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Final report

By Christina Schmudermayer

Modulation of DCAF14 expression and its impact in the progression of brain cancer

Practical Training Semester

WS 2017/2018

External Supervisor: Dr. David de Semir

Internal Supervisor: Dr. Christoph Wiesner

Date of Submission: 31.03.2018

Statutory Declaration

"I declare in lieu of an oath that I have written this bachelor paper myself and that I have not used any sources or resources other than stated for its preparation. I further declare that I have clearly indicated all direct and indirect quotations. This bachelor paper has not been submitted elsewhere for examination purposes.

Date: 31.03.2018 **Signature:** Christina Schmudermayer

Acknowledgment

First of all I want to thank the CPMC for giving me the opportunity to do an internship in their research institute and for having trust in me to be able to do the *in vivo* training. It was a great experience to work with mice in the Animal Care Facility and to see both sides of cancer research the basic *in vitro* research and the pre-clinical *in vivo* trials.
Furthermore I want to thank my supervisor Dr. David de Semir, who supported me through my whole internship. He was very helpful from the beginning on and I learned a lot from him. It was a pleasure working together with him.
As well I want to thank my internal supervisor Dr. Christoph Wiesner for supporting me with writing my Bachelor thesis 1 and 2.
Additionally a huge thank you goes to my colleague and friend Hannah Proetsch-Gugerbauer, who shared this 7-month adventure with me and was always there if I needed something.
I also want to thank the Marshall Plan Foundation and the Top NOE Stipendium for their financial support.
And finally I want to thank my family and my friends for their understanding, their mental support and the numerous and long phone calls.

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List of Abbreviations

CPMCRI	California Pacific Medical Center Research Institute
DCAF14	DDB1 and CUL4 Associated Factor 14
PHIP	Pleckstrin homology domain-interacting protein
GBM	Glioblastoma
shRNA	small hairpin RNA
BSA	Bovine serum albumin
WB	Western Blot
IF	Immunofluorescence
GAPDH	Glyceraldehyde-3-phosphate
FBS	Fetal bovine serum
siRNA	Small interfering RNA
APS	Ammonium Persulfate
SDS	Sodium Dodecyl Sulfate
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline + Tween 20
IGF1R	insulin-like growth factor 1 receptor
PDX	Patient-derived xenograft
cDNA	copy DNA
PCR	Polymerase Chain Reaction
KD	Knockdown
GFAP	glial fibrillary acidic protein
EGFR	epidermal growth factor receptor
pAKT	Phosphorylated AKT
CCND1	Cyclin D1
EtOH	Ethanol
kDa	Kilo Dalton

PBS	Phosphate buffered saline
SDS-Page	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
RISC	RNA induced silencing complex
Ago	Argonaute
mRNA	Messenger RNA
RT-PCR	Real-time PCR
CT	cycle at threshold
GOI	Gene of interest
MetOH	Methanol
ECM	Extracellular Matrix
AB	Antibody
AED	Antiepileptic Drugs
DAPI	4′,6-Diamidin-2-phenylindol

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1. Abstract

Brain cancer has an extremely poor prognosis, with only a few targets for therapy identified to date. It is one of the most resistant cancers to chemotherapy, radiation and targeted therapies. Therefore, biomarkers of prognosis and new targets for therapy are needed. Approximately 23,800 people will be diagnosed in 2016 with brain or nervous system cancer in the United States, and almost 68% of them will eventually die from the disease. [1] The average survival rate is 14.6 months. [1, 2]

Among children and adolescents brain cancer has surpassed leukemia as the leading cause of cancer death due to the improvement in targeted therapies in leukemia.

The expression of DCAF14 in 3 commercially available human glioblastoma cancer cells was recently detected. It is known that this protein is involved in the insulin receptor signaling axis which is part of a key cancer proliferation pathway. Very recently, DCAF14 has been reported as a key factor in the initiation of DNA replication in mammalian cells.[3]

The aim of this study is to evaluate the effects of the suppression of DCAF14 expression by cellular and molecular biology techniques, in the progression of the U251 human glioblastoma cell model *in vitro* and *in vivo.* After knocking down *DCAF14* gene, the impact on the cell proliferation, migration and invasion behavior of the cells was assessed *in vitro*. Furthermore *in vivo* mouse models using human tumor xenografts have become a very promising tool for oncology research as well. [4]

Although the knowledge about brain cancer has increased a lot in recent years, the progress in the treatment and cure of this disease remains still poor. Therefore the basic research *in vitro* and the development of *in vivo* models is important for the validation of new drug treatments and therapeutic approaches.

Previously the impact of modulating the expression of *DCAF14* gene was evaluated in different cancer types. This study is focused on the impact on the brain cancer progression.

2. Introduction

For the treatment of brain cancer there are only a few different treatment options and therapeutic approaches known until now.
Glioblastoma (GBM) is the most aggressive form of human brain cancer.
Among children and young people, brain cancer has surpassed leukemia as the leading cause of death due to the better therapies for leukemia. This study is focused on adult brain tumors which are called glioblastoma.

2.1. Brain cancer

A brain tumor is a collection of cells that grow abnormally in the brain. Any uncontrolled growth inside such a restricted area can cause problems and life-threatening conditions. Although brain tumors rarely spread to other parts of the body, most of them can spread within the brain parenchyma. Even benign tumors can, as they grow or press on and destroy normal brain tissue, causing damage that is often disabling and harmful.[5]

Brain tumors can be cancerous, which is called malignant or noncancerous which is called benign. Benign tumors do not spread into other nearby tissues, so there is no metastasis in other parts of the body. The reason why malignant tumors are so dangerous is due to their ability to spread throughout the body which is called metastasis.[5]
Due to that, doctors usually speak of “brain tumors” rather than “brain cancers.” The main concerns with brain and spinal cord tumors are how readily they spread through the rest of the brain or spinal cord and whether they can be removed and not come back. [5]

Brain and spinal cord tumors tend to be different in adults and children. They normally form in different areas, develop from different cell types, and might have another outcome and treatment.

Glioblastoma

Glioblastomas (GBM) are tumors that develop from astrocytes and supportive tissue of the brain. Glioblastomas are very rich in blood vessels and are therefore mostly the primary and malignant type of tumor that is affecting the brain or central nervous system (CNS) [6] [7]

Location in the brain

Glioblastomas are normally found in the cerebral hemispheres of the brain, but can be also located in the spinal cord or in other regions of the brain.
Towards the center of the tumor also dead cells can be seen. Due to the development of the tumor from normal brain cells it can easily spread into normal brain tissue and have a high invasion potential. However, glioblastoma normally do not migrate to some other parts in the body except the brain.[8]

Types of glioblastomas

Primary: These tumors tend to form quickly and show their presence in the brain. It is the most common form of glioblastoma and also very aggressive.
Secondary: This type of glioblastoma have a much longer and slower growth, but they are still aggressive. Normally these tumors begin as lower grade tumors and become higher grade over time. It is mostly found in people around 45 years old and younger and represents approximately 10% of glioblastomas.

Symptoms

Due to their rapid growth the most common symptom of glioblastomas is caused by increasing pressure in the brain. These symptoms can be headache, vomiting, and nausea. Patients can also have a weakness on one body side, speech or memory difficulties, depending on the location of the tumor.

Incidence

Glioblastoma represents about 15.4% of all primary brain tumors and approximately 70% of all astrocytomas. The frequency increases with age and more men than woman are affected. Only 3% of childhood brain tumors are glioblastomas.

Treatment of brain cancer

Treatment for brain tumors is based on many factors:

- Age, overall health, and medical history
- Type, location, and size of the tumor
- How likely the tumor is to spread or recur
- Tolerance for specific medications, procedures, or therapies

Treatment for these symptoms include:

Antiepileptic Drugs (AEDs)

Steroids

Surgery

Low-grade tumors (grade I and II), which are not aggressive, are treated with monitoring or surgery alone. Although all tumors are monitored with repeat scans, grade II tumors are watched more closely after surgery and over time to make sure there is no recurrence.

Higher grade tumors (grade III and IV), which are malignant and grow very quickly, are more difficult to remove and require additional treatments beyond surgery, such as radiation, chemotherapy, or a clinical trial if one is available. Microscopic tumor cells can remain after surgery and will eventually grow back. Therefore all treatment options are intended to prolong and improve life for as long as possible.[9]

Additional treatment options for high-grade tumors include:

Radiation therapy: X-rays and other forms of radiation can destroy tumor cells or delay tumor growth.

Chemotherapy: The use of drugs to kill rapidly dividing cells. It can be taken orally or intravenously.

Targeted therapy: The focus on a specific element of a cell, such as molecules or pathways required for cell growth, in order to use them as a target. [8] [9]

2.2. DCAF14-PHIP

The gene *DCAF14* is one of the gene alias for PHIP (Pleckstrin homology domain-interacting protein).
This protein binds to the Pleckstrin homology (PH) domain of insulin receptor substrate-1 (IRS1), which modulates the insulin signaling pathway and plays a role in pancreatic beta cell growth and survival.[10]
DCAF14 not only triggers cancer and promote tumor growth, but it also increases the metastatic potential, by increasing its invasiveness.
It was demonstrated that *DCAF14* overexpression stimulates the insulin-like growth factor 1. This growth factor is either dependent or independent on proliferation of beta-cells, an event which also correlates with transcriptional upregulation of Cyclin D2. [11]

2.3. DCAF14 role in cancer

ShRNA-mediated suppression of *DCAF14* resulted in significant suppression of cell invasion and metastatic potential. High levels of *DCAF14* expression were associated with significantly reduced survival in both *in vitro* experiments and *in vivo* mouse models. [12]
DCAF14 gene locus is 6q14 and it encodes a protein which is a regulator of insulin and insulin-like growth factor signaling pathways. Furthermore, *DCAF14* stimulates cell proliferation by regulation of cyclin transcription. *DCAF14* plays a role in regulating cell morphology and cytoskeletal organization. [13]
This gene also associates with insulin receptor substrate (IRS)-1 and IRS-2 and has also shown a stimulation of cell proliferation and an inhibition of apoptosis. Therefore, it can serve as a marker for aggressive metastatic potential. [14] [3]

Due to the same gene alias of DCAF14 and PHIP, in the further sections of this thesis PHIP is mentioned as the target gene of this study.

2.4. IRS-1 pathway and connection to PHIP

The insulin-like growth factor receptor type 1 (IGF1R) signaling pathway has been acknowledged to play an important role in tumorigenesis. [15] Binding of IGF1 or IGF2 to IGF1R results in phosphorylation of tyrosine and carboxyl-terminal serine residues that form binding sites for the insulin-receptor substrate (IRS) docking proteins. IRS activation results in AKT activation. [16] Efficient docking of IRS proteins is mediated via their pleckstrin homology domain. [17]
Pleckstrin homology domain-interacting protein, which is the main focus in this study, was identified through interactions with the pleckstrin homology domain of IRS proteins.
In recent years, *PHIP* was identified as the gene most highly overexpressed in metastatic melanomas, compared with primary tumors by cDNA microarray analysis. [18]

The insulin-like growth factor (IGF) pathway is a complex pathway involving interactions between membrane-bound receptors, ligands, binding proteins, downstream effectors, and other receptor tyrosine kinase signaling cascades. The IGF pathway has been identified as a potential therapeutic based on the following provocative factors. Preclinical observations have shown that this pathway is involved in tumor cell proliferation, survival, migration behavior and invasiveness. [19]
In addition, IGF1R protein expression is found in a significant number of cell tumor specimens. Initial therapeutic efforts involved the development of monoclonal antibodies and tyrosine kinase inhibitors that target IGF1R, a transmembrane receptor tyrosine kinase. [20]

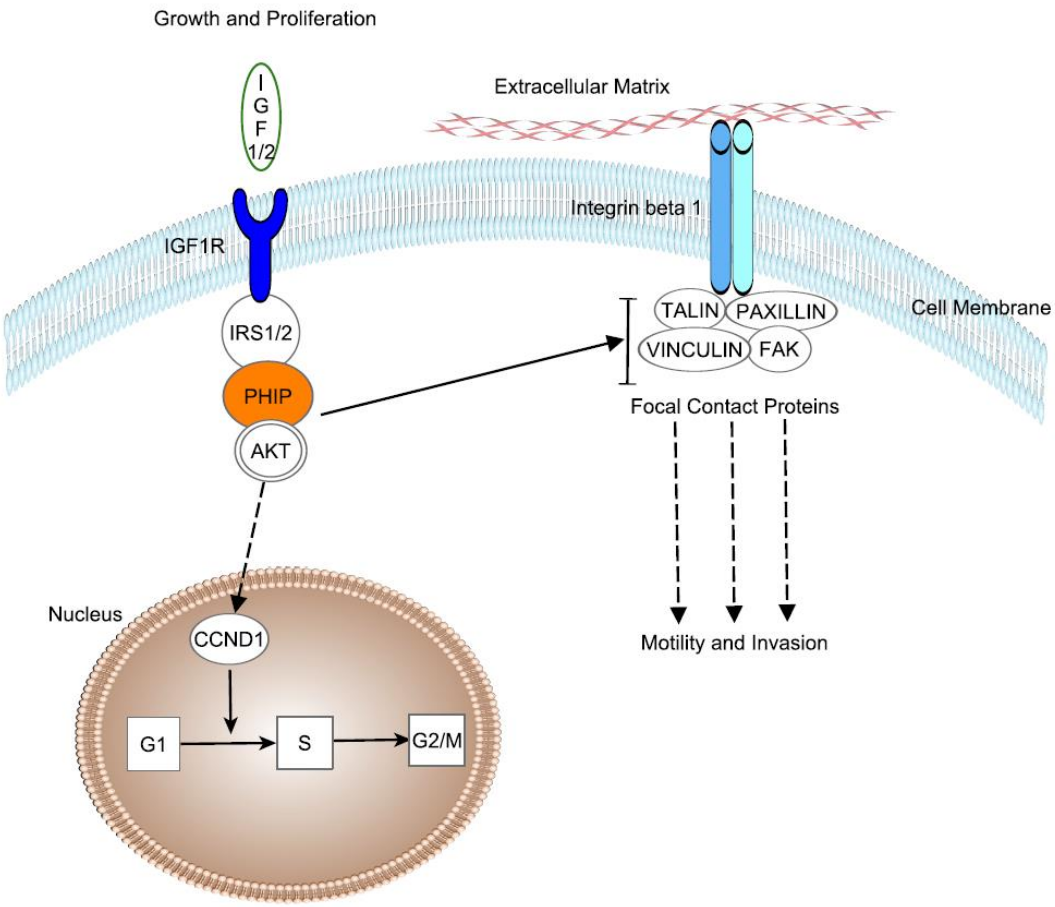


Figure 1: PHIP in IGF signaling pathway

This figure created with the Ingenuity Pathway Analysis software shows the signaling pathway of IRS 1. IGF 1/2 is binding to the IGF1R receptor embedded in the cell membrane. [21] To the receptor-ligand formation the Insulin receptor substrate 1/2 is binding. This interaction leads also to the binding of PHIP. PHIP has an impact on AKT, which drives the expression of CCND1 in the nucleus and has therefore an impact on the cell cycle and especially the S phase. PHIP plays a role in the phosphorylation of AKT. [22]

Furthermore PHIP has also an impact on the organization of focal contact proteins Vinculin, Talin, Paxillin and FAK, which are binding to integrin beta 1 in the cell membrane. This focal contact proteins have an impact on motility and invasion behavior.

Therefore, PHIP plays not only a role in cell cycle but also in the migration and invasion capabilities of tumor cells.

2.5. U251 cell line

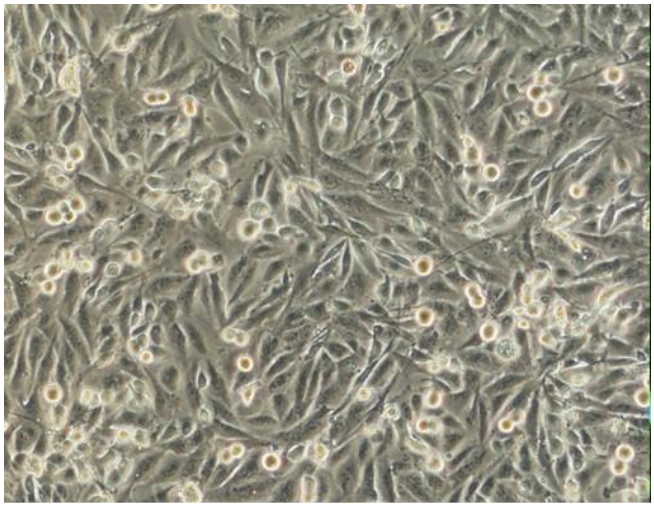


Figure 2: U251 cell line 40x magnification

Cell Line Description

U251 is a permanent cell line derived from a malignant glioblastoma tumor by explant technique. This cell line originates from a 75 year old male with astrocytoma, grade 3-4. [23] Astrocytoma is a type of brain cancer that developed from glial cells called astrocytes.
This cell line expresses the epidermal growth factor receptor (EGFR) and glial fibrillary acidic protein (GFAP) over many cell passages. [24] [25]

Properties

Table 1: Properties of U251 cell line

biological source	Brain from human
growth mode	Adherent
karyotype	2n = 46
morphology	Fibroblastic pattern
products	GFAP positive cells
receptors	EGFR, PDGFR alpha
application(s)	cell culture | mammalian: suitable
Storage	Liquid nitrogen

2.6. RNA interference- Mammalian shRNA tools

In the past years, the use of RNAi has become an important tool for the study of gene function in mammalian cells. The sequence-specific degradation is the basic mechanism of RNA interference. RNA interference (RNAi) is an RNA-mediated gene silencing mechanism. As a tool in mammalian cell systems, silencing is achieved through the delivery of a double-stranded RNA (dsRNA) that matches the mRNA target sequence.
The dsRNA can be delivered as a siRNA (short interfering RNA) via transfection, or shRNA (short hairpin RNA) via transfection or viral delivery of a plasmid. [26]
Luciferase shRNA control and PHIP shRNA were cloned into a plasmid. [26]
The introduction of shRNA into mammalian cells through infection with viral vectors allows a stable integration of shRNA and long-term knockdown of the targeted gene, which is in this case PHIP. [27]
RNA interference knockdown of PHIP in Insulin like growth factor 1 cells abrogates insulin receptor substrate 2 (IRS2)-mediated DNA synthesis, providing for a specific role for PHIP in the enhancement of IRS2-dependent signaling responses leading to beta-cell growth. [11] The degradation of host mRNA happens through the cytoplasmic delivery of double-stranded RNA (dsRNA) identical to the target sequence. The reduction of target gene expression is achieved through an enzymatic pathway involving the endogenous RNA-induced silencing complex (RISC). One strand of the siRNA duplex (the guide strand) is loaded into the RISC with the help of Argonaute (Ago) proteins and double-stranded RNA-binding proteins. The RISC then localizes the guide strand to the complementary mRNA molecule, which is subsequently cleaved by Ago. The cleaved mRNA is further degraded by other endogenous nucleases. The RISC also plays also an important cellular role in inhibiting endogenously derived mRNA through a related micro-RNA (miRNA) mechanism.
shRNAs may be transfected as plasmid vectors encoding shRNAs transcribed by RNA pol III or modified pol II promoters, but can also be delivered into mammalian cells through infection of the cell with virally produced vectors. shRNAs are capable of DNA integration and consist of two complementary 19–22 base pairs RNA sequences similar to the hairpin found in naturally occurring miRNA. Following transcription, the shRNA sequence is

exported to the cytosol where it is recognized by an endogenous enzyme, Dicer, which processes the shRNA into the siRNA duplexes.

Like the exogenously delivered synthetic siRNA oligonucleotides, this endogenously derived siRNA binds to the target mRNA and is incorporated into the RISC complex for target-specific mRNA degradation.

Although siRNA and shRNA ultimately utilize a similar cellular mechanism (RISC), the choice of which method to use depends on several factors such as cell type, time demands, and the need for transient *versus* stable integration. [27]

2.7. Transient (siRNA) *versus* stable (shRNA) integration

Transfection is the process of introducing DNA or RNA into a cell via a non-viral method such as a lipid or a polymer. In this process genes can be manipulated to be overexpressed or silenced based on the sequence specific targeting of genes (RNAi techniques).
The application of the transfection allows to analyze gene function or determine disease pathways.

Transient Transfection of siRNA, microRNA, mRNA, or plasmid DNA

Transient transfection occurs when the transfected DNA or RNA are introduced into the cell but are not consistently expressed or incorporated into the host cell genome.
The nucleic acids are expressed for a short time manner but are eventually recognized as foreign genetic material and ultimately degraded. The main advantage of the transient transfection is the rapid production of recombinant proteins that have a high quality and are activated and post-translationally modified.[28]

Gene Knockdown: Stable RNAi Cell Line Development

Stable RNAi cell lines, which are also known as knockdown stable cell lines, have a shRNA or a plasmid DNA construct integrated into the cellular genome to consistently silence the target genes.
Although only few of the transfected cells will incorporate the foreign nucleic acids into their genome, the use of antibiotic resistance marker genes has been demonstrated to be very effective for specific selection. The cells that show the ability to proliferate and grow in the presence of an antibiotic selecting agent can be isolated and characterized.

Although the generation of stable RNAi-expressing cell lines is a long and complex process, they are a very useful tool for analyzing effects of altered gene expression on cellular physiology, pathway signaling, and so on. [28]

3. Materials and Methods

First of all a lentivirus containing an anti-PHIP shRNA and a selectable marker gene (Puromycin) as well as a control virus targeting an irrelevant gene, in this case Luciferase, was generated and used to stably infect the brain cancer cells. [27, 29]
The cells that have integrated the desired shRNA were selected and PHIP expression/downregulation confirmed via quantitative RT-PCR, western-blot analysis and quantitative immunofluorescence.
As mentioned before, the reduction in protein expression should lead to a change in cell behavior which will be studied by cell proliferation assays such as cell cycle analysis, colony formation and cell survival and other assays including migration and invasion assays. The impact on the progression of brain cancer was monitored by the use of *in vivo* mouse models.

U251 cell line 127738 (code for PHIP shRNA) was bought from the company Open Biosystems. In the results section this cell line is described as U251 PHIP shRNA and the control cell lines a U251 Luc shRNA.

3.1. Lentivirus preparation

Lentiviruses are generally used to create a stable knockdown cell line by introducing small hairpin RNA (shRNA) into the host genome. The shRNA is designed to match the target sequence by forming a complementary strand to the desired sequence.
To ensure that the virus cannot replicate itself a few safety measures need to be taken by deleting genes from the original viral genome and by splitting the vectors.
A third generation lentiviral vector system consists of four vectors or plasmids. To simplify the transfer plasmid contains the shRNA (or cDNA) to be introduced into the cell. The Envelope Plasmid codes for envelope proteins in order to bind to the cell and thereby depends on the cell type to be infected but usually expresses the VSV-G gene due to its broad infectivity. The packaging vector is split into two plasmids in order to enhance safety and contains important genes such as Gag, Pol and Rev. Gag is encoding for the capsid,

matrix and nucleic capsid proteins. Pol encodes for the reverse transcriptase enzyme which is converting the single-stranded viral RNA to double-stranded DNA in order to integrate it into the genome. The rev gene is assuring the transportation and expression of the gene to be delivered. All promotors are CMV (human cytomegalovirus) instead of long terminal repeats. [29]

A commercially available lentiviral plasmid from Openbiosystems containing a Puromycin resistance gene and either a shRNA (short-hairpin RNA) targeting PHIP gene or a shRNA targeting an irrelevant gene (luciferase) as control to infect brain cancer cells was used to generate lentiviruses. This is accomplished by a quadruple transfection of 80-90% confluent HEK293T (human embryonic kidney) cells in 10 cm petri dishes with 3 helper plasmids (from Addgene) containing the viral capsid and reverse transcriptase genes (REV, VSVG and RSV) plus the plasmid coding for the respective shRNA by Lipofectamine 2000 reagent (from Invitrogen) in plain culture medium without fetal bovine serum (FBS, from JR Scientific). Five hours post-transfection, the plain culture medium was replaced by medium containing 5-10% FBS. Two days post-transfection, lentiviral particles were collected by centrifugation of the medium supernatant at 1000 rpm for 5 minutes.

Approximately 9 ml of culture medium containing the lentiviruses were aliquoted into 1.5 ml Eppendorf tubes and stored in the -80°C freezer. At the time of infection, when petri dishes with cells were confluent at approximately 50%, the polycation polybrene reagent (from Sigma) was used at 8µg/ml to increase the infection efficiency of previously thawed lentiviruses and medium containing FBS previously inactivated for 30 min at 56°C. One day post-infection, the culture medium was replaced by culture medium containing regular FBS. Three days post-infection, cells were exposed to puromycin at 10 µg/ml and only the infected cells should be resistant to the selectable drug. Then, after drug selection for a week, the expression of PHIP and a housekeeping gene by quantitative Real Time-PCR using TaqMan gene specific probes (from Lifetech) was monitored.

RNA extraction

For this purpose, RNA was collected first from infected cells by using a RNA extraction kit (from Qiagen), and its concentration will be quantified by a spectrophotometer (Nanodrop, from Thermofisher)

cDNA synthesis & TaqMan

A 1 µg of RNA input was used in the cDNA synthesis reaction that was performed in a PCR machine (from Applied Biosystems) by using a specific cDNA synthesis kit (from Biorad) in 200 µl PCR thin wall tubes. A 1:5 dilution with RNAse free water of the cDNA was used in the TaqMan reaction carried out in an optical 96-well plate in the 7900HT PCR machine (from Applied Biosystems). Relative gene expression was quantified by using the 2-dCT *100 formula as per manufacturer's recommendations and compared to the relative expression of a housekeeping gene (HPRT, UBC or GAPDH).
To carry out a TaqMan, the cDNA TaqMan is mixed with a master mix solution and the specific probe/primer set following manufacturer's protocol.

Mastermix: 18µl x number of samples
H_2O: 13µl x number of samples
20X probe/primer set: 1µl x number of samples

The PCR tubes already contained 5µl of cDNA solution. To this volume 32µl of the prepared master mix were added. Each sample from the PCR tubes was pipetted in the TaqMan plate in triplicates with a used volume of 11µl. Then the plate was sealed and put into the TaqMan machine for evaluation.

The 7500 Fast RT-qPCR machine gave the operator an output showing the amplification curves and their corresponding cycles at threshold (C_T – values). The threshold cycle is the intersection of a set threshold value and the amplification curve of every single sample.

An average C_T for the triplicates of every sample for every probe has to be calculated first. Then the difference in C_T between the housekeeping/control gene and the gene of interest (GOI) is calculated by substracting the C_T of the GOI from the C_T of the housekeeping gene. After this the relative expression normalized to the housekeeping gene was calculated using the formula:

$$\%_{rel.Expression} = 2^{-dC_T} * 100.$$

The Knockdown in percent is calculated with the help of the formula:

$$\%_{Knockdown} = 100 - (\frac{\%_{rel.Expression\ GOI\ in\ Knockdown}}{\%_{rel.Expression\ GOI\ in\ control}} * 100)$$

Finally to plot the bars the percentage of GOI expression in the two subjects is calculated by substracting the percent of expression from 100.

3.2. Transfections

Generally, 5000-10000 cells were plated in either a 96 well plate, 6- well plate or in small P60 plates. After 24 hours if the cells are fully attached a transfection can be done.
Therefore, different vectors are used to transfect the cells. Luciferase vector was used as a control vector and PHIP shRNA as test vector.
First of all media without FBS were used due to the reason that the FBS would interfere with the transfection process. A different amount of plasmid was used for transfections and upon this the amount of vectors or Lipofectamine was calculated.
Then the FBS free medium was transferred to Eppendorf tubes and the plasmids and Lipofectamine were added on top. After the corresponding DNA was put on top of the Lipofectamine the mixture was incubated for 20 minutes.
After the incubation time, the remaining media in the plate was aspirated and 100 µl of medium was put inside the well. Then 50 µl of the incubated mixtures of Lipofectamine and plasmid was pipetted into the well of a 96 well plate. After 5 hours, the media was changed to the respective FBS containing media of the cell line.

3.3. Western Blot

3.3.1. Protein extraction

For the extraction of the proteins the collected cell pellet was resuspended in 50-100 µl RIPA lysis buffer (Thermo Scientific, Prod# 89900) depending of the size of the pellet to which a 100x Protease and Phosphatase inhibitor (HALT, from Thermo Scientific, Prod# 1861281) was previously added in order to dilute it to 1x.
The mixture was then transferred to a new Eppendorf tube and by using a 30 G syringe the cells were again lysed carefully. The tube was then placed on ice for 20- 30 minutes and after that centrifuged for 10 minutes at maximum speed at 4 °C. The supernatant was transferred to a new Eppendorf tube and is either kept on ice if used directly or stored in -20°C if not used immediately.

3.3.2. Protein quantification - Bradford assay

After protein extraction, the samples were measured in triplicates in order to get more reliable results. 160µl HBS and 40µl of the Bradford reagent (Bio Rad, Cat# 500-0006) were added per well and also to the wells for the standards.
First the volume of the stock solution was determined by using the following formula: (number of samples x 3) + 10 (for the standard curve) = x?
With this result the needed amount of HBS and BioRad was calculated:
HBS = 160µl x ?
BioRad = 40µl x ?
Then 1µl of the samples were added to the mixture and every sample was distributed evenly in the well. The standard curve was prepared by a serial dilution of 40µg, 20µg, 10µg, 5µg, 2.5µg and 0µg (Blank) of BSA in duplicates. The concentration of the samples was measured via a spectrophotometer microplate reader and the SoftMax Pro software.

3.3.3. SDS-Page Electrophoresis

Before starting the electrophoresis the separation gel has to be prepared. Depending on the protein size to be detected, the percentage of acrylamide is adjusted.

Table 2: Composition of the different concentrations of separation gel

Concentration of gel	**5%**	**7.5%**	**15%**
H_20	5.7 ml	5.1 ml	3.5 ml
40% Acrylamide	1.125 ml	1.7 ml	3.375 ml
1.5M Tris	2 ml	2 ml	2 ml
10% SDS	80 µl	80 µl	80 µl
APS	75 µl	75 µl	50 µl
TEMED	8 µl	8 µl	8 µl

Table 3: Composition of the stacking gel (4%)

H_20	3.1 ml
40% Acrylamide	0.5 ml
0.5 M Tris-glycine	1.25 ml
10% SDS	55 µl
APS	50 µl
TEMED	5 µl

First the separating gel was filled into the tray, and after it solidifies the stacking gel has to be filled on top. After that the 10 well comb is inserted.

Then the proteins need to be prepared for the loading step. Therefore the amount of protein had to be calculated and has to be mixed with loading dye (Nu Page LDS Sample Buffer from Invitrogen, Cat# NP0007). The volume of dye added to the protein is ¼ of the volume of protein needed. This mixture was then heated in a heating block for 5 minutes at 95°C.

The samples as well as the protein marker (Page Ruler plus pre-stained from BioRad, Cat#26619) were loaded into the wells of the solidified gel. The gel in the tray has to be covered with 1x running buffer (TGS, 10x from BioRad, Cat#161-0772). An electrical field

between (80 and 120 V) was applied and the experiment was run until the bands of interest were well separated.

3.3.4. Protein Transfer

There are two different methods of protein transfer that were used in this project.
The wet transfer was used for larger proteins and semi-dry transfer for smaller proteins. For the semi-dry transfer the gel and the nitrocellulose membrane (from EMD Millipore) were placed in between 4 filter papers (2 on each side) and Transfer Buffer (1x TGS containing 30% Methanol) was used to make sure that every layer is wet, as well as the contact plates of the machine. The transfer was ran at 25V for 15 minutes.
For the wet transfer the gel and the membrane were covered with a filter paper and a sponge on each side and were put into a cassette. To assure a close connection between the membrane and the gel, a roller is used several times. It is very important to have the membrane and the gel in very close proximity to ensure a high transfer of proteins upon applying a specific voltage to be determined. The cassette was filled with transfer buffer and an ice tray to avoid overheating of the buffer. The run was performed in the cold room, another measure to avoid overheating, for 1 hour at 100V, or 2h at 75V, depending on the size of the protein of interest.

3.3.5. Antibodies and development

After the transfer was completed the membrane was blocked for 30-60 minutes in 5% milk made out of non-fat dry milk powder in 1xTBST (TBS 10X, Corning; Cat# 46-012CM), (Tween-20, Santa Cruz Cat# sc-29113).The membrane was then put into a plastic pouch into which the corresponding primary antibody was added in the correct dilution (given by supplier) in 5% milk in TBST. The pouch was then sealed and left on the shaker overnight in the cold room at 4°C.
After the overnight incubation three 10 minute washes with TBST were performed to remove all the unbound antibodies. Then, the HRP-conjugated secondary antibody is added. After an incubation of 1 hour, the antibody is removed and the membrane is washed again three times. The secondary antibodies used were either anti-mouse

(BioRad, Cat#170-6516) or anti-rabbit (BioRad, Cat# 172-1019) depending on the primary antibody.
As a last step, three 10 minute washes were done until the “Western Blotting Luminol Reagent” (Santa Cruz Biotechnology, Cat# sc-2048) was added to the membrane and then it was developed in an X-ray film developer in the dark room.

3.4. Proliferation

3.4.1. Cell cycle

To determine in which stages of the cell cycle the cells are, a cell cycle analysis was performed as soon as the plate was confluent. The media was aspirated and the cells were washed with PBS. Then the cells were trypsinized and the cell solution was put into a 15ml tube and centrifuged at 1,000 rpm for 2 mins. The supernatant was aspirated, roughly 1ml of PBS was added to the pellet to get rid of all the remaining media and the tube was centrifuged again at the same conditions. After this centrifugation step the supernatant was again aspirated except 10-20µl were left in the tube. Then 200µl of the cell cycle kit solution were added to the 15ml tube which were used to suspend the pellet and transfer it into an Eppendorf tube. The tube was left in the dark for about 30 mins. Then the lid was cut off and the tube was put into the cell cycle machine for its analysis.
The samples could also be stored in between to continue the processing at a later point of time. Therefore, the pellet was not suspended in the cell cycle kit solution but in 100% EtOH. The ethanol was added dropwise to the 15ml tube by using a vortexing machine. It was important to add the ethanol drop by drop to avoid the formation of cell clumps because once clumps appear, it is impossible to break them up again which would lead to a misleading of the results obtained by the cell cycle machine.

3.4.2. Colony formation

5000-10000 cells were plated in 6 well plates with the corresponding growth medium and were incubated under normal conditions for 6 to 10 days. After colonies were seen at the bottom of the wells with the bare eye the medium was removed and the colonies were stained with crystal violet solution (Sigma Aldrich, Cat# HT90132-1L) for 10 minutes and the wells were then washed with water until colonies can be counted.

3.4.3. Cell survival

For the cell survival assay 5000 or 10000 cells were plated per well in a 96-well plate and were grown in the corresponding culture media for 4 days, whereas every time point is measured in quadruplicates. The absorbance of cells was measured every day at the same time point by adding 10 µl of the Dojindo cell counting kit-8 reagent (Supplier DOJINDO, 500 tests, # CK04) and reading the plate in a spectrophotometer microplate readerat 450nm.

3.5. Migration

3.5.1. Scratch assay

When cells are fully confluent, a scratch is made by using a pipette tip along the diameter of the plae. Floating cells are removing by aspiration and fresh medium is added.
It is important to create scratches of approximately similar size in the test cells and control cells to minimize any possible variations caused by the difference in the width of the scratches.
The dish is placed in a tissue culture incubator at 37°C for 8–18 h. The time frame for incubation should be determined empirically for the particular cell type used. The dishes can be taken out of the incubator to be examined periodically and then returned to resume incubation. A time frame of incubation is chosen that allows the cells under the fastest migrating condition to just achieve the complete closure of the scratch.
After the incubation, the dish is placed under a phase-contrast microscope, and several images are acquired to assess the extent of wound closure.

3.5.2. Transwell

For this assay 5000-10000 cells are plated in a non-coated insert containing media with very low FBS. Then the lower chamber is filled with high-serum media, so the cells are trying to migrate through the pores in the insert and through the chamber in order to reach the high-serum media. The cells that migrated through are then stained and counted.

3.6. Invasion assay

Coating and loading of cells

In this case, the insert has to be coated with matrigel (its concentration varies depending and how aggressive the cells are, from 5-9µg/µl), therefore matrigel was diluted in the desired concentration in the appropriate media (without FBS). Next the matrigel was added onto every filter and distributed evenly, so that the thickness of the coating is equal all over the membrane. The amount of matrigel used in this step determines the overall thickness of the coating but is usually 15µl-17µl. After 45 minutes at RT the matrigel solidifies and a specific number of cells can be added.

The trypsinized cells were counted using a counting chamber and were diluted so that approximately 150 000 cells per 200µl media (without FBS) were placed into every transwell. In the lower chamber 300µl of the proper media containing 20% FBS were pipetted in. It is important that there are no bubbles between the two chambers, if so, the upper chamber has to be readjusted. The plate was then incubated ON in the incubator.

Fixation, staining and counting

Before taking the plate out of the incubator a 2.5% glutaraldehyde in PBS solution and a 0.5% toluidine blue/2% Na_2CO_3 in ddH_20 solution were prepared.

After that the media from both chambers was removed and 500µl of the glutaraldehyde solution was filled into each lower chamber. Then PBS was filled into new (empty) wells of the 24-well plate and the upper chambers were transferred to these wells containing PBS only. Then the PBS was removed and 500µl of the toluidine blue/Na2CO3 solution was filled into these wells and was incubated for 5 minutes at RT.

After these 5 minutes the upper chamber gets “washed” in a beaker containing PBS only. This washing step was performed by carefully dipping the chamber into the beaker several times using a forceps. To remove the cells that did not migrate through the Matrigel and filter to top of the filter was carefully wiped several times with a cotton cutips. The chambers were placed back into empty wells and cells were counted under the microscope.

3.7. Phosphoarray

We used the R&D Systems™ Human Phospho-Receptor Tyrosine Kinase (RTK) Array Kit (Catalog # ARY001B) which is a screening tool designed to simultaneously detect the relative phosphorylation of 49 different RTKs.

This kit eliminates the need for numerous immunoprecipitation (IP) and/or Western blot experiments. RTKs are captured by antibodies spotted on a nitrocellulose membrane. Levels of Phospho-RTK are then assessed using an HRP-conjugated pan phospho-tyrosine antibody followed by chemiluminescence detection.

Carefully selected capture antibodies have been spotted in duplicate on nitrocellulose membranes. Cell lysate samples were diluted and mixed with a mixture of biotinylated detection antibodies. The sample-antibody mixture was then incubated with the array. Any protein/detection antibody complex present was bound by its cognate immobilized capture antibody on the membrane. Streptavidin-Horseradish Peroxidase and chemiluminescent detection reagents were added, and a signal is produced in proportion to the amount of cytokine bound. Chemiluminescence was detected in the same manner as a Western blot. [30] The first step of the Phosphoarray was blocking each array by adding 1.5 ml of Array Buffer to each well of the multi-dish. By the usage of flat-tip tweezers each array was removed from the protective sheets.

One array was placed into each well of the Multi-dish. The array number should be facing upward. This was incubated for 1 hour on a shaker. The tray was oriented so that each array rocks end to end in its well.

The next step was to dilute the lysate to 1.5 ml with Array Buffer. Then the Array Buffer was removed from the 4-well Multi-dish and the diluted lysates were added.

An overnight incubation was following at 2 - 8° Celsius on a shaker.

Afterwards each array was carefully removed and placed into individual plastic containers with a minimum of 20 ml of 1X Wash Buffer. The dish was rinsed with deionized or distilled water and dry thoroughly.
Then the array was washed with 1X Wash Buffer by soaking for 10 minutes on a shaker. This was repeated two times for a total of three washes.
1.5 ml of freshly diluted Detection Antibody were added to each well.
Then each array was again carefully removed from its washing container and returned in the 4-well Multi-dish and covered with the lid.
An incubation for 2 hours at room temperature on a shaker was following. Afterwards the washing steps as described before were repeated again. And each array was removed from the wash container and placed on a plastic sheet protector.
Each array was exposed to chemiluminescent reagents. Then the plastic wrap was covered and exposed to X-ray film for 1-10 minutes.

3.8. Immunofluorescence staining

Immunofluorescence Microscopy is a method to visualize cells or some of their proteins by using antibodies coupled to a fluorophore. 4′,6-Diamidin-2-phenylindol (short DAPI) is a fluorescent blue counterstain that can bind to the DNA in A-T rich parts. First of all, the culture medium was aspirated from the 6 well plate and cells were washed with Phosphate Buffered Saline (PBS, from Thermofisher) three times for five minutes. Next 4% formaldehyde in PBS was added for 10 minutes at room temperature to fix cells. After fixation, cells are permeabilized to enable the detection of intracellular antigens by antibodies. For this purpose, Triton X-100 was used. After 3 washing steps with PBS for 5 minutes, a 0.2% Triton X-100 in PBS solution was added for 10 minutes. After a rinse in PBS, cells were blocked by 3% Bovine Serum Albumin (BSA, from Sigma) in PBS for 10 minutes to reduce the unspecific binding of antibodies. Then, a primary antibody was used against the specific target. This type of antibody directly binds to the desired antigen and it is diluted with background reducing antibody diluent (from DAKO). This primary antibody was incubated overnight at 4°C. The day after, a secondary antibody was used which can directly bind to the primary antibody. Secondary antibodies are conjugated with Alexa fluorophores (from Lifetech) which have a specific excitation and emission wave

lengths. This secondary antibody was also diluted with background reducing antibody diluent. Before adding it, the wells were washed 3 times with PBS for 5 minutes. The secondary antibody was added for 1 hour at room temperature on an orbital shaker and covered by aluminum foil. Then, 3 additional washing steps with PBS for 5 minutes were performed again. Afterwards, the cells were dehydrated in ethanol series with 70%, 90% and 100% ethanol. As a last step, cells was covered by a Vectashield mounting medium solution (from Vector Laboratories) to mount the coverslip onto the microscope glass slide previously washed with 70% ethanol. The Vectashield solution inhibits photobleaching, prolongs the storage time and contains Dapi for a staining of cell nuclei. After Vectashield was dried with a kimwipe paper, cells were ready to be visualized by using a fluorescence microscope (from Zeiss).

3.9. *In vivo* experiments

For all the procedures in the safety cabinet, the hood is turned on and the airflow equilibrates for a few minutes. Heating pads are required to keep the body temperature of the animal. All the surfaces before procedure are sprayed with 70% ethanol and upon completion the surfaces are sprayed with MB10 chlorine solution. Surgical instruments are previously autoclaved.

Six week old female athymic nude mice, which weigh about 17-20 g were purchased from Envigo (California). The mice were hold in the CPMC animal facility and were maintained in a temperature and light controlled environment with a variant 12 hour light/dark cycle. The mice are more active at night.

Subcutaneous implantations or injections are done to prove if the cells are growing and a tumor graft is developing in the mouse before doing an intracranial injection. [31]

The intracranial methods are done for evaluation of tumor growth and drug treatment of brain cancer. [32]

For brain cancer orthotopic implantation tumor cells or tissue samples are used.

The mice are kept in accordance to the guidelines for animal studies and the tumor volume is measured regularly using a caliper. The measurement is carried out double-blinded to avoid bias and a change of the size of the tumor.

For this experiment the mice were kept alive as long as it was ethically justifiable, and where then euthanized according to guidelines and protocols established.

The statistical calculation of the tumor volume using the output of the tumor measurement is carried-out using the formula. $\frac{Length*width^2}{2}$

3.9.1. Subcutaneous implantation

The tumor growth of brain cancer cells expressing either the shRNA targeting the *DCAF14* gene or the control gene will be assessed upon subcutaneous implantation *in vivo* in nude mice. [33] Briefly, between 1 to 10 million cells will be resuspended in the corresponding plain culture medium and they will be injected subcutaneously with or without Matrigel (from BD).

The tumor volume will be measured and monitored biweekly by caliper measurement (Caliper Life Sciences) by using the formula:

$$Volume = \frac{length * width^2}{2}$$

Until the day of sacrifice the California Pacific Medical Center Research Institute's animal care guidelines are followed.

3.9.1.1. Transplant of tumor tissue

A small, horizontal incision is made by using surgical scissors upon anesthesia with isofluorane. The tip of the scissor is inserted into the incision and by cutting a piece a pocket in the subcutaneous space is created.
Then the tumor tissue is inserted into the pocket by the use of forceps. While the pocket is open a drop of 100X penicillin/streptomycin is dropped in the opening.
Afterwards the incision is closed with suturing material.

3.9.1.2. Injection of cell suspension

The prepared single cell suspension generally containing 50% Matrigel is mixed before injection to prevent the cells from settling down. Then the solution is withdrawn in the needle of a syringe. By using a forceps, the skin is lifted and the syringe is introduced at the base and the cells are released into that space when the animal is fully anesthetized with isofluorane.
For the injection a 21 G needle is used and 0.1 ml of the cell suspension (1×10^6 cells) are injected subcutaneously.
After each procedure the animal is put in its corresponding cage and observed for the next 5-10 minutes. When fully awake, the cage is then put back in the cage rack.

3.9.2. Intracranial injections

Surgical Area Preparation

A sterile surgical drape is placed over the heating pad and the tubing supplying the Isoflurane to the stereotactic instrument.
The surgical tools are placed on the drape to avoid contamination. Two scalpels are used, one for bone wax and one for opening the skin to expose the skull. A 25-gauge needle is used to drill the skull and make a little hole in which the Hamilton syringe containing cells will be inserted.

Preparation of cells for Injection

A single cell suspension containing $3x10^5$ cells per 4 µl of injection media (NB w/o growth factors or serum) is prepared. Afterwards the cells should be kept on ice prior to injection.

Preparation of Hamilton syringes

After the syringe is removed from the packet, it is sterilized by drawing and dispensing ethanol three times and then the ethanol is washed off with autoclaved water. The syringe is dried by pushing air through the needle several times until no more liquid comes out.
Then the plunger is removed and the syringe body is placed into the repeating dispenser frame. The plunger holder is loosened with a flathead screwdriver and the plunger is inserted into the body of the syringe.
The plunger is secured by tightening the screw on the repeating dispenser frame arm with a flathead screwdriver.

Preoperative Preparation of Animals

The animal is weighed and 10µl/g of the ketamine/xylazine solution is injected intraperitoneally. Afterwards the mouse is placed into an Isoflurane chamber (1-3% constant flow pressure of isoflurane mixed with oxygen).
After the anesthesia is completed, it is checked by a toe pinch on both paws of the mouse. The animals will be considered fully anesthetized when they do not withdraw their hind

paws when pressure is being applied. Then the mouse is placed into the stereotactic device.

Stereotactic Immobilization and Preparation of Injection Area

The snout of the mouse is secured by inserting the front teeth into a tooth holder by opening the mouth and displacing the tongue. The snout clamp is tightened and the ear bars are moved to touch the mouse one each side and then tightened 1 mm at a time until proper fixation has been achieved.
An eye lubricant is used due to the missing eye lashes of mice to not dry out during anesthesia. Afterwards the skull is wiped down with betadine and ethanol three times.
The scalp will be cleaned with Betadine. A skin incision about 15 mm in length will be made over the middle frontal bone, medial to lateral towards the side where the cells will be injected.
The connective tissue, that overlays the skull is cut away and allows the scalp to be more easily moved out of the way.
Then the bregma is located. A hole is drilled in the skull 2 mm mediolateral and 2 mm caudal (posterior to the coronal suture) to the bregma using a small gauge needle. The goal is to inject all the cells into the supratentorial region of the brain. [3]

Injection of the Cells

Before starting the cell suspension tube is mixed properly. Then 4.5µl of cell suspension are pulled up using the Hamilton syringe.
Afterwards 0.5 µl of the solution are dispensed by clicking the repeating dispenser to make sure there are no air bubbles in the needle or syringe.
The syringe is placed into the clamp and tighten down to ensure the syringe volume gradations are facing the user.
Then it is moved over the opening of the skull and slowly the needle is descended until the bevel passes the skull.
The electronic coordinate tracker is set to zero and within 1 minute the needle is descended down to 3.1 mm below the skull and then up to 3 mm. The release of a 0.1 mm gap allows the cell suspension to stay inside the brain.

The needle will pierce the dura and be lowered to a final depth of 3 mm. The cell suspension in 4 µl of sterile culture media will be injected very slowly (3-5 minutes).
Then the needle is raised 0.5 mm and the timer is started again. After one minute and thirty seconds the needle is raised another 0.5 mm. It is proceeded in that way until the needle can be removed from the skull.
During the surgery an optical saline solution will be periodically added to the open eyes of the animals, as needed due to prevent dryness.

Post Injection Animal Preparation

To seal the burr hole some bone wax is placed at the tip of a scalpel to prevent reflux of cells.
The incision is closed by pulling up on the skin and then pulling it over the injection site on each side until the wound is closed. Finally the scalp will be closed using a single surgical suture in addition to administration of 5-10 µl of liquid suture. Surgical suture is a medical device used to hold body tissues together after an injury or surgery.
The skin is brought together by the use of forceps and veterinary grade surgical glue is applied.
The mouse should be placed in a recovery cage on a warming pad until the animal exhibits consciousness and normal motility. The heating pad is necessary due to their body temperature loss and hypothermia.
The animals are monitored for signs of neurological deficits such as ataxia, head tilt and severe weight loss.
The following days the mice will be inspected for general health and to assure the wound has healed correctly. The mouse will be injected subcutaneously with 5 mg/kg Carprofen as needed to treat postoperative pain.

4. Results

4.1. Downregulation of PHIP using Lentiviral delivery systems

In order to confirm the gene knockdown two different molecular and cellular techniques such as TaqMan assay and western blotting were performed. Gene knockdown means that upon shRNA targeting, the *PHIP* mRNA is expressed at a lower level in a cell line compared to the control cell line.

This knockdown assessment was performed by culturing the cells in media containing Puromycin as a selectable marker to which the cells become resistant after infection with the specific plasmid.

As a negative control the U251 Luciferase shRNA cell line was used for the TaqMan analysis.

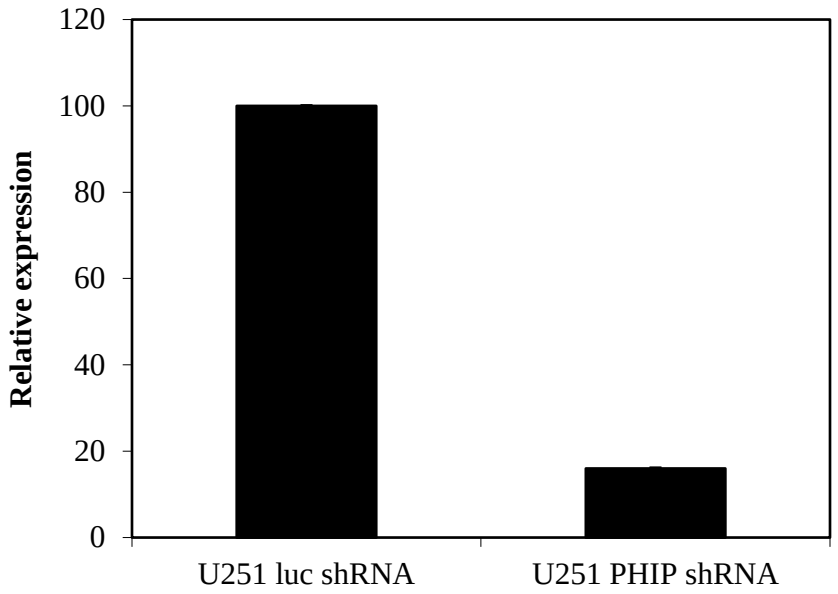


Figure 3: TaqMan Results showing PHIP knockdown

The relative expression of the TaqMan describes the control cell line which is 100 % relative expression and U251 PHIP shRNA which demonstrates a knockdown of 83%.

The *PHIP* mRNA levels of the luc shRNA infected U251 cells are considered as 100%. The standard deviation of three measurements of U251 *PHIP* shRNA tested for *PHIP* is 0.174 and for the used control gene the standard deviation is 0.037.

4.2. Western Blot

The figure above shows that both cell lines were starved for 3 days, which means grown without FBS inside the medium in order to synchronize them at the same cell cycle phase. The U251 *PHIP* shRNA cell line shows a 48% knockdown of PHIP as well a 67% Knockdown of phosphorylated AKT when compared to control cells.
The focal contact protein phosphorylated Paxillin and Cyclin D1showed a 47% and 42% knockdown respectively. The housekeeping protein GAPDH is used for normalization and

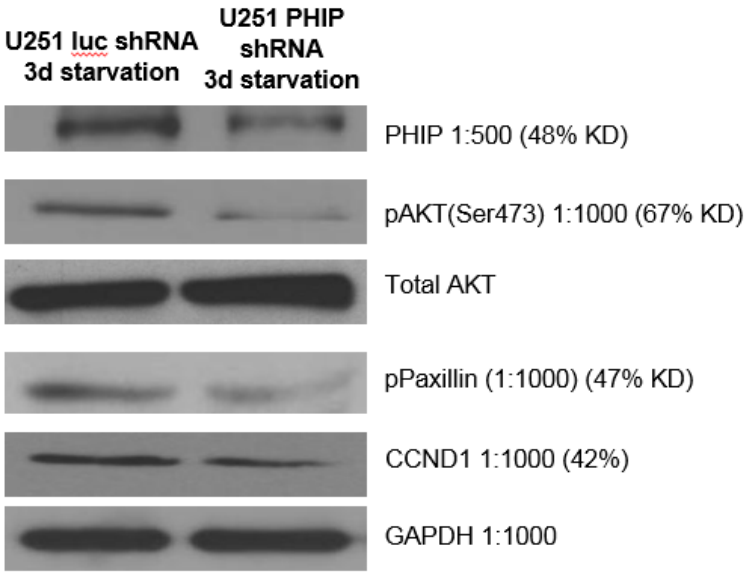


Figure 4: Western Blot of several proteins upon gene targeting in U251 PHIP shRNA cells when compared to control cells. The decreased bands indicates the percentage of knockdown. The knockdown is given as % KD

shows the same bands in the control cell line and the *PHIP* shRNA cell line. The decreased bands also indicate the percentage of knockdown
The shRNA cell line shows a decrease of phosphorylated protein bands compared to the total protein or housekeeping proteins.
1:500 or 1:1000 states the concentration of the antibody that was used for this Western Blot.

4.3. PHIP knockdown decreased cell proliferation

4.3.1. Cell cycle

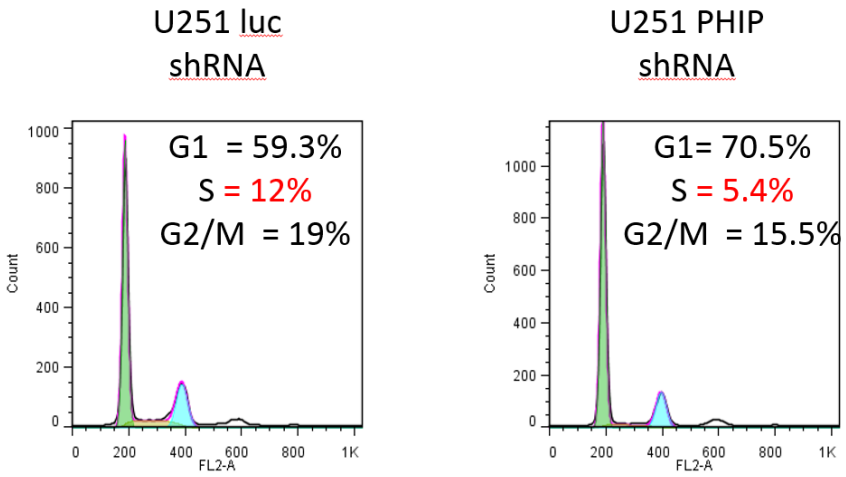


Figure 5: Cell cycle result comparison between U251 luc shRNA and PHIP shRNA

The figure above shows the cell cycle results comparing G1, S and G2 phase. The important parameter to consider is the S phase. This result shows a decrease from 12% to 5.4% in the S-phase. G2 phase or Mitosis Phase decreases as well. And the G1 phase shows a slight increase from the control cell line to the shRNA PHIP knockdown sample. Thus, reduced PHIP expression decreased the cell division of targeted cells.

4.3.2. Colony formation confirming the proliferation decrease

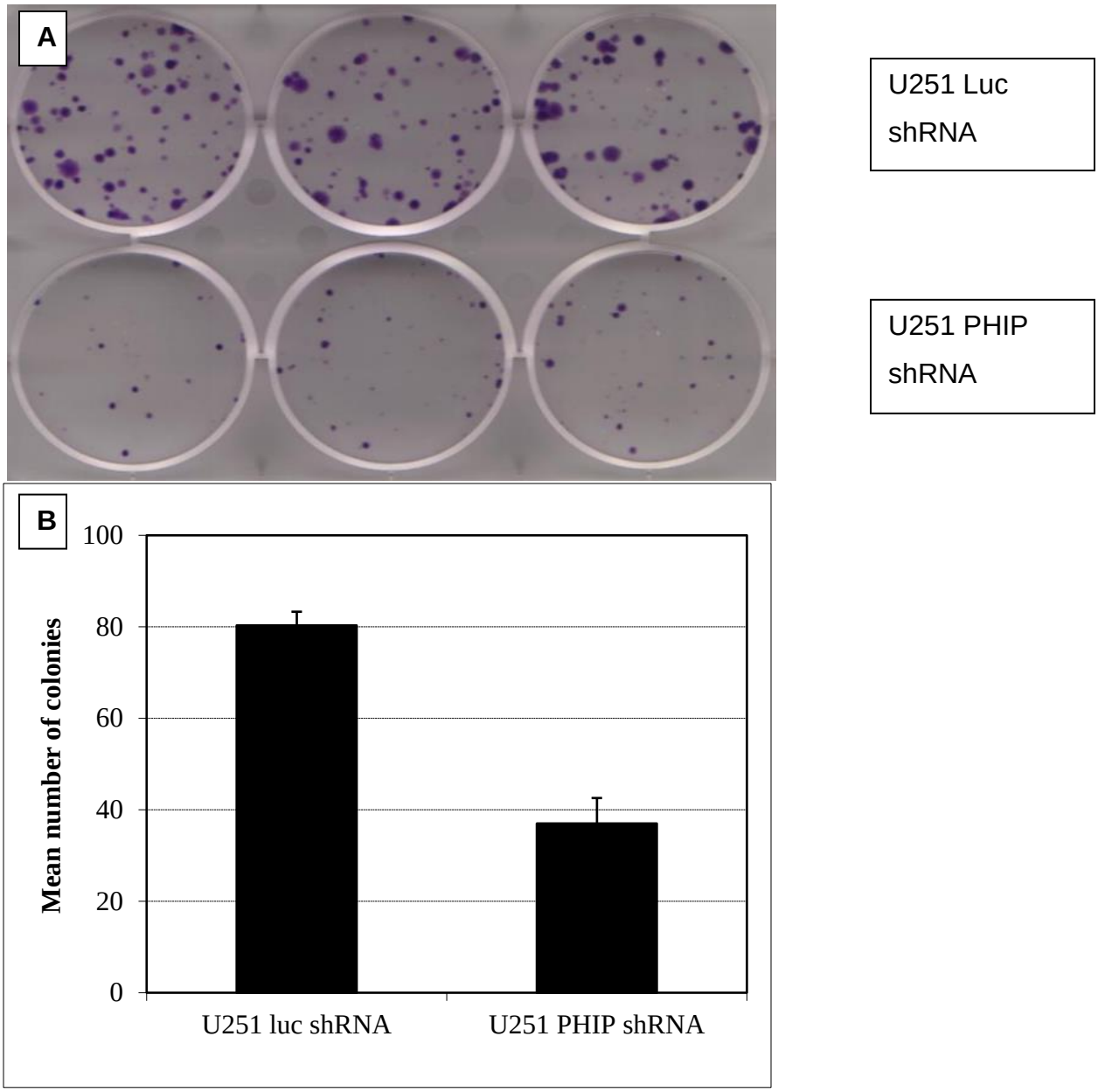


Figure 6: Colony formation results demonstrated on petri dishes and plotted in a diagram

The Colony formation assay was done to prove that the gene knockdown has an effect on the proliferation of the U251 cell line. The cell were grown for 7 days and then stained with a crystal violet stain.

Figure A shows the Colony formation after crystal violet stain comparing U251 Luc shRNA and U251 PHIP shRNA cell lines.
Figure B demonstrates the above mentioned results plotted in a diagram.
The diagram shows the difference in the mean number of colonies which indicates the same result as the picture of the 6-well plate. The control cell line U251 luc shRNA has a mean value of 80.3 and the U251 PHIP shRNA a mean value of 37.
The table also states the standard deviation and the TTest result of this numbers. P values are considered statistically significant when $p<0.05$

Table 4: Colony formation mean values

Cell lines	Mean value	Standard deviation	TTest
U251 luc shRNA	80.3	2.96	0.002351
U251 PHIP shRNA	37.0	5.57	Not measureable

4.3.3. Cell survival

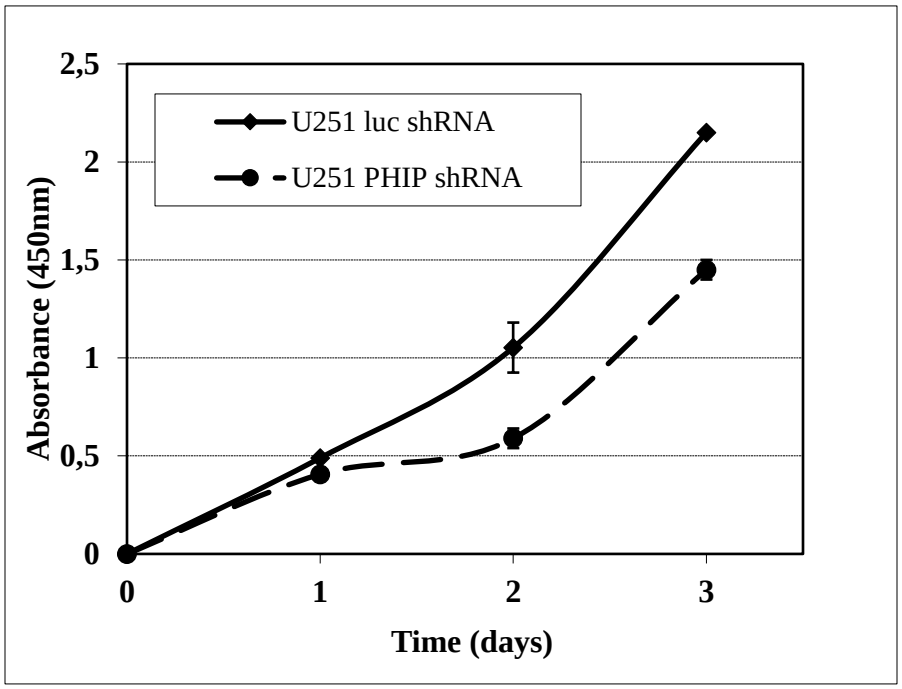


Figure 7: Cell survival curve of U251 cell line

For the cell survival assay either 5000 or 100000 cells were plated and grown over night. If the cells are attached on the following day the transfection is done. This procedure was described above.
After the cells were treated with the Dojindo reagent (Cell survival kit) at a concentration of 10 µl/100µl in a 96 well plate, the cells have to be incubated for 3-4 hours.
The cells were treated every 24 hours to create a timeline with 3 time points. Then the 96 well plate is read by using a microplate reader at an absorbance of 450 nm.
The outcome result shows less cell survival of the PHIP knockdown cell line compared to the control cell line. The U251 luc shRNA shows an increased cell survival. This is more visible after more than 2 days of incubation.

4.4. Migration

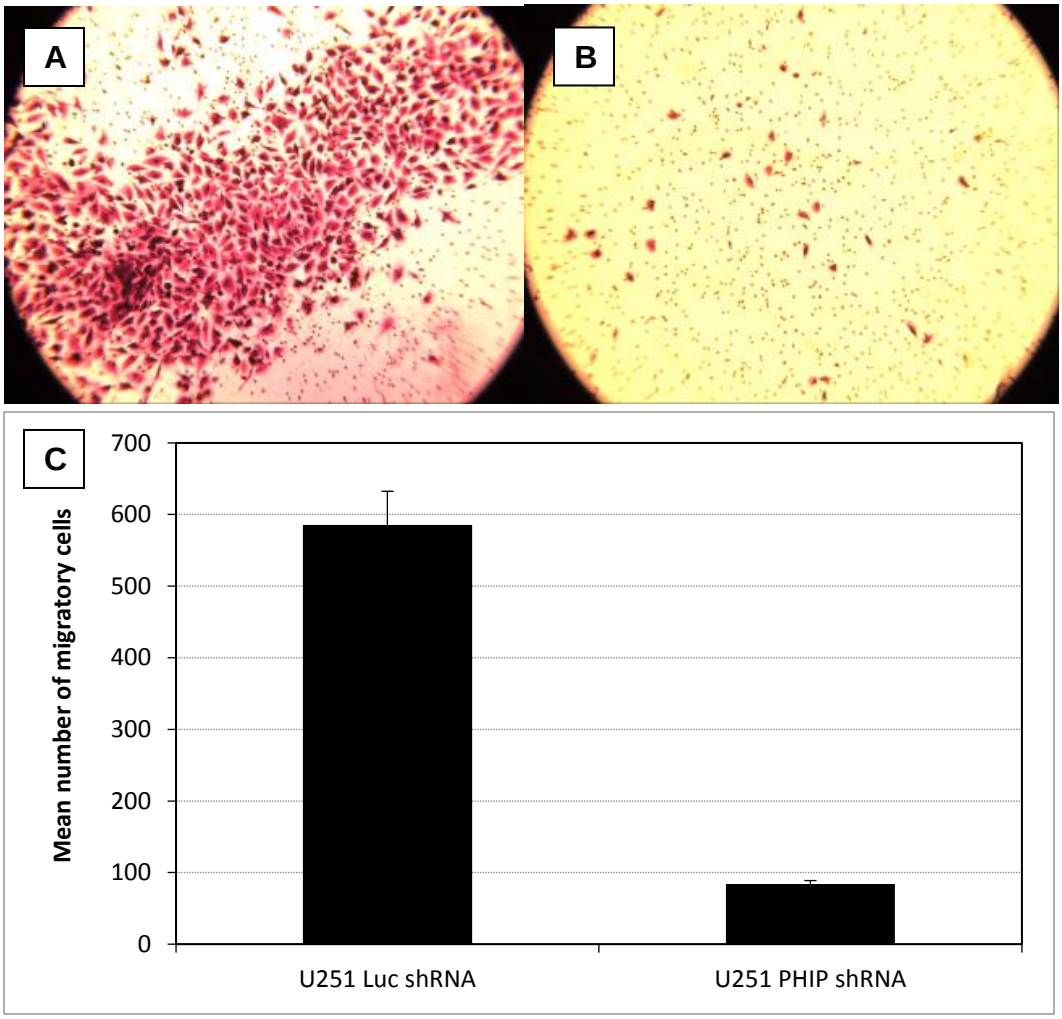


Figure 8: Migration assay results and diagram showing the effect on control cell line compared to U251 luc shRNA

Figure A and B demonstrate the effect of the migration behavior of the control cell line and the U251 luc shRNA.

Figure A is the control cell line after 24 hours of incubation and Figure B the U251 PHIP shRNA cell line also after 24 hours.

In the diagram C the mean number of migratory cells in the control cell line and the U251 cell line is compared to each other.

The mean number of the control cell line is 585 cells and the mean number of U251 PHIP shRNA is 83 cells ($p<0.05$).

4.4.1. Scratch assay

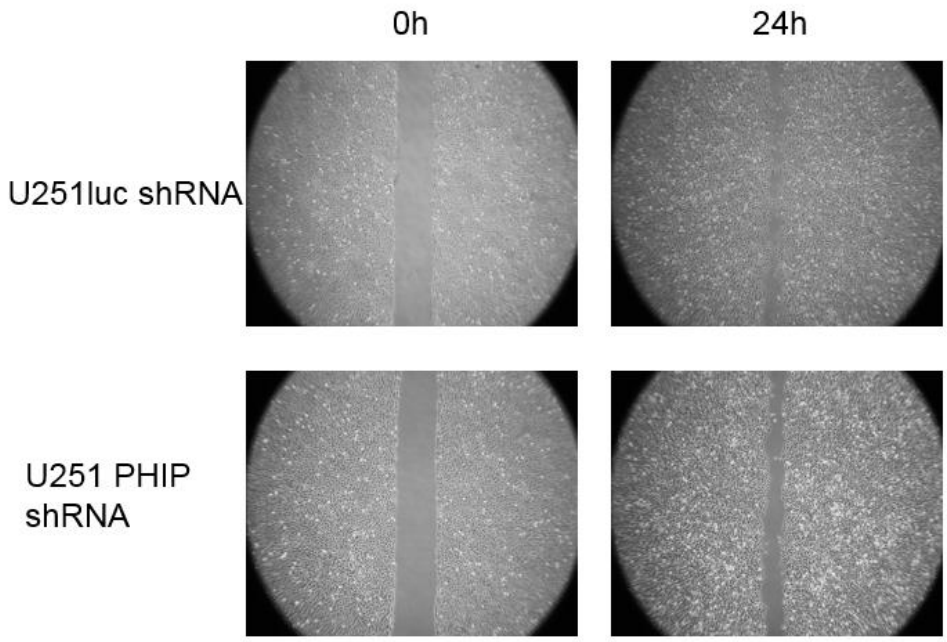


Figure 9: Scratch Assay results comparing 0 hours and 24 hours after scratch. Control cell line is U251 Luc shRNA and U251 PHIP shRNA is test cell line.

The scratch assay shows 2 different cell lines compared at two time points. One picture was taken right after performing the scratch assay and one after 24 h incubation.
It can be clearly seen that the control cell line is closing the scratch faster than the *PHIP* shRNA. The knockdown cell line leaves a bigger scratch in between. This indicates a decreased movement of the cells towards the center of the cell to close the scratch and therefore a decreased cell growth.

4.5. Invasion assay

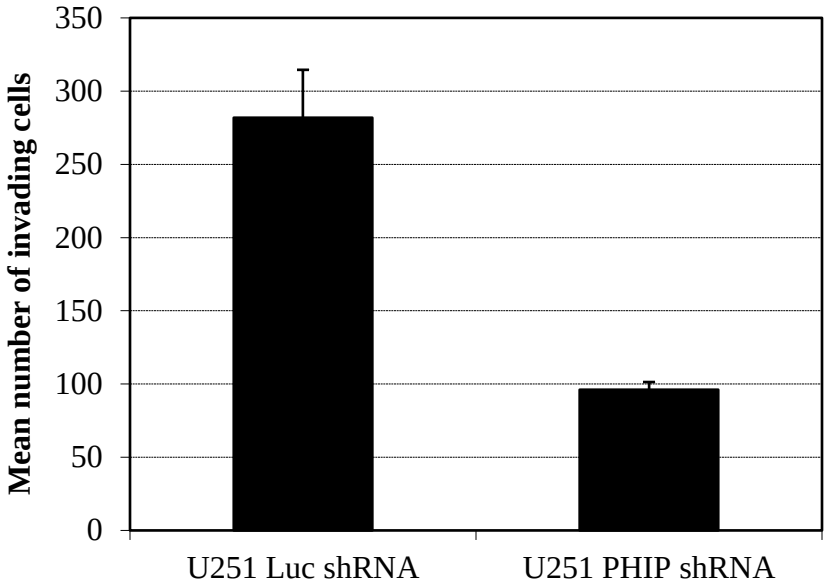


Figure 10: Mean number of invading cells comparing U251 Luc and PHIP shRNA cell line

The mean number of invaded U251 Luciferase cells is 280 cells per plate and the mean number of U251 PHIP cells that were able to migrate through the Matrigel is 98 cells per plate. This means there is around 65% less invasion in the U251 PHIP cells.

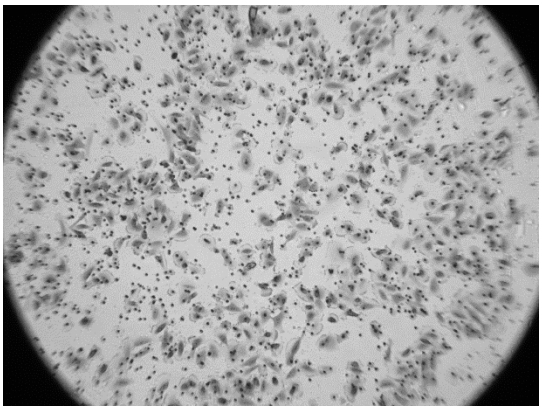


Figure 12: U251 luc control Invasion assay

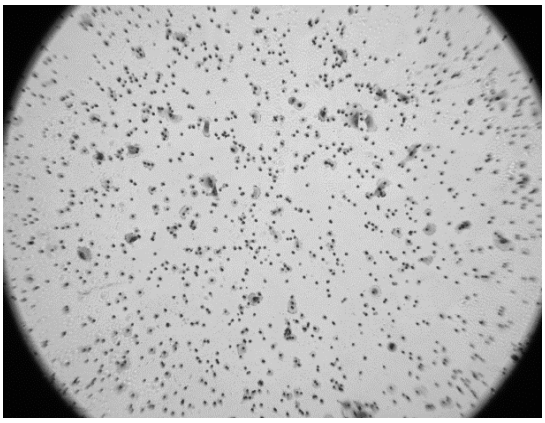
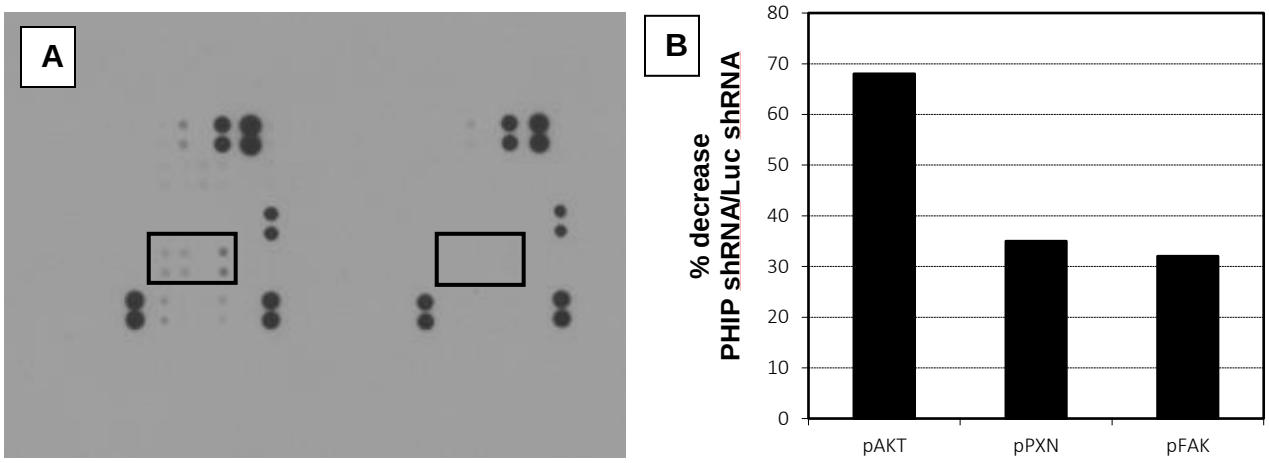


Figure 11: U251 PHIP shRNA cell line

The pictures above were taken after 24 h incubation and are showing the same outcome as the graph. The U251 PHIP cells indicates as well a reduction of 65% of the cells that are invading through the Matrigel.

4.6. Phosphoarray



U251 control *U251 PHIP shRNA*

Figure 13: Phosphoarray results demonstration a reduction of the arrays for all 3 tested targets

Figure A is a picture showing the decrease of the arrays in pAKT, pPXN and pFAK. This figure was plotted in the Figure B. It can be seen that there are no dots on the right side, which also confirms the decrease of PHIP shRNA cells when compared to the control cell line. Figure B demonstrates the Phosphoarray result showing the % decrease of PHIP shRNA vs. Luc shRNA compared to pAKT, pPXN, pFAK

4.7. Immunofluorescence staining

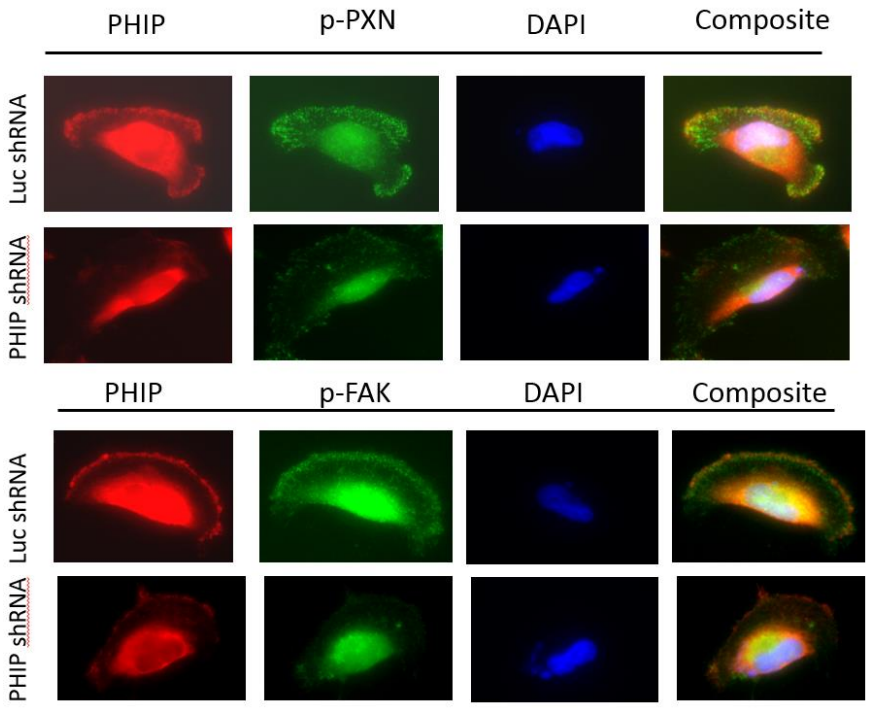


Figure 14: Immunofluorescence staining of U251 Luciferase shRNA and PHIP shRNA

The pictures above demonstrate the outcome of the Immunofluorescence staining. The cells were treated with different antibodies.

The primary antibody anti-mouse PHIP (Abnova) needs to be treated with a secondary antibody which corresponds to the anti-mouse. This antibody is labelled red. Therefore the PHIP image is shown in red. The concentration of the mouse PHIP antibody is 1:250.

The green picture in the second column was treated with primary antibody anti-rabbit Paxillin (Cell Signaling Technology) with a concentration of 1:500. The secondary antibody was an anti-rabbit corresponding, secondary antibody at a concentration of 1:1000.

pFAK was treated with primary antibody anti-rabbit pFAK (Cell Signaling Technology) with a concentration of 1:250 and the secondary antibody was anti-rabbit, which is labelled in green.

The blue DAPI stain is used for the localization of the nucleus.

The composite image on the right side shows a yellow merging image. This happens when the green and red part are overlaying so it induces a yellow merging image suggesting of a co-localization of both proteins.

The result of the immunofluorescence stain confirmed a decreased PHIP expression and especially less localization at the leading edge. This explains that the cells have less invading and moving potential which was confirmed by the invasion, migration and scratch assays.
All in all the results show an impaired movement of the cells compared to the control cell line.

4.8. In vivo experiments

4.8.1. Subcutaneous injections

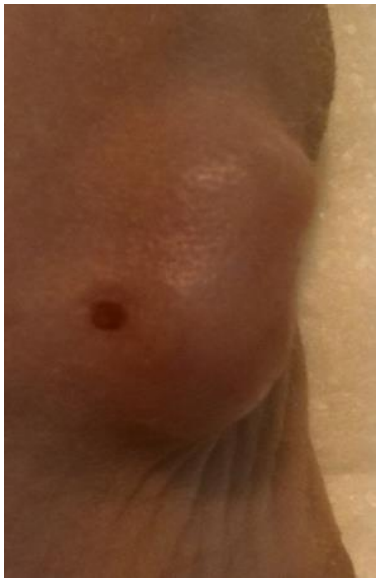


Figure 15: U251 luciferase control tumor size of a subcutaneous injection in the right flank of a nude mouse

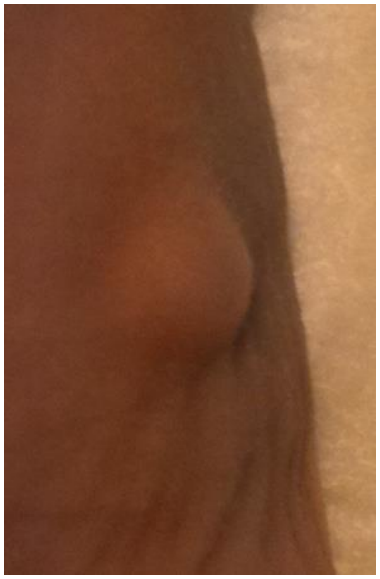


Figure 16: U251 PHIP shRNA tumor size of a subcutaneous injection in the right flank of a nude mouse

The two pictures were taken from the right flank of a nude mouse. Subcutaneous injections are done before intracranial injections in the brain, because the growth of the cell line in nude mice has to be confirmed first. Therefore brain cancer cells are injected beneath the skin and are growing on the flank of the mouse.
After confirmation of the cell growth in nude mice subcutaneously the more complex and orthotopic intracranial injections are done.

The pictures taken from the subcutaneous injection represents the tumor growth measurement which is done by a caliper. Then the tumor growth is calculated by the formula:

$$Volume = \frac{length * width^2}{2}$$

In this case it can be clearly seen that the control cell line tumor sample is bigger than the U251 *PHIP* shRNA.

This is another confirmation that the gene knockdown of PHIP decreases the tumor growth in this case also *in vivo*.

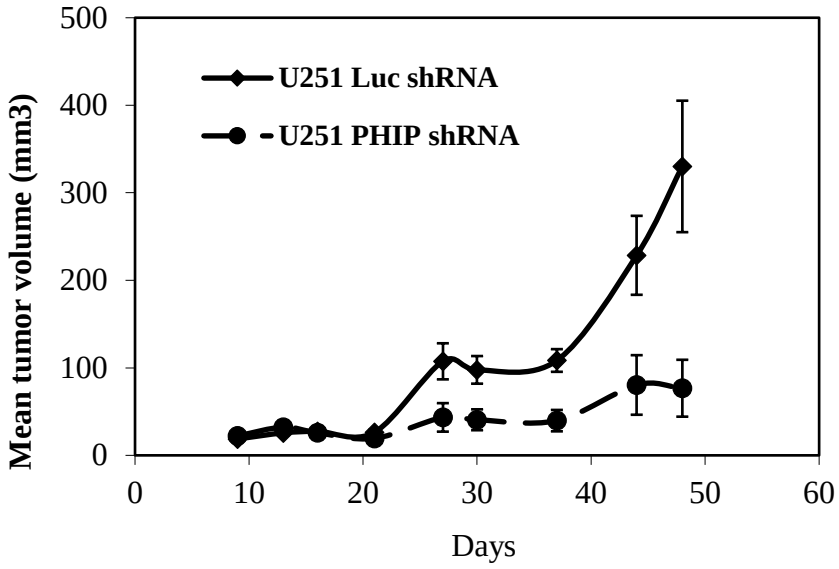


Figure 17: Mean tumor volume measurement of a subcutaneous injected U251 Luc control cell line and U251 PHIP shRNA

After calculating the mean tumor volume, which is given in mm^3, a tumor growth graph is plotted.

This graph demonstrates a steep growth of the control cell line after 35 days and a stagnating and low growth of the U251 *PHIP* shRNA.

4.8.2. Intracranial injection

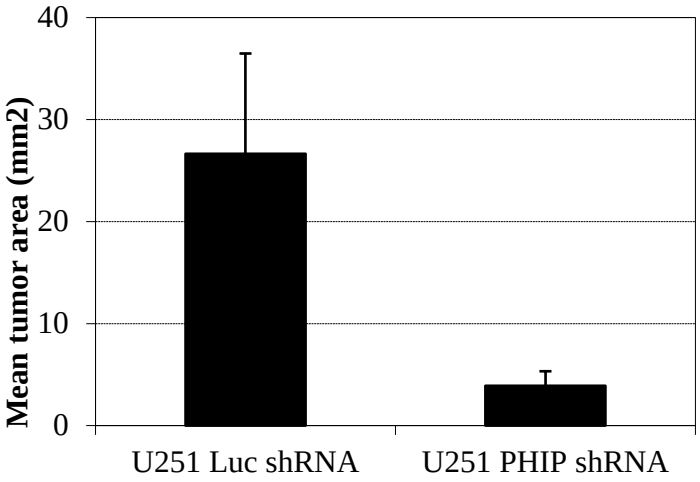


Figure 18: Mean tumor area from brain cancer

Figure 24 shows a mean tumor area plot of the intracranial injection including the standard deviation. The mean tumor area of the control cell line is 26.6 mm^2 and the U251 *PHIP* shRNA is 3.8 mm^2. This confirms a knockdown of 85% ($P<0.05$).

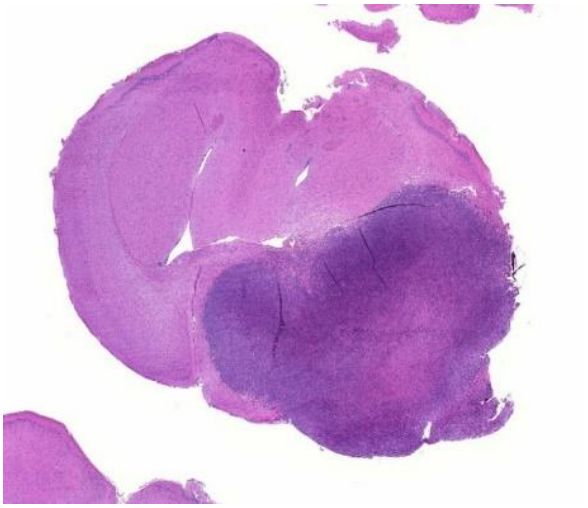


Figure 20: U251 Luc control cell line showing a stained brain tumor slice

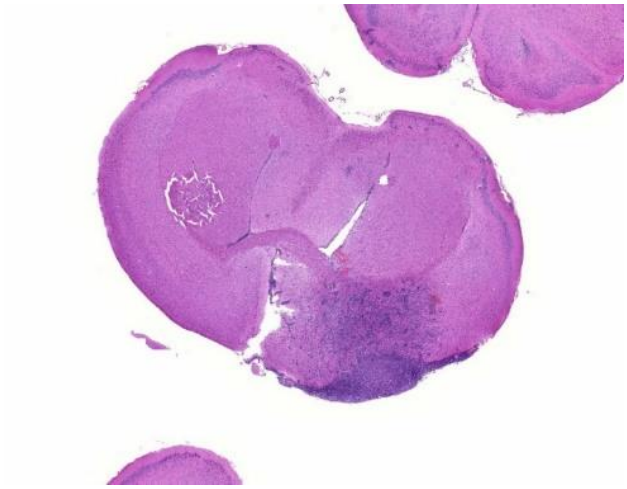


Figure 19: U251 PHIP shRNA cell line demonstrating a stained brain tumor slice

In pictures 25 and 26 tumor slices of the harvested brains are shown.
After euthanasia of the laboratory mice, their brains are taken out of the carcasses and a Hematoxilin and Eosin stain was done on thin slices of the brain and the tumor

section. This histological stain is a technique in histopathology. This uses a combination of two dyes, Hematoxylin and Eosin used for demonstration of nucleus and cytoplasmic inclusions in clinical specimens.
Pathologic review also confirmed that the dark part of this slices is a tumor.
Picture 25 on the left side from the control cell line U251 Luc shows a very big dark part which is in this case the tumor. In comparison picture 26 indicates cell line U251 *PHIP* shRNA where PHIP is downregulated and the dark tumor part of the brain is smaller.
The figure above states the mean tumor area of intracranial tumors which is given in mm^2.

5. Discussion

Generation of a gene knockdown

The first step was the generation of knockdown cell lines to assure that the gene knockdown was performed and to prove it with the following experiments.
The successful knockdown of the *PHIP* gene and following also the PHIP protein was proven with the TaqMan assay and Western blots.
Due to the reason that only knockdown and not a complete knockout of the *PHIP* gene was performed the levels of PHIP shRNA expression are the point of interest in this study. Figure 5 shows an 83.9% reduction in PHIP mRNA levels in U251 PHIP shRNA cells when compared to the U251 Luciferase shRNA control cell line whose PHIP levels were assumed to be 100% as a knockdown of luciferase is not having any effect on the other proteins. A knockdown level of 83.9% is very high and therefore the experiment was continued with this cell line. The standard deviation of 0.174 for three measurements of U251 PHIP shRNA and a standard deviation of 0.037 when testing them for the control gene is low and due to this the knockdown level is confirmed.

Western Blot

The Western Blot result in Figure 6 showed that PHIP shRNA produced a protein knockdown of 48%. This was compared to the knockdown of phosphorylated AKT to confirm that this knockdown had also an impact on the signaling pathway of AKT. With 67% knockdown PHIP plays a key role in the phosphorylation of AKT. As control, the total AKT band shows no difference to the control cell line.
This indicates that PHIP is modulating AKT expression and has an impact on the signaling pathway that leads to Cyclin D1 in the nucleus and plays a role in the cell cycle. This is also confirmed by a 42% knockdown of CCND1.
Another important aspect is that phosphorylated Paxillin is also showing a knockdown of 47%.
Therefore PHIP is not only a key modulator of AKT and CCND1 expression and following the cell cycle but also for the focal contact proteins that bind to an integrin beta 1 in the cell membrane.

Furthermore PHIP has also an impact on the phosphorylation of focal contact proteins, Paxillin and FAK. Those proteins have been implicated in cell motility and invasion behavior.
Additionally this is observed below by the confirmation of migration and invasion assays.

Proliferation

Cell Cycle

The results of the cell cycle assay show a clear reduction of the S-Phase from 12% in the control cell line to 5.4% in the PHIP shRNA cell line.
This confirmed the above statement that PHIP has an impact on CCND1, which is an important factor in the cell cycle and especially the S phase.

Colony formation

The colony formation assay is as well a test for the proliferation impact on the gene knockdown. After growing the cells for 7 days and staining those with crystal violet solution the control cell line had a mean value of 80.3 colonies and U251 PHIP shRNA showed a mean value of 37 colonies.

Cell survival

The third part of the cell proliferation tests was the cell survival assay.
Figure 10 demonstrates that there is a huge difference in cell growth comparing the control cell line U251 Luc shRNA and the U251 PHIP shRNA.
Connecting these results of the cell cycle assay, the colony formation and the cell survival assay it can be clearly seen that the knockdown of PHIP has a huge impact in the cell proliferation and finally also in cancer proliferation.

Migration assay

The migration assays includes transwell and scratch assay, which both give an insight in the migration behavior of the cells.

For the transwell migration assay the cells had to migrate through small pores of a well and the control cell line showed a mean number of 585 cells that migrates. The PHIP shRNA cell line only had a mean number of 83 cells that were migrating through the well. This was also confirmed with picture 12, where a huge difference can be seen after 24 hours.
The scratch assay was performed by making a scratch with the tip of a pipette into the plate of 80-90% confluent cells. Then the growth of the cells was recorded by pictures 13 and 14
It can be clearly seen that the control cell line showed a higher cell growth and movement to close the scratch than the PHIP shRNA cell line. These results indicate as well the impact on the migration behavior and an impaired movement behavior of the knockdown cell line.
The control cell line shows a huge number of cells that migrate through the well. Instead the *PHIP* shRNA indicates less migration behavior through the well and therefore less cells on the picture of the bottom of the well.
As in the theory section already explained, PHIP plays a role in the phosphorylation of focal contact proteins FAK, and Paxillin. All these proteins are key players for migration and invasion behavior.
With the different migration assays the movement of the two compared cell lines can be monitored very well and confirms as well the impact of PHIP in this signaling pathway.

Invasion assay

The invasion assay is similar to the Migration assay as already described previously. The only difference is that Matrigel (basal membrane protein extract) is used for the invasion assay. This makes it more difficult for the cells to invade through the Matrigel and the transwell at the bottom of the well.
Figure 15 demonstrates that for the control cell line (280 cells) and for the PHIP shRNA only 98 cells were counted at the bottom of the well. This is a reduction of 65%, which again indicates that PHIP knockdown has an impact on the migration and also invasion behavior of the cells.

Relating these results to cancer progression in the brain it can be an indication that the knockdown of PHIP decreases the migration and invasion behavior of brain cancer cells.
This can be an important aspect when looking into *in vivo* models as the invasive ability of cells is directly linked with metastasis as the cells first need to invade through ECM and tissue surrounding the tumor before they can metastasize in different parts of the body.

Phosphoarray

We analyzed the phosphorylation profiles of kinases and their protein substrates using a proteome profiler array on U-251 stable cells expressing either an anti-luc shRNA or an anti-*PHIP* shRNA following the manufacturer's protocol. We detected marked reduction in the phosphorylation level of several kinases that promote tumor progression, including phosphorylated AKT (pAKT), showing ~70% inhibition, which indicates the highest level of suppression of any of the proteins included in this array.
Phosphorylation levels of Paxillin and FAK were also inhibited (by 35% and 32%, respectively) in U-251 cells expressing anti-*PHIP* shRNA. AKT and PXN are involved in promoting tumor cell motility and invasion following IGF1R pathway activation, and pAKT was significantly downregulated following PHIP knockdown. Thus, PHIP reduced expression lead to the decrease in expression of key players in motility and cell proliferation.

Immunofluorescence staining

IF-staining is another technique to visually demonstrate protein knockdown and the localization in the cell.
Furthermore the images can be quantified to have a scientific result showing protein levels.
As already described above for IF-staining the cells are stained with two different antibodies against PHIP and another protein to be investigated.
The nucleus is stained as well with a DAPI stain and appears in blue.
The IF results confirm that with the knockdown of PHIP also p-FAK and p-PXN and as well CCND1 are downregulated.

As described above these 3 proteins do have an important role in cancer progression and in the regulation of different factors important in the formation and development of cancer cells.
As seen in Figure 20 knockdown of PHIP is also inducing a slightly downregulated level of these 3 proteins in the cells, either through a direct or indirect effect.
After proving a significant knockdown of PHIP in this experiment, the question how the downregulation of PHIP is changing the cell behavior can be answered with the results of the IF, and the proliferation assays that were described above.

***In vivo* experiments**

After successfully proving the effect of PHIP knockdown *in vitro* it was also confirmed in different *in vivo* experiments.
First of all a subcutaneous injections were done to show if the cell line is growing in nude mice. Figure 21 and 22 demonstrate the tumor growth of the control cell line and the U251 PHIP shRNA on the flank of a nude mouse.
It is clearly visible that the control tumor is bigger than the one with knockdown PHIP cell line. The tumor growth was measured with the use of a caliper and a formula to calculate the mean tumor volume.

The tumor growth diagram above indicates a steep tumor growth of the control cell line after 30 days. The other cell line grows not really much and stayed stable (Figure 23).

Due to the growth confirmation of the U251 cell line in nude mouse an orthotopic injection experiment was done afterwards to have more reliable results on the growth of this brain cancer cell line in the brain. Furthermore the results produced by intracranial injections observing the brain tumor progression improves the understanding of brain tumor biology and the use in preclinical studies. [34]
Orthotopic injections mean an injection in the anatomical correct area of the body.
The procedure of the intracranial injection was already mentioned above and after the experiment ended the brains of the injected mice were taken out, sliced and stained with a Hematoxilin and Eosin histological stain and was pathologically reviewed as positive on cancer. Figure 24 shows the quantification of the area occupied by the control tumor and compared to the tumor area of the U251 PHIP shRNA cells.
Figure 25 and 26 demonstrate the stained brain slices in a light violet color, the darker part of this slice indicates the tumor area of the tissue.
The control cell line shows a bigger tumor area than the PHIP knockdown cell line. Therefore this histological stain of the brain demonstrates another evidence of the impact of PHIP knockdown in the progression of brain cancer *in vivo.*
Additionally the mean tumor size of the intracranial injection was measured and confirms this statement with an 85% knockdown of PHIP. Finally this leads to the conclusion that PHIP shows not only an effect *in vitro* but also *in vivo*, which can be linked to pre-clinical studies of brain tumor progression.

To sum it up the knockdown of PHIP leads to a series of impacts in the cells' behavior as well as in the interacting signaling pathways of the brain cancer cells.
After a gene knockdown there is a significant decrease in the brain tumor progression and growth. Also additional signaling genes as CCND1 and focal contact proteins like Fax and Paxillin show a decrease in migration and invasion potential and in the cell cycle of the cell.

6. Conclusion

The study proposed did identify *DCAF14* alias *PHIP* as an important target for glioblastoma. Having shown an effect of its modulation in melanoma and lung cancer previously, this research could make DCAF14 a broad cancer target for solid tumors. In previous years the role of the *PHIP* gene was tested in breast, lung, and ovarian cancer. With this study it was also proven that PHIP plays an important role in the progression of brain cancer.
This outcome was also confirmed with two more commercially available brain cancer cell lines (LN18 and U87 not shown). All of the three cell lines indicates the same result and it was also proven with the same assays as in this study.
Due to the big extent of the whole study with all three cell lines, this Bachelor thesis is focusing on the results of U251 cell line.
To sum up upon the results above it was confirmed that the modulation of DCAF14 has as well an impact on the progression of brain cancer.

After the gene knockdown confirmation, the Western blot results demonstrates that DCAF14 plays a role in the insulin-growth receptor pathway. The proliferation assays indicates the effects on the cell cycle and cell survival behavior of the cells.

Given the effects on the migration and invasion assays, the modulation of DCAF14 expression decreased the invasiveness and migratory capabilities of brain cancer cells. Therefore metastasis is not that likely to happen.

And finally the *in vivo* mouse experiments demonstrated a significant difference in the tumor growth of control cells in respect to DCAF14 targeted cells in the brain of the mice.

Outlook

The downregulation of DCAF14 has proven to have an impact on cell proliferation, migration, invasion behavior and in tumor progression in an animal model not only in the gliblastoma context as shown in this study but also in breast, lung and ovarian cancers. Altogether, these results make DCAF14 as a rational target for the treatment of the above mentioned cancers. Given the recent discovery of a small molecule with affinity to PHIP, it is possible to envisage a drug able PHIP for the treatment of several cancers.

7. References

1. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2016.* CA Cancer J Clin, 2016. **66**(1): p. 7-30.
2. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer Statistics, 2017.* CA Cancer J Clin, 2017. **67**(1): p. 7-30.
3. Zhang, Y., et al., *A replicator-specific binding protein essential for site-specific initiation of DNA replication in mammalian cells.* Nat Commun, 2016. **7**: p. 11748.
4. Azar, H.A., et al., *Human tumor xenografts in athymic (nude) mice: chemotherapy trials in serially transplanted tumors.* Ann Clin Lab Sci, 1982. **12**(1): p. 51-9.
5. https://www.cancer.gov/types/brain, *Brain cancer.* National Cancer Institute, 2017.
6. Miao, C., et al., *A novel molecular agent for glioma angiogenesis imaging.* Nucl Med Commun, 2017.
7. Carlson, B.L., et al., *Establishment, maintenance and in vitro and in vivo applications of primary human glioblastoma multiforme (GBM) xenograft models for translational biology studies and drug discovery.* Curr Protoc Pharmacol, 2011. **Chapter 14**: p. Unit 14 16.
8. http://braintumor.org/brain-tumor-information/treatment-options/, *Brain tumor treatment options.* National Brain Tumor Society, 2017(4): p. 1616-1628.
9. https://www.cancer.gov/about-cancer/treatment/types, *Brain Cancer Treatment types.* NIH National Cancer Institute, 2017. **117**(12): p. 2243-2260.
10. Farhang-Fallah, J., et al., *The pleckstrin homology (PH) domain-interacting protein couples the insulin receptor substrate 1 PH domain to insulin signaling pathways leading to mitogenesis and GLUT4 translocation.* Mol Cell Biol, 2002. **22**(20): p. 7325-36.
11. Podcheko, A., et al., *Identification of a WD40 repeat-containing isoform of PHIP as a novel regulator of beta-cell growth and survival.* Mol Cell Biol, 2007. **27**(18): p. 6484-96.

12. Bezrookove, V., et al., *Prognostic impact of PHIP copy number in melanoma: linkage to ulceration.* J Invest Dermatol, 2014. **134**(3): p. 783-790.
13. https://medical-dictionary.thefreedictionary.com/DCAF14, *DCAF14-PHIP.* 2017. **12**(12): p. e0187018.
14. Webster, E., et al., *De novo PHIP-predicted deleterious variants are associated with developmental delay, intellectual disability, obesity, and dysmorphic features.* Cold Spring Harb Mol Case Stud, 2016. **2**(6): p. a001172.
15. Han, C.H., et al., *Clinical significance of insulin receptor substrate-I down-regulation in non-small cell lung cancer.* Oncol Rep, 2006. **16**(6): p. 1205-10.
16. Sun, X.J., et al., *Expression and function of IRS-1 in insulin signal transmission.* J Biol Chem, 1992. **267**(31): p. 22662-72.
17. Farhang-Fallah, J., et al., *Cloning and characterization of PHIP, a novel insulin receptor substrate-1 pleckstrin homology domain interacting protein.* J Biol Chem, 2000. **275**(51): p. 40492-7.
18. De Semir, D., et al., *Pleckstrin homology domain-interacting protein (PHIP) as a marker and mediator of melanoma metastasis.* Proc Natl Acad Sci U S A, 2012. **109**(18): p. 7067-72.
19. Wang, Y. and Y. Sun, *Insulin-like growth factor receptor-1 as an anti-cancer target: blocking transformation and inducing apoptosis.* Curr Cancer Drug Targets, 2002. **2**(3): p. 191-207.
20. Fidler, M.J., et al., *Targeting the insulin-like growth factor receptor pathway in lung cancer: problems and pitfalls.* Ther Adv Med Oncol, 2012. **4**(2): p. 51-60.
21. Alberts B, et al., *Molecular Biology of the Cell.* 1989: p. 872-876.
22. http://slideplayer.com/slide/6872622/, *Control of beta-cell function by PHIP.* Implications for Type I and II Diabetes Laboratory Medicine and Pathobiology Faculty of Medicine University of Toronto, 2017.
23. Bigner, S.H., et al., *Relationship of in vitro morphologic and growth characteristics of established human glioma-derived cell lines to their tumorigenicity in athymic nude mice.* J Neuropathol Exp Neurol, 1981. **40**(4): p. 390-409.

24. Zhang, S., et al., *Identification of U251 glioma stem cells and their heterogeneous stem-like phenotypes.* Oncol Lett, 2013. **6**(6): p. 1649-1655.
25. Bullard, D.E., et al., *Growth and chemotherapeutic response in athymic mice of tumors arising from human glioma-derived cell lines.* J Neuropathol Exp Neurol, 1981. **40**(4): p. 410-27.
26. Moore, C.B., et al., *Short hairpin RNA (shRNA): design, delivery, and assessment of gene knockdown.* Methods Mol Biol, 2010. **629**: p. 141-58.
27. Rubinson, D.A., et al., *A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference.* Nat Genet, 2003. **33**(3): p. 401-6.
28. http://altogenlabs.com/rna-interference-rnai-services/sirna-and-shrna-transfection-services/, *Transient (siRNA) and Stable (shRNA) Transfection.* 2017.
29. Manjunath, N., et al., *Lentiviral delivery of short hairpin RNAs.* Adv Drug Deliv Rev, 2009. **61**(9): p. 732-45.
30. https://www.rndsystems.com/products/human-phospho-receptor-tyrosine-kinase-array-kit, *Human Phospho-Receptor Tyrosine Kinase Array Kit.* Nat Methods, 2017.
31. Morton, C.L. and P.J. Houghton, *Establishment of human tumor xenografts in immunodeficient mice.* Nat Protoc, 2007. **2**(2): p. 247-50.
32. Ozawa, T. and C.D. James, *Establishing intracranial brain tumor xenografts with subsequent analysis of tumor growth and response to therapy using bioluminescence imaging.* J Vis Exp, 2010(41).
33. Pompili, L., et al., *Patient-derived xenografts: a relevant preclinical model for drug development.* J Exp Clin Cancer Res, 2016. **35**(1): p. 189.
34. Simeonova, I. and E. Huillard, *In vivo models of brain tumors: roles of genetically engineered mouse models in understanding tumor biology and use in preclinical studies.* Cell Mol Life Sci, 2014. **71**(20): p. 4007-26.
35. http://ezbiosystems.com/view2.asp?d_id=315, *U251 cell line phenotype.* EZ Biosystems, 2017.

7.1. Figure References

Figure 1: IRS pathway: 2000-2012 Ingenuity Systems, Inc.
Figure 2: U251 cell line [35]

All the other figures and tables were produced by the California Pacific Medical Center Research Institute.