ROMANIAN ACADEMY

School of Advanced Studies of the Romanian Academy

Institute of Biochemistry of the Romanian Academy

SUMMARY OF THE DOCTORAL THESIS

GENETIC EDITING FOR THE STUDY OF CELLULAR PROTEINS INVOLVED IN PATHOLOGIES

PhD ADVISOR

Dr. Norica Nichita

PhD STUDENT

Patriche David-Sebastian

2024

Contents

Thanks

I would like to thank my PhD advisor Dr. Norica Nichita for her constant guidance and patience throughout these years, during which she helped me overcome the difficult moments that arose during any PhD.

I also want to thank my PhD supervisor, Dr. Costin-Ioan Popescu, for his guidance at the beginning of my training period in the Institute of Biochemistry, in the study of the interaction of HCV with the host cell, which continued with the study on HBV, presented in this doctoral thesis.

I thank Dr. Florentina Pena for giving me the chance to participate in her research project, which allowed me to study another pathology and progress even more in my research career.

I would like to thank Dr. Ștefana Petrescu and Dr. Andrei Petrescu for helping me obtain the necessary funding for my involvement in the projects carried out in the Institute of Biochemistry.

I also thank my colleagues, Dr. Ioana Popa and Dr. Robi Tăcutu, for giving me the chance to develop by participating in their research projects and for the useful advice I received.

I would like to thank all my colleagues who contributed with me to the creation of the papers that I published as part of this doctorate: Dr. Mirela Popescu, Dr. Olivia Dobrică, Dr. Ana Pantazică. Dr. Petruța Alexandru, Andreea Anghel, Dr. Rodica Badea, Dr. Gabriela Chirițoiu. Without their contribution this would not have been possible.

I thank my friend Dr. Eliza Martin for her suggestions and technical help in writing the thesis.

I thank all past and present colleagues at the Institute of Biochemistry who have helped me, for valuable advice and encouragement over the years to carry out experiments.

I would like to thank my family who supported me throughout this PhD.

The purpose of the study

Diabetes mellitus and hepatitis B virus (HBV) infection have an enormous impact on public health. Globally, their cumulative incidence is about 1/10, affecting 800 million people and causing more than 8 million deaths annually (World Health Organization). Understanding the molecular mechanisms of these pathologies is essential for treatment and leads to scientific progress for control and ultimately eradication. In this sense, the development of new technologies and relevant experimental models are crucial for the advancement of mechanistic studies. The exponential progress in the field of genetics in the last decades has allowed the facilitation of these studies, opening new horizons regarding the manipulation of the expression of some proteins of interest, through the rapid and efficient modification of the coding genetic information.

In this work, two methods of altering the expression of genes of interest were implemented: the "Clustered Regularly Interspaced Short Palindromic Repeats"-CRISPR/Cas9 system and retroviral transduction. Clonal cell lines derived from Huh7 hepatoma cells with reduced expression of certain enzymes involved in lipid metabolism were obtained by CRISPR/Cas9: Sac1 (a phosphatase whose substrate is phosphatidyl inositol-4-phosphate), PI3KC2α and PI3KC2β (phosphatidyl inositol -3-kinases, class 2 isoforms). In the case of the PI3KC2β and PI3KC2α enzymes, the doctoral study is a continuation of the my own dissertation thesis in which the single guide sequences that target the PI3KC2β genes and were cloned and where an inhomogeneous cell population with low expression of the PI3KC2β protein (bulk). This population was subjected to a cloning process, successfully obtaining cell clones completely devoid of PI3KC2β expression (knockout). For gene editing in order to reduce the synthesis of proteins of interest, an experimental design was carried out that led to the generation of several single guide sequences targeting the SACM1L gene, followed by their cloning into the pSpCas9-2A-Puro vector and obtaining such clones with reduced expression (knockdown) of Sac1 and PI3KC2α. The lines generated in this study were characterized at the molecular level by sequencing, RT-qPCR and Western Blot. For studies in which recovery of target protein expression was sought, the Sac1 coding gene was cloned in fusion with the Green Fluorescent Protein (GFP) coding sequence in the retroviral vector pLNCX2. Using this

plasmid, new Huh7 cell lines expressing the GFP-Sac1 fusion protein were obtained by retroviral transduction, starting from a clone with reduced expression of Sac1. At the same time, cell clones were also obtained that express the PI3KC2β gene again (rescue), starting from clones without expression (knockout). These newly generated clonal cell lines formed the basis of functional studies on the role of Sac1 phosphatase and PI3KC2α and PI3KC2β kinases in the HBV life cycle. The detailed analysis of the role of Sac1 in the process of replication, assembly and intracellular trafficking of HBV particles was investigated and presented in another doctoral thesis, by my colleague, Dr. Mirela Popescu, starting from expression plasmids and cell clones with modulated Sac1expression obtained by me (Popescu et al, 2022). My studies, presented in this thesis, focused on the functional analysis of PI3KC2α and PI3KC2β kinases and their role in HBV infection, using genetically modified cell lines generated as investigational systems. Furthermore, the observed effects were confirmed using another method of transiently reducing gene expression through RNA interference (Patriche et al, 2022).

My expertise in molecular biology also contributed to the realization of another project within the Institute of Biochemistry, in which the identification of new natural compounds with modulatory activity on GLP-1 secretion was sought, with a potential role in improving the treatment of type 2 diabetes . In this study we obtained by lentiviral transduction a GluTag cell line, which provides cytosolic expression of luciferase. In conjunction with a GluTag reporter cell line that secretes GLP-1 and luciferase in a 1:1 molar ratio, this line has been successfully used by my colleagues for rapid screening and validation of modulators of GLP-secretion 1, leading to the publication of a paper where I am co-author (Anghel et al, 2022). These data will be used and presented in another doctoral thesis by my colleague, Andrea Anghel.

General introduction

The technological progress registered in recent years in biochemistry, genetics and medicine has led to an exponential increase in the number of studies, results and experimental data with new medical perspectives. Genetic editing techniques represent a group of molecular biology methods that allow the modification of the genetic material in an organism, by adding, removing or specifically altering some DNA sequences, thus

opening new horizons for the in vivo study of the functioning mechanism of proteins key involved in certain pathologies.

This doctoral thesis aims to implement genetic editing techniques such as the CRISPR system ("Clustered Regularly Interspaced Short Palindromic Repeats") and lentiviral transduction to facilitate the study of two distinct pathologies that have a devastating impact on the human population: hepatitis B and diabetes sugary

Globally, in the year 2022, hepatitis B virus (HBV) infection led to 1.1 million deaths, mainly from cirrhosis and liver cancer. About 1.2 million new cases of infection are registered annually. It is estimated that about 250 million people are infected with this virus, representing about 3.2% of the world's population (World Health Organization). Moreover, approximately 30% of the global population show serological markers of passage through HBV infection (Trépo et al, 2014). Also, in 2021 it was estimated that approximately 530 million people suffered from diabetes (10% of the world's population) and there were 6.7 million deaths. The incidence of this pathology is increasing, the number of people affected is expected to increase to 640 million by 2030. Another 540 million people are estimated to have an increased risk of developing diabetes due to glucose tolerance (Diabetes Atlas). In the same year, there were more than 800,000 patients in Romania (National Institute of Public Health).

The study presented in this thesis contributes to the understanding of HBV-host cell interaction, by identifying new cellular factors involved in its life cycle. These factors are enzymes that are involved in phosphatidylinositol metabolism: Sac1 and the α and β isoforms of phosphatidylinositol 3 kinase class 2 (PI3KC2α and PI3KC2β). These studies were based on obtaining clonal cell lines with low expression (for Sac1) or no expression (PI3KC2β and PI3KC2α), using CRISPR technology, but also modulating expression transiently through overexpression and gene silencing experiments, through RNA interference (Popescu et al, 2022; Patriche et al, 2022). These enzymes have been identified as having an important role in the life cycle of several different viruses (Polachek et al, 2016; Abere et al, 2018; O'Hanlon et al, 2019). Because of the interference of HBV infection with host cell lipid metabolism, our working hypothesis was that phosphoinositides might play a major role in distinct stages of the HBV life cycle, both directly (through a structural role at the level of cell membranes) and indirectly, through cellular signaling. Thus, after the generation of genetically modified cell lines, I focused on the functional analysis of the

effects of modulating the expression of target proteins on HBV replication, assembly and trafficking (PI3KC2α and PI3KC2β).

At the same time, we contributed to a study that focused on obtaining a cellular system based on luciferase secretion in a 1:1 molar ratio with the GLP-1 hormone, in order to more quickly identify compounds with modulatory activity on GLP- secretion 1. This hormone plays a key role in the pathology of diabetes, regulating glucose homeostasis. A cell line expressing cytosolic luciferase was obtained by lentiviral transduction, which was used to confirm the activity of the selected compounds (Anghel et al, 2022).

1. Theoretical synopsis

1.1. Theoretical bases of the methodology of biochemistry and molecular biology investigations used

The CRISPR system is part of the "immune system" of the prokaryotic cell, which evolved to protect the cell against phage infection. Thus, after infection of the host cell with a phage, fragments of its genome are digested and integrated into the bacterial genome in CRISPR-type loci, interspersed by repetitive palindromic sequences (Hille et al, 2018, Strich et al, 2019). Later, they are transcribed together with the palindromic sequences, processed by cleavage and subsequently guide the enzyme complex to specifically cleave sequences from the phage genome, recognized on the basis of complementarity with the sequences inserted into the bacterial genome within the CRISPR locus. Thus, the progression of the phage life cycle is prevented based on a type of immunological memory (Hille et al, 2018). An RNA sequence is required in this process, called tracrRNA (CRISPR "trans activator" RNA). This RNA hybridizes with the repetitive ("spacer") sequence, forming a complex and thereby guiding the Cas9 enzyme to cleave any sequence in the phage genome that contains the complementary 20-nucleotide sequence and the adjacent PAM sequence. (Jiang et al., 2017). The Cas9 enzyme contains two domains with nuclease activity that cleave the complementary strand and the non-complementary strand with the single guide sequence (named RuvC and HNH) (Ma et al, 2014) There are mutations that inactivate one catalytic site each (D10A in the RuvC domain and H840A in the HNH domain) and thus transforms the enzyme by partial loss of the hydrolysis function into half-active forms, called "nickases" (Cas9n), capable of cleaving only one strand, not both, like the wild-type form. This system shows a more specific large because two "single guide" sequences are required (Ran et al, 2013).

1.2. The study of HBV

1.2.1. Introduction

Approximately 250 million people suffer from chronic HBV infection, and annually there are more than one million new cases of infection globally and more than one million deaths from cirrhosis and liver cancer caused by HBV infection, despite the fact that that there is a highly effective vaccine (World Health Organization).

Viral hepatitis B is currently not curable. Treatment for chronic infection consists of taking antiviral drugs such as tenofovir or entecavir. Once started, antiviral treatment generally must be continued throughout life. (World Health Organization).

1.2.2. HBV structure

HBV is an enveloped DNA genome virus of the Hepadnaviridae family. Two different types of particles are distinguished in the blood of infected patients, 42 nm particles that contain the viral genome and are infectious and 22 nm particles that do not contain the viral genome and are not infectious. (Tsukuda et al, 2020, Chai et al, 2008). The genome is approximately 3.2 kbp in length and is in the form of circular relaxed DNA that is partially double-stranded, with a complete minus strand and an incomplete plus strand. (Tong et al., 2016).

1.3. Phosphatidylinositol 3-kinases and phosphatidylinositol phosphatases in HBV study

Phosphatidylinositol 3-kinases (PI3K) are enzymes that phosphorylate phosphatidyl inositol (PI), changing the composition of cell membranes and therefore having major roles in processes such as cell signaling or cell trafficking. They are divided into 3 classes, according to structure and substrate specificity. (Thibault et al., 2023). Class II phosphatidyl inositol 3-kinases (PI3KC2) remain the least studied class. However, new studies show the increasing importance of this class in various processes such as proliferation, migration or viral replication. There are 3 isoforms of these enzymes, named PI3KC2α, PI3KC2β, and PI3KC2γ, which have distinct, non-overlapping functions. The reaction products are different from the reaction products of class I enzymes. In contrast to the latter, PI3KC2 produces PI 3-monophosphate and PI 3,4-diphosphate, while class I enzymes produce PI 3,4 , 5-triphosphate, which indicates their different action through other pathways. In general, PI3KC1 has a direct role in cell signaling through plasma membrane-bound receptors and GTPases, while PI3KC2 and PI3KC3 have predominantly roles in intracellular trafficking (Yoshioka et al, 2021).

Phosphatases involved in PI metabolism also have crucial signaling functions at the level of cell membranes. Through its unique phosphatidyl inositol-4-phosphate (PI4P) dephosphorylation activity, Sac1 contributes decisively to maintaining the intracellular level of PI4P, regulating multiple cellular processes, including cytoskeleton organization, intermembrane vesicular traffic, cell signaling (Del Bel et al, 2018). All these processes are relevant for the interaction between a pathogen and the host cell, which is why Sac1 has been considered a possible target for modulating HBV infection.

1.4. The study of the GLP1 peptide in the pathology of diabetes

Diabetes mellitus is a chronic disease that occurs either due to autoimmune-mediated destruction of β-cells in the islets of Langerhans, resulting in a lack of insulin production (type I diabetes) or due to resistance of the target tissues to the insulin produced (type II diabetes). Hyperglycemia is the consequence of these two conditions in the absence of treatment and leads to serious effects on the body such as peripheral neuropathy, retinopathy, nephropathy or cardiovascular complications (Krause et al 2023). In 2014, 8.5% of adults had diabetes. In 2019, diabetes was the cause of death of 1.5 million people (WHO).

GLP-1 and GLP-2 peptides are peptides secreted by enteroendocrine L cells located predominantly in the distal small intestine and colon following nutrient ingestion. Together with glucagon, they originate from the same precursor molecule, proglucagon, but have completely divergent functions. GLP-1 has insulinotropic and glucagonostatic function (Marathea et al, 2013).

GLP-1 secretion can be stimulated by various nutrients, such as glucose, other saccharides, fatty acids, essential amino acids, or fiber. (Baggio et al., 2007). After secretion, the half-life of GLP-1 is very short, 60-90 seconds, due to its rapid inactivation by the enzyme DPP-4 (dipeptidyl peptidase 4) (Hui, et al, 2002).

2. Materials and methods

We achieved stable modulation of target gene expression by implementing gene editing techniques such as the CRISPR/Cas9 system and retroviral transduction in the Huh7 cell line. Sequences targeting these genes have been cloned, as well as the GFP-Sac1 fusion gene. Analysis of the expression of these genes was performed at the messenger RNA level by RT-qPCR and at the protein level by Western Blot. The obtained cell lines were used as a model to study the role of PI3KC2β in the HBV life cycle. Subviral particle levels were analyzed by ELISA and viral DNA levels were analyzed by qPCR.

Retroviral transduction was also used to obtain the GluTag line with cytosolic luciferase expression.

3. Results and Discussion

3.1. Investigating the role of Sac1 phosphatase and kinases (PI3KC2β and PI3KC2α) in the HBV life cycle

Phosphatidylinositol derivatives have been identified as having an important role in the life cycle of many positive-sense RNA viruses, such as members of the flavivirus or picornavirus families (Roulin et al., 2014; Li et al., 2014). Within the group, Sac1 phosphatase and phosphatidylinositol 3-kinases were studied in the context of the impact on the HCV life cycle (unpublished data) using the Huh7 cell line as a model. This previous experience with these targets facilitated the study of their role in the HBV life cycle.

3.1.1. Stable modulation of Sac1 expression in Huh7 cells using CRISPR/Cas9 technology

As homozygous deletion of the Sac1 gene has previously been described as lethal (Liu et al, 2008), we aimed to obtain cell clones with low expression rather than knockout cell clones without expression. For this purpose we used single guide sequences that target

the 5' untranscribed region (5'UTR), before the ATG start codon of the SACM1L gene, starting from the hypothesis that this way only clones with lower expression, viable, could be obtained. For this, 4 single guide sequences were selected because from an experimental point of view, the ability of a single guide sequence to produce the desired mutation cannot be anticipated with certainty, only by analyzing the reference genome databases. One of the reasons for this could be the large number of mutations that cell lines can have, especially due to the fact that most of them are derived from different forms of cancer. The 4 sequences were cloned into the pSpCas9-2A-Puro vector. For gain of function studies, Sac1 was cloned in fusion with GFP, resulting in pLNCX-GFP and pLNCX-GFP-Sac1 vectors.

3.1.2. Cloning of cell lines with low expression of Sac1

Among the 4 single guide sequences cloned in the pSpCas-2A-Puro vector, the sequence TAGAGTTGTAGCCGAGGTGG was selected because it had the lowest probability of introducing non-specific mutations in genomic DNA compared to the other 3 single guide sequences described previously. A CRISPR system based on the Cas9 enzyme derived from wild-type *S. pyogenes* was used, which cleaves both DNA strands and produces a straight-ended cut. During and after puromycin selection after transfection a significant percentage of cells did not survive, which could be explained by the lethality of the SACM1L gene deletion. Untransfected cells did not survive puromycin treatment, and transfected cells were not viable due to the homozygous deletion. After removing the selection antibiotic from the environment, the cell population obtained was cloned by the method of serial dilutions, obtaining a number of cell clones that were later tested for the expression of Sac1, both at the level of RNA and protein expression (Popescu et a, 2022).The low-expressing Sac(-)c1 clone was transduced with the obtained retroviral particles and following geneticin-based selection a cell line expressing the GFP-Sac1 fusion protein was obtained. Expression was confirmed by Western Blot, yielding a band at a mass of approximately 75 kDa, yielding clones with differential expression of the fusion protein. Expression was confirmed in biological triplicate by Western Blot using both anti-Sac1 and anti-GFP antibodies.

The functional analysis of stable lines with modulated expression of Sac1 and the consequences of this expression on the HBV life cycle were investigated in another doctoral thesis, by my colleague, Popescu Mirela. Thus, it was demonstrated that Sac1 is an important

cellular factor in the HBV life cycle, these data being also confirmed by transient gene expression modulation experiments. The data obtained in these studies were published in the journal Farmacia (Popescu et al, 2022).

3.1.3. Stable modulation of PI3KC2β expression in Huh7 cells using CRISPR/Cas9 technology

Obtaining knockout clones for the PI3KC2β gene

A cell population derived from the parental Huh7 bulk line with reduced expression of the PI3KC2β gene was obtained using a CRISPR/Cas9n (nickcase-type) system in my own dissertation thesis, starting from the design and cloning of single guide sequences. Starting from this heterogeneous low-expressing population, cloning was performed by serial dilutions and a number of low-expressing clones were obtained. The expression level of this gene at the protein level was analyzed by Western Blot using tubulin as a loading control and it was shown to obtain several clones without PI3KC2β expression of the knockout type: b3, b5 and b6, as well as a clone with reduced expression (b1) (**Figure 3.1**,) (Patriche et al, 2022).

Analysis of PI3KC2ß expression in Huh7 clonal cell lines

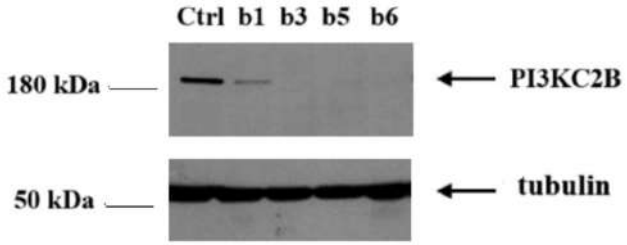

Figure 3.1. Analysis of PI3KC2ß expression in Huh7 clonal cell lines. Absence or reduced expression of PI3KC2β was confirmed by Western Blot in the analyzed clones by comparison with control cells (Huh7). Tubulin was used as a loading control. Adapted from Patriche et al., 2022.

Protein extracts were obtained from control Huh7 cells and four CRISPR-derived cell clones: b1, b3, b5, and b6. Equal amounts of these extracts were loaded on a polyacrylamide gel and protein expression was determined by Western blot using specific antibodies. The absence of the band at approximately 190 kDa corresponding to the PI3KC2β gene is highlighted in the case of clones b3, b5 and b6 and a lower intensity in the case of clone b1 (**Figure 3.1**).

3.1.4. Analysis of the role of PI3KC2β in the HBV life cycle

Analysis of the secretion of viral and subviral particles.

The cell lines obtained, as well as clones without expression of PI3KC2β, were transfected with the pTriEx-HBV1.1 plasmid containing the entire HBV genome, and it was analyzed whether this deletion has an impact on the HBV life cycle. The Huh7 cell line is permissive for HBV replication, but cannot facilitate the complete life cycle of the virus. Therefore, for studies using the Huh7 cell line as a model, it is necessary to transfect the entire viral genome. Using the parental Huh7 line as a control, the amount of secreted subviral particles was analyzed by ELISA at two time intervals: 7 and 9 days after transfection. An approximately 50% decrease in the amount of secreted subviral particles was observed at both time intervals in the knockout b3 and b5 clones, but also in the bulk population (**Figure 3.2**).

At the same time, the secretion of encapsulated viral DNA was quantified on day 9 after transfection by qPCR using a standard curve, both secreted (**Figure 3.3**) and intracellular (Figure 3.4B B). The same approximately 50% decrease in viral DNA levels was observed. Analysis of viral DNA at shorter time intervals after transfection may be less relevant due to possible contamination with plasmid DNA that is amplified because it contains the entire HBV genome, including the region targeted by the primers used for analysis. It was determined by ELISA and the level of intracellular HBs antigen also on day 9 (**Figure 3.4. A**) (Patriche et al, 2022).

Secretion of HBsAg from PI3KC2β knockout Huh7 cells.

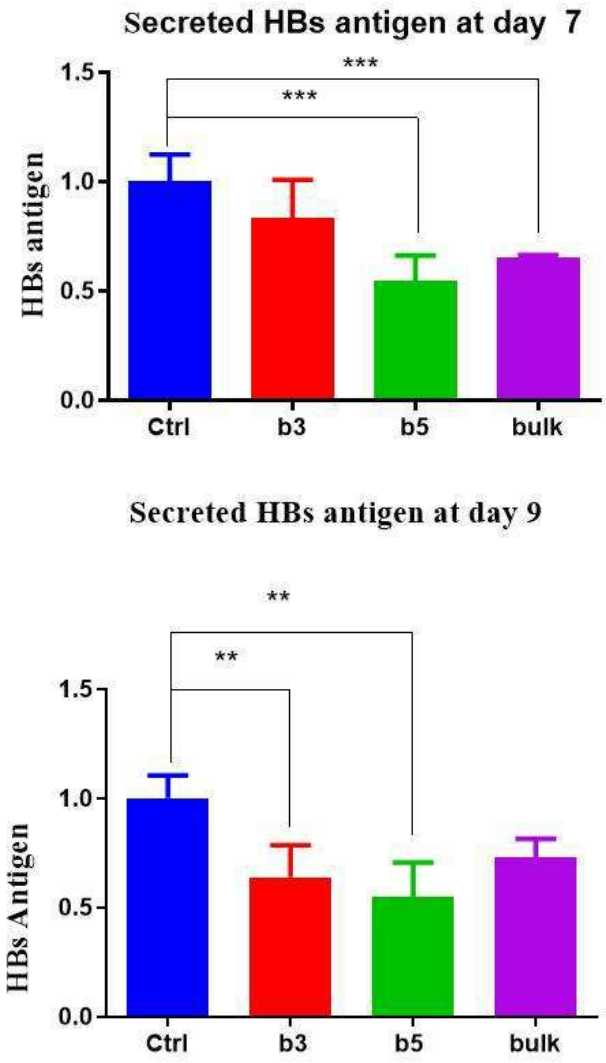

Figure 3.2. HBsAg secretion from PI3KC2β knockout Huh7 cells. Control cells and knockout clones b3, b5 and the low-expressing bulk population were transfected with pTriEx-HBV1.1 plasmid and HBs antigen secretion was analyzed by ELISA on days 7 and 9 post-transfection and expressed as a fraction of the control. p-values were calculated using the Student's t-test for groups of biological replicates. Adapted from Patriche et al., 2022

Secretion of HBV viral particles from PI3KC2β "knockout" Huh7 cells

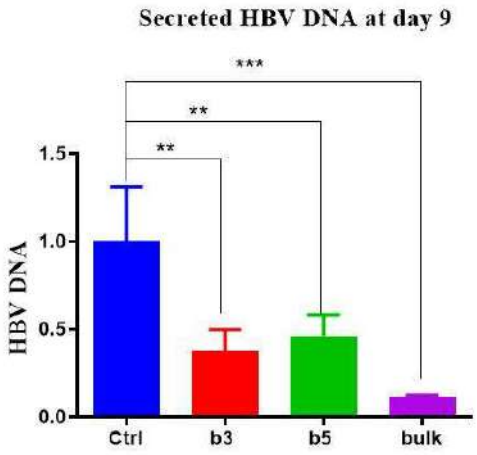

Figure 3.3. Secretion of HBV viral particles from PI3KC2β knockout Huh7 cells. HBV nucleocapsids were purified and levels of secreted viral DNA were determined by qPCR at day 9 post-transfection and expressed as a percentage of control. Knockout clones b3, b5 and the low-expressing population bulk were compared with control cells, represented by the Huh7 parental line (Ctrl). p-values for groups of biological replicates were calculated using Student's t-test. Adapted from Patriche et al., 2022

Intracellular HBs antigen analysis and quantification of HBV viral particles

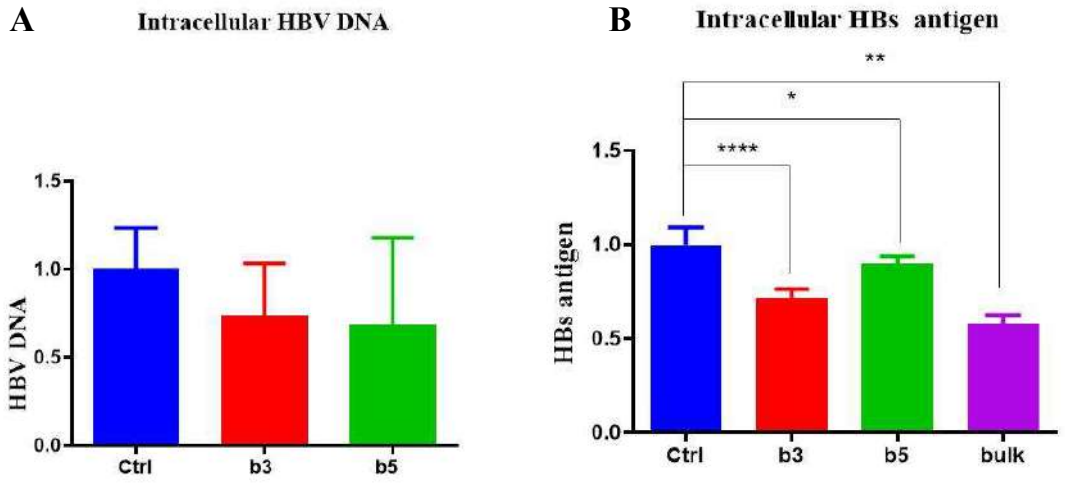

Figure 3.4. Intracellular HBs antigen analysis and quantification of HBV viral particles. PI3KC2β knockout Huh7 cells were transfected with pTriEx-HBV1.1 plasmid and analyzed for HBs antigen levels by ELISA at day 9 (A) and HBV viral particle levels by

qPCR after transfection (B) and s -expressed as a fraction from the control (Ctrl). p-values were calculated using the Student's t-test for groups of biological replicates. Adapted from Patriche et al., 2022

3.1.5. Analysis of the role of PI3KC2α in the HBV life cycle

The impact on the life cycle of HBV and a deletion of another isoform of the PI3KC2 gene, PI3KC2α, was analyzed. Clones without expression for PI3KC2α were also obtained by CRISPR/Cas9, starting from cloning in the pSpCas-2A-Puro vector and the design of single guide sequences targeting the α isoform. After confirming the obtaining of cell clones with reduced or absent expression, functional studies were performed on the role in the HBV life cycle, but no effect could be demonstrated, probably due to the compensation phenomenon often encountered during the selection of stable clones with expression reduced or no expression by CRISPR/Cas9.

3.1.6. Transient modulation of PI3KC2β expression in Huh7 cells by silencing and overexpression

Cellular cloning can often have the undesired effect of selecting clones with random mutations, many of which have a potential effect on the viral life cycle. To validate these results from CRISPR-derived clones, the effect of knockdown of PI3KC2β gene expression was studied in a transient system using small interfering RNA (siRNA) silencing.

Thus, Huh7 cells were transfected with a control siRNA, which has no gene silencing effects, and a siRNA targeting the PI3KC2β gene. At the same time, they were also transfected with the plasmid pTriEx-HBV1. On day 5 of the experiment they were trypsinized, resuspended and transfected again with siRNA to keep the expression level low. The levels of secreted HBs antigen (**Figure 3.5 A**), intracellular HBs antigen (**Figure 3.5 B**) and viral DNA were analyzed, obtaining decreases of approximately 25-40%, **Figure 3.6**). These smaller decreases can be attributed to the fact that by RNA interference the expression levels are not reduced to zero, as in the case of the knockout lines, but only reduced.

Analysis of secreted and intracellular HBs antigen in the Huh7 cell line following siRNA transfection

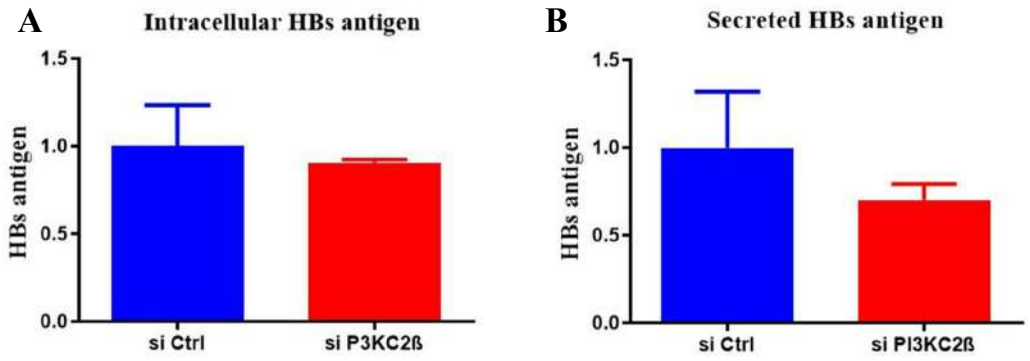

Figure 3.5. Analysis of secreted and intracellular HBs antigen in the Huh7 cell line following siRNA transfection. Huh7 cells were transfected with pTriExHBV1.1 plasmid and control siRNA (Ctrl) and siRNA targeting the PI3KC2β gene. Secreted (A) and intracellular (B) HBs antigen levels were analyzed by ELISA and expressed as fraction of control.

Analysis of intracellular HBV viral particles in the Huh7 cell line following siRNA transfection

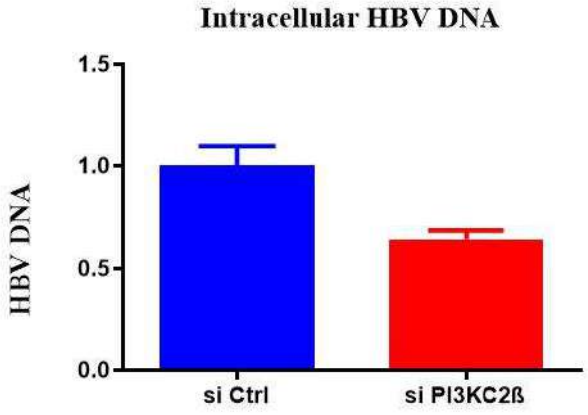

Figure 3.6. Analysis of intracellular HBV viral particles in the Huh7 cell line following siRNA transfection. Huh7 cells were transfected with pTriEx-HBV1.1 plasmid and control

siRNA (Ctrl) and siRNA targeting the PI3KC2β gene, and intracellular HBV particle levels were analyzed by qPCR and expressed as fraction of control .

Protein extracts were obtained from Huh7 cells following transfection with controlsiRNA (siCtrl) and a sequence targeting the PI3KC2β gene (siPI3KC2β). Equal amounts were loaded on a polyacrylamide gel and protein expression was quantified by Western Blot. Note the decrease in intensity of the band at approximately 190 kDa (corresponding to the gene of interest) compared to the intensity of the control band. (**Figure 3.7**).

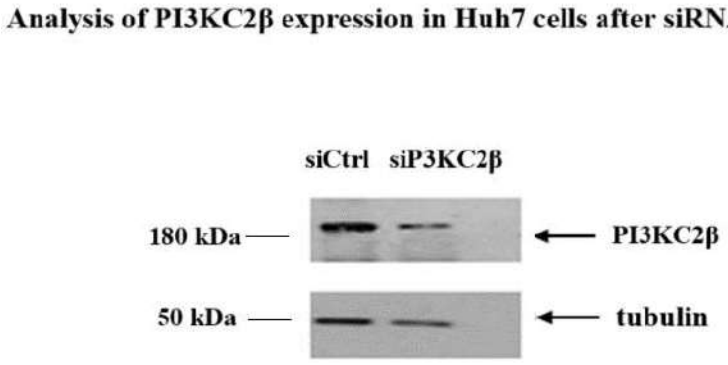

Figure 3.7. Analysis of PI3KC2β expression in the Huh7 clonal cell line following siRNA transfection. Reduction of PI3KC2β expression was confirmed by Western Blot in cells transfected with siRNA targeting the gene of interest. Huh7 cells transfected with control siRNA were used as a control. Tubulin was used as a reference gene.

To further validate these results, a rescue phenotype recovery experiment was performed in a transient system, through which I aimed to reintroduce the PI3KC2β gene into Huh7 cells without expression. Thus, clone b3 was selected (due to similarity in phenotype and proliferation rate) and transfected with increasing amounts of the plasmid pMyc-DDK-PI3KC2β (Origene, catalog no. RC218354), which expresses the gene of interest and levels of secreted subviral particles and viral DNA were analyzed. For normalization, the total concentration of transfected DNA per well was brought to 2µg using the pcDNA3.1 empty vector. The control is represented by parental Huh7 cells transfected only with the pcDNA3.1 empty vector. An increase in both subviral particles and viral DNA is observed directly proportional to the amount of transfected PI3KC2β-expressing plasmid (**Figure 3.8, 3.9**).

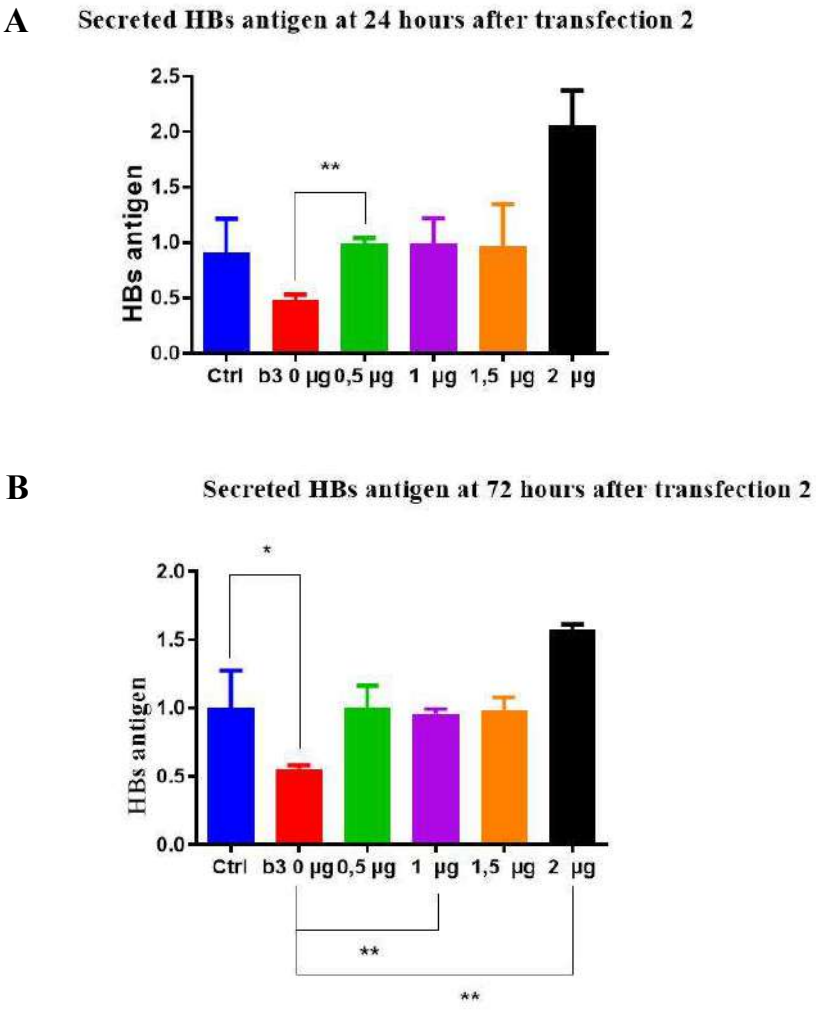

Figure 3.8. Analysis of secreted HBs antigen in the Huh7 cell line following the rescue phenotype experiment. Huh7 cells lacking PI3KC2β expression (clone b3) were co-transfected with the pTriEx-HBV1.1 plasmid and with increasing amounts of the pMyc-DDK-PI3KC2β plasmid. The amount of total transfected DNA was adjusted to 2 µg/well using the pcDNA3.1 empty vector. Parental Huh7 cells transfected with 2 µg of pcDNA3.1 empty vector were used as a control. The secretion of secreted HBs antigen at 24(A) and 72(B) hours post-transfection was analyzed by ELISA. p-values for groups of biological replicates were determined using Student's t-test.

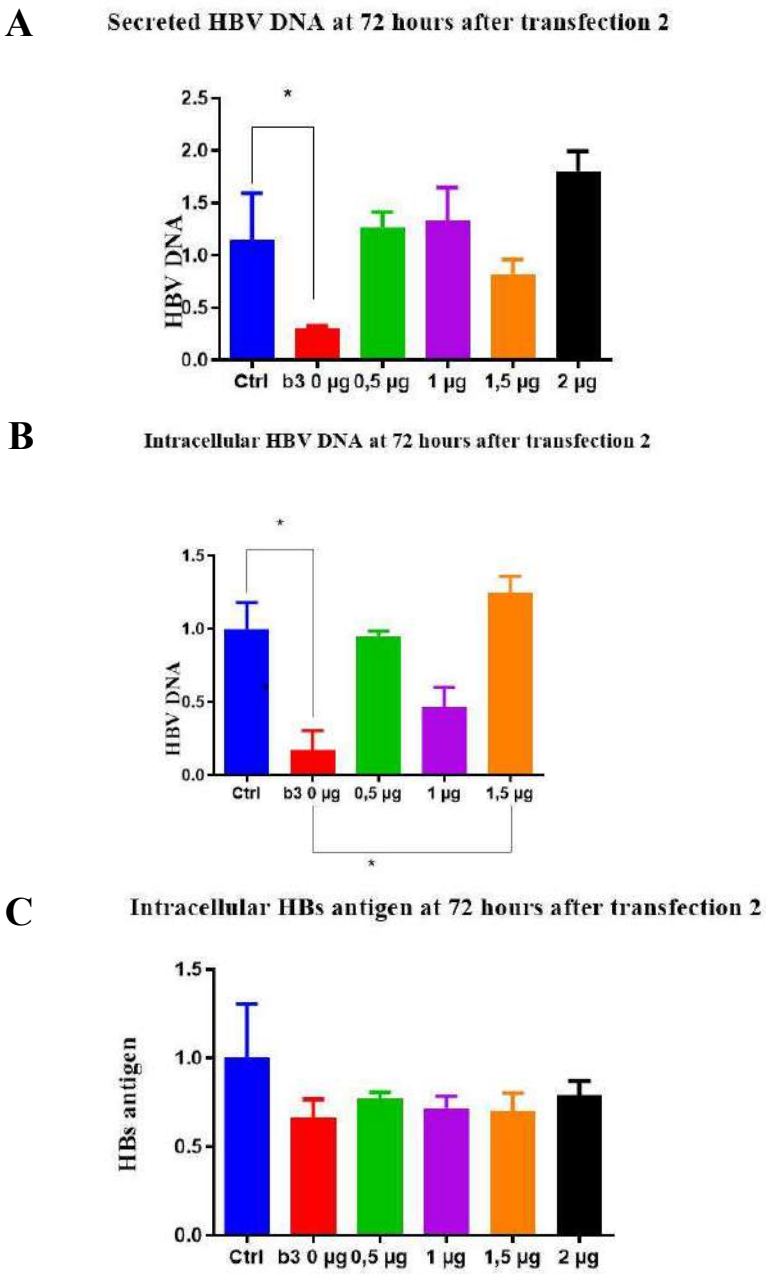

Figure 3.9. Analysis of intracellular HBs antigen as well as secreted and intracellular HBV viral particles following the rescue phenotype experiment. Huh7 cells lacking PI3KC2β expression (clone b3) were co-transfected with the pTriEx-HBV1.1 plasmid and with increasing amounts of the PI3KC2β-expressing plasmid pMyc-DDK-PI3KC2β. The amount of total transfected DNA was adjusted to 2 µg/well using the pcDNA3.1 empty vector. Parental Huh7 cells transfected with 2 µg of pcDNA3.1 empty vector were used as a

control. The amount of both extracellular (A) (secreted) and intracellular (B) HBV was analyzed by qPCR (after 72 h post-transfection), as well as the amount of intracellular HBs antigen by ELISA (C). p-values for groups of biological replicates were determined using Student's t-test. Adapted from Patriche et al., 2022.

Exogenous expression of PI3KC2β leads to the recovery of viral and subviral particle levels from the control and in the case of the 1.5 or 2 μg points even exceeding them.

In conclusion, it was determined that the PI3KC2β enzyme has an important role in the HBV life cycle, while the α isoform is not involved. This role was confirmed by several methods of decreasing expression, stably or transiently. Moreover, the recovery of the phenotype by introducing exogenous expression further strengthens this conclusion of its involvement in the HBV viral life cycle. The mTOR/Akt signaling pathway is a negative regulator of autophagy, an essential process in the life cycle of this virus. Thus, this would be a possible explanation for the observed decreases in viral and subviral particles, but further studies on this hypothesis are needed, as a perspective of this work (Patriche et al, 2022). At the same time, the role of the third isoform, the γ isoform, must be investigated.

3.2. A new system based on the fusion of luciferase with the GLP-1 peptide - with applications in the discovery of new natural compounds with anti-diabetic potential

GLP-1 hormone is an important therapeutic target in diabetic pathology, its synthetic analogues such as semaglutide have proven an important therapeutic role (Rubino et al, 2021). Another approach is to find molecules that induce the secretion of GLP-1. For this, an important cell model for testing molecules with modulatory action is represented by the murine enteroendocrine cell line GLUTag, derived from an intestinal tumor due to the fact that it faithfully reproduces the physiological secretion of GLP-1 in response to various substances (Gil- Lozano et al., 2015). NanoLuc luciferase is the newest commercially available luciferase. It is a 19.1 kDa protein that uses the furimazine substrate and produces luminescence with significant intensity (England et al, 2016).

The GLUTag line represents a useful cell model in the study of compounds with modulatory activity on GLP-1 secretion. But the impediments of these studies are represented by the more laborious protocol for determining this peptide, by the high cost (ELISA kit) (Anghel et al, 2022). It was necessary to develop another system to facilitate these secretion experiments, to reduce costs and working time, and to increase the level of confidence in the data obtained. Thus, a cell line expressing the preproglucagon-NanoLuc fusion gene and showing proteolytic cleavage sites was created by lentiviral transduction, the protein being cleaved so that NanoLuc luciferase and GLP-1 are secreted in a 1:1 molar ratio (Anghel et al , 2022). These results were obtained by my colleagues, Andreea Anghel, Dr. Rodica Badea, Dr. Gabriela Chirițoiu, Dr. Petruța Alexandru and Dr. Florentina Pena and will be detailed in another doctoral thesis. This experimental concept of using a reporter line based on a luciferase was previously described to study the secretion of another peptide involved in the pathology of diabetes (Burns et. al., 2015).

The measurement of luciferase activity is directly proportional to the level of GLP-1 secretion within this system. But testing new chemical compounds using cell cultures as a model raises the issue of toxicity that can lead to erroneous results by cell lysis and nonspecific release of luciferase into the environment along with other cellular proteins. To address this problem, in the framework of the doctoral work, we obtained the GLUTagNanoLuc line (with cytosolic expression of luciferase) by lentiviral transduction using the pLenti6.2-Nanoluc-ccdB vector. Thus, if a compound exhibits such toxicity, it will also increase cytosolically expressed luciferase activity. This line is in turn a convenient system for testing the toxicity of compounds. Moreover, it is all the more important as it allows testing this in experimental conditions that faithfully reproduce those during GLP-1 secretion experiments (Anghel et al, 2022).

In conclusion, genome editing using transduction was again applied to obtain the control line expressing cytosolic luciferase, which was further used in the experimental workflow in a study that led to the identification of new GLP-1 secretagogue natural compounds and to obtain a system of cell lines that allow the screening of chemical compounds. Moreover, a lentiviral transduction protocol was optimized for this cell line, which allows applications for the study of other cellular target proteins in the pathology of diabetes, but also in other pathologies.

4. Final conclusions

In the framework of the thesis, genetic manipulation techniques were applied, such as lentiviral transduction and the CRISPR/Cas9 system in order to obtain genetically modified cell lines, which were used as important tools in studies of hepatitis B and diabetes, these two pathologies having a huge cumulative incidence, they affect about 800 million people, representing about 1/10 of the planet's population.

Clones with reduced or no expression of the studied genes were obtained by means of CRISPR in the Huh7 cell line. These produced cell lines led to the discovery of two cellular factors that are involved in the HBV life cycle: Sac1 and the β isoform of PI3K.

At the same time, single guide sequences for CRISPR as well as the GFP-Sac1 fusion gene in a retroviral vector were cloned and validated, which can also be used to generate clones with altered expression in other lines for further studies. The next step would be to obtain cell clones with reduced expression of PI3KC2β derived from the HepG2-NTCP line, which is permissive for HBV infection and thus allows studying its entire life cycle, in contrast to the Huh7 line, which does not allow infection.

Research on these cellular enzymatic factors can be extended by searching for their inhibitors in the context of HBV infection, given that there is currently no drug treatment capable of completely eliminating the infection. Another disadvantage of antiviral drugs is that they target viral enzymes, and viral genes very frequently undergo mutations leading to resistance, while host genes have a very low mutation frequency. At the same time, research on these factors can also be extended to VHD, since co-infection with these two viruses represents the most serious form of viral hepatitis.

In parallel, we also produced a cell population derived from the enteroendocrine GLUTag line with cytosolic luciferase expression (GLUTag-NLuc), which was used to validate an easy and economical cell system in a study that identified natural molecules from plants that induce secretion of GLP-1 (quercetin). As perspectives, this system can be used to identify new compounds with modulatory action on GLP-1 secretion, which can subsequently be used as antidiabetic drugs.

Personal contributions

During the doctoral thesis I personally obtained the following original results:

1. Cloning into the pSpCas9BB2A-Puro vector of single guide sequences with an original design, which target the coding gene for the Sac1 protein, using CRISPR technology.
2. Obtaining by CRISPR clones with reduced expression (knockdown) of Sac1 derived from the Huh7 line, highlighting the mutations produced by sequencing, and molecular characterization of the clones by quantitative PCR verification of the decrease in mRNA level and verification of the decrease in expression proteins by Western Blot.
3. Cloning of the GFP-Sac1 fusion gene into the pLNCX2 vector, production of retroviral particles and their use to produce lines expressing this fusion protein.
4. Obtaining clones with low expression (knockdown) or without expression (knockout) of PI3KC2β starting from a heterogeneous cell population with low expression previously obtained by CRISPR technology in the Huh7 line, in the framework of my dissertation thesis and verifying the level of protein expression by Western Blot.
5. Identification of PI3KC2β as a cellular factor involved in the HBV life cycle using these clones and phenotype recovery experiments by transient expression of PI3KC2β in knockout cells. These results were also validated by transient silencing via RNA interference.
6. Obtaining a GLUTag-derived cell population with cytosolic luciferase expression by transduction using lentiviral particles produced from the pLenti6.2ccdB-Nanoluc vector.

List of papers published within the doctoral thesis

Publications in ISI indexed journals

1. **Sac1 phosphatidylinositol 4-phosphate phosphatase is a novel host cell factor regulating hepatitis B virus particles assembly and release.**

Popescu MA*, Patriche DS*, Dobrica MO, Pantazica AM, Flintoaca Alexandru PR, Rouillé Y, Popescu CI, Branza-Nichita N. FEBS J. 2022 Jul 11. doi: 10.1111/febs.16575. [IF: 5,622]

1. **Class II phosphatidylinositol 3-kinase 2ß is a novel target for the potential development of antiviral drugs against the Hepatitis B virus.**

Patriche DS, Popescu MA, Popescu CI, Nichita N. Farmacia J, 2022 [IF: 1,6]

1. **Novel luciferase-based glucagon-like peptide 1 reporter assay reveals naturally occurring secretagogues.**

Anghel SA, Badea RA, Chiritoiu G, Patriche DS, Alexandru PR, Pena F. Br J Pharmacol. 2022 Oct;179(19):4738-4753. doi: 10.1111/bph.15896. Epub 2022 Jul 21. PMID: 35736785. [IF: 9.47]

*co-first authors with equal contribution

National conference participations

1. **Class II phosphatidylinositol 3-kinases regulate HBV life cycle in hepatoma cell lines**

Patriche DS, Popescu MA, Popescu CI, Nichita N. RSBBM Conference, Iasi, 2019

1. **The role of phosphatidylinositol (Ptdins) phosphatase Sac1 in the HBV life cycle**

Popescu MA, Patriche DS, Popescu CI, Nichita N. RSBBM Conference, Iasi, 2019.

References

Abere B, Samarina N, Gramolelli S, Rückert J, Gerold G, Pich A, Schulz TF. Kaposi's Sarcoma-Associated Herpesvirus Nonstructural Membrane Protein pK15 Recruits the Class II Phosphatidylinositol 3-Kinase PI3K-C2a To Activate Productive Viral Replication. J Virol. 2018 Aug 16;92(17):e00544-18. doi: 10.1128/JVI.00544-18. PMID: 29950425; PMCID: PMC6096797.

Anghel SA, Badea RA, Chiritoiu G, Patriche DS, Alexandru PR, Pena F. Novel luciferase-based glucagon-like peptide 1 reporter assay reveals naturally occurring secretagogues. Br J Pharmacol. 2022 Oct;179(19):4738-4753. doi: 10.1111/bph.15896. Epub 2022 Jul 21. PMID: 35736785.

Baggio LL, Drucker DJ. Biology of incretins: GLP-1 and GIP. Gastroenterology. 2007 May;132(6):2131-57. doi: 10.1053/j.gastro.2007.03.054. PMID: 17498508.

Chai N, Chang HE, Nicolas E, Han Z, Jarnik M, Taylor J. Properties of subviral particles of hepatitis B virus. J Virol. 2008 Aug;82(16):7812-7. doi: 10.1128/JVI.00561-08. Epub 2008 Jun 4. PMID: 18524834; PMCID: PMC2519590.

Del Bel LM, Brill JA. Sac1, a lipid phosphatase at the interface of vesicular and nonvesicular transport. Traffic. 2018 May;19(5):301-318. doi: 10.1111/tra.12554. Epub 2018 Mar 25. Erratum in: Traffic. 2019 Feb;20(2):181. doi: 10.1111/tra.12626. PMID: 29411923.

England CG, Ehlerding EB, Cai W. NanoLuc: A Small Luciferase Is Brightening Up the Field of Bioluminescence. Bioconjug Chem. 2016 May 18;27(5):1175-1187. doi: 10.1021/acs.bioconjchem.6b00112. Epub 2016 Apr 19. PMID: 27045664; PMCID: PMC4871753.

Gil-Lozano M, Brubaker PL. Murine GLUTag Cells. In: Verhoeckx K, Cotter P, López-Expósito I, Kleiveland C, Lea T, Mackie A, Requena T, Swiatecka D, Wichers H, editors. The Impact of Food Bioactives on Health: in vitro and ex vivo models [Internet]. Cham (CH): Springer; 2015. Chapter 21. PMID: 29787050

Hille, F., Richter, H., Wong, S. P., Bratovic, M., Ressel, S., & Charpentier, E. (2018). Review of The Biology of CRISPR-Cas : Backward and Forward]. Cell, 172(6), 1239–1259. https://doi.org/10.1016/j.cell.2017.11.032

Hui H, Farilla L, Merkel P, Perfetti R. The short half-life of glucagon-like peptide-1 in plasma does not reflect its long-lasting beneficial effects. Eur J Endocrinol. 2002 Jun;146(6):863-9. doi: 10.1530/eje.0.1460863. PMID: 12039708.

Jiang F, Doudna JA. CRISPR-Cas9 Structures and Mechanisms. Annu Rev Biophys. 2017 May 22;46:505-529. doi: 10.1146/annurev-biophys-062215-010822. Epub 2017 Mar 30. PMID: 28375731.

Krause M, De Vito G. Type 1 and Type 2 Diabetes Mellitus: Commonalities, Differences and the Importance of Exercise and Nutrition. Nutrients. 2023 Oct 7;15(19):4279. doi: 10.3390/nu15194279. PMID: 37836562; PMCID: PMC10574155.

Li W. The hepatitis B virus receptor. Annu Rev Cell Dev Biol. 2015;31:125-47. doi: 10.1146/annurev-cellbio-100814-125241. Epub 2015 Oct 2. PMID: 26436705

Liu Y, Boukhelifa M, Tribble E, Morin-Kensicki E, Uetrecht A, Bear JE, Bankaitis VA. The Sac1 phosphoinositide phosphatase regulates Golgi membrane morphology and mitotic spindle organization in mammals. Mol Biol Cell. 2008 Jul;19(7):3080-96. doi: 10.1091/mbc.e07-12-1290. Epub 2008 May 14. PMID: 18480408; PMCID: PMC2441670.

Ma Y, Zhang L, Huang X. Genome modification by CRISPR/Cas9. FEBS J. 2014 Dec;281(23):5186-93. doi: 10.1111/febs.13110. Epub 2014 Nov 7. PMID: 25315507.

Marathe CS, Rayner CK, Jones KL, Horowitz M. Glucagon-like peptides 1 and 2 in health and disease: a review. Peptides. 2013 Jun;44:75-86. doi: 10.1016/j.peptides.2013.01.014. Epub 2013 Mar 20. PMID: 23523778.

O'Hanlon R, Leyva-Grado VH, Sourisseau M, Evans MJ, Shaw ML. An Influenza Virus Entry Inhibitor Targets Class II PI3 Kinase and Synergizes with Oseltamivir. ACS Infect Dis. 2019 Oct 11;5(10):1779-1793. doi: 10.1021/acsinfecdis.9b00230. Epub 2019 Sep 9. PMID: 31448902.

Patriche, David. (2022). Class Ii Phosphatidylinositol 3-Kinase 2ß Is A Novel Target For The Potential Development Of Antiviral Drugs Against The Hepatitis B Virus. Farmacia. 70. 266-271. 10.31925/Farmacia.2022.2.11.

Polachek WS, Moshrif HF, Franti M, Coen DM, Sreenu VB, Strang BL. High-Throughput Small Interfering RNA Screening Identifies Phosphatidylinositol 3-Kinase Class II Alpha as Important for Production of Human Cytomegalovirus Virions. J Virol. 2016 Aug 26;90(18):8360-71. doi: 10.1128/JVI.01134-16. PMID: 27412598; PMCID: PMC5008103.

Popescu MA, Patriche D, Dobrica MO, Pantazica AM, Flintoaca Alexandru PR, Rouillé Y, Popescu CI, Branza-Nichita N. Sac1 phosphatidylinositol 4-phosphate phosphatase is a novel host cell factor regulating hepatitis B virus particles assembly and release. FEBS J. 2022 Dec;289(23):7486-7499. doi: 10.1111/febs.16575. Epub 2022 Jul 22. PMID: 35816160.

Ran FA, Hsu PD, Lin CY, Gootenberg JS, Konermann S, Trevino AE, Scott DA, Inoue A, Matoba S, Zhang Y, Zhang F. Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. Cell. 2013 Sep 12;154(6):1380-9. doi: 10.1016/j.cell.2013.08.021. Epub 2013 Aug 29. Erratum in: Cell. 2013 Oct 10;155(2):479-80. PMID: 23992846; PMCID: PMC3856256.

Rubino D, Abrahamsson N, Davies M, Hesse D, Greenway FL, Jensen C, Lingvay I, Mosenzon O, Rosenstock J, Rubio MA, Rudofsky G, Tadayon S, Wadden TA, Dicker D; STEP 4 Investigators. Effect of Continued Weekly Subcutaneous Semaglutide vs Placebo on Weight Loss Maintenance in Adults With Overweight or Obesity: The STEP 4 Randomized Clinical Trial. JAMA. 2021 Apr 13;325(14):1414-1425. doi: 10.1001/jama.2021.3224. PMID: 33755728; PMCID: PMC7988425.

Strich JR, Chertow DS. CRISPR-Cas Biology and Its Application to Infectious Diseases. J Clin Microbiol. 2019 Mar 28;57(4):e01307-18. doi: 10.1128/JCM.01307-18. PMID: 30429256; PMCID: PMC6440769.

Thibault B, Ramos-Delgado F, Guillermet-Guibert J. Targeting Class I-II-III PI3Ks in Cancer Therapy: Recent Advances in Tumor Biology and Preclinical Research. Cancers (Basel). 2023 Jan 27;15(3):784. doi: 10.3390/cancers15030784. PMID: 36765741; PMCID: PMC9913247.

Tong S, Revill P. Overview of hepatitis B viral replication and genetic variability. J Hepatol. 2016 Apr;64(1 Suppl):S4-S16. doi: 10.1016/j.jhep.2016.01.027. PMID: 27084035; PMCID: PMC4834849.

Tsukuda S, Watashi K. Hepatitis B virus biology and life cycle. Antiviral Res. 2020 Oct;182:104925. doi: 10.1016/j.antiviral.2020.104925. Epub 2020 Aug 28. PMID: 32866519.

Yoshioka K. Class II phosphatidylinositol 3-kinase isoforms in vesicular trafficking. Biochem Soc Trans. 2021 Apr 30;49(2):893-901. doi: 10.1042/BST20200835. PMID: 33666217; PMCID: PMC8106491.