Huh7.5_hNTCP cells appeared to be more susceptible to HBV infection compared to HepG2_hNTCP cells, and this may be justified by the differences in regulation, subsequent expression and conformation of the hNTCP protein on the plasma membrane. Therefore, it is highly possible that different cells will generally have diverse susceptibility efficiencies to HBV infection. *Mock, refers to the equivalent parental cell lines that were also infected with the virus (negative control samples). ND; Nondetermined.*

To confirm that the results we were observing were specific, we decided to repeat the previous infection experiment precisely in the same manner, but only infecting the HepG2_hNTCP and the H2.35_chimNTCP (and the equivalent parental) cells, and analysed the samples with a commercial human phospho-kinase antibody array. Our aim was to examine possible different signalling pathways induced in these cell lines upon infection with HBV, and interestingly we confirmed a number of factors that were either up- or down-regulated in either of the cells upon HBV infection (*Table 3.4* and *3.5*).

HepG2_hNTCP	H2.35_chimNTCP
Upregulated	
- FAK, JNK 1/2/3, GSK-3a/b, STAT5b, EGF R, b-Catenin, PDGF Rb, STAT6, Lck, STAT2, STAT5a, STATa/b, PRAS40, Fyn, Fgr, Hck, Akt 1/2/3, MSK1/2, AMPka2, Src, Lyn, CREB and Chk-2	- ERK1/2, AMPka1, RSK1/2/3, p27, p70 S6 Kinase, STAT3, p53, TOR, WNK1, PYK2 and eNOS

Table 3.4; Phospho-kinase signalling factors activated in either a human or a mouse cell line, overexpressing different forms of the NTCP receptor, upon infection with HBV.

HepG2_hNTCP		
Gene	**Name**	**Uniprot Accession**
Akt 1/2/3	RAC-alpha serine/threonine-protein kinase	P31749, P31751, Q9Y243
AMPka2	5'AMP-activated protein kinase catalytic subunit	Q28948
b-Catenin		Q90424
Chk-2	Serine/threonine-protein kinase	O96017
CRB	Protein crumbs homolog 1	P82279
EGFR		P00533
FAK	Focal adhesion kinase 1	Q05397
Fgr	Tyrosine-protein kinase	P09769
Fyn	Tyrosine-protein kinase	P06241
GSK-3a/b	Glycogen synthase kinase-3	P49840, P49841
Hck	Tyrosine-protein kinase	P08631
JNK 1/2/3	Mitogen-activated protein kinase	P45983, P45984, P53779
Lck	Tyrosine-protein kinase	P06239
Lyn	Tyrosine-protein kinase	P07948
MSK1/2	Ribosomal protein S6 kinase	O75582, O75676
PRAS40	Proline-rich AKT1 substrate	Q96B36
Src	Proto-oncogene tyrosine-protein kinase	P12931
STAT2	Signal transducer and activator of transcription 2	P52630
STAT5a	Signal transducer and activator of transcription 5a/b	P42229, P51692
STAT6	Signal transducer and activator of transcription 6	P42226

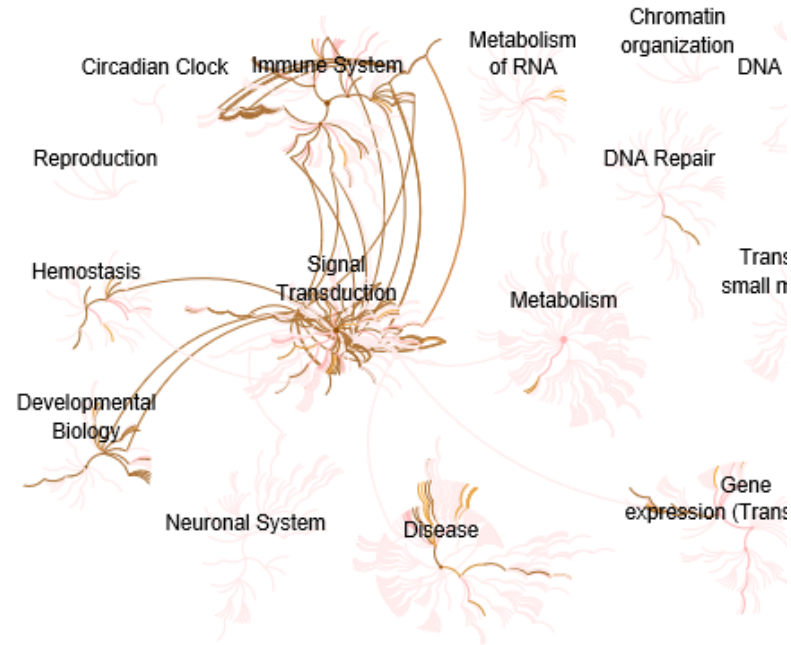
H2.35_hNTCP		
Gene	**Name**	**Uniprot Accession**
AMPka1	5'AMP-activated protein kinase catalytic subunit	Q13131
eNOS	Endothelial nitric oxide synthase	P29474
ERK1/2	Mitogen-activated protein kinase	P27361, P28482
p53	Cellular tumour antigen	P04637
p70 S6	Ribosomal protein S6 kinase beta	P23443
PYK2	Protein-tyrosine kinase	Q14289
RSK1/2/3	Ribosomal protein S6 kinase alpha	Q15418, P51812, Q15349
STAT3	Signal transducer and activator of transcription 3	P40763
mTOR	Serine/Threonine-protein kinase	P42345
WNK1	Serine/Threonine-protein kinase	Q9H4A3

***Table 3.5*; Genes, Names and Uniprot Accession numbers of the different phospho-kinase signalling factors activated in either a human or a mouse cell line, overexpressing different forms of the NTCP receptor, upon infection with HBV.**

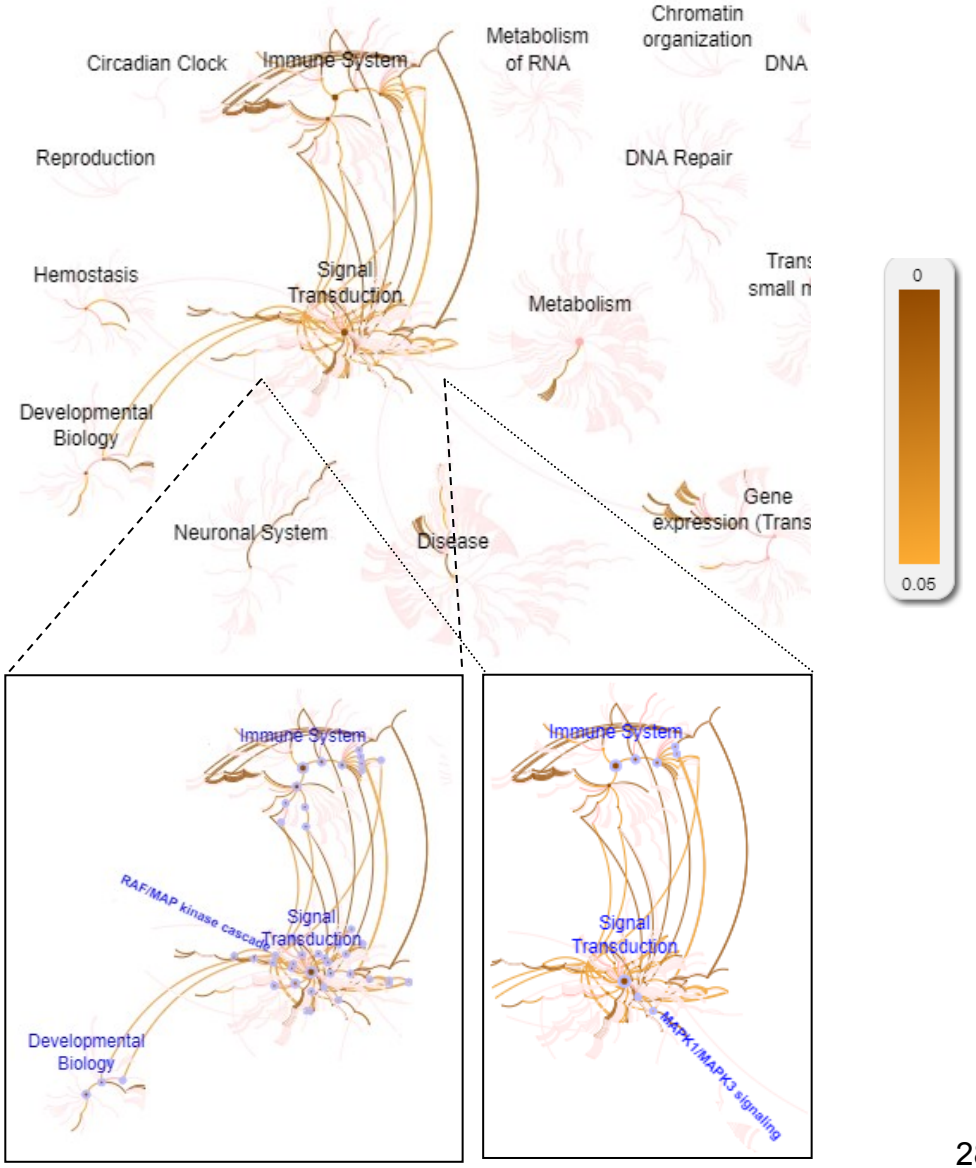
Next, we went on to perform a signalling pathway analysis utilising the Reactome pathway database browser (www.reactome.org; version v61). Interestingly, this analysis revealed that, in the stable mouse hepatic cell line overexpressing the chimNTCP receptor, after infection with HBV the RAF/MAP kinase cascade and MAPK1/MAPK3 signalling are primarily activated; this was not apparent in the stable human hepatic cell line overexpressing the hNTCP receptor (*Figure 3.54*). These particular pathways, in the infected mouse hepatocytes, appear to be involved principally in processes regulating the immune system, as well as development. In general, the RAF/MAP pathway is known to regulate many cellular processes such as proliferation, differentiation, senescence and is essential for the survival of any organism. Similarly, the MAPK1/MAPK3 pathway becomes activated in response to a wide range of extracellular stimuli, so as to promote proliferation, differentiation and survival, amongst other [306-308].

Signalling Pathway Analysis:

HepG2_hNTCP



H2.35_hNTCP



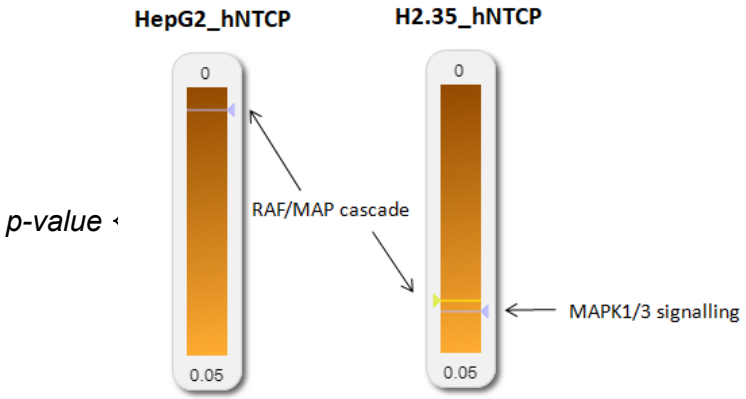


Figure 3.54; Signalling pathway analysis activated in either a human or a mouse cell line, overexpressing different forms of the NTCP receptor, upon infection with HBV.

Upon infection of the stable mouse hepatic cell line overexpressing the chimNTCP receptor with HBV two signalling pathways were predominantly activated; the RAF/MAP kinase cascade and the MAPK1/MAPK3 signalling pathway. These pathways are shown to be particularly involved in regulating the immune system responses, as well as developmental biology processes. Interestingly, these two pathways did not appear to become activated, at least to the same extent, in the stable human hepatic cell line overexpressing the hNTCP receptor. Analysis was performed with the Reactome pathway database browser (v61).

In summary; DMSO appears not to regulate a chimeric form of the NTCP receptor in the same way as it does the human NTCP receptor in the HepG2 and H2.35 stably overexpressing cell lines, thus also confirming the extent of the difference between the human and mouse forms of the receptor and possibly explaining partially the difference between human and mouse hepatocytes in regards to their susceptibility to HBV infection. Most interestingly, however, upon overexpression of the designed chimeric NTCP receptor, mouse cells consistently became susceptible to HBV infection. As a consequence, different phospho-kinase signalling pathways were found to be activated in the two cell culture systems (human vs. mouse hNTCP overexpressing cell lines).

3.2.3 Expression of the chimeric NTCP receptor renders mice susceptible to low-level HBV infection

After repeating analogous HBV infection experiments a number of times *in vitro*, we decided to attempt a preliminary *in vivo* HBV infection experiment. ***The animal, in vivo, side of this experiment was performed by Dr. Marcus Dorner****, while the analysis of the samples was performed by me****.*** This was only a very preliminary experiment, however, it may provide useful information towards the creation of a small animal model suitable for studying HBV infection.

We specifically made use of NOD.Rag1-/-IL2gR-/- mice (https://www.jax.org/strain/007799), also known as NRG mice, which are severely immunocompromised. We started off with a cohort of five, male and female, mice and we hydrodynamically delivered separately three plasmids; a control plasmid expressing Fluc (1x mouse), a plasmid expressing the hNTCP receptor (2x mice) and a plasmid expressing the chimNTCP receptor (2x mice). At day four post-hydrodynamic delivery of the different plasmids, we infected the mice with HBV infectious supernatants collected from confluent HepG2.2.15 cells at an MOI of 1000. We took the experiment up to a total of 16 days, and monitored the weight of the mice every day up till 12 days, as everything looked normal (*Figure 3.55*).

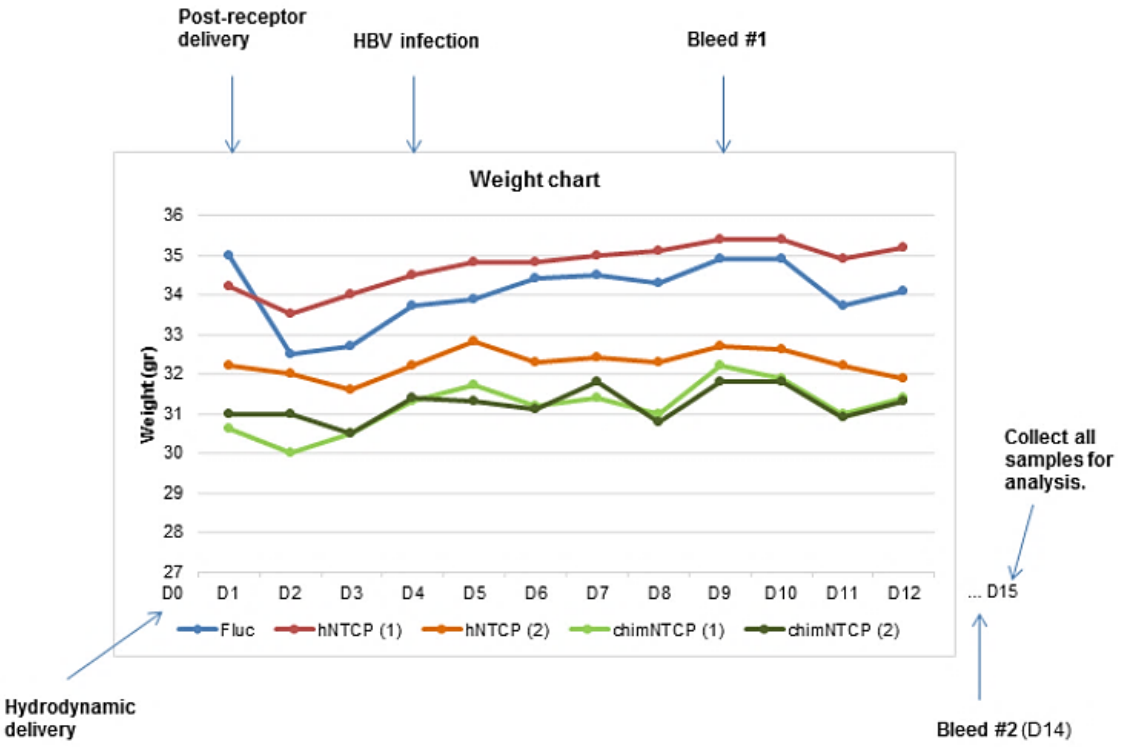


Figure 3.55; Weight chart of mice into which two forms of the NTCP receptor were separately hydrodynamically delivered, a human and a chimeric form, so as to evaluate if they would facilitate HBV entry further allowing the mice to support viral infection.

The weight of the mice was monitored from the first day (D1) post-delivery of the plasmids up till day twelve (D12), and no abnormalities were observed. In only a few cases there was a small drop in the animal's weight post-delivery but it wasn't considered a serious drop, and even post-HBV infection all the animals appeared normal.

Initially, we bled the mice at days five and ten post-infection with HBV, so as to determine any level of circulating virus within their blood. We specifically measured the HBV DNA copies/ng of extracted DNA sample (*Figure 3.56, A*), while we also directly measured the levels of the secreted HBsAg (*Figure 3.56, B*). At both time points, only one out of the two mice (i.e. chimNTCP, 2) in which we had hydrodynamically delivered the chimNTCP receptor was considered to be positively infected with the administered virus, as we were clearly detecting higher levels of HBV DNA copies and HBsAg compared to the background infection levels.

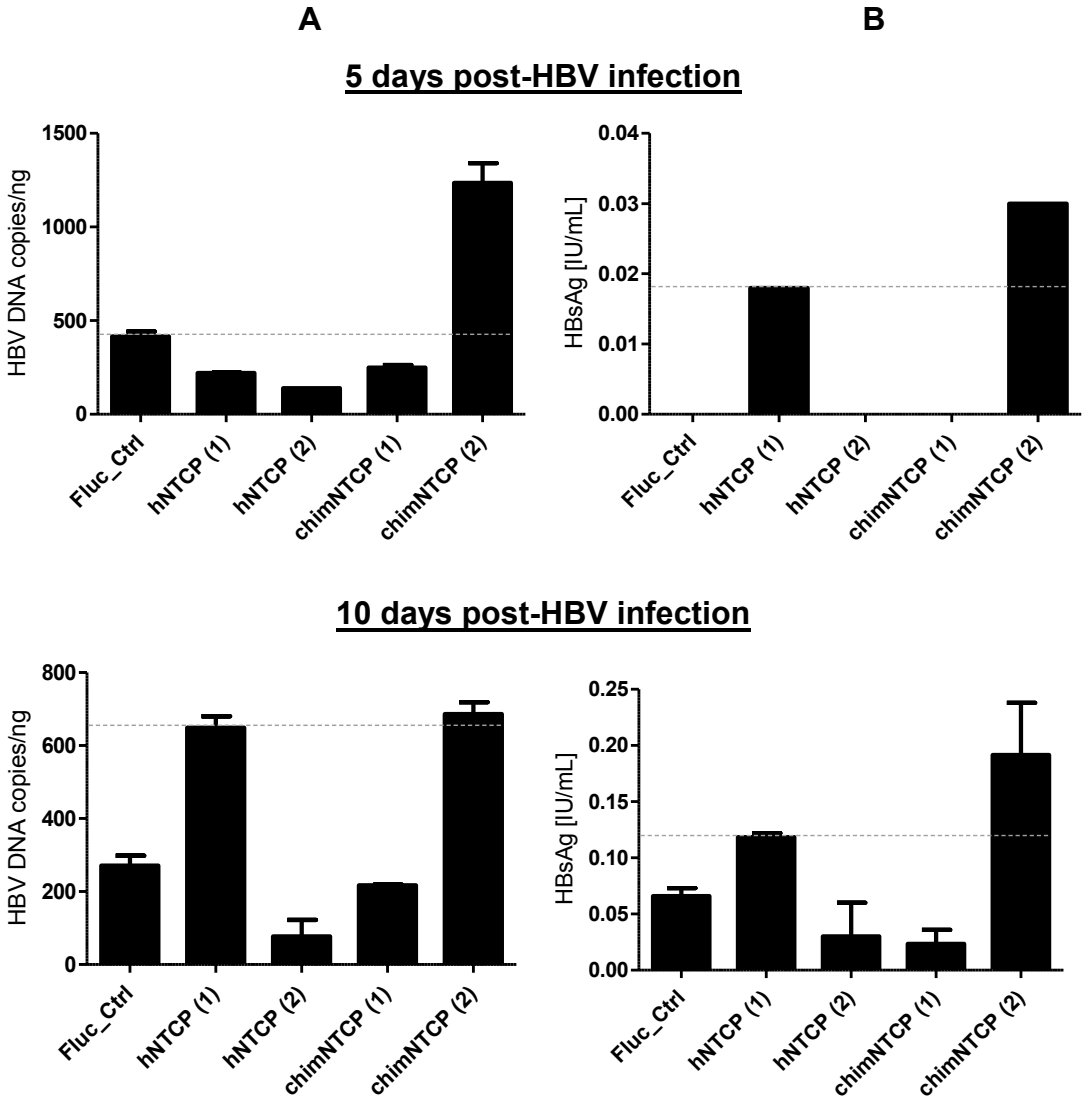


Figure 3.56; Evaluating the susceptibility of mice, overexpressing either the human NTCP receptor or the chimNTCP form of the receptor, to HBV infection (day 5 and 10).

The HBV DNA copies/ng of DNA **(A)** and the secreted HBsAg levels **(B)** of the virus circulating in the animal's blood were particularly measured. The dotted lines indicate the limit of background infection (*mice expressing the hNTCP receptor cannot theoretically support HBV infection*), and values above the dotted lines are considered positive for HBV infection. Therefore, the mouse expressing the chimNTCP (2) receptor was probably supporting HBV infection from day 5 at least up until day 10, nevertheless the infection levels were low and probably the animal was slowly resolving infection over time.

We culled the animals on day 11 post-infection and collected any available samples, such as blood and liver. Unfortunately, liver samples could not be collected from all animals (i.e. a liver sample could not be collected for animal "hNTCP(2)", due to certain problem when culling the animal), and therefore only the equivalent blood samples were used for subsequent analysis. We extracted DNA from the blood and liver and measured the total HBV DNA copies/ng of DNA sample (*Figure 3.57; A* and *B,* respectively), while we additionally extracted RNA but only from the liver so as to measure the levels of NTCP mRNA expression (*Figure 3.57, C*). We determined that both mice that had been hydrodynamically injected with the chimNTCP receptor plasmid had low but detectable levels of HBV infection present, as compared to the background infections levels and as seen in both their blood and liver samples (*Figure 3.57; A* and *B,* respectively). Interestingly, when we quantified the levels of NTCP mRNA expression present in the corresponding liver samples, we determined that only the mouse expressing the "chimNTCP (2)" receptor had detectable and considerably higher levels of NTCP mRNA expression. This was the mouse that had positive levels of HBV infection measured also at day five and day ten post-infection.

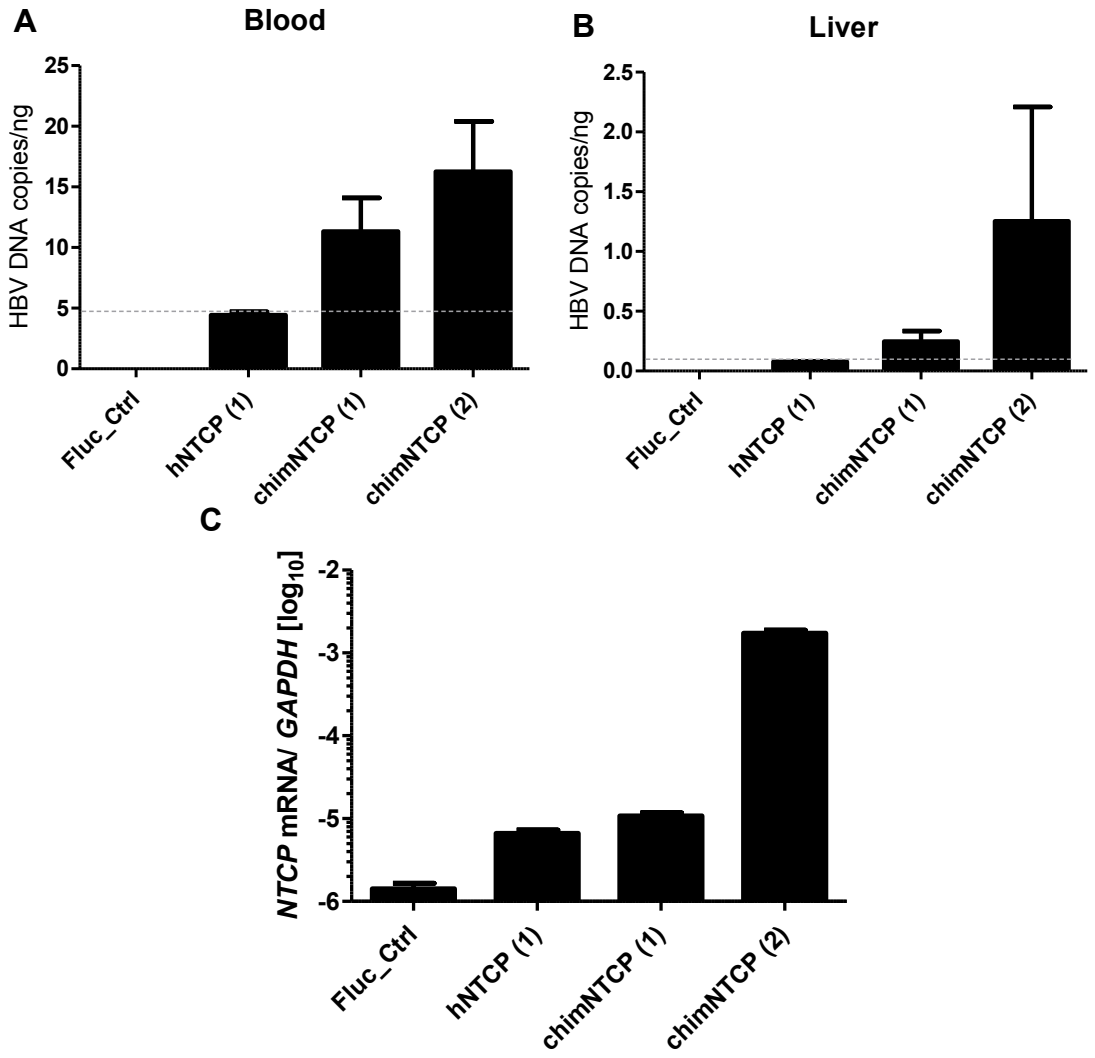


Figure 3.57; Evaluating the susceptibility of mice, overexpressing either the human NTCP receptor or the chimNTCP form of the receptor, to HBV infection (day 11).

The HBV DNA copies/ng of sample DNA in the blood **(A)** and in the liver **(B)** of the mice were particularly measured. Both in the blood and the liver of the two mice expressing the chimNTCP receptor we detected low but positive levels of HBV infection. **(C) mRNA analysis of the human or mouse NTCP receptor in mice hydrodynamically injected with the analogous constructs.** Only one of the mice expressing the chimNTCP receptor (i.e.chimNTCP(2)) actually had high expression levels of that receptor. The Fluc_Ctrl was the negative control sample.

In summary; In a preliminary *in vivo* experiment we performed, upon overexpression of a designed chimeric NTCP receptor, one mouse was considered to have become susceptible and briefly supported HBV infection. This requires further investigation before any final conclusions can be drawn (*see suggestions in Discussion chapter; Section 4.3, "Future work"*).

3.3 Enhancing HBV susceptibility by identifying novel host factors restricting HBV replication in low-susceptibility or altogether non-susceptible hosts, *in vitro*

For detailed information on "Lentiviral cyclic repackaging screening for the identification of novel host factors required for HBV entry", please refer to Section 2.2.2.4.1.3.1.

It should be noted, however, that for this procedure the generation of the HBV- & HDV-mimicking lentiviral pseudoparticles (*as already discussed in Section 3.1.5.3.1*), as well as the generation of recombinant HBV viruses, which will be discussed below, played a very important role.

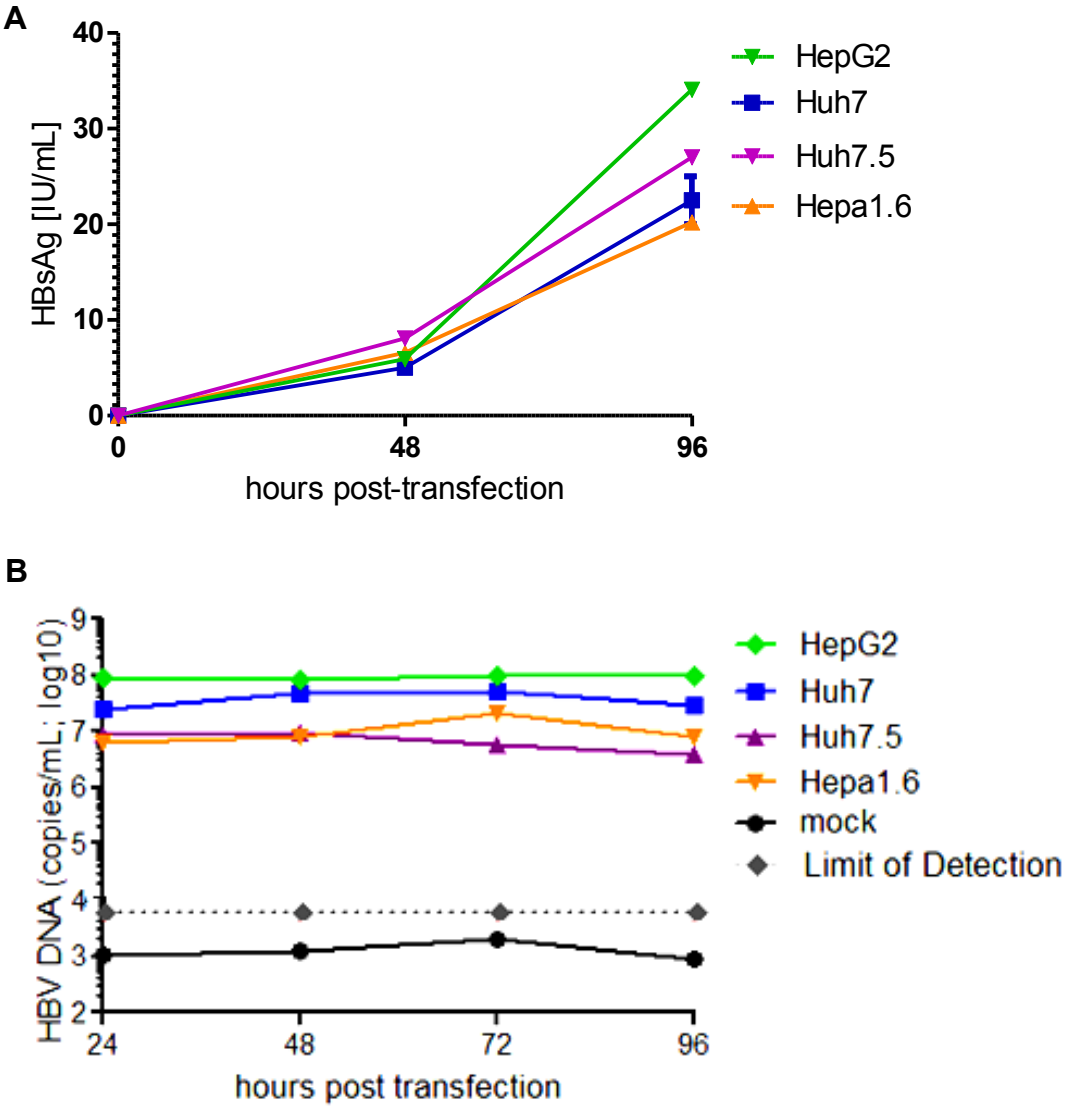
3.3.1 Generation and characterisation of recombinant HBV viruses

HBV replication-competent genomes encoding reporter genes have over the years become invaluable tools for easy and quantitative monitoring of virus infection and replication [266, 309]. Therefore, we were interested in testing this out for ourselves. Before, however, attempting to create such constructs, we needed to confirm the functionality of an original 1.3kb overlength HBV plasmid in regards to being able to efficiently replicate within cells.

3.3.2.1 Characterisation of a pCMV_HBV plasmid

The HBV plasmid we had available, as already mentioned, contained a HBV overlength (1.3kb) sequence under the control of a CMV promoter. In order to characterise this construct we simply transfected it into different types of cells so as to determine whether or not it could establish replication and hence could functionally infect those cells.

In particular, we utilised parental human hepatoma HepG2, Huh7 and Huh7.5 cells, while we additionally included the mouse hepatoma Hepa1.6 cell line, as we thought it would be interesting to evaluate whether this HBV plasmid could also establish replication/ infection within mouse cells after bypassing the entry step. We transfected the plasmid into the cells and took this experiment up to 96 hours post-transfection with the HBV plasmid. Samples were collected every 24 hours and were analysed for secretion of HBsAg by ELISA, production of HBV DNA copies by quantitative PCR and formation of cccDNA by a typical PCR procedure (*Figure 3.58; A, B* and *C,* respectively). Interestingly, comparable levels of HBV replication were seen in all cell lines, in all different types of analysis performed.



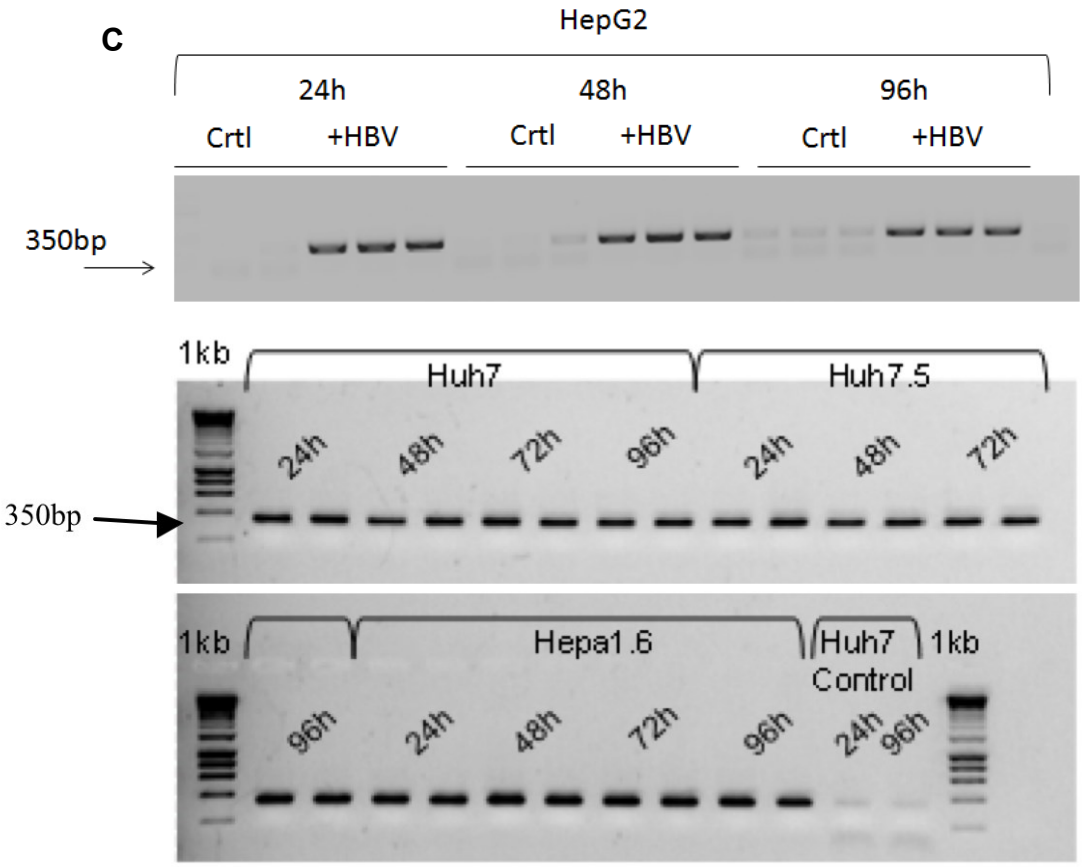


Figure 3.58; Characterisation of a 1.3kb overlength HBV plasmid.
Transfection of HepG2, Huh7, Huh7.5 and Hepa1.6 cells with the pCMV_HBV plasmid resulted in highly comparable levels of HBsAg being secreted **(A)** and HBV DNA copies being expressed **(B)** over time, while we were also able to distinguish cccDNA formation in the transfected cells **(C)**.

In summary; We confirmed the functionality of an original pCMV_HBV construct in terms of its ability to infect and replicate within cells. Furthermore and most interestingly, we also confirmed that, as well as establishing HBV infection and replication in human cells, we could also do so in mouse cells but after virus entry was bypassed by direct transfection of the HBV plasmid into the cells. These results were validated up to 10 days post-transfection with the HBV plasmid.

3.3.2.2 Characterisation of the newly created HBV constructs encoding transgenes

For full details in regards to the cloning strategy performed for the creation of the HBV constructs expressing either a fluorescent or a selection marker, please refer to Section 2.2.1.1.2.

To begin with, once we had created the replication-competent HBV genomes encoding the various reporter genes, we were interested in characterising their exact replication-competency and specifically to verify that it compared to that of the original pCMV_HBV. From the four newly created HBV constructs we initially decided to continue only with two of them; the ones expressing either the BFP or BsdR transgene. As all plasmids had been now cloned from an original pCMV vector backbone into a pCDNA3.1(-) vector, with their expression once again under the control of a CMV promoter, we additionally included the pCDNA3.1(-)_HBV plasmid so as to confirm that different vector backbones should not have an effect on the replication-competency of these HBV plasmids. The set up of the experiment was similar to the one previously described; specifically, parental HepG2 cells were transfected with either one of the plasmids, the cells were then maintained for 144 hours post-transfection and samples were collected every third day. The samples from the two time points were finally analysed for HBsAg and HBeAg secretion by ELISA, HBV DNA copies by quantitative PCR (*Figure 3.59; A, B, and C,* respectively), as well as for production of viral RNAs. For viral RNAs, we particularly analysed the 3.5kb pgRNA and total HBV sgRNA levels by qRT-PCR (*Figure 3.59; D and E,* respectively). The replication efficiencies of all transfected plasmids were highly comparable, as determined by the similar levels of HBV antigens, DNA copies and viral RNAs being produced from the transfected cells.

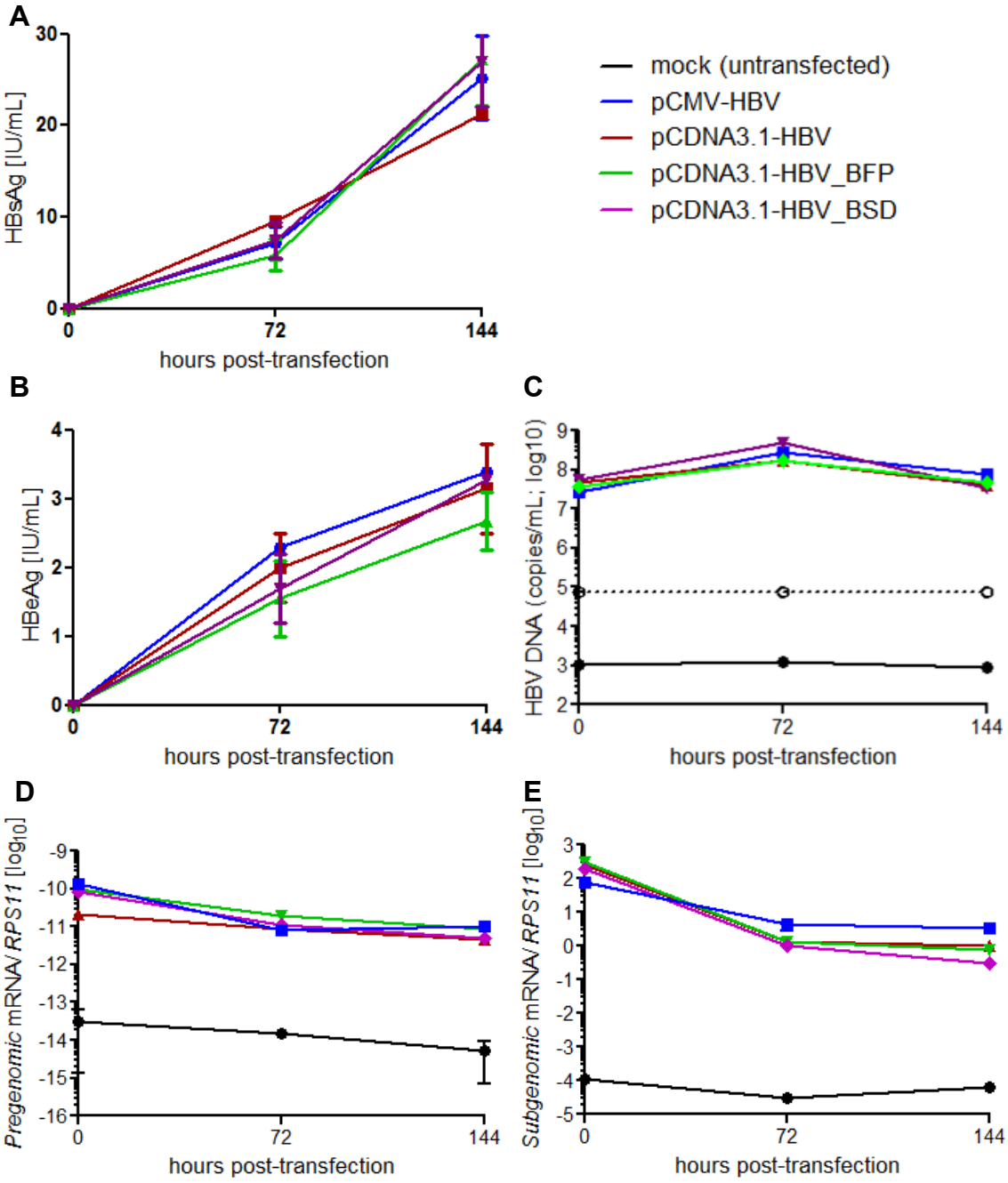


Figure 3.59; Characterisation of two newly generated recombinant HBV genomes encoding transgenes.

Transfection of the two recombinant HBV constructs encoding the different transgenes (BFP or BsdR) into HepG2 cells, generally, resulted in comparable levels of secreted HBsAg **(A)** and HBeAg **(B)**, equivalent levels of HBV DNA copies being expressed **(C),** as well as comparable levels of pregenomic **(D)** and subgenomic **(E)** RNA copies being produced. In addition, these replication levels were comparable to the ones produced by the two non-transgene encoding HBV constructs, pCMV_HBV and pCDNA3.1_HBV. Similar outcomes were obtained when these plasmids were transfected into Huh7.5 or Hepa1.6 cells. *The mock (untransfected) samples, indicated by the black lines, for the secreted HBs/eAg were equal to zero for all time points analysed. The dashed line indicates the limit of detection.*

Following on, we also performed immunofluorescence of the HepG2 cells particularly transfected with the BFP-expressing HBV plasmid (*Figure 3.60*). An increasing number of BFP-positive cells were observed over time, suggesting that there was ongoing HBV replication within these cells.

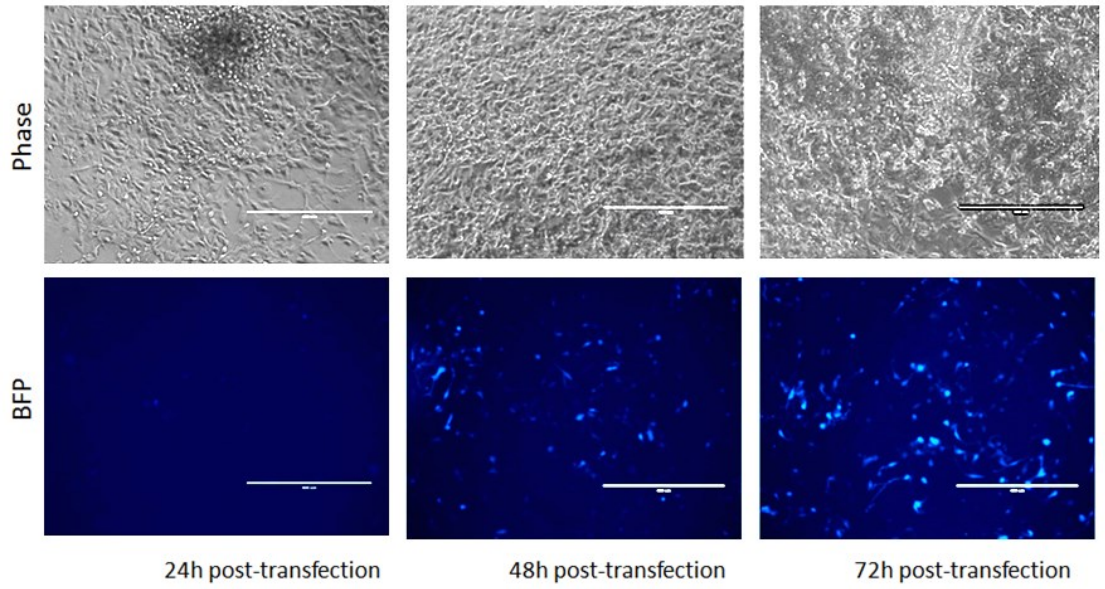


Figure 3.60; Immunofluorescence analysis of the HepG2 cells after transfection with the HBV plasmid encoding for BFP.
Over time more cells became BFP-positive, and hence these cells would have had active HBV replicating within them. A similar outcome was obtained when this plasmid was transfected into Huh7 cells. The image scale bar is 400µm.

In summary; We confirmed the functionality of the newly created replication-competent HBV plasmids encoding the various reporter genes, specifically in terms of them replicating as efficiently as the original non-transgene encoding HBV plasmids. However, as the final aim was to create replication-competent HBV virus encoding the transgenes of interest that could be further used to re-infect cells, we were interested in testing whether there were any other possible ways of improving the transfection efficiency and hence the virus yield for use in subsequent infection experiments.

3.3.2.3 Further evaluation of virus production from the newly created HBV constructs encoding transgenes

Having established that all HBV plasmids, the originals and the newly created, replicated comparably well within cells, we decided for this next experiment to only make use of the BFP-encoding HBV construct, as the transfection efficiency of the plasmid into the cells could be easily visualised by immunofluorescence. Initially, we thought of testing virus production after transfection of this specific plasmid into different stable human hepatic Huh7.5 cell lines, meaning cell lines either stably overexpressing hNTCP or both hNTCP/hASGPR, as well as the parental controls. We decided to use these variations of the Huh7.5 cells to examine whether the presence of the receptors would promote cell-to-cell spread and hence enhance virus production. Additionally, we thought of performing this experiment in a Huh7.5 cell background, rather than the HepG2 human hepatic cell lines, due to the fact that the former generally resulted in higher transfection efficiencies.

Therefore, similarly to before, we transfected the HBV_BFP plasmid into the three different Huh7.5 cell lines and carried out the experiment up till 120 hours post-transfection. The samples were collected at three time points (48, 72 and 120 hours post-transfection) and were analysed for HBsAg and HBeAg secretion by ELISA (*Figure 3.61; A* and *B,* respectively). Immunofluorescence analysis of the cells was performed up till 72 hours (*Figure 3.61, C*). In the case of the Huh7.5 hNTCP/hASGPR cells transfected with the HBV_BFP plasmid, we also treated a subset of these cells with 2% DMSO pre- and during transfection so as to determine whether addition of DMSO could possibly increase the virus yield.

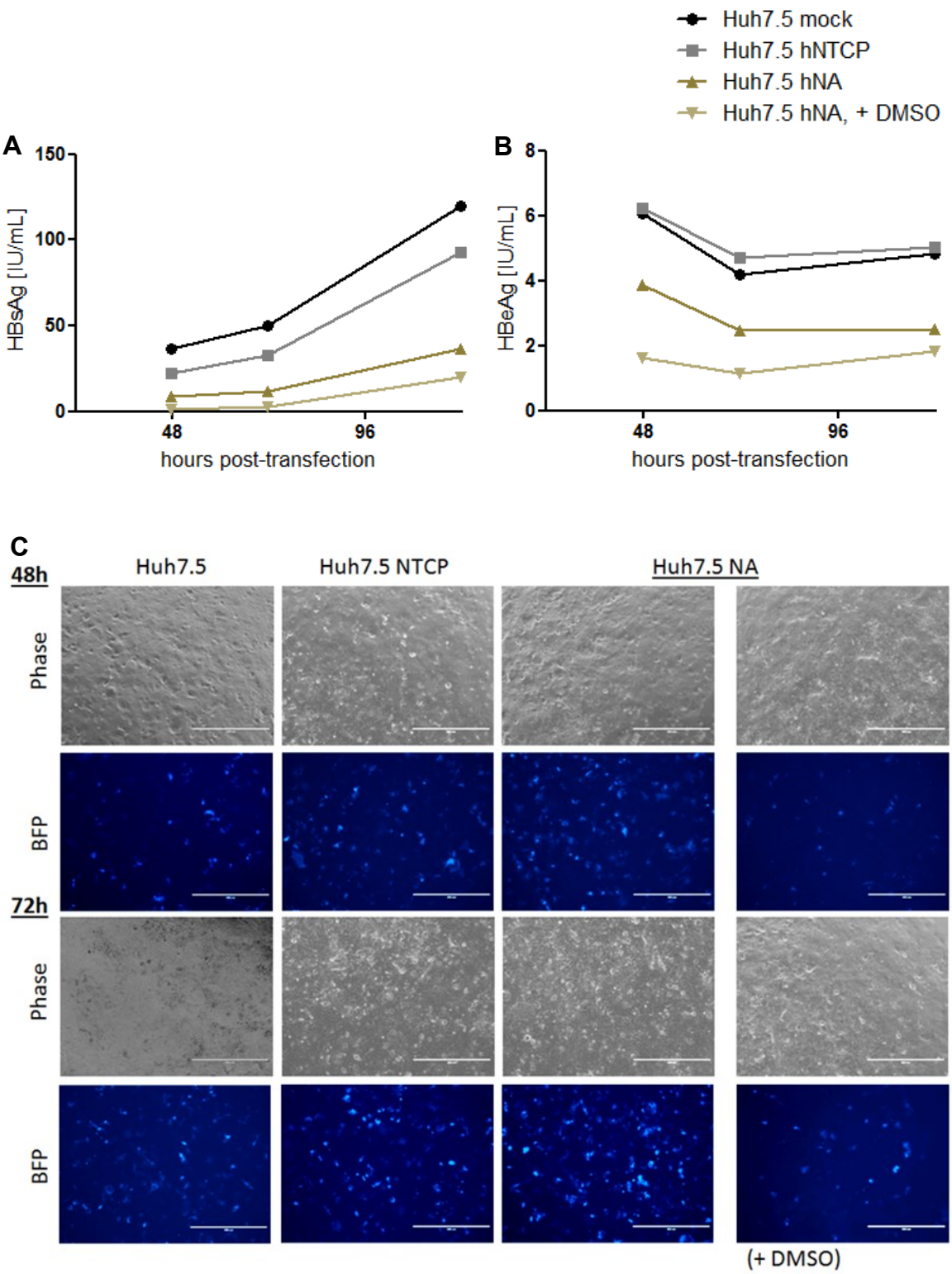


Figure 3.61; Characterisation of the BFP-expressing HBV genome in Huh7.5 and stable Huh7.5 cell lines overexpressing HBV-related receptors.

Positive replication was established in all three cell lines but to considerably varying degrees, as determined by HBsAg **(A)** and HBeAg **(B)** secretion, as well as immunofluorescence analysis **(C)**. *Mock, refers to the parental cells. hNA: hNTCP/hASGPR.* The image scale bar is 400µm.

In particular, the outcomes of this experiment were as follows:

1) We established positive replication up to 5 days post-transfection in the three different cell lines tested, yet to very different levels.

2) The presence of the receptors did not result in the anticipated enhanced virus yield, as the parental cells were still better at permitting virus production. However, virus replication in the presence of hNTCP was not that far off the levels established in the parental cells, whereas the presence of both HBV-related receptors seemed to substantially inhibit it.

3) The presence of DMSO definitely inhibited even greater the transfection efficiency and hence the replication of HBV.

4) Unexpectedly, however, the secretion levels of the two HBV antigens and the immunofluorescent data did not match and were somewhat contradictory to each other. For example, even though the Huh7.5 double-receptor-expressing cells after transfection with the HBV_BFP plasmid resulted in very low secretion of both HBsAg and HBeAg, yet when visualising the cells under the microscope there were definitely more BFP-positive cells in that cell line compared to the rest. A possible explanation could be that the receptors may have been binding the HBs/eAgs and hence preventing their proper secretion.

As the results from the last experiment were rather inconclusive, we decided to transfect the BFP and this time also the BSD HBV-encoding plasmids into the HepG2.2.15 cell line, previously used for the collection of infectious HBV virus for subsequent infection experiments. The idea was that transfecting these plasmids into a cell line that already replicates HBV and produces a stable level of infectious viral particles could possibly enhance production of virus that additionally encodes transgenes of interest. Therefore, we transfected individually the two plasmids, HBV_BFP/_BSD, into the HepG2.2.15 cells and carried out the experiment up till 144 hours post-transfection. Samples were

collected every 48 hours and were analysed for HBsAg and HBeAg secretion by ELISA (*Figure 3.62; A* and *B,* respectively). Immunofluorescence analysis of the cells transfected with the HBV_BFP plasmid was also performed (*Figure 3.62, C*). Interestingly, transfecting the HepG2.2.15 cells with the HBV plasmids encoding for the two different reporter genes resulted in the recovery of a somewhat higher virus yield.

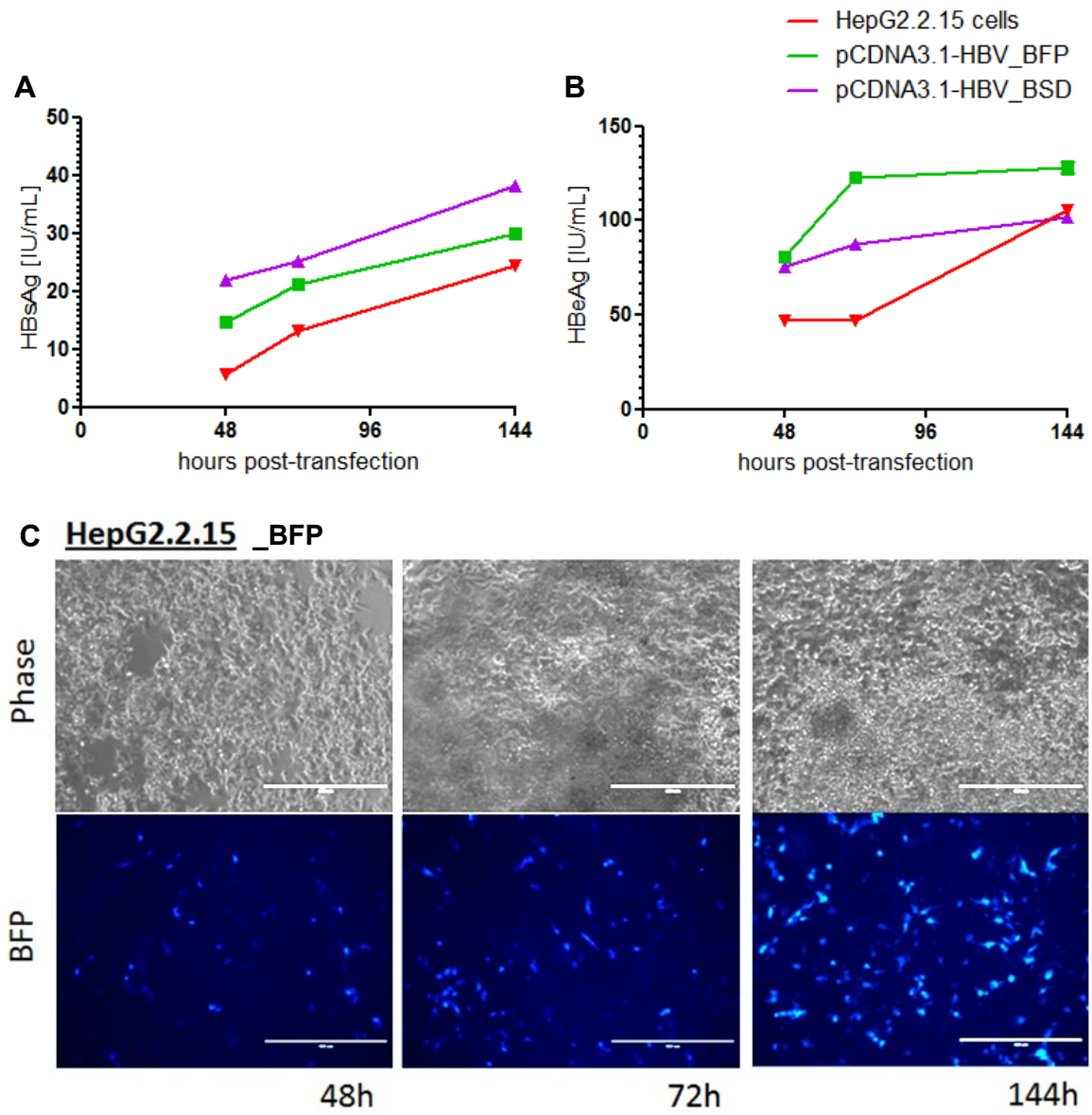
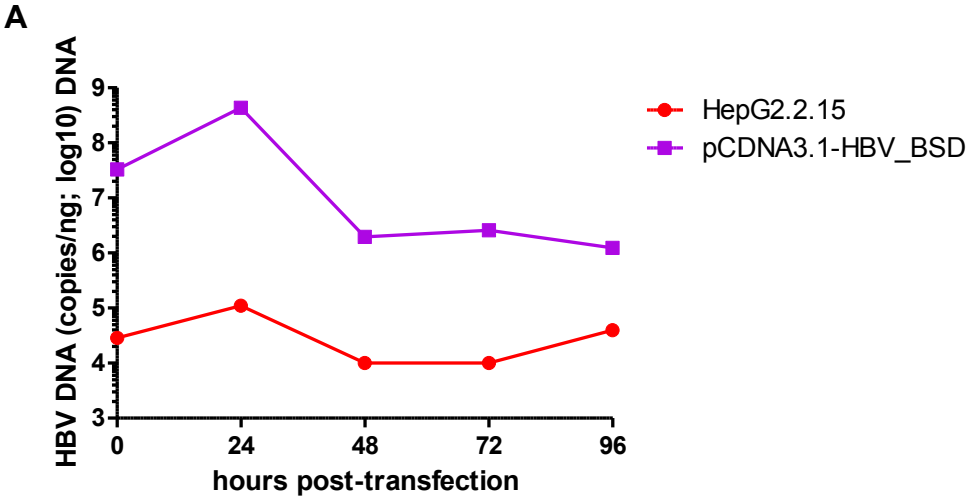


Figure 3.62; Characterisation of the BFP- and BsdR- expressing HBV genomes in HepG2.2.15 cells.

Positive replication of the HBV plasmids encoding the reporter genes was established in the HepG2.2.15 cells, with the transfected cells producing even higher levels of virus compared to the parental cells, as specifically determined by the secreted HBV antigens **(A and B)**. Increasing HBV replication was also observed over time by immunofluorescence of the cells transfected with the HBV_BFP plasmid **(C)**. The image scale bar is 400µm.

As a consequence, we decided to produce a bigger quantity of the virus encoding the BsdR for use in subsequent re-infection experiments. We transfected the HepG2.2.15 cells with the HBV_BSD plasmid, as well as a plasmid encoding GFP (pTRIP_Venus; *Table 2.4*) so as to be able to visualise the efficiency of the transfection. The experiment was carried out for 96 hours and samples were collected every 24 hours. This time we analysed the samples for expression of HBV DNA copies (*Figure 3.63, A*) as we had already evaluated secretion of the HBV antigens, while we also examined the transfected cells by immunofluorescence (*Figure 3.63, B*). At the end of the experiment, once we had once again confirmed positive replication of the HBV_BSD plasmid within the HepG2.2.15 cells, producing higher levels of virus compared to the parental cells, we collected all supernatants and stored them for future use in a single re-infection experiment.



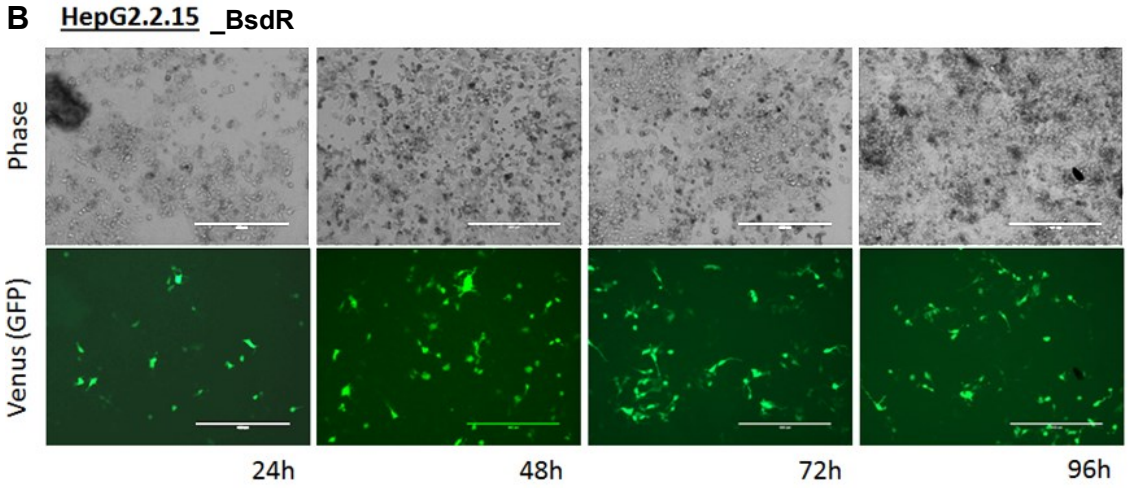


Figure 3.63; Additional characterisation of the BsdR-expressing HBV genome in HepG2.2.15 cells.

(A) Analysis of the HBV DNA copies revealed positive replication of the HBV_BSD plasmid within the HepG2.2.15 cells, with the transfected cells producing higher levels of virus compared to the parental cells. **(B)** Immunofluorescence analysis confirmed ongoing positive replication of the HBV_BSD plasmid within the cells. The number of GFP-positive cells should theoretically be equivalent to the number of cells positively transfected with the HBV_BSD plasmid. The image scale bar is 400μm.

Finally, to confirm that what we were detecting in our analysis was newly produced virus and not residual plasmids from the transfection procedure, we digested a small fraction of the supernatants with either DNAseI or DpnI (both of these enzymes typically digest input DNA plasmids present within samples). After we digested the samples, we re-analysed them and were able to detect the exact same levels of virus being present. Therefore, what we had been detecting all along must have been newly synthesised virus.

In summary; We confirmed that transfection of the reporter-encoding HBV plasmids into a cell line that already stably replicates HBV and produces a constant level of infectious viral particles, such as the HepG2.2.15 cells, resulted in enhanced production of virus that should additionally express the transgene of interest. This recombinant virus can now be used in subsequent re-infection experiments further allowing for selection of the cells that have been positive infected with the virus either by selection with a specific antibiotic

(e.g. BsdR) or, in other occasions, by sorting the cells according to the expression of a particular fluorescent marker.

3.3.2.3.1 Additional attempts to produce functional recombinant HBV virus encoding transgenes

During the course of this project, many different attempts were made to produce high yield, functional virus from the replication-competent HBV genomes encoding the reporter genes. One of the first ideas we tested was whether transfecting additional HBV plasmids (i.e. plasmids expressing different HBV genes) together with the full-length, reporter-expressing HBV plasmids would aid virus replication and hence improve virus production. For example, we simultaneously transfected the reporter-expressing HBV plasmids with a construct specifically expressing the full-length HBsAg sequence. By doing so we hoped that we could improve certain stages of the virus' life cycle. Nevertheless, the extra availability of HBsAg had varying effects on virus production (*Figure 3.64*); in particular, the additional presence of HBsAg significantly enhanced virus production when the plasmid was co-transfected with the wild-type and BsdR reporter-expressing HBV plasmids (*p-values* of 0.005 and 0.02, respectively), while when co-transfected with the BFP reporter-expressing HBV plasmid no significant effect could be observed. Similarly, a plasmid expressing the full-length HBV polymerase sequence could have also been utilised and co-transfected with either one of the replication-competent HBV constructs, so as to determine its effects on improving HBV replication and further virus production.

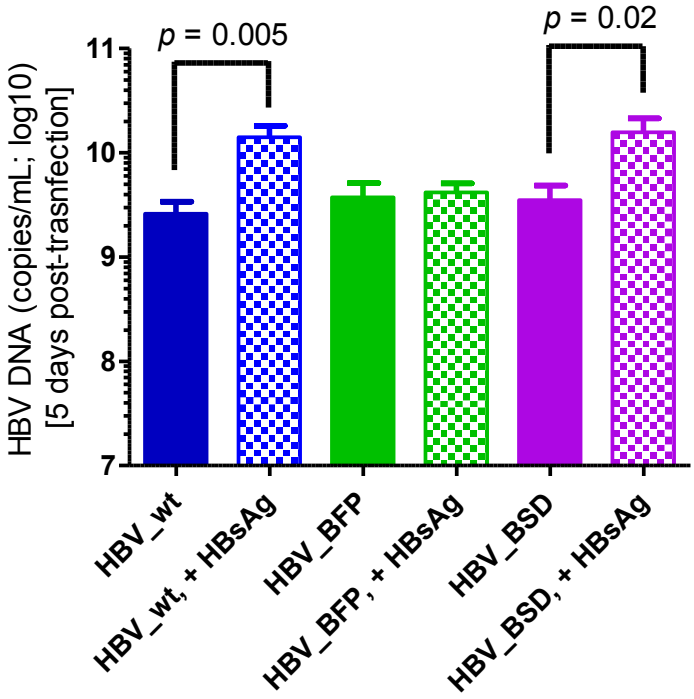


Figure 3.64; Analysis of the effect of additional HBsAg being co-transfected with the different HBV genomes, wild-type (wt) or reporter-expressing, on virus production.

The presence of additional HBsAg expression demonstrated to have a significant effect on enhancing HBV production when the plasmid was particularly co-transfected with the wt or BSD-expressing HBV genomes. No significant effect was determined when HBsAg was co-transfected with the BFP-expressing HBV genome.

Following on, we attempted electroporating the reporter-expressing HBV plasmids, rather than transfecting them, into cells. We particularly tested electroporating the cells with different plasmid concentrations, while also culturing them under different FBS concentrations so as to enhance cell viability (*Figure 3.65*; *A a*nd *B, respectively*). The results were extremely variable.

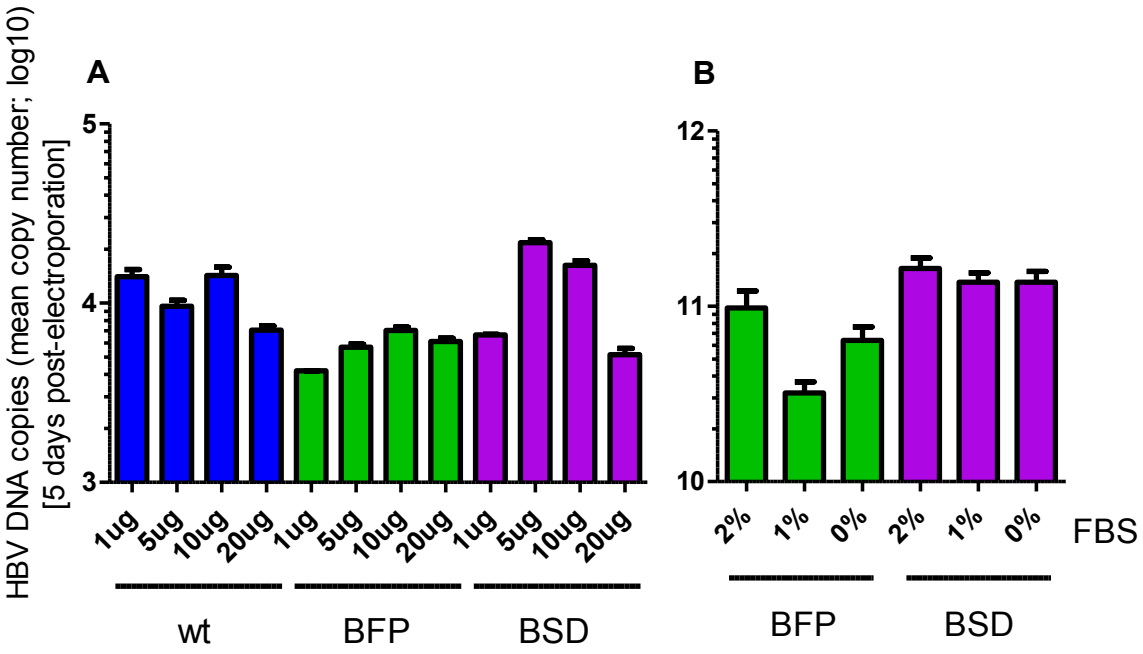


Figure 3.65; Analysis of the effect of different plasmid and FBS concentrations during HBV plasmid electroporation on virus production.

The different conditions tested during HBV plasmid electroporation into cells had variable outcomes.

Furthermore, we tested various virus concentration and purification techniques. We began by testing Amicon® filters for concentration of the HepG2.2.15 virus supernatants and determined that, even though we were concentrating the virus to some extent, we were also losing some of the viral particles in the flow-through (*Appendix A.3.VI*). Instead we tried testing virus concentration through an iodixanol gradient, specifically for virus collected from cells transfected with the BSD-expressing HBV plasmid, and we managed to recover some fractions of media containing possibly infectious virus. The fractions collected were the ones with a simultaneous peak for HBsAg secretion and HBV DNA copy expression (*Figure 3.66*).

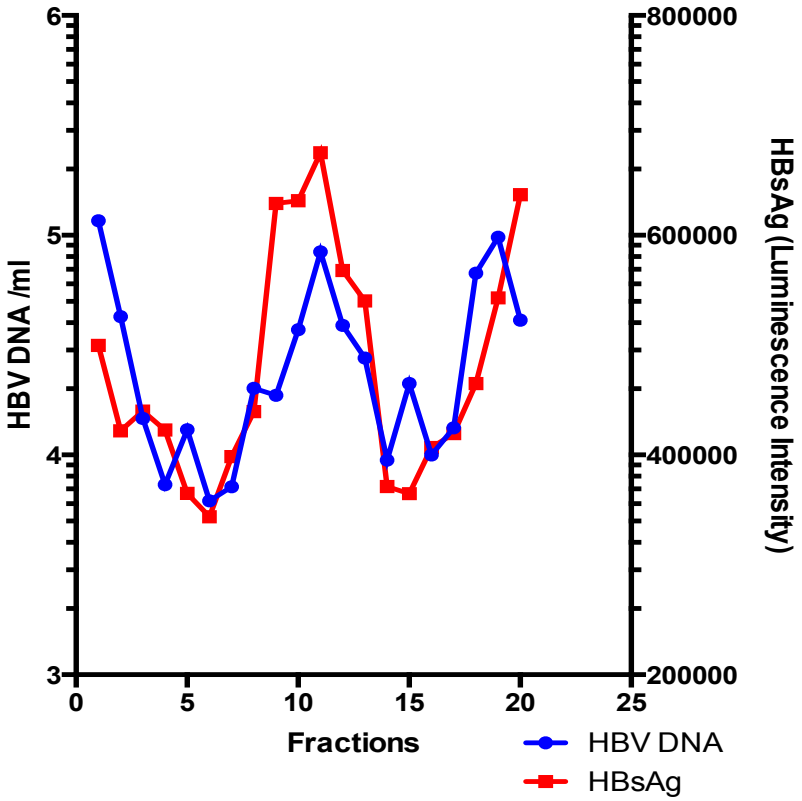


Figure 3.66; Analysis of the levels of secreted HBsAg and HBV DNA copies expressed in twenty fractions collected after an iodixanol gradient of supernatants collected from cells transfected with the BSD-expressing HBV plasmid. The samples regarded as infectious and thus collected for future use were fractions 9 to 13.

Finally, we tested the effect of antibiotic selection on long-term virus production of cells transfected with the reporter-expressing HBV plasmids and determined that, even though there was clearly attenuated HBV virus production during the course of antibiotic treatment of the these cells (as seen by the levels of secreted HBsAg and the HBV DNA copies expressed; *Figure 3.67, A* and *B* respectively), there was possibly active HBV replication occurring (as seen by the production of pregenomic and subgenomic RNA copies; *Figure 3.67, C* and *D* respectively). This outcome could be of importance during the cyclic repackaging rescue screen.

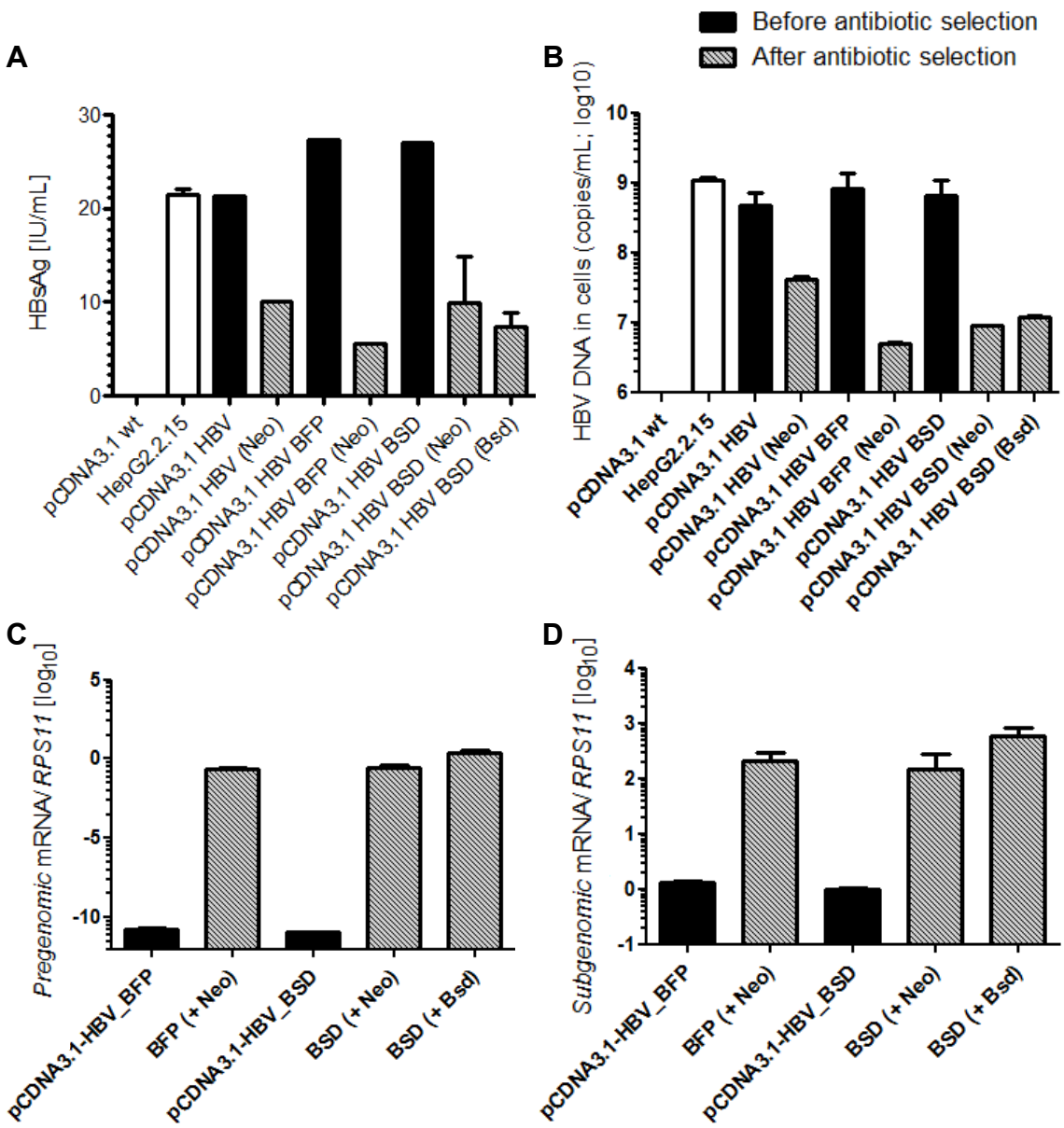


Figure 3.67; Analysing the effect of antibiotic selection on the replication and production of HBV virus from cells transfected with reporter-expressing HBV plasmids.

Virus production is definitely attenuated in the presence of antibiotic selection, as seen by decreased levels of secreted HBsAg **(A)** and HBV DNA copies **(B)**, however, there still appears to be ongoing active replication, as seen by the increased levels of viral RNAs being produced **(C and D)**. The original HBV plasmid, as well as the BFP-expressing HBV plasmid, were both selected for with Neomycin antibiotic. A neomycin antibiotic cassette is found within the pCDNA3.1 backbone vector. The BSD-encoding HBV plasmid was additionally selected for with Blasticidin. Theoretically, even though the neomycin and blasticidin resistance cassettes are found in different locations within the particular construct, selection with either antibiotic should results in a very similar outcome. Samples transfected with an empty pCDNA3.1_wt vector were regarded as negative controls, while HepG2.2.15 cells were used as positive control samples.

In summary; Numerous attempts were made to improve virus production from the cells transfected with the reporter-expressing HBV plasmids; some of them resulted in the production of higher yield virus, while others had extremely variable outcomes.

3.3.2 Isolation and clonal analysis of cells supporting HBV infection

After establishing the necessary tools, we went on to perform the altered version of the cyclic repackaging rescue screen (*CRRS; Figure 2.14*). We particularly made use of the Huh7.5 hNTCP/hASGPR overexpressing cell line and further transduced these cells with the already established lentiviral cDNA library, as described in *Section 2.2.2.4.1.3/1*.

The first round of HBV infection selection, after culturing the cells for at least 72 hours post-transduction with the cDNA library, was to infect these with the Type I HBSpp. We allowed the latter lentiviruses to infect the cells for a further 72 hours after which we FACS sorted single cell clones positive for GFP fluorescent signal. FACS sorting was performed by the staff at St. Mary's Flow Cytometry, and an example of the procedure used to FACS sort these cells can be found in *Appendix A.3.VII.*

After nearly two months of expanding the single cell clones that had survived, we were able to examine the cells we had recovered for GFP expression by immunofluorescence. The cells that had no or very low levels of GFP signal were discarded. A small number of the GFP-positive clones selected, indicating cells infected with the HBSpp at varying degrees, can be seen in *Figure 3.68* (*for more examples of positive clones, please refer to Appendix A.3.VIII*).

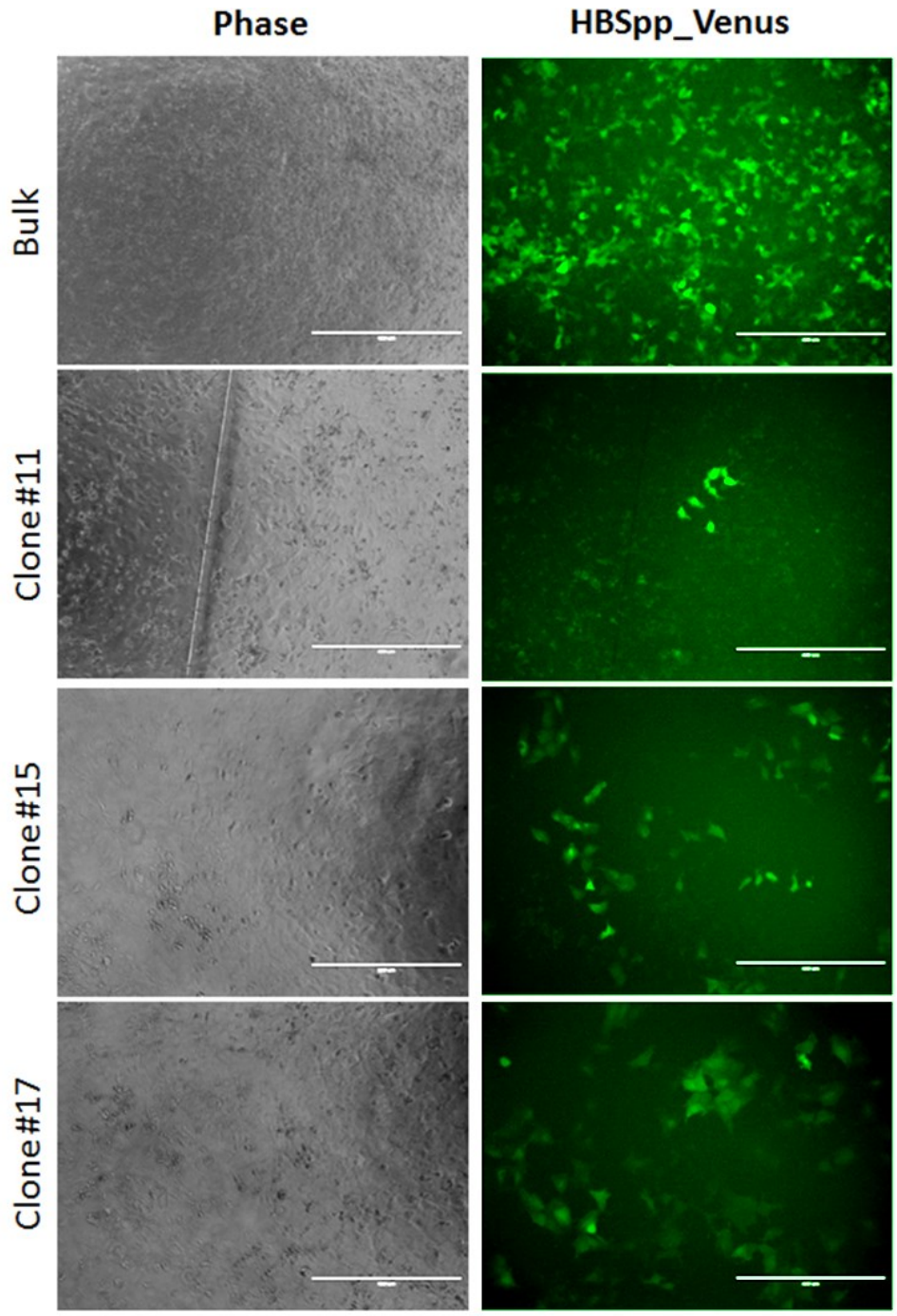


Figure 3.68; GFP-positive single cell clones of stable Huh7.5 cells overexpressing hNTCP/hASGPR, further transduced with a particular lentiviral cDNA library and infected with Type I HBSpp.

Even though all single cell clones had originated from the same cell line, they expressed very different levels of GFP signal. The scale bar is 400µm.

Next, we infected the GFP-positive clones we had selected with fresh HBV infectious supernatants collected from HepG2.2.15 cells, following the normal experimental procedure. At day seven post infection we analysed the clones for

HBsAg and HBeAg secretion (*Figure 3.69; A* and *B,* respectively) and selected only those clones that secreted high enough levels of both HBV antigens. These were maintained in culture, while the rest were discarded.

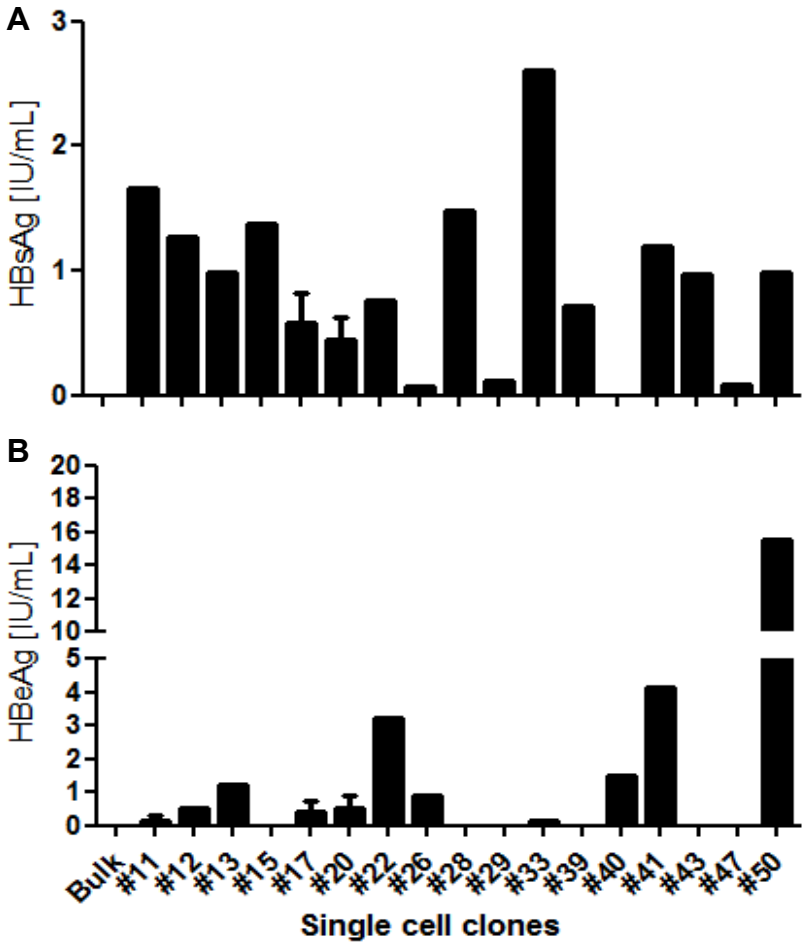


Figure 3.69; Single cell clones of stable Huh7.5 cells overexpressing hNTCP/hASGPR, further transduced with a particular lentiviral cDNA library and infected with initially Type I HBSpp and then HepG2.2.15 infectious supernatants.

Even though all single cell clones had originated from the same cell line, they expressed very different levels of secreted HBsAg **(A)** and HBeAg **(B)**.

Surprisingly, apart from the fact that the levels of secreted HBsAg and HBeAg were rather random between the clones examined, they also did not match the corresponding levels of GFP-positive signal. However, these assays are depicting different stages of the HBV life cycle; from entry to infection and replication, and it is quite possible that these different stages vary between the different clones. Below is a summary of the clones selected during this second

round of HBV infection (*Table 3.6*). Some clones were frozen down at this point, for future use.

Clones	11	12	13	15	17	20	22	26	28
GFP	~	-	-	+	+	+	++	+	+++
HBsAg	+++	+++	+ ++	+ ++	++	++	+ +	+	+++
HBeAg	+	+	+	-	+	+	+ +	+	-
Clones	**29**	**33**	**39**	**40**	**41**	**43**	**47**	**50**	
GFP	+++	-	+++	+++	++	+++	++	++	
HBsAg	+	++++	++	-	+++	+++	+	+++	
HBeAg	-	+	-	+	++	-	-	+++	

Table 3.6; Summary of the single cell clones selected according to their GFP expression (indicating infection with HBSpp) and levels of HBsAg/ HBeAg secretion (after infection with HepG2.2.15 supernatants).
No infection ('-'), Low infection ('+') towards higher levels of infection ('++++').

Following on, we decided to analyse the particular single cell clones for expression of hNTCP (*Figure 3.70, A*) and hASGPR (*Figure 3.70, B*) by western blot, while we also quantified the individual protein bands by densitometry and calculated their expression over the levels of the housekeeping protein, *Actin*.

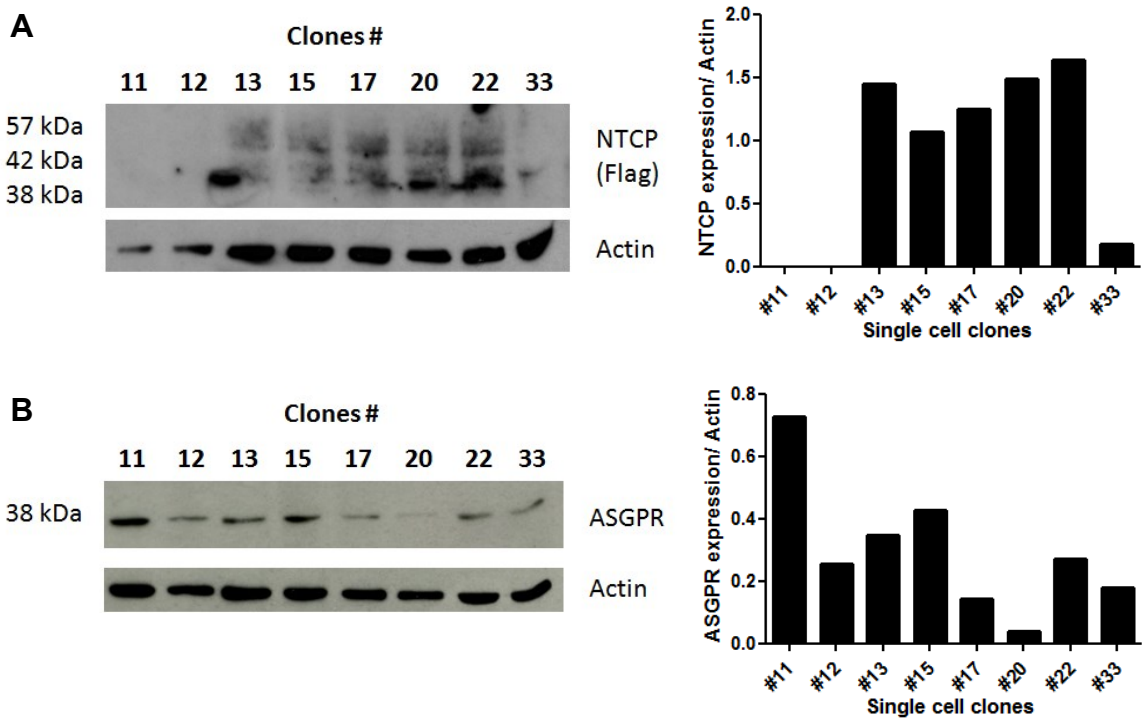


Figure 3.70; Analysis of the expression levels of hNTCP (A) and hASGPR (B) in stable Huh7.5 cells overexpressing hNTCP/hASGPR, further transduced with a particular lentiviral cDNA library and infected with Type I HBSpp and HepG2.2.15 infectious supernatants.

Even though all single cell clones had originated from the same cell line, they expressed different levels of hNTCP **(A)** and hASGPR **(B)**.

Unexpectedly, even though the clones of interest had been selected because they had been determined to have positively been infected with either HBSpp or HBV derived from the HepG2.2.15 cells, some of them appeared not to express hNTCP. This could simply be due to technical problems during the actual western blot procedure; for example, maybe the proteins did not transfer properly onto the membrane blot, or not equal concentrations of the protein were actually loaded. All single cell clones had been derived from Huh7.5 cells stably overexpressing the hNTCP receptor so theoretically they shouldn't have completely lost expression of the receptor. Nevertheless, the clones that were determined to express hNTCP seemed to have comparable levels of expression. The hASGPR expression levels varied slightly, but not significantly, between the clones; an outcome that could be attributed to slightly inconsistent protein loading concentrations. Regardless, we decided to continue onto the

third and final round of HBV selection with the following single cell clones (*Table 3.7*).

Clones	11	12	13	15	17	20	22	33
GFP (IF)	~	-	-	+	+	+	++	-
HBsAg	+++	+++	+ ++	+ ++	++	++	+ +	++++
HBeAg	+	+	+	-	+	+	+ +	+
NTCP	-	-	++	++	++	++	++	~
ASGPR	+++	++	++	++	+	~	++	+

Table 3.7; Summary of the single cell clones selected according to their expression of hNTCP and hASGPR receptors.
No infection ('-'), Inconclusive infection ('~'), Low infection ('+') towards higher levels of infection ('++++').

The above selected clones were finally infected with the recombinant HBV virus produced after transfection of the HepG2.2.15 cells with the BSD-encoding HBV plasmid. After a considerable period of antibiotic selection of the single cell clones with blasticidin, one of the clones appeared to have survived antibiotic selection with at least two individual cell colonies expanding very slowly (*Figure 3.71*). These colonies were eventually rescued and frozen down for future analysis, which will especially involve their sequencing with primers specific for detection of the lentiviral cDNA library so as to see if any additional factors detrimental for HBV infection of cells can be uncovered besides the already known ones.

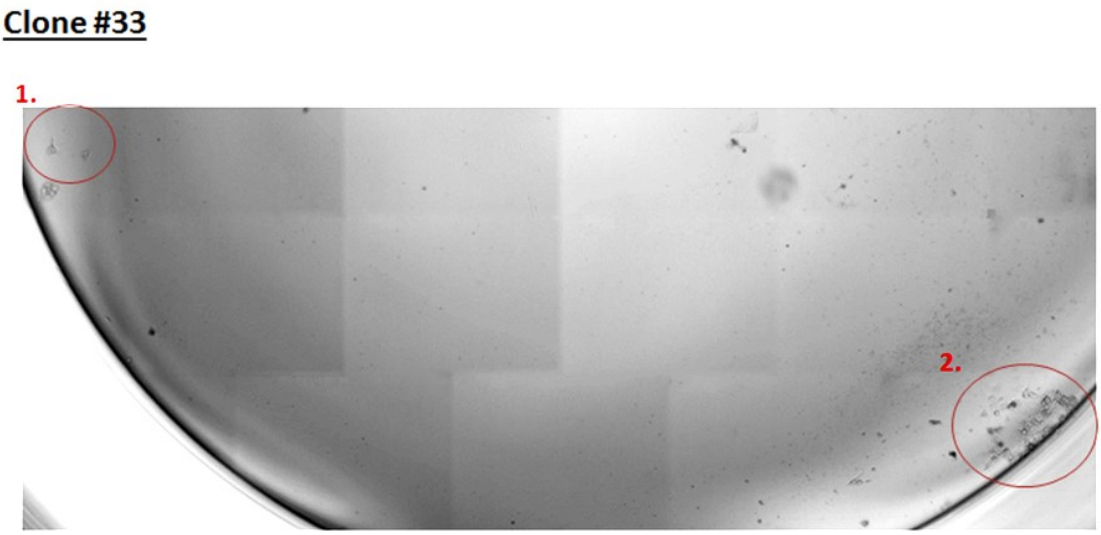


Figure 3.71; Two blasticidin-positive cell colonies observed after clone #33 was infected with HBV virus expressing BsdR and subsequently selected with the particular antibiotic.
Multiple bright field images stitched together.

In summary; We performed a modified version of a cyclic repackaging screen previously used successfully for the discovery of Occludin as one of the receptors utilised by HCV for virus entry and infection of cells [267]. During this experimental plan we especially made use not only of HBV- & HDV- mimicking lentiviral particles, but also of a recombinant HBV virus specifically produced by transfecting cells with a replication-competent HBV genome that we purposefully created to encode an antibiotic resistance cassette for subsequent selection of the cells permissive to this virus. We determined that the recombinant virus produced was in fact functional but possibly we required much higher MOIs for a considerable number of cells to become infected, as is in any case required for infection of human hepatic cells overexpressing the NTCP receptor. Therefore, this technique can definitely be used in the near future so as to hopefully uncover crucial host factors utilised by HBV for entry and infection of cells *in vitro*, further paving the way for the creation of a small *in vivo* model for the study of HBV.

4. Discussion

4.1 Importance of this study

The aim of the present study was to dissect the molecular determinants and pathways involved in HBV entry into hepatocytes. Previous research has shown that HBV entry is, in general, mediated through a precisely orchestrated interaction of certain pre-S1 glycoprotein domains of the virus with its recently discovered functional receptor, NTCP, on the surface of hepatocytes [32, 116, 131, 132, 136]. However, the exact details of the virus/host interactions leading to productive entry and further spread of the infection are still enigmatic, while there are still many questions surrounding the virus/host recognition and attachment sites even prior to entry.

Over the years a lot of research has been focused on determining the active recognition sites within the virus envelope glycoproteins and most of this work has verified that the pre-S1 domain, and particular regions within this domain, is indispensable for virus recognition by the host cells [113, 121-125]. Since the discovery of NTCP in 2012 other studies have tried to identify the precise regions within the receptor's sequence that recognise and interact with the virus' active envelope glycoprotein domains. So far two NTCP domains have been revealed as important for binding to the virus [30, 32], and these have also been found to vary greatly amongst species, hence at least partially explaining the HBV narrow host and organ tropism. All these and subsequent studies, however, have yet to determine how these limitations can be fully overcome, and thus it is possible that other domains or regions within the virus' envelope glycoproteins or the NTCP sequence are also essential for virus recognition and attachment to host cells in humans as well as other, naturally susceptible or altogether non-susceptible, species. For example, it has been demonstrated that substituting the important virus binding regions of the mouse NTCP sequence with the human counterparts still does not confer full HBV susceptibility to mouse hepatocytes [30]. Susceptibility of mice to HBV infection, that can then recapitulate the entire life cycle of the virus, has been a goal of researchers for years, as the creation of a small animal model for the

study of HBV biology and the analysis of therapeutic options under more physiological conditions would be beyond ideal.

Furthermore, it is possible that this virus naturally utilises supplementary binding sites, such as other receptors, on the host plasma membrane, in addition to NTCP, for its productive entry through the cell surface. If this is the case, HBV will not be the first reported virus to use more than one host cell surface receptor for recognition, attachment and efficient entry into its target cells. HCV has previously been reported to recognise and bind to between eight to ten different receptors on hepatocytes [134], while HIV has also been shown to interact with several receptors for successful infection of host cells, which is also characteristic of its viral tropism [310, 311]. A number of possible HBV receptors have been reported over the years, however, apart from NTCP, none of them have been proven to support functional virus infection on their own; a typical example being the ASGPR receptor [89, 115, 121, 133, 135, 136, 140]. In regards to the HBV interspecies transmission barrier, nevertheless, further host factors could be crucial for entry as well as post-entry events that are naturally missing in non-susceptible species. Such additional host factors critical for the infection of cells with HBV could possibly be uncovered for example with the use of a cyclic repackaging rescue screen in conjunction with HBV-/HDV- mimicking pseudoparticles or a recombinant-type virus, as previously performed successfully for HCV when Occludin was discovered as an additional receptor fundamental for virus entry into hepatocytes [267].

The gaps in our knowledge and understanding of HBV entry into its target cells have also had a significant impact on the availability and generation of efficient *in vitro* cell culture systems, with the currently more widely used ones (i.e. NTCP overexpressing human hepatic cell lines) typically relying on two major points for successful virus entry into its target cells:

a) High multiplicities of infection, and even then only a small majority of these hepatocytes become effectively infected with the virus, as well as

b) The addition of cellular differentiation agents (i.e. DMSO) and certain other reagents that promote fusion (i.e. PEG), as opposed to the virus naturally attaching to and entering the cell.

Certain studies have attempted to overcome these problems of productive HBV entry into cells by making use of for example HBV-mimicking lentiviral pseudoparticles, which only express the HBV virus envelope glycoprotein in a HIV background [166, 260]. Even these HBV-like particle models, nevertheless, are not remarkably successful at entering cells *in vitro*, but they are preferable to utilise as they are non-infectious and hence do not require special training for handling of highly infectious material or the need of highly contained laboratories.

In vitro cell culture systems, apart from small animal models, efficiently recapitulating all stages of the virus life cycle are of immense importance not only for studying virus/host interactions and all subsequent events leading to viral spread and infection, but also for developing and easily testing possible new drug inhibitors against the virus that could eventually be clinically tested and used to cure the disease. Moreover, *in vitro* cell culture systems are simpler to handle, less timely to work with and the results are easily reproducible, making them a more than desirable system to utilise and study HBV biology and further potential treatments.

Therefore, the main scope of this project was, by dissecting the molecular determinants and pathways involved in HBV entry into hepatocytes *in vitro*, to primarily gain a better understanding of some of the very early events that take place during HBV infection and especially the dynamic interplay between HBV and the NTCP receptor. The discovery of NTCP as the long-sought receptor

for HBV undeniably opened up the door for HBV research and allowed the understanding of certain vital aspects of HBV entry, however the receptor itself has still not been fully characterised in human hepatic cells in regards to its precise permissivity to HBV infection. Aspects such as the receptors differential regulation in hepatocytes and its necessary conformation for efficient binding to the virus and infection have so far only been studied in rat hepatocytes [173-183, 185, 186], presumably due to their vast availability and because they are not as expensive to obtain as human primary hepatocytes. The rat NTCP is approximately 75% identical to the hNTCP protein sequence (*Figure 1.21*), therefore it is possible that some similarities may exist between them as to how there expression is controlled and regulated [156, 162].

Only very recently did a group of researchers establish that proper glycosylation of NTCP in a human hepatic cell line is crucial for efficient HBV infection, as it particularly determines the receptor's functional trafficking and stability on the plasma membrane where the virus effectively recognises it and binds to it for entry [158]. However, there are still numerous aspects of the NTCP receptor regulation that need to be studied, which will further allow a better understanding of HBV entry into hepatocytes. In the case of our study and towards this end, experiments were initially performed in human hepatocytes, and for comparison purposes certain equivalent experiments were then performed in mouse hepatocytes, in the hope of not only collecting important information relating to the complex virus/host interactions in these two very different *in vitro* systems, but additionally in the hope of gaining some understanding in regards to the HBV interspecies transmission barrier and the role NTCP may play in this. A chimeric NTCP receptor was moreover assessed for its functionality in rendering mouse hepatocytes susceptible to HBV infection.

For the interest of this study, we also attempted to partially characterise the ASGPR receptor, which has previously been reported as a potential receptor for HBV [25, 188, 190, 191], and which may traffic together with NTCP to the

plasma membrane. Even though there is no direct evidence supporting the latter point, there is a possibility that the two receptors may traffic together in the same endocytic vesicles back to the plasma membrane. Previous research has specifically demonstrated that NTCP and TfR are recycled back to the plasma membrane in the same endocytic vesicles, while we know from other studies that ASGPR and TfR undergo very similar trafficking kinetics [207, 208]. To assess viral entry leading to infection, a set of tools were generated, including HBV-mimicking lentiviral pseudoparticles and HBV recombinant viruses encoding reporter genes.

The anticipated outcome from this study was primarily to further characterise the human NTCP regulation and the receptors subsequent expression, and therefore gain a better understanding of how these mechanisms may affect the susceptibility of cells to HBV infection *in vitro*. Nevertheless, all information gathered during the course of this project is of importance and will hopefully contribute towards a better understanding of the complex virus/host interplay leading to infection, as well as provide some understanding related to the HBV narrow species tropism. Some of the knowledge acquired from this study could perhaps now be employed to improve the currently used *in vitro* cell culture systems, thus allowing for more effective HBV entry and infection of cells. This will hopefully open the road towards identifying novel targets that are urgently required for blocking viral infection at the very early stages of the virus' life cycle.

4.2 Results of this study

As already mentioned, the aim of the present study was to dissect the molecular determinants and pathways involved in HBV entry into hepatocytes, *in vitro.* In particular, two receptor proteins one fully verified while the other thought to be potentially involved in the fundamental process of HBV entry into its host cells were investigated; the hepatic bile acid transporter NTCP and the asialoglycoprotein receptor ASGPR, respectively, both of which are typically

expressed on the basolateral hepatic membrane [25, 30, 32, 116, 120, 155-157, 188, 189]. The main purpose of the study was to examine the properties of these two receptors, while additionally characterising their function and how this is affected under certain cell culturing conditions, hence further affecting susceptibility of cells to HBV entry and infection. For this reason, we specifically created *in vitro* cell culture systems overexpressing individually or simultaneously these two receptors, while different sources of non-infectious or infectious HBV, previously discussed, were utilised for conducting infection experiments and hence studying specific aspects of the virus' biology.

Additionally, we created *in vitro* cell culture systems overexpressing a chimeric form of the mouse NTCP receptor, where the particular sites for recognition and binding to HBV were replaced with their functional human counterparts, as previously suggested [30, 32, 170], for the purpose of studying HBV entry and infection under these unusual conditions after, initially, characterising the properties of this chimeric NTCP receptor. This cell culture system we created and further used for characterisation of its permissiveness to HBV infection is, to our knowledge, the first successful attempt at infecting mouse hepatic cells overexpressing such a chimeric form of the NTCP receptor with HBV. A very preliminary *in vivo* experiment generated optimistic results, further confirming our *in vitro* data.

Upon completion of this study, it was anticipated that a better understanding of some of the early stages in the virus' life cycle would be gained, knowledge which throughout the years has been hampered first and foremost due to the unavailability of a functional HBV receptor which further resulted in a lack of suitable *in vitro* cell culture systems recapitulating the entire life cycle of the virus. Our prime interest, however, was to determine the precise regulation of the long-sought HBV receptor, NTCP, and how this affected expression of the receptor on the plasma membrane hence further affecting susceptibility of cells to virus infection. In the final stages of the project, an altered version of a cyclic repackaging rescue screen, previously described by Ploss and his colleagues

[267], was attempted in the hope of uncovering any additional host factors indispensable for effective HBV entry into hepatocytes. This experimental procedure demonstrated the possibilities of performing such a technique, in a similar manner to how it was previously used for HCV, for gathering important knowledge on HBV entry in the near future.

4.2.1 HBV tools generated for this study

4.2.1.1 HBV-mimicking lentiviral pseudoparticles

To assess HBV entry, especially in the cell lines overexpressing the human ASGPR receptor, we purposefully designed and produced HBV-mimicking lentiviral pseudoparticles, similarly to what has previously been described by other research groups [166, 258-260]. These lentiviral pseudoparticles specifically expressed the HBV envelope glycoproteins on their cell surface, as well as GFP for visualisation of the subsequently infected cells by immunofluorescence. For validation purposes, we initially evaluated their expression of HBsAg and determined that they would most likely be more effective at infecting cells after being concentrated by ultracentrifugation (*Figure 3.46*). In particular, when we analysed the freshly collected HBV-like lentiviruses or ones that had been concentrated in centrifugal filters, they generally expressed low levels of HBsAg compared to the total levels expressed by the ultracentrifuged particles. In general, however, all lentiviral pseudoparticles, concentrated or non-concentrated, only infected a very minute number of cells, as determined by a positive GFP signal with the use of fluorescent microscopy. As a result, we went on to use the ultracentrifuged HBV-like particles to further characterise the efficiency by which they infected hepatocytes, which will be further discussed below.

4.2.1.2 HBV recombinant viruses encoding reporter genes

HBV replication-competent genomes expressing reporter transgenes have over the years become invaluable tools for studying and monitoring HBV

infection and particularly the different stages of the virus' life cycle within cell culture systems and animal models [263-266, 309]. Therefore, before attempting to create similar HBV recombinant viruses encoding different transgenes of interest, we initially characterised the available 1.3kb overlength HBV plasmid in regards to its ability to replicate and hence infect human as well as mouse hepatic cells. We established positive and comparable infection levels in all cell lines tested for up to ten days, as specifically determined by analysis of the secreted HBsAg, the expression of HBV DNA copies and the formation of cccDNA (*Figure 3.58*). Of interest, however, was the fact that we observed replication and infection of the mouse hepatic cell line (Hepa1.6) tested, with the levels particularly being highly comparable to the replication and infection levels detected in all other human hepatic cells (HepG2, Huh7 and Huh7.5) tested. HBV replication and infection of mice and other naturally non-susceptible species, after bypassing virus entry by direct delivery of the HBV plasmid into the cells, has previously been reported [30, 43, 88, 225, 226].

Following on, we designed and created the various HBV genomes encoding particular transgenes by performing a similar procedure as previously described by Wang and his colleagues [266]. In brief, we isolated the core and the polymerase genes from the HBV genome, reinserted them back into the virus' genome but in a linear manner so they would no longer overlap, and inserted the reporter genes of interest between them, further separating everything with two minimal 22nt IRES sequences so as to drive expression of the appropriate genes. The reporter genes we specifically chose to individually express within the HBV genome were the: BFP, Fluc, Gluc and BsdR (or else referred to as BSD). *Please refer to "Example of a cloning strategy designed for the construction of replication-competent HBV genomes encoding reporter genes", Section 2.2.1.1.2, for further details of the cloning strategy undertaken.*

We focused primarily on characterising the HBV genomes encoding the BFP and BsdR reporter genes in terms of their replication-competency initially in

one human hepatic cell line (HepG2), and we established positive and comparable infection between both transfected plasmids expressing the particular transgenes (*Figure 3.59*). Most interestingly, their replication-competency was also comparable to the original HBV overlength genomes, as determined by analysis of the secretion of HBsAg and HBeAg, the expression of HBV DNA copies, but also the production of pgRNA and sgRNA. The background of the vector into which the HBV genomes were cloned into did not appear to have any adverse effects on the replication-competency of these plasmids. Furthermore, we analysed the cells transfected with the HBV genome encoding for BFP by immunofluorescence, and evaluated that over time the particular recombinant virus was replicating in an increasing number of cells (*Figure 3.60*).

Consequently, we verified the functionality of the newly constructed replication-competent HBV genomes encoding the reporter transgenes under the particular conditions tested. Nevertheless, as the final goal was to produce functional infectious virus from cell lines transfected with these constructs that could be used to further re-infect the cell lines overexpressing the HBV receptors of interest, we wanted to further evaluate HBV production under a series of different conditions. Wang and his colleagues were able, in their study, to verify the functionality of the virus they produced by showing proper formation of enveloped virions, which were then used to further infect HepaRG cells [266].

Many attempts were made to produce fully infectious replication-competent virus from these newly created recombinant HBV genomes encoding the reporter genes, as previously discussed (*refer to Section 3.3.2.3.1*), however they resulted in variable outcomes. Additionally, the precise virus yield and the subsequent infectivity of human hepatic cell lines overexpressing hNTCP could not be confirmed. The latter reason, however, could have also been due to the fact that the dependence of hNTCP functional expression on the presence of

DMSO had not been established at that point, or simply due to the fact that much higher MOIs were required for detectable viral infection.

Subsequently, we only utilised the HBV genome encoding the BFP, as we had already established comparable levels of replication between the two recombinant constructs tested (i.e. BFP and BSD), and especially as the results generated from this construct could easily be monitored by immunofluorescence of the live transfected cells. We particularly transfected the plasmid into human hepatic cells (Huh7.5, as they generally exhibit higher transfection efficiencies compared to HepG2 cells) overexpressing either the hNTCP alone or the hNTCP and hASGPR receptors simultaneously. We established positive replication in all cell lines but to greatly varying degrees (*Figure 3.61*).

In particular, the presence of the receptors did not enhance the production of the infectious virus as anticipated, but on the contrary it appeared on occasions to have a negative impact; transfected Huh7.5 cells secreted more HBsAg compared to the other receptor-expressing cell lines, Huh7.5 and Huh7.5_NTCP cells secreted similar levels of HBeAg, while Huh7.5 double receptor-transduced cell lines revealed more BFP-positive replicating cells by immunofluorescence. As the results were rather ambiguous, no final conclusions could be drawn about the presence of the HBV receptors in regards to promoting enhanced infectious virus production.

In addition, in this study we demonstrated that the presence of DMSO during the process of HBV transfection and further infection definitely obliterated virus replication and hence production of infectious viral particles. This is somewhat in contrast to a previous study which demonstrated that the presence of DMSO does enhance the production of cell culture-derived HBV, but only to a moderate extent [312]. A possible explanation for this could be the difference in the cells' origin used in these experiments, which could have generally

resulted in a difference in the HBV plasmids replication kinetic, with the presence of DMSO having varying effects in either case. However, it is also possible that this outcome was as a result of differences in the experimental procedure, as well as the final concentration of the agent utilised; for example, in our experimental procedure DMSO may have interfered with the transfection reagents and hence resulted in much lower efficiencies of HBV replication. Another study proved that addition of DMSO during electroporation procedures for the direct transfer of plasmids into cells actually enhanced the transfection efficiency. DMSO was demonstrated to do so by interfering with the cells' membrane integrity [313].

Finally, we decided to transfect the recombinant plasmids expressing either BFP or BSD into a variation of the HepG2 cells, the HepG2.2.15 cell line, specifically created to stably integrate and express the full HBV genome. The hypothesis was that by transfecting these plasmids individually into a cell line that already produces infectious HBV, this could possibly additionally result in enhanced production of the HBV recombinant virus encoding the reporter genes of interest. Under these transfection conditions, we established indeed higher recombinant virus yields from the transfection of the recombinant HBV genomes into these cells, as especially determined by increased secretion of HBsAg and HBeAg compared to the levels produced by the parental cells (*Figure 3.62*). Furthermore, we analysed the cells transfected with the HBV genome encoding for BFP by immunofluorescence, and similarly to previously evaluated that over time the particular recombinant virus was replicating in an increasing number of cells. Interestingly, however, in this particular case we could actually observe a number of positively replicating cells from 24 hours post-transfection of the BFP-expressing HBV genome, compared to 48 hours when this construct was transfected into the HepG2 cells. This further proves that the recombinant HBV genomes have enhanced replication efficiency in the HepG2.2.15 cells.

As a consequence, we went on to similarly produce a larger quantity of the virus encoding only for BSD for use in follow up re-infection experiments, and further confirmed its ability to produce higher levels of HBV DNA copies compared to the virus produced from the parental HepG2.2.15 cells (*Figure 3.63*). Moreover, simultaneous transfection with a plasmid expressing GFP, for visualisation purposes, demonstrated that as early as 24 hours post-transfection and over time this particular recombinant virus was also replicating in an increasing number of cells. Interestingly, we confirmed that transfecting the replication-competent HBV constructs encoding transgenes into a cell line that naturally stably replicates and produces HBV certainly resulted in enhanced levels of recombinant virus production. Incubation of a small virus sample with enzymes whose function is to digest away residual plasmids left behind from the transfection procedure, revealed that the virus we were detecting during our analysis was in fact newly produced virus. The infectivity of this virus, however, would still require to be properly and entirely assessed for its functionality. Following on, this virus was eventually used during the cyclic repackaging rescue screen experimental procedure and determined to be functional, even though it only infected a minute number of cells.

4.2.2 Properties of the human NTCP receptor, and how they affect susceptibility of cells to HBV infection

Creation of cell lines stably overexpressing the human NTCP receptor, and further analysis of the receptor's precise expression levels

For the first part of this study, we principally focused on the functional HBV receptor, NTCP. A lentiviral plasmid expressing the human NTCP receptor under the control of a CMV promoter was specifically purchased for this reason. Lentiviral pseudoparticles expressing the hNTCP were produced following standard techniques, as previously described. These were then used to further transduce various other cell lines; human and mouse hepatic cells. Stably hNTCP overexpressing cell lines were selected for with a suitable antibiotic as polyclonal populations.

Currently, in the field of HBV there are two accepted ways of producing NTCP overexpressing cell lines: either by transiently transfecting the NTCP-expressing plasmid into the cells or by stably transducing the cells of interest with lentiviral pseudoparticles expressing the NTCP receptor. In the first case, the receptor is not integrated within the cell's genome and hence requires constant antibiotic selection so as not to lose its expression, while in the second case the receptor is stably integrated into the genome of the cell after undergoing a period (approximately a full month) of antibiotic selection [158, 163]. Therefore, stable integration of the receptor into the cells of interest seems more advantageous, while it additionally results in higher integration efficiencies as after the one off antibiotic selection period ideally there is no problem of losing expression of the gene of interest, which continues to express indefinitely without any further requirements and is inheritable to all future cell generations. There are certain situations, nevertheless, when genes of interest can in fact be silenced even if they have been stably integrated within the cell's genome; for example, when a gene is toxic to a cell or when the position of a normally active gene within the genome is altered – known as position-effect variegation (PEV), the cell itself may regulate their epigenetic silencing.

Moreover, in the field, some research groups use polyclonal populations of these NTCP overexpressing cells while others use monoclonal, single cell, clones that have been specifically selected for their high expression levels of the NTCP receptor and their higher ability to become susceptible to HBV infection [226, 228, 232, 255, 256]. Initially, we decided to characterise the polyclonal populations of cells we had generated to overexpress hNTCP, before deciding whether it was necessary to go ahead and single cell clone them.

The polyclonal populations of cells, human and mouse hepatic, were initially assessed for hNTCP expression at the mRNA level following standard techniques, as previously discussed. This analysis demonstrated that the

generated cell lines overexpressed the receptor of interest between three to five log higher compared to the equivalent untransduced parental cells (*Figure 3.1 and 3.49*). Therefore, we considered that our lentiviral transduction protocol for hNTCP expression had worked successfully in all cell lines regardless of their origin, and hence they all overexpressed the receptor of interest at high levels. Interestingly, however, in spite of the high hNTCP expression at the mRNA level, no hNTCP expression could be detected at the protein level (*Figure 3.2; A*). A number of different antibodies were even tested in the hope of overcoming any receptor antibody specificity problems but none of them could detect any hNTCP protein. These antibodies were eventually demonstrated to recognise a recombinant form of the hNTCP receptor, when it was directly run on a western blot (*Figure 3.2; B* and *C*). Consequently, even though all the generated cell lines overexpressed the receptor at the mRNA level, clearly something unusual was happening at the protein level, between transcription of the gene and its translation to a fully functional protein, seemingly causing hNTCP expression to be suppressed or lost. Otherwise, we were simply having technical problems detecting the receptor protein (*see below; Section 4.2.2.1*).

As there was still some uncertainty surrounding the suitability of the hNTCP-specific antibodies, however, we decided to purchase a similar but also slightly different lentiviral plasmid expressing the hNTCP; in particular, expression of the gene was still under the control of a CMV promoter, but a FLAG-tag had additionally been added to the intracellular C-terminus of the hNTCP gene. The reason for this being, to specifically aid with protein detection of the receptor. Many research groups typically make use of fluorescent or other detection tags for similar purposes [30, 32, 158, 169, 183]. New lentiviral pseudoparticles expressing the hNTCP (Flag-tag) were produced and were further used to transduce the same cell lines, as well as a human non-hepatic cell line. Stably hNTCP (Flag-tag) overexpressing cell lines were once again selected for with a suitable antibiotic as polyclonal populations. The new polyclonal populations of cells were once again assessed for hNTCP expression at the mRNA level. This analysis demonstrated that the generated

cell lines similarly overexpressed the receptor of interest between three to six log higher compared to the equivalent untransduced parental cells (*Figure 3.4*). Therefore, we still believed that there was nothing unusual happening during the lentiviral transduction protocol for overexpression of hNTCP.

Nonetheless, to verify this and further confirm that absolutely nothing unusual was going on when producing and subsequently when transducing the cells with the hNTCP-expressing lentiviruses that could be possibly resulting in undetectable hNTCP protein expression, we decided to analyse protein expression of the hNTCP_FLAG-tag specifically in transiently transfected cells (HEK293T), before moving on to analysing protein expression in the new stable polyclonal populations. Fortunately, hNTCP_FLAG-tag protein expression could be detected by both western blot and immunofluorescence (*Figure 3.3*), and thus we concluded that it was highly unlikely that there was something wrong with the hNTCP-lentiviral pseudoparticles we were producing.

Negative impact of antibiotic selection (and possibly time) on hNTCP protein expression within a stable human hepatic cell line

Following on, we could now attempt to analyse the stable polyclonal population for protein expression of the hNTCP_FLAG-tag, and as there appeared not to be a problem with the lentiviral transduction protocol per se, we decided to begin with checking the effect antibiotic selection was having only on some of the created stable cell lines. We specifically analysed the cells over a period of a month by immunofluorescence, and, to our surprise, even though hNTCP was highly expressed at the mRNA level, antibiotic selection over the course of that month was considerably downregulating/ suppressing hNTCP_FLAG-tag expression at the protein level (*Figure 3.5*). In particular, protein expression could be detected for up to 15 days post-antibiotic selection but rapidly disappeared after that. Also surprisingly, this appeared to be the case only in the human hepatic cells (HepG2) analysed, while particular human non-hepatic

cells (Hek293) maintained expression of the receptor throughout the course of antibiotic selection and thereafter.

Suppression of transgenes delivered via lentiviral vectors by antibiotic selection, under similar conditions, is not something that, to our knowledge, has previously been reported, and due to the high levels of hNTCP mRNA expression it cannot be the case that we have selected for a resistant subpopulation of cells. There has been, however, one reported case of transgenes being suppressed directly following integration via lentiviral vectors in a promoter-dependent manner particularly in human embryonic stem (ES) cells. The same study revealed that mouse ES cells were not affected to the same extent [314].

In our case, however, the process of suppression did not occur directly post-transduction but rather it appeared to be a slow and gradual process that took place post-gene integration, throughout the duration of antibiotic selection and over the course of a month. Additionally, as already mentioned, in our case there was suppression of expression only at the protein level and not at the mRNA level, so the selected CMV promoter was capable at successfully driving transgene expression in these cells. Consequently, maybe antibiotic selection was somehow causing the host cell to actively suppress productive translation of the hNTCP protein or was negatively impacting on post-translational processes necessary for proper expression of the receptor. The fact that this was only the case in the hepatic and not in the non-hepatic cell line, clearly illustrates that NTCP is differentially regulated in different cell types and possibly in the specific hepatic cell line NTCP requires to undergo complex regulation processing for accurate expression on the plasma membrane. It is probable that in the HepG2 cells, unlike Hek293 cells, hNTCP protein expression requires a particular amount of time for accurate regulation and overexpression on the plasma membrane; a process which in fact may be independent from the receptor's silencing due to antibiotic selection. Nevertheless it is also possible that these processes required for accurate

hNTCP expression are just not fully functional in the specific human hepatic cell line.

After losing considerable hNTCP protein expression in the hepatic cell line post-antibiotic selection during the creation of the stable polyclonal cells, we thought that it could possibly be advantageous to select for single cell clones that had maintained a positive level of hNTCP protein expression throughout the course of antibiotic selection. Unfortunately, though, we were not able to select for any such clones. All the single cell clones we had cultured were determined to once again be negative for hNTCP protein expression (*Figure 3.6*); either they were not originally positive or they had all managed to further suppress the receptors protein expression. In addition, this suppression or loss of hNTCP protein expression could possibly explain the problems we were facing when attempting to infect these cells with cell culture-derived HBV virus. In particular, these cells were constantly demonstrated to be refractory to viral infection. *An example of such an infection experiment can be seen in Figure 3.9*.

Effect of optimised culturing media on the vast recovery of hNTCP protein expression in the same stable human hepatic cell line

Following on, we were interested in figuring out if hNTCP protein expression could possibly be restored by culturing the cells under different conditions in the presence of other types of media or reagents, especially since hepatic cells and primary hepatocytes typically require specialised formulated media for proper and effective culturing and maintenance. We initially decided to test primary human maintenance media (PHMM), which is typically used for culturing and maintaining primary human hepatocytes and is thus supplemented with a number of important growth factors, differentiation agents, and other essential substances and nutrients. This cell culturing solution has been the media of chose for a number of groups performing HBV infectious experiments in NTCP overexpressing cell lines [32, 253, 255, 256].

We specifically cultured the cells for a total of 48 hours in this media, which was commercially purchased and further supplemented with appropriate quantities of DMSO and hydrocortisone [295]. Surprisingly, all protein expression in the hepatic cell line was fully restored, while the non-hepatic cells remained unaffected (*Figure 3.5 and 3.6*). In addition, in the case of the hepatic cell line, regardless of whether they were polyclonal of monoclonal populations of cells, they appeared to behave in a similar manner in regards to expression of hNTCP on both the mRNA and the protein level (*Figure 3.6 and 3.7*), so there is possibly no major advantage in single cell cloning these particular cell lines on the basis of their hNTCP expression.

The obvious question then was which ingredient within the extra supplemented HMM media was actually responsible for restoring hNTCP protein expression, specifically in the hepatic cell line. By process of elimination we eventually determined that the crucial ingredient for efficient hNTCP protein expression was DMSO, which was determined to have an effect both on the transcriptional and on the translational level (*Figure 3.11 and 3.12*), with its effect on the latter being possibly more pronounced. Additionally, as a differentiation agent it characteristically helped the hepatic cells obtain a more hepatocyte-like morphology (*Figure 3.8*).

<u>The positive impact of the presence of DMSO, and the characterisation of the reagents full effect, on the recovery of hNTCP protein expression in the stable human hepatic cell line</u>

Only very recently, was DMSO reported by another group to promote hNTCP protein expression in the same hepatic cell line, even though the expression of the transgene was also driven by a CMV promoter; a fact that partially explains the ability of these cells to become susceptible to HBV infection in the presence of this agent [254]. However, this group did not go on to further characterise the full effect of DMSO on the NTCP receptor. Their study and our study are, to our knowledge, the first reported cases of DMSO having been

described to have such an effect on a receptor protein. The effect of DMSO in regards to enhancing HBV infection efficiency has been well documented for a long time [229], while the agents effect on differentiation of cells has also been studied in HepaRG cells, PHH [268], rat hepatocytes [272-274], and more extensively in stem cells [269-271]. The effect of DMSO on the hNTCP receptor, nevertheless, appears to lie beyond differentiation for correct protein regulation and expression.

Next, we aimed at fully characterising the effect of DMSO on hNTCP, on both the transcriptional and translational levels, and we determined the following important points:

1. DMSO, apart from having a positive effect on the ectopically expressed hNTCP in the stable human hepatic cell lines created, also had a similar effect on the endogenous levels of the receptor in the equivalent human hepatic parental cells (*Figure 3.11, C*).

2. DMSO exerted its full effect on the hNTCP receptor between 10 to 15 hours after the reagents addition into the cell culturing media (*Figure 3.13*). However, by immunofluorescence analysis we could observe obvious *de novo* translation of the receptor taking place in the ER from just 4 hours post-DMSO addition, with the receptor reaching the plasma membrane by 24 hours (*Figure 3.14; A*). A subcellular fractionation experiment additionally revealed that by 24 hours, cultured in the presence of DMSO, hNTCP is principally translocated to the plasma membrane – with low levels of the receptor remaining stuck in the nucleus (*Figure 3.14; B*).

3. Higher concentrations of DMSO (4%), even though they resulted in even higher hNTCP protein expression levels, they were also responsible for considerably reduced cell viability (50%) (*Figure 3.15*). Lower concentrations of DMSO (0.5%), however, did not support hNTCP overexpression in the stable hepatic cell line. Therefore, 2% DMSO was sufficient for hNTCP overexpression. As expected, non-

hepatic cells were unaffected by the different concentrations of DMSO tested.

4. The effect of DMSO on hNTCP could be reversed by removal of the agent from the culturing media (*Figure 3.16*). Interestingly, at the mRNA level the effect was reversed immediately while at the protein level the effect was only fully reversed after 48 hours post-DMSO removal, indicating that the hNTCP protein (particularly when the receptor becomes integrated within the cell membrane) is in general a more stable product compared to the hNTCP mRNA transcripts, as previously shown [28]. As expected, non-hepatic cells again remained unaffected by the addition, followed by the removal, of DMSO primarily at the protein level. At the mRNA level, there was a slight decrease in the expression of the receptor, but the expression levels quickly recovered. This could be as a result of cell stress or due to the fact that as soon as DMSO was removed cells were able to start proliferating and dividing again and hence hNTCP expression required some time to reach normal levels.

5. DMSO directly affected the hNTCP expression levels, and it had no effect on the CMV promoter driving expression of the receptor. This was determined by transiently transfecting the human hepatic cells of interest with a plasmid expressing GFP under the control of a CMV promoter, followed by culturing these cells in the absence or presence of DMSO (2%). No difference in the GFP expression levels could be observed (*Figure 3.17*).

In summary; we demonstrated that hNTCP expression in the specific human hepatic cell line was highly dependent on the presence of DMSO in the cell culturing media, with DMSO having a direct effect on hNTCP overexpression that was both time and concentration dependent, while the effect of this agent was easily reversible by its removal from the cell culturing media. In addition, we demonstrated that this was not the case in a non-hepatic cell line, where

hNTCP overexpression was high and remained at constant levels regardless of the absence or presence of DMSO.

Therefore, having established certain aspects of how hNTCP overexpression was regulated by DMSO in the particular hepatic and non-hepatic cell lines, we were curious to determine whether hNTCP was regulated in a similar manner by DMSO in other stable hepatic, human and mouse, cell lines (*Figure 3.18 and 3.50*). Interestingly, the effect of DMSO on hNTCP did not appear to be human or mouse specific, while at the same time it appeared to be a bit random as the cell lines in which hNTCP expression was affected by the presence of DMSO did not have any apparent similarities between them. This probably just comes back to prove that generally NTCP is differentially regulated in different cell types originating from diverse backgrounds, with certain cell lines possibly expressing fully functional endogenous processing mechanisms necessary for the accurate expression of NTCP that are naturally lacking in others. However, it is likely that hNTCP expression in certain cell lines requires to undergo more complex processing and regulation, and these mechanisms may be unnecessary for its proper expression in other cell lines. In particular, from all the cell lines tested, DMSO affected hNTCP expression only in HepG2 human hepatic cells and H2.35 mouse hepatic cells, but was not affected in Huh7.5 human hepatic cells and Hepa1.6 mouse hepatic cells, or the HEK293 human non-hepatic cells.

Characterising hNTCP receptor stability in the stable human hepatic cell line

Having now established that the presence of DMSO was required in certain situations, such as certain cell lines, possibly for accurate hNTCP regulation, processing and expression, we wanted to understand what exactly was happening to the stability of the receptor and why it was being suppressed during the course of antibiotic selection.

In terms of the receptors stability on the plasma membrane, we now know that accurate post-translational *N*-glycosylation processing is of major importance, while this process is additionally responsible for correct trafficking of the receptor to the plasma membrane [158]. Therefore, we needed to confirm that this process was intact in the stable hNTCP overexpressing cell lines generated, and indeed we established that hNTCP was being glycosylated precisely as expected in the overexpressing cell lines of interest (*Figure 3.19*). We specifically determined this by culturing the hepatic and non-hepatic cell lines (i.e. HepG2_ and Hek293_hNTCP, respectively) in the presence of DMSO (always 2%, as previously suggested [315]), after which the cells were collected and treated with the deglycosylation enzyme, PNGaseF, that cleaves complex sugars from *N*-linked glycoproteins. Our results demonstrated that hNTCP glycosylation was being processed as anticipated and, interestingly, in a very similar manner in both hepatic and non-hepatic cell lines. Therefore, it could be suggested that DMSO probably does not affect the regulation of hNTCP glycosylation. An experiment with Tunicamycin, an inhibitor of *de novo* protein glycosylation, revealed that hNTCP glycosylation took place at approximately eight hours post-addition of DMSO, since no glycosylated hNTCP was produced in the cells when tunicamycin was present for longer periods of time (*Figure 3.21*).

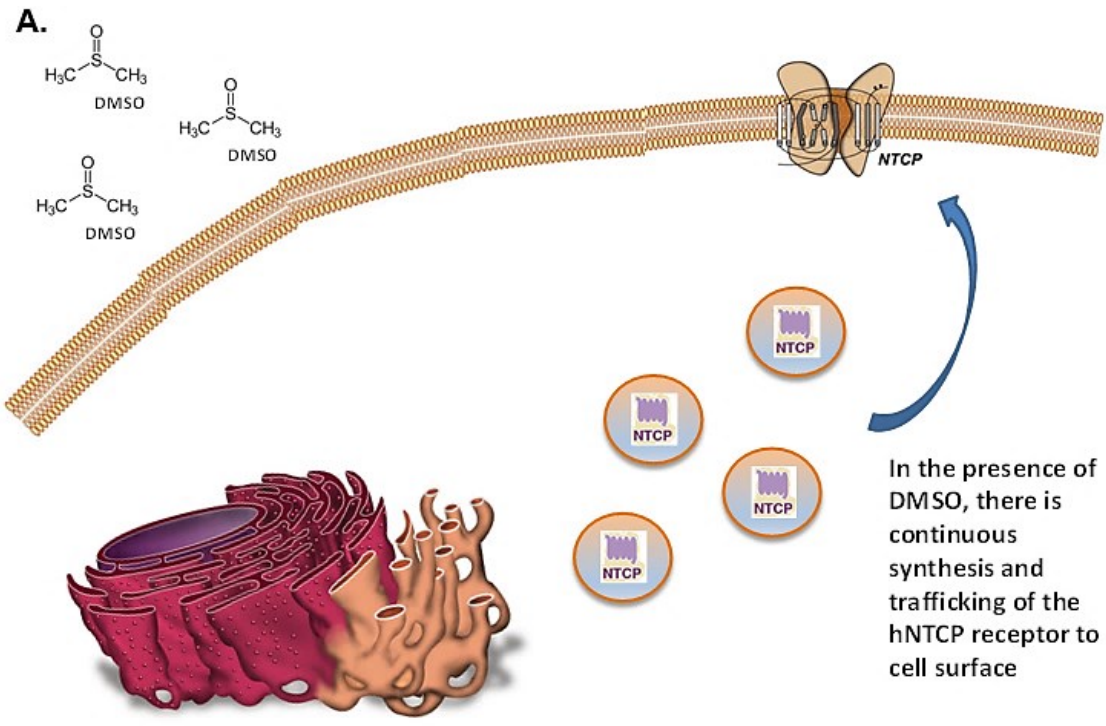
To further investigate the status of the hNTCP protein's stability, we decided to examine the regulation of the receptor on the transcriptional and translational levels, prior to any post-translational modifications taking place. To specifically study what was happening at the transcriptional level, we used Actinomycin D to block all *de novo* transcription in the absence or presence of DMSO (*Figure 3.22*). The HEK293 non-hepatic cell line overexpressing hNTCP behaved as expected; in the presence of actinomycin D there was an evident decrease in the expression of hNTCP at both the mRNA and the protein levels, which however was unaffected by the presence of DMSO. In the HepG2 hepatic cell lines, on the other hand, even though there was a considerable decrease in hNTCP expression at the mRNA level in the presence of both actinomycin D and DMSO compared to when only DMSO was present and actinomycin D

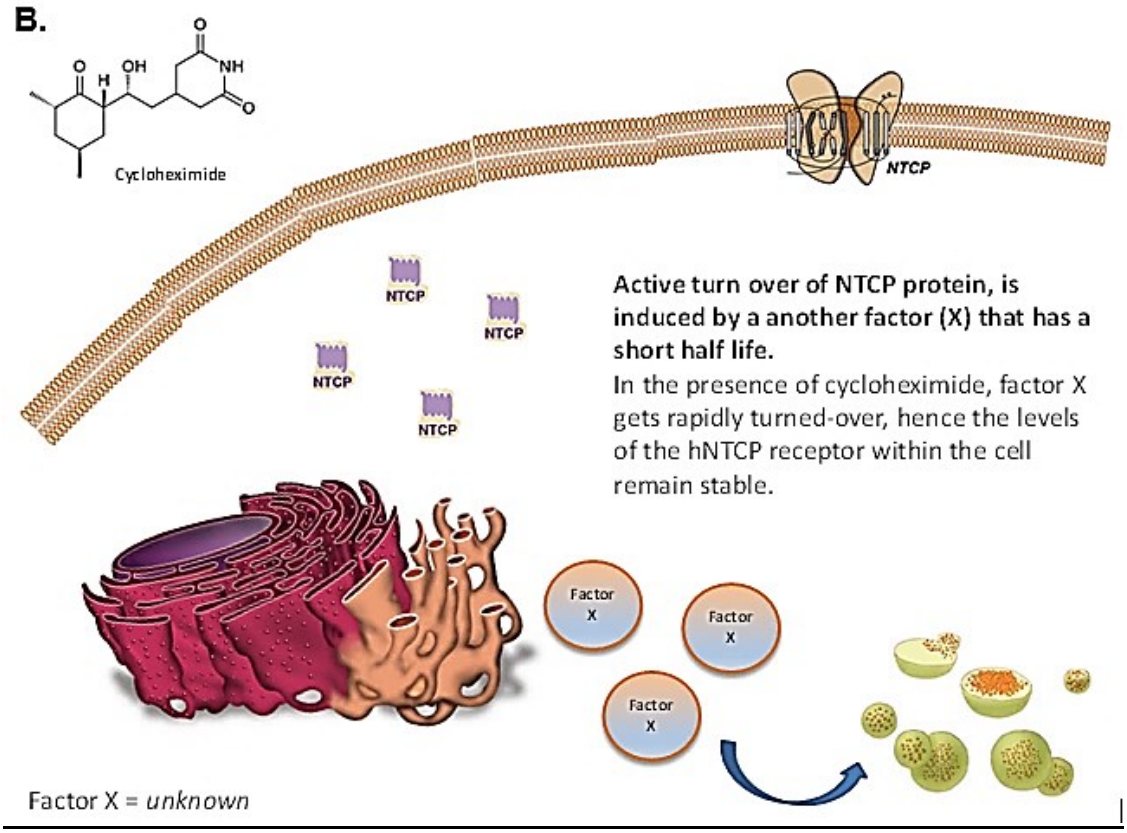
was absent, at the protein level that decrease was not obvious with the protein expression levels seemingly being comparable in those two samples. This most likely demonstrates that the effect of DMSO on hNTCP is primarily on the translational, while it could still be at a post-translational, level.

To particularly study what was happening on the translational level, we used Cycloheximide to inhibit *de novo* protein synthesis. The hNTCP overexpressing cells of interest were initially cultured in the presence of DMSO for an appropriate amount of time after which the regulatory agent was removed and cycloheximide was added to the culture for a further period of time. Our data illustrated that induction of hNTCP protein expression by DMSO was stable for at least 24 hours after the agent had been removed from the cells, while concurrently inhibiting *de novo* protein synthesis with the particular inhibitor (*Figure 3.23*). However, in the absence of both DMSO and Cycloheximide, the receptor's protein expression levels were only stable for approximately 6 hours after which there was a gradual decline (*as also demonstrated in Figure 3.16*).

Finally, we checked whether hNTCP was being degraded in the proteasome due to problems during protein synthesis, such as protein misfolding. We specifically used the MG132 proteasome inhibitor to culture the cells, in the absence of any DMSO, so as to see if we could recover any protein expression. No hNTCP protein expression could be recovered when using the MG132 drug in cell culture, thus we concluded that the receptor was possibly not being degraded in the proteasome. However, this experiment would require further investigation using proper controls to assure complete inhibition of the proteasome (looking, for example, at the p53 protein), while other proteasome inhibitors/drugs could have also been tested for their function.

Our hypothesis up till this point as to how DMSO has been possibly affecting hNTCP protein expression is summarised in the model below.


A.
DMSO
DMSO
DMSO
NTCP
NTCP
NTCP
NTCP
NTCP
In the presence of DMSO, there is continuous synthesis and trafficking of the hNTCP receptor to cell surface


B.
Cycloheximide
NTCP
NTCP
NTCP
NTCP
Active turn over of NTCP protein, is induced by a another factor (X) that has a short half life.
In the presence of cycloheximide, factor X gets rapidly turned-over, hence the levels of the hNTCP receptor within the cell remain stable.
Factor X
Factor X
Factor X
Factor X = unknown

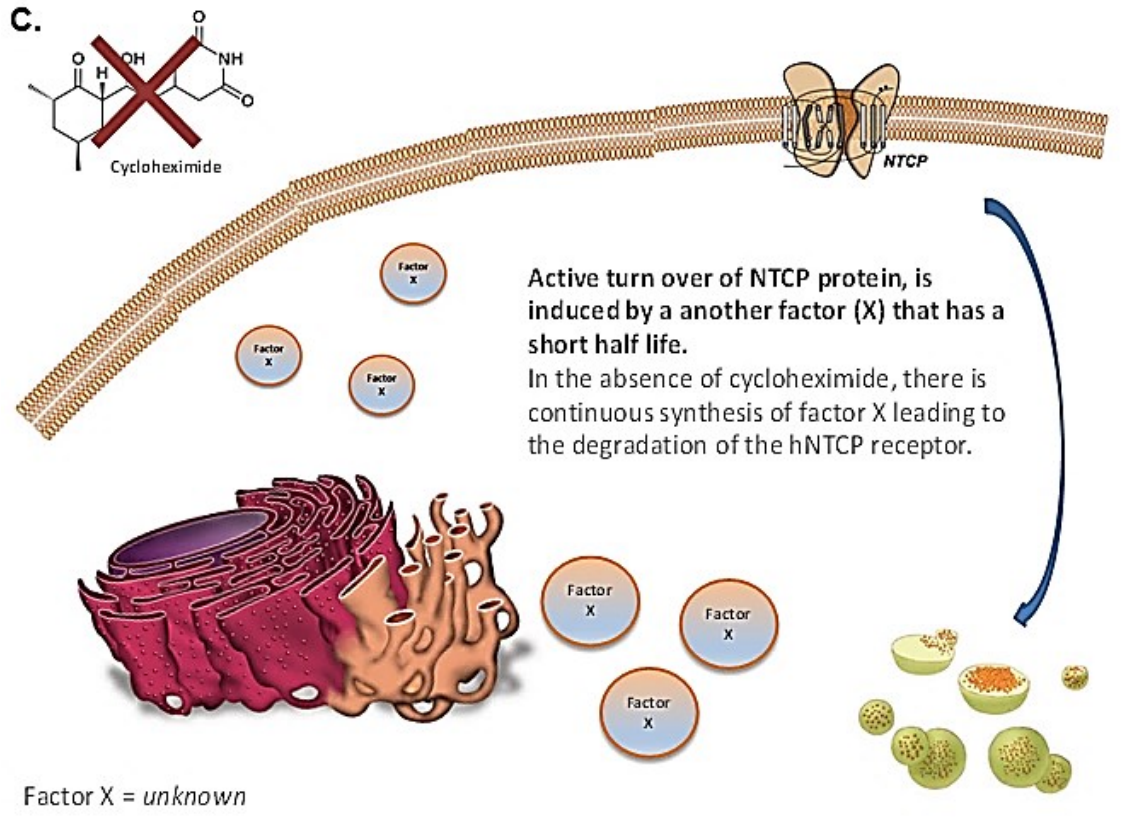


Figure 4.1; Possible model of DMSO's mechanism of action, and comparison to the effect of Cycloheximide in inducing hNTCP overexpression and translocation to the cell surface.

Effect of the presence of DMSO on the susceptibility of hNTCP overexpressing cells to HBV infection

The logical next step of our experimental plan was to evaluate how the presence of DMSO in the hNTCP overexpressing hepatic cell lines, by specifically enhancing the expression of the receptor, was actually affecting susceptibility of these cells to HBV infection. For this reason, we cultured the cells in the absence and presence of DMSO for an appropriate period of time prior to infecting them with cell culture-derived infectious virus, in the further absence or presence of DMSO. We quantified the samples for the presence of HBV DNA copies at 7 days post-HBV infection and, to our surprise, the susceptibility of cells to HBV infection in the presence of DMSO had considerably increased compared to when DMSO was not added to the cell culture media during viral infection (*Figure 3.24*). This increase in the number of cells that became permissive to HBV in the presence of DMSO, was

determined to be statistically significant (*p=0.009*). So clearly, DMSO was actively responsible for enhancing susceptibility of cells to HBV infection, as originally reported by Gripon and his colleagues [229], as well as others [163, 254], and this was possibly directly linked to the increased levels of hNTCP protein expression on the plasma membrane.

Cytoskeletal reorganisations caused in the presence of DMSO may result in the accurate hNTCP overexpression on the plasma membrane of the stable human hepatic cell line, further enhancing the susceptibility of these cells to HBV infection

One of our original observations when culturing the cells in the presence of DMSO was that their morphology was drastically changing. When specifically staining the cells with Phalloidin, which binds to actin filaments, it was evident that in the presence of DMSO the actin filaments did not look as usual (*Figure 3.26*). As a result, we started to wonder whether DMSO was somehow interfering with the cells cytoskeleton, further resulting in the cells obtaining a more hepatocyte-like morphology. In addition, we started wondering whether that was how DMSO was actually promoting hNTCP translocation to the plasma membrane. The effect of DMSO on microfilament organisation has in the past been suggested to occur, for example, in cultured mouse B16 melanoma cells [316] and mouse oocytes [317]. Proteomic analysis, *performed in Professor Anne Dell's laboratory,* also revealed the effect of DMSO in inducing cytoskeletal modifications. This analysis also revealed the effect of the agent in inducing expression of certain epigenetic markers, however, these did not appear to have any effect on the hNTCP protein overexpression (*Figure 3.25*) and therefore there was no further investigation into DMSO's possible epigenetic regulation.

To test our first hypothesis, that DMSO was actively interfering with cytoskeletal reorganisation and hence further enhancing hNTCP translocation to the plasma membrane, we made use of a number of drugs some targeting

actin (i.e. Cytochalasin D, Latrunculin A) while some targeted tubulin (i.e. Colchicine, Demecolchine), and these were either polymerisation inhibitors (Cytochalasin D, Colchicine) or active depolymerisers (Latrunculin A, Demecolchine). We treated the cells in the absence or presence of these drugs, and additionally in the absence or presence of DMSO, and we confirmed that disruption of the cytoskeleton by either of these drugs resulted in an obvious increase in the hNTCP protein expression, which on occasions was even more pronounced compared to when DMSO was added to the cell culture media alone (*Figure 3.27*). Simultaneous addition of either of these drugs with DMSO had an even more pronounced effect. Nevertheless, disruption of actin filaments had in general a more evident effect on hNTCP protein expression, compared to when tubulin filaments were interfered with and disrupted; possibly suggesting that hNTCP translocation to the plasma membrane requires intact microtubules for the proper transportation of the endocytic vesicles containing the receptor.

In summary; enhancement of hNTCP protein expression resulting in augmented translocation of the receptor to the plasma membrane, appeared to involve disruption of the cells' cytoskeleton and DMSO appears to be directly involved in this mechanism. Interestingly, it has previously been reported that cAMP also indirectly induces remodelling of the cells' cytoskeleton by specifically phosphorylating actin and hence interfering with its polymerisation [303-305, 318, 319]. Consequently, it is probable that both DMSO and cAMP may be participating together in this particular pathway involving actin depolymerisation, which results in the translocation of hNTCP to the plasma membrane. This is in contrast to what has previously been reported to be the case for rat NTCP, which was found to require intact actin filaments for its translocation to the plasma membrane; a process that was specifically reported to be a microfilament/microtubule-sensitive process [179].

The model of DMSO's mechanism of action, according to our hypothesis so far, is as follows:

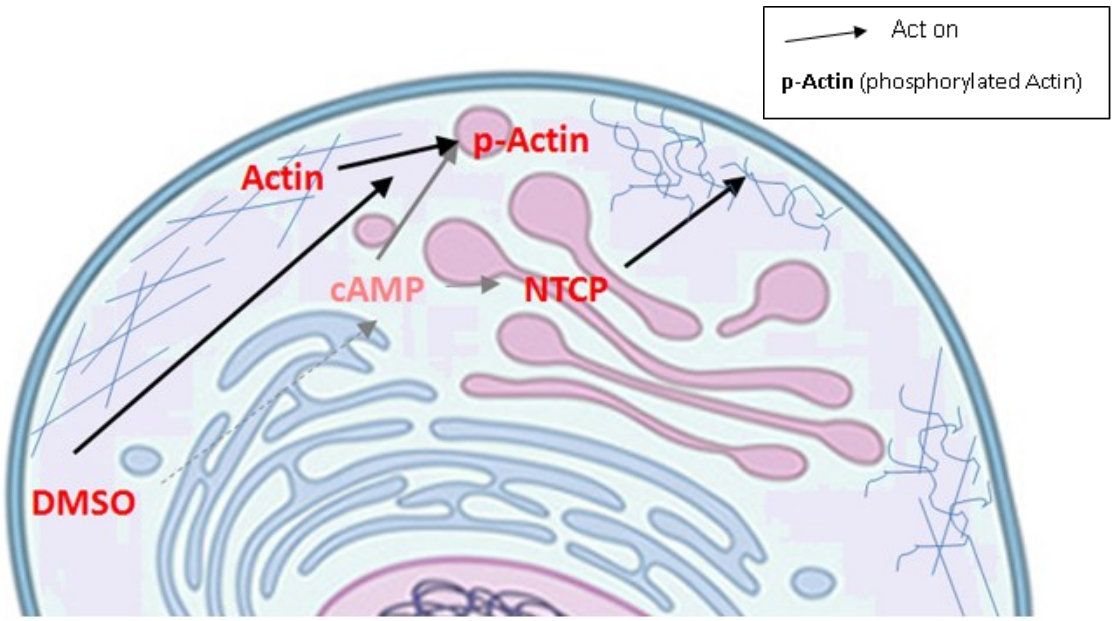


Figure 4.2; DMSO's mechanism of action #1; Induction of cytoskeletal modifications further permit direct hNTCP translocation to the plasma membrane.

DMSO appears to be directly responsible for cytoskeletal changes within the cell, affecting both the actin and tubulin polymerisation, which may further aid the translocation of the hNTCP receptor to the plasma membrane. cAMP is also known to be involved in cytoskeletal reorganisations by specifically phosphorylating actin and thus interfering with its polymerisation status. This, however, may be one of the final stages in the regulation of the hNTCP receptor's expression.

Effect of cAMP, and the characterisation of the molecules full effect, on the accurate hNTCP overexpression on the plasma membrane of the stable human hepatic cell line. Investigating the possibility that cAMP and DMSO may be indirectly "co-operating" during this process.

Subsequently, we decided to look a bit further into the post-translational modifications of the NTCP receptor. NTCP as well as being a well known glycoprotein, is also a serine/ threonine phosphoprotein. Most interestingly, however, is the fact that cAMP has been demonstrated to play yet again an important role in the process of regulating the phosphorylation status of this receptor. This molecule has specifically been reported to dephosphorylate rat NTCP on its serine residue and hence promote its translocation to the plasma membrane, where it stabilises [30-32, 116, 155-158, 174, 177, 178]. It should be noted that a lot of research has been performed up to date about the effect of cAMP on NTCP regulation/ processing (including its trafficking) and expression on the plasma membrane, however it has all been based on rat

NTCP in rat hepatocytes (*See Section 1.2.7.1*) [175, 180, 181]. Unfortunately, there is currently no available knowledge on how this mechanism is regulated in human hepatocytes.

For the purposes of this project, we initially wanted to assess the effect the presence of DMSO in the cell culturing media had on the endogenous levels of cAMP (*Figure 3.29*). We particularly confirmed that the presence of DMSO, when culturing the hNTCP overexpressing hepatic cell line, increased the endogenous levels of cAMP within those cells. Our hypothesis now was that most likely DMSO and cAMP were involved in the same regulatory mechanism in regards to hNTCP processing and expression on the cell surface.

Moreover, we were interested in assessing the effect of exogenous cAMP when added to the cell culturing media. When we cultured the hNTCP overexpressing hepatic cell line in the absence or presence of DMSO, while additionally adding exogenous cAMP, we observed that, regardless of the presence of DMSO, the presence of cAMP alone was resulting in the induction of hNTCP protein expression (*Figure 3.30*). However, the presence of DMSO had still a more potent effect compared to cAMP alone, but when added together the two reagents had an obvious additive effect, hence partially explaining the increase in hNTCP protein induction and verifying the fact that the two reagents are somehow involved in a common mechanistic/ regulatory pathway resulting in this effect.

To further confirm that the effect of cAMP on the protein expression levels of hNTCP was specific, we additionally treated the cells with Wortmannin, a PI3K pathway inhibitor that typically targets cAMP-downstream signalling events, and hence any effect seen on hNTCP as a result of cAMP should in theory be blocked in the presence of this inhibitor. In the case that DMSO and cAMP are indeed working together in the same signalling pathway then the presence of wortmannin should have the same outcome, and thus block the effect of

DMSO on hNTCP protein expression. Interestingly, the presence of wortmannin while culturing the cells of interest did in fact block hNTCP protein overexpression (*Figure 3.31*). The presence of the inhibitor specifically appeared to suppress the trafficking of the receptor to the plasma membrane by causing its retention within the ER/Golgi complex. This general role of wortmannin has previously been confirmed in rat hepatocytes where the inhibitor has specifically been demonstrated to block the specific cAMP-mediated translocation of rat NTCP to the cell surface [179-182].

At this point, our working model in regards to DMSO's full mechanism of action and the regulatory pathway this agent is involved in, together with cAMP, for induction of hNTCP protein expression, is as follows:

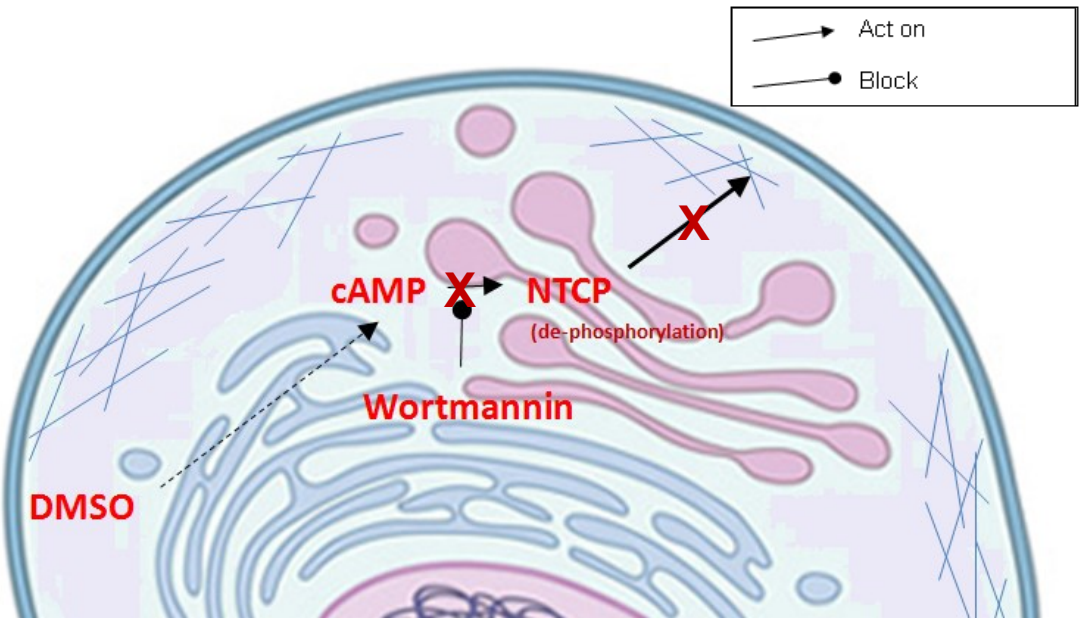


Figure 4.3; DMSO and cAMP are involved in the same regulatory pathway resulting in hNTCP translocation to the plasma membrane.
Their function is specific and can be blocked by certain downstream signalling inhibitors, such as Wortmannin.

We further hypothesised that DMSO may be indirectly acting on cAMP by specifically targeting a PDE. In this way, DMSO may be blocking the breakdown of cAMP, as a result of the action of a PDE, and hence indirectly be

involved in enhancing the cAMP-mediated hNTCP receptors translocation to the plasma membrane. This hypothesis is illustrated in the model below.

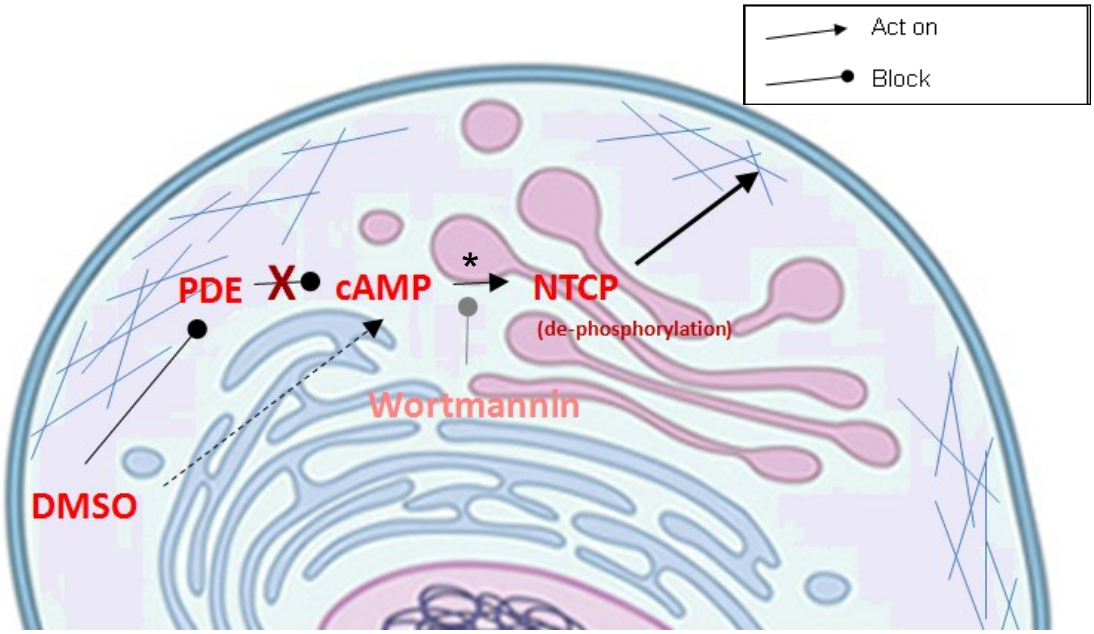


Figure 4.4; DMSO's hypothetical mechanism of action #2; DMSO targets and blocks a PDE, which consequently permits cAMP to perform its function and promote hNTCP translocation to the plasma membrane.

* cAMP is indirectly responsible for NTCP dephosphorylation. The molecule specifically particulates in this process in two different ways (a*s described in Figure 1.22*): it either activates the PI3K signalling pathway or it increases the intracellular calcium levels, which in turn result in the activation of the PP2B signalling pathway. Both these pathways are directly responsible for dephosphorylating the receptor, hence resulting in its retention within the plasma membrane.

To test our second hypothesis, we made use of two particular drugs: a) MDL12, an inhibitor of adenylyl cyclase that blocks the *de novo* synthesis of cAMP, and b) Caffeine, a pan-PDE inhibitor. To verify our hypothesis we would expect that the presence of MDL12, while culturing the cells of interest, would result in no hNTCP protein overexpression, as even in the presence of DMSO targeting a PDE there would still be no cAMP being produced to indirectly induce expression of the receptor (*Figure 4.5*). This phenotype, however, could theoretically be overcome with the presence of exogenous cAMP in the cell culture media. On the other hand, caffeine would block all PDEs within the cell and hence the cAMP-mediated translocation of the hNTCP receptor should occur uninterrupted (*Figure 4.6*). According to our hypothesis, DMSO and

caffeine should have the same mechanism of action. Our results revealed the following points (*Figure 3.34*):

1. MDL12 blocked hNTCP protein expression in a very similar manner to what was previously seen in the presence of wortmannin (even though the drugs act on very different stages of this pathway they are predicted to result in a very similar outcome). Addition of exogenous cAMP in the presence of the MDL12 inhibitor did in fact moderately rescue the phenotype, indicating the importance of newly formed cAMP in the process of hNTCP translocation to the cell surface. This effect was, nevertheless, more profound when DMSO was also simultaneously added to the cell culture media.

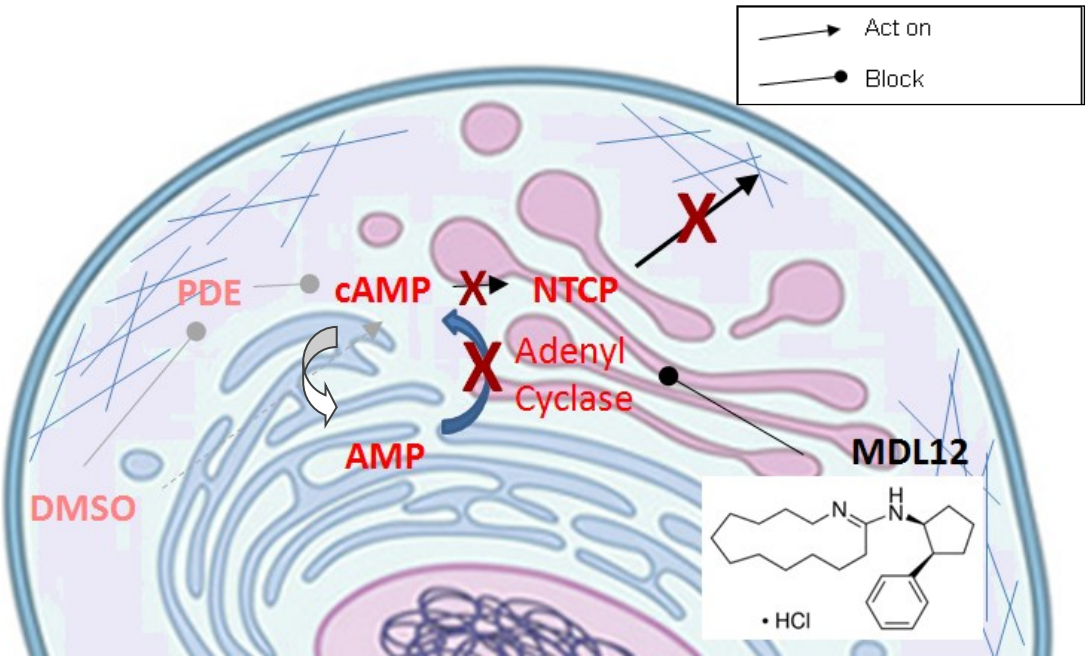


Figure 4.5; Effect of the MDL12 inhibitor on the subsequent hNTCP receptor translocation to the plasma membrane.

2. Caffeine enhanced the hNTCP protein expression in a similar manner to when exogenous cAMP was previously added to the cell culture media alone. However, the effect of DMSO was still more pronounced, which probably provided further evidence that DMSO, unlike caffeine that

targets all PDEs, was having a strong affinity against a very particular PDE, which it targeted and completely blocked its action. Interestingly, however, when caffeine was added to the cell culture media in combination with DMSO there was a strikingly vast overexpression of hNTCP on the cell surface. Even more interestingly, it has previously been shown that caffeine increases cAMP expression [320], while it also has been reported to play a role in actin depolymerisation [321]. These two properties of caffeine sound very similar to DMSO's mechanism of action, as shown so far from our experimental data. This provides further proof that these two reagents quite likely function in a very similar manner in regards to hNTCP overexpression and its subsequent translocation to the plasma membrane.

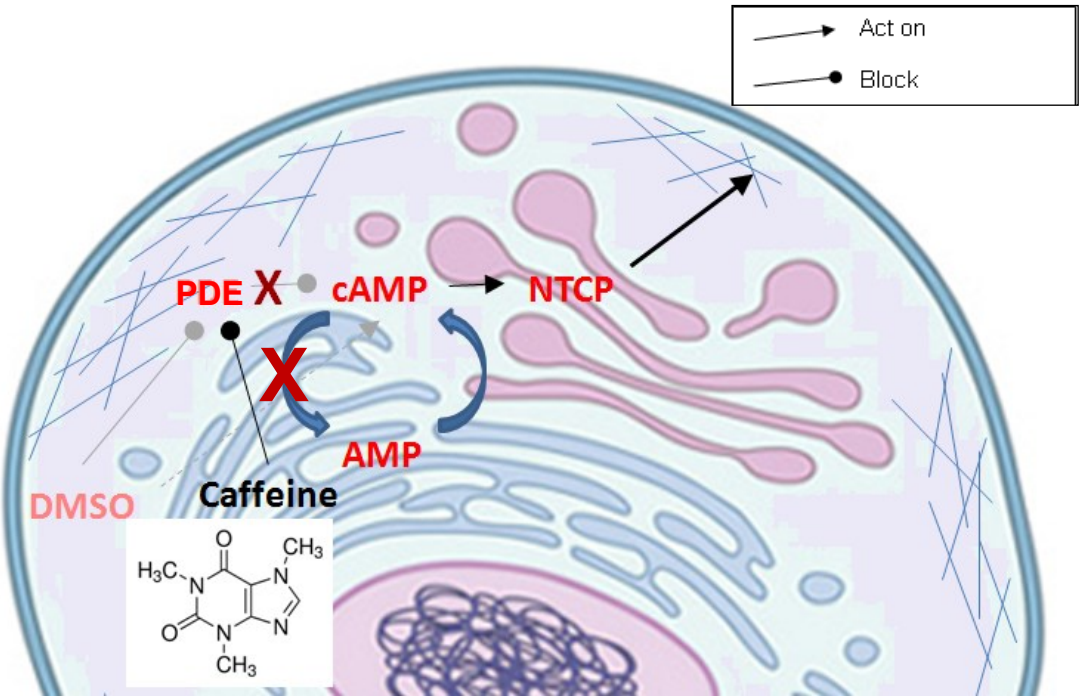


Figure 4.6; Effect of Caffeine on the subsequent hNTCP receptor translocation to the plasma membrane.

DMSO particularly inhibits PDE4(D), hence allowing cAMP to perform its function in regards to hNTCP receptor translocation and retention within the plasma membrane

Following on, we wanted to determine if possible which exact PDE was possibly being targeted by DMSO. We specifically chose a small number of PDEs to investigate; these were found to be enriched either in the liver or in HepG2 hepatoma cells, and they particularly were: PDE1A, PDE1C, PDE4D, and PDE12. We initially purchased commercial siRNAs to silence their expression in the hepatic cell line of interest, so as to study whether we could observe an increase in the protein expression of hNTCP even in the absence of additional DMSO. By doing so, we hoped to verify our hypothesis that DMSO's mechanism of action involved blocking the function of a certain PDE, further preventing the breakdown of cAMP, and hence resulting in the cAMP-mediated translocation of hNTCP to the plasma membrane.

The siRNAs were transfected into the cells following standard procedures, as previously described. After a period of time, enough to result in considerable silencing of the PDEs, the cells were cultured in the absence or presence of DMSO. The percent of each PDE silencing was confirmed at the mRNA level, and was found to vary between 60-96% (*Figure 3.37*). However, the additional presence of DMSO actually improved the silencing efficiency, which was not a surprise if our theory was correct and DMSO was in fact targeting their expression. PDE4D, which was the one that exhibited the least silencing at the mRNA levels, was confirmed to be completely suppressed at the protein level (*Figure 3.38; B and C*). Moreover, at the protein level, we further established that the silencing of both PDE4D and PDE12 had a slight positive effect on induction of hNTCP protein expression in the absence of DMSO, while the simultaneous presence of DMSO had an obvious increase in the receptors protein expression (*Figure 3.38; A*). Unfortunately, as there are currently no commercial PDE12 inhibitors, we could only go on to further study PDE4D. An example of a similar effect of DMSO on PDE4D, as well as other PDE4s, has

previously been described in HL60 cells during differentiation with DMSO so as to acquire a more neutrophil-like phenotype [322].

We next utilised Roflumilast, a specific PDE4 inhibitor, and cultured the hNTCP overexpressing hepatic cell line of interest in the presence of this drug and simultaneously in the absence or presence of DMSO. The results were clear; hNTCP protein expression was enhanced in the presence of roflumilast in a dose dependent manner, while the addition of DMSO did not obviously further enhance this outcome (*Figure 3.39*). As a result, we could conclude that roflumilast and DMSO appeared to have the same mechanism of action, and hence our second hypothesis appeared to be correct; DMSO specifically targets PDE4(D), indirectly blocking the breakdown of cAMP, and hence resulting in the cAMP-mediated translocation of hNTCP to the plasma membrane. This process simultaneously involves cAMP-mediated dephosphorylation of the receptor protein, as well as cAMP-mediated phosphorylation of actin filaments that become destabilised, hence aiding the transport of hNTCP to the plasma membrane. Nevertheless, it could still be the case that DMSO targets other PDEs within the cell, something that requires further investigation in the future.

The model of DMSO's full mechanism of action, according to our experimental data, is as follows:

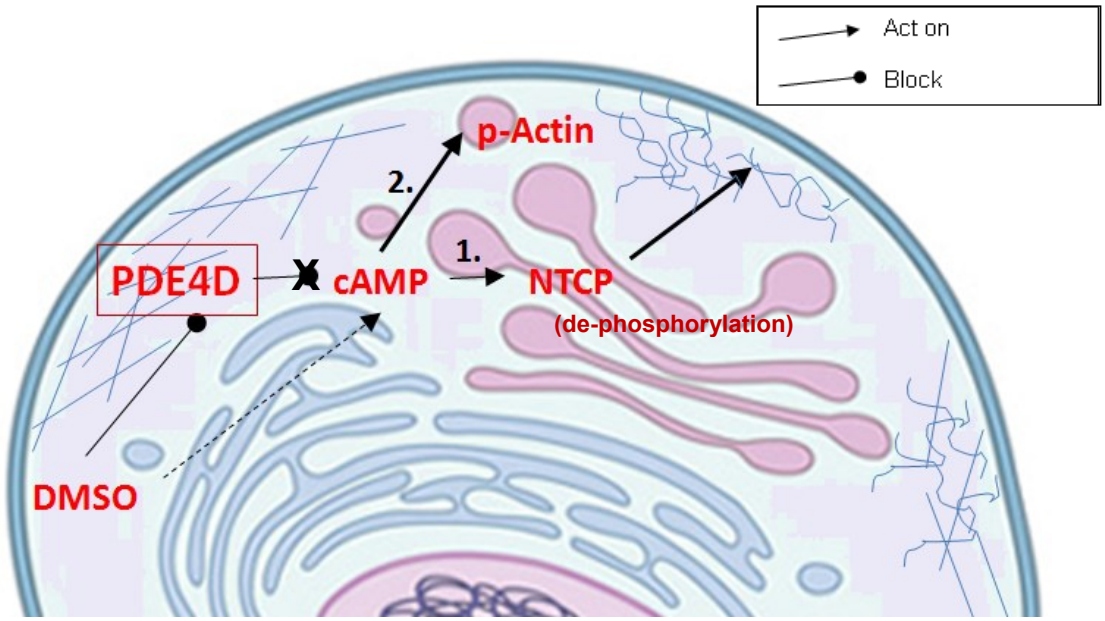


Figure 4.7; DMSO's mechanism of action #2; DMSO targets PDE4(D), eventually resulting in indirect translocation of hNTCP to the plasma membrane.

DMSO is possibly indirectly acting on cAMP by specifically targeting PDE4(D), and hence preventing cAMP degradation. This in turn enhances hNTCP receptor translocation to the cell surface mediated by cAMP, by: 1) dephosphorylation of the hNTCP receptor, and 2) phosphorylation of actin, causing its depolymerisation and thus providing easier access for hNTCP transportation to the plasma membrane.

Enhanced expression of the hNTCP receptor on the plasma membrane correlates with enhanced susceptible of these cells to HBV infection

While performing the experiments discussed above, we additionally evaluated how susceptibility of the hNTCP overexpressing hepatic cell lines to HBV infection was affected under all the different culturing conditions, and particularly in the presence of all exogenously added reagents/ drugs/ inhibitors, as well as DMSO. We quantified all samples for the presence of HBV DNA copies and additionally analysed the experiments by HBcAg staining for visualisation of positively infected cells. The conditions tested were exactly the same as the ones previously performed for analysis of the effect of these compounds on the hNTCP protein expression levels and the receptors translocation to the cell surface. Our data particularly demonstrated the following points:

1. The presence of cytochalasin D had a similar effect to DMSO, while addition of the two reagents together had a highly pronounced effect on the number of cells becoming susceptible to viral infection. This was the case also for latrunculin A were a significant number of cells became susceptible to infection (*p=0.001*), regardless of the presence of DMSO. Colchicine and demecolchine had rather similar outcomes, which were comparable to the effect of DMSO. Nevertheless, when either of the inhibitors was added to the cell culturing media simultaneously with DMSO, then there was an additive effect, with an even greater number of cells becoming susceptible to viral infection (*Figure 3.28*).

   Subversion of the actin cytoskeleton during viral infection is a process that has been discussed amongst virologists over the years [323]; therefore, it cannot be disregarded that by additionally using these drugs that vitally affect the actin polymerisation of the cells we are in a way promoting virus entry and subsequent infection of these cells under non-natural conditions.

2. The presence of exogenous cAMP while culturing the cells prior to and during the infection protocol with HBV significantly increased the susceptibility of cells to HBV infection, more so than what DMSO alone did (*p=0.005*). However, when cAMP was added together with DMSO an even significantly greater number of cells became susceptible to infection with the virus (*p=0.001*). The presence of wortmannin generally reduced susceptibility of cells to HBV infection, and so did MDL12. Caffeine, on the contrary, had a similar effect to DMSO, with the simultaneous addition of the two reagents having an even greater and additive effect (*Figure 3.32-3.33 and Figure 3.35-3.36*).

3. The presence of roflumilast, the PDE4 inhibitor, resulted in an enhanced susceptibility of cells to HBV infection, which appeared to be independent of the presence of DMSO. Thus, further supporting our hypothesis that roflumilast and DMSO have the exact same mechanism of action, which is to target PDE4(D) eventually resulting in an

enhanced hNTCP protein expression that is further linked to increased susceptibility of cells to HBV infection (*Figure 3.40*).

As silencing PDE12 had a comparable effect to inhibition of PDE4D in regards to overexpression of hNTCP, it would definitely be interesting to investigate whether a similar effect would be seen when silencing PDE12 on increasing susceptibility of cells to HBV infection. However, as already mentioned there are currently no available small-molecule PDE12 inhibitors, so in the future when one becomes available it will be interesting to further investigate the full effect of its silencing on both hNTCP cell surface overexpression and subsequent susceptibility of cells to HBV infection, while additionally comparing it to the effect of Roflumilast. At the same time, it would also be interesting to investigate whether the effect we hypothesised that cycloheximide had on inducing hNTCP overexpression (*Figure 4.1*), by specifically targeting an NTCP-inhibitory factor for rapid turn-over and hence stabilising the levels of hNTCP within and on the cell, is in any way associated with blocking any of these PDEs. Previous research has in fact demonstrated that cycloheximide blocks cAMP PDE activity by specifically having an inhibitory effect on PDE4(D) [324-326]. Finally, it should be restated that currently the preferred hNTCP overexpressing human hepatic cell lines are monoclonal populations of cells that have predominantly been selected on the basis of facilitating HBV infection upon treatment with DMSO as well as PEG [226, 228, 232, 254-256]. Therefore, it is very likely that these single cell clones may in fact have characteristically low baseline expression levels of distinct PDEs, something which would be interesting to further investigate.

4.2.2.1 Human NTCP protein analysis; Technical & Experimental difficulties faced particularly during western blot analysis

In our lab, we principally made use of the NuPAGE® SDS-PAGE and Western Blot technology as supplied by ThermoFisher Scientific; meaning that we

particularly used ready-made gels and the company's running buffers, as per their suggestion, while we additionally utilised the iBlot 2 dry blotting system (*ThermoFisher Scientific*) for transferring the proteins onto appropriate membranes. The remaining process was performed following standard procedures for blocking and staining the membranes with appropriate antibodies, and finally for detection of the proteins by ECL technology, as previously described.

The ThermoFisher Scientific system seemed to work well, for example, when originally detecting the hASGPR receptor overexpression in whole protein lysates prepared from different cell lines, or for detecting endogenous levels of human EGFR and TfR. However, when it came to detecting the hNTCP receptor, as previously mentioned, no protein could be detected regardless of the conditions tested. Initially, we believed this to be due to problems with the anti-hNTCP antibody specificity, as previously discussed, but we later figured out that that was not the only reason. Towards this discovery an important role played the switch from modern to conventional protein analysis methods; explicitly, we began to follow traditional protocols for gel preparation, sample running and transfer of the proteins.

Analysing the hNTCP protein samples by conventional methods, we specifically determined the following points:

a. hNTCP was in fact significantly overexpressed at the protein level, but only in the presence of DMSO (2%) – a conclusion which on its own took a very long time to be reached. In the absence of DMSO, no protein could be detected whatsoever regardless of the precise method used for analysis. However, for some unexplained reason the hNTCP protein samples could not be analysed optimally, even in the presence of DMSO, when using the NuPAGE® or iBlot technologies compared to when the samples were analysed by traditional methods, as predominantly determined by considerably lower protein expression

levels (*Appendix A.4.I*). Therefore, any conclusions made when performing the commercial western blot analysis procedure would have most likely been skewed.

b. Denaturing the hNTCP protein prior to running on the SDS-PAGE gel by boiling the RIPA-lysed samples resulted in a significantly degraded form of the receptor being detected by western blot. However, this degradation could in the majority be overcome by lysing the cells directly in Laemmli buffer, rather than performing a RIPA protein extraction (*Appendix A.4.I*). In the end, we resulted in lysing the cells in RIPA buffer, followed by addition of Laemmli sample buffer and running the samples after briefly sonicating them, rather than heat denaturing them, to ensure everything within the samples was completely fragmented.

c. The anti-NTCP antibodies were finally demonstrated to have a much lower detection efficiency compared to the Flag-tag (*Appendix A.4.II*), which we ended up utilising precisely for the purpose of aiding recognition of the receptor protein. Therefore, NTCP antibody specificity would have also been a problem even if everything else worked optimally during the analysis of hNTCP protein expression under the different conditions and cell lines investigated, probably also resulting in rather skewed conclusions being reached.

Another general problem we were facing when performing western blotting was the somewhat unequal quantities of proteins being, on occasions, loaded onto the SDS-PAGE gels, as determined by slightly irregular actin loading control bands between different samples. We typically used a nanodrop to quantify the protein concentrations, which most likely could not have always been extremely accurate. Time permitting, it would have been ideal if some of the blots could have been repeated after more accurately quantifying the concentration of the protein samples by some other method, such as BSA or BCA protein assays.

4.2.3 Effect of DMSO on the human ASGPR receptor, and how this further affects susceptibility of cells overexpressing this receptor to HBV infection

Similarly to the production of cell lines overexpressing the hNTCP receptor, we purchased an identical commercial lentiviral plasmid expressing the human ASGPR receptor under the control of a CMV promoter. This was used for the production of lentiviruses that were further utilised to transduce only human hepatic cells (HepG2 and Huh7.5). Stably hASGPR overexpressing cell lines were selected for with a suitable antibiotic as polyclonal populations. These were initially assessed for hASGPR expression at the mRNA level, and were determined to overexpress the receptor of interest between three to five log higher compared to the equivalent untransduced parental cells (*Figure 3.41*). At the protein level, hASGPR overexpression could be observed in both stable cell lines when compared to the parental controls (*Figure 3.41*). However, the stable HepG2 cell line had definitely significantly higher levels of hASGPR overexpression compared to the stable Huh7.5 cell line.

Having determined that the lentiviral transduction protocol for hASGPR worked successfully in the cell lines generated, next we decided to generate double transduced cell lines; meaning cell lines overexpressing both the hNTCP and hASGPR receptor. For this reason, we utilised the previously generated hNTCP overexpressing cell lines, human hepatic and non-hepatic, and further transduced them with the lentiviruses expressing the hASGPR receptor, following exactly the same process as previously described. New stable hNTCP/hASGPR overexpressing cell lines were created as once again polyclonal populations. Our primary interest was to determine the effect of DMSO on hASGPR in these double transduced cell lines, and to clarify whether the effect we were seeing on hNTCP was actually unique to this receptor or whether the effect of DMSO was broader, affecting similarly other receptors. Therefore, we treated the newly created double-receptor overexpressing cell lines with various concentrations of DMSO for different

periods of time, while we also eventually removed the agent to see whether the effect if any could be reversed. Our data illustrated that (*Figure 3.42*):

1. The presence of DMSO surprisingly affected the hASGPR expression in a somewhat similar manner to how hNTCP expression was being affected in the reagents presence, on the mRNA and protein level, while the results were confirmed to be independent of the simultaneous presence of hNTCP. The extent of DMSO's effect, however, on occasions was obviously more pronounced on hNTCP compared to hASGPR (*Figure 3.43*), as especially at the protein level it was not always easy to detect differences in hASGPR expression. Interestingly, it appeared that the presence of DMSO in these cell lines affected in a similar manner also the endogenous levels of hASGPR, further confirming the fact that the effect DMSO was responsible for was independent of the CMV promoter driving expression of these receptors.
2. Similarly to previous observations, this effect was only obvious in one human hepatic cell line tested (HepG2) and not in a second human hepatic cell line (Huh7.5) (*Figure 3.44*), or in the non-hepatic cell line (HEK293); once again validating that both these receptors are differentially regulated in the specific human hepatic cell line and possibly require to undergo more complex processing mechanisms for their accurate expression, a process that appears to be properly regulated in the presence of DMSO, and these processes are just not as complex or are generally not necessary for the receptors' proper expression in other human hepatic cells or in the non-hepatic cell line.

The effect of DMSO on hASGPR, nonetheless, requires further investigation for a precise comparison to be made between the two receptors and how their expression on the plasma membrane is being regulated in the presence of this reagent. It would be particularly interesting to identify whether DMSO follows similar mechanisms of action to promote expression of hASGPR, as the ones it participates in for the purpose of enhancing expression of hNTCP on the plasma membrane; a fact that would further provide proof that the two

receptors possibly traffic together to the cell surface in the same endocytic vesicles [207, 208]. Our data further illustrates that DMSO has a thus far unknown mechanism of action on possibly a number of other receptors, which are yet to be investigated. We additionally decided to briefly look into the effect of DMSO on the expression of human EGFR and TfR receptors, and our findings are discussed below (*Section 4.2.3.1*).

Following on, for the purpose of characterising susceptibility of these cells to HBV infection, we purposefully designed and produced the HBV-mimicking lentiviral pseudoparticles, as discussed in *Section 4.2.1*, and we particularly used the ultracentrifuged HBV-like particles to further infect human hepatic cell lines (HepG2) overexpressing hNTCP or hASGPR, or hNTCP/hASGPR. Unfortunately, however, we were only able to establish very low efficiency of HBV entry in the double-receptor transduced cells (*Figure 3.47; A*). Testing the effect of different sodium concentrations during the infection protocol, in the anticipation of enhancing the general levels of infection, revealed that the infection efficiency of these pseudoparticles into the hNTCP/hASGPR overexpressing human hepatic cells did indeed increase in a dose dependent manner, but again only resulted in especially low infection efficiencies (*Figure 3.47; B*). Presumably influx of higher sodium concentrations into the cells may have simultaneously allowed the particles' entry, however this requires further investigation before any final conclusions can be made. The presence of DMSO, on the other hand, enhanced even greater the infection efficiency of the cells with these HBV pseudoparticles, compared to the effect detected when the different sodium concentrations were tested (*Figure 3.48*). Our data particularly revealed:

1. In the human hepatic cell line overexpressing both receptors, approximately 15% extra cells were infected with the HBV-like particles in the presence of DMSO ($p = 0.02$), compared to the number of cells infected when only hNTCP was overexpressed. All numbers were determined by counting the GFP positive cells under a fluorescent microscope.

2. In the human non-hepatic cell lines the infection efficiency was evidently improved compared to the hepatic cell lines, verifying the fact that in the hepatic cell line there is probably an additional host factor missing or maybe there is a post-entry block negatively influencing subsequent infection of these cells. In the double-receptor transduced cells between 60-70% of cells became infected (*p = 0.01 – 0.017, when compared to the infection levels obtained in the parental cells*), regardless of the presence of DMSO, with lower levels of infection being seen in the cells overexpressing only hNTCP (~35% GFP positive cells; *p = 0.02 – 0.03*). It should be noted, however, that even the parental cells not overexpressing any of the receptors became quite readily infected with these HBV pseudoparticles (~ 20% GFP positive cells), possibly indicating non-specific entry of these particles into these cells. Regardless, the presence of both HBV-related receptors resulted in an increased number of cells becoming susceptible to infection.

Consequently, our results indicated that entry of the HBV-mimicking particles relied on the simultaneous presence of both hNTCP and hASGPR receptors, while the presence of DMSO had an apparent positive effect, as anticipated. ASGPR has in the past been reported as a possible receptor utilised by HBV [25, 188, 190, 191], however its precise functionality to support HBV entry and infection has not been verified. Therefore, further investigations are definitely required for the purpose of determining the precise susceptibility of these cells overexpressing either hASGPR or hNTCP/hASGPR to HBV infection. In addition, even though previous studies have shown that similar HBV-mimicking particles can infect PHH or human hepatic cell lines overexpressing hNTCP [259, 260], further investigations into the effectiveness of these HBV-mimicking pseudoparticles at infecting cells overexpressing the two receptors of interest are also necessary.

4.2.3.1 The human EGFR and TfR receptors, and their differential regulation by DMSO

Previous research, as already mentioned, has demonstrated that NTCP and TfR travel to the plasma membrane in the same endocytic vesicles, and that the presence of EGFR is essential for regulating these trafficking processes, while from other studies we know that ASGPR and TfR undergo very similar trafficking kinetics [207, 208].

For the purpose of analysing the effect of DMSO on these two additional receptors, we used the original human hepatic and non-hepatic cells overexpressing hNTCP and evaluated the endogenous expression levels of human EGFR and TfR after the cells had been cultured in the absence or presence of DMSO over time. We determined that the expression levels, mRNA and protein, of hEGFR and hTfR decreased in the presence of DMSO, with hTfR being more visibly affected compared to hEGFR (*Figure 3.45*). The results were confirmed to be independent of the simultaneous presence of hNTCP, as the same outcome was demonstrated in the parental human hepatic cell lines that did not overexpress hNTCP.

The precise effect of DMSO on these receptors requires further investigation for a more accurate understanding of its mechanism of action; however, it is interesting that the reagent exerts an opposite effect on hEGFR and hTfR compared to its effect on hNTCP and hASGPR, demonstrating that DMSO possible has a broader and at the same time diverse effect on a number of other yet uncharacterised receptors.

4.2.4 Properties of the chimeric NTCP receptor, its particular regulation by DMSO, and subsequent susceptibility of cells overexpressing this receptor to HBV infection

The design and creation of the chimeric NTCP receptor has been previously discussed (*see Section 3.2.2 and Figure 3.51*); in brief, amino acids 157-165 of the mouse NTCP receptor were substituted with their human counterpart. The gene of interest was inserted into lentiviral plasmids, under the control once again of a CMV promote, and lentiviral pseudoparticles expressing the chimNTCP were produced. These were then used to further transduce various other cell lines; human and mouse hepatic cells, as well as non-hepatic cells. Stably chimNTCP overexpressing cell lines were finally selected for with a suitable antibiotic.

Initially, the stable polyclonal populations of cells, human and mouse hepatic (HepG2 and H2.35, respectively) and non-hepatic (HEK293), were assessed for chimNTCP (i.e. mouse NTCP) expression at the mRNA level, and this analysis demonstrated that the generated cell lines overexpressed the receptor of interest between three to five log higher, depending on the cell line, compared to the equivalent untransduced parental cells; similarly to the levels previously determined for hNTCP. Nevertheless, what we were particularly curious to assess was whether DMSO had a similar effect on chimNTCP like the one it had on hNTCP in the specific human and mouse hepatic cell lines.

Therefore, we treated the newly created chimNTCP overexpressing cell lines with various concentrations of the agent for different time periods, while we also eventually removed the agent to see whether the effect, if any, could be reversed. Interestingly, however, the expression of chimNTCP on the mRNA and protein level was completely unaffected by the presence of DMSO (the antibody specificity is however rather questionably; *Figure 3.52*), revealing that human and mouse NTCP receptors are definitely differentially regulated hence possibly partially explaining the inability of mouse hepatocytes to become

susceptible to HBV infection. We know for a fact from previous research that protein glycosylation is differentially regulated in humans and in mice [327-329], and interestingly this was also obvious when analysing the chimNTCP protein samples by western blot; the chimNTCP was obviously differentially glycosylated in the stable human HepG2 versus the stable mouse H2.35 cell lines (*Figure 3.52; B*). In particular, in the HepG2 cells the chimNTCP seemed to be glycosylated rather similarly to hNTCP, in the specific cell line; meaning that a smeary band was visible. However, in the H2.35 cells, and on the contrary to hNTCP glycosylation, the chimNTCP appeared not be glycosylated in the same manner, as a distinct band rather than a smear was noticeable; possibly suggesting lack of glycosylation. Therefore, this could still be an additional explanation in regards to the HBV interspecies transmission barrier further suggesting that the mouse NTCP differential glycosylation pattern may not be able to support HBV infection.

Following on, we attempted to perform some HBV infection experiments in these human and mouse hepatic cells overexpressing the chimNTCP receptor. Equivalent cells expressing the hNTCP receptor were used as positive controls for infection. We specifically used cell culture-derived virus, and quantified the samples for HBV DNA copies. To our immense surprise, the mouse hepatocyte cell line overexpressing the chimNTCP receptor became susceptible to HBV infection to comparable levels as the human hepatoma cell lines overexpressing the hNTCP receptor (*Figure 3.53*). This outcome was reproducible every time the experiment was repeated. To our knowledge, this is the first reported case of mouse hepatocytes, expressing this particular chimeric form of the NTCP receptor, supporting HBV entry leading to infection *in vitro*. We additionally demonstrated that infection of human and mouse cells resulted in the activation of different signalling factors and pathways (*Table 3.4* and *3.5*), with two signalling pathways being predominantly activated in the stable mouse hepatic cell line overexpressing the chimNTCP receptor upon infection with HBV; the RAF/MAP kinase cascade and the MAPK1/MAPK3 signalling pathway (*Figure 3.54*). These two pathways, interestingly, were not

as obviously activated in the stable human hepatic cell line overexpressing the hNTCP.

Previous research to date has failed to demonstrate that mouse cells can become infected with HBV when expressing the same chimeric NTCP receptor [30]. These studies have, nonetheless, shown that two domains of the receptor are definitely indispensable for HBV binding and subsequent infection in human cells (aa 84-87 and 157-165) [30, 163]. Both studies particularly demonstrated that replacing the mouse NTCP aa 84-87 with the human counterparts converted the mNTCP into a fully functional receptor supporting HBV infection but only in human hepatic cells. On the contrary, it has been verified that mouse cells overexpressing the human form of the NTCP receptor are actually capable of supporting HDV infection [30, 163, 223, 224]. HDV utilises the HBV envelope glycoproteins for virus entry and therefore it is probable that in mice there is also some sort of a post-entry block that renders them non-susceptible to HBV infection [171, 172].

Recently, another group demonstrated that a particular mouse hepatic cell line reconstituted with the human NTCP receptor did in fact become susceptible to HBV infection [164]. Consequently, in conjunction with the fact that the NTCP receptor is differential regulated in different cell lines, maybe it is the case that generally cell lines of diverse backgrounds have varied susceptibilities to viral infection; meaning that reconstitution of the entire human NTCP receptor or only certain of its functional domains in specific, and not all, mouse cell lines with different backgrounds of origin may be enough to somewhat render them susceptible to HBV. However, this requires further investigation.

Moreover, a preliminary *in vivo* experiment, performed by Dr Marcus Dorner, confirmed susceptibility of one mouse (out of two), that had been hydrodynamically injected with the lentiviral plasmid expressing the chimNTCP receptor, to HBV. For the infection of the mice, cell culture-derived infectious

virus was once again used, and infection was confirmed by positive levels of HBV DNA copies and HBsAg at day five and ten post infection (*Figure 3.56*). HBV DNA copies were also measured and found to be positive at the end of the experiment (eleven days post infection) in the samples isolated from the blood and the liver of the same animal (*Figure 3.57*). Nevertheless, infection was most likely naturally resolving over time.

Previous *in vivo* research, further confirming the results of some *in vitro* studies [30, 163, 223, 224], has revealed that substituting aa 84-87 of the endogenous mouse NTCP with their human counterparts, by specifically using transgenic mice expressing exogenous human NTCP in their liver, renders these mice susceptible to HDV infection, not HBV [330]. Moreover, another study has shown that mice with a humanised liver can become fully susceptible to HBV infection, and the spread of the infection can be effectively blocked by targeting the endogenous NTCP receptor [331]. Our *in vivo* study, however, was only a very preliminary experiment, and even though the results were optimistic in regards to one mouse supporting HBV infection when exogenously expressing a chimeric form of the NTCP receptor, no significant conclusions should be drawn until confirmed by further investigations.

4.2.5 The cyclic repackaging rescue screen for identification of additional factors vital for HBV entry and subsequent infection

A cyclic repackaging rescue screen technology, for the subsequent identification of additional receptors or host factors required for efficient HBV entry *in vitro* and *in vivo*, can in theory be performed similarly to what was previously performed for HCV, when Occludin was identified as another functional receptor for virus entry and infection [267]. Even though NTCP has been identified as the long-sought functional receptor for HBV, it is still quite likely that HBV requires the presence of additional host factors for productive infection of susceptible or otherwise non-susceptible systems or hosts, further

explaining why we still require very high MOIs to infect hNTCP overexpressing human cells as well as the limited host range of the virus. Host factors that block entry at a post-entry step, could possibly also be uncovered during this procedure.

For our experimental purposes, we slightly altered the protocol described by Ploss and his colleagues (*for full details refer to Section 2.2.2.4.1.3.1, and Figure 2.14*), and utilised the previously generated HBV-mimicking pseudoparticles as well as the available recombinant HBV virus specifically encoding for BSD, to aid the selection of the positively infected cells.

In particular, we began by making use of a human hepatic cell line (Huh7.5) overexpressing both HBV receptors of interest, hNTCP and hASGPR. This was transduced with the already established cDNA library, originally provided by P. Bieniasz (*Aaron Diamond AIDS, US*) to A. Ploss (*Rockefeller University, US*) and, which was then provided to us by C. Rice (*Rockefeller University, US*). These cells expressing the lentiviral cDNA library where then further transduced with the HBV-mimicking particles expressing GFP, which were single-cell FACS sorted by the staff at St. Mary's Flow Cytometry Core Facility. All single cell clones, even though sorted for specific GFP positive signal, surprisingly once they began to grow they expressed varying levels of GFP within the same cell population samples (*Figure 3.68 and A.3VIII*). In addition, there were other cells that, even though they had originated from a single GFP positive cell, where negative for GFP expression. Confirming that cells possibly have an ability to switch off or suppress selectively expression of genes following integration via lentiviral vectors, as previously discussed.

For the first round of selection of these single cell populations, we infected them with infectious cell culture-derived virus and analysed the samples for secretion of HBsAg and HBeAg. We continued only with those clones that were characterised by production of high levels of the two HBV antigens

(*Figure 3.69*). Interestingly, however, the high levels of HBV infection did not necessarily match the levels of GFP positive expression or the levels of hASGPR expression (*Figure 3.70; B*), which also varied amongst the different cell populations. Moreover, expression of hNTCP was rather odd, with cells either expressing the receptor at comparable levels or not expressing it at all (*Figure 3.70; A*). As these cells were originally selected for the stable expression of both HBV receptors, while in the specific cell line NTCP expression has previously been shown not to be reliant on the presence of DMSO, it is therefore highly unlikely that they had fully lost or even suppressed hNTCP expression. For these reasons, this outcome could most likely be attributed to some technical problems during analysis of the particular protein samples; such as protein transfer problems, non-equal concentrations of protein being loaded on the gel or limited antibody availability for full protein detection.

For the second and final round of selection, the already selected single cell populations were infected with the cell culture-derived recombinant virus expressing the BSD selection marker (*as previously discussed in Section 4.2.2*) and were eventually cultured under antibiotic selection until only a very small number of cells had survived. Specifically, in one of all the clones we had selected, two individual cell colonies appeared to have fully survived antibiotic selection (*Figure 3.71*). Possibly if we had infected these cells with much higher MOIs of this recombinant virus more cells would have become infected and survived, nevertheless, this was an initial confirmation that the recombinant virus we had produced was moderately functional at re-infecting cells.

Once fully confluent, the selected cell clones were rescued and frozen down. These will eventually be sequenced and hopefully, apart from hNTCP, some other host factor(s) necessary for effective HBV infection will be uncovered. The identification of any additional host factors necessary for effective HBV entry, should hopefully lead to the establishment of more efficient HBV

infection systems *in vitro*, as well as the development of a small *in vivo* model for the complete study of the HBV life cycle.

4.3 Future work

In general, there is a lot more experimental work that could have additionally been performed if there had been more time available, especially in regards to the further characterisation of all the tools created during the course of this project. Some of the important next steps for this project, as well as various experimental questions of interest, are mentioned below.

Susceptibility of human hepatic cell lines overexpressing the hNTCP receptor to HBV infection

Now that we have established and confirmed the necessity of DMSO for hNTCP overexpression on the plasma membrane, and its precise mechanism of action, which subsequently results in enhanced susceptibility of the cells to HBV infection, it would be interesting to evaluate whether the presence of PEG is really required any longer. Maybe it is the case that PEG further enhances the susceptibility of these cells to HBV infection, possibly working in combination with DMSO, but DMSO may also be validated to be adequate on its own for enhancing the susceptibility of cells to HBV. Nevertheless, if the presence of PEG is still determined to be essential in the HBV infection process, possibly further suggesting that additional host factors are definitely necessary for productive HBV entry, then it could also be interesting to investigate whether the reagent has a direct effect on the NTCP receptor and subsequent HBV infection, rather than just permitting fusion of the virus to the cell membrane.

Following on, having confirmed that the stable human hepatic hNTCP overexpressing cell lines generated can indeed support HBV infection, it would also be interesting to test infections at different MOIs and specifically

determine what is the lowest MOIs that can be used for detectable HBV infection under different cell culturing conditions. The main goal always being to improve HBV infection efficiencies, *in vitro*. Some binding competition assays, for example with Myrcludex or other preS1 peptides, or even [^{3}H] taurocholate (TC) uptake assays, would have also been advantageous to perform, so as to clarify whether the observed HBV infection was indeed specific and hence could be actually blocked under certain situations.

Furthermore, it would have been ideal if certain of the already performed HBV infection experiments could have been repeated yet again or just re-analysed, primarily so as to be able to acquire larger sets of complementary data; including secretion of HBsAg and HBeAg, production of pgRNA and sgRNA, and even possibly quantification of cccDNA. Such an example, amongst others, is the HBV infection experiment in the presence of Roflumilast, while a HBV infection experiment of hNTCP overexpressing cell lines in the presence of the PDE4D siRNA ideally should have also been performed. Analysing the endogenous levels of the PDEs of interest in the different cell lines would have also been informative. Ideally, it would have also been interesting to confirm the results from this study in a different *in vitro* cell culturing system, such as in 3D plated PHH.

<u>Susceptibility of human hepatic cell lines overexpressing the hASGPR receptor to HBV infection</u>

Further infection experiments of the human hepatic cell lines overexpressing either hASGPR alone or both hNTCP and hASGPR are definitely required, so as to confirm the precise susceptibility of these cells to HBV infection and whether ASGPR is definitely required for this process or not. For this purpose, it would be preferable to use cell culture-derived virus, as previously performed for HBV infection of the cell lines overexpressing hNTCP alone. For these HBV infection experiments, however, it would possibly be advantageous to further

investigate the precise mechanism of action of DMSO on hASGPR receptor regulation and expression.

Moreover, the HBV-mimicking lentiviral pseudoparticles require further characterisation and their precise infectivity needs to be re-assessed, while at the same time susceptibility of cells to these particles could be tested under diverse culturing conditions. Further characterisation could involve the use of HIV-based assays, such as measuring p24 with the use of particular ELISAs or even a SG-PERT (SYBR-green, product-enhanced reverse transcriptase) assay that measures the reverse transcriptase activity of these particles. During the course of this project, we specifically produced two different types of HBV-like particles, but unfortunately only had time to characterise as best as possible one of the two. Therefore, it could still be the case that the second type of HBSpp may in fact be more efficient at infecting hepatocytes compared to the first type that we characterised.

In vitro and *in vivo* susceptibility of mouse hepatocytes overexpressing a chimeric NTCP receptor to HBV infection

Having established susceptibility of one mouse cell line to HBV infection when overexpressing a chimeric form of the NTCP receptor, it would initially be interesting to verify the susceptibility of other mouse cell lines originating from different backgrounds and overexpressing the same chimNTCP to HBV infection, while at the same time also evaluating the full effect, if any, of DMSO on the chimNTCP expression in these additional cell lines. This should hopefully help elucidate the differential regulation of chimNTCP in different cell backgrounds, as well as the potential differential susceptibility of mouse cells to HBV infection, hence further permitting identification of differential factors influencing efficient HBV infectivity under diverse situations. If there had been more time available, it would have also been interesting to further analyse in much greater depth the different signalling pathways activated upon HBV infection in human versus mouse cells and perform follow up experiments.

Further proteomic and bioinformatics analysis in regards to the different backgrounds of the cell lines utilised during this project would also definitely be advantageous.

On the other hand, the *in vivo* mouse experiment (i.e. mice hydrodynamically injected with the plasmid overexpressing the chimNTCP receptor and subsequently infected with cell culture-derived HBV) requires repetition and further confirmation. Primarily, a larger cohort of animals needs to be included in any follow up experiments, while initially any experiment should be performed under completely the same conditions. Follow up experiments, once the original experiment has been fully confirmed, may include testing once again mice of different backgrounds for their ability to support HBV infection under similar conditions. In addition, instead of hydrodynamic delivery of the receptor of interest, this could instead be directly delivered into the livers of the animals via AAVs expressing the particular receptor, as this would result in a more stable expression of the chimNTCP over time and hence longer periods of HBV infection could be studied. The particular AAV plasmids expressing the chimNTCP have already been constructed/ cloned during the course of this project and were subsequently used for the commercial generation of AAV particles (*Vector Biolabs, US*).

General susceptibility of mouse hepatocytes

Having determined the regulation and signal transduction pathways for accurate hNTCP expression and stability on the plasma membrane of human hepatic cell lines, and especially the importance of PDE expression regulation by DMSO, it would now be interesting to characterise this regulatory pathway on mNTCP in murine cells. In particular, it would be of great interest to investigate the precise signal transduction pathways regulated by DMSO, if any, and fully characterise the possible impact of PDEs on regulation of mNTCP in mouse cells. This should provide us with a better insight into the HBV narrow species tropism and hopefully offer possible mechanisms by

which this could be overcome in the near future for the creation of a much needed small animal model to study HBV.

Recombinant HBV, for subsequent infection experiments and use in CRRS

Having had time to characterise two out of the four different recombinant HBV constructs encoding the various reporter genes in regards to their replication-competency, it would be interesting to have the chance to go back and characterise the remaining two HBV genomes specifically encoding for Fluc and Gluc, and evaluate whether their replication efficiency compares to the ones already studied, as well as the original construct they originated from. According to our data, they would be anticipated to replicate in a very comparable way. The fully functional infectivity of all these recombinant HBV constructs, however, certainly requires much further investigation, with higher MOIs possibly being necessary for detectable infection.

Finally, it would have been great to be able to repeat and this time actually finalise the CRRS, in the hope of uncovering any possible factors crucial for productive HBV infection. Any such factor(s) could then be further characterised both *in vitro* and *in vivo.*

4.4 Conclusions

In this study, we have primarily described the mechanistic regulation of the human NTCP receptor by DMSO and the agent's subsequent impact on the susceptibility of human hepatic cells overexpressing this particular receptor to HBV infection, with the number of cells becoming susceptible to HBV infection nearly doubling in the presence of DMSO. The effect of DMSO on human NTCP, in that its presence results in enhanced levels of the receptor's protein expression, has previously been reported and was specifically found to be independent of the CMV promoter driving expression of the receptor [254], as also confirmed by our data. To our knowledge, however, this study presented

here is the first extensive study performed with the aim of dissecting the precise way by which DMSO is exerting its action by tightly regulating the expression of the NTCP receptor, in the hope of first and foremost gaining some better understanding of the complex virus/host interactions established during the early events of HBV infection. The information collected during the course of this project should also assist in improving the currently available cell culture systems for studying HBV, while at the same time offering a broader understanding of the vast effects and the importance of DMSO on different biological processes.

Our data has specifically demonstrated that the presence of DMSO enhances expression of human NTCP on a transcriptional but also principally on a translational and post-translational level, especially promoting precise NTCP translocation to the plasma membrane. DMSO's mechanism of action on expression of NTCP appears to be a very precisely orchestrated procedure, which is particularly time and dose dependent, while the absence of DMSO easily and rapidly results in the reversal of its enhancing effect on NTCP caused when the reagent is present in the cell culture media. This means that in the absence of DMSO the levels of NTCP expression quickly diminish, however, we have also demonstrated that when this occurs the NTCP protein is definitely more stable than the receptor's mRNA expression.

In addition, we have shown that the presence of DMSO in cell culture results in an altered cellular cytoskeleton and morphology, mainly observed by rearrangement of actin microfilaments. This could just be an indirect effect caused by the induction of cell stress; however, interestingly this directly coincides with accumulation of intracellular cAMP. Inhibition of particular cellular processes by specific drugs confirmed the importance of cAMP accumulation, and its downstream signalling events, for enhanced NTCP expression and translocation to the plasma membrane. cAMP has been known to be involved in various cellular processes, including differentiation of cells. In particular, cAMP has previously been determined to play an important role in

rearranging cortical actin through phosphorylation procedures [303-305, 318, 319, 332]. Intentional disruption of actin microfilaments *in vitro*, resulted in the elevation of NTCP expression and trafficking to the plasma membrane, even in the absence of accumulated cAMP, possibly revealing that this is one of the final stages in facilitating accurate regulation of NTCP. Therefore, upon destabilisation of cortical actin filaments, NTCP is more easily shuttled to the plasma membrane where the receptor is stabilised.

In fact, we believe to have provided enough evidence to show that one of the initial stages responsible for the observed effect of DMSO on the expression of NTCP is the inhibition of certain intracellular PDEs by this agent. We specifically considered PDE4, which has been reported to be highly expressed in the liver, as one of the enzymes whose function is blocked or downregulated in the presence of DMSO, with PDE12 possibly being another PDE negatively regulated by DMSO. Our data specifically demonstrated a scenario where the presence of DMSO in cell culture directly blocks PDE4. This was determined to be the case as inhibition of PDE4 with the use of Roflumilast completely abolished the previously observed effect of DMSO, further suggesting that the DMSO-mediated block of PDE4 activity is possibly the main mechanism by which DMSO exerts its effect on NTCP expression. Furthermore, in the absence or suppression of PDE4, cAMP no longer gets degraded by this enzyme and hence can continue to regulate expression and trafficking of NTCP to the plasma membrane. Therefore, DMSO most likely has an indirect effect on cAMP accumulation. The impact of PDEs on the NTCP receptor regulation, to our knowledge, has never been described before now.

The different cell culture conditions tested for the validation of the effect of DMSO on NTCP expression, were also tested precisely in the same manner for validation of the effect of DMSO on susceptibility of the cells overexpressing hNTCP to HBV infection. In general, elevated NTCP expression and translocation to the plasma membrane resulted in elevated susceptibility of those cells to HBV infection.

Consequently, we have herein described a novel mechanism where NTCP expression is regulated primarily post-translationally in the presence of DMSO. This effect is mediated via inhibition of particular PDEs, such as PDE4, hence revealing a thus far unknown role of cAMP in the regulation of human NTCP expression and transport to the plasma membrane. A similar mechanism of action for cAMP has previously been reported but only in regards to rat NTCP [156, 174, 176-182]. This data can possibly be utilised for improving HBV infection efficiencies *in vitro*, even though certain culturing conditions may be characterised as unnatural. Moreover, our data has also allowed the identification of novel targets for blocking entry of HBV by specifically targeting the cAMP-mediated transport of NTCP to the plasma membrane and hence diminishing the cell surface accumulation of the HBV functional receptor, NTCP. This should result in restricted HBV access to its receptor and thus prevent further infection events.

Finally, the effect of DMSO was determined not to be entirely specific to human NTCP, as other cellular receptor proteins were also affected in its presence but to different extents. Some examples include:

- The human ASGPR receptor, which in this study has been demonstrated to be affected by DMSO in a similar manner to human NTCP but not necessarily to the same extent.

  Interestingly, preliminary infection experiments of cell lines overexpressing the HBV receptors of interest with HBV-mimicking pseudoparticles revealed that entry of these particles did somewhat depend on the simultaneous presence of both human NTCP and ASGPR receptors, while, as anticipate, the presence of DMSO had an apparent positive effect on the efficiency of HBV entry.

- The human EGFR and TfR proteins, which were specifically demonstrated to be downregulated in the presence of DMSO. TfR was

nevertheless more highly affected compared to EGFR, where the effect of DMSO was only slightly detectable.

On the contrary, a chimeric form of the NTCP receptor was not found to be affected whatsoever by the presence of DMSO in the cell lines tested. However, of prime interest was the fact that one mouse hepatic cell line overexpressing this receptor was validated to be consistently susceptible and able to support HBV infection. This outcome was further confirmed by a very preliminary *in vivo* experiment. To our knowledge, this is the first study to have established positive infection of mouse hepatocytes overexpressing the particular chimeric form of the NTCP receptor, hopefully opening the road for further research and understanding of the HBV interspecies transmission barrier, as well as identification of those host factors vital for productive HBV infection *in vitro* and *in vivo*. To this end, a cyclic repackaging rescue screen, as previously utilised for HCV [267], could also be performed for HBV; while making use of HBV recombinant viruses encoding reporter genes for monitoring infection, could additionally prove to be highly advantageous.

In conclusion; The discovery of NTCP as the long-awaited, functional receptor for HBV infection has finally opened up the field of HBV research, making it possible to investigate stages of the virus' life cycle that were practically impossible to study up till recently, that being principally virus entry into liver cells. However, up to date, the entry of the virus into hepatocytes still remains rather elusive, with a number of research groups trying to fully understand the virus/host interactions and how precisely these interactions result in virus entry. Even though we now know that NTCP is definitely one of the receptors the virus utilises for its entry into cells, the full interaction between HBV and NTCP has not been characterised from either the virus' or the receptors' point. The current research and knowledge acquired from recent studies has undoubtedly been priceless for getting a step closer to understanding; unfortunately, though, there are still numerous important aspects of the entry stage of the virus' life cycle that the field is lacking full understanding of. For

example, it is still not clear whether NTCP is the first point of contact for the virus or whether apart from HSPGs the virus requires to recognise and interact with other host factors for efficient entry. Therefore, it is quite possible that other co-receptors or host entry factors exist that together with NTCP are responsible for virus entry. This would possibly explain the low infection efficiencies of the hepatic cell lines overexpressing solely NTCP, while any additional factors may also be responsible for limiting HBV infection in naturally non-susceptible hosts. Factors like this still remain to be discovered, however, once they are they will hopefully shed some further light into understanding the complex interplay between HBV and its host cells while additionally also providing important information on the HBV interspecies transmission barrier, which needs to be resolved for the creation of a more physiological small animal model to study the full spectrum of HBV biology, as well as investigating urgently needed treatment strategies. Identification of these factors limiting HBV infection, can additionally further be assessed for the development of potential therapeutic candidates, which are urgently required. Curing HBV should automatically eradicate and HDV.

The study discussed here, we believe, has definitely provided very useful information essential for understanding different aspects of HBV biology and, especially, the complex virus/host interplay during HBV infection, as well as the important role that precise and orchestrated regulation and expression of the human NTCP receptor plays during this process. In addition, this study has also provided some important information in regards to the HBV interspecies transmission barrier, and once again the imperative role of the NTCP receptor towards this complex restriction.

Bibliography

1. Mason, A.L. and R.P. Perrillo, *The A to Z of new hepatotropic agents: human hepatitis viruses and monkey business.* Liver Transpl Surg, 1996. **2**(5): p. 395-405.

2. Hoofnagle , J.H. and A.M. Di Bisceglie *The Treatment of Chronic Viral Hepatitis.* New England Journal of Medicine, 1997. **336**(5): p. 347-356.

3. Michielsen, P.P., S.M. Francque, and J.L. van Dongen, *Viral hepatitis and hepatocellular carcinoma.* World J Surg Oncol, 2005. **3**: p. 27.

4. Davis, G.L., et al., *Hepatocellular carcinoma: management of an increasingly common problem.* Proc (Bayl Univ Med Cent), 2008. **21**(3): p. 266-80.

5. Lang, K., et al., *The burden of illness associated with hepatocellular carcinoma in the United States.* J Hepatol, 2009. **50**(1): p. 89-99.

6. Shebl, F.M., et al., *Socioeconomic status and hepatocellular carcinoma in the United States.* Cancer Epidemiol Biomarkers Prev, 2012. **21**(8): p. 1330-5.

7. Pinter, M., et al., *Cancer and liver cirrhosis: implications on prognosis and management.* ESMO Open, 2016. **1**(2): p. e000042.

8. Stanaway, J.D., et al., *The global burden of viral hepatitis from 1990 to 2013: findings from the Global Burden of Disease Study 2013.* Lancet, 2016. **388**(10049): p. 1081-1088.

9. Helsper, C.W., et al., *Real-life costs of hepatitis C treatment.* Neth J Med, 2012. **70**(3): p. 145-53.

10. Chhatwal, J., et al., *Cost-effectiveness and budget impact of hepatitis C virus treatment with sofosbuvir and ledipasvir in the United States.* Ann Intern Med, 2015. **162**(6): p. 397-406.

11. Snydman, D.R., B.Y. Tung, and K.V. Kowdley, *Hepatitis B and Liver Transplantation.* Clinical Infectious Diseases, 2005. **41**(10): p. 1461-1466.

12. Pellicoro, A., et al., *Liver fibrosis and repair: immune regulation of wound healing in a solid organ.* Nat Rev Immunol, 2014. **14**(3): p. 181-94.

13. Blumberg, B.S., et al., *A serum antigen (Australia antigen) in Down's syndrome, leukemia, and hepatitis.* Ann Intern Med, 1967. **66**(5): p. 924-31.

14. Blumberg, B.S., *Australia antigen and the biology of hepatitis B.* Science, 1977. **197**(4298): p. 17-25.

15. Dane, D.S., C.H. Cameron, and M. Briggs, *Virus-like particles in serum of patients with Australia-antigen-associated hepatitis.* Lancet, 1970. **1**(7649): p. 695-8.

16. Gerlich, W.H., *Medical virology of hepatitis B: how it began and where we are now.* Virol J, 2013. **10**: p. 239.

17. Seeger, C. and W.S. Mason, *Molecular biology of hepatitis B virus infection.* Virology, 2015. **479-480C**: p. 672-686.

18. Brown, A. and Z. Goodman, *Hepatitis B-associated fibrosis and fibrosis/cirrhosis regression with nucleoside and nucleotide analogs.* Expert Rev Gastroenterol Hepatol, 2012. **6**(2): p. 187-98.

19. Franco, E., et al., *Hepatitis B: Epidemiology and prevention in developing countries.* World J Hepatol, 2012. **4**(3): p. 74-80.

20. Gill, U.S. and P.T. Kennedy, *New insights in the management of chronic hepatitis B.* Clin Med (Lond), 2015. **15**(2): p. 191-6.

21. Hou, J., Z. Liu, and F. Gu, *Epidemiology and Prevention of Hepatitis B Virus Infection.* Int J Med Sci, 2005. **2**(1): p. 50-57.

22. Shepard, C.W., et al., *Hepatitis B virus infection: epidemiology and vaccination.* Epidemiol Rev, 2006. **28**: p. 112-25.

23. El-Serag, H.B., *Epidemiology of viral hepatitis and hepatocellular carcinoma.* Gastroenterology, 2012. **142**(6): p. 1264-1273.e1.

24. *EASL 2017 Clinical Practice Guidelines on the management of hepatitis B virus infection.* J Hepatol, 2017.

25. Treichel, U., et al., *The asialoglycoprotein receptor mediates hepatic binding and uptake of natural hepatitis B virus particles derived from viraemic carriers.* Journal of General Virology, 1994. **75**: p. 3021-3029.

26. Block, T.M., G. Haitao, and G. Ju-Tao, *Molecular virology of hepatitis B virus for clinicians.* Clin Liver Dis, 2007. **11**(4): p. 685-706.

27. Venook, A.P., et al., *The incidence and epidemiology of hepatocellular carcinoma: a global and regional perspective.* Oncologist, 2010. **15 Suppl 4**: p. 5-13.

28. Meier, A., et al., *Myristoylated preS1-domain of the hepatitis B virus L-protein mediates specific binding to differentiated hepatocytes.* Hepatology, 2013. **58**(1): p. 31-42.

29. Mittal, S. and H.B. El-Serag, *Epidemiology of hepatocellular carcinoma: consider the population.* J Clin Gastroenterol, 2013. **47 Suppl**: p. S2-6.

30. Yan, H., et al., *Molecular determinants of hepatitis B and D virus entry restriction in mouse sodium taurocholate cotransporting polypeptide.* Journal of Virology, 2013. **87**(14): p. 7977-7991.

31. Zhong, G., et al., *Sodium taurocholate cotransporting polypeptide mediates woolly monkey hepatitis B virus infection of Tupaia hepatocytes.* Journal of Virology, 2013. **87**(12): p. 7176-7184.

32. Yan, H., et al., *Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus.* eLife, 2012. **1**(e00049): p. 1-28.

33. Dienstag, J.L., *Hepatitis B virus infection.* N Engl J Med, 2008. **359**(14): p. 1486-500.

34. MacLachlan, J.H. and B.C. Cowie, *Hepatitis B virus epidemiology.* Cold Spring Harb Perspect Med, 2015. **5**(5): p. a021410.

35. Norder, H., A.M. Courouce, and L.O. Magnius, *Molecular basis of hepatitis B virus serotype variations within the four major subtypes.* J Gen Virol, 1992. **73 (Pt 12)**: p. 3141-5.

36. Ohba, K., et al., *Relationships between serotypes and genotypes of hepatitis B virus: genetic classification of HBV by use of surface genes.* Virus Res, 1995. **39**(1): p. 25-34.

37. Locarnini, S., *Molecular virology of hepatitis B virus.* Semin Liver Dis, 2004. **24 Suppl 1**: p. 3-10.

38. Grimm, D., R. Thimme, and H.E. Blum, *HBV life cycle and novel drug targets.* Hepatol Int, 2011. **5**(2): p. 644-53.

39. Yano, Y., T. Azuma, and Y. Hayashi, *Variations and mutations in the hepatitis B virus genome and their associations with clinical characteristics.* World J Hepatol, 2015. **7**(3): p. 583-92.

40. Enomoto, M., A. Tamori, and S. Nishiguchi, *Hepatitis B virus genotypes and response to antiviral therapy.* Clin Lab, 2006. **52**(1-2): p. 43-7.

41. Schaefer, S., *Hepatitis B virus taxonomy and hepatitis B virus genotypes.* World J Gastroenterol, 2007. **13**(1): p. 14-21.

42. Tanaka, Y. and M. Mizokami, *Genetic diversity of hepatitis B virus as an important factor associated with differences in clinical outcomes.* J Infect Dis, 2007. **195**(1): p. 1-4.

43. Lamontagne, R.J., S. Bagga, and M.J. Bouchard, *Hepatitis B virus molecular biology and pathogenesis.* Hepatoma Res, 2016. **2**: p. 163-186.

44. Zhang, Z.H., et al., *Genetic variation of hepatitis B virus and its significance for pathogenesis.* World J Gastroenterol, 2016. **22**(1): p. 126-44.

45. Chu, C.J. and A.S. Lok, *Clinical significance of hepatitis B virus genotypes.* Hepatology, 2002. **35**(5): p. 1274-6.

46. Scott, R.M., et al., *Experimental transmission of hepatitis B virus by semen and saliva.* J Infect Dis, 1980. **142**(1): p. 67-71.

47. Ganem, D. and A.M. Prince, *Hepatitis B virus infection--natural history and clinical consequences.* N Engl J Med, 2004. **350**(11): p. 1118-29.

48. Li, Z., X. Hou, and G. Cao, *Is mother-to-infant transmission the most important factor for persistent HBV infection?* Emerg Microbes Infect, 2015. **4**(5): p. e30.

49. Yi, P., et al., *Management of mother-to-child transmission of hepatitis B virus: Propositions and challenges.* J Clin Virol, 2016. **77**: p. 32-9.

50. Shapiro, C.N., et al., *Hepatitis B virus transmission between children in day care.* Pediatr Infect Dis J, 1989. **8**(12): p. 870-5.

51. Williams, I.T., J.F. Perz, and B.P. Bell, *Viral hepatitis transmission in ambulatory health care settings.* Clin Infect Dis, 2004. **38**(11): p. 1592-8.

52. Kwon, S.Y. and C.H. Lee, *Epidemiology and prevention of hepatitis B virus infection.* Korean J Hepatol, 2011. **17**(2): p. 87-95.

53. Samal, J., M. Kandpal, and P. Vivekanandan, *Molecular mechanisms underlying occult hepatitis B virus infection.* Clin Microbiol Rev, 2012. **25**(1): p. 142-63.

54. Pearce, N., A. Milne, and C. Moyes, *Hepatitis B virus: the importance of age at infection.* N Z Med J, 1988. **101**(858): p. 788-90.

55. Mahoney, F.J., *Update on diagnosis, management, and prevention of hepatitis B virus infection.* Clin Microbiol Rev, 1999. **12**(2): p. 351-66.

56. Pungpapong, S., W.R. Kim, and J.J. Poterucha, *Natural history of hepatitis B virus infection: an update for clinicians.* Mayo Clin Proc, 2007. **82**(8): p. 967-75.

57. Carneiro de Moura, M. and R. Marinho, *[Natural history and clinical manifestations of chronic hepatitis B virus].* Enferm Infecc Microbiol Clin, 2008. **26 Suppl 7**: p. 11-8.

58. Liang, T.J., *Hepatitis B: the virus and disease.* Hepatology, 2009. **49**(5 Suppl): p. S13-21.

59. Chisari, F.V., M. Isogawa, and S.F. Wieland, *Pathogenesis of hepatitis B virus infection.* Pathol Biol (Paris), 2010. **58**(4): p. 258-66.

60. Navabakhsh, B., et al., *Hepatitis B Virus Infection during Pregnancy: Transmission and Prevention.* Middle East J Dig Dis, 2011. **3**(2): p. 92-102.

61. Croagh, C.M. and J.S. Lubel, *Natural history of chronic hepatitis B: phases in a complex relationship.* World J Gastroenterol, 2014. **20**(30): p. 10395-404.

62. Liaw, Y.F., M.R. Brunetto, and S. Hadziyannis, *The natural history of chronic HBV infection and geographical differences.* Antivir Ther, 2010. **15 Suppl 3**: p. 25-33.

63. Feitelson, M.A., *Biology of hepatitis B virus variants.* Lab Invest, 1994. **71**(3): p. 324-49.

64. Moyer, L.A. and E.E. Mast, *Hepatitis B: virology, epidemiology, disease, and prevention, and an overview of viral hepatitis.* Am J Prev Med, 1994. **10 Suppl**: p. 45-55.

65. Grob, P.J., *Hepatitis B: virus, pathogenesis and treatment.* Vaccine, 1998. **16 Suppl**: p. S11-6.

66. Chang, J.J. and S.R. Lewin, *Immunopathogenesis of hepatitis B virus infection.* Immunol Cell Biol, 2007. **85**(1): p. 16-23.

67. Payne, R.J., M.A. Nowak, and B.S. Blumberg, *The dynamics of hepatitis B virus infection.* Proc Natl Acad Sci U S A, 1996. **93**(13): p. 6542-6.

68. Shiffman, M.L., *Management of acute hepatitis B.* Clin Liver Dis, 2010. **14**(1): p. 75-91; viii-ix.

69. Lok, A.S., *Chronic hepatitis B.* N Engl J Med, 2002. **346**(22): p. 1682-3.

70. Rehermann, B. and M. Nascimbeni, *Immunology of hepatitis B virus and hepatitis C virus infection.* Nat Rev Immunol, 2005. **5**(3): p. 215-29.

71. Lok, A.S. and B.J. McMahon, *Chronic hepatitis B.* Hepatology, 2007. **45**(2): p. 507-39.

72. Tran, T.T., *Immune tolerant hepatitis B: a clinical dilemma.* Gastroenterol Hepatol (N Y), 2011. **7**(8): p. 511-6.

73. Seto, W.-K. and M.-F. Yuen, *Viral hepatitis: 'Immune tolerance' in HBV infection: danger lurks.* Nat Rev Gastroenterol Hepatol, 2016. **13**(11): p. 627-628.

74. *WHO Guidelines Approved by the Guidelines Review Committee*, in *Guidelines for the Prevention, Care and Treatment of Persons with Chronic Hepatitis B Infection*. 2015, World Health Organization

Copyright (c) World Health Organization 2015.: Geneva.

75. Krajden, M., G. McNabb, and M. Petric, *The laboratory diagnosis of hepatitis B virus.* Can J Infect Dis Med Microbiol, 2005. **16**(2): p. 65-72.

76. Taylor, B.C., et al., *Clinical outcomes in adults with chronic hepatitis B in association with patient and viral characteristics: A systematic review of evidence.* Hepatology, 2009. **49**(5 Suppl): p. S85-95.

77. Prince, A.M. and B. Brotman, *Perspectives on hepatitis B studies with chimpanzees.* Ilar j, 2001. **42**(2): p. 85-8.

78. Wieland, S.F., *The chimpanzee model for hepatitis B virus infection.* Cold Spring Harb Perspect Med, 2015. **5**(6).

79. Murray, J.M., et al., *Dynamics of hepatitis B virus clearance in chimpanzees.* Proc Natl Acad Sci U S A, 2005. **102**(49): p. 17780-5.

80. Wieland, S.F., et al., *Expansion and contraction of the hepatitis B virus transcriptional template in infected chimpanzees.* Proc Natl Acad Sci U S A, 2004. **101**(7): p. 2129-34.

81. Tillmann, H.L., *Antiviral therapy and resistance with hepatitis B virus infection.* World J Gastroenterol, 2007. **13**(1): p. 125-40.

82. De Clercq, E., et al., *Antiviral treatment of chronic hepatitis B virus (HBV) infections.* Viruses, 2010. **2**(6): p. 1279-305.

83. Papatheodoridis, G.V., et al., *Therapeutic strategies in the management of patients with chronic hepatitis B virus infection.* Lancet Infect Dis, 2008. **8**(3): p. 167-78.

84. Santantonio, T.A. and M. Fasano, *Chronic hepatitis B: Advances in treatment.* World J Hepatol, 2014. **6**(5): p. 284-92.

85. Koumbi, L., *Current and future antiviral drug therapies of hepatitis B chronic infection.* World J Hepatol, 2015. **7**(8): p. 1030-40.

86. Gust, I.D., et al., *Taxonomic classification of human hepatitis B virus.* Intervirology, 1986. **25**(1): p. 14-29.

87. Hu, X., et al., *Identification of hepatitis B virus indigenous to chimpanzees.* Proc Natl Acad Sci U S A, 2000. **97**(4): p. 1661-4.

88. Seeger, C. and W.S. Mason, *Hepatitis B virus biology.* Microbiol Mol Biol Rev, 2000. **64**(1): p. 51-68.

89. Glebe, D. and S. Urban, *Viral and cellular determinants involved in hepadnaviral entry.* World J Gastroenterol, 2007. **13**(1): p. 22-38.

90. Datta, S., et al., *Molecular biology of the hepatitis B virus for clinicians.* J Clin Exp Hepatol, 2012. **2**(4): p. 353-65.

91. Bonvicino, C.R., M.A. Moreira, and M.A. Soares, *Hepatitis B virus lineages in mammalian hosts: potential for bidirectional cross-species transmission.* World J Gastroenterol, 2014. **20**(24): p. 7665-74.

92. Yugo, D.M., et al., *Hepatitis Virus Infections in Poultry.* Avian Dis, 2016. **60**(3): p. 576-88.

93. Park, N.H., I.H. Song, and Y.H. Chung, *Molecular Pathogenesis of Hepatitis-B-virus-associated Hepatocellular Carcinoma.* Gut Liver, 2007. **1**(2): p. 101-17.

94. Gao, W. and J. Hu, *Formation of hepatitis B virus covalently closed circular DNA: removal of genome-linked protein.* J Virol, 2007. **81**(12): p. 6164-74.

95. Moolla, N., M. Kew, and P. Arbuthnot, *Regulatory elements of hepatitis B virus transcription.* J Viral Hepat, 2002. **9**(5): p. 323-31.

96. Nassal, M., *HBV cccDNA: viral persistence reservoir and key obstacle for a cure of chronic hepatitis B.* Gut, 2015.

97. Torres, C., et al., *Influence of overlapping genes on the evolution of human hepatitis B virus.* Virology, 2013. **441**(1): p. 40-8.

98. Chisari, F.V., *Rous-Whipple Award Lecture. Viruses, immunity, and cancer: lessons from hepatitis B.* Am J Pathol, 2000. **156**(4): p. 1117-32.

99. Bouchard, M.J. and R.J. Schneider, *The enigmatic X gene of hepatitis B virus.* J Virol, 2004. **78**(23): p. 12725-34.

100. Beck, J. and M. Nassal, *Hepatitis B virus replication.* World J Gastroenterol, 2007. **13**(1): p. 48-64.

101. Murakami, S., *Hepatitis B virus X protein: structure, function and biology.* Intervirology, 1999. **42**(2-3): p. 81-99.

102. Kumar, V. and D.P. Sarkar, *Hepatitis B Virus X Protein: Structure-Function Relationships and Role in Viral Pathogenesis*, in *Transcription Factors*, M. Gossen, J. Kaufmann, and S.J. Triezenberg, Editors. 2004, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 377-407.

103. Le Duff, Y., M. Blanchet, and C. Sureau, *The pre-S1 and antigenic loop infectivity determinants of the hepatitis B virus envelope proteins are functionally independent.* J Virol, 2009. **83**(23): p. 12443-51.

104. Ni, Y., et al., *The pre-s2 domain of the hepatitis B virus is dispensable for infectivity but serves a spacer function for L-protein-connected virus assembly.* J Virol, 2010. **84**(8): p. 3879-88.

105. Nassal, M. and H. Schaller, *Hepatitis B virus replication.* Trends Microbiol, 1993. **1**(6): p. 221-8.

106. Carman, W.F., *The clinical significance of surface antigen variants of hepatitis B virus.* J Viral Hepat, 1997. **4 Suppl 1**: p. 11-20.

107. Boulant, S., M. Stanifer, and P.Y. Lozach, *Dynamics of virus-receptor interactions in virus binding, signaling, and endocytosis.* Viruses, 2015. **7**(6): p. 2794-815.

108. Ganem, D. and H.E. Varmus, *The molecular biology of the hepatitis B viruses.* Annu Rev Biochem, 1987. **56**: p. 651-93.

109. Nguyen, D.H., L. Ludgate, and J. Hu, *Hepatitis B virus-cell interactions and pathogenesis.* J Cell Physiol, 2008. **216**(2): p. 289-94.

110. Will, H., et al., *Replication strategy of human hepatitis B virus.* J Virol, 1987. **61**(3): p. 904-11.

111. Nisole, S. and A. Saib, *Early steps of retrovirus replicative cycle.* Retrovirology, 2004. **1**: p. 9.

112. Shafritz, D.A. and H.M. Lieberman, *The molecular biology of hepatitis B virus.* Annu Rev Med, 1984. **35**: p. 219-32.

113. Barrera, A., et al., *Mapping of the hepatitis B virus pre-S1 domain involved in receptor recognition.* J Virol, 2005. **79**(15): p. 9786-98.

114. Schieck, A., et al., *Hepatitis B virus hepatotropism is mediated by specific receptor recognition in the liver and not restricted to susceptible hosts.* Hepatology, 2013. **58**(1): p. 43-53.

115. De Meyer, S., et al., *Organ and species specificity of hepatitis B virus (HBV) infection: a review of literature with a special reference to preferential attachment of HBV to human hepatocytes.* J Viral Hepat, 1997. **4**(3): p. 145-53.

116. Watashi, K., et al., *NTCP and beyond: opening the door to unveil hepatitis B virus entry.* Int J Mol Sci, 2014. **15**(2): p. 2892-905.

117. Lempp, F.A., et al., *Evidence that hepatitis B virus replication in mouse cells is limited by the lack of a host cell dependency factor.* J Hepatol, 2015.

118. Lempp, F.A., et al., *Sodium taurocholate cotransporting polypeptide is the limiting host factor of Hepatitis B Virus infection in macaque and pig hepatocytes.* Hepatology, 2017.

119. Yang, P.L., et al., *Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13825-30.

120. Zhong, G., et al., *Sodium taurocholate cotransporting polypeptide mediates woolly monkey hepatitis B virus infection of Tupaia hepatocytes.* J Virol, 2013. **87**(12): p. 7176-84.

121. Cooper, A., N. Paran, and Y. Shaul, *The earliest steps in hepatitis B virus infection.* Biochim Biophys Acta, 2003. **1614**(1): p. 89-96.

122. Schulze, A., et al., *Fine mapping of pre-S sequence requirements for hepatitis B virus large envelope protein-mediated receptor interaction.* J Virol, 2010. **84**(4): p. 1989-2000.

123. Paran, N., B. Geiger, and Y. Shaul, *HBV infection of cell culture: evidence for multivalent and cooperative attachment.* Embo j, 2001. **20**(16): p. 4443-53.

124. Paran, N., A. Cooper, and Y. Shaul, *Interaction of hepatitis B virus with cells.* Rev Med Virol, 2003. **13**(3): p. 137-43.

125. Lu, X. and T. Block, *Study of the early steps of the Hepatitis B Virus life cycle.* Int J Med Sci, 2004. **1**(1): p. 21-33.

126. Chandran, K., D.L. Farsetta, and M.L. Nibert, *Strategy for nonenveloped virus entry: a hydrophobic conformer of the reovirus membrane penetration protein micro 1 mediates membrane disruption.* J Virol, 2002. **76**(19): p. 9920-33.

127. Maurer-Stroh, S. and F. Eisenhaber, *Myristoylation of viral and bacterial proteins.* Trends Microbiol, 2004. **12**(4): p. 178-85.

128. De Falco, S., et al., *N-terminal myristylation of HBV preS1 domain enhances receptor recognition.* J Pept Res, 2001. **57**(5): p. 390-400.

129. Le Seyec, J., et al., *Infection process of the hepatitis B virus depends on the presence of a defined sequence in the pre-S1 domain.* J Virol, 1999. **73**(3): p. 2052-7.

130. Blanchet, M. and C. Sureau, *Infectivity determinants of the hepatitis B virus pre-S domain are confined to the N-terminal 75 amino acid residues.* J Virol, 2007. **81**(11): p. 5841-9.

131. Lempp, F.A. and S. Urban, *Inhibitors of hepatitis B virus attachment and entry.* Intervirology, 2014. **57**(3-4): p. 151-7.

132. Leistner, C.M., S. Gruen-Bernhard, and D. Glebe, *Role of glycosaminoglycans for binding and infection of hepatitis B virus.* Cell Microbiol, 2008. **10**(1): p. 122-33.

133. Xie, Y., et al., *Entry of hepatitis B virus: mechanism and new therapeutic target.* Pathol Biol (Paris), 2010. **58**(4): p. 301-7.

134. Lindenbach, B.D. and C.M. Rice, *The ins and outs of hepatitis C virus entry and assembly.* Nat Rev Microbiol, 2013. **11**(10): p. 688-700.

135. Verrier, E.R., et al., *A targeted functional RNAi screen uncovers Glypican 5 as an entry factor for hepatitis B and D viruses.* Hepatology, 2015.

136. Schulze, A., P. Gripon, and S. Urban, *Hepatitis B virus infection initiates with a large surface protein-dependent binding to heparan sulfate proteoglycans.* Hepatology, 2007. **46**(6): p. 1759-68.

137. Huang, H.C., et al., *(-)-Epigallocatechin-3-gallate inhibits entry of hepatitis B virus into hepatocytes.* Antiviral Res, 2014. **111**: p. 100-11.

138. Giroglou, T., et al., *Human papillomavirus infection requires cell surface heparan sulfate.* J Virol, 2001. **75**(3): p. 1565-70.

139. Saphire, A.C., et al., *Syndecans serve as attachment receptors for human immunodeficiency virus type 1 on macrophages.* J Virol, 2001. **75**(19): p. 9187-200.

140. Petit, M.A., et al., *PreS1-specific binding proteins as potential receptors for hepatitis B virus in human hepatocytes.* Virology, 1992. **187**(1): p. 211-22.

141. Urban, S., et al., *Strategies to inhibit entry of HBV and HDV into hepatocytes.* Gastroenterology, 2014. **147**(1): p. 48-64.

142. Kann, M., et al., *Phosphorylation-dependent binding of hepatitis B virus core particles to the nuclear pore complex.* J Cell Biol, 1999. **145**(1): p. 45-55.

143. Daub, H., et al., *Identification of SRPK1 and SRPK2 as the major cellular protein kinases phosphorylating hepatitis B virus core protein.* J Virol, 2002. **76**(16): p. 8124-37.

144. Hayes, C.N., et al., *Early events in hepatitis B virus infection: From the cell surface to the nucleus.* J Gastroenterol Hepatol, 2015.

145. Zeisel, M.B., et al., *Towards an HBV cure: state-of-the-art and unresolved questions--report of the ANRS workshop on HBV cure.* Gut, 2015. **64**(8): p. 1314-26.

146. Fay, N. and N. Pante, *Nuclear entry of DNA viruses.* Front Microbiol, 2015. **6**: p. 467.

147. Russnak, R. and D. Ganem, *Sequences 5' to the polyadenylation signal mediate differential poly(A) site use in hepatitis B viruses.* Genes Dev, 1990. **4**(5): p. 764-76.

148. Jeong, J.K., G.S. Yoon, and W.S. Ryu, *Evidence that the 5'-end cap structure is essential for encapsidation of hepatitis B virus pregenomic RNA.* J Virol, 2000. **74**(12): p. 5502-8.

149. Hu, J. and L. Lin, *RNA-protein interactions in hepadnavirus reverse transcription.* Front Biosci (Landmark Ed), 2009. **14**: p. 1606-18.

150. Kock, J., et al., *Generation of covalently closed circular DNA of hepatitis B viruses via intracellular recycling is regulated in a virus specific manner.* PLoS Pathog, 2010. **6**(9): p. e1001082.

151. Yang, H.C. and J.H. Kao, *Persistence of hepatitis B virus covalently closed circular DNA in hepatocytes: molecular mechanisms and clinical significance.* Emerg Microbes Infect, 2014. **3**(9): p. e64.

152. Yan, H. and W. Li, *Sodium taurocholate cotransporting polypeptide acts as a receptor for hepatitis B and d virus.* Dig Dis, 2015. **33**(3): p. 388-96.

153. Yan, H., et al., *NTCP opens the door for hepatitis B virus infection.* Antiviral Res, 2015.

154. Budkowska, A., et al., *Hepatitis B virus (HBV) binding factor in human serum: candidate for a soluble form of hepatocyte HBV receptor.* J Virol, 1993. **67**(7): p. 4316-22.

155. Alrefai, W.A. and R.K. Gill, *Bile acid transporters: structure, function, regulation and pathophysiological implications.* Pharm Res, 2007. **24**(10): p. 1803-23.

156. Claro da Silva, T., J.E. Polli, and P.W. Swaan, *The solute carrier family 10 (SLC10): beyond bile acid transport.* Mol Aspects Med, 2013. **34**(2-3): p. 252-69.

157. Anwer, M.S. and B. Stieger, *Sodium-dependent bile salt transporters of the SLC10A transporter family: more than solute transporters.* Pflugers Arch, 2014. **466**(1): p. 77-89.

158. Appelman, M.D., et al., *N-Glycosylation of the Na+-Taurocholate Cotransporting Polypeptide (NTCP) Determines Its Trafficking and Stability and Is Required for Hepatitis B Virus Infection.* PLoS One, 2017. **12**(1): p. e0170419.

159. Bijsmans, I.T., et al., *Homo- and hetero-dimeric architecture of the human liver Na(+)-dependent taurocholate co-transporting protein.* Biochem J, 2012. **441**(3): p. 1007-15.

160. Stieger, B., *The role of the sodium-taurocholate cotransporting polypeptide (NTCP) and of the bile salt export pump (BSEP) in physiology and pathophysiology of bile formation.* Handb Exp Pharmacol, 2011(201): p. 205-59.

161. Molina, H., et al., *Localization of the Sodium-Taurocholate cotransporting polypeptide in membrane rafts and modulation of its activity by cholesterol in vitro.* Biochim Biophys Acta, 2008. **1778**(5): p. 1283-91.

162. Stanca, C., et al., *Hepatocellular transport proteins and their role in liver disease.* World J Gastroenterol, 2001. **7**(2): p. 157-69.

163. Ni, Y., et al., *Hepatitis B and D viruses exploit sodium taurocholate co-transporting polypeptide for species-specific entry into hepatocytes.* Gastroenterology, 2014. **146**(4): p. 1070-83.

164. Lempp, F.A., et al., *Hepatitis B Virus infection of a mouse hepatic cell line reconstituted with human sodium taurocholate cotransporting polypeptide.* J Virol, 2016.

165. Fu, L., et al., *Woodchuck sodium taurocholate cotransporting polypeptide supports low-level hepatitis B and D virus entry*. Virology, 2017. **505**: p. 1-11.

166. Taylor, J.M., *Virus entry mediated by hepatitis B virus envelope proteins.* World J Gastroenterol, 2013. **19**(40): p. 6730-4.

167. Wang, Y. and X. Chen, *How the key finds its door - identification of HBV receptor.* Virol Sin, 2013. **28**(1): p. 1-2.

168. Xiao, F., J.A. McKeating, and T.F. Baumert, *A bile acid transporter as a candidate receptor for hepatitis B and D virus entry.* J Hepatol, 2013. **58**(6): p. 1246-8.

169. Konig, A., et al., *Kinetics of the bile acid transporter and hepatitis B virus receptor Na+/taurocholate cotransporting polypeptide (NTCP) in hepatocytes.* J Hepatol, 2014. **61**(4): p. 867-75.

170. Yan, H., et al., *Viral entry of hepatitis B and D viruses and bile salts transportation share common molecular determinants on sodium taurocholate cotransporting polypeptide.* J Virol, 2014. **88**(6): p. 3273-84.

171. Watashi, K. and T. Wakita, *Hepatitis B Virus and Hepatitis D Virus Entry, Species Specificity, and Tissue Tropism.* Cold Spring Harb Perspect Med, 2015. **5**(8).

172. Winer, B.Y. and A. Ploss, *Determinants of hepatitis B and delta virus host tropism.* Curr Opin Virol, 2015. **13**: p. 109-116.

173. Hagenbuch, B., et al., *Functional expression cloning and characterization of the hepatocyte Na+/bile acid cotransport system.* Proc Natl Acad Sci U S A, 1991. **88**(23): p. 10629-33.

174. Mukhopadhyay, S., et al., *Sodium taurocholate cotransporting polypeptide is a serine, threonine phosphoprotein and is dephosphorylated by cyclic adenosine monophosphate.* Hepatology, 1998. **28**(6): p. 1629-36.

175. Sun, A.Q., et al., *The rat liver Na(+)/bile acid cotransporter. Importance of the cytoplasmic tail to function and plasma membrane targeting.* J Biol Chem, 2001. **276**(9): p. 6825-33.

176. Xu, D. and G. You, *Loops and layers of post-translational modifications of drug transporters.* Adv Drug Deliv Rev, 2016.

177. Mukhopadhayay, S., et al., *cAMP increases liver Na+-taurocholate cotransport by translocating transporter to plasma membranes.* Am J Physiol, 1997. **273**(4 Pt 1): p. G842-8.

178. Anwer, M.S., et al., *Dephosphorylation of Ser-226 facilitates plasma membrane retention of Ntcp.* J Biol Chem, 2005. **280**(39): p. 33687-92.

179. Dranoff, J.A., et al., *Short-term regulation of bile acid uptake by microfilament-dependent translocation of rat ntcp to the plasma membrane.* Hepatology, 1999. **30**(1): p. 223-9.

180. Webster, C.R. and M.S. Anwer, *Role of the PI3K/PKB signaling pathway in cAMP-mediated translocation of rat liver Ntcp.* Am J Physiol, 1999. **277**(6 Pt 1): p. G1165-72.

181. Webster, C.R., C. Blanch, and M.S. Anwer, *Role of PP2B in cAMP-induced dephosphorylation and translocation of NTCP.* Am J Physiol Gastrointest Liver Physiol, 2002. **283**(1): p. G44-50.

182. Sarkar, S., et al., *PKCzeta is required for microtubule-based motility of vesicles containing the ntcp transporter.* Traffic, 2006. **7**(8): p. 1078-91.

183. Sommerfeld, A., et al., *Regulation of plasma membrane localization of the Na+-taurocholate cotransporting polypeptide (Ntcp) by hyperosmolarity and tauroursodeoxycholate.* J Biol Chem, 2015. **290**(40): p. 24237-54.

184. Kuhlkamp, T., et al., *Degradation of the sodium taurocholate cotransporting polypeptide (NTCP) by the ubiquitin-proteasome system.* Biol Chem, 2005. **386**(10): p. 1065-74.

185. Stross, C., et al., *Protein kinase C induces endocytosis of the sodium taurocholate cotransporting polypeptide.* Am J Physiol Gastrointest Liver Physiol, 2010. **299**(2): p. G320-8.

186. Stross, C., et al., *A dileucine motif is involved in plasma membrane expression and endocytosis of rat sodium taurocholate cotransporting polypeptide (Ntcp).* Am J Physiol Gastrointest Liver Physiol, 2013. **305**(10): p. G722-30.

187. Ashwell, G. and A.G. Morell, *The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins.* Adv Enzymol Relat Areas Mol Biol, 1974. **41**(0): p. 99-128.

188. Zhang, X., et al., *Asialoglycoprotein receptor interacts with the preS1 domain of hepatitis B virus in vivo and in vitro.* Arch Virol, 2011. **156**(4): p. 637-45.

189. Shi, B., M. Abrams, and L. Sepp-Lorenzino, *Expression of asialoglycoprotein receptor 1 in human hepatocellular carcinoma.* J Histochem Cytochem, 2013. **61**(12): p. 901-9.

190. Treichel, U., et al., *Receptor-mediated entry of hepatitis B virus particles into liver cells.* Arch Virol, 1997. **142**(3): p. 493-8.

191. Rehman, Z., A. Fahim, and H. Sadia, *Deciphering the mystery of hepatitis B virus receptors: A historical perspective.* Virusdisease, 2015. **26**(3): p. 97-104.

192. Maverakis, E., et al., *Glycans in the immune system and The Altered Glycan Theory of Autoimmunity: a critical review.* J Autoimmun, 2015. **57**: p. 1-13.

193. Ponka, P. and C.N. Lok, *The transferrin receptor: role in health and disease.* Int J Biochem Cell Biol, 1999. **31**(10): p. 1111-37.

194. Qian, Z.M., et al., *Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway.* Pharmacol Rev, 2002. **54**(4): p. 561-87.

195. De Domenico, I., D. McVey Ward, and J. Kaplan, *Regulation of iron acquisition and storage: consequences for iron-linked disorders.* Nat Rev Mol Cell Biol, 2008. **9**(1): p. 72-81.

196. Franco, A., et al., *Transferrin receptor mediates uptake and presentation of hepatitis B envelope antigen by T lymphocytes.* J Exp Med, 1992. **175**(5): p. 1195-205.

197. Harari, P.M., *Epidermal growth factor receptor inhibition strategies in oncology.* Endocr Relat Cancer, 2004. **11**(4): p. 689-708.

198. Herbst, R.S., *Review of epidermal growth factor receptor biology.* Int J Radiat Oncol Biol Phys, 2004. **59**(2 Suppl): p. 21-6.

199. Zheng, K., K. Kitazato, and Y. Wang, *Viruses exploit the function of epidermal growth factor receptor.* Rev Med Virol, 2014. **24**(4): p. 274-86.

200. Menzo, S., et al., *Trans-activation of epidermal growth factor receptor gene by the hepatitis B virus X-gene product.* Virology, 1993. **196**(2): p. 878-82.

201. Tokarev AA, A.A., Segev N., *Overview of Intracellular Compartments and Trafficking Pathways.* In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013., Available from: https://www.ncbi.nlm.nih.gov/books/NBK7286/.

202. Perlmutter, D.H., *Chemical chaperones: a pharmacological strategy for disorders of protein folding and trafficking.* Pediatr Res, 2002. **52**(6): p. 832-6.

203. Braakman, I. and D.N. Hebert, *Protein folding in the endoplasmic reticulum.* Cold Spring Harb Perspect Biol, 2013. **5**(5): p. a013201.

204. Conn, P.M., et al., *G protein-coupled receptor trafficking in health and disease: lessons learned to prepare for therapeutic mutant rescue in vivo.* Pharmacol Rev, 2007. **59**(3): p. 225-50.

205. GM., C., *The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. Microtubule Motors and Movements. Available from: https://www.ncbi.nlm.nih.gov/books/NBK9833/.*

206. Hunt, S.D. and D.J. Stephens, *The role of motor proteins in endosomal sorting.* Biochem Soc Trans, 2011. **39**(5): p. 1179-84.

207. Murray, J.W., S. Sarkar, and A.W. Wolkoff, *Single vesicle analysis of endocytic fission on microtubules in vitro.* Traffic, 2008. **9**(5): p. 833-47.

208. Wang, X., et al., *The Na -Taurocholate Cotransporting Polypeptide Traffics with the Epidermal Growth Factor Receptor.* Traffic, 2015.

209. Rizzetto, M., et al., *Immunofluorescence detection of new antigen-antibody system (delta/anti-delta) associated to hepatitis B virus in liver and in serum of HBsAg carriers.* Gut, 1977. **18**(12): p. 997-1003.

210. Taylor, J.M., *Hepatitis delta virus.* Virology, 2006. **344**(1): p. 71-6.

211. Hughes, S.A., H. Wedemeyer, and P.M. Harrison, *Hepatitis delta virus.* Lancet, 2011. **378**(9785): p. 73-85.

212. Heidrich, B., M.P. Manns, and H. Wedemeyer, *Treatment options for hepatitis delta virus infection.* Curr Infect Dis Rep, 2013. **15**(1): p. 31-8.

213. Negro, F., *Hepatitis D virus coinfection and superinfection.* Cold Spring Harb Perspect Med, 2014. **4**(11): p. a021550.

214. Radjef, N., et al., *Molecular phylogenetic analyses indicate a wide and ancient radiation of African hepatitis delta virus, suggesting a deltavirus genus of at least seven major clades.* J Virol, 2004. **78**(5): p. 2537-44.

215. Weiner, A.J., et al., *A single antigenomic open reading frame of the hepatitis delta virus encodes the epitope(s) of both hepatitis delta antigen polypeptides p24 delta and p27 delta.* J Virol, 1988. **62**(2): p. 594-9.

216. Abbas, Z. and R. Afzal, *Life cycle and pathogenesis of hepatitis D virus: A review.* World J Hepatol, 2013. **5**(12): p. 666-75.

217. Alves, C., C. Branco, and C. Cunha, *Hepatitis delta virus: a peculiar virus.* Adv Virol, 2013. **2013**: p. 560105.

218. Noureddin, M. and R. Gish, *Hepatitis delta: epidemiology, diagnosis and management 36 years after discovery.* Curr Gastroenterol Rep, 2014. **16**(1): p. 365.

219. !!! INVALID CITATION !!! {}.

220. Li, Y.J., et al., *RNA-templated replication of hepatitis delta virus: genomic and antigenomic RNAs associate with different nuclear bodies.* J Virol, 2006. **80**(13): p. 6478-86.

221. Tseng, C.H. and M.M. Lai, *Hepatitis delta virus RNA replication.* Viruses, 2009. **1**(3): p. 818-31.

222. Flores, R., S. Ruiz-Ruiz, and P. Serra, *Viroids and hepatitis delta virus.* Semin Liver Dis, 2012. **32**(3): p. 201-10.

223. Li, H., et al., *HBV life cycle is restricted in mouse hepatocytes expressing human NTCP.* Cell Mol Immunol, 2014. **11**(2): p. 175-83.

224. He, W., et al., *Hepatitis D Virus Infection of Mice Expressing Human Sodium Taurocholate Co-transporting Polypeptide.* PLoS Pathog, 2015. **11**(4): p. e1004840.

225. Allweiss, L. and M. Dandri, *Experimental in vitro and in vivo models for the study of human hepatitis B virus infection.* J Hepatol, 2016. **64**(1 Suppl): p. S17-31.

226. Witt-Kehati, D., M. Bitton Alaluf, and A. Shlomai, *Advances and Challenges in Studying Hepatitis B Virus In Vitro.* Viruses, 2016. **8**(1).

227. Chen, J., et al., *New insights into hepatitis B virus biology and implications for novel antiviral strategies.* National Science Review, 2015. **2**(3): p. 296-313.

228. Hayes, C.N. and K. Chayama, *HBV culture and infectious systems.* Hepatol Int, 2016.

229. Gripon, P., et al., *Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide.* J Virol, 1988. **62**(11): p. 4136-43.

230. Gripon, P., C. Diot, and C. Guguen-Guillouzo, *Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration.* Virology, 1993. **192**(2): p. 534-40.

231. Dandri, M. and M. Lutgehetmann, *Mouse models of hepatitis B and delta virus infection.* J Immunol Methods, 2014. **410**: p. 39-49.

232. Verrier, E.R., et al., *Cell Culture Models for the Investigation of Hepatitis B and D Virus Infection.* Viruses, 2016. **8**(9).

233. Dandri, M., et al., *Animal models for the study of HBV replication and its variants.* J Clin Virol, 2005. **34 Suppl 1**: p. S54-62.

234. Winer, B.Y., et al., *In vivo models of hepatitis B and C virus infection.* FEBS Lett, 2016.

235. Sells, M.A., M.L. Chen, and G. Acs, *Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA.* Proc Natl Acad Sci U S A, 1987. **84**(4): p. 1005-9.

236. Sells, M.A., et al., *Replicative intermediates of hepatitis B virus in HepG2 cells that produce infectious virions.* J Virol, 1988. **62**(8): p. 2836-44.

237. Ladner, S.K., 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**(8): p. 1715-20.

238. Zhou, T., et al., *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**(2): p. 116-24.

239. Cai, D., et al., *Establishment of an inducible HBV stable cell line that expresses cccDNA-dependent epitope-tagged HBeAg for screening of cccDNA modulators.* Antiviral Res, 2016. **132**: p. 26-37.

240. Shimizu, Y., et al., *Replication of hepatitis B virus in culture systems with adult human hepatocytes.* J Med Virol, 1986. **20**(4): p. 313-27.

241. Ochiya, T., et al., *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**(6): p. 1875-9.

242. Kock, J., et al., *Efficient infection of primary tupaia hepatocytes with purified human and woolly monkey hepatitis B virus.* J Virol, 2001. **75**(11): p. 5084-9.

243. Gripon, P., et al., *Infection of a human hepatoma cell line by hepatitis B virus.* Proc Natl Acad Sci U S A, 2002. **99**(24): p. 15655-60.

244. Cerec, V., et al., *Transdifferentiation of hepatocyte-like cells from the human hepatoma HepaRG cell line through bipotent progenitor.* Hepatology, 2007. **45**(4): p. 957-67.

245. Guillouzo, A., et al., *The human hepatoma HepaRG cells: a highly differentiated model for studies of liver metabolism and toxicity of xenobiotics.* Chem Biol Interact, 2007. **168**(1): p. 66-73.

246. Andersson, T.B., K.P. Kanebratt, and J.G. Kenna, *The HepaRG cell line: a unique in vitro tool for understanding drug metabolism and toxicology in human.* Expert Opin Drug Metab Toxicol, 2012. **8**(7): p. 909-20.

247. Shlomai, A., et al., *Modeling host interactions with hepatitis B virus using primary and induced pluripotent stem cell-derived hepatocellular systems.* Proc Natl Acad Sci U S A, 2014. **111**(33): p. 12193-8.

248. March, S., et al., *Micropatterned coculture of primary human hepatocytes and supportive cells for the study of hepatotropic pathogens.* Nat Protoc, 2015. **10**(12): p. 2027-2053.

249. Si-Tayeb, K., et al., *Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells.* Hepatology, 2010. **51**(1): p. 297-305.

250. Ma, X., et al., *Highly efficient differentiation of functional hepatocytes from human induced pluripotent stem cells.* Stem Cells Transl Med, 2013. **2**(6): p. 409-19.

251. Sun, P., et al., *Modeling human liver biology using stem cell-derived hepatocytes.* Int J Mol Sci, 2013. **14**(11): p. 22011-21.

252. Palakkan, A.A., et al., *Polarisation and functional characterisation of hepatocytes derived from human embryonic and mesenchymal stem cells.* Biomed Rep, 2015. **3**(5): p. 626-636.

253. Iwamoto, M., et al., *Evaluation and identification of hepatitis B virus entry inhibitors using HepG2 cells overexpressing a membrane transporter NTCP.* Biochem Biophys Res Commun, 2014. **443**(3): p. 808-13.

254. Li, J., et al., *Unusual Features of Sodium Taurocholate Cotransporting Polypeptide as a Hepatitis B Virus Receptor.* J Virol, 2016. **90**(18): p. 8302-13.

255. Okuyama-Dobashi, K., et al., *Hepatitis B virus efficiently infects non-adherent hepatoma cells via human sodium taurocholate cotransporting polypeptide.* Scientific Reports, 2015. **5**: p. 17047.

256. Yan, R., et al., *Spinoculation Enhances HBV Infection in NTCP-Reconstituted Hepatocytes.* PLoS One, 2015. **10**(6): p. e0129889.

257. Cui, X., J.T. Guo, and J. Hu, *Hepatitis B Virus Covalently Closed Circular DNA Formation in Immortalized Mouse Hepatocytes Associated with Nucleocapsid Destabilization.* J Virol, 2015. **89**(17): p. 9021-8.

258. Bruss, V. and D. Ganem, *The role of envelope proteins in hepatitis B virus assembly.* Proc Natl Acad Sci U S A, 1991. **88**(3): p. 1059-63.

259. Chai, N., et al., *Assembly of hepatitis B virus envelope proteins onto a lentivirus pseudotype that infects primary human hepatocytes.* J Virol, 2007. **81**(20): p. 10897-904.

260. Meredith, L.W., et al., *Lentiviral hepatitis B pseudotype entry requires NTCP and additional hepatocyte-specific factors.* J Gen Virol, 2015.

261. Somiya, M., et al., *Cellular uptake of hepatitis B virus envelope L particles is independent of sodium taurocholate cotransporting polypeptide, but dependent on heparan sulfate proteoglycan.* Virology, 2016. **497**: p. 23-32.

262. Bai, W., et al., *Engineering Hepadnaviruses as Reporter-Expressing Vectors: Recent Progress and Future Perspectives.* Viruses, 2016. **8**(5).

263. Nishitsuji, H., et al., *Novel reporter system to monitor early stages of the hepatitis B virus life cycle.* Cancer Sci, 2015. **106**(11): p. 1616-24.

264. Nishitsuji, H., et al., *Development of a Hepatitis B Virus Reporter System to Monitor the Early Stages of the Replication Cycle.* J Vis Exp, 2017(120).

265. Hong, R., et al., *Novel recombinant hepatitis B virus vectors efficiently deliver protein and RNA encoding genes into primary hepatocytes.* J Virol, 2013. **87**(12): p. 6615-24.

266. Wang, Z., et al., *Replication-competent infectious hepatitis B virus vectors carrying substantially sized transgenes by redesigned viral polymerase translation.* PLoS One, 2013. **8**(4): p. e60306.

267. Ploss, A., et al., *Human occludin is a hepatitis C virus entry factor required for infection of mouse cells.* Nature, 2009. **457**(7231): p. 882-6.

268. Schulze, A., et al., *Hepatocyte polarization is essential for the productive entry of the hepatitis B virus.* Hepatology, 2012. **55**(2): p. 373-83.

269. Martin, S.J., J.G. Bradley, and T.G. Cotter, *HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis.* Clin Exp Immunol, 1990. **79**(3): p. 448-53.

270. Skerjanc, I.S., *Cardiac and skeletal muscle development in P19 embryonal carcinoma cells.* Trends Cardiovasc Med, 1999. **9**(5): p. 139-43.

271. Pal, R., et al., *Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells.* Arch Toxicol, 2012. **86**(4): p. 651-61.

272. Isom, H.C., et al., *Maintenance of differentiated rat hepatocytes in primary culture.* Proc Natl Acad Sci U S A, 1985. **82**(10): p. 3252-6.

273. Arterburn, L.M., et al., *A morphological study of differentiated hepatocytes in vitro.* Hepatology, 1995. **22**(1): p. 175-87.

274. Olsavsky Goyak, K.M., E.M. Laurenzana, and C.J. Omiecinski, *Hepatocyte differentiation.* Methods Mol Biol, 2010. **640**: p. 115-38.

275. Huang, H.C., et al., *Entry of hepatitis B virus into immortalized human primary hepatocytes by clathrin-dependent endocytosis.* J Virol, 2012. **86**(17): p. 9443-53.

276. Jacob, S.W. and R. Herschler, *Pharmacology of DMSO.* Cryobiology, 1986. **23**(1): p. 14-27.

277. Santos, N.C., et al., *Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects.* Biochem Pharmacol, 2003. **65**(7): p. 1035-41.

278. Chisari, F.V., *Hepatitis B virus transgenic mice: models of viral immunobiology and pathogenesis.* Curr Top Microbiol Immunol, 1996. **206**: p. 149-73.

279. Reifenberg, K., et al., *Long-term expression of the hepatitis B virus core-e- and X-proteins does not cause pathologic changes in transgenic mice.* J Hepatol, 1997. **26**(1): p. 119-30.

280. Huang, L.R., et al., *An immunocompetent mouse model for the tolerance of human chronic hepatitis B virus infection.* Proc Natl Acad Sci U S A, 2006. **103**(47): p. 17862-7.

281. Dion, S., et al., *Adeno-associated virus-mediated gene transfer leads to persistent hepatitis B virus replication in mice expressing HLA-A2 and HLA-DR1 molecules.* J Virol, 2013. **87**(10): p. 5554-63.

282. Ye, L., et al., *Adeno-Associated Virus Vector Mediated Delivery of the HBV Genome Induces Chronic Hepatitis B Virus Infection and Liver Fibrosis in Mice.* PLoS One, 2015. **10**(6): p. e0130052.

283. Zhang, G., V. Budker, and J.A. Wolff, *High levels of foreign gene expression in hepatocytes after tail vein injections of naked plasmid DNA.* Hum Gene Ther, 1999. **10**(10): p. 1735-7.

284. Ketzinel-Gilad, M., et al., *The use of the hydrodynamic HBV animal model to study HBV biology and anti-viral therapy.* Hepatol Res, 2006. **34**(4): p. 228-37.

285. Ilan, E., et al., *The hepatitis B virus-trimera mouse: a model for human HBV infection and evaluation of anti-HBV therapeutic agents.* Hepatology, 1999. **29**(2): p. 553-62.

286. Dandri, M., et al., *Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus.* Hepatology, 2001. **33**(4): p. 981-8.

287. Dandri, M., et al., *Chronic infection with hepatitis B viruses and antiviral drug evaluation in uPA mice after liver repopulation with tupaia hepatocytes.* J Hepatol, 2005. **42**(1): p. 54-60.

288. Petersen, J., et al., *Prevention of hepatitis B virus infection in vivo by entry inhibitors derived from the large envelope protein.* Nat Biotechnol, 2008. **26**(3): p. 335-41.

289. Meuleman, P. and G. Leroux-Roels, *The human liver-uPA-SCID mouse: a model for the evaluation of antiviral compounds against HBV and HCV.* Antiviral Res, 2008. **80**(3): p. 231-8.

290. Strom, S.C., J. Davila, and M. Grompe, *Chimeric mice with humanized liver: tools for the study of drug metabolism, excretion, and toxicity.* Methods Mol Biol, 2010. **640**: p. 491-509.

291. Lan, P., et al., *Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34+ cell transplantation.* Blood, 2006. **108**(2): p. 487-92.

292. Dorner, M., et al., *A genetically humanized mouse model for hepatitis C virus infection.* Nature, 2011. **474**(7350): p. 208-11.

293. Bility, M.T., et al., *Hepatitis B virus infection and immunopathogenesis in a humanized mouse model: induction of human-specific liver fibrosis and M2-like macrophages.* PLoS Pathog, 2014. **10**(3): p. e1004032.

294. Strick-Marchand, H., et al., *A novel mouse model for stable engraftment of a human immune system and human hepatocytes.* PLoS One, 2015. **10**(3): p. e0119820.

295. Tropberger, P., et al., *Mapping of histone modifications in episomal HBV cccDNA uncovers an unusual chromatin organization amenable to epigenetic manipulation.* Proc Natl Acad Sci U S A, 2015. **112**(42): p. E5715-24.

296. Murray, J.L., P. Hu, and D.A. Shafer, *Seven novel probe systems for real-time PCR provide absolute single-base discrimination, higher signaling, and generic components.* J Mol Diagn, 2014. **16**(6): p. 627-38.

297. Bartenschlager, R. and T. Pietschmann, *Efficient hepatitis C virus cell culture system: what a difference the host cell makes.* Proc Natl Acad Sci U S A, 2005. **102**(28): p. 9739-40.

298. Guo, H., et al., *Characterization of the intracellular deproteinized relaxed circular DNA of hepatitis B virus: an intermediate of covalently closed circular DNA formation.* J Virol, 2007. **81**(22): p. 12472-84.

299. Werle-Lapostolle, B., et al., *Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy.* Gastroenterology, 2004. **126**(7): p. 1750-8.

300. Tiscornia, G., O. Singer, and I.M. Verma, *Production and purification of lentiviral vectors.* Nat Protoc, 2006. **1**(1): p. 241-5.

301. Ortega-Prieto, A.M. and M. Dorner, *The expanding toolbox for hepatitis C virus research.* J Viral Hepat, 2016. **23**(5): p. 320-9.

302. Molden, R.C., et al., *Multi-faceted quantitative proteomics analysis of histone H2B isoforms and their modifications.* Epigenetics Chromatin, 2015. **8**: p. 15.

303. Hamachi, T., M. Hirata, and T. Koga, *Effect of cAMP-elevating drugs on Ca2+ efflux and actin polymerization in peritoneal macrophages stimulated with N-formyl chemotactic peptide.* Biochim Biophys Acta, 1984. **804**(2): p. 230-6.

304. Gerits, N., et al., *Modulation of F-actin rearrangement by the cyclic AMP/cAMP-dependent protein kinase (PKA) pathway is mediated by MAPK-*

activated protein kinase 5 and requires PKA-induced nuclear export of MK5. J Biol Chem, 2007. **282**(51): p. 37232-43.

305. Nadella, K.S., et al., *Regulation of actin function by protein kinase A-mediated phosphorylation of Limk1.* EMBO Rep, 2009. **10**(6): p. 599-605.

306. Roskoski, R., Jr., *RAF protein-serine/threonine kinases: structure and regulation.* Biochem Biophys Res Commun, 2010. **399**(3): p. 313-7.

307. Roskoski, R., Jr., *MEK1/2 dual-specificity protein kinases: structure and regulation.* Biochem Biophys Res Commun, 2012. **417**(1): p. 5-10.

308. Roskoski, R., Jr., *ERK1/2 MAP kinases: structure, function, and regulation.* Pharmacol Res, 2012. **66**(2): p. 105-43.

309. Li, S.H., et al., *High expression of hepatitis B virus based vector with reporter gene in hepatitis B virus infection system.* World J Gastroenterol, 2007. **13**(17): p. 2490-5.

310. Clapham, P.R. and A. McKnight, *HIV-1 receptors and cell tropism.* Br Med Bull, 2001. **58**: p. 43-59.

311. Wilen, C.B., J.C. Tilton, and R.W. Doms, *HIV: cell binding and entry.* Cold Spring Harb Perspect Med, 2012. **2**(8).

312. Glebe, D., et al., *Optimised conditions for the production of hepatitis B virus from cell culture.* Intervirology, 2001. **44**(6): p. 370-8.

313. Melkonyan, H., C. Sorg, and M. Klempt, *Electroporation efficiency in mammalian cells is increased by dimethyl sulfoxide (DMSO).* Nucleic Acids Res, 1996. **24**(21): p. 4356-7.

314. Xia, X., et al., *Transgenes delivered by lentiviral vector are suppressed in human embryonic stem cells in a promoter-dependent manner.* Stem Cells Dev, 2007. **16**(1): p. 167-76.

315. Sumida, K., et al., *Effects of DMSO on gene expression in human and rat hepatocytes.* Hum Exp Toxicol, 2011. **30**(10): p. 1701-9.

316. Lampugnani, M.G., et al., *Effects of dimethyl sulfoxide (DMSO) on microfilament organization, cellular adhesion, and growth of cultured mouse B16 melanoma cells.* Exp Cell Res, 1987. **172**(2): p. 385-96.

317. Vincent, C., et al., *Dimethylsulphoxide affects the organisation of microfilaments in the mouse oocyte.* Mol Reprod Dev, 1990. **26**(3): p. 227-35.

318. Whitsett, J.A., et al., *cAMP dependent actin phosphorylation in developing rat lung and type II epithelial cells.* Exp Lung Res, 1985. **9**(3-4): p. 191-209.

319. Kim, M.O., et al., *cAMP Promotes Cell Migration Through Cell Junctional Complex Dynamics and Actin Cytoskeleton Remodeling: Implications in Skin Wound Healing.* Stem Cells Dev, 2015. **24**(21): p. 2513-24.

320. Shim, S.G., et al., *Caffeine attenuates liver fibrosis via defective adhesion of hepatic stellate cells in cirrhotic model.* J Gastroenterol Hepatol, 2013. **28**(12): p. 1877-84.

321. Tazzeo, T., et al., *Caffeine relaxes smooth muscle through actin depolymerization.* Am J Physiol Lung Cell Mol Physiol, 2012. **303**(4): p. L334-42.

322. Jacob, C., et al., *DMSO-treated HL60 cells: a model of neutrophil-like cells mainly expressing PDE4B subtype.* Int Immunopharmacol, 2002. **2**(12): p. 1647-56.

323. Taylor, M.P., O.O. Koyuncu, and L.W. Enquist, *Subversion of the actin cytoskeleton during viral infection.* Nat Rev Microbiol, 2011. **9**(6): p. 427-39.

324. Kovala, T., et al., *Protein kinase A regulation of cAMP phosphodiesterase expression in rat skeletal myoblasts.* J Biol Chem, 1994. **269**(12): p. 8680-5.

325. Liu, H., et al., *Expression of phosphodiesterase 4D (PDE4D) is regulated by both the cyclic AMP-dependent protein kinase and mitogen-activated protein kinase signaling pathways. A potential mechanism allowing for the coordinated regulation of PDE4D activity and expression in cells.* J Biol Chem, 2000. **275**(34): p. 26615-24.

326. Campos-Toimil, M., et al., *Short-term or long-term treatments with a phosphodiesterase-4 (PDE4) inhibitor result in opposing agonist-induced Ca(2+) responses in endothelial cells.* Br J Pharmacol, 2008. **154**(1): p. 82-92.

327. Raju, T.S., et al., *Species-specific variation in glycosylation of IgG: evidence for the species-specific sialylation and branch-specific galactosylation and importance for engineering recombinant glycoprotein therapeutics.* Glycobiology, 2000. **10**(5): p. 477-86.

328. Urita, A., et al., *Alterations of high-mannose type N-glycosylation in human and mouse osteoarthritis cartilage.* Arthritis Rheum, 2011. **63**(11): p. 3428-38.

329. Antonopoulos, A., et al., *Glycosylation of mouse and human immune cells: insights emerging from N-glycomics analyses.* Biochem Soc Trans, 2011. **39**(5): p. 1334-40.

330. He, W., et al., *Modification of Three Amino Acids in Sodium Taurocholate Cotransporting Polypeptide Renders Mice Susceptible to Infection with Hepatitis D Virus In Vivo.* J Virol, 2016. **90**(19): p. 8866-74.

331. Nakabori, T., et al., *Sodium taurocholate cotransporting polypeptide inhibition efficiently blocks hepatitis B virus spread in mice with a humanized liver.* Sci Rep, 2016. **6**: p. 27782.

332. Kostenko, S., M. Johannessen, and U. Moens, *PKA-induced F-actin rearrangement requires phosphorylation of Hsp27 by the MAPKAP kinase MK5.* Cell Signal, 2009. **21**(5): p. 712-8.

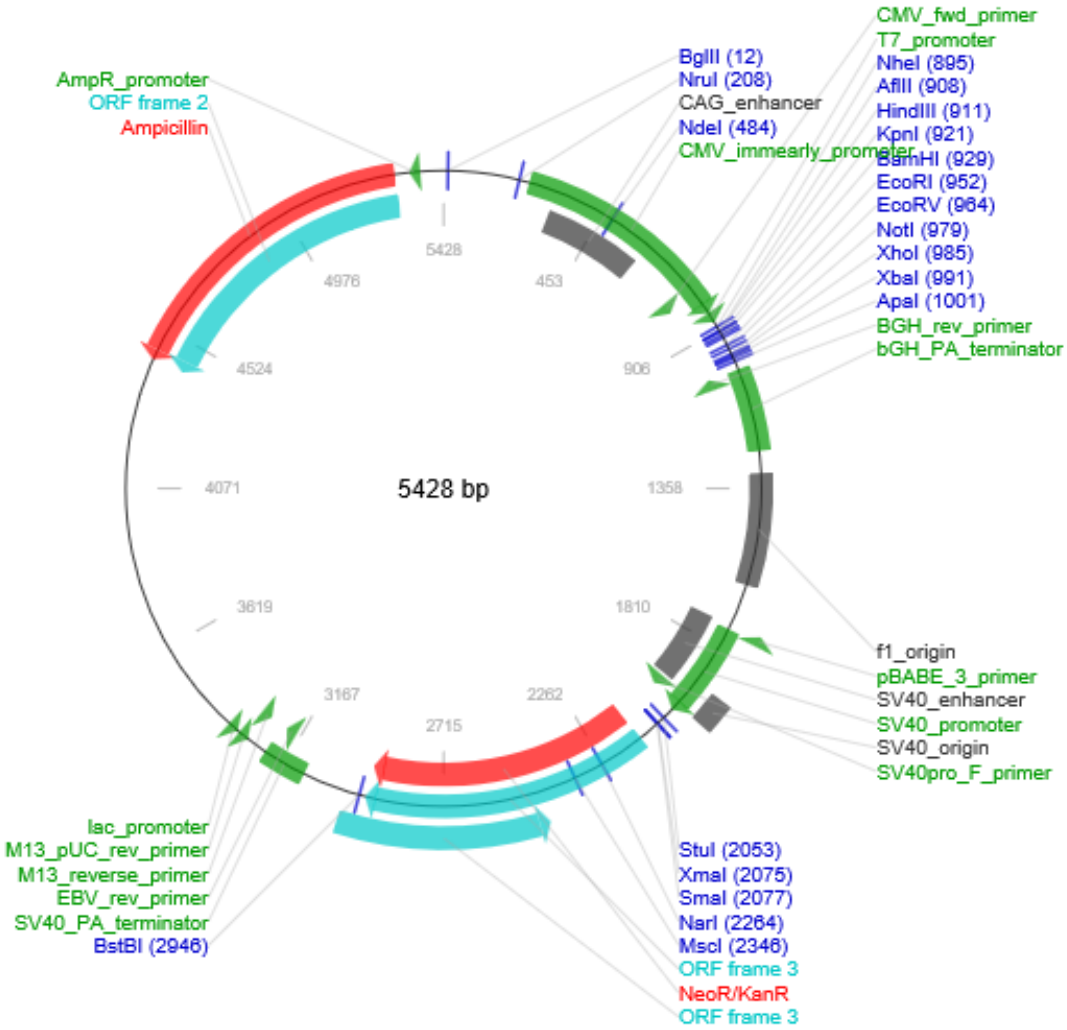
Appendix & Supplementary Data

2. Materials and Methods

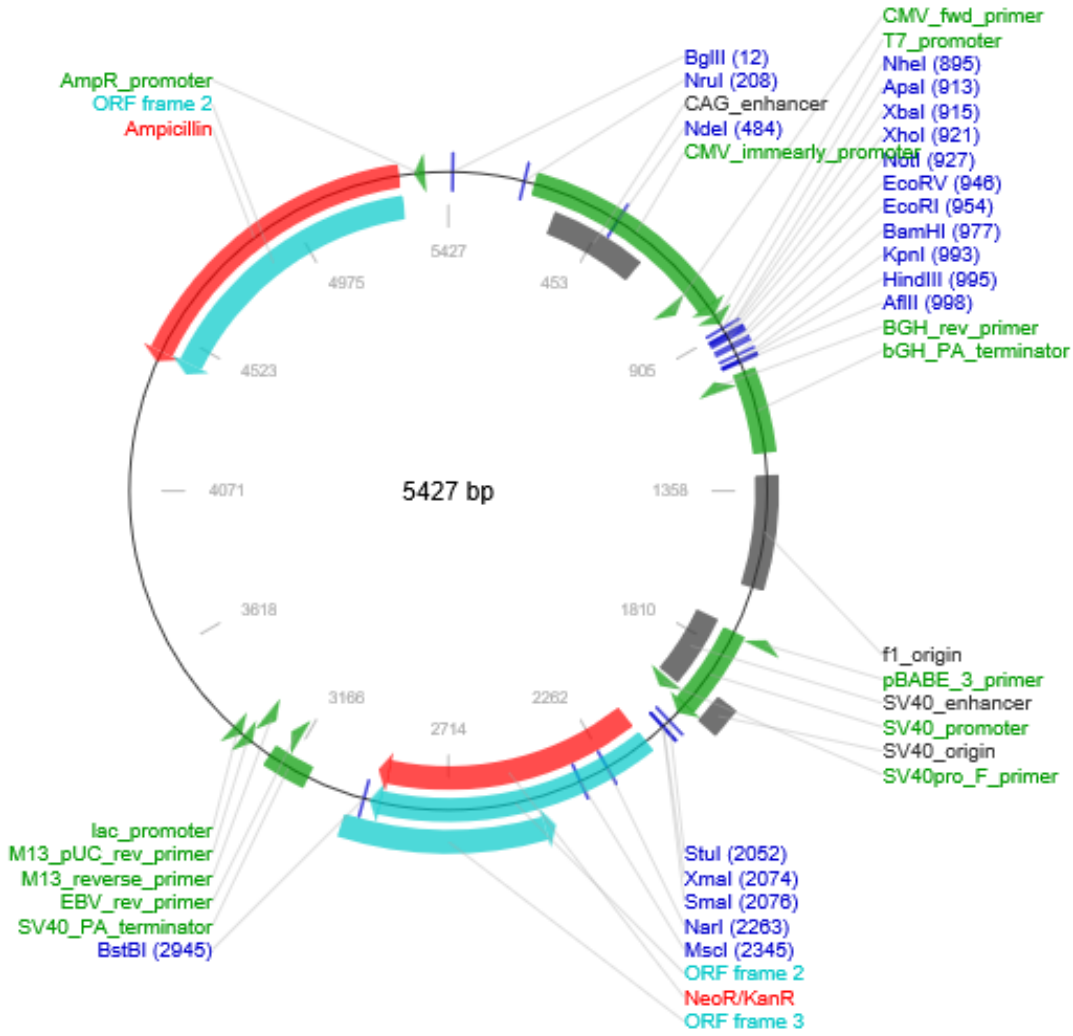
Vector Maps

2.I. Various empty vectors

i. pCDNA3.1(+)

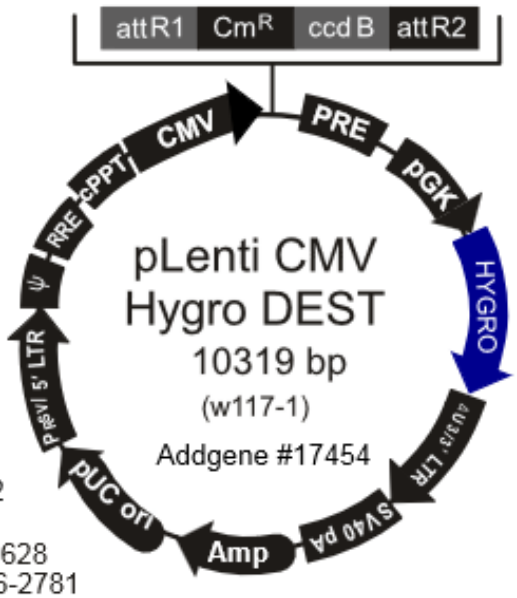


ii. pCDNA3.1(-)



pCDNA3.1 (+) and (-): Mammalian expression vector, containing a CMV promoter. In the (+) vector the MCS is in the forward orientation, while in the (-) vector the MCS is in the reverse orientation. *Source:* Addgene.

iii. pLenti_CMV_Hygromycin



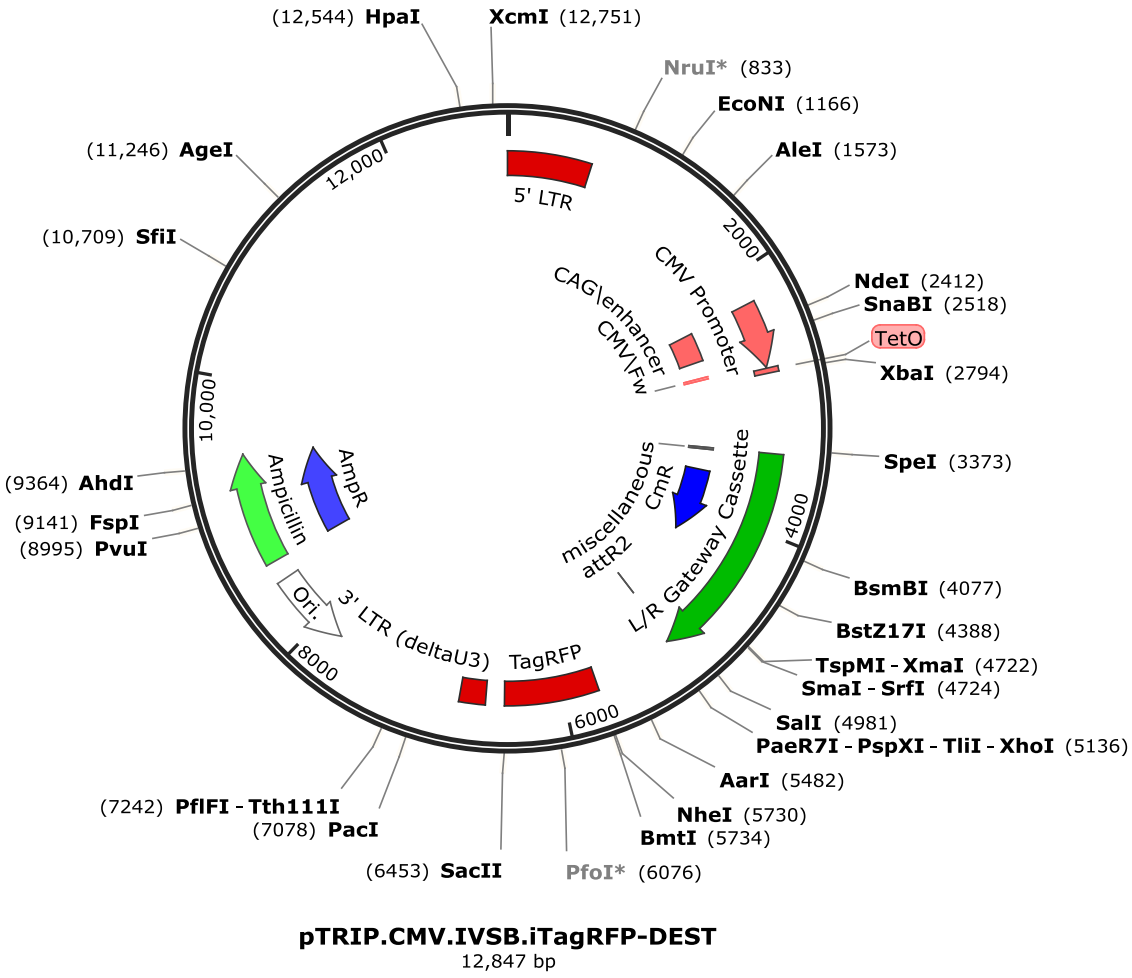
Comments for pLenti CMV Hygro DEST
10319 nucleotides

bla promoter: bases 32-131
ampicillin resistance gene: bases 132-992
pUC origin: bases 1138-1810
RSV/5LTR hybrid promoter: bases 2218-2628
HIV-1 psi (ψ)packaging signal: bases 2736-2781
HIV-1 Rev response element (RRE): bases 3272-3525
3' splice acceptor: base 3862
3' splice acceptor: base 3901
Central polypurine tract (cPPT): bases 4011-4073
CMV promoter: bases 4145-4702
*att*R1 site: bases 4740-4864
Chloramphenicol resistance gene (Cm): bases 4974-5633
*ccd*B gene: bases 5975-6280
*att*R2 site: bases 6320-6446
Woodchuck post-transcriptional element (PRE): bases 6475-7067
Murine phosphoglycerate kinase (pGK) promoter: bases 7133-7642
Hygromycin resistance gene: bases 7660-8686
ΔU3/3'LTR: bases 9058-9293
SV40 polyadenylation signal: bases 9365-9450

Recommended *E.coli* strain for propagation: DB3.1 or ccDB survival'

pLenti_CMV_Hygro_Dest: 3rd generation lentiviral Gateway® destination vector, containing a CMV promoter and a Hygromycin selection cassette. *Source:* Addgene.

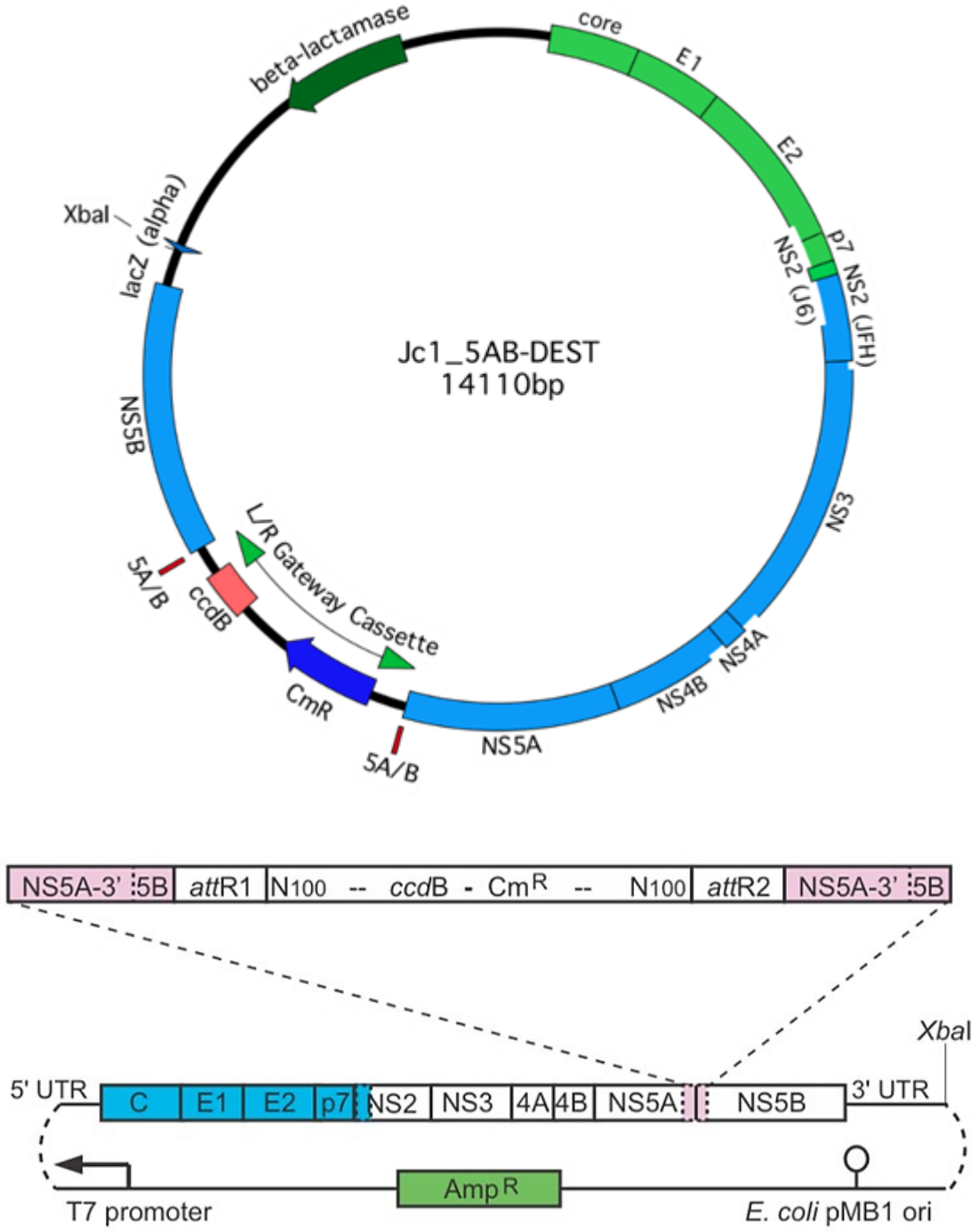
iv. pTRIP_CMV_IRES_RFP



pTRIP.CMV.IVSB.iTagRFP-DEST
12,847 bp

pTRIP_CMV_IRES_RFP: Lentiviral Gateway® destination vector containing a CMV promoter, as well as an RFP under the control of an independent IRES promoter element.

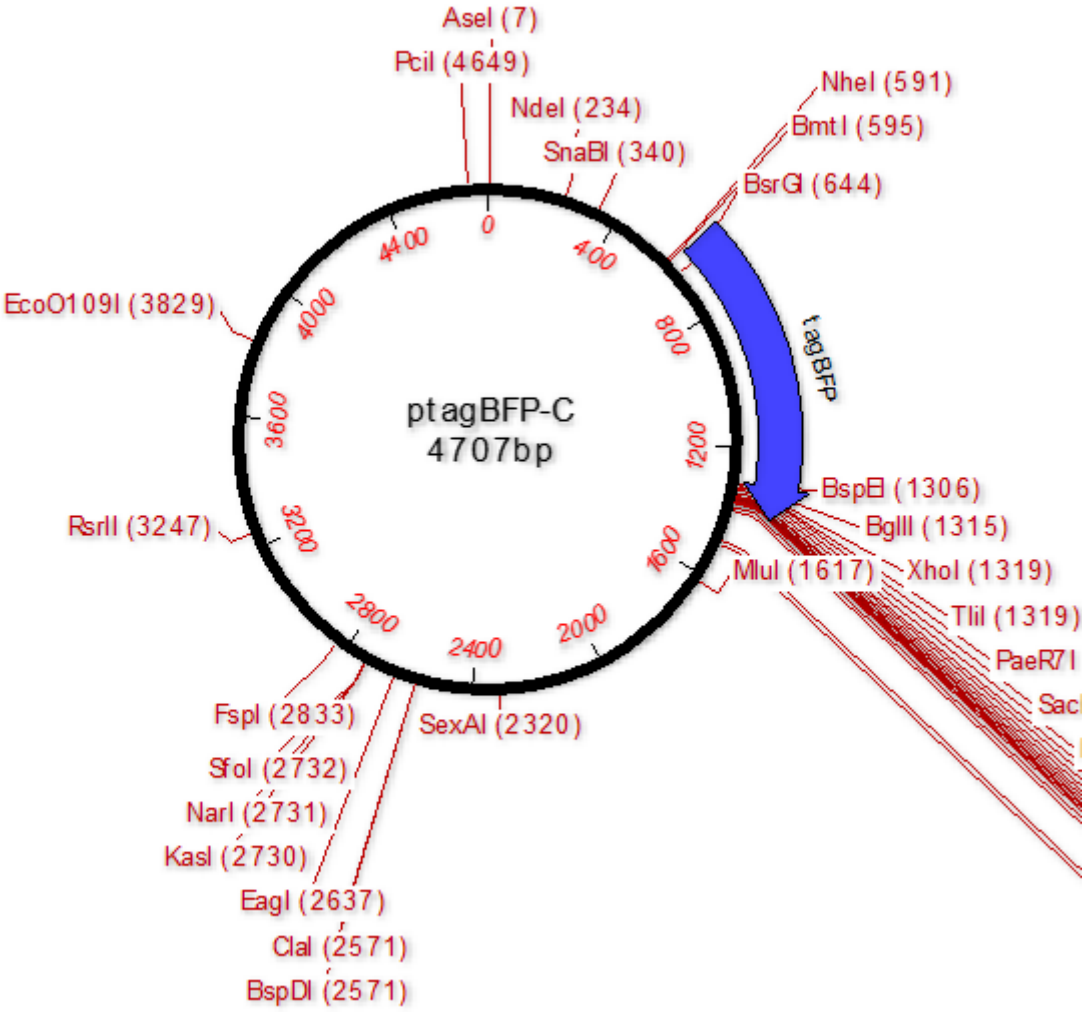
2.II. Origin of destination cassette for use during the cloning strategy for the creation of recombinant HBV genomes



Jc1-5AB-DEST: This construct contains the full length monocistronic Jc1 plus-strand genome with a Gateway® destination cassette inserted between duplicate NS5A/NS5B cleavage motifs. This vector does not encode a functional genome.

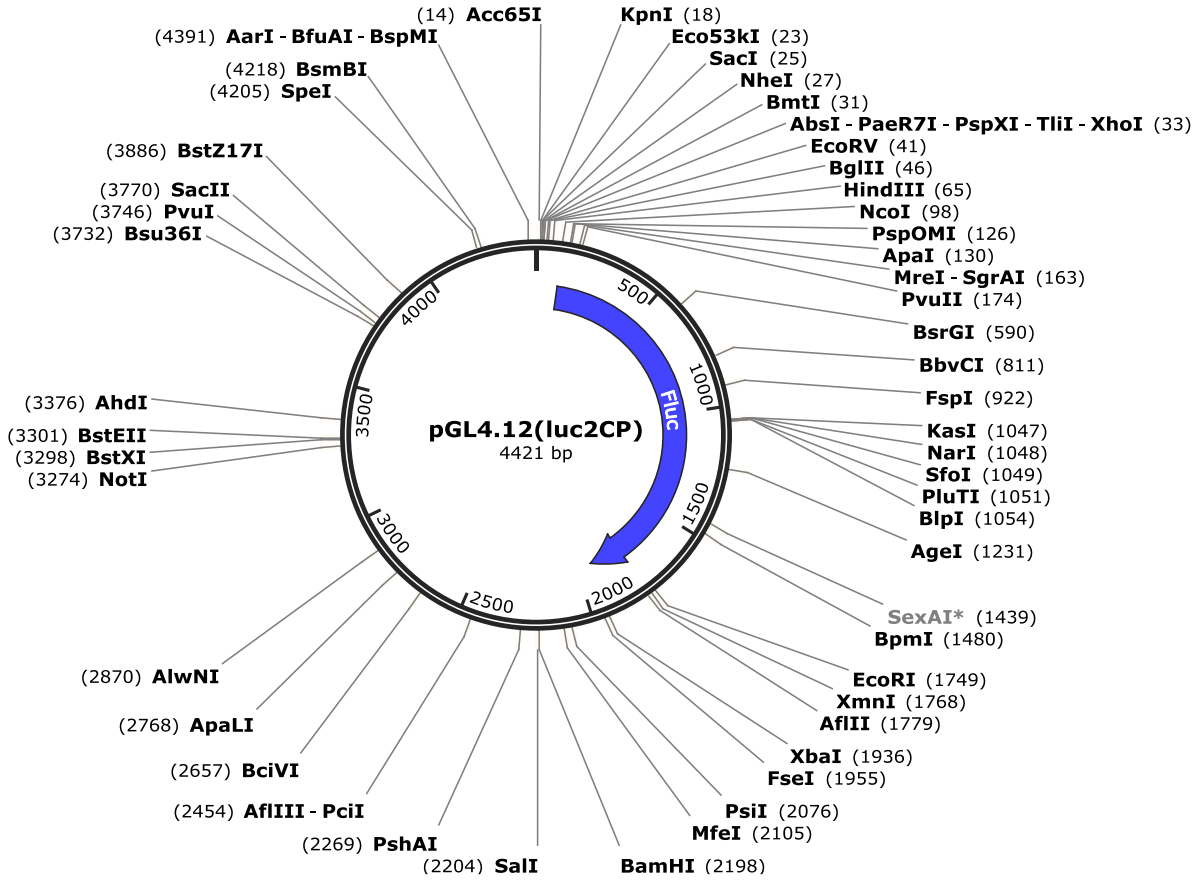
2.III. Constructs expressing reporter genes isolated for use during the cloning strategy for the creation of recombinant HBV genomes

i. pTagBFP-C



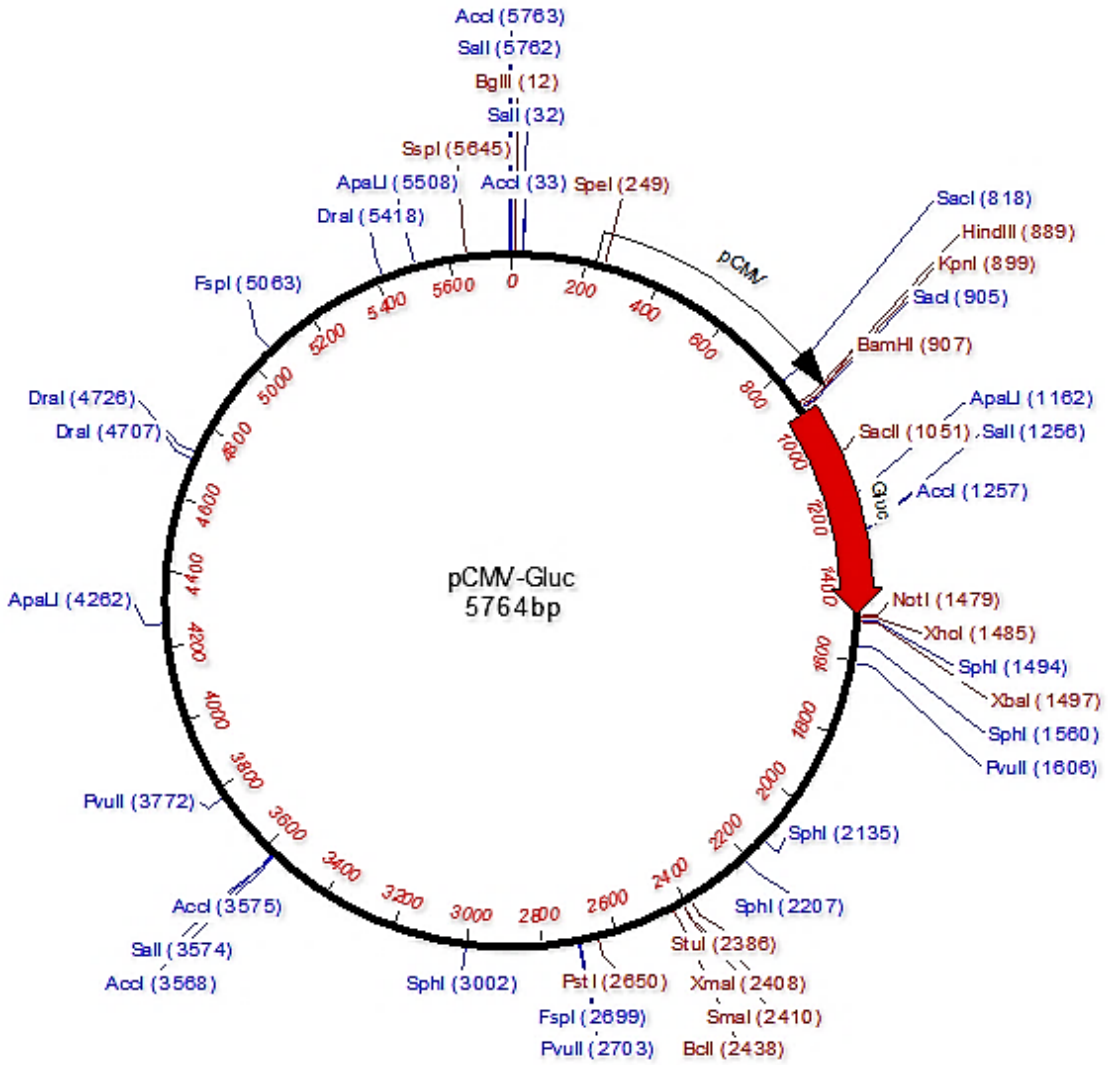
pTagBFP-C: Basic vector containing a BFP sequence.

ii. pGL4.12(luc2CP)



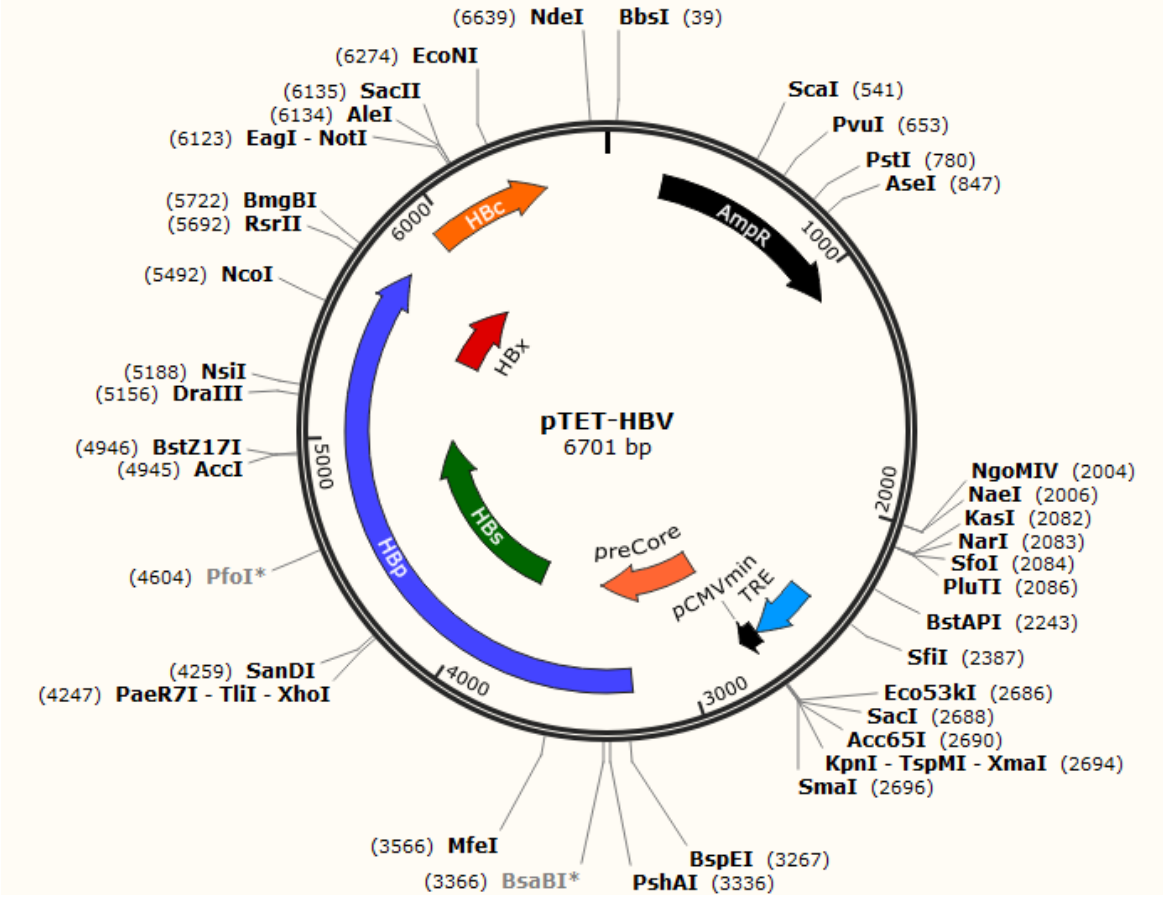
pGL4.12(luc2CP): Basic vector containing a Fluc sequence.

iii. pCMV_Gluc



pCMV_Gluc: Mammalian vector containing a CMV promoter and encoding for a Gluc sequence.

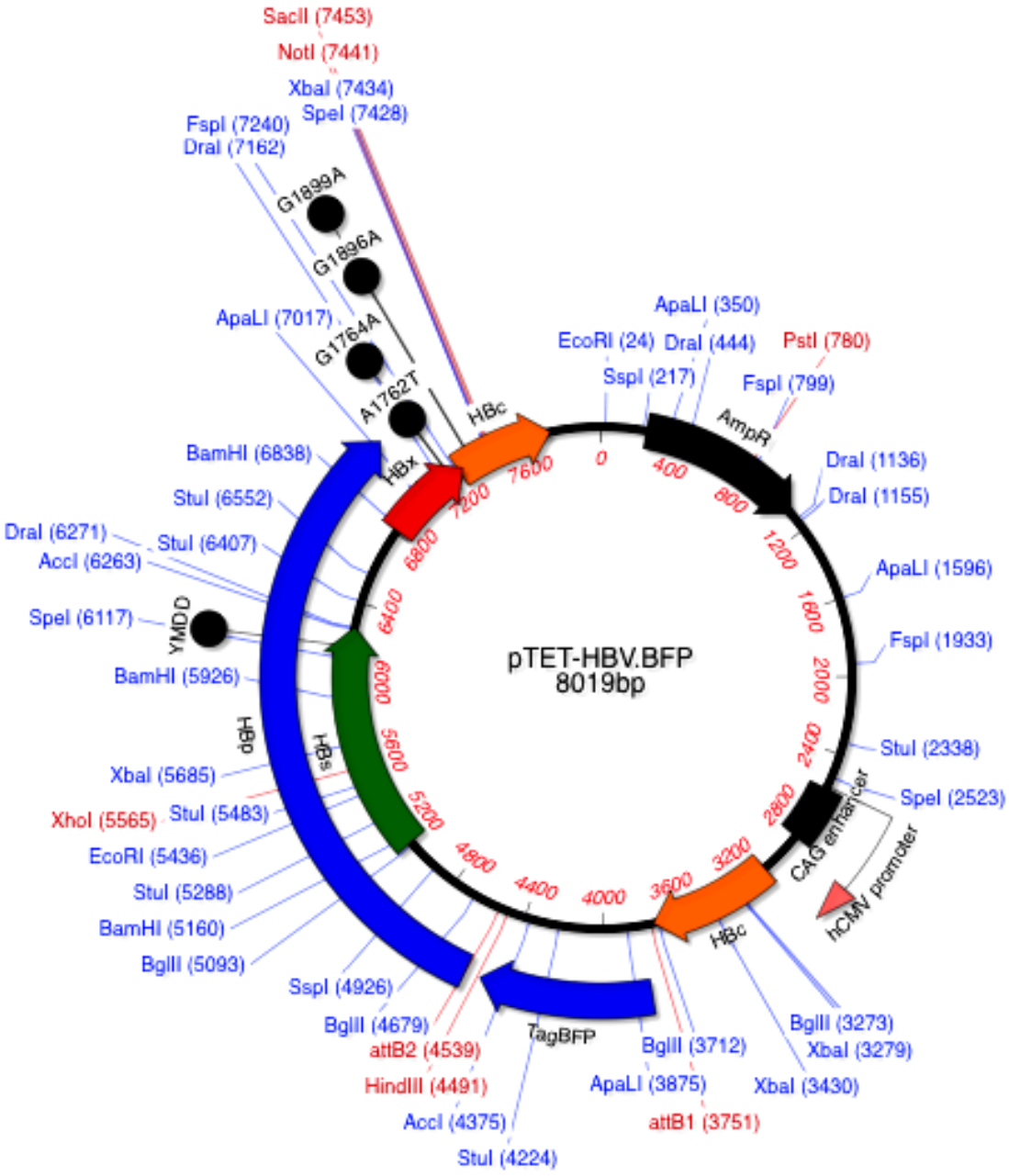
2.IV. Original pTET(CMV)_HBV construct



pCMV_HBV: Mammalian expression vector with a CMV promoter. Contains the wild-type HBV 1.3kb overlength genomic sequence.

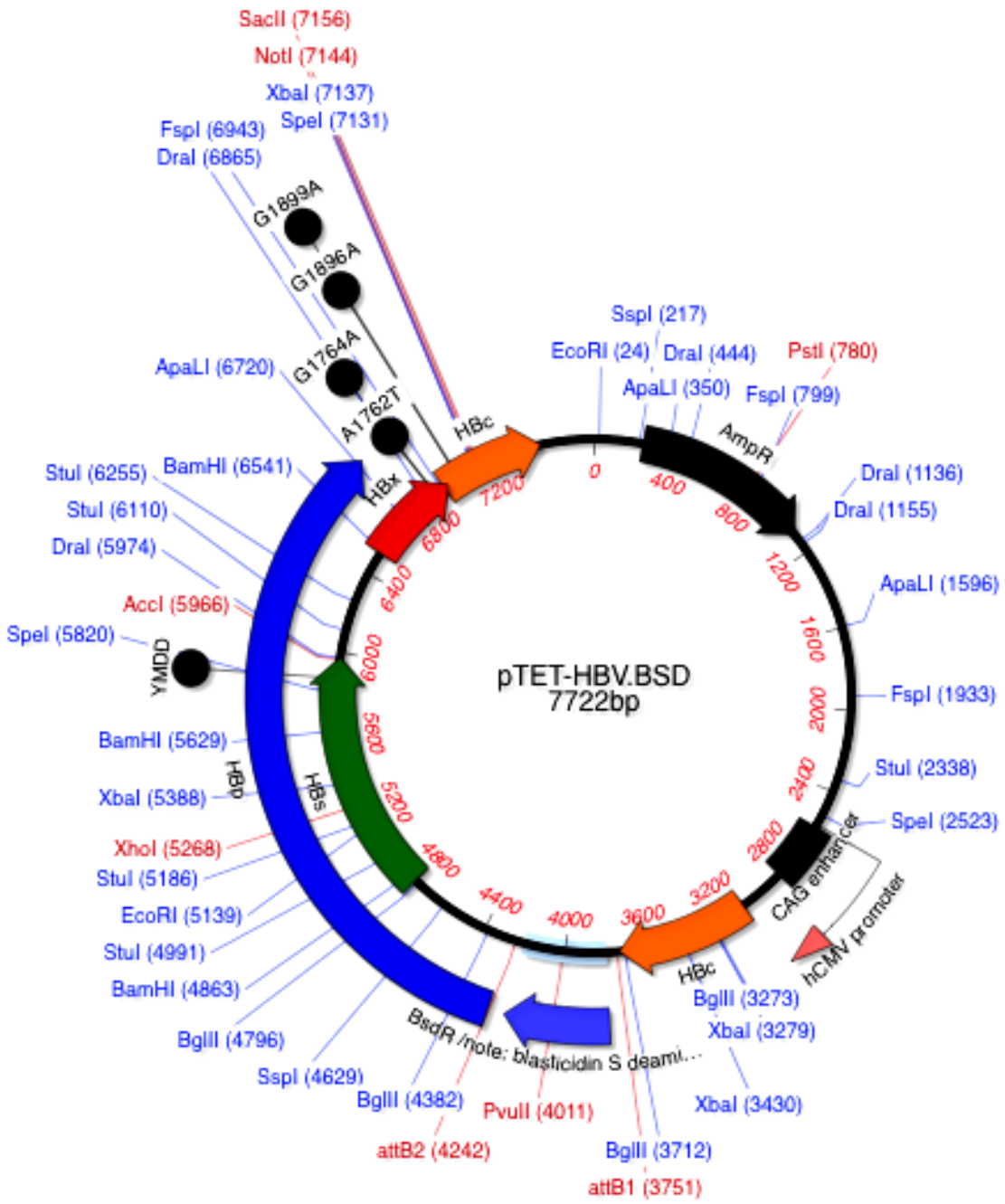
2.V. Replication-competent HBV genomes encoding reporter genes, in a pCMV vector backbone

i. pCMV_HBV_BFP



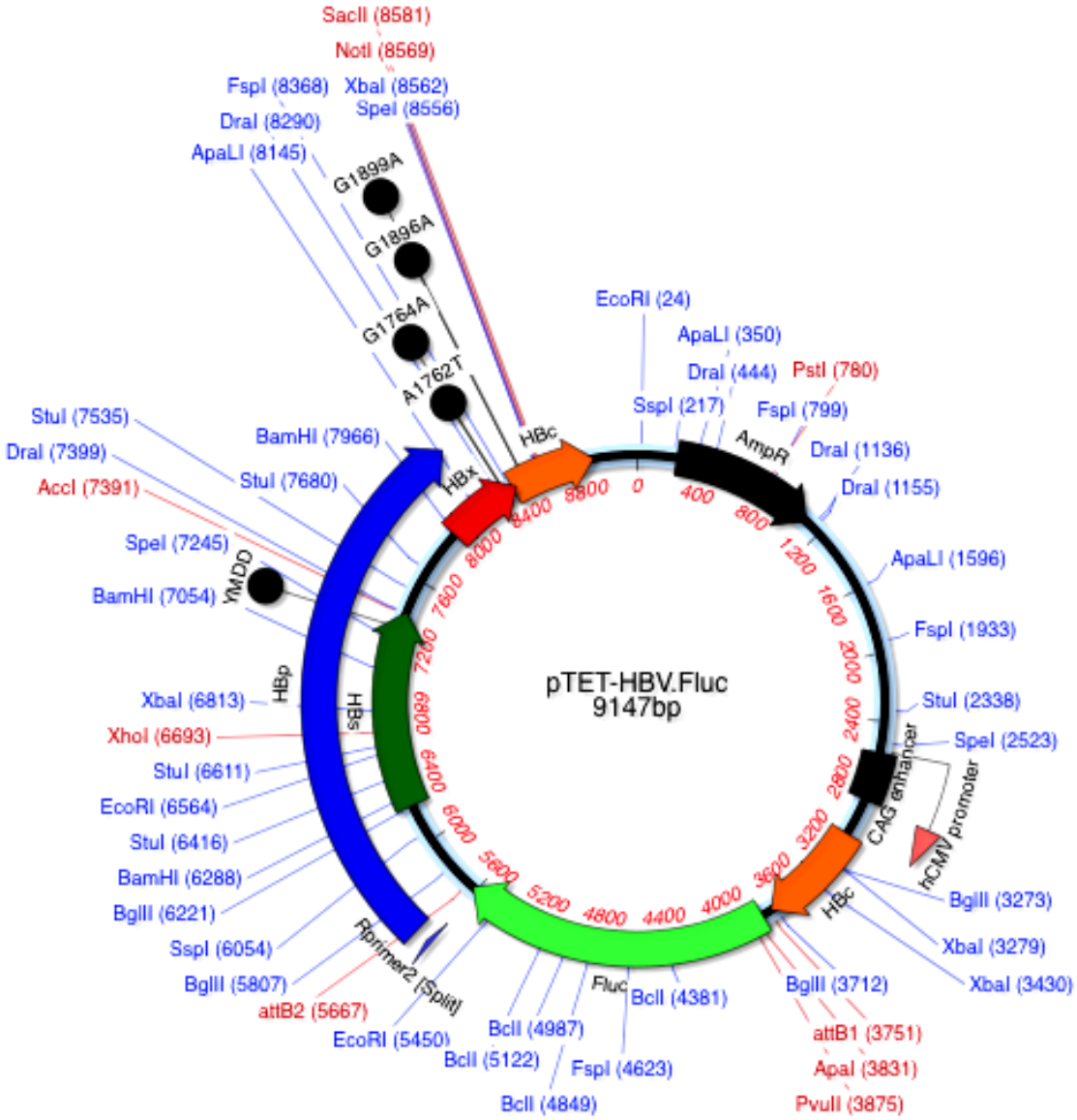
pCMV_HBV_BFP: Mammalian expression vector with a CMV promoter. Contains an altered HBV 1.3kb overlength genomic sequence, specifically encoding a BFP sequence further inserted between the core and the polymerase sequences.

ii. pCMV_HBV_BSD



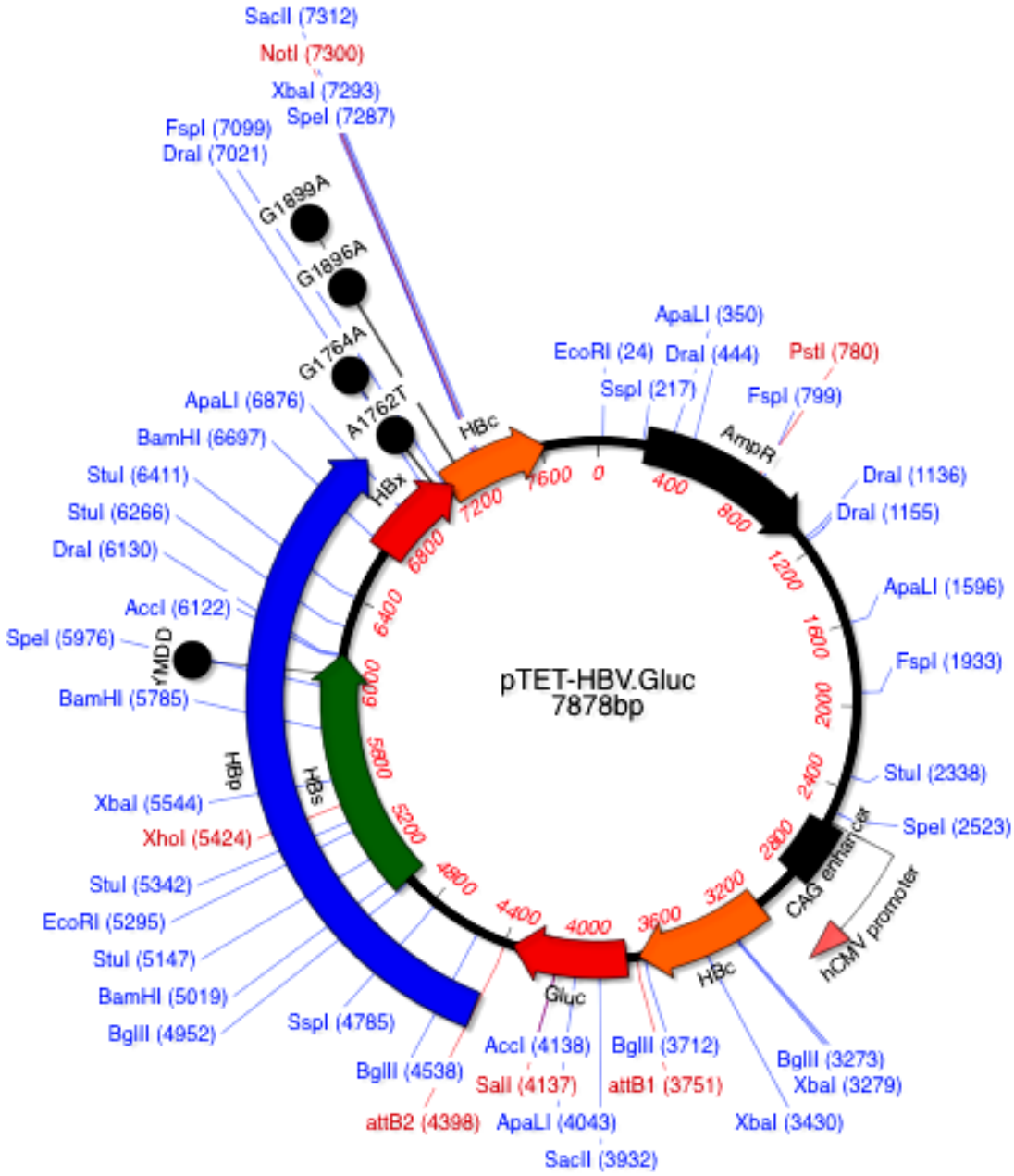
pCMV_HBV_BSD: Mammalian expression vector with a CMV promoter. Contains an altered HBV 1.3kb overlength genomic sequence, specifically encoding a BsdR sequence further inserted between the core and the polymerase sequences.

iii. pCMV_HBV_Fluc



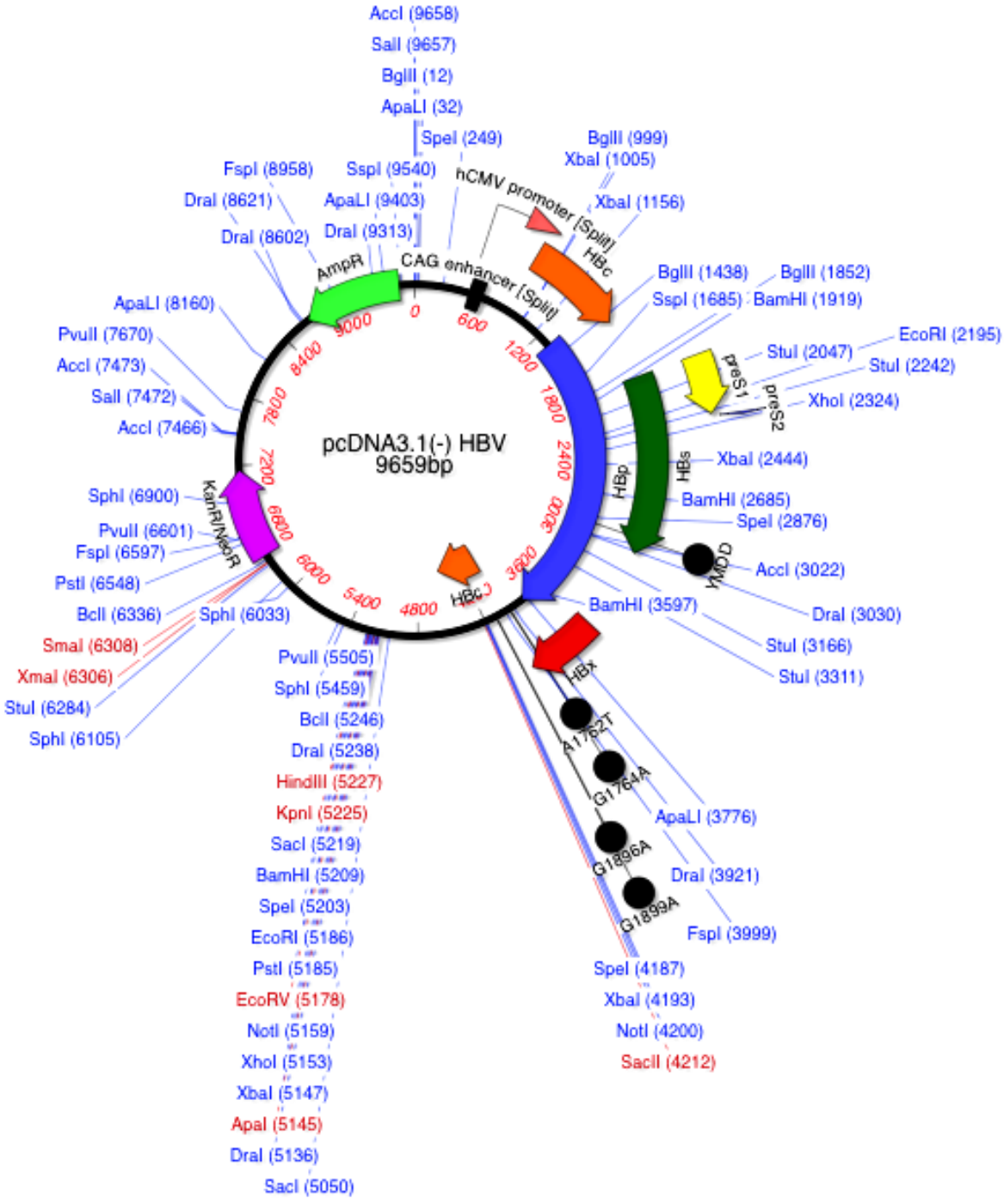
pCMV_HBV_Fluc: Mammalian expression vector with a CMV promoter. Contains an altered HBV 1.3kb overlength genomic sequence, specifically encoding a Fluc sequence further inserted between the core and the polymerase sequences.

iv. pCMV_HBV_Gluc



pCMV_HBV_Gluc: Mammalian expression vector with a CMV promoter. Contains an altered HBV 1.3kb overlength genomic sequence, specifically encoding a Gluc sequence further inserted between the core and the polymerase sequences.

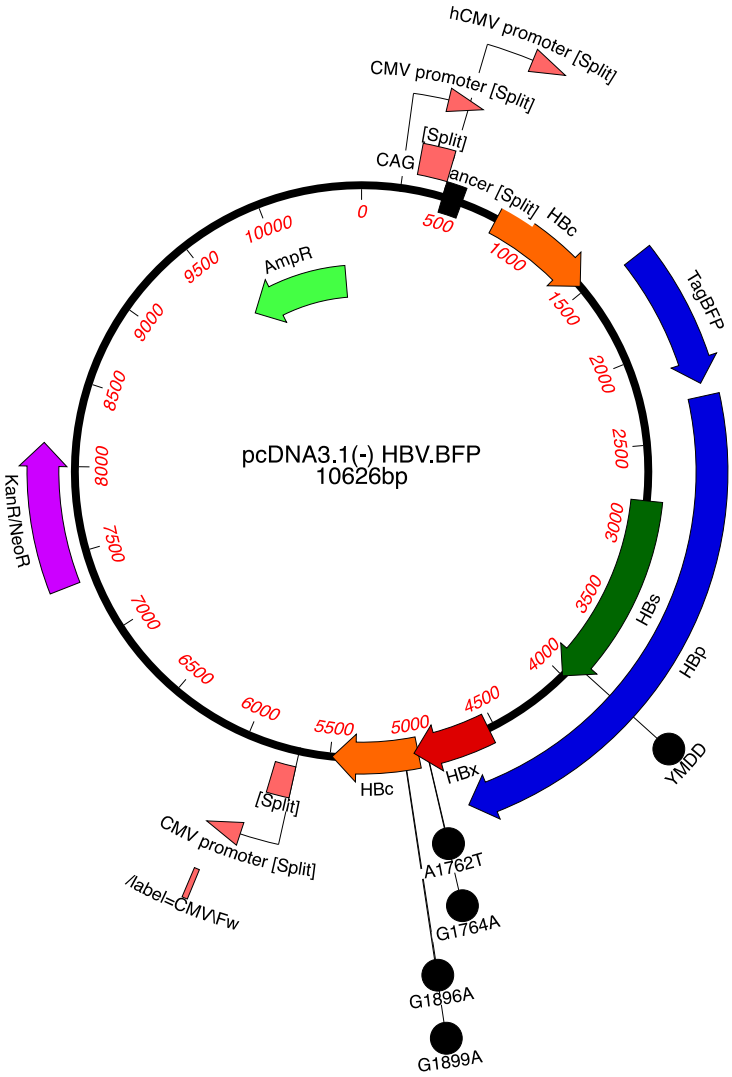
2.VI. Newly created pCDNA3.1(-)_HBV construct



pCDNA3.1(-)_HBV: Mammalian expression vector with a CMV promoter. Contains the wild-type HBV 1.3kb overlength genomic sequence.

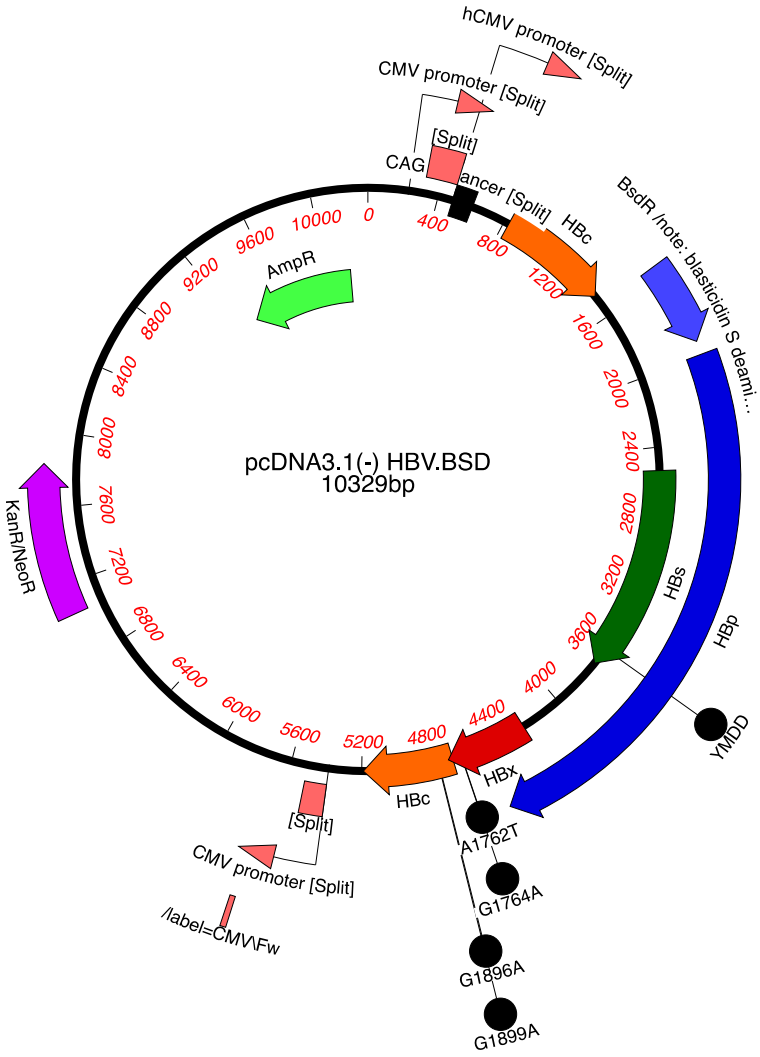
2.VII. Additional replication-competent HBV genomes encoding reporter genes, in a pCDNA3.1(-) vector backbone

i. pCDNA3.1(-)_HBV_BFP



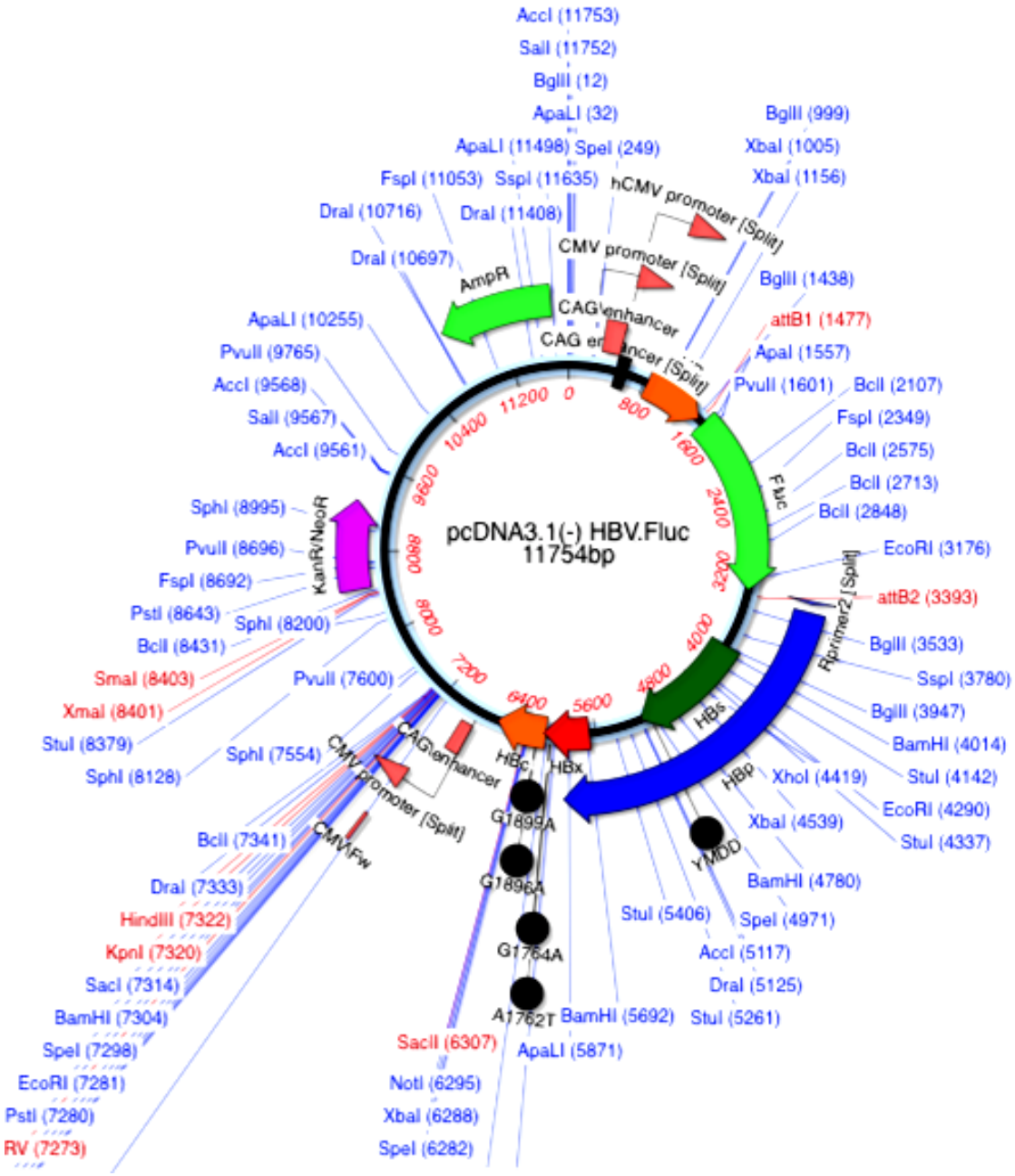
pCDNA3.1(-)_HBV_BFP: Mammalian expression vector with a CMV promoter. Contains an altered HBV 1.3kb overlength genomic sequence, specifically encoding a BFP sequence further inserted between the core and the polymerase sequences.

ii. pCDNA3.1(-)_HBV_BSD



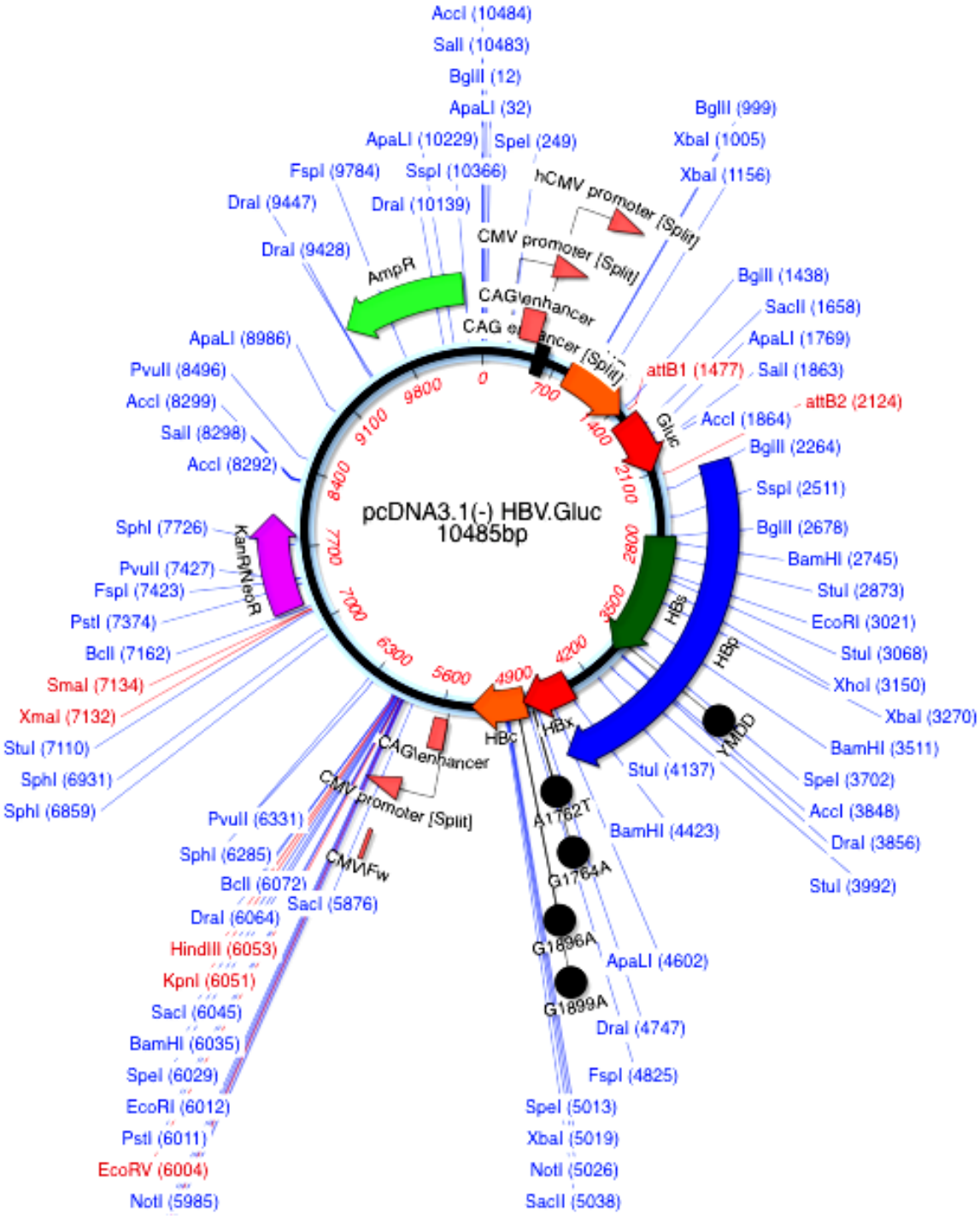
pCDNA3.1(-)_HBV_BSD: Mammalian expression vector with a CMV promoter. Contains an altered HBV 1.3kb overlength genomic sequence, specifically encoding a BsdR sequence further inserted between the core and the polymerase sequences.

iii. pCDNA3.1(-)_HBV_Fluc



pCDNA3.1(-)_HBV_Fluc: Mammalian expression vector with a CMV promoter. Contains an altered HBV 1.3kb overlength genomic sequence, specifically encoding a Fluc sequence further inserted between the core and the polymerase sequences.

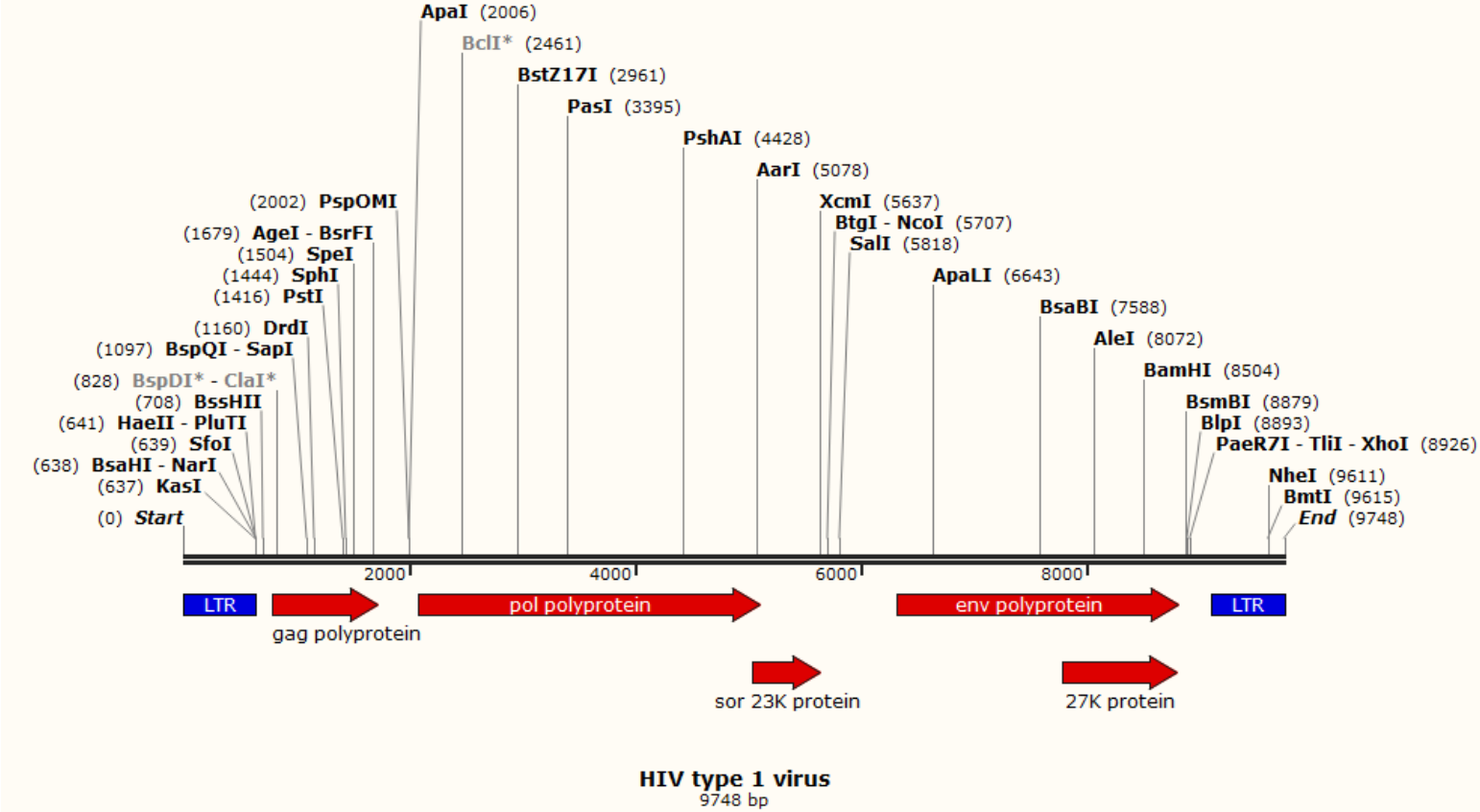
iv. pCDNA3.1(-)_HBV_Gluc



pCDNA3.1(-)_HBV_Gluc: Mammalian expression vector with a CMV promoter. Contains an altered HBV 1.3kb overlength genomic sequence, specifically encoding a Gluc sequence further inserted between the core and the polymerase sequences.

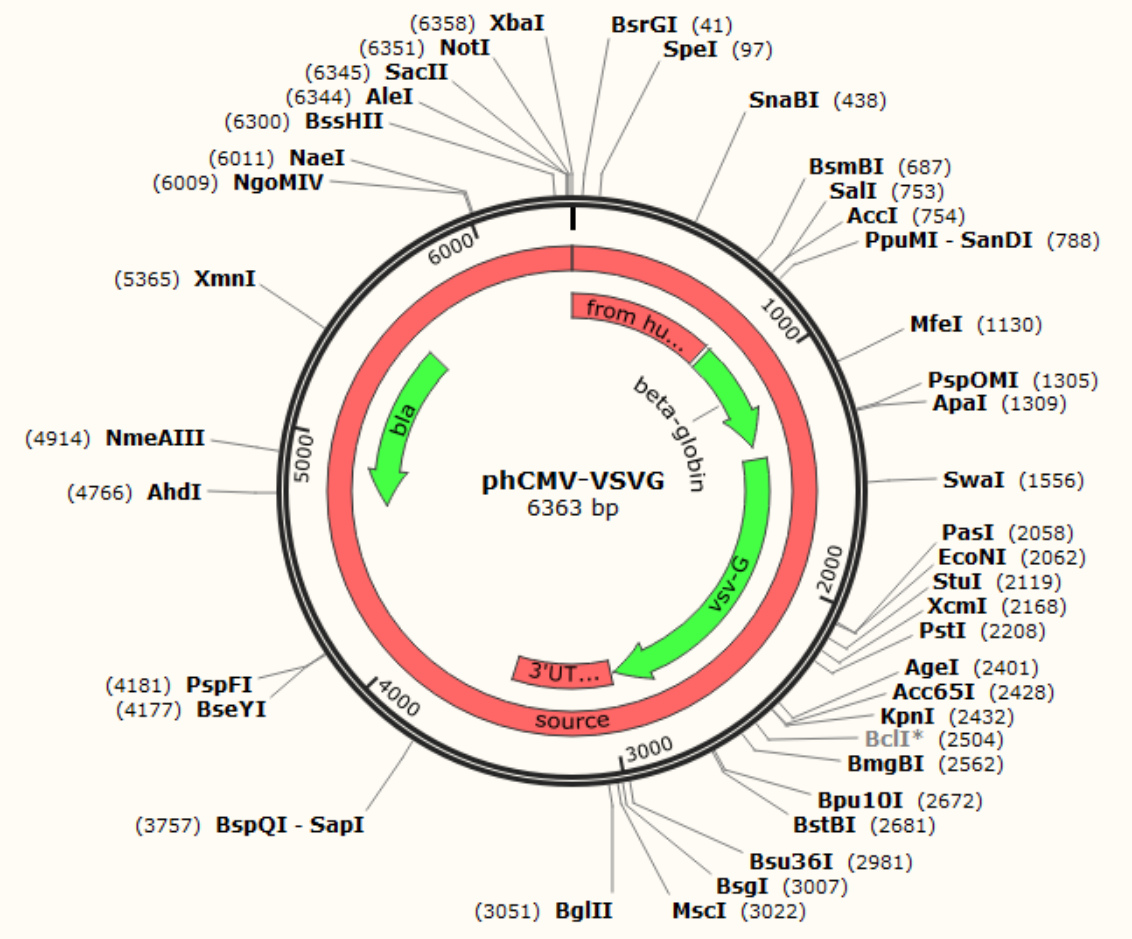
2.VIII. Plasmids used during production of lentiviral pseudoparticles

i. HIV gag/pol



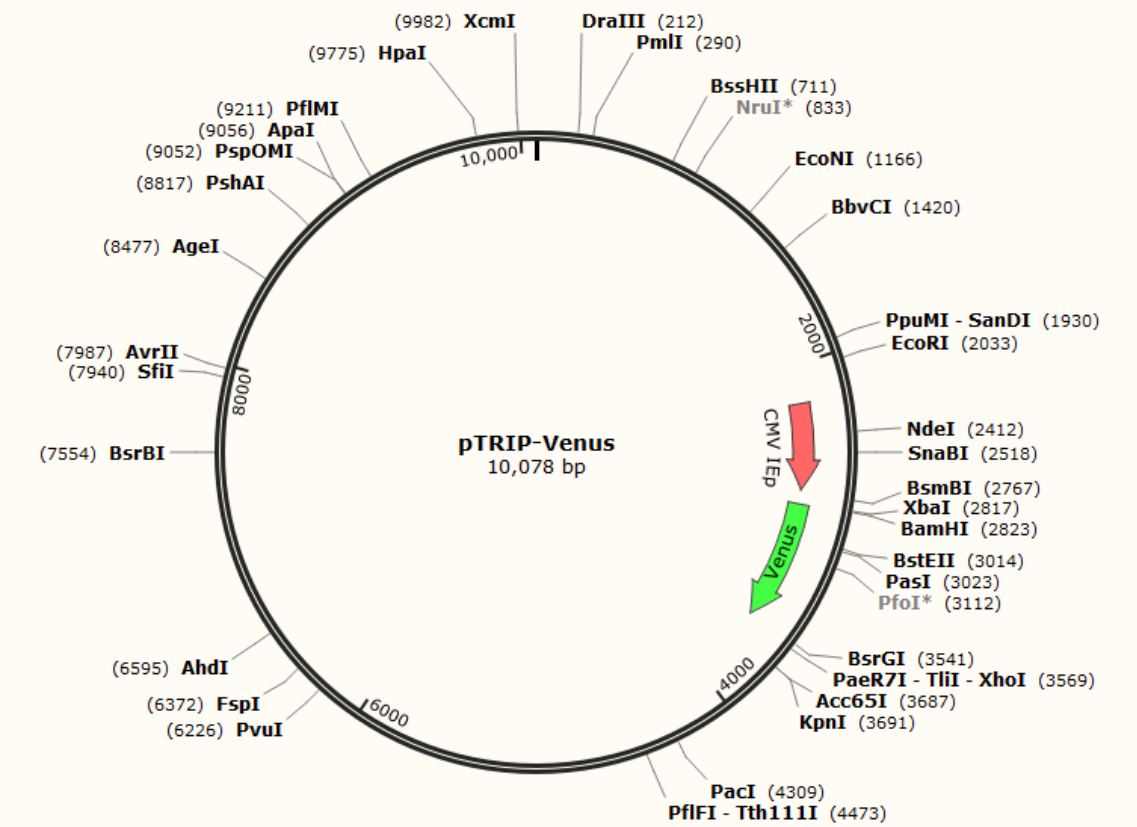
HIV gag/pol: Basic vector expressing the HIV type I virus.

ii. VSVg



phCMV_VSVg: Mammalian expression vector encoding for the VSVg under the control of a CMV promoter.

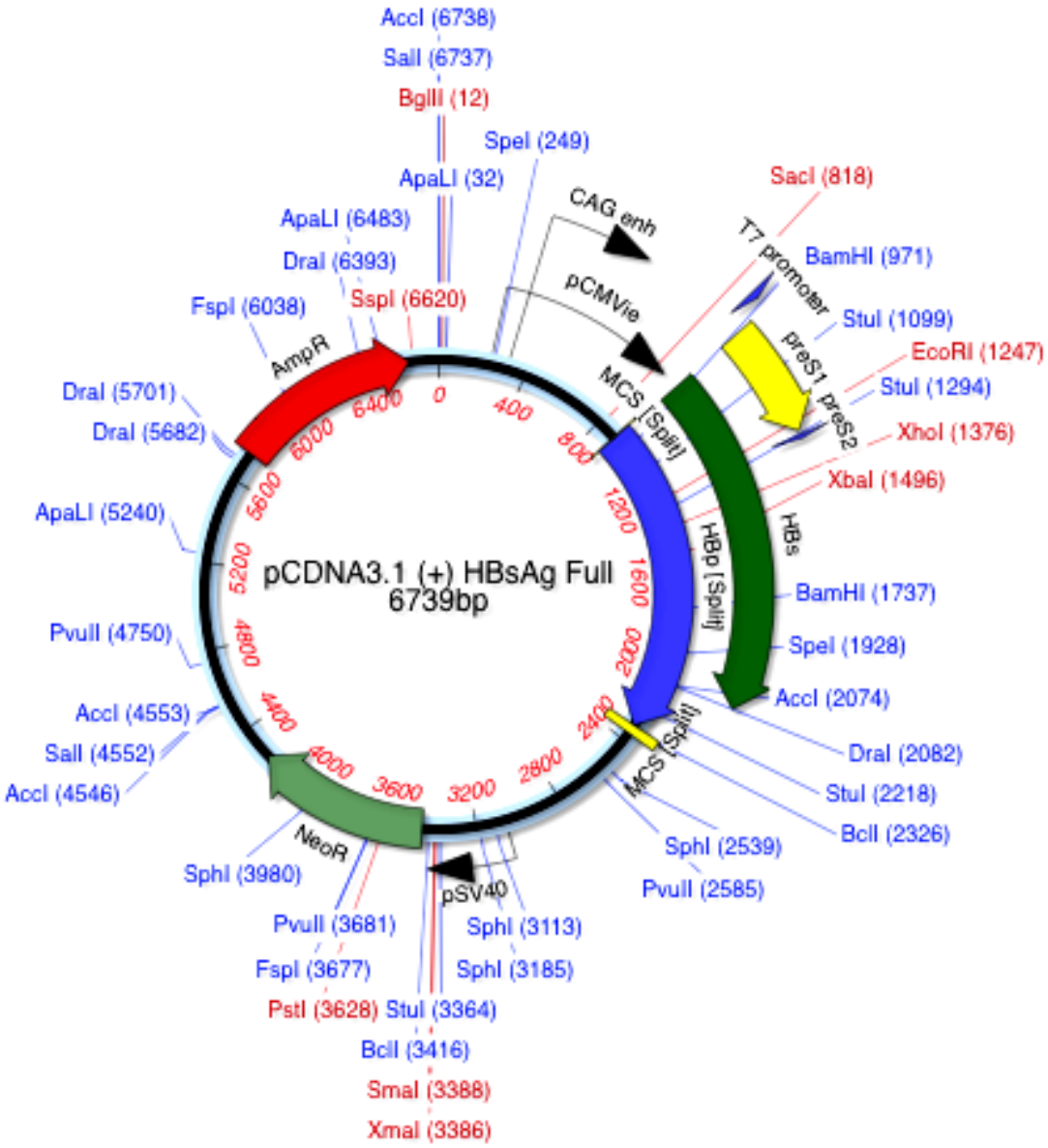
iii. pTRIP_Venus



pTRIP_Venus: Lentiviral vector expressing a Venus fragment, as an alternative to a GFP sequence, under the control of a CMV promoter.

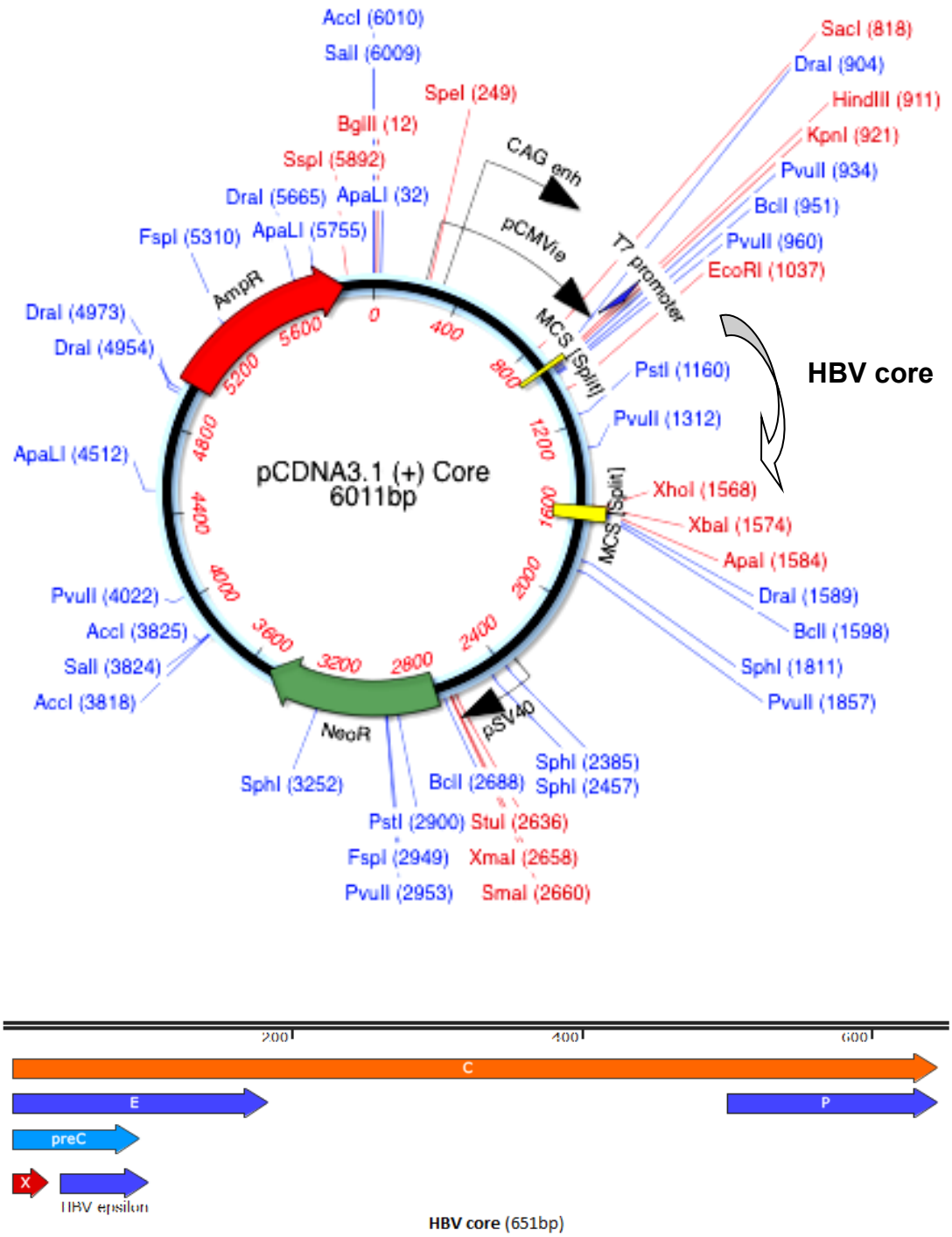
2.IX. Constructs used for the production of the HBSpp

i. pCDNA3.1(+)_HBsAg



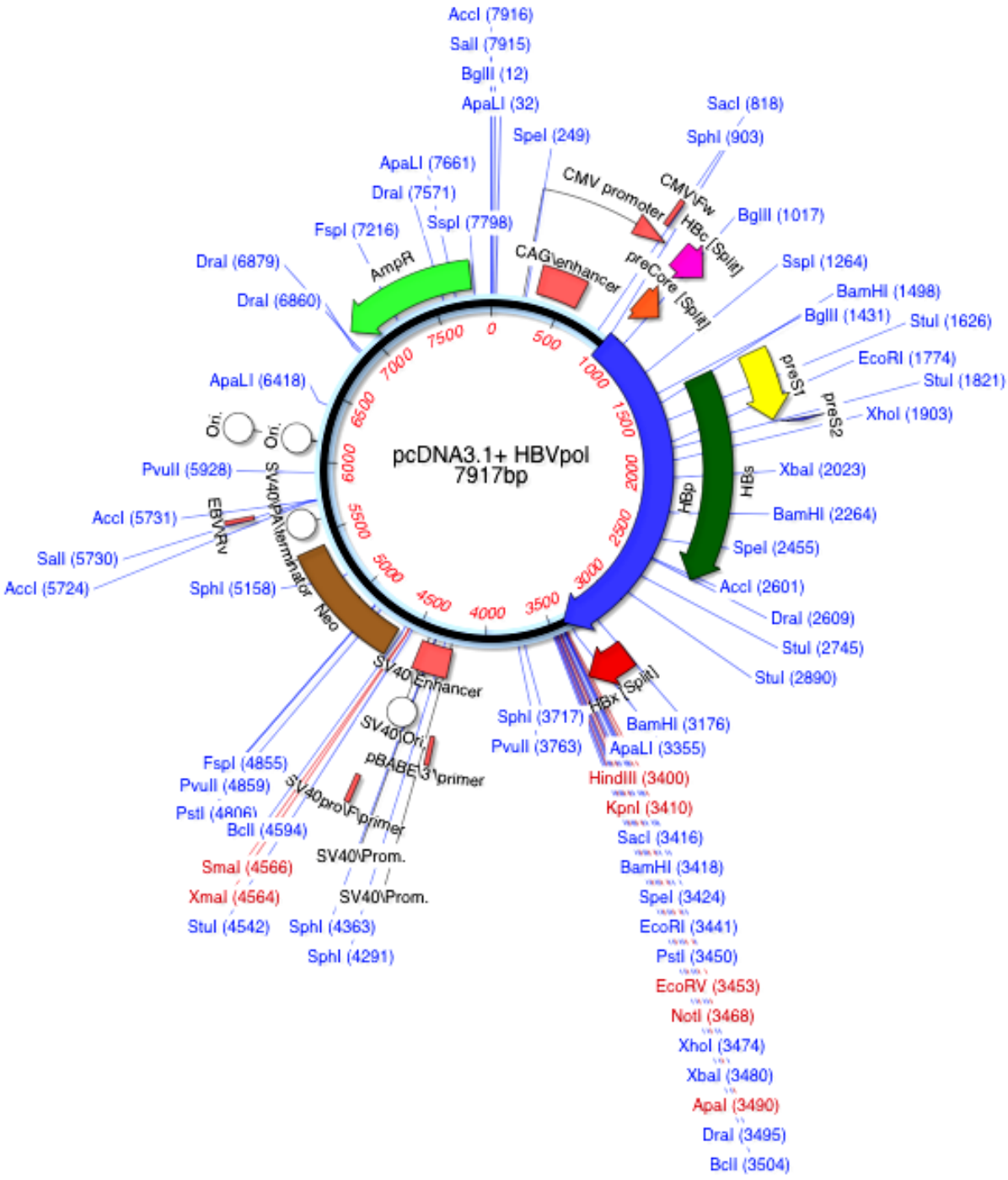
pCDNA3.1 (+)_HBsAg: Mammalian expression vector containing a CMV promoter, driving expression of the full HBsAg sequence isolated from the HBV genome.

ii. pCDNA3.1(+)_HBVcore



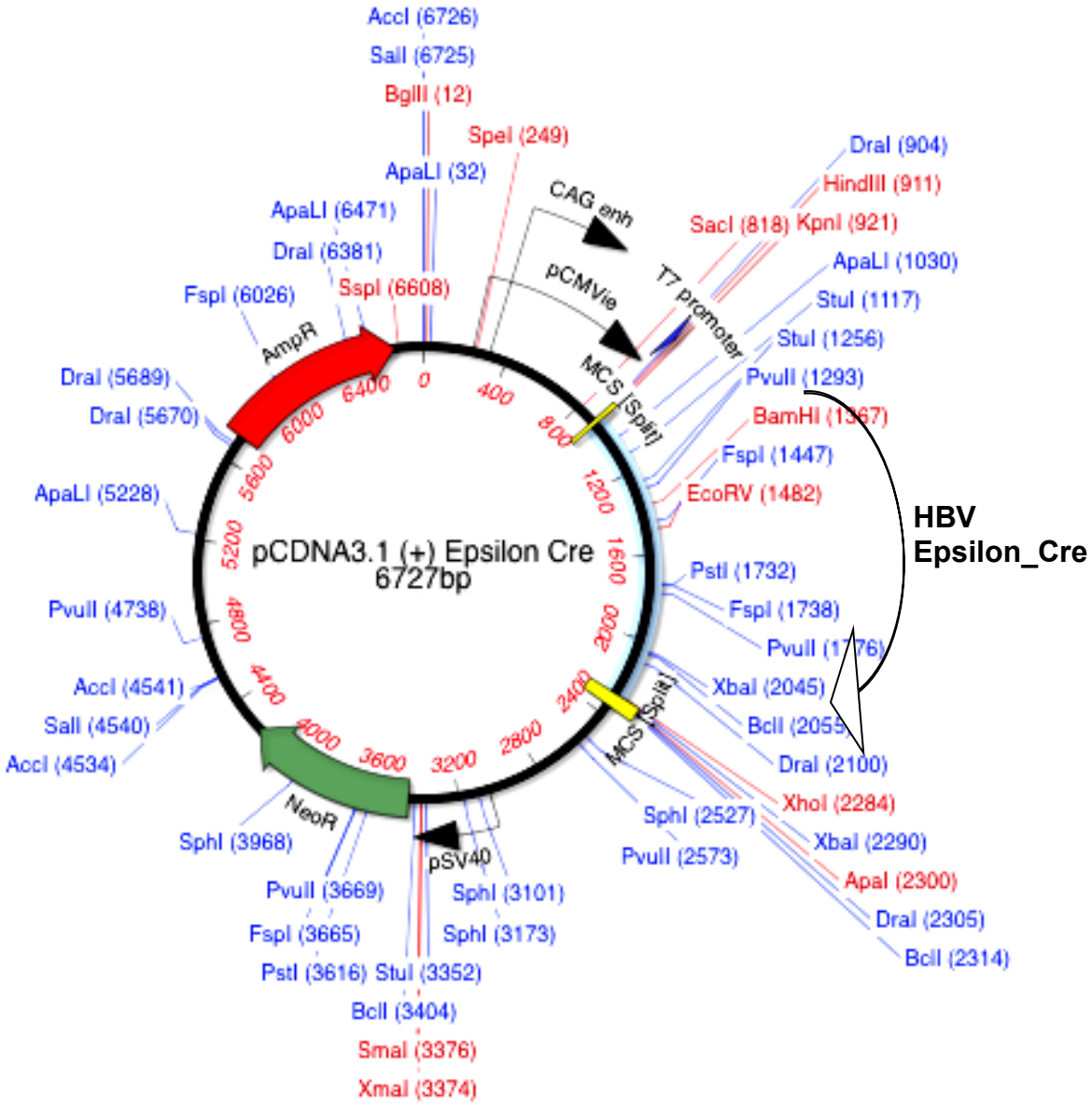
pCDNA3.1 (+)_HBVcore: Mammalian expression vector containing a CMV promoter, driving expression of the full HBV core sequence.

iii. pCDNA3.1(+)_HBV polymerase



pCDNA3.1 (+)_HBVpolymerase: Mammalian expression vector containing a CMV promoter, driving expression of the full HBV polymerase sequence.

iv. pCDNA3.1(+)_HBVepsilonCre



pCDNA3.1 (+)_HBVepsilonCre: Mammalian expression vector containing a CMV promoter, driving expression of the HBV RNA encapsidation signal, Epsilon, as well as a Cre-recombinase.

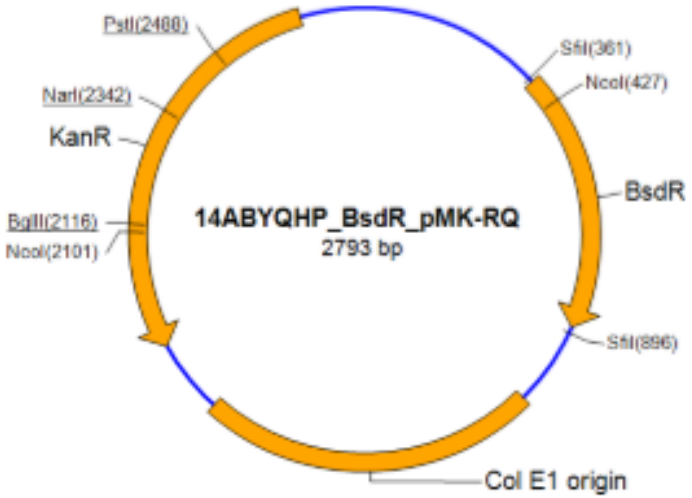
2.X. Purchased GeneArt constructs (*ThermoFisher Scientific*)

i. BsdR

Plasmid DNA Description:

The synthetic gene BsdR was assembled from synthetic oligonucleotides and/or PCR products. The fragment was cloned into pMK-RQ (kanR) using SfiI and SfiI cloning sites. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the used restriction sites was 100%. See the accompanying data sheets for sequences and find the original ABI trace files as well as the assembled sequences electronically on disk.
5 µg of the plasmid preparation were lyophilized for shipping.

Plasmid Map:



life technologies™

Quality Assurance Documentation: 14ABYQHP

Ref. No.: 1557204

Designation:	E.coli K12 (dam+ dcm+ tonA rec-)
Gene name:	**BsdR**
Gene size:	515 bp
Vector backbone:	pMK-RQ (kanR)
Cloning sites:	SfiI / SfiI
Quantity:	~5 µg Plasmid DNA
Note:	Please dissolve lyophilized DNA in 50 µl distilled water or 10 mM Tris-HCl (pH 8.0). We recommend sequence verification after each transformation step.
Date:	4 August 2014

Carmen Billerbeck
Quality control

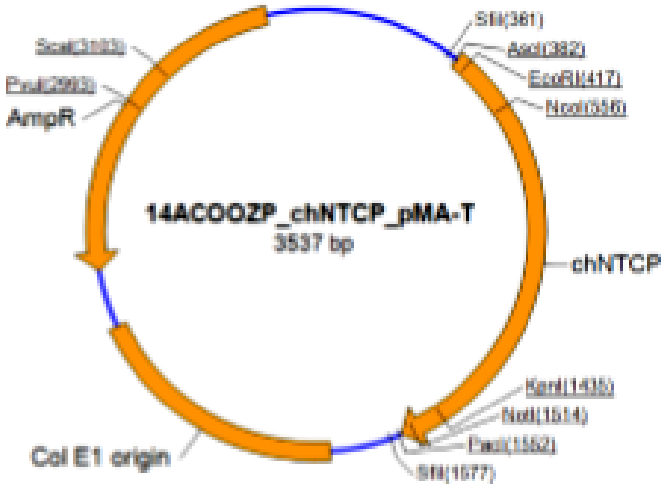
GeneArt AG **www.lifetechnologies.com** **GeneArtSupport@lifetech.com**

ii. chimNTCP

Plasmid DNA Description:

The synthetic gene chNTCP was assembled from synthetic oligonucleotides and/or PCR products. The fragment was inserted into pMA-T. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the insertion sites was 100%. See the accompanying data sheets for sequences and find the original ABI trace files as well as the assembled sequences electronically on disk.
5 µg of the plasmid preparation were lyophilized for shipping.

Plasmid Map:



life technologies™

Quality Assurance Documentation: 14ACOOZP

Ref. No.: 1607030

Designation:	E.coli K12 (dam+ dcm+ tonA)
Gene name:	**chNTCP**
Gene size:	1157 bp
Vector backbone:	pMA-T
Cloning sites:	SfiI / SfiI
Quantity:	~5 µg Plasmid DNA
Note:	Please dissolve lyophilized DNA in 50 µl distilled water or 10 mM Tris-HCl (pH 8.0). We recommend sequence verification after each transformation step.
Date:	6 November 2014

Meike Brodt
Quality control

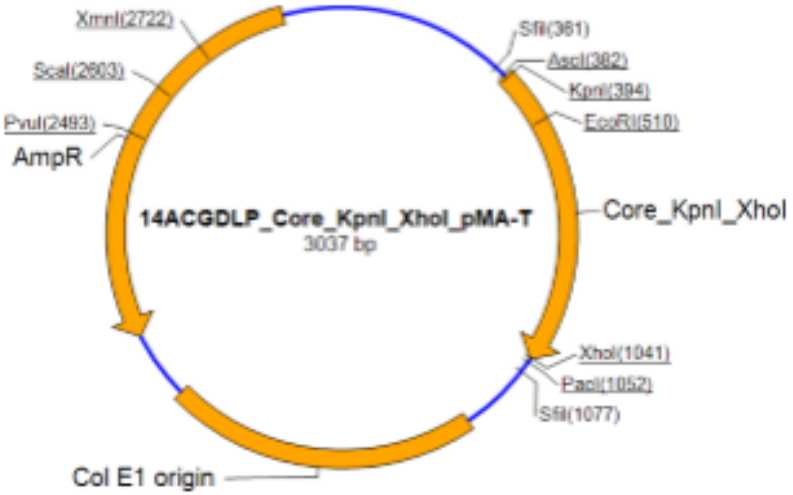
GeneArt AG **www.lifetechnologies.com** **GeneArtSupport@lifetech.com**

iii. HBV Core

Plasmid DNA Description:

The synthetic gene Core_KpnI_XhoI was assembled from synthetic oligonucleotides and/or PCR products. The fragment was inserted into pMA-T. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the insertion sites was 100%. See the accompanying data sheets for sequences and find the original ABI trace files as well as the assembled sequences electronically on disk.
5 µg of the plasmid preparation were lyophilized for shipping.

Plasmid Map:



life technologies™

Quality Assurance Documentation: 14ACGDLP

Ref. No.: 1585776

Designation:	E.coli K12 (dam+ dcm+ tonA)
Gene name:	**Core_KpnI_XhoI**
Gene size:	657 bp
Vector backbone:	pMA-T
Cloning sites:	SfiI / SfiI
Quantity:	~5 µg Plasmid DNA
Note:	Please dissolve lyophilized DNA in 50 µl distilled water or 10 mM Tris-HCl (pH 8.0). We recommend sequence verification after each transformation step.
Date:	26 September 2014

Patricia Wyant
Quality control

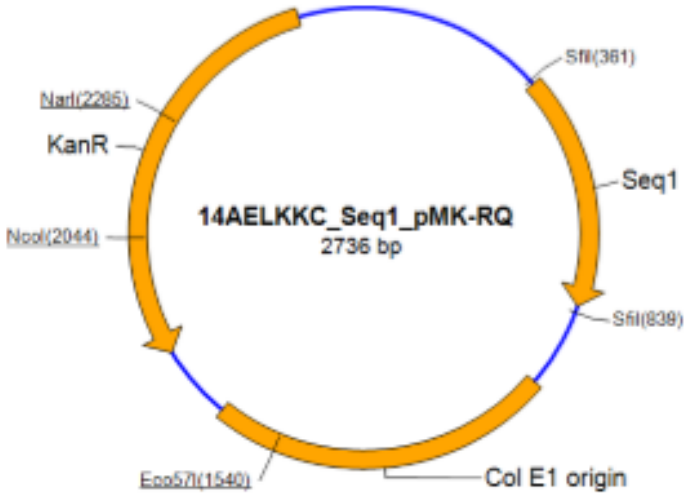
GeneArt AG **www.lifetechnologies.com** **GeneArtSupport@lifetech.com**

iv. HBV Core/Polymerase

Plasmid DNA Description:

The synthetic gene Seq1 was assembled from synthetic oligonucleotides and/or PCR products. The fragment was cloned into pMK-RQ (kanR) using SfiI and SfiI cloning sites. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the used restriction sites was 100%. See the accompanying data sheets for sequences and find the original ABI trace files as well as the assembled sequences electronically on disk.
5 µg of the plasmid preparation were lyophilized for shipping.

Plasmid Map:



life technologies™

Quality Assurance Documentation: 14AELKKC

Ref. No.: 1498631

Designation:	E.coli K12 (dam+ dcm+ tonA rec-)
Gene name:	**Seq1**
Gene size:	458 bp
Vector backbone:	pMK-RQ (kanR)
Cloning sites:	SfiI / SfiI
Quantity:	~5 µg Plasmid DNA
Note:	Please dissolve lyophilized DNA in 50 µl distilled water or 10 mM Tris-HCl (pH 8.0). We recommend sequence verification after each transformation step.
Date:	4 April 2014

Patricia Wyant
Quality control

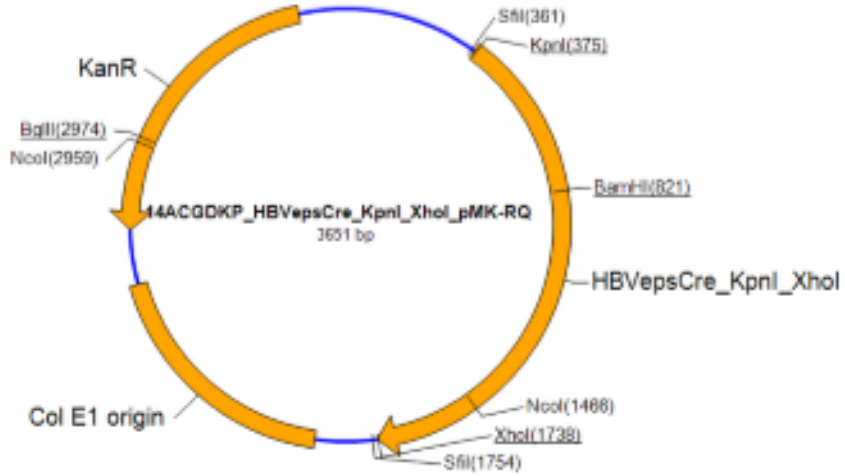
GeneArt AG **www.lifetechnologies.com** **GeneArtSupport@lifetech.com**

v. HBVepsilonCre

Plasmid DNA Description:

The synthetic gene HBVepsCre_KpnI_XhoI was assembled from synthetic oligonucleotides and/or PCR products. The fragment was inserted into pMK-RQ (kanR). The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the insertion sites was 100%. See the accompanying data sheets for sequences and find the original ABI trace files as well as the assembled sequences electronically on disk.
5 µg of the plasmid preparation were lyophilized for shipping.

Plasmid Map:



life technologies™

Quality Assurance Documentation: 14ACGDKP

Ref. No.: 1585777

Designation:	E.coli K12 (dam+ dcm+ tonA rec-)
Gene name:	**HBVepsCre_KpnI_XhoI**
Gene size:	1373 bp
Vector backbone:	pMK-RQ (kanR)
Cloning sites:	SfiI / SfiI
Quantity:	~5 µg Plasmid DNA
Note:	Please dissolve lyophilized DNA in 50 µl distilled water or 10 mM Tris-HCl (pH 8.0). We recommend sequence verification after each transformation step.
Date:	1 October 2014

Meike Brodt
Quality control

GeneArt AG **www.lifetechnologies.com** **GeneArtSupport@lifetech.com**

2.XI. Purchased lentiviral vectors containing the HBV-related receptors

i. Vector information for EX-C0391-Lv105 (GeneCopoeia)

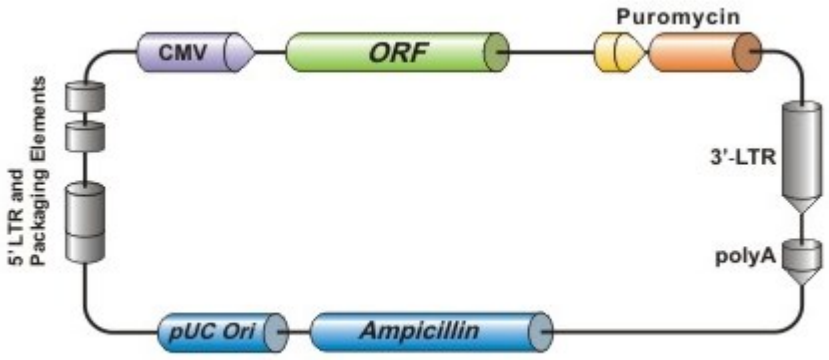
OmicsLink™ Expression Clone Datasheet of EX-C0391-Lv105

Clone Information

Catalog No.: EX-C0391-Lv105
Accession No.: NM_003049
ORF Length: 1050 bp
Whole Plasmid Size: 9090 bp
Description: Homo sapiens solute carrier family 10 (sodium/bile acid cotransporter family), member 1 (SLC10A1), mRNA.
Vector: pReceiver-Lv105
Antibiotic: Ampicillin
Stable Selection Marker: Puromycin

Suggested Sequencing Primers:
Forward: 5'-GCGGTAGGCGTGTACGGT-3'
Reverse: 5'-ATTGTGGATGAATACTGCC-3'

Vector Information for EX-C0391-Lv105





>EX-C0391-Lv105 [with 457F_F10]- NTCP

CAGGCTTGGAAGGAGTTCGAACCATGGAGGCCCACAACGCGTCTG
CCCCATTCAACTTCACCCTGCCACCCAACTTTGGCAAGCGCCCCACAGAC
CTGGCACTGAGCGTCATCCTGGTGTTCATGTTGTTCTTCATCATGCTCTC
GCTGGGCTGCACCATGGAGTTCAGCAAGATCAAGGCTCACTTATGGAAGC
CTAAAGGGCTGGCCATCGCCCTGGTGGCACAGTATGGCATCATGCCCCT
CACGGCCTTTGTGCTGGGCAAGGTCTTCCGGCTGAAGAACATTGAGGCA
CTGGCCATCTTGGTCTGTGGCTGCTCACCTGGAGGGAACCTGTCCAATG
TCTTCAGTCTGGCCATGAAGGGGGACATGAACCTCAGCATTGTGATGACC
ACCTGCTCCACCTTCTGTGCCCTTGGCATGATGCCTCTCCTCCTGTACAT
CTACTCCAGGGGGATCTATGATGGGGACCTGAAGGACAAGGTGCCCTAT
AAAGGCATCGTGATATCACTGGTCCTGGTTCTCATTCCTTGCACCATAGG
GATCGTCCTCAAATCCAAACGGCCACAATACATGCGCTATGTCATCAAGG
GAGGGATGATCATCATTCTCTTGTGCAGTGTGGCCGTCACAGTTCTCTC
TGCCATCAATGTGGGGAAGAGCATCATGTTTGCCATGACACCACTCTTGA
TTGCCACCTCCTCCCTGATGCCTTTTATTGGCTTTCTGCTGGGTTATGTTC
TCTCTGCTCTCTTCTGCCTCAATGGACGGTGCAGACGCACTGTCAGCATG
GAGACTGGATGCCAAAATGTCCAACTCTGTTCCACCATCCTCAATGTGGCC
TTTCCACCTGAAGTCATTGGACCACTTTTCTTCTTTCCCCTCCTCTACATG
ATTTTCCAGCTTGGAGAAGGGCTTCTCCTCATTGCCATATTTTGGTGCTAT
GAGAAATTCAAGACTCCCAAGGATAAAACAAAAATGATCTACACAGCTGCC
ACAACTGAAGAAACAATTCCAGGAGCTCTGGGAAATGGCACCTACAAAGG
GGAGGACTGCTCCCCTTGCACAGCCTAGCTCGAGTGCGGCCGCAACCCAG

>EX-A0379-Lv105 (NM_001671, **ASGPR ORF**)

ATGACCAAGGAGTATCAAGACCTTCAGCATCTGGACAATGAGGAGAGTG
ACCACCATCAGCTCAGAAAAGGGCCACCTCCTCCCCAGCCCCTCCTGCA
GCGTCTCTGCTCCGGACCTCGCCTCCTCCTGCTCTCCCTGGGCCTCAGC
CTCCTGCTGCTTGTGGTTGTCTGTGTGATCGGATCCCAAAACTCCCAGC
TGCAGGAGGAGCTGCGGGGCCTGAGAGAGACGTTCAGCAACTTCACAG
CGAGCACGGAGGCCCAGGTCAAGGGCTTGAGCACCCAGGGAGGCAAT
GTGGGAAGAAAGATGAAGTCGCTAGAGTCCCAGCTGGAGAAACAGCAG
AAGGACCTGAGTGAAGATCACTCCAGCCTGCTGCTCCACGTGAAGCAGT
TCGTGTCTGACCTGCGGAGCCTGAGCTGTCAGATGGCGGCGCTCCAGG
GCAATGGCTCAGAAAGGACCTGCTGCCCGGTCAACTGGGTGGAGCACG
AGCGCAGCTGCTACTGGTTCTCTCGCTCCGGGAAGGCCTGGGCTGACG
CCGACAACTACTGCCGGCTGGAGGACGCGCACCTGGTGGTGGTCACGT
CCTGGGAGGAGCAGAAATTTGTCCAGCACCACATAGGCCCTGTGAACAC
CTGGATGGGCCTCCACGACCAAAACGGGCCCTGGAAGTGGGTGGACGG
GACGGACTACGAGACGGGCTTCAAGAACTGGAGGCCGGAGCAGCCGGA
CGACTGGTACGGCCACGGGCTCGGAGGAGGCGAGGACTGTGCCCACTT
CACCGACGACGGCCGCTGGAACGACGACGTCTGCCAGAGGCCCTACCG
CTGGGTCTGCGAGACAGAGCTGGACAAGGCCAGCCAGGAGCCACCTCT
CCTTTAA

ii. Vector information for EX-C0391-Lv158 (GeneCopoeia)

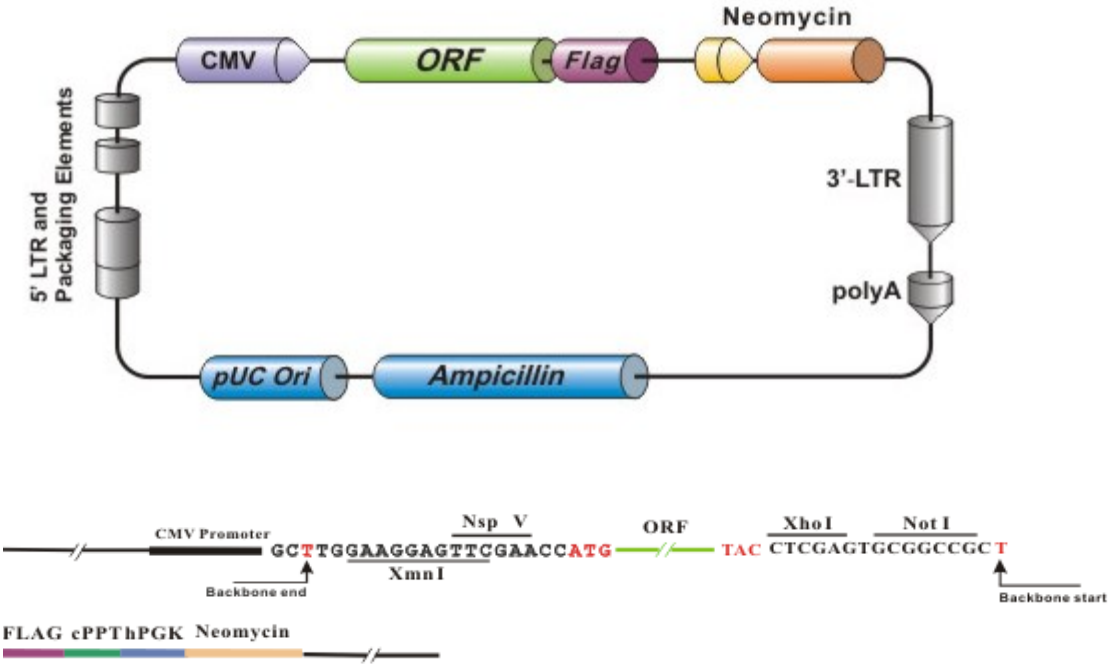
OmicsLink™ Expression Clone Datasheet of EX-C0391-Lv158

Clone Information

Catalog No.: EX-C0391-Lv158
Accession No.: NM_003049
ORF Length: 1050 bp
Whole Plasmid Size: 8577 bp
Description: Homo sapiens solute carrier family 10 (sodium/bile acid cotransporter), member 1 (SLC10A1), mRNA.
Vector: pReceiver-Lv158
Antibiotic: Ampicillin
Stable Selection Marker: Neomycin

Suggested Sequencing Primers:
Forward: 5'-GCGGTAGGCGTGTACGGT-3'
Reverse: 5'-ATTGTGGATGAATACTGCC-3'

Vector Information for EX-C0391-Lv158

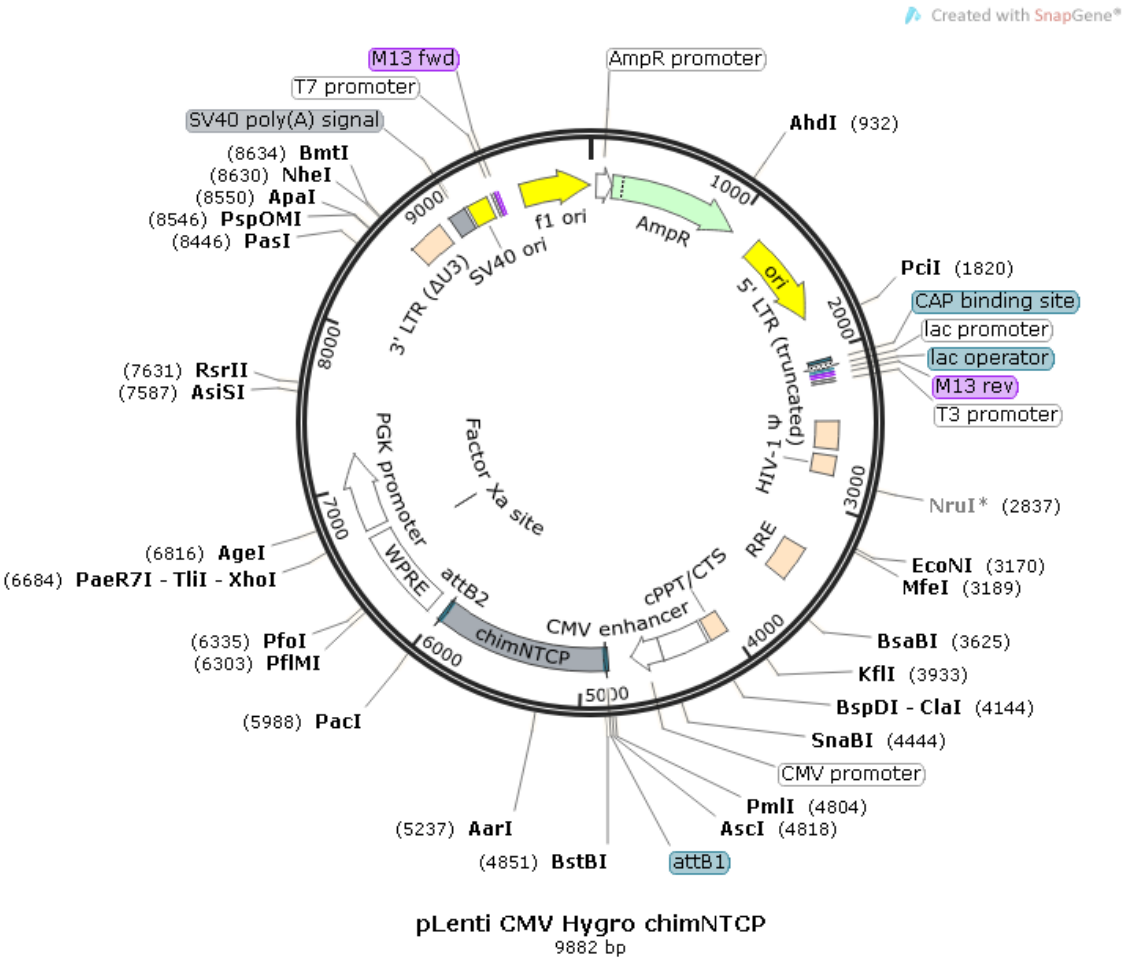


>EX-C0391-Lv158 [with G62256]- NTCP

TGGAAGGAGTTCGAACCATGGAGGCCCACAACGCGTCTGCCCCATTCAA
CTTCACCCTGCCACCCAACTTTGGCAAGCGCCCCACAGACCTGGCACTG
AGCGTCATCCTGGTGTTCATGTTGTTCTTCATCATGCTCTCGCTGGGCTG
CACCATGGAGTTCAGCAAGATCAAGGCTCACTTATGGAAGCCTAAAGGG
CTGGCCATCGCCCTGGTGGCACAGTATGGCATCATGCCCCTCACGGCC
TTTGTGCTGGGCAAGGTCTTCCGGCTGAAGAACATTGAGGCACTGGCCA
TCTTGGTCTGTGGCTGCTCACCTGGAGGGAACCTGTCCAATGTCTTCAG
TCTGGCCATGAAGGGGGACATGAACCTCAGCATTGTGATGACCACCTGC
TCCACCTTCTGTGCCCTTGGCATGATGCCTCTCCTCCTGTACATCTACTC
CAGGGGGATCTATGATGGGGACCTGAAGGACAAGGTGCCCTATAAAGG
CATCGTGATATCACTGGTCCTGGTTCTCATTCCTTGCACCATAGGGATCG
TCCTCAAATCCAAACGGCCACAATACATGCGCTATGTCATCAAGGGAGG
GATGATCATCATTCTCTTGTGCAGTGTGGCCGTCACAGTTCTCTCTGCCA
TCAATGTGGGGAAGAGCATCATGTTTGCCATGACACCACTCTTGATTGCC
ACCTCCTCCCTGATGCCTTTTATTGGCTTTCTGCTGGGTTATGTTCTCTCT
GCTCTCTTCTGCCTCAATGGACGGTGCAGACGCACTGTCAGCATGGAGA
CTGGATGCCAAAATGTCCAACTCTGTTCCACCATCCTCAATGTGGCCTTT
CCACCTGAAGTCATTGGACCACTTTTCTTCTTTCCCCTCCTCTACATGATT
TTCCAGCTTGGAGAAGGGCTTCTCCTCATTGCCATATTTTGGTGCTATGA
GAAATTCAAGACTCCCAAGGATAAAACAAAAATGATCTACACAGCTGCCA
CAACTGAAGAAACAATTCCAGGAGCTCTGGGAAATGGCACCTACAAAGG
GGAGGACTGCTCCCCTTGCACAGCCTACCTCGAGTGCGGCCGC

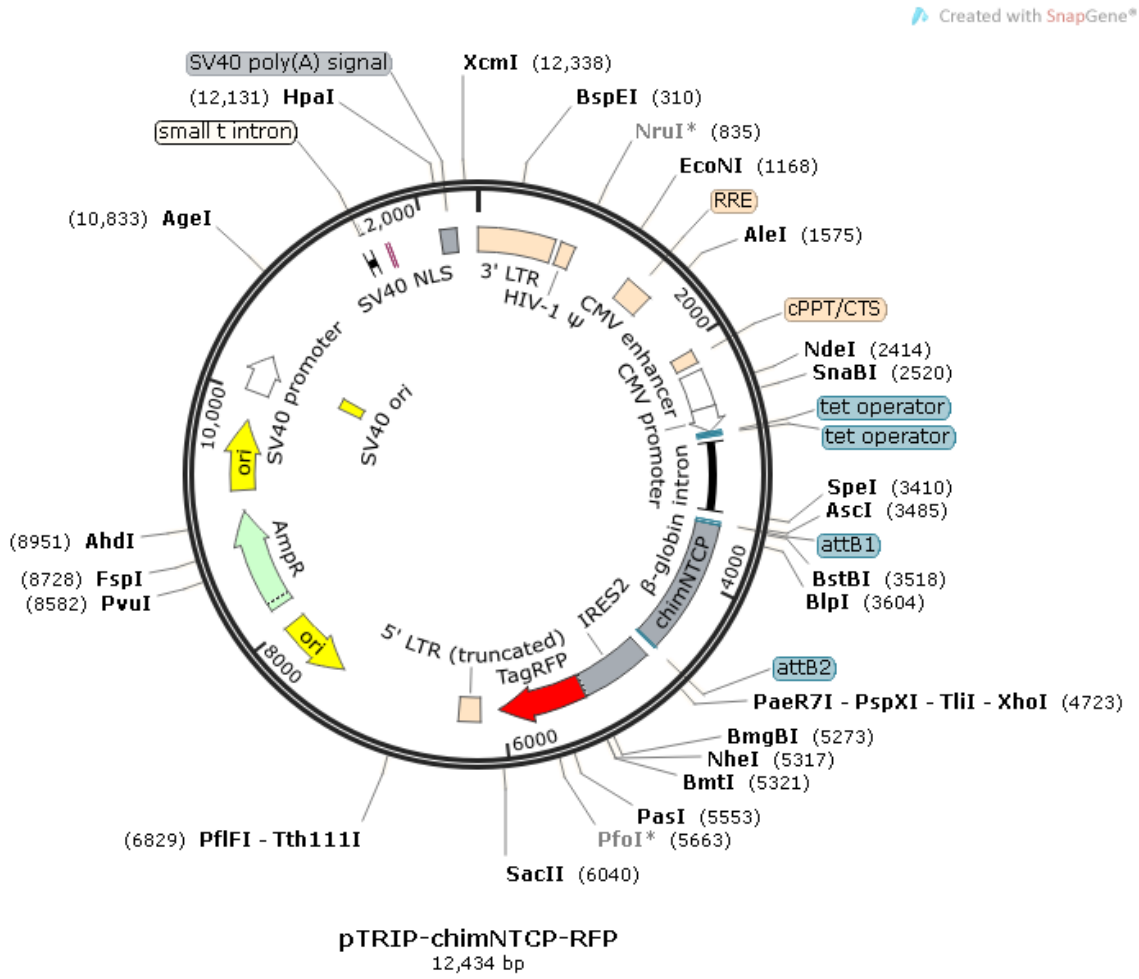
2.XII. Cloned chimNTCP constructs

i. pLenti_CMV_Hygromycin_chimNTCP



pLenti_CMV_chimNTCP_Hygro: 3rd generation lentiviral plasmid expressing a chimeric form of the NTCP receptor (i.e. the amino acids mapping to the important HBV pre-S1 binding domains of the mouse NTCP have been replaced with their human counterparts) under the control of a CMV promoter. It also expresses a Hygromycin selection cassette.

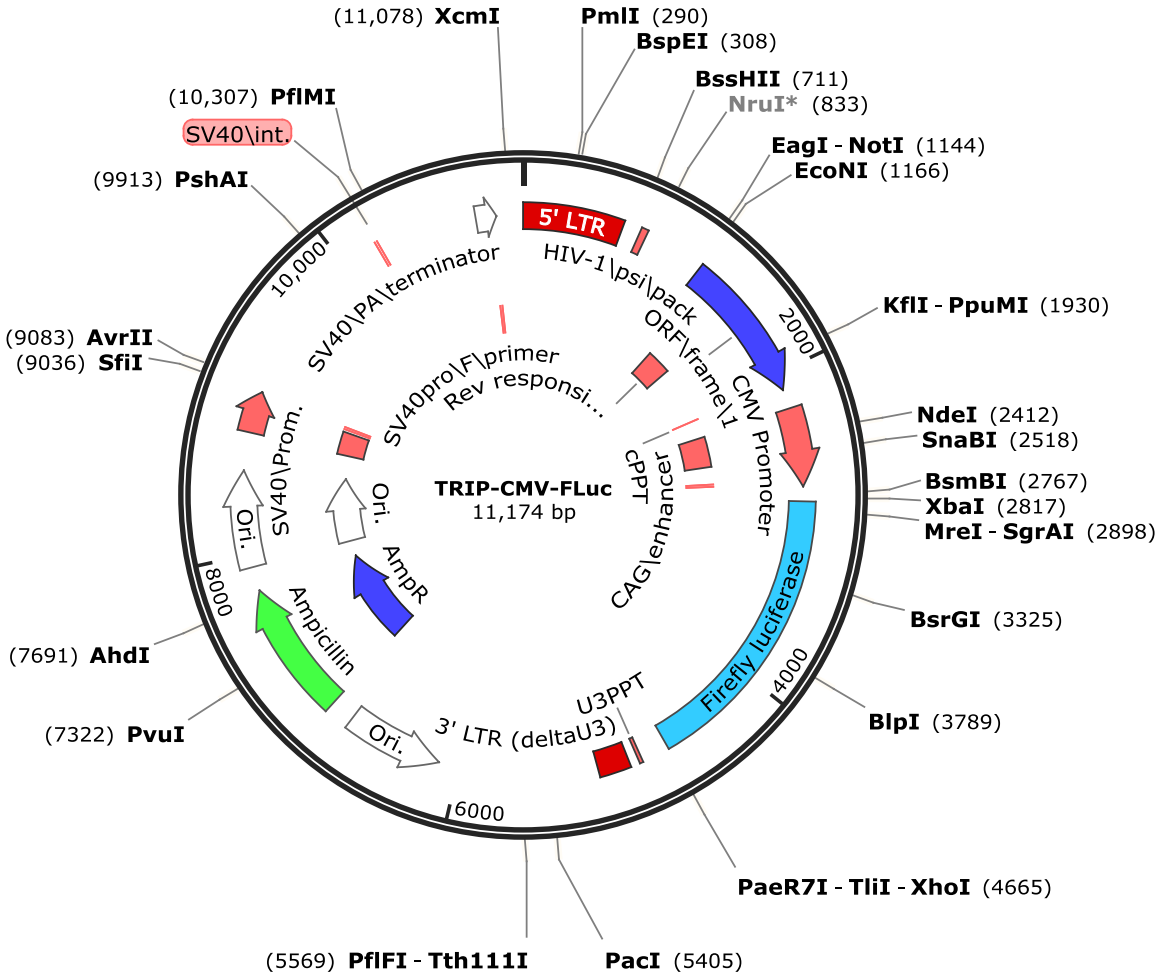
ii. pTRIP_CMV_IRES_RFP_chimNTCP



pTRIP_CMV_chimNTCP_RFP: Lentiviral plasmid expressing a chimeric form of the NTCP receptor (i.e. the amino acids mapping to the important HBV pre-S1 binding domains of the mouse NTCP have been replaced with their human counterparts) under the control of a CMV promoter. It also expresses a RFP sequence, after the receptor sequence, under the control of an IRES promoter element.

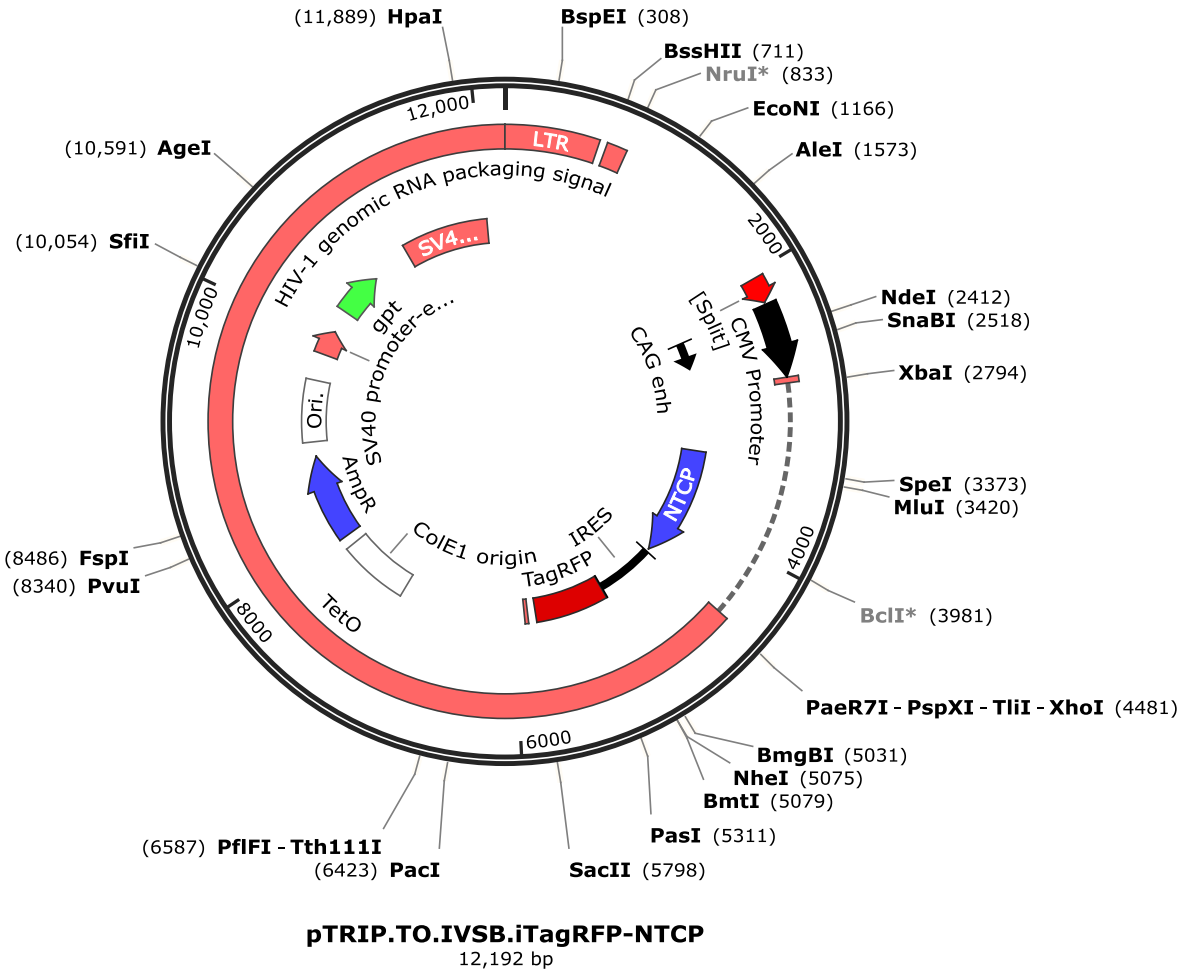
2.XIII. Other pTRIP_CMV_IRES_RFP constructs, specifically used during the preliminary *in vivo* murine experiment

i. pTRIP_Fluc_RFP



pTRIP_CMV_Fluc: Lentiviral plasmid expressing a Fluc sequence under the control of a CMV promoter.

ii. pTRIP_human NTCP_RFP



pTRIP_CMV_hNTCP_RFP: Lentiviral plasmid expressing the full-length human NTCP receptor under the control of a CMV promoter. It also expresses a RFP sequence, after the receptor sequence, under the control of an IRES promoter element.

Purchased siRNAs

2.XIV. PDE siRNAs; Product details

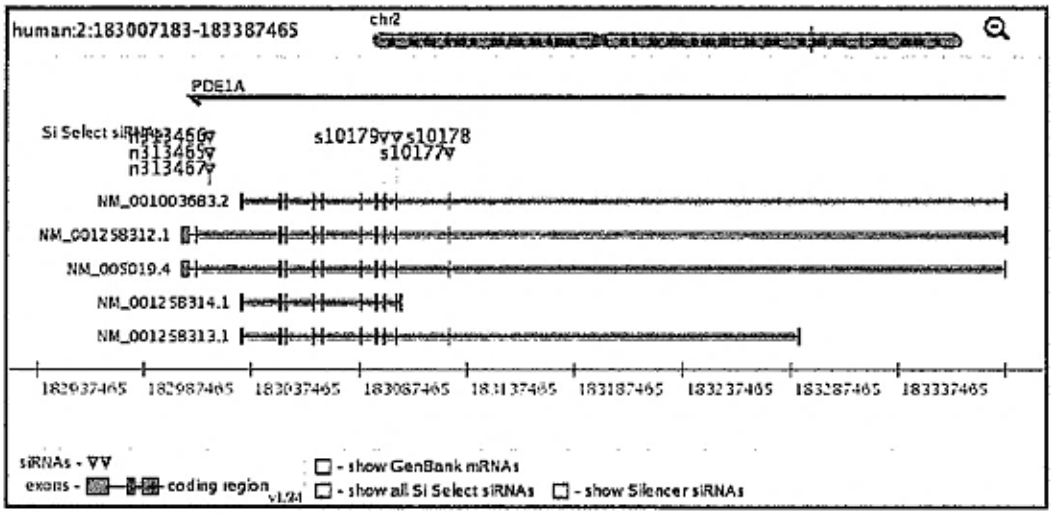
***Silencer*® Select Pre-designed (Inventoried) siRNA product information**

i. **PDE1A** (# s10179)

Gene Alias(es):	CAM-PDE-1A, HCAM-1, HCAM1, HSPDE1A
Full Gene Name:	phosphodiesterase 1A, calmodulin-dependent
Organism:	human
Species:	Homo sapiens
Locus ID:	5136

siRNA INFORMATION

	Sense	Antisense
Sequence (5'->3')	GGAUGUUGAUAAAUGGUCUtt	AGACCAUUUAUCAACAUCCtt
Length	21	21
Percent G/C	33%	33%
Molecular Weight	6800	6600
Molar Extinction Coefficient	217500	207900
Annealed Molecular Weight	13300 (40 µg/OD was used to calculate concentration)	



Detailed Target Information

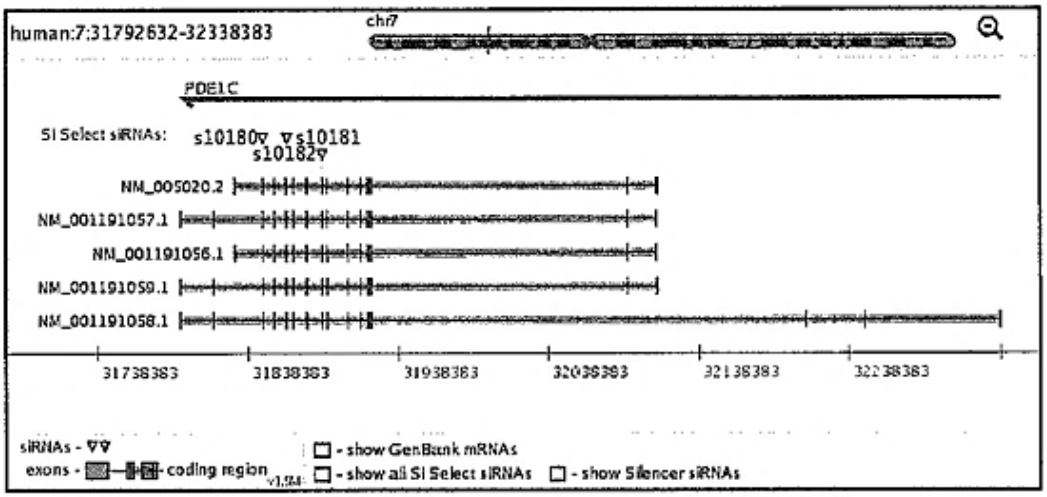
Target Transcripts:	NM_001003683U40370.1, AL110263.1, AJ401610.1, BC022480.1, DC396228.1, AK294239.1, AK295657.1, AK301720.1
NCBI UniGene ID:	Hs.191046
OMIM ID:	171890
Functional Class(es) of Target Gene (Gene Ontology):	calmodulin-dependent cyclic-nucleotide phosphodiesterase activity calmodulin binding metal ion binding
Related TaqMan® Gene Expression Assay:	Hs00897273_m1

ii. **PDE1C** (# s10181)

Gene Alias(es):	Hcam3
Full Gene Name:	phosphodiesterase 1C, calmodulin-dependent 70kDa
Organism:	human
Species:	Homo sapiens
Locus ID:	5137

siRNA INFORMATION

	Sense	Antisense
Sequence (5'->3')	CCUUCACUGUGCUUACGGAtt	UCCGUAAGCACAGUGAAGGtg
Length	21	21
Percent G/C	38%	52%
Molecular Weight	6700	6800
Molar Extinction Coefficient	195500	213500
Annealed Molecular Weight	13400 (40 µg/OD was used to calculate concentration)	



Detailed Target Information

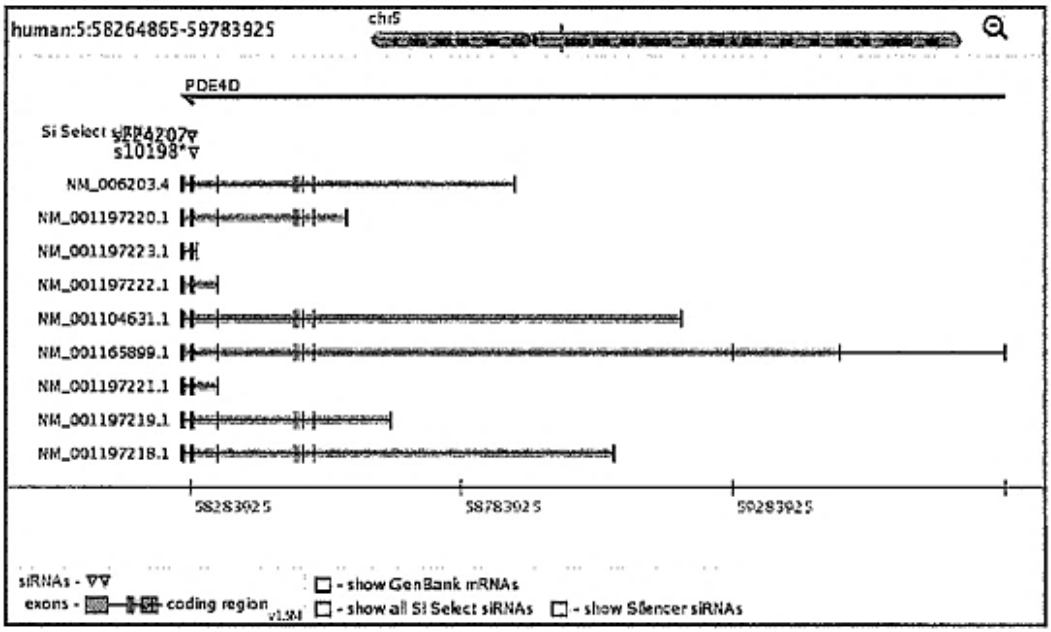
Target Transcripts:	NM_005020U40372.1, U40371.1, AK056170.1, BC022479.1, BC022525.1, AK091734.1, AK094180.1
NCBI UniGene ID:	Hs.728862
OMIM ID:	602987
Functional Class(es) of Target Gene (Gene Ontology):	calmodulin-dependent cyclic-nucleotide phosphodiesterase activity calmodulin binding metal ion binding
Related TaqMan® Gene Expression Assay:	Hs01095682_m1

iii. PDE4D (# s10200)

Gene Alias(es):	ACRDYS2, DPDE3, HSPDE4D, PDE43, PDE4DN2, STRK1
Full Gene Name:	phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)
Organism:	human
Species:	Homo sapiens
Locus ID:	5144

siRNA INFORMATION

	Sense	Antisense
Sequence (5'->3')	GUGUCAGAGUUUAUAUCAAtt	UUGAUAUAAACUCUGACACtt
Length	21	21
Percent G/C	29%	29%
Molecular Weight	6700	6600
Molar Extinction Coefficient	221700	209800
Annealed Molecular Weight	13300 (40 µg/OD was used to calculate concentration)	



Detailed Target Information

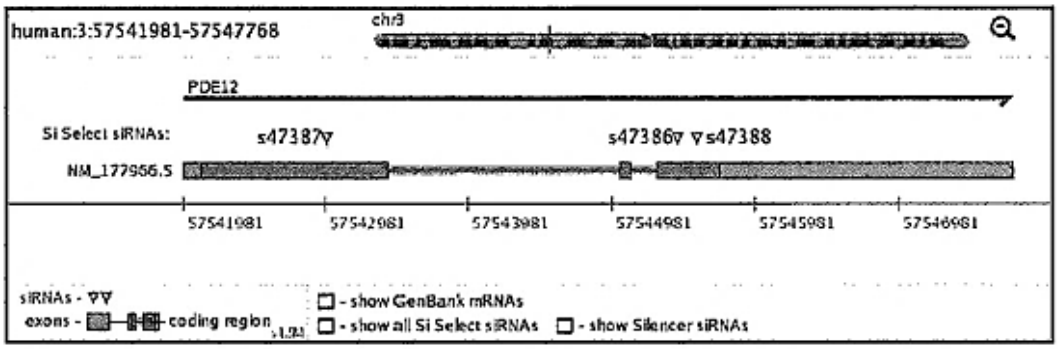
Target Transcripts:	NM_006203AY388960.1, L20970.1, U50157.1, U50158.1, U50159.1, AF012073.1, AF012074.1, U02882.1, U79571.1, AJ250852.1	
NCBI UniGene ID:	Hs.117545	
OMIM ID:	600129	
Functional Class(es) of Target Gene (Gene Ontology):	3,5-cyclic-nucleotide phosphodiesterase activity drug binding cAMP binding beta-2 adrenergic receptor binding metal ion binding scaffold protein binding	3,5-cyclic-AMP phosphodiesterase activity enzyme binding ubiquitin protein ligase binding ion channel binding ATPase binding
Related TaqMan® Gene Expression Assay:	Hs01579625_m1	

iv. PDE12 (# s47388)

Gene Alias(es):	2-PDE
Full Gene Name:	phosphodiesterase 12
Organism:	human
Species:	Homo sapiens
Locus ID:	201626

siRNA INFORMATION

	Sense	Antisense
Sequence (5'->3')	GGAUGUCUAGAUUACAUUUtt	AAAUGUAAUCUAGACAUCCat
Length	21	21
Percent G/C	29%	29%
Molecular Weight	6700	6600
Molar Extinction Coefficient	216600	220900
Annealed Molecular Weight	13300 (40 µg/OD was used to calculate concentration)	



Detailed Target Information

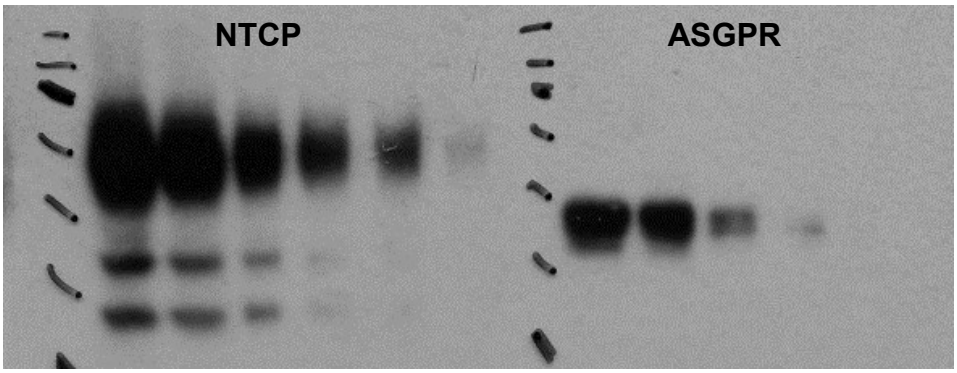
Target Transcripts:	NM_177966AK001526.1, AB115695.1, BX647169.1, AL831824.1, AK300374.1
NCBI UniGene ID:	Hs.572993
Functional Class(es) of Target Gene (Gene Ontology):	hydrolase activity
Related TaqMan® Gene Expression Assay:	Hs00698272_m1

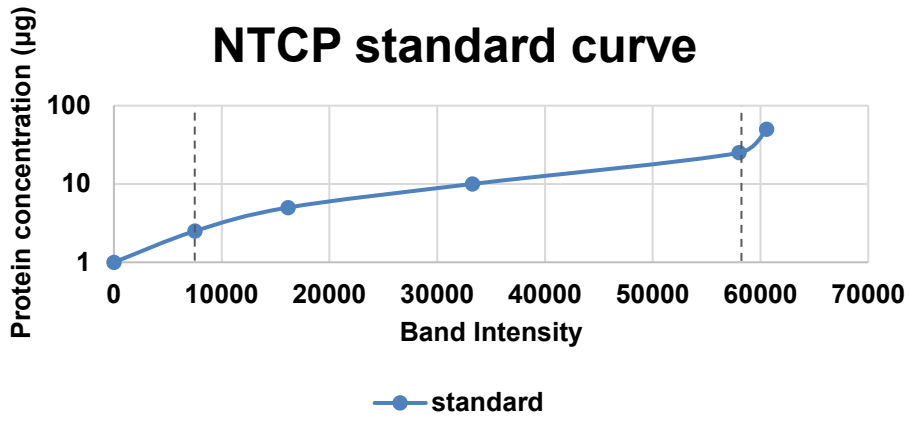
© 2010 Life Technologies Corporation. All rights reserved.

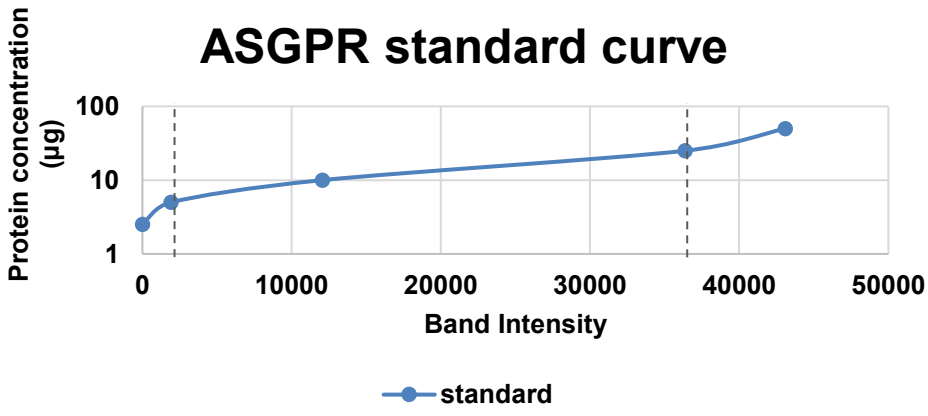
Analysis of ECL versus LICOR® technology

2.XV. Determination of linear range differences between ECL and LI-COR® technologies, using a-FLAG (NTCP) and a-ASGPR antibodies; *indicated by the dashed lines on the various graphs.*

ECL

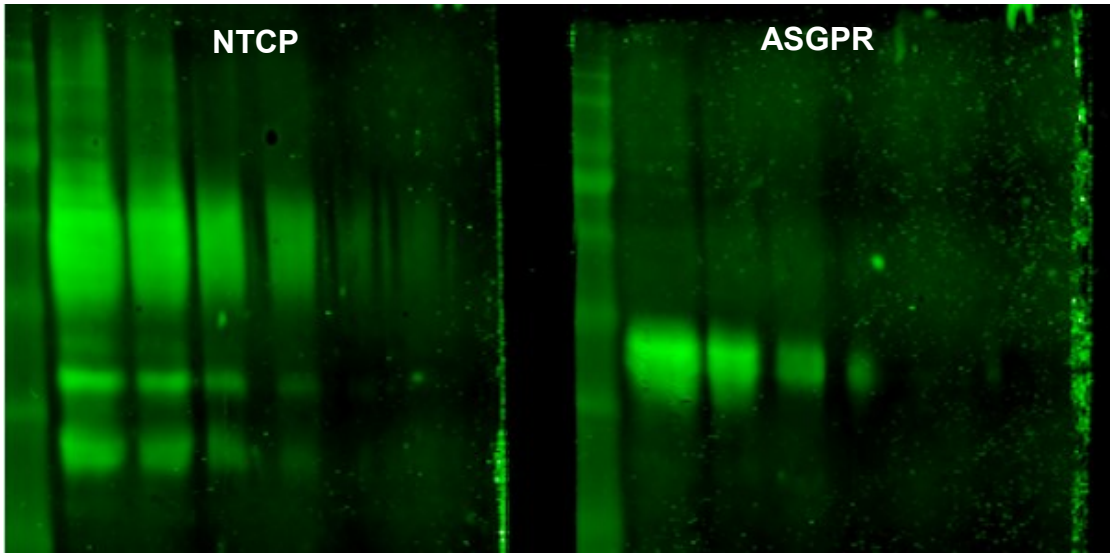


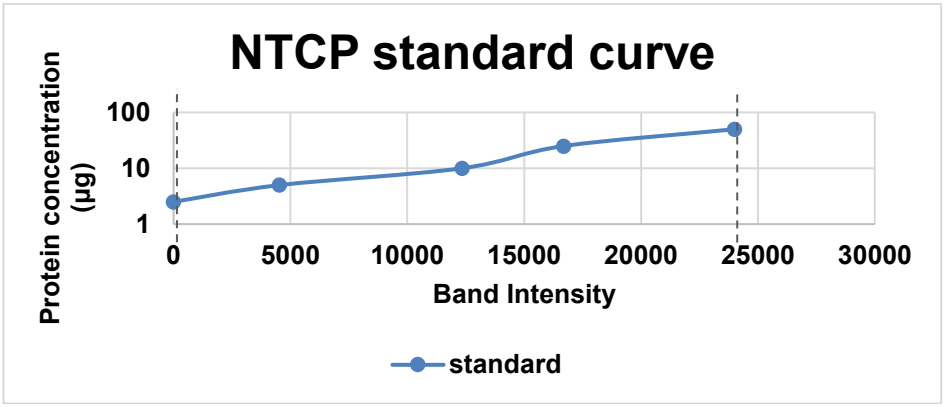


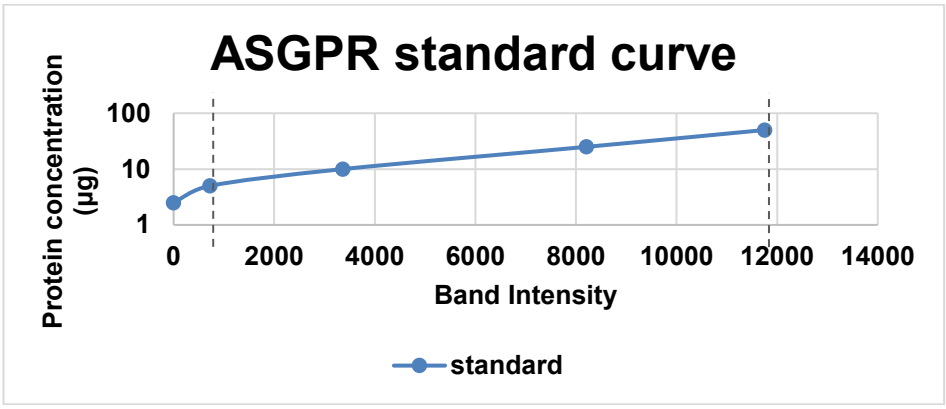


ECL linear range for NTCP/ ASGPR: 5-25µg

LI-COR®





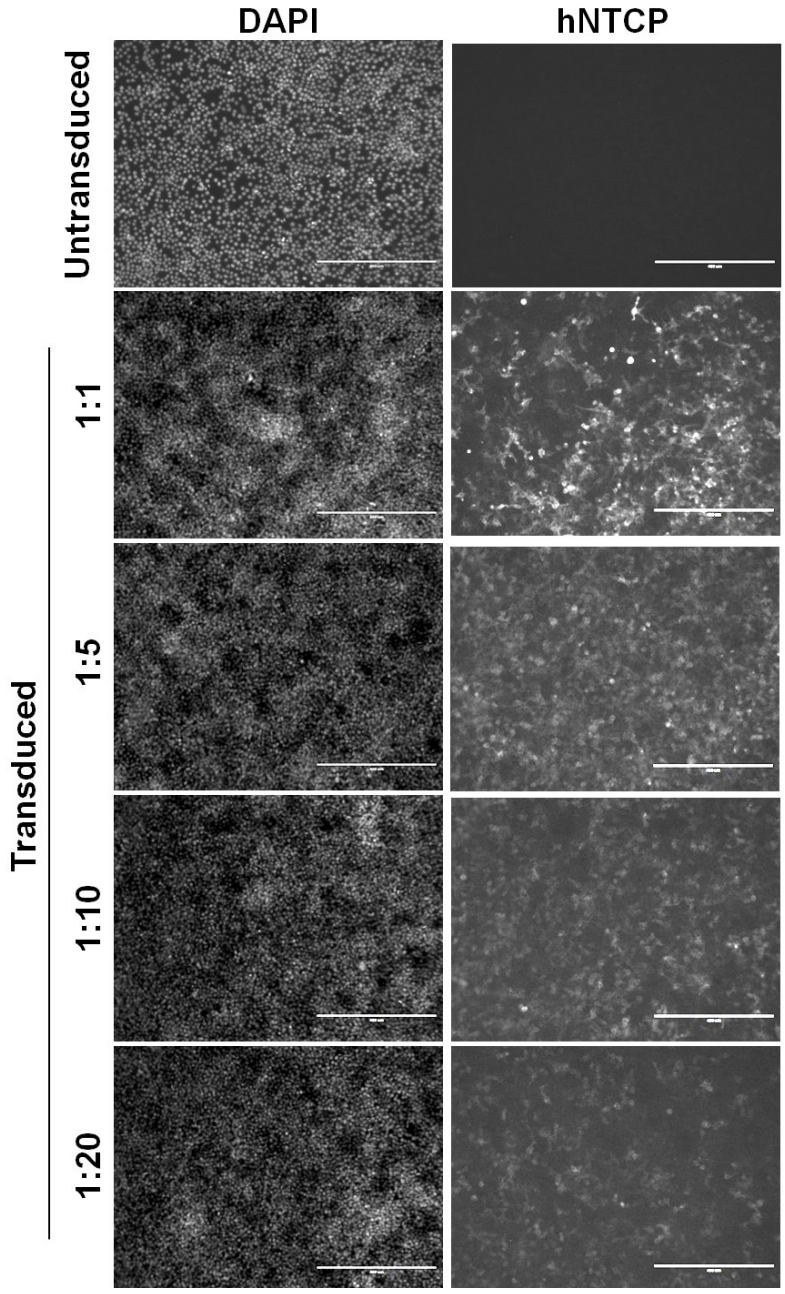


LI-COR® linear range for:

NTCP: 2.5-50µg
ASGPR: 5-50µg

Analysis of the generated hNTCP lentiviral pseudoparticles

2.XVI. Titration of hNTCP_FLAG-tag lentiviral pseudoparticles in HEK293T cells

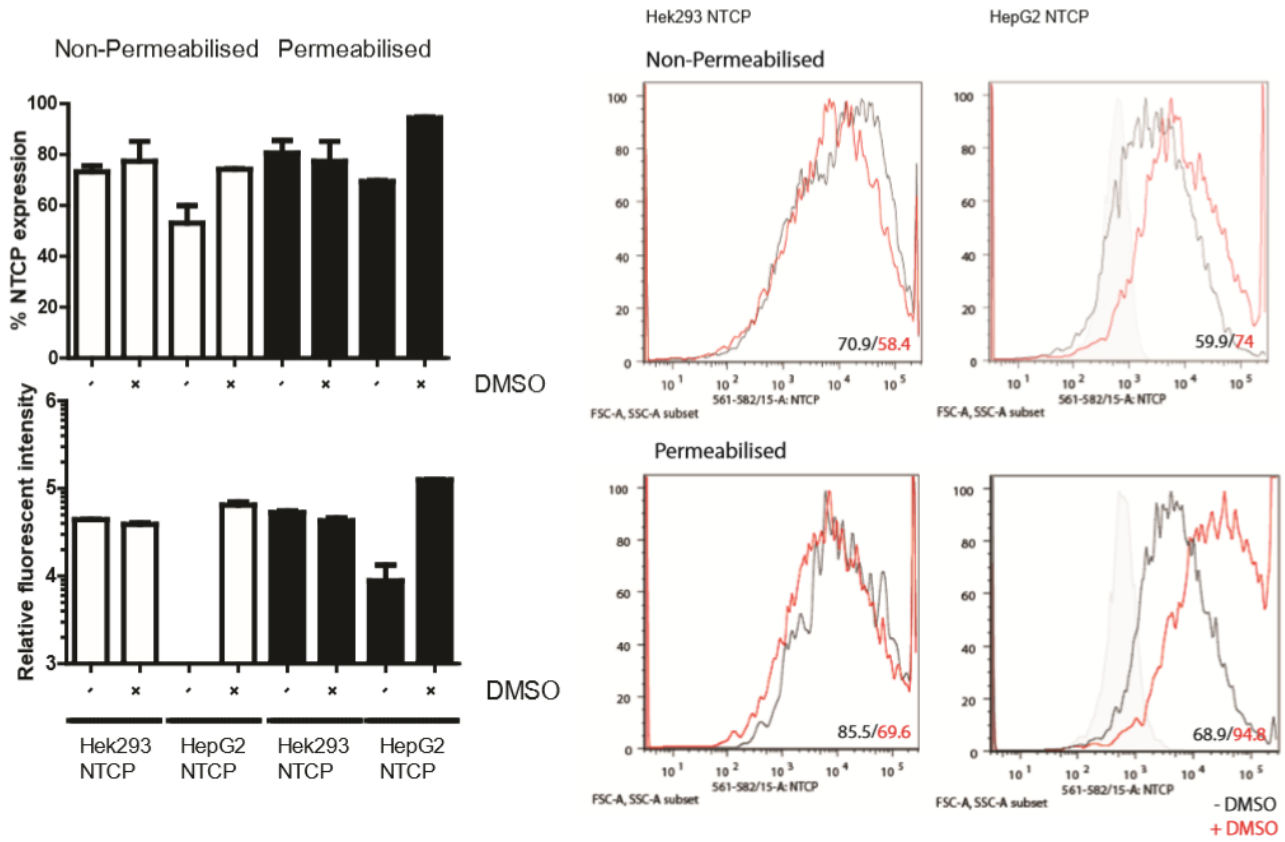


The image scale bar is 400μm.

We concluded that the optimum dilution of lentiviruses to cell culture media was a 1:1 ratio, and that was used for further cell transductions particularly for the creation of hNTCP overexpressing stable cell lines. *Analysis was performed at 48 hours post-transduction of the cells.*

3. Results

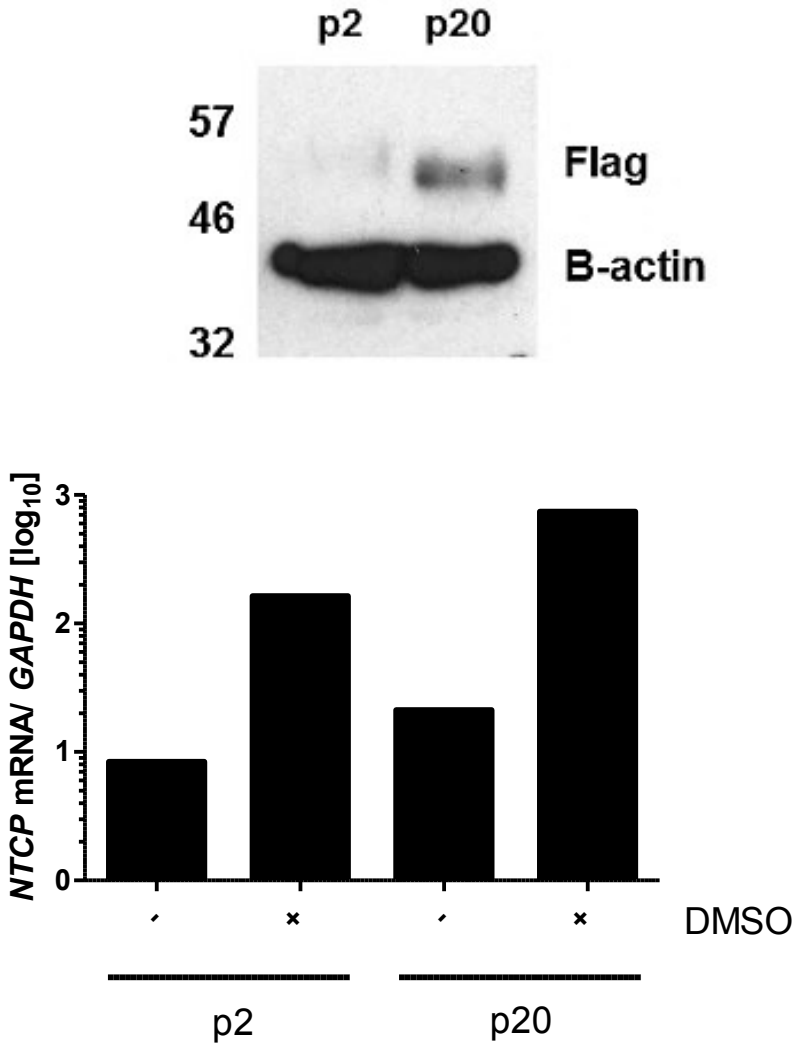
3.I Flow cytometry analysis of hNTCP_FLAG-tag in stable hepatic and non-hepatic cell lines



Detection of hNTCP_FLAG-tag in the stable human hepatic cell line is enhanced in the presence of DMSO, when cultured in the presence of the agent for a total of 24 hours.

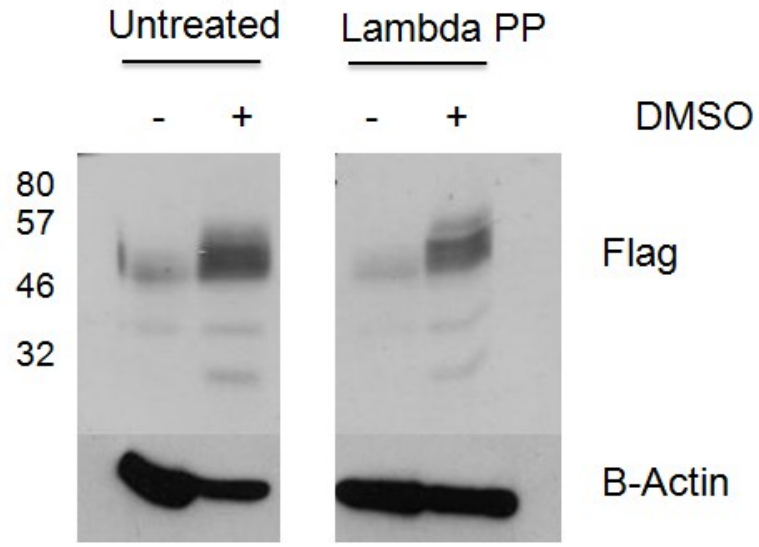
Our data also indicates that the C-terminus of the human NTCP receptor may in fact be located extracellularly, as permeabilisation of the cells was not necessary for its detection.

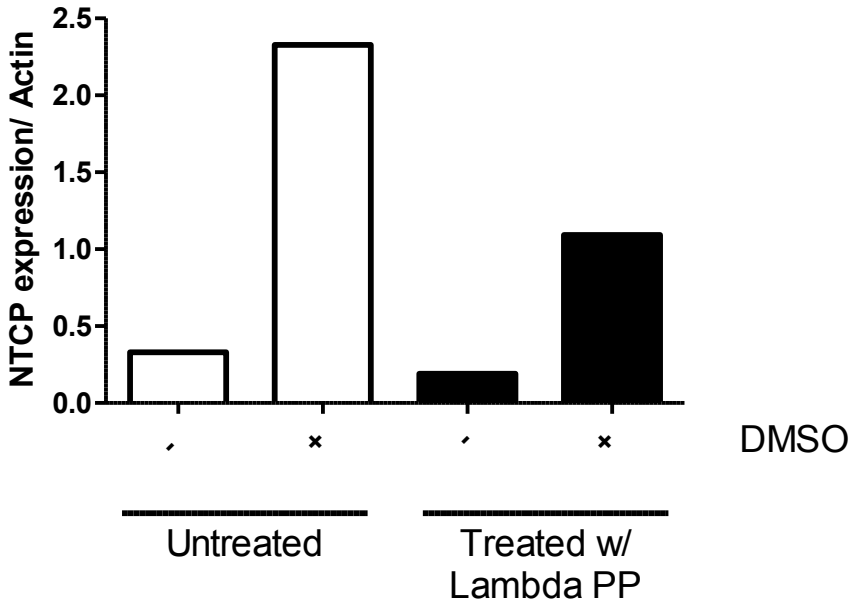
3.II Baseline hNTCP expression in a stable human hepatic cell line (HepG2), in the absence of DMSO



The baseline mRNA and protein expression of hNTCP naturally increases over time as the cells are passaged in the absence of DMSO. However, the presence of DMSO always has an enhanced effect on hNTCP expression in these cell lines, indicating that some regulatory process governing expression of this receptor in this cell line is not functioning optimally. *p refers to the passage number of the cells, post-completion of antibiotic selection.*

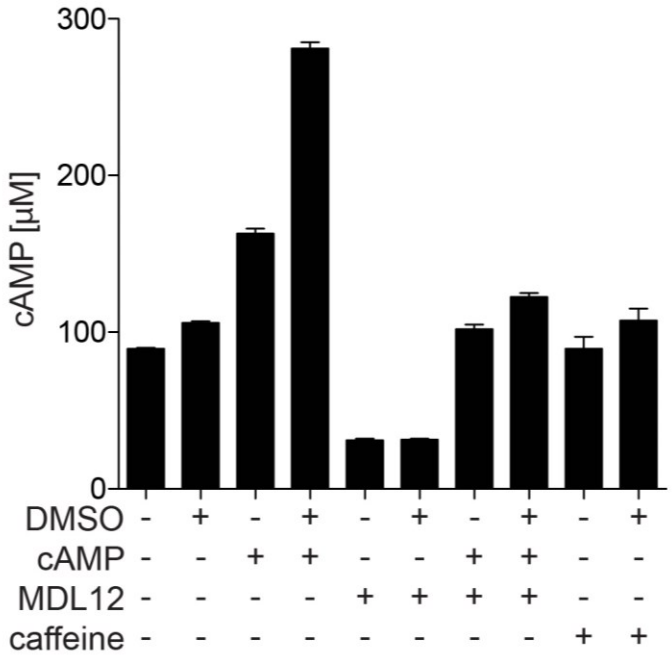
3.III hNTCP dephosphorylation by a Lambda phosphatase enzyme, illustrating particular differences between hNTCP protein band levels and patterns.





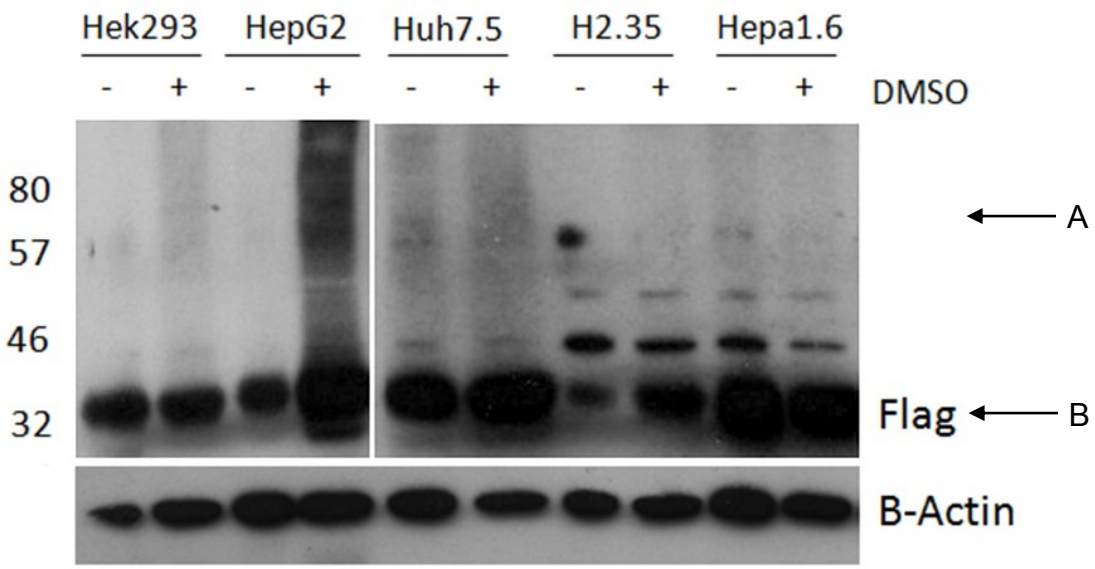
Treatment of protein samples with Lambda phosphatase results in a less smeary hNTCP protein band compared to the band obtained for the untreated samples. The less smeary band in addition expresses lower protein levels, as determined by densitometry analysis of the western blot results.

3.IV Effect of various drugs on cAMP levels within cells



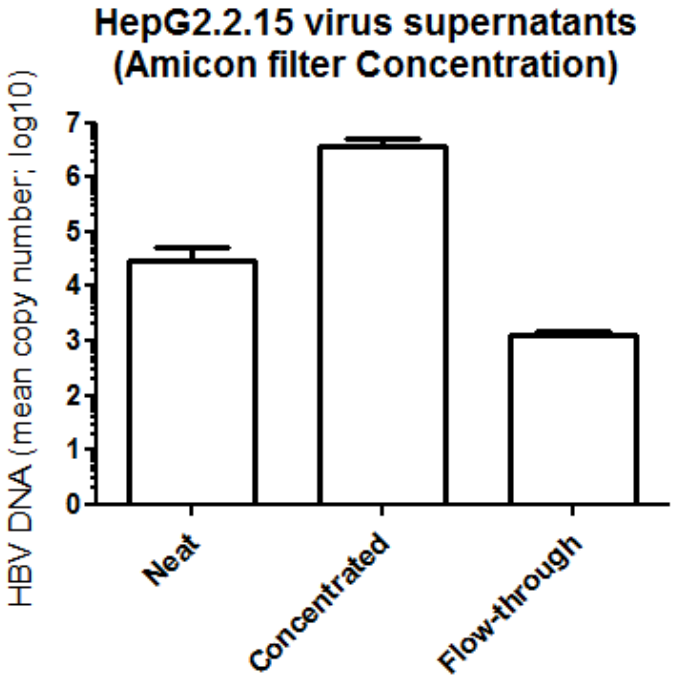
DMSO increased endogenous cAMP levels within the cells, while the exogenous cAMP was readily detectable. Addition of MDL12 blocked production of cAMP by inhibiting adenylyl cyclase, however, the presence of exogenous cAMP and DMSO somewhat rescued the phenotype. Caffeine, in contrast to what was expected, did not greatly affect the endogenous levels of cAMP, possibly suggesting that DMSO has a more specific mechanism of action.

3.V PNGaseF deglycosylation of stable human and mouse cell lines overexpressing hNTCP



PNGaseF digestion results in the full deglycosylation of the glycosylated hNTCP protein (~ 50KDa) in both human and mouse cells. The hNTCP deglycosylated protein band is approximately 38kDa, in all samples tested. *A; glycosylated band; B. deglycosylated band.*

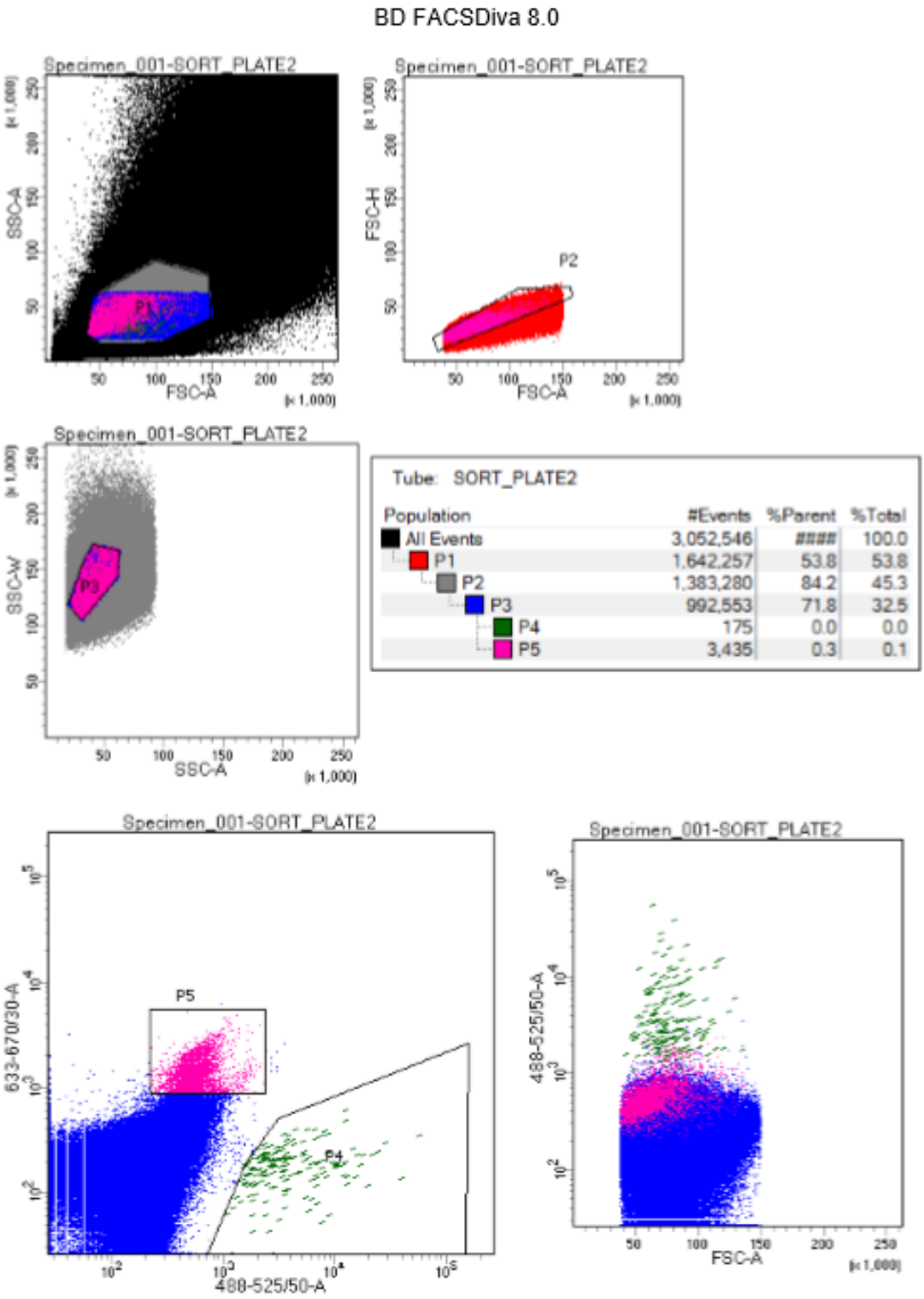
3.VI The result of Amicon® filter concentration on HepG2.2.15 infectious sups



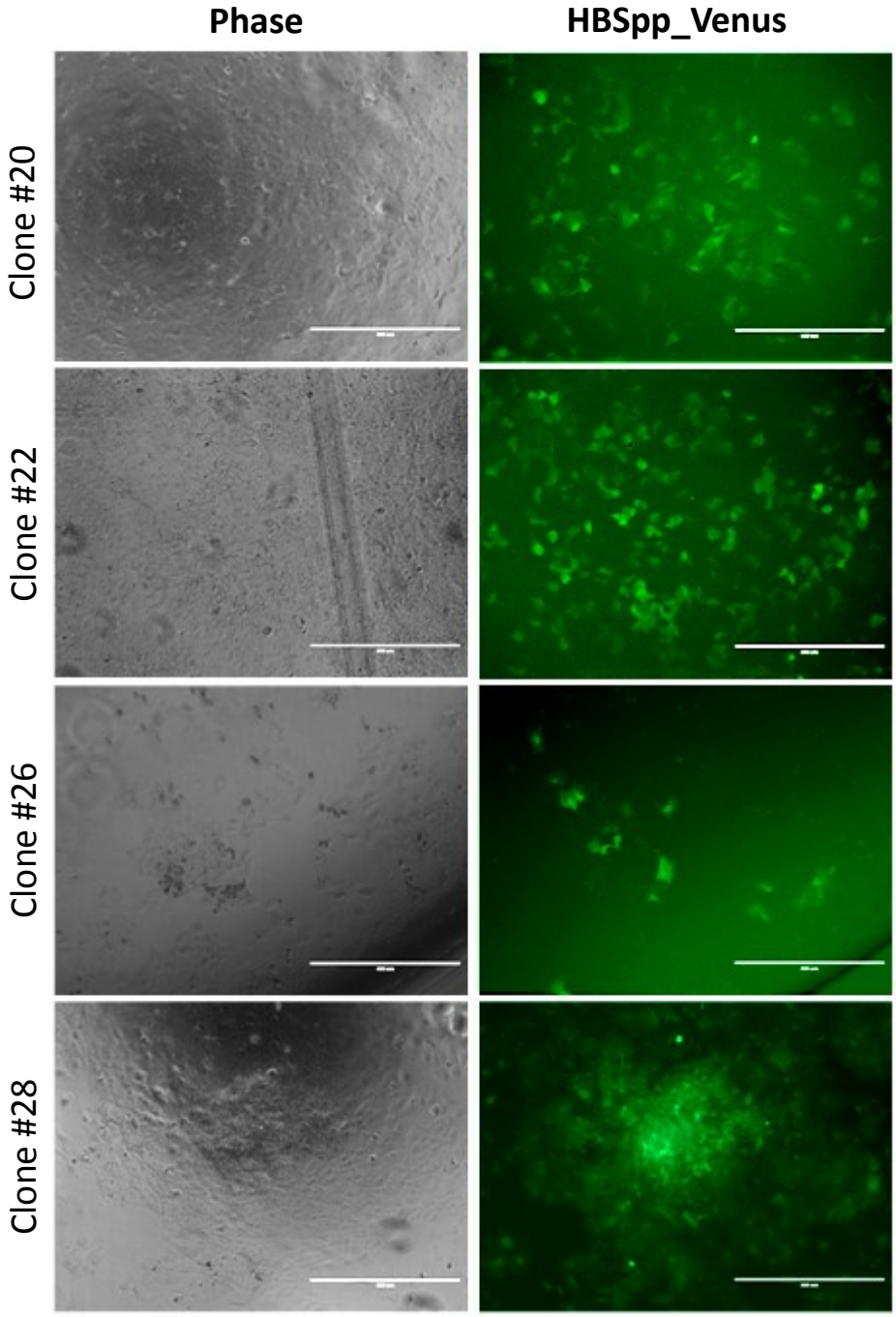
During Amicon® filter concentration it appeared that quite a bit of the infectious virus was able to pass through the filter and thus a considerable amount of it was being lost in the flow-through.

3.VII FACS sorting protocol of single cell clones positive for GFP expression

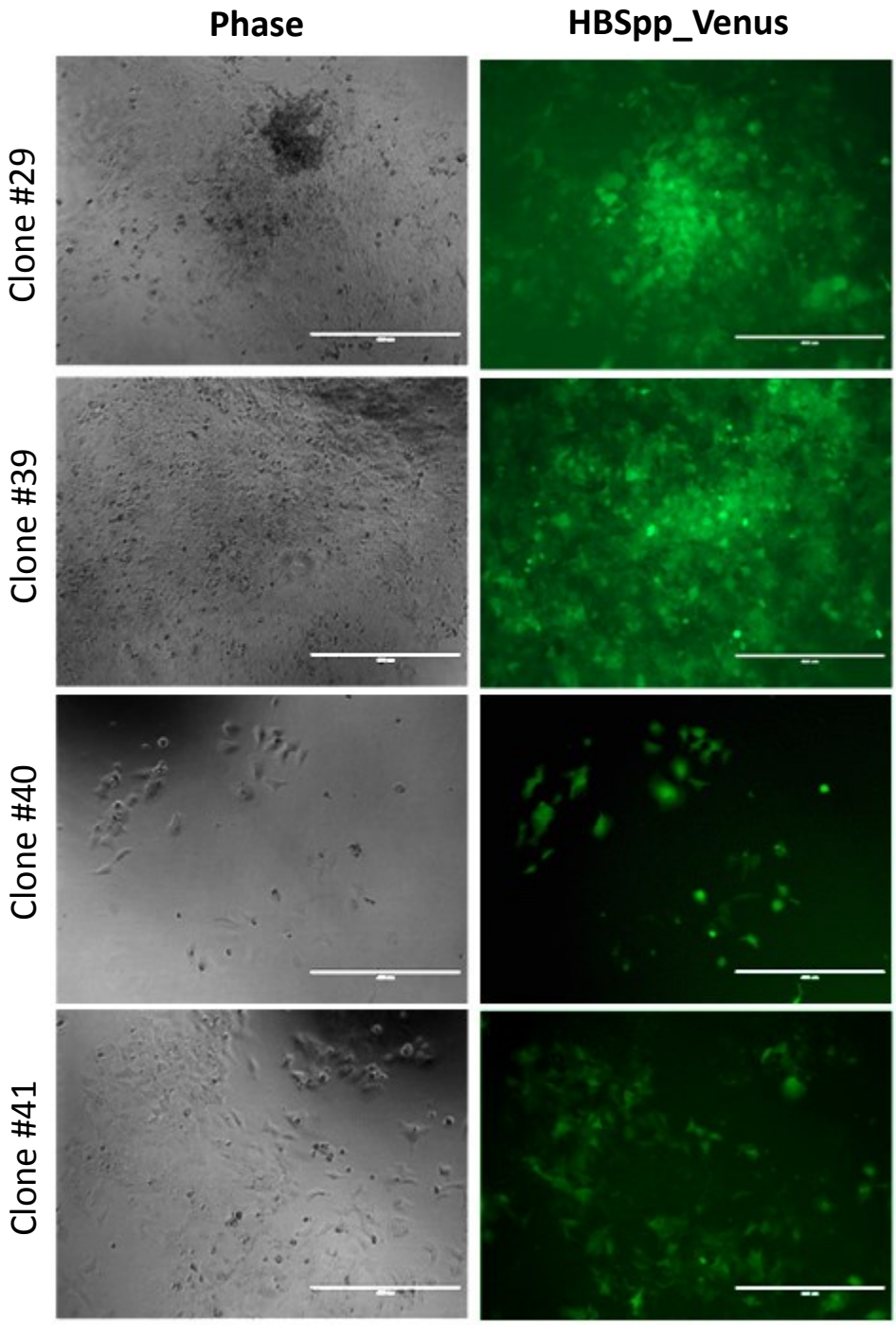
(As performed by staff at St. Mary's Flow Cytometry Core Facility)



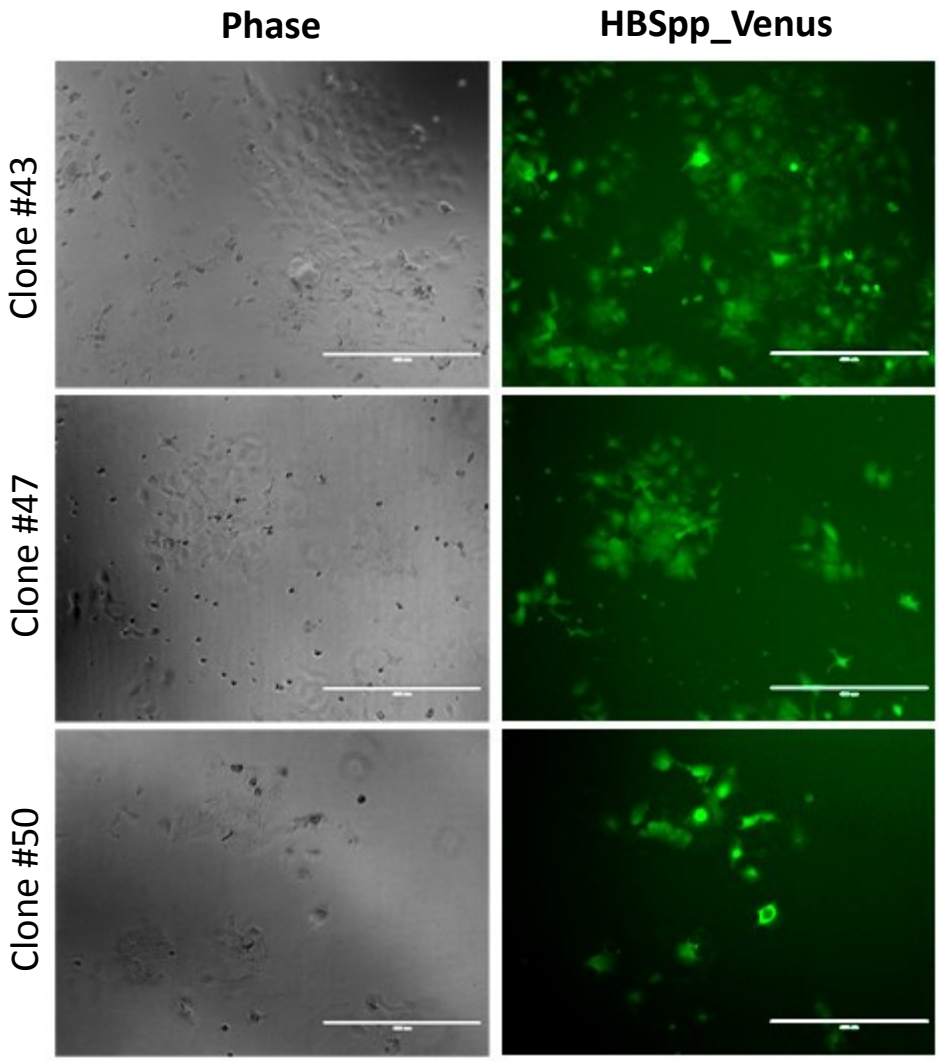
3.VIII *Cont.* Single cell clones that have been infected with the Type I HBSpp and FACS sorted for GFP expression



The image scale bar is 400μm.



The image scale bar is 400μm.



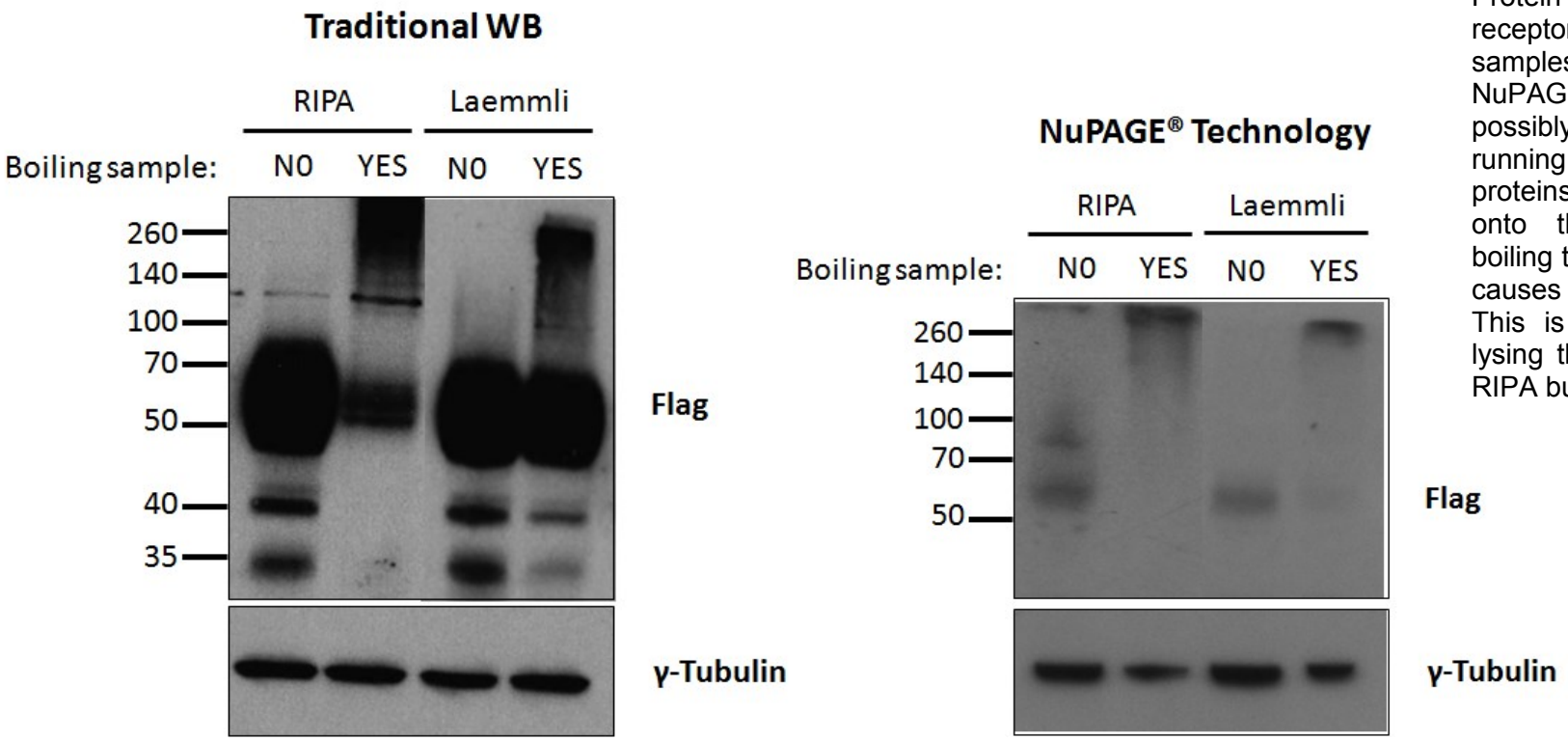
The image scale bar is 400µm.

Visualisation of GFP expression in the different single cell clones, utilised during the cyclic repackaging rescue screen.

4. Discussion

4.I Human NTCP protein expression differences between conventional western blotting procedures and the NuPAGE® technology (*ThermoFisher Scientific*).

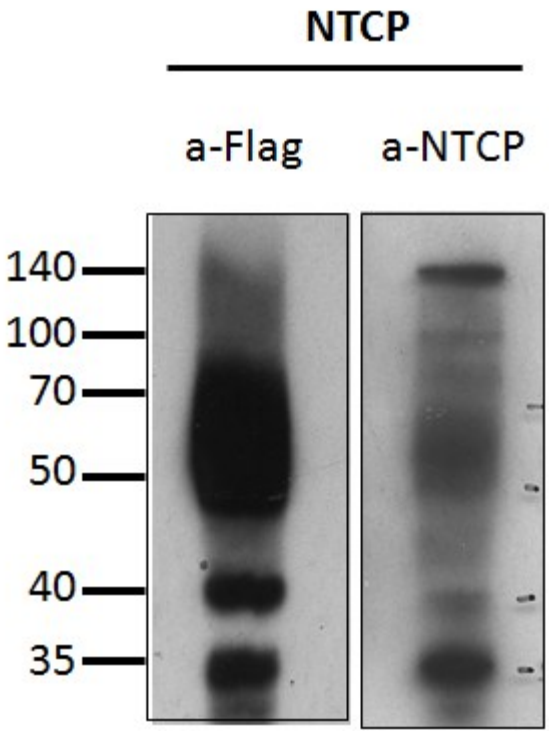
In addition; Human NTCP protein expression differences when utilising different protein extraction procedures, as well as the effect of heat denaturation on the protein's expression.



Traditional WB experiment, as performed by Dr. Quentin Bazot.

Protein expression of the human NTCP receptor appears to get lost when the samples are analysed by the NuPAGE® technology. This could possibly be due to the samples not running properly or, after running, the proteins are not properly transferred onto the membranes. In addition, boiling the samples prior to running also causes significant protein degradation. This is in the majority overcome by lysing the cells in Laemmli rather than RIPA buffer.

4.II Analysis of the anti-hNTCP antibody specificity by conventional western blotting



The specificity of the a-NTCP antibody is not optimal when compared to the detection of the human NTCP protein by an a-Flag antibody. *HepG2_hNTCP cells, in which hNTCP protein expression was induced in the presence of DMSO for 48 hours, were used during this experiment. 5µg of protein sample were loaded onto the gel for subsequent analysis.*