Ovarian cancer

Assessment of modulating Pleckstrin Homology domain-Interacting Protein (PHIP) expression in ovarian cancer

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Abstract

Cancer is the second deadliest disease in Western Countries. In the last decades the treatment possibilities for patients have increased, but there are still many types of cancer that can only be targeted by general methods like chemotherapy or radiation.

Personal Medicine is used to customize and adapt the therapy on the individual patient. Cancers can arise from many different tissues and cell types. Even in the same type of cancer the mutations can be completely different. This variety of cancer types need different types of drugs, which specifically target their individual mutations. Therefore it is important to uncover proteins of cancer cells and their functions in all types of tumors.

The research group headed by Dr. Kashani investigates the novel protein Pleckstrin Homology domain-Interacting Protein (PHIP) in ovarian cancer. It interacts with the PH Domain of the IRS-1 or IRS-2. PHIP has been identified as the top gene overexpressed in metastatic *versus* primary melanomas. It also regulates many cellular roles like the morphology and cytoskeletal organizations. PHIP increases cell proliferations by regulating the transcription of cyclin proteins. Another function is that it has anti-apoptotic activity through activation and phosphorylation of AKT. Latest data suggest that PHIP has influence on Talin1 (TLN1). TLN1 is involved in the building of actin filaments. This directly correlates with invasion and metastasis through AKT-dependent effects.

We generate ovarian cancer cell lines (PA-1 and SKOV-3) with PHIP protein knockdown. These cells express shRNAs targeting the PHIP mRNA. We observed that our cell lines, which contain PHIP knockdown, have significantly lower proliferation rates and invasiveness compared to control groups. Additionally several focal adhesion proteins like Talin are down-regulated in PHIP knockdown cells.

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List of abbreviations

APS	Ammonium persulfate
BSA	Bovine Serum Albumin
BRD	Bromodomain
C	Celsius
cDNA	Copy Deoxyribonucleic acid
Cat#	Catalog number
Dapi	4',6-diamidino-2-phenylindole
ddH2O	Double-distilled water
DMEM	Dulbecco’s Modified Eagle Medium
DMEM/F12	Dulbecco’s Modified Eagle Medium, Nutrient mixture F12
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EM	Extracellular matrix
FBS	Fetal Bovine Serum
FISH	Fluorescence In Situ Hybridization
FRET	Fluorescence Resonance Energy Transfer
GFP	Green fluorescent protein
HBSS	Hank's Balanced Salt Solution
IgG	Immunoglobulin G
Kb	Kilobases
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
Ml	Milliliter
miRNA	MicroRNA
mRNA	Messenger RNA
NRAS	Neuroblastoma RAS viral oncogene homolog
P/S	Penicillin/Streptomycin
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor

RNA	Ribonucleic acid
RTK	Receptor tyrosine kinases
RIPA	Radioimmunoprecipitation Assay
RPM	Rounds per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SDS	(Sodium dodecyl sulfate)
shRNA	short hairpin RNA
TBST	Tris-Buffered Saline and Tween 20
TLN1	Talin 1
TNF	Tumor necrosis factor
V	Volt
β-ME	β-mercaptoethanol

1. Introduction

1.1. Facts about Cancer

According to the World Health Organization (WHO) cancer is the second leading cause of death after cardiovascular diseases in Western Countries. In 2008 more than 7.6 million people died as a result of cancer. The most common types are colon, liver, breast, lung, ovarian and stomach cancer. The World Health Organization uncovered the five leading risks that cause approximately 30% of all cancers. These risks are: low physical activity, a high body mass index, a low uptake of vitamins and fruits, alcohol and tobacco. Tobacco itself causes 71% of all lung cancers. These numbers show that cancer is one of the leading causes of death, but still many of cases are untreatable because of a lack in knowledge and therapies. Therefore research in cancer is very important to fill the gaps and provide better ways of treatments in future (http://www.who.int/cancer/en/).

Until the 19th century scientists believed that cancers could be foreign bodies. The discovery that all cells of a body descend from a single fertilized egg led to the conclusion that tumors are not foreign bodies. It also became clear that cancer is a disease of malfunctioning cells. There are two types of tumors: benign and malignant. Benign tumors are localized and therefore noninvasive. On the other side malignant tumors are invasive and metastatic. The metastasis released by malignant tumors cause almost all cancer deaths. Depended on the origin, cancer can be classified into epithelial, mesenchymal, hematopoietic and neuroectodermal cancers. Every body cell has the possibility to transform into a cancer cell, but the most common type of cancer originate from epithelial cells. This type of cancer is better known as carcinoma. Carcinomas can be classified into squamous cell carcinoma (derived from the epithelia that function as protective cell layers) and adenocarcinoma (derived from the epithelia that function as secretory cells)(Bickers and Lowy 1989).

Nonepithelial cancers are sarcomas (from mesenchymal cells), hematopoitec cancers (from circulatory and immune system cells) and neuroectodermal tumors

(from nervous system cells). But of course there are several types of cancers which are out of these definitions. In some cases the origin of tumors cannot be identified because these cancer cells have totally dedifferentiated and show no connections to a specific tissue anymore. This cancer type is called anaplastic (Weinberg 2007).

A tumor develops in a progressive way and goes through various stages. First the tumors are hyperplastic or metaplastic. In a hyperplastic tissue only the cell number is increased and in a metaplastic tissue normal cells are displaced by other cell types which are usually not encountered at their location. A tumor in this first two stages is still considered as benign. In the next stage tumors are termed dysplastic. Here the cells are already cytologically abnormal. This stage is located between totally benign and premalignant. Dysplastic tumors still do not go through the basement membrane boundary and are therefore benign. If these tumors breach through it and invade the underlying tissues they become malignant. These tumors are able to spread through metastasis. Metastasis is the ability to seed tumor colonies on different sides in the human body. Cells of these tumors have to gain new abilities like motility and adaption to new environments. Usually tumors are monoclonal and not polyclonal. Monoclonal tumors are derived from a single ancestor cell that has an altered DNA and therefore became a cancer cell.

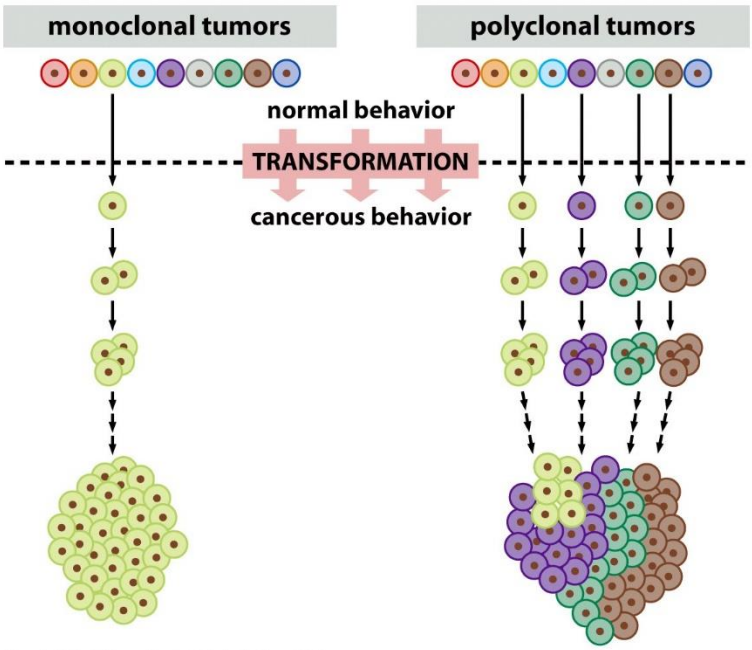


Figure 1: Difference between monoclonal and polyclonal tumors

(Weinberg 2007)

One big question in the understanding of cancer is the cause that transform a regular cell to a tumor cell. Scientists found two main groups that can lead to cancer: radiation and chemicals. Radiation like X-Rays causes mutations in the DNA, which can lead to the birth of a cancer cell. To find chemicals that can cause cancer the Ames test is used. In this test Salmonella bacteria with a mutation in their DNA are used. This mutation disenables them to synthesize the important amino acid histidine. Therefore they have to take it up from the culture medium to survive. In the Ames test these bacteria are put onto a histidine lacking culture medium together with possible mutagenic compounds. If these compounds can alter the DNA of the Salmonella, there is a high chance that the specific mutation in their DNA is reversed in some of the bacteria. Therefore they will be able to synthesize their own histidine and ultimately grow in the histidine lacking media. If the compound cannot affect the DNA no bacteria will grow at all (Weinberg 2007).

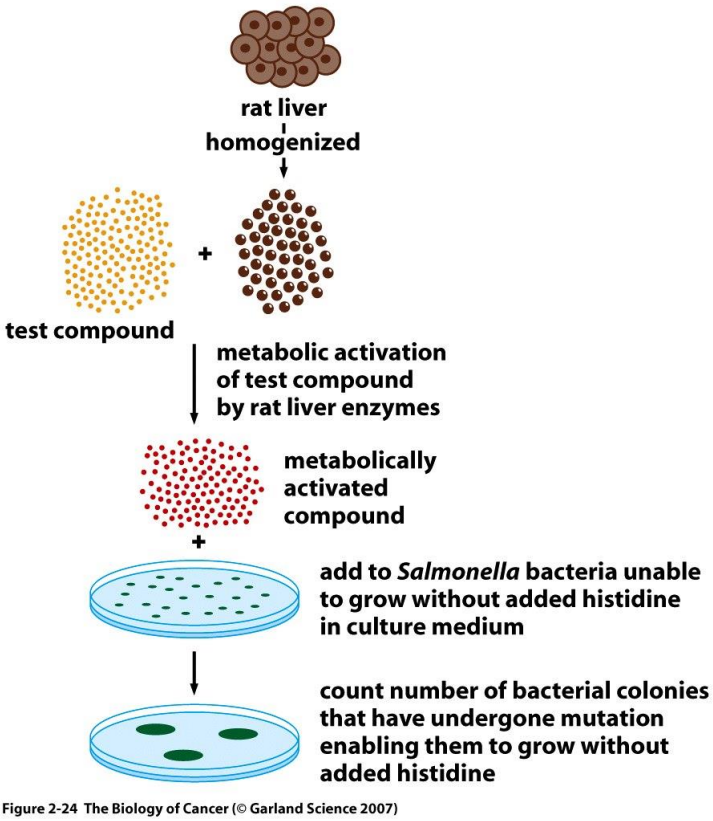


Figure 2-24 The Biology of Cancer (© Garland Science 2007)

Figure 2: The Ames test for detection of mutagenicity
In the Ames test a special Salmonella bacteria strain is used which has a mutation. This mutation affects the synthesis of cell own histidine and therefore they have to take it up through the cell culture media. In this test the bacteria strain is plated onto a histidine lacking culture medium together with a test compound. If this compound is mutagenic it will reverse the DNA mutation and therefore enables some bacteria to synthesize own histidine and to grow.

(Weinberg 2007)

This test revealed mutagenic compounds like tobacco, vinyl chloride, arsenic, asbestos, coal dust and many more (Weinberg 2007).

1.2. The Hallmarks of cancer

Cancer is a disease caused by the uncontrolled division of the own body cells (Preston-Martin et al. 1990). In the last quarter of the century a lot of knowledge about cancer has been generated which states that cancer cells have plenty of dynamic changes in their genome. On the one side cell cycle promoting pathways (oncogenes) are promoted by mutations, on the other side cell cycle arresting or even apoptotic pathways (tumor suppressor genes) are down-regulated or switched off by them. In fact there are a lot of different pathways altered that can dramatically vary in tumors derived from different tissue. These mutations can range from a single point mutation at the right amino acid to changes in chromosome complements. Often transformation from a normal cell to a cancer cell requires many of these genetic changes before it is able to form a tumor. This is analogous to Darwinian evolution, in which only favorable mutations lead to an uncontrolled and quicker proliferation than in normal body cells.

In 2011 Douglas Hanahan and Robert A. Weinberg published the second edition of their well-known paper “The Hallmarks of Cancer”. In this paper they describe ten unique hallmarks that cancer cells can have. They do not necessary have all of them but at least some very important ones like self-sufficiency in growth signals and insensitivity to anti-growth signals (Hanahan, Weinberg, and Francisco 2000).

Ten hallmarks of cancer

- **Sustaining proliferative signaling**
- **Evading growth suppressors**
- **Resisting cell death**
- **Genome instability and mutation**
- **Activating invasion and metastasis**
- **Tumor promoting inflammation**
- **Enabling replicative immortality**

- **Avoiding immune destruction**
- **Deregulating cellular energetics**
- **Inducing of angiogenesis**

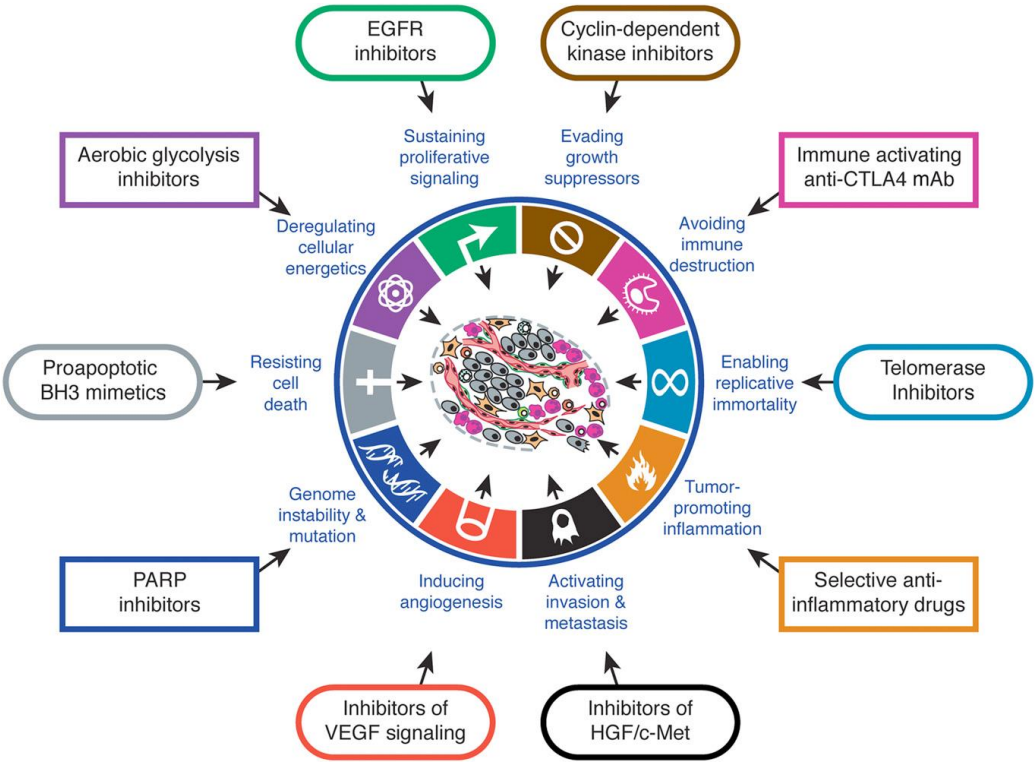


Figure 3: Hanahan and Weinbergs Hallmarks of Cancer and clinical treatment options

(Hanahan and Weinberg 2011)

1.3. Sustaining in proliferative signaling

As shown in figure 3 there are many cellular processes that tumor cells are able to change and therefore achieve independence from the body governed control of proliferation, apoptosis and survival (Hanahan, Weinberg, and Francisco 2000). One of these ten hallmarks is the self-sufficiency in growth signals. Usually normal body cells are tightly regulated by signals transmitted by different types of cells. Cells that produce a specific signal like PDGF (platelet-derived growth factor) have no or less receptors for their signal. Therefore they are not able to stimulate themselves by their own signals like a mitogenic growth signal. There are different types of signaling molecules like diffusive growth factors, cell to cell adhesion molecules and extracellular matrix components. As experienced until now no normal cell can proliferate in absence of them.

However cancer cells are possible to proliferate even in the absence of these specific growth factors. This gives them autonomy from the rest of the human body. There are three possible strategies for cancer cells to gain independence. The first is called autocrine stimulation. In this strategy cancer cells can produce growth factors, which stimulate their own receptors. This creates a positive feedback loop, which drives unregulated proliferation. Usually all cells recognize a signal through binding to a cell surface transmembrane receptor. Upon binding of a ligand, this receptors are able to transmit the signal into the interior of a cell. Cancer cells often overexpress these receptors (like the EGF receptor) and therefore are able to respond to very small doses of signaling molecules. This even allows ligand independent signaling through to random collision between them, that generates a signal in cells (Di Fiore et al. 1987).

On the other side cancer cells often undergo structural changes in their receptors. These alterations can lead to a ligand independent and permanent signal transduction. Permanent signals allows cells to proliferate even without any signals in their environment. The third strategy is to adapt their extracellular matrix receptors. These receptors are important to transmit signals that promote proliferation. Of course there are many other functions they can have. It has been found that tumor cells can switch these types to special types that give them benefits and get rid of those that does not (Lukashev 1998). These cell surface receptors (integrins) allows cells to physically link to extracellular matrix (ECM) structures. Integrins are beside their signaling function also important for motility. Therefore cells without them cannot move proper in the ECM. A lack of them also can lead to cell cycle arrest or even apoptosis (Giancotti 1999). This shows why cancer cells usually have lots of integrins. Growth factors and integrins are able transmit their signals through the MAP-Kinase pathway, which will be explained later.

These three strategies given are often used by cancer cells to provide themselves with unlimited growth factors to assure unlimited proliferation.

1.4. Receptor tyrosine kinases

One of the most prominent type of cell surface receptors are the receptor tyrosine kinases (RTK). Among all other receptors like G-protein coupled receptors, receptor tyrosine kinases are assumed to be the most mutated receptors in cancer cells.

Usually they consist of single subunits, but like the insulin receptor they can form multimeric complexes. Single subunit receptors consist of an extracellular domain (called N-terminal region), a transmembrane domain (between 25 and 38 hydrophobic amino acids) and an intracellular domain (called C-terminal region). The N-terminal region is very unique in each receptor as it provides a ligand-binding side for their specific substrate. However the C-terminal region is highly conserved and has a kinase that phosphorylates other kinases (Weinberg 2007).

When a receptor tyrosine kinase binds a ligand, a second receptor is attracted to bind to it too. This induces dimerization and brings the kinases of each receptor close to the other C-terminal region. These kinases will start to transphosphorylate the tyrosine residues of the other receptor. This dimerization explains why overexpression of growth factor receptors is beneficial for cancer cells. As they are high in number and can freely move in the plasma membrane, these receptors will collide more frequent. This collision provides the same result as ligand attracted dimerization. The collided receptors will transphosphorylate and produce a mitotic signal (Weinberg 2007).

Mutations in three different receptor tyrosine kinase domains can cause a ligand independent firing. In the N-terminal domain mutations can lead to an attraction and dimerization, independent of substrate binding. Deletions or amino acid substitutions in the cytoplasmic domain are also very prominent in tumor cells (Weinberg 2007).

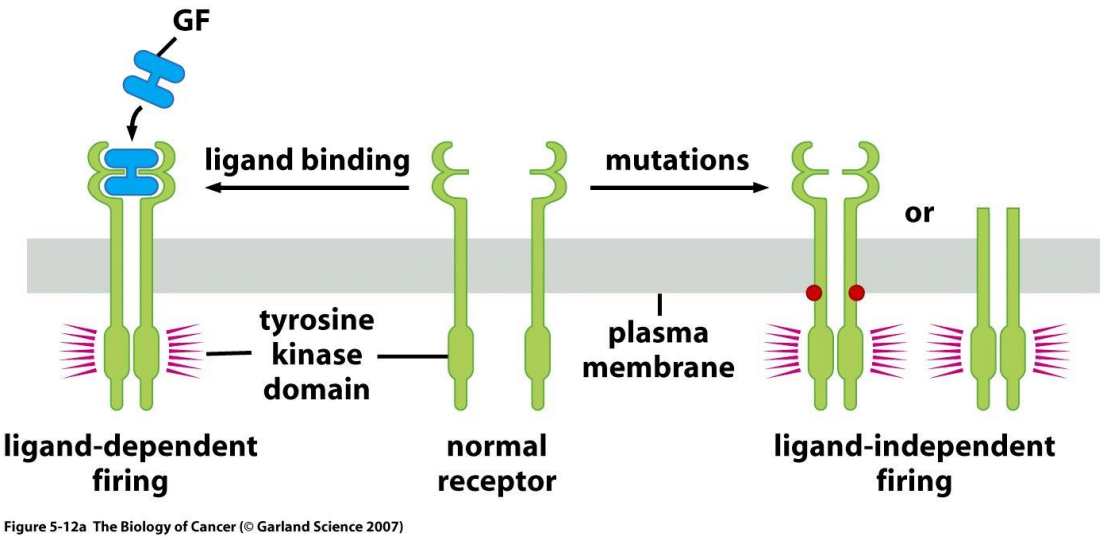


Figure 4: Dimerization of receptor tyrosine kinase monomers and ligand-independent firing in the absence of growth factors (GF)

(Weinberg 2007)

1.5. Receptor tyrosine kinase signaling pathways

After the finding that receptors transphosphorylate themselves on tyrosine residues the question arose how are they able to spread the signal with these phosphorylations? Soon scientists discovered the involvement of the protein Src with these receptor phosphorlyations on the tyrosine residues. Src has three homology domains: SH1 has catalytic functions, SH2 binds to phosphotyrosines, whose unique identities are defined by their surrounding amino acid sequence, and SH3 that can bind to proline rich domains of substrates (Pawson 2004). Therefore proteins with a SH2 domain are able to recognize and to bind to the unique phosphorylated tyrosine residues on activated receptors (Weinberg 2007).

The protein Grb2 has one SH2 group and two SH3 groups. This allows it to bind with the SH2 domain to the receptor and with the SH3 group to the proline rich residues of the protein Sos. Grb2 builds a physical bridge between the receptors and Sos and therefore brings Sos into close position with the cell membrane (Egan et al. 1993). There it has the possibility to interact with the very important membrane bound protein Ras (Malumbres and Barbacid 2003).

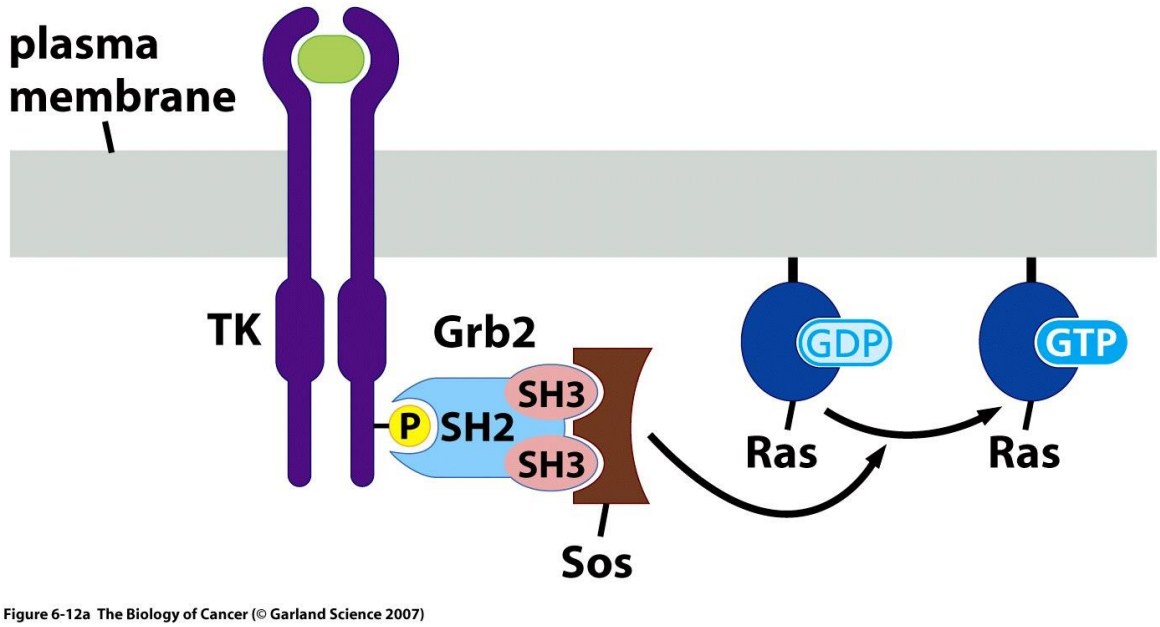


Figure 5: Receptor tyrosine kinase downstream signaling to Ras via the bridge protein Grb2 and the Ras activator protein Sos.

(Egan et al. 1993; Weinberg 2007)

Ras can switch between two different states. An activated one where it is bound to GTP and an inactive when the GTP is hydrolyzed to GDP (Lowy and Willumsen 1993). Sos has the ability to function as a guanine nucleotide exchange factor (GEF) for Ras. As long as it is in the cytoplasm it is unlikely that it encounters a Ras protein and therefore activates Ras. But when it is linked to the plasma membrane via Grb2 it exchanges the GDP to a GTP in Ras and therefore triggers signal transduction.

Ras is controls many different signaling pathways. It has influence on cytoskeletal integrity, differentiation, cell adhehsion, migration, proliferation and apoptosis. Therefore a mutation in Ras is observed in many types of cancer. In a mutated form Ras has the possibility to stay in its activated state because of a defect in the hydrolysis of the GTP. Three important downstream signaling cascades origin of Ras: The PI3 kinase pathways, the Map kinase pathway and Ral pathway (Egan et al. 1993).

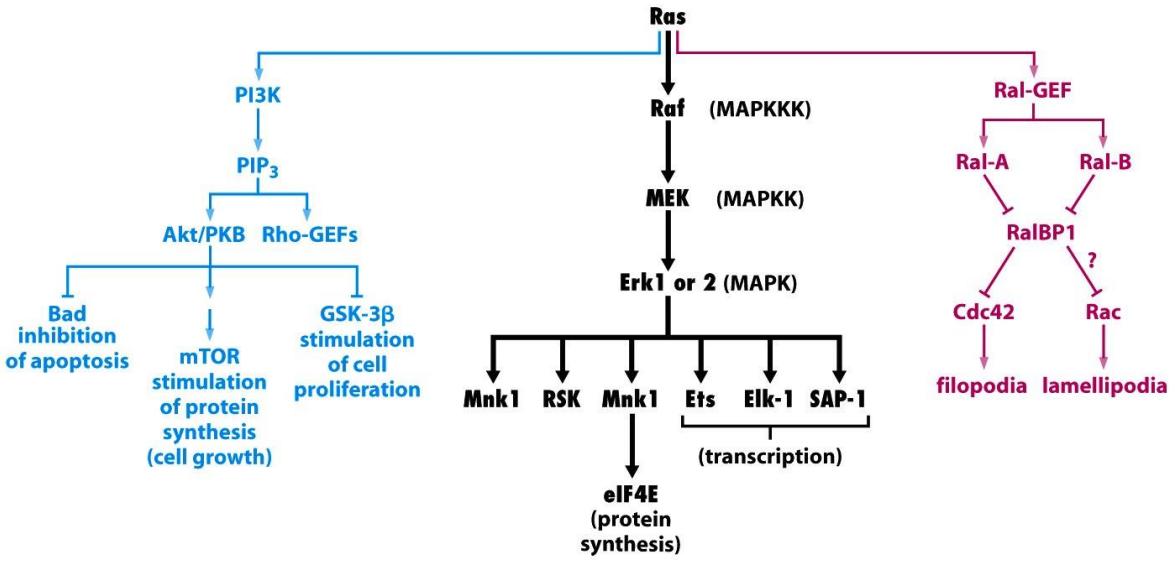


Figure 6: The three pathways that can be activated by Ras: the MAP kinase pathway, the PI3 kinase pathway and the Ral pathway.

(Weinberg 2007)

The mitogen-activated protein kinase pathway (MAP) is the most famous pathway. Ras helps to activate the protein Raf. Raf phosphorylates the next protein and so on. In the end a lot of transcription factors are activated like Bad, mTor and GSK-3β (Chang and Karin 2001). Bad is an inhibitor of apoptosis, mTOR stimulates protein synthesis and GSK-3β the cell proliferation. In certain cancers this pathway is strongly activated even without Ras. It is sufficient if the Raf protein has a specific mutation that allows it to be active in the absence of Ras.

1.6. The PI3 kinase pathway

Another very important pathway is the PI3 kinase pathway. In context with cancer, this pathway is mainly responsible for suppression of cellular apoptosis. The cellular plasma membrane consists of phospholipids that usually act as a barrier between the aqueous environments inside and outside of a cell. Phospholipids consist of a hydrophilic head and a hydrophobic tail. This results in a tight barrier between the environment and the cell interior. But some phospholipids contain at their hydrophilic head an inositol group. This inositol group consists out of a water-soluble carbohydrate molecule. The inositol can be modified by addition of a phosphate

group which results in the generation of a phosphoinositol. This phosphoinositol can be cleaved off from the remaining phospholipid. These phosphoinositols (short PI3) can travel in the cell interior and therefore serve as an intracellular hormone, which transmit signals from the plasma membrane into the cell interior. These intracellular hormones are called second messengers (Weinberg 2007).
Alternatively there is also a possibility that phosphorylated inositols remain attached to their phospholipid and thereby create an anchoring point for other cytosolic proteins.

The inositol moiety can be targeted and modified by several distinct kinases, each of them can phosphorylate a specific hydroxyl group of the inositol molecule. The very important Phosphatidylinositol 3-kinase (PI3K) targets the 3´hydroxyl group of the inositol moiety. This inositol molecule is still membrane-embedded. There are several types of PI3 kinases but the most important one is the Phosphatidylinositol 3-kinase that phosphorylates the phosphoidylinositol (4,5) P2 (short PIP2). Before PIP2 gets phosphorylated by the PI3K it already has two phosphate groups at the 4´and 5´hydroxyl groups. But after the phosphorylation by PI3K it receives an additional phosphate group and PIP2 is converted to phosphatidylinositol (3´,4´,5´)-triphosphate (short PIP3) (Weinberg 2007).
The main difference between this pathway and the Ras-Raf-MEK-Erk pathway is that PI3K phosphorylate a phospholipid instead of a protein substrate like the Ras-Raf-MEK-Erk pathway kinases.

The Phosphatidylinositol 3-kinase is closely linked to the activated Ras protein. If Ras is active it allows the PI3K to attach at it via SH2 binding. This enhances the kinase activity of it and PI3K becomes closely associated with the plasma membrane where the desired phosphoinositol molecules are located. The PI3K can be activated by several signaling agents like PDGF and the insulin-like growth factor-1 (Weinberg 2007).

The phosphorylation of the inositol groups has no effect on the overall function of the plasma membrane. But a phosphorylated inositol head can be recognized and

of course bound by specific proteins, which are usually floating in the cytoplasm. The attached proteins are able to start a signaling cascade. The PIP3 appears to be the most important head group. Many proteins, which contain a pleckstrin homology (PH) domain are attracted to PIP3. The PH domain strongly interacts with the triply phosphorylated inositol head group. The most important protein PH domain containing protein is the well-known serine-threonine kinase Akt (sometimes called protein kinase B). Therefore an activated PI3K converts PIP2 to PIP3, which is then recognized by Akt (Weinberg 2007).

The association between PIP3 and Akt leads to functional activation of the Akt kinase. Akt can phosphorylate many protein substrates that have multiple effects on the cell. One of these effects is that the cell survival is increased by a down regulation of the cells apoptotic program. Other effects are the stimulation of cell proliferation and the increase of cell size. The activation of Akt is tightly controlled. Regular cells have only a very low amount of PIP3. One of the major inhibitors is PTEN. It reverses the effect of PI3K by removing the 3´phosphate of PIP3 and therefore converting it to PIP2 (Weinberg 2007).

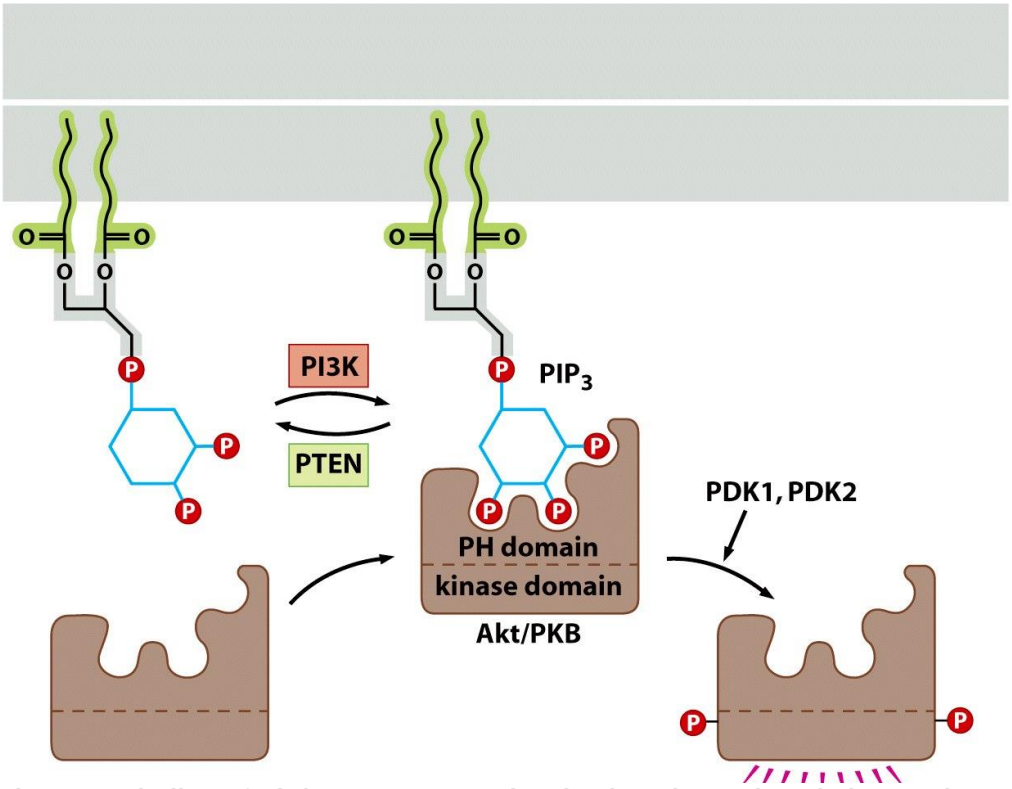


Figure 7: Binding of Akt/PKB to PIP3 and activation via PH domain interaction

(Weinberg 2007)

1.7. The Insulin Receptor

Insulin is a peptide hormone that transmits its signal via binding to the insulin receptor (IR). The insulin receptor is a multi-subunit glycoprotein that acts as a tyrosine kinase due to activation by insulin binding (Kido, Nakae, and Accili 2001). The insulin receptor signaling is important for glucose, lipid, and protein metabolism. Very common diseases affecting the insulin receptor pathways are Diabetes Type I and II.

The gene locus of the insulin receptor is on the human chromosome 19 and is more than 150 kilobases (kb) large (Seino et al. 1989). The translated and spliced form of the insulin receptor gene results in 22 exons. They range from 4.2 to 9.5 kb depending on their 3' untranslated regions (Goldstein and Kahn 1989).

The structure of the insulin receptor consists of two α-subunits and two ß-subunits. Each α-subunit is linked to a ß-subunit via disulfide bonds. Experiments showed that an reduction of these crosslinks between the two subunits lead to an reduced insulin binding to the receptor and consequently the tyrosine kinase is deactivated (Sweet, Morrison, and Pessin 1987).

The α-subunit consists of either 719 or 731 amino acids, depending on the mRNA splicing on exon 11. The molecular mass is 130 kDa. The α-subunit is located entirely in the extracellular space and functions as the binding side for insulin.
The ß-subunit contains of 620 amino acids and has a molecular mass of 95 kDa. This subunit is a transmembrane protein and therefore relies the signal of the exterior α-subunit through the plasma membrane. The ß-subunit consists of extracellular, transmembrane and cytosolic components. The tyrosine kinase is located on the cytosolic side and can be activated by insulin binding. This kinase is structurally very homologous to other tyrosine kinases (Ullrich et al. 1985). Additionally to the tyrosine kinase there is also an ATP binding side and an activation loop located in the cytosolic part.

After the translation of both subunits, they get glycosylated and contain a complex N-linked carbohydrate chain. This chain is essential for proper folding and function of the receptor (Elleman et al. 2000). Additionally the ß-subunit contains O-linked oligosaccharides (Sparrow et al. 2008).

As mentioned before, binding of insulin to its receptor leads to autophosphorylation in the tyrosine residues. The activated tyrosine kinase is then able to phosphorylate tyrosine on the insulin receptor substrates (IRS-1, IRS-2, and IRS-3). The phosphorylation of IRS-1 enables many proteins to bind via the Src homology 2 domains. One of these proteins can be the previously discussed phosphoinositol 3 kinase (PI3K). Other proteins that can bind are Grb2 and Shp2. Grb2 is an important adaptor protein that translocate Sos to the plasma membrane where it interacts and activates Ras. Another protein that binds to the IRS-1 protein is the Pleckstrin-Homolgy Domain Interacting Protein (PHIP).

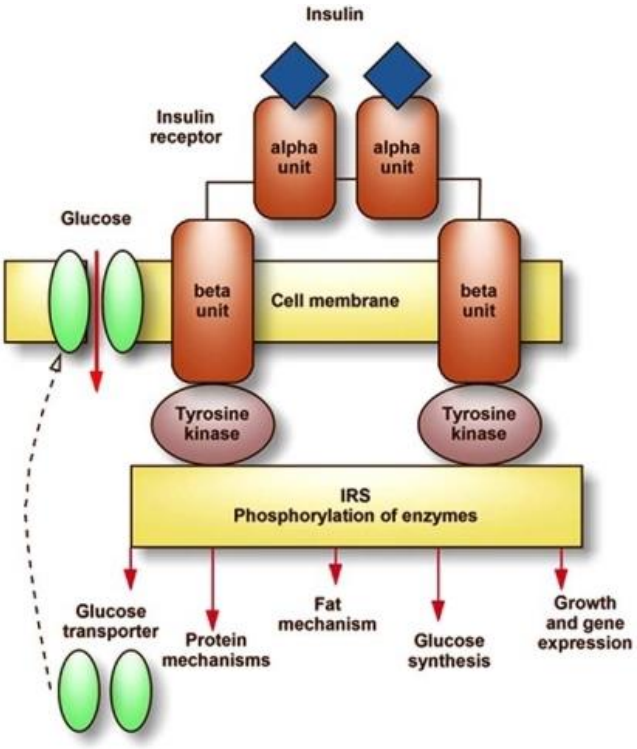


Figure 8: Structure of the insulin receptor and IRS-1 binding
(http://openi.nlm.nih.gov/detailedresult.php?img=3044890_TOBEJ-4-236_F1&req=4)

1.8. Pleckstrin-Homology domain-interacting protein

The Pleckstrin Homology Domain-Interacting Protein or PHIP is a novel discovered protein that interacts with the PH Domain of the IRS-1 or IRS-2. It has been identified as the top gene overexpressed in metastatic *versus* primary melanomas (Haqq et al. 2005).

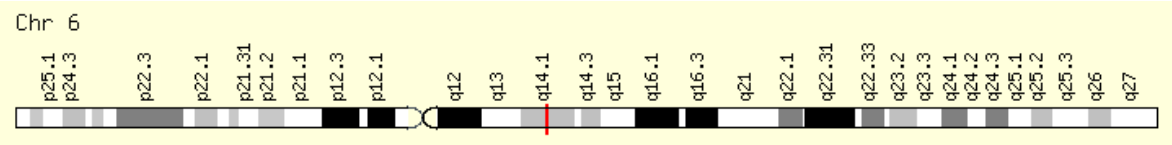


Figure 9: Location of the Pleckstrin-Homology domain-interacting protein on chromosome number 6 at the q14.1 locus.

(http://www.genecards.org/cgi-bin/carddisp.pl?gene=PHIP)

PHIP is located on chromosome 6 at the q14 position. It consists of 1821 amino acids and has an approximate size of 206 kDa.

PHIP has a role in regulation of different cellular functions. It can change the morphology and the cytoskeletal organization of cells (Bai et al. 2011; Farhang-Fallah et al. 2002). It also stimulates cell proliferation by regulating cyclin transcription. Additionally PHIP has an anti-apoptotic activity through the activation and phosphorylation of AKT. Latest data suggest that PHIP has influence on Talin1 (TLN1). TLN1 is involved in the building of actin filaments. This directly correlates with invasion and metastasis through AKT-dependent effects (De Semir et al. 2012). Therefore PHIP may directly activate TLN1 and AKT.

In 2012 it was shown that high PHIP protein expression leads to a significantly increased risk of metastasis and death due to melanoma (De Semir et al. 2012). The Pleckstrin-Homology domain-Interacting Protein is also involved in transcription responses in pancreatic islet cells and in postnatal growth (Li et al. 2010; Podcheko et al. 2007)(Li et al. 2010). Despite these findings it is unknown how PHIP regulates these pathways, therefore further experiments and investigations are necessary.

1.9. Bromodomains on the Pleckstrin Homology domain-interacting protein

A bromodomain (BRD) is a special protein interaction module that specifically recognizes and binds ε-N-lysine acetylation motifs. This is considered as a key event during the reading process of epigenetic marks. ε-N-lysine acetylation of lysine is the most frequent occurring posttranslational modification of proteins (Choudhary et al. 2009). These acetylations have effects on the physicochemical properties of lysine residues, due to the neutralization of positives charges of the ε-amino group (Kouzarides 2000). Many acetylations can be found in macromolecular complexes that are involved in chromatin remodeling, DNA damage and cell cycle control (Choudhary et al. 2009).

Bromodomains can find and interact with acetylation motifs. They are conserved in several different proteins including PHIP, helicases nuclear-scaffolding proteins, transcriptional coactivators and many more (Muller, Filippakopoulos, and Knapp 2011). All bromodomain modules share, despite their large sequence variation, four left handed α helices. The helices are linked by loop regions of variable length together.

Dysfunction of the bromodomains in proteins are related to several diseases. It even can cause an aggressive form of human squamous carcinoma (French 2010). Also deregulation of transcription as a result of altered protein acetylation is a hallmark of cancer. Therefore inhibition of bromodomains by selective inhibitors is a possible option in recent research of cancer.

PHIP is closely related to the proteins BRWD1 and BRWD3. They all have in common a number of WD40 repeats that are followed by two bromodomains. In a druggability analysis and structural classification of bromodomains, the two bromodomains of PHIP were investigated. The first bromodomain sequence had a low score and was therefore classified as not able to be targeted by selective inhibitors. However the second bromodomain of PHIP scored highest in its group

and was placed in the druggable range (Vidler et al. 2012). Therefore we decided to target PHIP with JQ1, a drug that targets bromodomains.

1.10. The bromodomain inhibitor JQ1

The inhibitor JQ1 is related to benzodiazepine derivatives and was shown to be a selective inhibitor of the bromodomains in the BRDT protein (Filippakopoulos et al. 2010). The protein is linked to chromatin remodeling during spermatogenesis. JQ1 has also been investigated for future treatments of HIV infection, cancer and heart diseases (Anand et al. 2013; Banerjee et al. 2012; Ott et al. 2012).

As the targets of JQ1 can be many bromodomain containing proteins, we tried to investigate if this inhibitor can target PHIP in ovarian cancer cell lines.

2. Aims

- Generation of ovarian cancer cell lines stably expressing shRNAs targeting PHIP and assessment of PHIP mRNA and protein levels.
- Characterization of stable ovarian cancer transformants *in vitro*.
- Evaluation of the impact of modulating PHIP expression in ovarian cancer cells.

3. Materials and Methods

3.1. Cell lines

Four cell lines derived from human ovarian cancer specimens were used in this study. All the cell lines mentioned have the capability of forming a tumor when injected subcutaneously in nude mice.

3.1.1. SKOV-3

SKOV-3 is a human cancer cell line derived from an ovarian tumor of a Caucasian woman (64 year old) in 1973. These cells have an epithelial-like morphology and therefore can be used in invasion and migration assays. SKOV-3 cells have a resistance to Tumor Necrosis Factor (TNF), diphtheria toxin, cis- platinum, Adriamycin and other cytotoxic drugs. They are able to grow in soft agar and have a high colony forming capability.

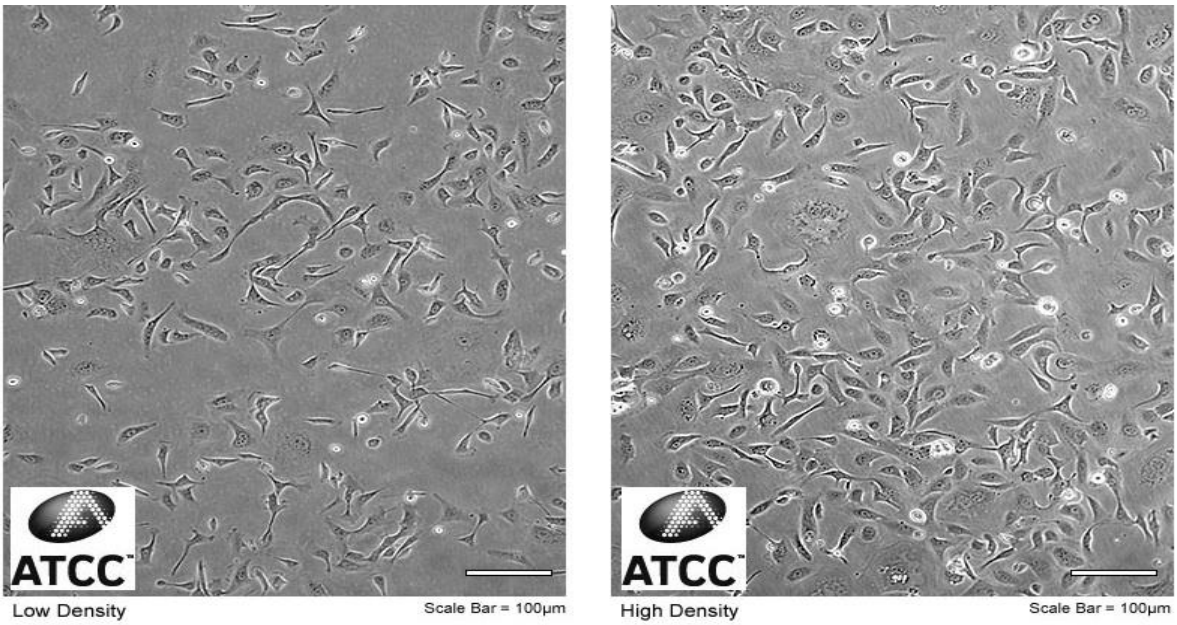


Figure 10: Morphology of SKOV-3 cells in low confluency (left) and in high confluency (right).

(http://www.lgcstandards-atcc.org/~/media/Attachments/6/5/E/8/25938.ashx)

3.1.2. Caov-3

Caov-3 cells were derived from an adenocarcinoma of a 54 year old Caucasian woman in 1973. Their morphology is epithelial and they behave adherent to each

other. Caov-3 cells are hypodiploid and their chromosome number ranges from 42 to 45 chromosomes. A higher number of chromosomes only occurs in 32% of all cells. They are resistant to Tumor Necrosis Factor (TNF) and to other cytotoxic drugs like cis-platinum or diphtheria toxin.

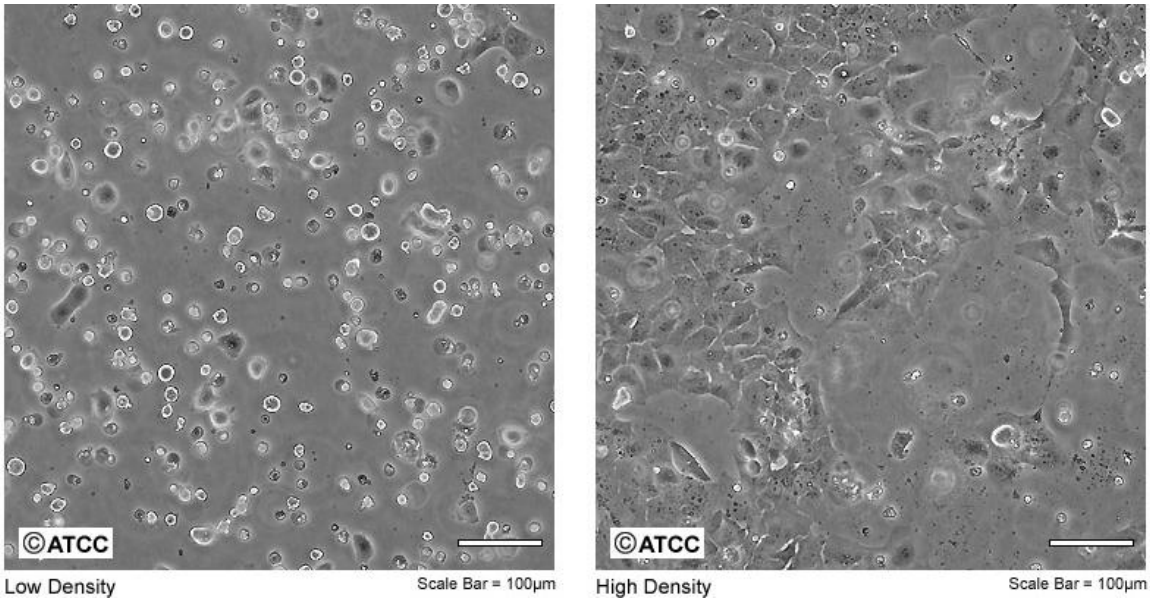


Figure 11: Morphology of Caov-3 cells in low density (left) and in high density (right).

(http://www.lgcstandards-atcc.org/~/media/Attachments/9/8/6/1/1993.ashx)

3.1.3. SW-626

SW-626 cells are epithelial ovary cancer cells derived from a grade III adenocarcinoma of a 46 year old Caucasian woman. These cells were isolated in 1974 in the Scott and White Clinic in Temple/Texas. They are hypertetraploid and have an average chromosome number of 104.

3.1.4. PA-1

PA-1 cells are derived from a 12 year old female Caucasian with a teratocarcinoma. They are ovarian cancer cells and show an epithelial morphology. One of their main characteristics is the activated oncogene N-ras.

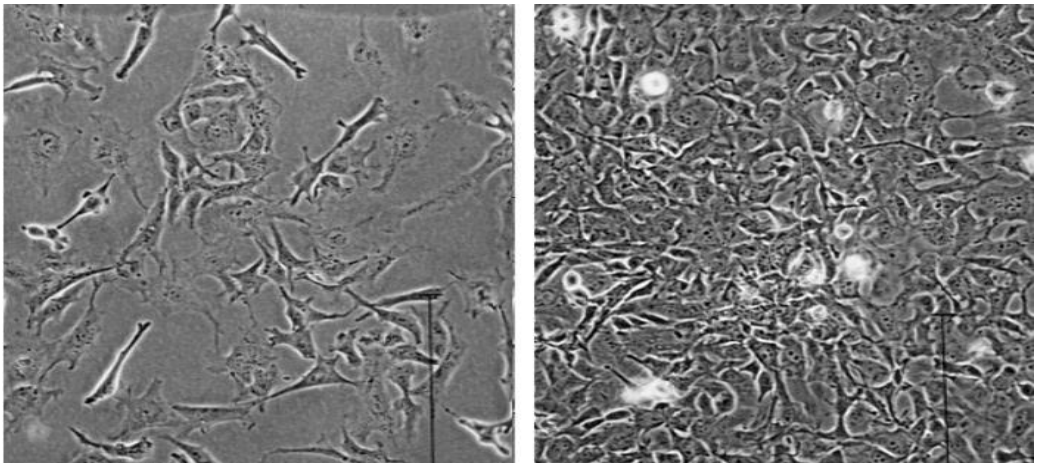


Figure 12: Morphology of PA-1 cells in low density (left) and in high density (right)

(http://cellbank.nibio.go.jp/legacy/pictures/clp06542.jpg)

3.1.5. Cell culture media

Table 1: Cell culture media for all used cell lines and their compositions

Cell line	Media	FBS (%)	P/S (%)	Puromycin [optional] (µg/ml)
SKOV-3	RPMI	10	1	1.5
Caov-3	RPMI	10	1	0.25
SW-626	RPMI	10	1	1.25
PA-1	DMEM/F12	10	1	0.75

3.2. Basic Cell Culture Techniques

All of listed cell culture techniques are performed in laminar hoods under sterile conditions.

3.2.1. Growth conditions of cancer cells

All used cancer cell lines are derived from humans, therefore their environment should be as similar as possible to the human body. All used incubators maintain a temperature of 37°C and a carbon dioxide level of 5%. A metal tray with sterile water at the bottom ensures that the air inside is moist.

3.2.2. Passaging of cancer cells

Cancer cell lines are growing in specific containers like dishes or flasks. These containers usually have a special surface to allow cells to attach. Otherwise most of

them would float and die as result of missing extracellular signals. The cover of the surface provides certain structures which are used by the surface proteins of cancer cells to bind. Usually cancer cells grow fast and need always space to proliferate. Therefore a specific amount of cells has to be removed on a regular basis. The following procedure is used for the harvesting and passaging of all cancer cell lines:

As a first step the media covering the cells has to be aspirated. 4 milliliters of DPBS (Gibco, Cat# 14040-117) are carefully added to wash dead cells, FBS and remaining proteins off. Next the DPBS is aspirated and 1 ml Trypsin-EDTA (0.25%) (Gibco, Cat# 25200-056) is put into the dish. The cells are now incubated for approximately 5 to 10 minutes at 37°C until they detach. A short look at the cells trough a microscope helps to find out how many cells have already detached. To neutralize the Trypsin 5 ml of the cell line specific media are added into the dish. The media is used to wash multiple times the surface of the dish and to detach all cells. Afterwards the liquid (containing detached cells) is sucked into the pipette. Only the desired amount of cells is put back into the old dish or a new one. As a last step the required media is added to a total amount of 10 ml in each 100mm dish. 1 ml out of 6 ml is the regular split ratio to passage cells for two to three days. Passaging is usually performed on Monday, Wednesday and Friday.

3.2.3. Thawing of cancer cells

All frozen cells are stored in liquid nitrogen. Dry ice is used for the transport of frozen cells from the liquid nitrogen tank to the cell culture room.

Cells are thawed in a 37°C warm water bath. When the cancer cells are completely thawed, they are transferred into a 15 milliliter tube (Falcon, Cat# 14-959-49D). Then immediately 1 ml of their specific media is added to counteract the toxic effect of DMSO. Next a centrifugation (1000 rpm for 2 minutes) is performed to collect all cells in a pellet. The remaining supernatant, which is containing DMSO, is removed. The pellet is resuspended in 10 ml media and then transferred into a cell culture dish. Finally the cancer cells are put into the incubator. Cancer cells need a couple of days to gain their normal growth pattern.

3.2.4. Freezing of cancer cells

The freezing media contains 10% DMSO, 40% FBS and 50% cell specific media. The first steps are the same as stated in the protocol of passaging of cancer cells in 2.2.2. .

When the cells are fully detached the liquid is sucked into the pipette and is transferred into 15 ml tube. After a centrifugation (1000 rpm for 2 minutes) the supernatant is removed. Then depending on the size of the cell pellet several milliliters of freezing medium are added to each tube. After the resuspension of the pellet in the freezing media 1 ml of it is transferred into each cryovial. The vials are immediately put onto ice for 30 minutes. They have to be stored in a -80°C fridge afterwards. On the next day the vials are transported into the liquid nitrogen tank. In it the storage time is unlimited.

3.3. Quantitative PCR

Quantitative PCR or real time polymerase chain reaction (rtPCR) uses the principle of the polymerase chain reaction (PCR). The specificity of it is that the amount of synthesized DNA is always monitored. This permanent monitoring shows the reaction process in real time. Compared to the classical PCR the final amount of product can be detected at any time. Another often used feature is that quantitative PCR shows how much starting DNA was in the different samples. This enables the comparison between different treatments and their effect on the RNA or DNA levels. For real time PCR different probes (that are labelled with fluorophores) can be used. These probes bind to DNA and therefore emit a detectable signal if the amount increases.

For a real time PCR DNA is required as the working material. If RNA should be analyzed it has to be extracted and converted into cDNA. If the starting material has a high DNA concentration an early signal will be detected. This is reflected as an increase of the curve in the amplification plot (Figure 4). The opposite is observed for a low concentration of DNA in the starting sample. The curve will rise during the late PCR cycles and therefore will be one of the delayed curves.

3.3.1. Cell pellet formation

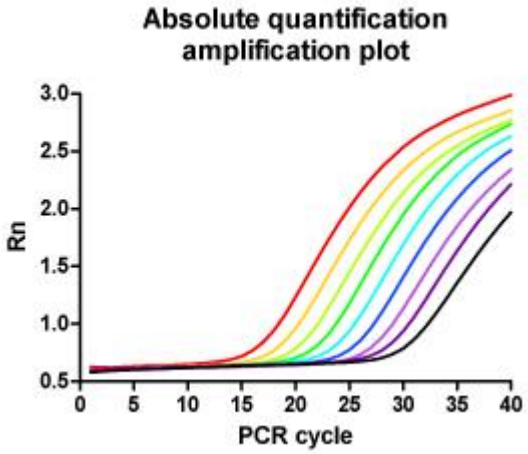


Figure 13: Example for a final read of a real time PCR

This figure shows nine unique samples (in different colors) in a real time PCR amplification plot. Each of these samples had a different amount of starting DNA. The red sample had the highest amount of DNA and the black sample the lowest amount. Therefore a clear difference in the number of PCR cycles can be seen in the plot.

(http://gerichtsmedizin.at/assets/images/forensic_molecular/rtpcr_aluyb8_rn_vs_cycleno.jpg)

Cells are harvested according to the protocol in 2.2.2.After the addition of 5 ml media they are centrifuged (1000 rpm for 2 minutes). The supernatant is removed and 3 ml of DPBS are put onto the pellet. After another centrifugation (1000 rpm for 2 minutes) the supernatant is removed again. The tube with the pellet is put in ice until further use.

3.3.2. mRNA extraction

For mRNA extraction the RNeasy Mini Kit (Qiagen, Cat# 74106) is used according to manufacturer's instructions.

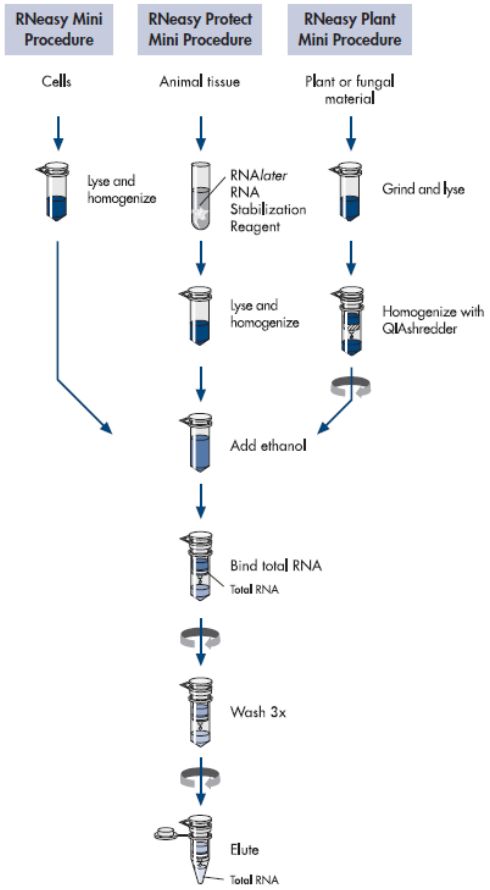


Figure 14: Simple overview of the different steps during the process of RNA extraction

(RNeasy Mini Handbook - (EN))

As a first step a lysis buffer for the cells is prepared. Dependent on the number of cells 350 ml (for <5 x 10^6 cells) or 600ml (>5 x 10^6 cells) of RLT buffer is used. To each milliliter of RLT buffer 10 µl β-ME have to be added. After the addition of RLT buffer (+β-ME) to the pellet, the whole suspension is transferred into a QIAshredder spin column (Qiagen, Cat# 79656). These columns homogenize the sample and remove the insoluble debris. The columns are centrifuged for 2 minutes at full speed. The flow-through is now mixed with the same amount of Ethanol. The Ethanol can cause precipitations in some special cases, but this does not affect the yield or purity of RNA extraction. The whole solution is transferred into an RNeasy spin column. After a centrifugation at 1000 rpm for 15 seconds the flow-through is discarded. Now 700 µl of the RW1 buffer are added into the spin column. Again it is centrifuged for 15 seconds at 1000 rpm. Then 500 µl of the RPE buffer are put into the spin column

and it is centrifuged for 15 seconds at 1000 rpm. The same step is repeated with 500 µl of the RPE buffer and a centrifugation of 1000 rpm for 2 minutes. These buffer additions are necessary to wash undesired product from the RNeasy spin column membrane. Now the collection tube below the filter is replaced by a new one and then the filter is centrifuged at maximum speed for 1 minute. This ensures that all residual flow-through remains are removed from the filter. The last step is to put the filter in new 1.5 ml collection tube and to add 30 to 50 µl of RNase-free water (the volume depends on the size of the starting cell pellet) directly onto the membrane. After a centrifugation for 1 minute at 1000 rpm the filter is removed and the pure RNA is gathered in the collection tube. The RNA should be always kept on ice to avoid degradation.

Kit Contents

RNeasy Mini Kit	(50)	(250)
Catalog no.	74104	74106
Number of preps	50	250
RNeasy Mini Spin Columns (pink)	50	250
Collection Tubes (1.5 ml)	50	250
Collection Tubes (2 ml)*	50	250
Buffer RLT*†	45 ml	220 ml
Buffer RW1†	45 ml	220 ml
Buffer RPE‡ (concentrate)	11 ml	65 ml
RNase-Free Water	10 ml	50 ml
Quick-Start Protocol	1	1

Figure 15: Components of the RNeasy Mini Kit

(RNeasy Mini Handbook - (EN))

3.3.3. mRNA purity

The extracted mRNA has to be tested for purity and quantity. If the mRNA does not meet the specifications it cannot be further used. Therefore a UV-Vis spectrophotometer called Nanodrop 2000 has been used.

The 260/230 ratio is used to indicate the presence of unwanted organic compounds such as Trizol or phenol. Generally acceptable 260/230 ratios are in the range from 2.0-2.2. Values higher than this may indicate contamination with the aforementioned compounds. 260/280 ratios are routinely used to determine the purity of nucleic acid

measurements. This ratio is most commonly used to determine the presence of protein in the isolated nucleic acid sample. Generally acceptable 260/280 ratios are in the range from 1.8 for DNA and 2.0 for RNA.

3.3.4. cDNA Synthesis

For a quantitative PCR of the harvested and purified mRNA, it has to be converted into cDNA. The cDNA does not contain noncoding sequences anymore (because of the already spliced mRNA). The mRNA is combined with the enzyme Reverse Transcriptase, primers and the protein DNA polymerase. The mix is first incubated for 5 minutes at 25°C, then it is heated up for 30 minutes at 42°C and finally to 85°C for 5 minutes. Afterwards it gets cooled down to 4°C until further usage.

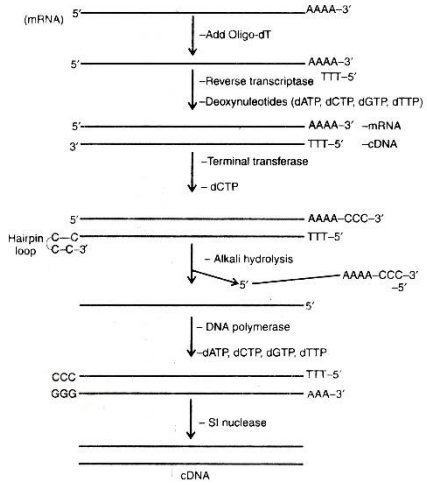


Figure 16: Schematic draw of the generation of cDNA

(http://www.biocyclopedia.com/index/images/Biotechnology/chapter02/013_large.jpg)

Usually 1 µg of mRNA is used. The following table shows the exact composition:

Table 2: Components and reaction set up for mRNA transcription to cDNA

RNA	x µl (final volume has to contain 1 µg of RNA)
5x iScript reaction mix	2 µl
Nuclease-free water	x µl (depended on the amount of RNA)
iScript reverse transcriptase	0.5 µl
Total volume	10 µl

The final cDNA has to be stored on ice or can be frozen at -20°C.

3.3.5. Quantitative PCR with TaqMan reagent

The principle of quantitative TaqMan PCR is that the TaqMan probe is an oligonucleotide probe with an attached fluorophore on the 5'-end. On the 3'-end is a quencher located that suppresses the fluorescence of the fluorophore, which gets excited by a light source via FRET (Fluorescence Resonance Energy Transfer). Therefore the signal is suppressed by the quencher as long as they share the same oligonucleotide.

The specific Taq polymerase can only bind to regions where the specific primer has bound. Therefore the added primer defines what regions are amplified. After the polymerase starts it will encounter the bound Taqman probes somewhere on the DNA strand. The exonuclease activity of the Taq polymerase will degrade the TaqMan probe. This degradation leads to a release of the fluorphore and therefore is not any longer suppressed by the quencher molecule. With FRET the released flourophore is able to emit light that can be detected. This amount of emitted light is proportional to the amount of generated DNA.

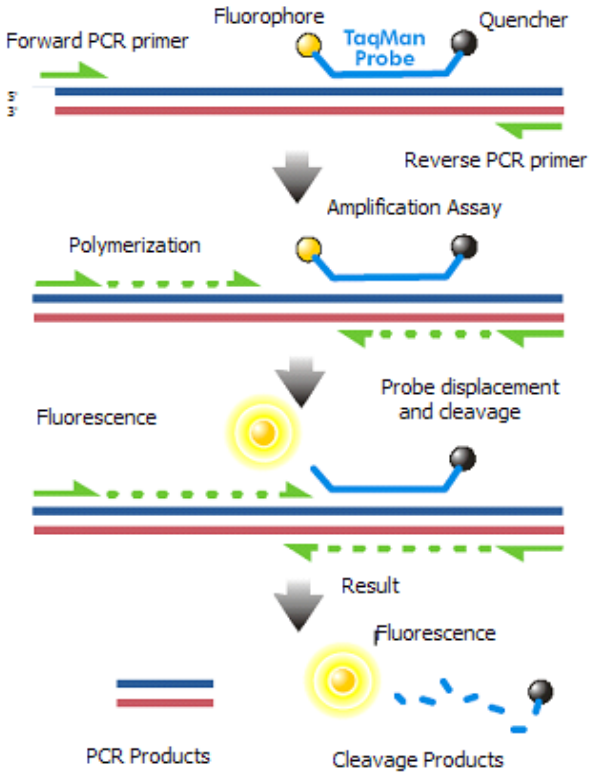


Figure 17: Principle of the quantitative PCR by using a TaqMan probe

First the TaqMan probes bind on different spots of the DNA. During the polymerization by the polymerase the oligonucleotide is degraded and the flourophore is released. The generated light by the flourophore is detected.

(http://upload.wikimedia.org/wikipedia/en/0/07/Taqman.png)

3.4. Knockdown of PHIP via shRNA

Small hairpin RNA (shRNA) is a short sequence of RNA in form of a tight hairpin turn. It is used to suppress a targeted gene by degrading mRNA via RNA interference. We used special designed shRNA to target PHIP mRNAs and reduce the final amount of PHIP protein.

3.4.1. Generation of shRNA lentiviruses

A shRNA PHIP target set (RHS4533-NM_017934) proven to knockdown PHIP in melanoma cell lines was used (Openbiosystems). shRNAs 127738 and 130419 lentiviruses, plus the filled-in ligated pLKO1 containing a shRNA targeting the GFP gene, were prepared by using a three-plasmid packaging system. Briefly, shRNAs cloned into the pLKO1-puro vector were cotransfected into 293T cells along with expression vectors containing the GAG/POL, REV, and VSVG genes by using a Lipofectamine 2000 reagent and following manufacturer's instructions.

3.4.2. Lentiviral infection and generation of stable transformants

Lentiviruses are harvested 48h after transfection by collecting the supernatant of transfected 293T cells and either used immediately or frozen as aliquots in -80°C fridge. As the pLKO-1 plasmid contains a puromycin resistance gene, this allows for the selection of stable transformants upon adding the drug to cells after 72h infection.

Subconfluent PA-1 or SKOV-3 cells are infected with each harvested lentivirus in the presence of 8 µg/mL polybrene and are selected in their respective puyromycin concentration at 72h postinfection.

3.4.3. Mechanism of action

The lentiviruses integrate the shRNA and puromycin resistance vector directly into the cell's DNA. This prevents the loss of it during cell division. After the incorporation of the viral vectors, the shRNA gets transcribed. This transcription of shRNA leads to pre-shRNA, which is exported out of the nucleus via the protein

Exportin 5. In the cytoplasm the pre-shRNA gets further processed by the endoribonuclease Dicer and finally bound to the RNA-induced silencing complex (RISC). The specific sequence of the attached shRNA binds to PHIP mRNA and RISC cleaves it or represses the translation. The result is a lower level of PHIP mRNA, which is translated. This directly leads to lower levels of PHIP proteins.

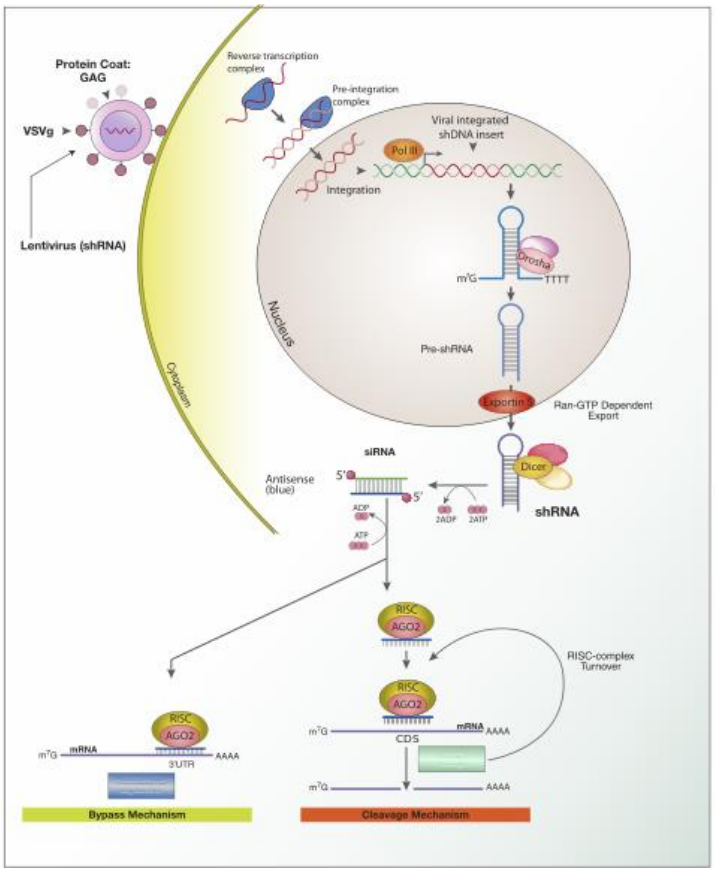


Figure 18: Overview of the lentiviral infection and the mechanism of shRNA

The lentivirus docks on the cell membrane and releases its shRNA containing vectors and the reverse transcriptase into the cytoplasm. The vector is incorporated into the cellular DNA in the nucleus. Then pre-shRNA is generated via translation. After the transfer from the nucleus to the cytoplasm and posttranslational modifications by DICER, the shRNA binds to the RISC complex. The shRNA binds to its target mRNA and the RISC complex either cleaves it or inhibits further usage. Therefore the translation gets inhibited and the final protein levels decreased.

(http://upload.wikimedia.org/wikipedia/commons/thumb/e/e4/ShRNA_Lentivirus.svg/2000px-ShRNA_Lentivirus.svg.png)

3.5. Western Blot

3.5.1. Cell Lysis

For cell lysis the sample buffer RIPA Buffer (Sigma-Aldrich, Cat# R0278) is used. This buffer contains 150mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 50 mM Tris and 0.1% SDS. SDS (Sodium dodecyl sulfate) gives proteins an overall negative charge. This negative charge is responsible that the protein subunits get separated according to their size and not their shape.

Cells are harvested according to the protocol in 2.2.2. . After the addition of 5 ml media they are centrifuged (1000 rpm for 2 minutes). The supernatant is removed and 3 ml of DPBS are put onto the pellet. After another centrifugation (1000 rpm for 2 minutes) the supernatant is removed again. The tube with the pellet is put on ice until it is further proceeded.

Before the RIPA buffer can be used, the HALT (Thermo Scientific, Cat# 78440) protease and phosphatase inhibitor (100x) has to bed added. This provides a protection for the proteins of the lysates from degradation. Dependent on the size of the pellet either 50 µl (for smaller ones) or 100 µl (for larger ones) of RIPA buffer are added and everything has to be well mixed. Then the mixture i pipetted several times through a syringe needle. After an incubation on ice for 30 minutes the whole lysate is centrifuged for 10 minutes at maximum speed. The supernatant is carefully transferred to a new tube without the pellet. This is the final lysate which can be stored at -20°C if needed.

3.5.2. Protein Quantification

For final analysis of the western blot it is very important that in all wells of the gel the same amount of proteins is loaded. Therefore a quantification of the different lysates is performed. Usually the Bradford Assay is the first choice for this task.
Each well of this assay is loaded with 160 µl of HBSS and 40 µl of the Bradford Reagent (Bio-Rad, Cat# 500-0006). For each sample in three wells 1µl of the protein lysis mix is added. Additionally a standard curve with 40 µg, 20 µg, 10 µg, 5 µg and

2.5 µg of BSA has to be performed. The scanning and calculation of the protein amounts is done by a mulitplate reader.

3.5.3. SDS page

For this step the lysed samples are put into a polyacrylamide gel in an electric field. As a result of their negative charges, they have to travel to the anode (positive electrode). Their speed of travelling is depending on their length because proteins, which are longer, can have more negative charges. The gel consists of polyacrylamide and SDS which denatures proteins and gives them negative charges. Our SDS page consists of a tank, lid with power cables, running buffer, stands and gels.

Table 3: Composition of the different separation gels and the stacking gel

	5%	7.5%	10%	Stacking Gel
H_2O	5.7 ml	5.1 ml	4.6 ml	3.1 ml
40% Acrylamide	1.125 ml	1.7ml	2ml	0.5ml
1.5M Tris	2 ml	2 ml	2ml	1.25ml (0.5M Tris)
APS (10%)	75 µl	50 µl	50 µl	50 µl
Temed	8 µl	8 µl	8 µl	5 µl

First the lysed samples are thaw and then mixed with loading dye (1:4). Afterwards they get put in a heating block (95°C) for 5 minutes. Then they are homogenized and centrifuged at 10000 rpm for 30 seconds. Now the samples are ready. Next the gels are put into the stands and then into the tank. The tank gets filled with 1x running buffer (Bio-Rad, Cat# 161-0772) until it covers the gel. Usually 50 µg of proteins (calculated with the Bradford Assay) and ¼ of marker are loaded into the wells. Then the top of the tank is covered by the lid and get connected to the anodes. Last the SDS page is run at 100V until the marker bands are well separated.

Afterwards the gels are taken into a bath of transfer buffer (1x running buffer with 20% methanol).

3.5.4. Immunoblotting

After the proteins are separated in the gel, they have to be transferred onto a membrane. This method also uses an electric field but this time it is perpendicular to the gel. Therefore proteins don't travel in the gel anymore, but out of it. Also here the membrane has to be between the anode and the gel. Very important is that the membrane and the gel are very close together to assure a high amount of transferred proteins.
For blots with high molecular weight proteins a wet blot and for all other proteins a semi dry blot is used.

As the first step the gels are taken out of the running buffer bath and are put onto the nitrocellulose membrane. The membrane and gel are covered by wet filter papers (sucked with transfer buffer). To assure a closely connection between the membrane and the gel, a roller are used several times. For the semi dry blot 25V for 25 minutes are used to transfer the proteins on the membrane. For the wet blot 75V for 120 minutes in a 4°C cold environment have to be used.
Afterwards a blocking of the membrane with TBST (Corning, Cat# 40012043) (Santa Cruz Biotechnology, Cat# sc-29113) plus 5% milk powder is performed for 30 minutes. Blocking assures that no antibodies can bind nonspecifically on the membrane. Then after three washing steps with TBST for 5 minutes the primary antibody is added. The dilutions of the antibody are done according to the technical bulletin and by using TBST. Afterwards the membrane is incubated with the primary antibody overnight in a cold room at 4°C. On the next day the membrane is washed again three times for 10 minutes with TBST to remove all unbound antibodies. Then the secondary antibody is added. After an incubation of 1 hour the antibody is removed and the membrane is washed again three times. As a last step the membrane is ready for the detection by the addition of the secondary antibody specific substrate and an X-ray film.

3.6. Immunofluorescence

Immunofluorescence Microscopy is a method to visualize cells or some of their proteins by using antibodies coupled to a fluorophore. Most times beside an antibody against a special protein also 6-Diamidin-2-phenylindol (Dapi) and Phalloidin are used. Dapi is a fluorescent stain that can bind to the DNA in A-T rich parts. It travels through cell membranes and therefore is used in dead and living cells. Phalloidin is a toxin that binds to F-actin and stains the actin filaments (Capani et al. 2001).

3.6.1. Fixation of cells and proteins

First the medium is removed from the plate and cells are washed with PBS three times for five minutes. Next 4% formaldehyde in PBS is added for 10 minutes at room temperature.

3.6.2. Permeablization

After fixation of the cells with a cross-linking agent cells have to be permeabilized to enable the detection of intracellular antigens by antibodies. For this purpose Triton X-100 is used. After 3 washing steps with PBS for 5 minutes a 0.2% Triton X-100 solution is added for 10 minutes.

3.6.3. Quenching

Afterwards the samples have been blocked by 3% BSA in 1x PBS for 10 minutes to reduce the intensity of fluorescence.

3.6.4. Primary Antibody

This type of antibody directly binds to the desired antigen. Antigens can be proteins, carbohydrates, peptides or other intracellular molecules. The antibody is diluted according to the technical bulletin in an antibody diluent (DAKO, Cat# S0809). Then the antibody is added and incubated overnight at 4°C.

3.6.5. Secondary Antibody

Secondary antibodies can directly bind primary antibodies. They are conjugated with fluorophores which have a specific excitation and emission wave length. Also this antibody has been diluted according to the technical bulletin in the antibody diluent. Before adding the secondary antibody the wells are washed 3 times with PBS for 5 minutes. The secondary antibody is added for 1h at room temperature. Then 3 additional washing steps with PBS for 5 minutes are performed.

3.6.6. Detection by Laser Scanning Microscopy

Afterwards the cells are dehydrated in ethanol series with 70%, 90% and 100% ethanol. As last step cells get covered by a solution called “Vectashield” (Vecta Laboratories, Cat# H-1500). This inhibits photobleaching, prolongs the storage time and contains Dapi for a staining of nuclei. After “Vectashield” is dried the samples can be analyzed by usage of Confocal Microscopy.

3.7. Invasion Assay

An invasion assay is used to determine if a specific cell line has the ability to invade and move through extracellular matrix (EM). In this experiment matrigel is used as an EM. The invasion assay consists out of an upper chamber and a lower chamber. Both chambers are separated by a filter, which is coated by a layer of matrigel. In the upper chamber cells are loaded in a 0% FBS containing media. In contrast the lower chamber is filled with a 20% FBS containing media. This triggers the cells to migrate through the matrigel and towards the lower end of the filter.

3.7.1. Coating with matrigel

The matrigel (BD, Cat# 354234) has to be thawed on ice overnight. Then the matrigel is diluted with media (RPMI or DMEM) to reach the desired concentration. Usually 6 mg/ml or 7 mg/ml (depends on the cell line) are used. Now 15 µl of the diluted matrigel are added onto each filter (Beckon-Dickenson, Cat# 354578). It has to be spread evenly on the top of filter and no bubbles have to be in the matrigel coat. Bubbles would create cavities that will adulterate the result. After 30 to 45

minutes at room temperature the gel should have been solidified. During this time period the next step can be done.

3.7.2. Preparation and loading of cells

The cancer cells are harvested according to the protocol in 2.2.2. . Afterwards they are counted in a Neubauer Chamber. Approximately 150000 cells are needed per upper chamber in 200 µl of plain media. Therefore a proper dilution has to be done. In the lower chamber 300 µl of media containing 20% FBS are loaded. Then the upper chamber is put onto the lower chamber. As a last step the cells are loaded in it. After an overnight incubation continue with the next step.

3.7.3. Fixation, staining and counting

First a 2.5% glutaraldehyde containing solution has to be prepared in PBS. Next a 0.5% toluidine blue / 2% Na_2CO_3 (sodium carbonate) containing solution has to be prepared in ddH_2O.

After preparing these solutions the media is removed (from both chambers) and 500 µl of 2.5% glutaraldehyde/PBS is put into each lower chamber. This fixes the cells. Then the upper chamber is transferred into a new lower chamber containing 500 µl of PBS. Afterwards remove the PBS add 500 µl of the 0.5% toluidine blue / 2% Na_2CO_3 in ddH_2O solution for 5 minutes at RT. This step stains the cells. Now the filters get rinsed by PBS to get rid of the unused dye. Wiping of the top of the filter dry with cotton cutips removes all cells that did not move through the matrigel. Now the cells can be counted under the microscope and the data analyzed.

3.8. Rescue Assay

Rescue Assays are performed to determine if the reduced ability to invade can be restored. In this case shRNA-treated cancer cells, which have significantly reduced invasion into matrigel, are transfected with plasmids containing DNA for the cytoskeletal protein Talin 1 (TLN1). TLN1 has a very important role in the assembly of actin filaments, cell-cell contacts and migration. After the transfection they are compared with cells of the same cell line which have been transfected with an empty plasmid.

To transfect the plasmids into cells, the popular transfection reagent Lipofectamine 2000 (Invitrogen, Cat# 11668027) is used. Lipofectamine uses a process called lipofection. The DNA plasmids are packed into liposomes, which form in aqueous environments like media. The surface of these liposomes is positively charged and can therefore easily interact with the negatively charged plasma membrane of cancer cells. This leads to a fusion of both and a release of the plasmids into the cytoplasm. As a result of the multitude of TLN1 DNA, the number of TLN1 proteins increases. This finally leads to a better invasion potential.

First two 70 to 90% confluent 100 mm plates with cancer cells have to be provided. Then the 5 µg of TLN1 plasmid and 5 µg of empty plasmid (in this case pcDNA) are diluted in two separate 1.5 ml plain media containing tubes. Each of these tubes is added to a 1.5 ml plain media and 15 µl lipofectamine 2000 containing tube. After 20 minutes of incubation each of these two tubes containing either pcDNA or TLN1 plasmids plus Lipofectamine is added together with 7 ml of plain media onto the cells. After another incubation of 5 hours the Lipofectamine containing medium is replaced by regular complete medium containing FBS. On the next day these cells are used for an invasion assay as described in 2.7. .

3.9. Colony Formation Assay

A colony formation assay is used to determine the abilities of cancer cells to create colonies out of a single cell. A colony usually has at least 50 cells. It is a well-

established method to find out the rate of cell proliferation and survival after a treatment with interfering mRNA or a cytotoxic agent. Usually a colony formation assay takes up to one week.

First the cells are harvested according to the protocol stated in 2.2.2. . It is essential to seed the same amount of cells in all wells. Therefore the cancer cell concentrations are determined by counting them with a Neubauer Chamber. Usually a concentration of 500 cells per milliliter is used because each well of a six well plate needs two milliliter of media. After the seeding of the cells in all wells they are incubated at 37°C for up to one week. A regular monitoring of the wells should done on a daily base to find the optimal time point for the read out.
If the single cells created colonies a crystal violet stain is added into each well for 30 minutes. After several washes with water the single colonies can be observed in blue and counted.

3.10. Generation of metaphases for FISH

FISH (Fluorescence *in situ* hybridization) is a method to detect and localize desired DNA sequences in chromosomes. For FISH, fluorescent probes are used that can only bind to specific parts of the chromosomes. For the detection of FISH a fluorescence microscope is used. FISH can be used to show the amount of chromosomes and specific sequences on them. Before FISH can be performed the cells have to be prepared. For this preparation the cells are blocked in metaphase. During the metaphases DNA is compacted as chromosomes which are perfectly aligned and condensed. This is a perfect condition for the performance of a FISH.

To a confluent cancer cell dish 0.5 µg/ml of KaryoMAX Colcemid Solution (Gibco, Cat# 15212) are added. Colcemid has the ability to depolymerise mircotubules and limit the formation of them. This leads to an arrest in the cell cycle during the metaphase. After the addition of Colcemid the cells are incubated from 5h to overnight. Then the cells are harvested and centrifuged at 1000 rpm for 5 minutes. Next the supernatant is removed and the cells are resuspended in a hypotonic 0.075M KCl solution (Gibco, Cat# 10575-090). This mixture is incubated at 37°C for

10 minutes. Again a centrifugation is performed at 1000 rpm for 5 minutes. After the removal of the supernatant a fresh, ice-cold fixative is added drop by drop. This fixative consists out of 25% acetic acid and 75% methanol. After the addition of the mixture it is put on ice for 10 minutes. Afterwards it is incubated at 37°C for 5 minutes and then again centrifuged at 1000 rpm for 5 minutes. Then the supernatant is removed and again 1 ml of the ice cold fixative is added drop by drop onto the pellet. Next this mixture is put as single drops onto clean microscopic slides. After they are dried by air, the chromosomes can be seen in the microscope.

3.11. Senescence Assay

Cells can switch from active proliferation to senescence if they have encountered different events like changes in the protein amounts or DNA damage. Senescent cells lose the ability to divide and change their morphology, shape and gene expression. At the end usually apoptosis occurs.

With the Senescence Cells Histochemical Staining Kit (Sigma, Cat# CS0030) the amount of cells are determined that undergo senescence after shRNA treatment targeting PHIP. This assay is based on a histochemical stain for β-galactosidase activity at a pH value of 6. At this pH value β-galactosidase activity can be easily detected in senescent cells, but not in proliferating or quiescent ones. The kit consists of three important solutions: fixation buffer, 1x PBS and staining mixture. The following protocol is used according to the technical bulletin:

10000 cells are plated in 6 well plates in their representing media three days before the assay is applied to them. This gives them time to settle, start proliferation or transform into a senescent form.
On the next day the medium is removed from the wells containing the plated cells. The wells get washed two times with 1 ml of 1x PBS to remove all undesired particles. The wash gets removed carefully by aspiration, so the plated cells don't detach. Then 1.5 ml of fixation buffer (containing 20% formaldehyde) is added per well. The plate gets incubated for 6 to 7 minutes at room temperature. Afterwards

the wells get washed carefully three times with 1 ml 1x PBS. Now 1 ml of the staining mixture is added per well. The plates get incubated overnight at 37°C. The cells cannot be put in a CO2 incubator because the staining is pH dependent. On the next day senescent cells appear blue under a microscope. They can be counted and compared to the numbers of different shRNA treated cell lines.

4. Results

4.1. Determination of intrinsic puromycin resistance

As one of the first steps our ovarian cancer cell lines will be infected with a plasmid that contains a shRNA targeting PHIP and a resistance gene against the antibiotic puromycin. Before we infect the cells it is important to determine the natural resistance of each cell. We observed that specific cell lines can handle three times more than others. Therefore it is important to start with an experiment that determines the intrinsic puromycin resistance.
The aim of this experiment was to find the perfect concentration in which the cells struggle to grow and slowly enter the process of apoptosis.

Table 4: Determination of the intrinsic puromycine resistance of ovarian cancer cell lines

In this experiment the four ovarian cancer cell lines are treated with six different puromycin concentrations. The aim is to find the optimum concentration for further treatment of plasmid infected cells, which carry a puromycin resistance. Orange shows that all cells survived the treatment without any impairment. Green shows the perfect concentration at which the cells start to undergo apoptosis. Red shows that all of the cells are dead.

Cell line	0.25 µg/µl	0.5 µg/µl	0.75 µg/µl	1 µg/µl	1.25 µg/µl	1.5 µg/µl
PA-1	Alive	Optimum	Dead	Dead	Dead	Dead
SKOV-3	Alive	Alive	Alive	Alive	Alive	Optimum
CAOV-3	Optimum	Dead	Dead	Dead	Dead	Dead
SW-626	Alive	Alive	Alive	Optimum	Dead	Dead

The outcome of this experiment shows us that the puromycin concentration varies strongly between the individual cell lines. For example the PA-1 cells need 3 times less puromycin than the SKOV-3 cells.

Based on this experiment, the proliferation rate and the PHIP expression levels by western blot, we decided to continue our study with an infection of the PA-1 cells and the SKOV-3 cells. The infection introduces either one of our two shRNAs (127 and 130) against PHIP or a shRNA against GFP (green fluorescence protein).

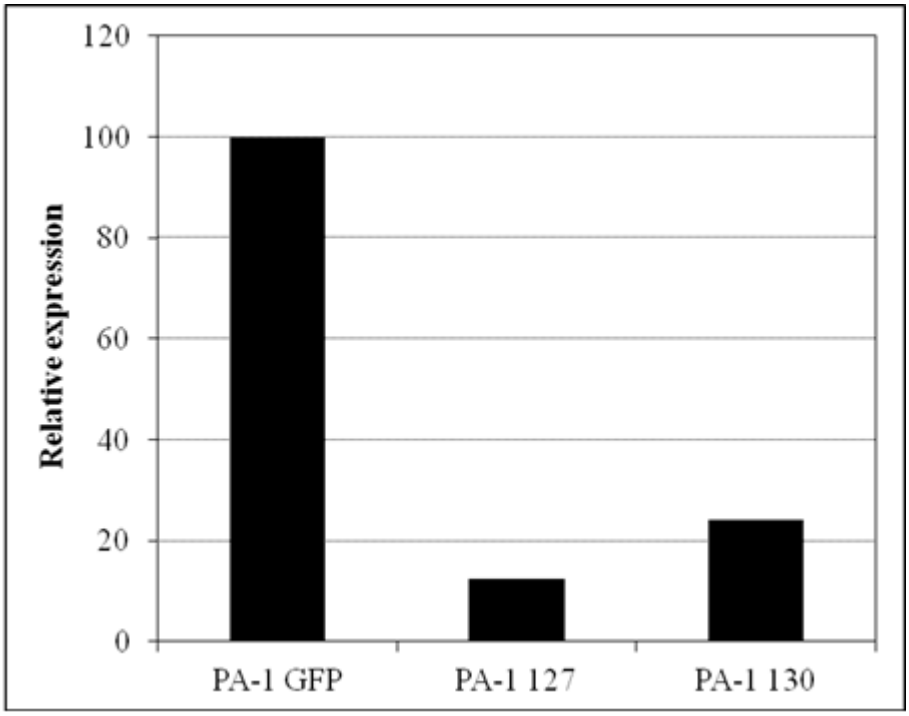
4.2. Determination of PHIP mRNA knockdown by a quantitative PCR

After the infection of the PA-1 and SKOV-3 cells with the plasmid containing the short hairpin RNA locus, the amount of PHIP mRNA in the cells has to be determined. The cancer cells with 127 and 130 shRNA interfering the PHIP mRNA are supposed to have significantly lower mRNA levels of PHIP than those of the cells with the GFP shRNA. To test this a quantitative PCR is performed and the CT values are compared.

Table 5: Percentage of PHIP mRNA knockdown in 127 and 130 shRNA treated cells compared to GFP shRNA treated cells

After the infection of the PA-1 and SKOV-3 cells with either a 127 or 130 shRNA targeting PHIP or a shRNA targeting GFP the amount of knockdown in RNA expression has to be determined by quantitative PCR. This table shows all three PA-1 and SKOV-3 cell lines with their relative percent expression of PHIP normalized to the housekeeping gene HPRT. The 127 and 130 values are compared to the GFP cells to determine the difference in percentage between the actual knockdown cell lines and the control group.

		Relative Percent Expression of PHIP normalized to HPRT	Knockdown of PHIP mRNA (%)
1	PA-1 GFP	90.1	
2	PA-1 127	11.25	87,52
3	PA-1 130	21.79	75,82
4	SKOV-3 GFP	215.56	
5	SKOV-3 127	36,63	83.01
6	SKOV-3 130	36,20	83.21



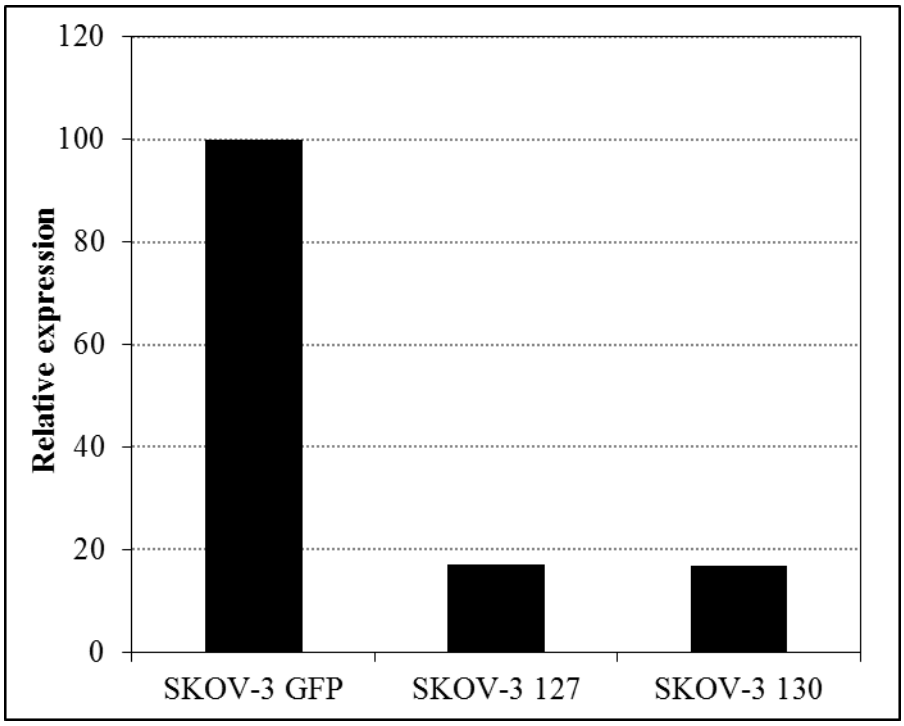


Figure 19: Illustration of the results shown in table 5

In both cell lines a significant difference between the cancer cells containing the 127 or 130 shRNAs and the cells containing the GFP shRNA can be observed.

The tested shRNAs 127 and 130 reduced the amount of PHIP mRNA in PA-1 and SKOV-3 cells significantly. The knockdown levels range from 75.8 to 87.5%. This data confirms that the viral infections and the puromycin selection worked.

4.3. Protein Quantification

To determine if the reduced mRNA levels of PHIP correlate with a reduced protein expression Western Blots are performed.

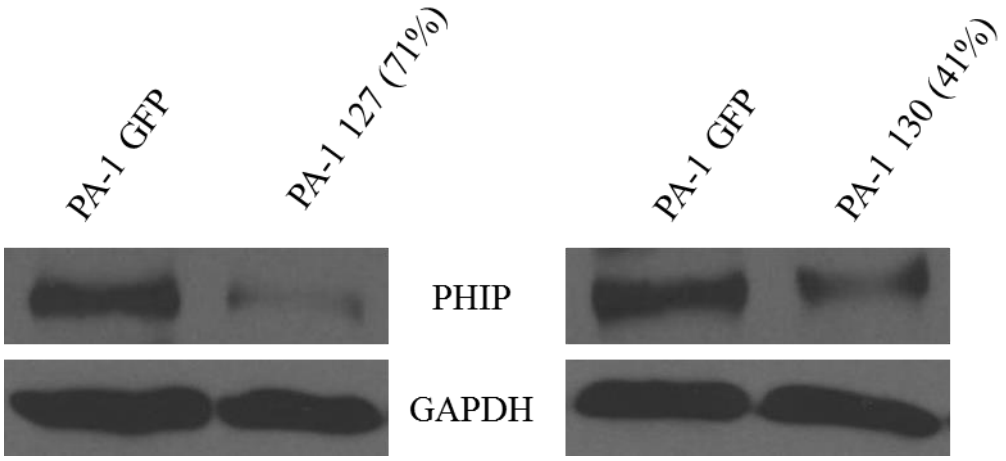


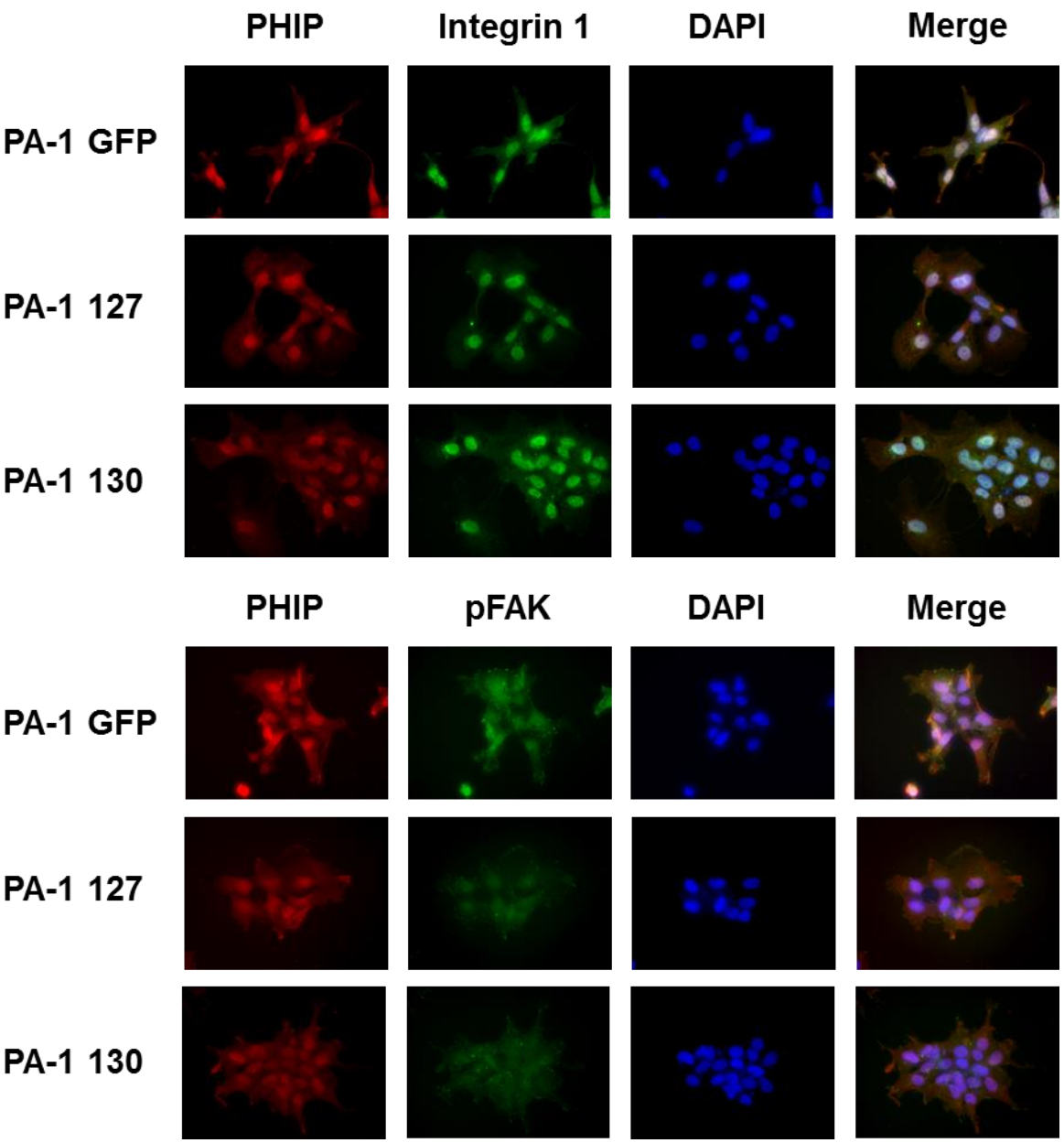
Figure 20: Western Blot for the proteins PHIP and GAPDH

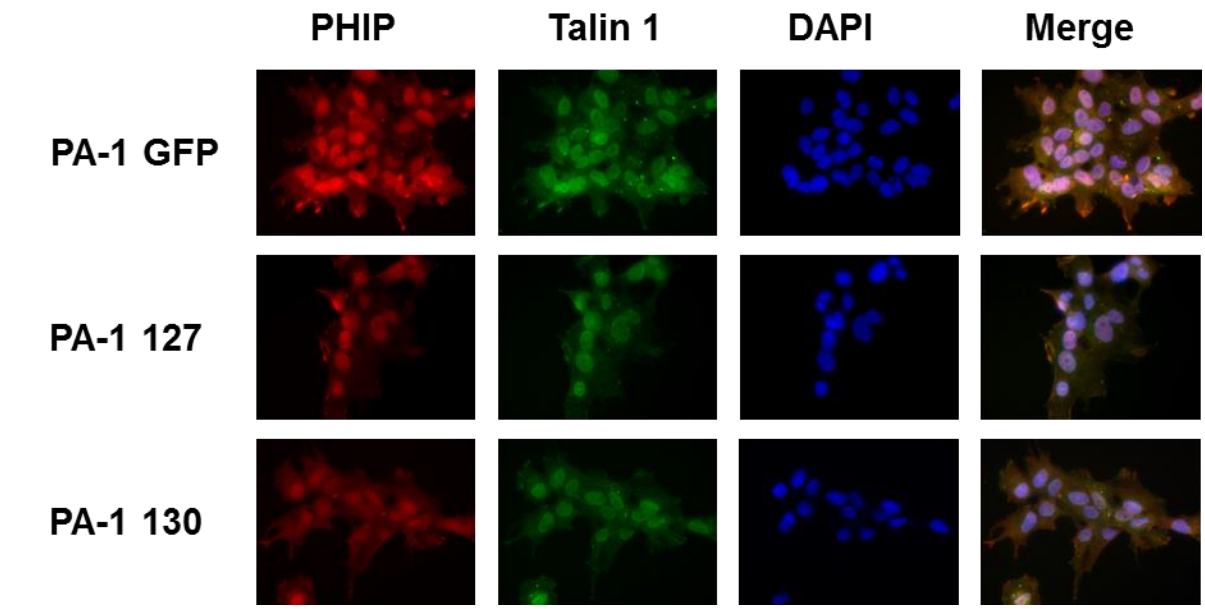
The proteins of our cell lines PA-1 GFP, 127 and 130 are used to perform Western Blots, that visualize the amount PHIP and GAPDH proteins. GAPDH is a house keeping gene that is used to show that the same amounts have been loaded in all slots of the gels.

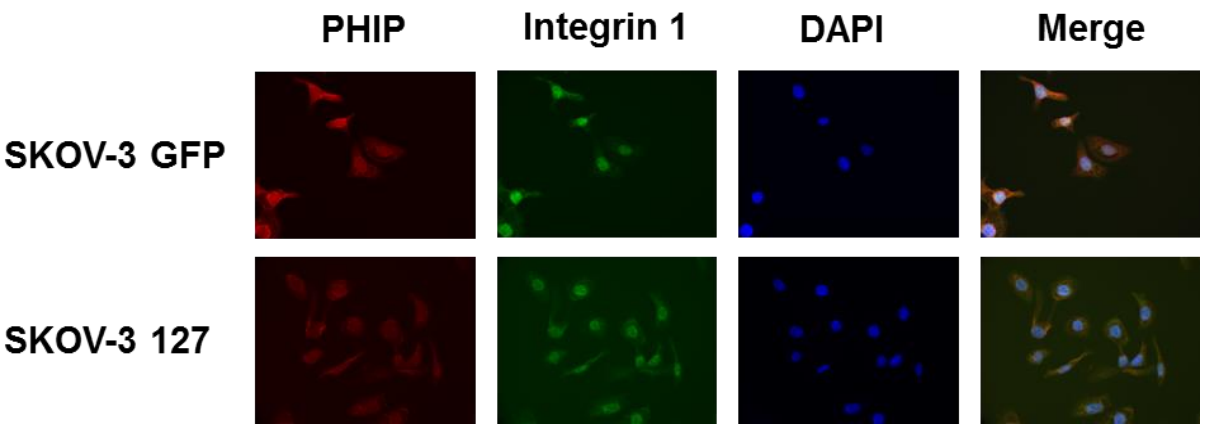
PHIP is expressed 71% less in PA-1 127 and 41% less in PA-1 130 cells compared to the control cells PA-1 GFP. This shows that the shRNA infection not only reduces the mRNA levels of PHIP but also the protein levels.

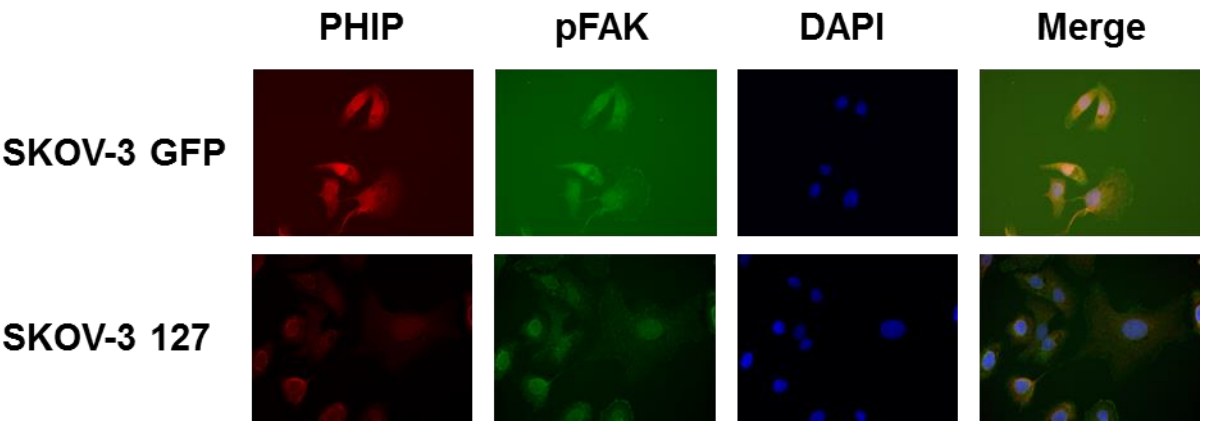
4.4. Immunostaining of PHIP and focal adhesion proteins

With the help of immunostaining it is possible to detect the location of different proteins in the cell and also to quantify them. This allows us to observe the effect of PHIP on important focal adhesion proteins like phospho-FAK (Focal adhesion kinase), Integrin B1 and Talin 1.


PHIP
Integrin 1
DAPI
Merge
PA-1 GFP
PA-1 127
PA-1 130
PHIP
pFAK
DAPI
Merge
PA-1 GFP
PA-1 127
PA-1 130


PHIP
Talin 1
DAPI
Merge
PA-1 GFP
PA-1 127
PA-1 130


PHIP
Integrin 1
DAPI
Merge
SKOV-3 GFP
SKOV-3 127


PHIP
pFAK
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Merge
SKOV-3 GFP
SKOV-3 127

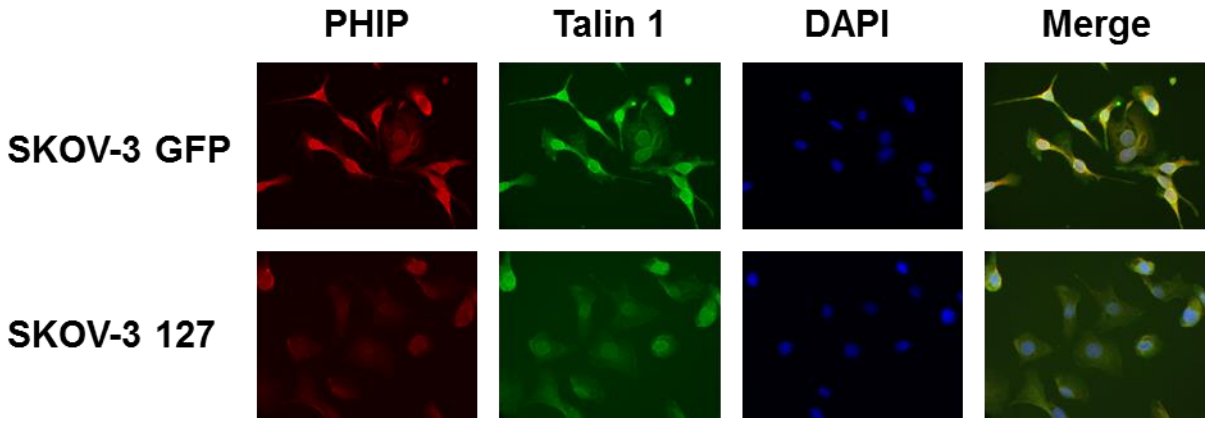
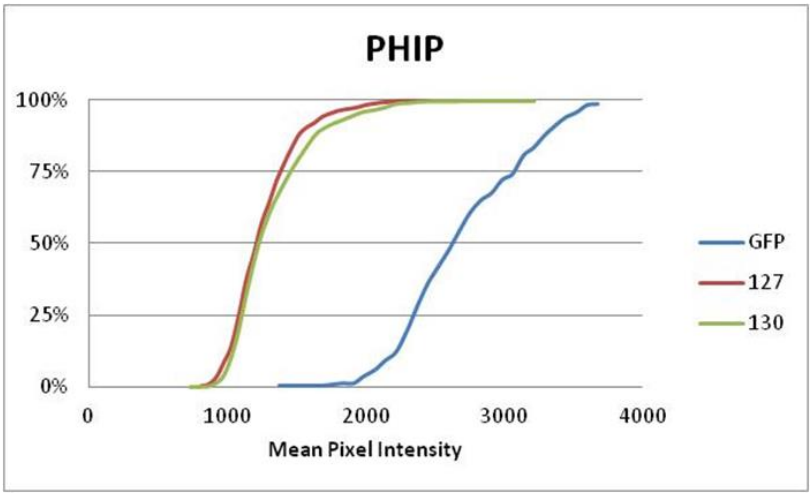
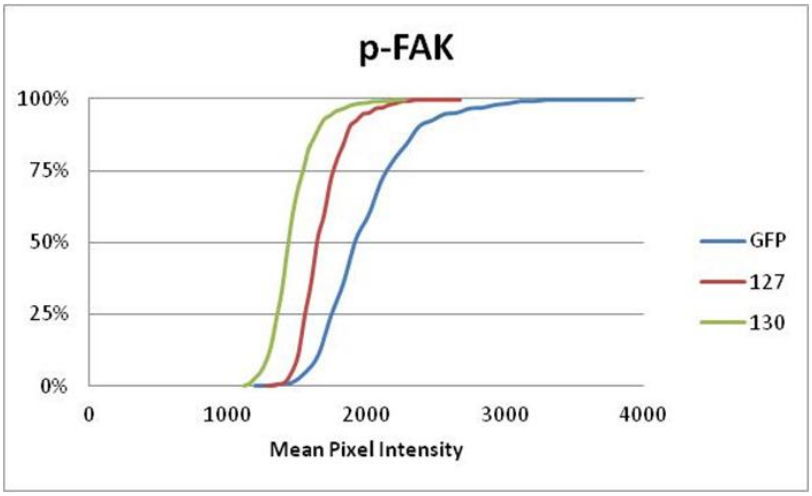


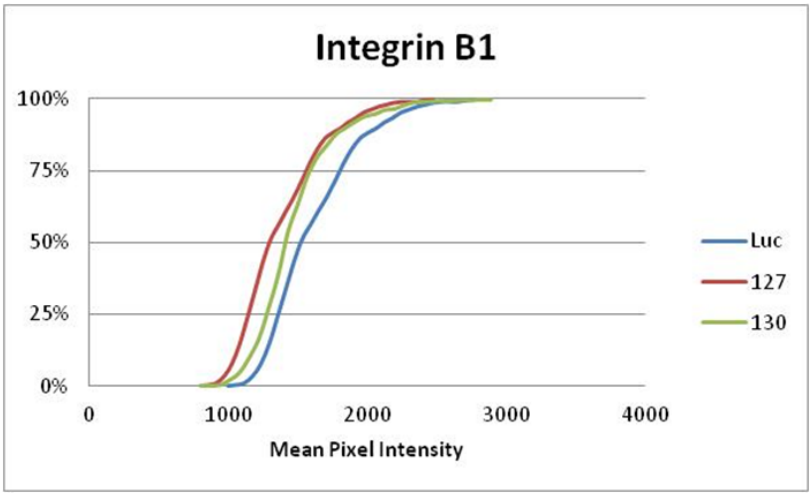
Figure 21: Immunostainings of the shRNA GFP, 127 and 130 containing PA-1 cells and GFP and 127 containing SKOV-3 cells

Blue indicates nuclei, red PHIP and green one of the three focal adhesion proteins p-FAK, Integrin B1 and Talin 1.

As shown in figure 20 PHIP is present in the nucleus as well as in the cytoplasm. In the cell lines with shRNA targeting PHIP (127 and 130) the focal adhesion proteins appear to be reduced in comparison with the control group. In the PA-1 GFP pFAK clusters can be clearly observed whereas they are less abundant in PA-1 127 and PA-1 130 cells. Almost the same occurs for Talin 1 expression. To determine the amount of reduction a quantitative analysis has been performed and the results are shown in figure 21.







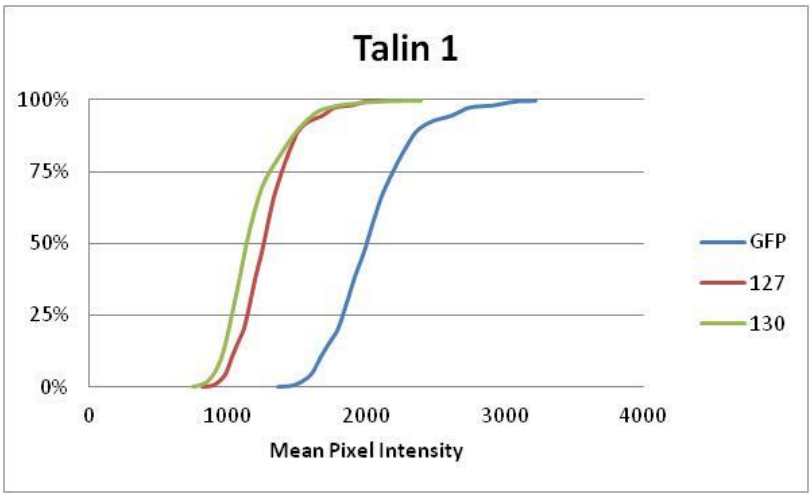
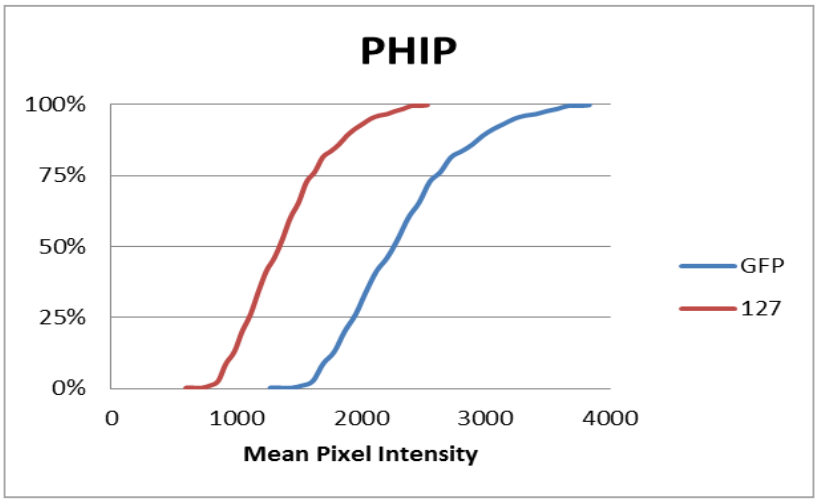
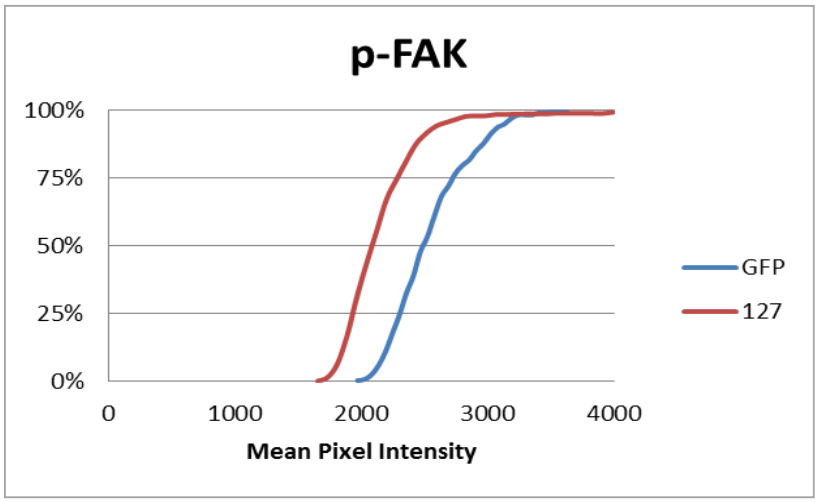
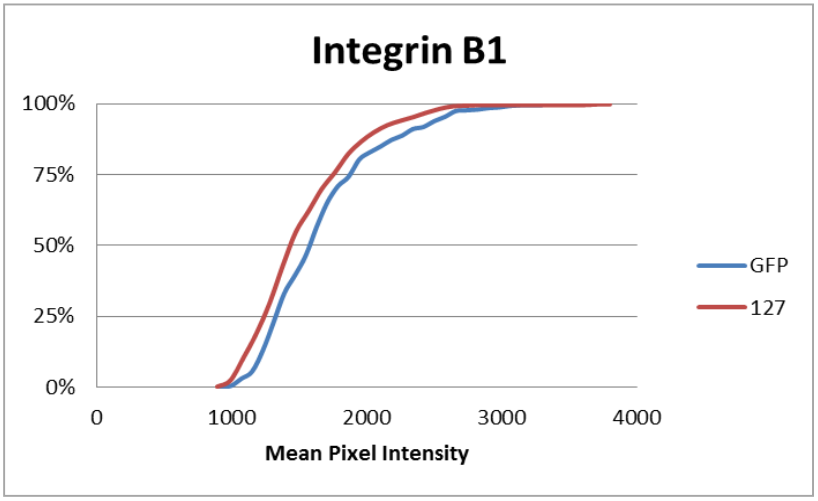


Figure 22: Quantification of the amount of PHIP, pFAK, Integrin B1 and Talin 1 in GFP, 127 and 130 shRNA containing PA-1 cells


PHIP
100%
75%
50%
25%
0%
0
1000
2000
3000
4000
Mean Pixel Intensity
GFP
127


p-FAK
100%
75%
50%
25%
0%
0
1000
2000
3000
4000
Mean Pixel Intensity
GFP
127


Integrin B1
100%
75%
50%
25%
0%
0
1000
2000
3000
4000
Mean Pixel Intensity
GFP
127

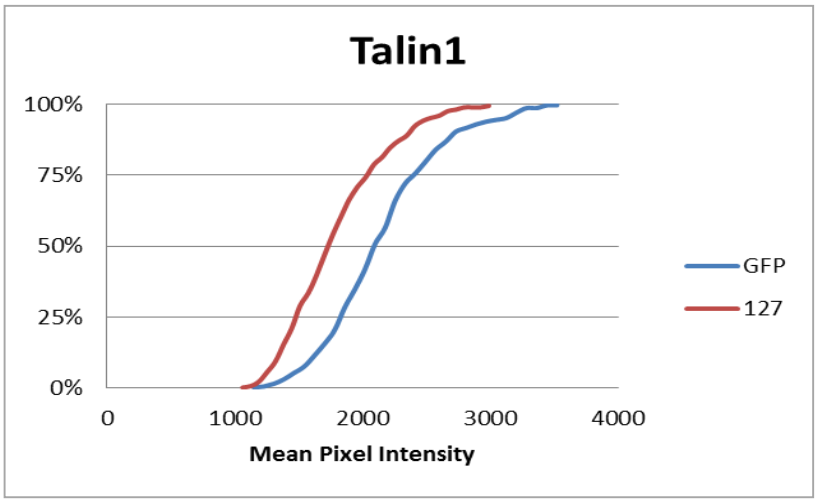


Figure 23: Quantification of the amount of PHIP, pFAK, Integrin B1 and Talin 1 in GFP, 127 and 130 shRNA containing SKOV-3 cells

The quantification of these four proteins reveals the possible effect of PHIP onto the amount of p-FAK, Integrin B1 and Talin 1 in PA-1 cells. As indicated in figure 21 the amount of PHIP is clearly decreased in the 127 and 130 shRNA containing cells compared to the GFP shRNA containing PA-1 cells. The levels of p-FAK and Talin 1 appear to be significantly reduced in PHIP knockdown cells. Also Integrin B1 has slightly lower levels than in the control cells.

4.5. Colony Formation

After the successful visualization and quantification of the PHIP knockdown, the next step is to determine its effects on the proliferation rate of the knockdown cells. Therefore a colony formation experiment has been performed with 1000 cells seeded in triplicate wells for 6 days.

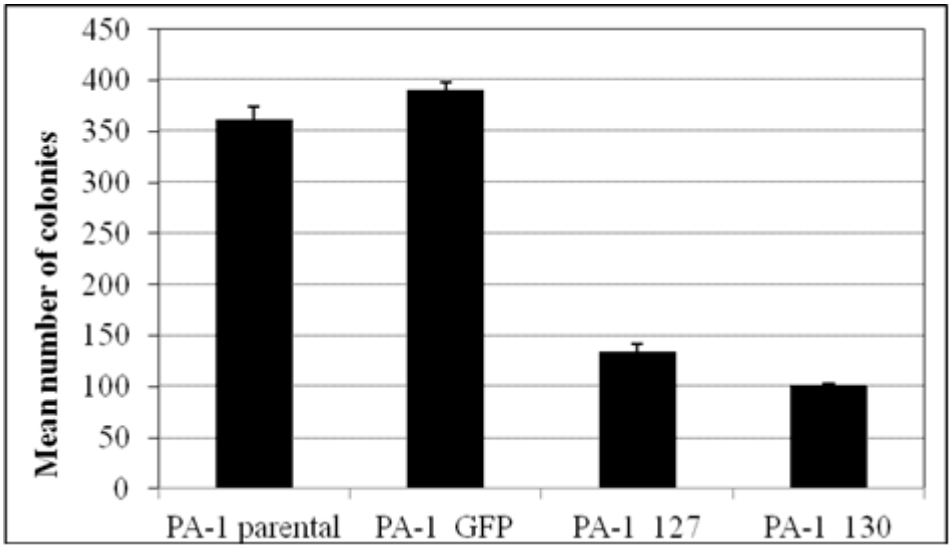


Figure 25: Mean number of colonies counted for PA-1 parental, PA-1 GFP, PA-1 127 and PA-1 130 cells after 6 days of incubation

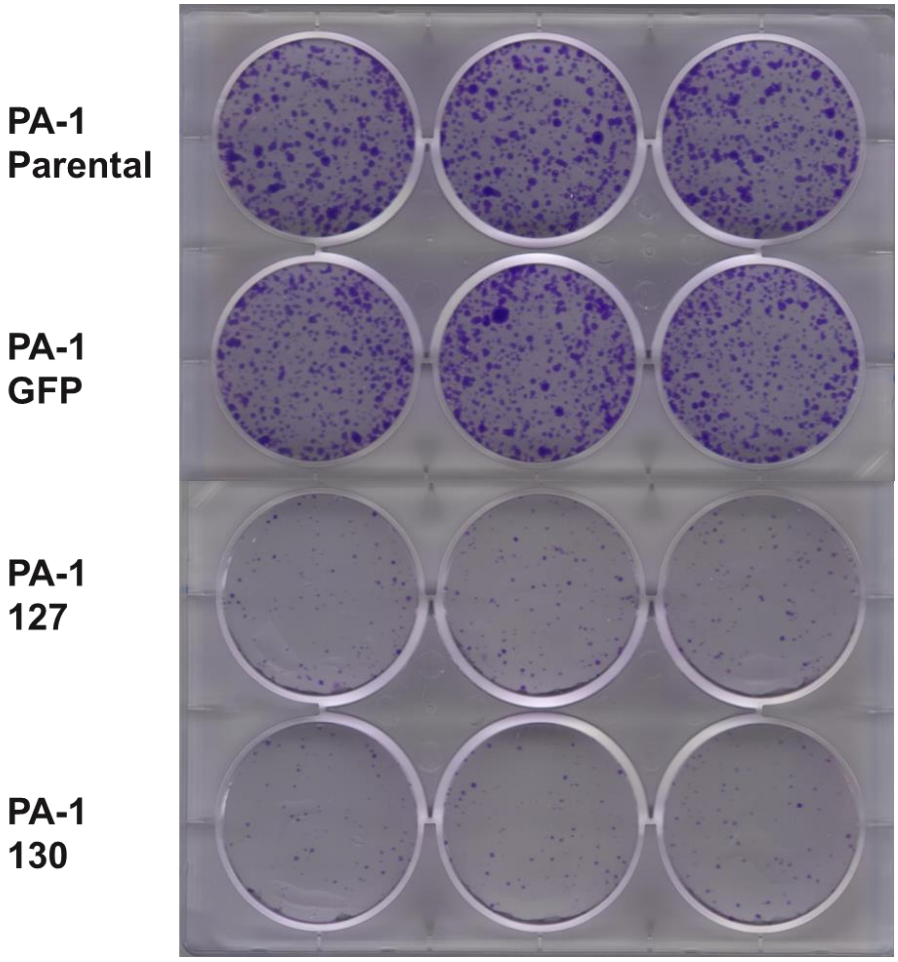
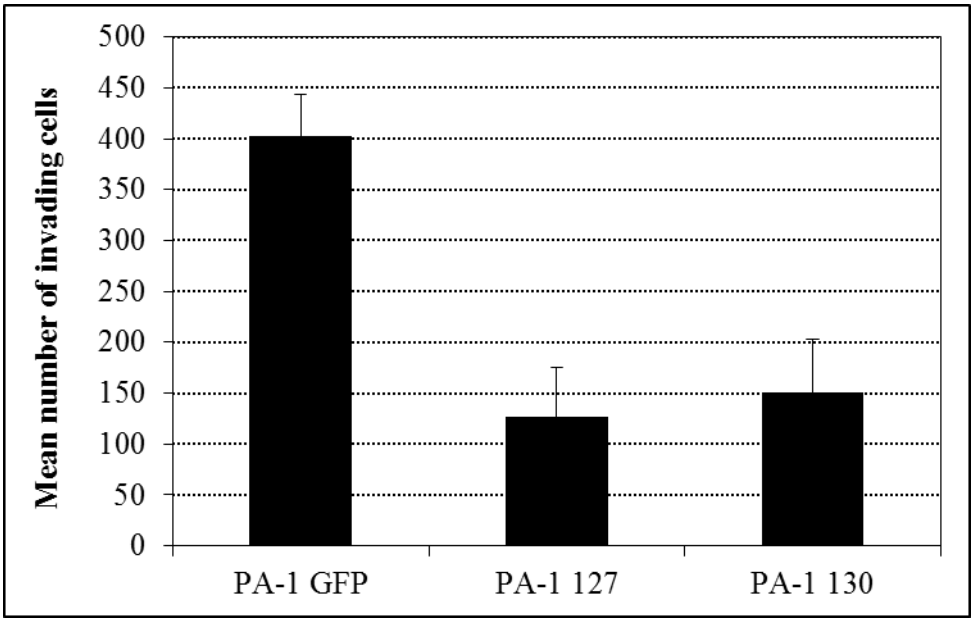


Figure 24: Image of the stained colonies of the four cell lines after 6 days of growth

Figure 21 and 22 clearly show that PHIP has an effect on the proliferation rate of cells. There is a huge difference between the knockdown and the control/parental cells. This arises the question if only the proliferation is impaired in absence of PHIP or their movement ability in extracellular matrix too.

4.6. Invasion Assay

The extracellular matrix is an important scaffold and barrier in tissues. If cancer cells can move through it by the use of digestion enzymes this can lead to higher rates of invasiveness and metastasis. To determine the effect of PHIP on the invasion capability of cancer cells in the extracellular matrix, an invasion assay is performed.



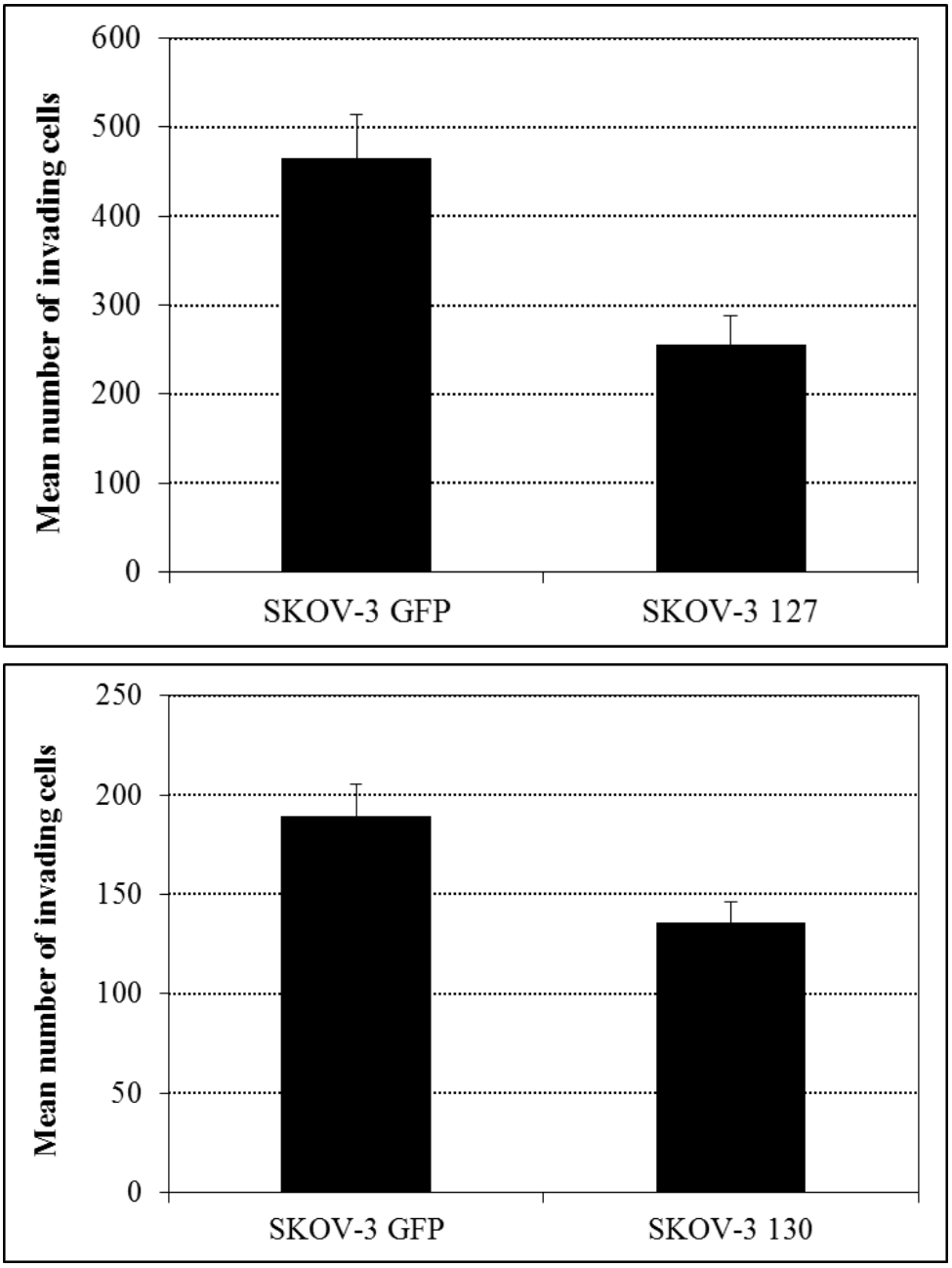


Figure 26: Results of the invasion assay performed with the cell lines PA-1 and SKOV-3 GFP, 127 and 130

This assay has been performed with 150000 cells per well onto 17 µl of matrigel with a concentration of 9 mg/ml. The cells have been incubated overnight. On the next day the migrated cells have been counted.

PA-1 shRNA 127 and 130 against PHIP cells show significantly less migration through the matrigel compared to the control cell line PA-1 GFP. Also the SKOV-

3 127 and 130 cells have a significantly decreases invasion potential than their GFP control cells.

4.7. Rescue Assay

To confirm that the reduced ability of invasiveness can be reversed, a Talin 1 containing plasmid is transfected into the shRNA treated cancer cells. TLN1 has a very important role in the assembly of actin filaments, cell to cell contacts and migration.

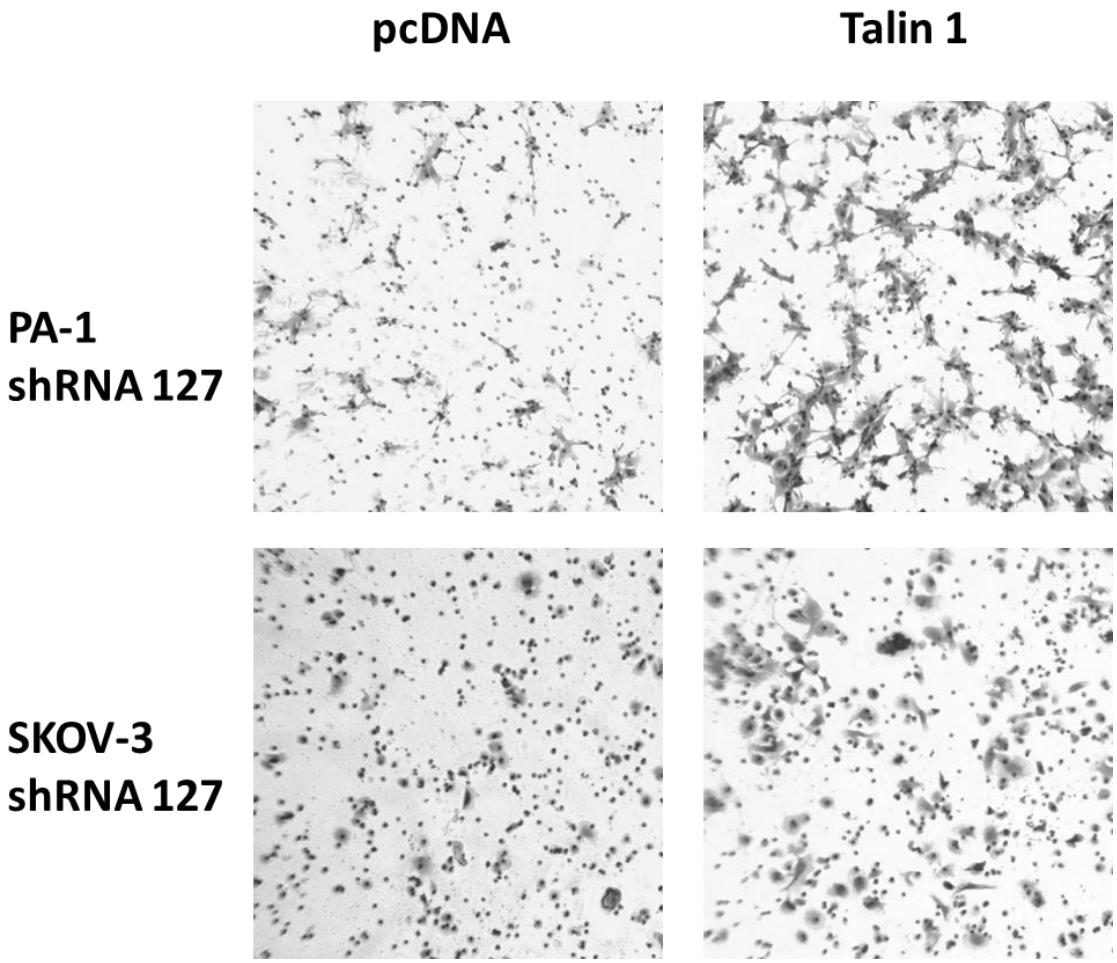
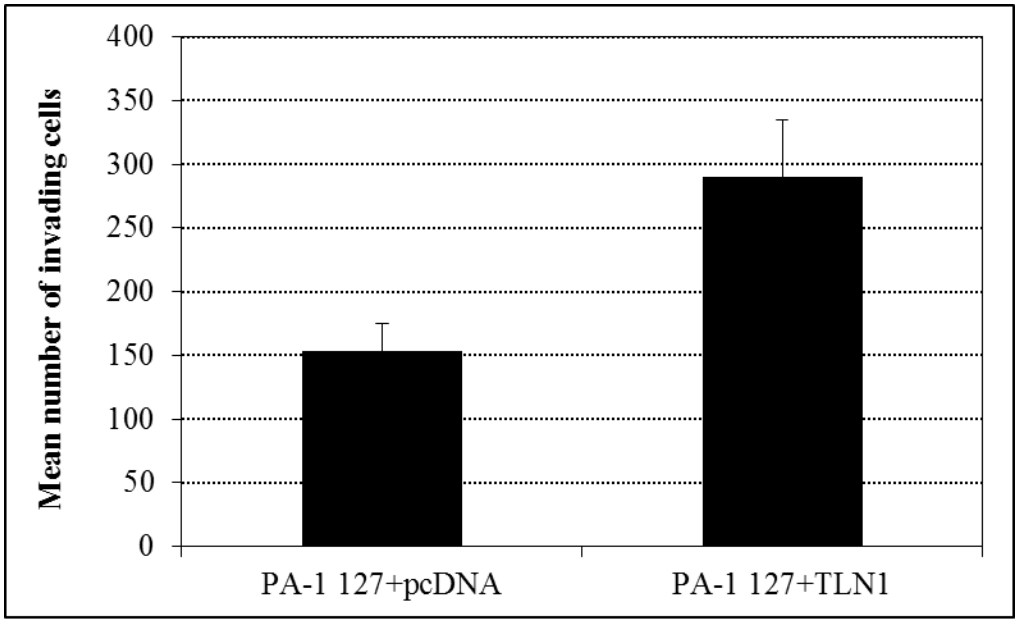


Figure 27: Rescue assay of shRNA 127 cells transfected with either the plasmids containing pcDNA (empty vector) or Talin 1.

PA-1 and SKOV-3 shRNA 127 cancer cells are transfected with the plasmids containing pcDNA (control) or Talin 1. The cells are plated on a matrigel coated filter overnight. On the next day they are fixed, stained and counted.



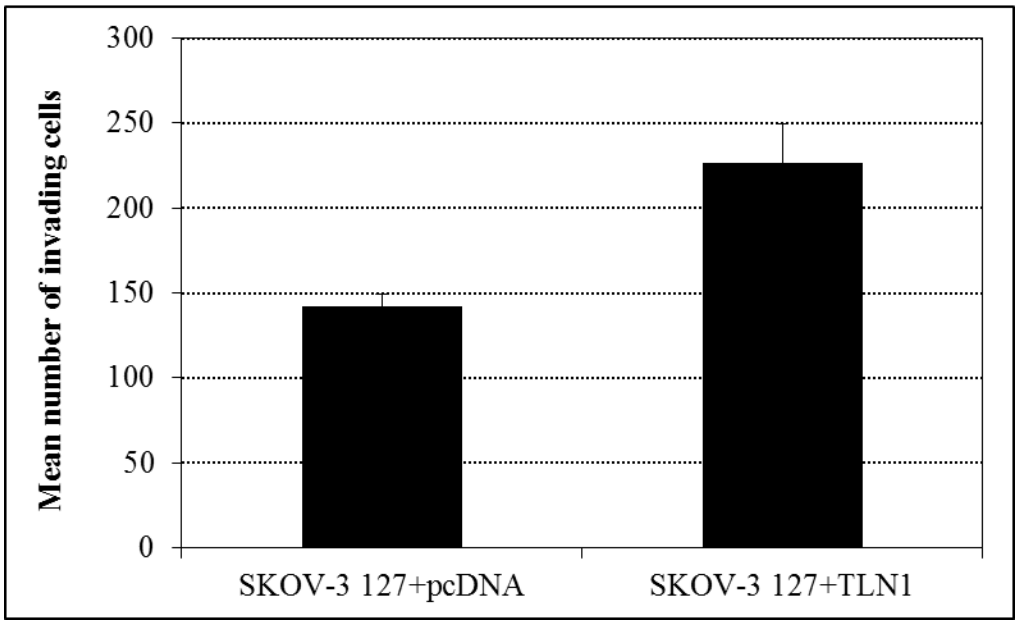


Figure 28: Mean cell numbers of pcDNA and Talin 1 plasmid containing PA-1 and SKOV-3 cells

5. JQ1 Sensitivity of PA-1 ovarian cancer cells

JQ1 is a selective inhibitor of bromodomains. PHIP contains two bromodomains that are theoretically targetable by inhibitors. We decided to investigate if lower PHIP levels would influence the sensitivity to JQ1 in the ovarian cancer cell line PA-1.

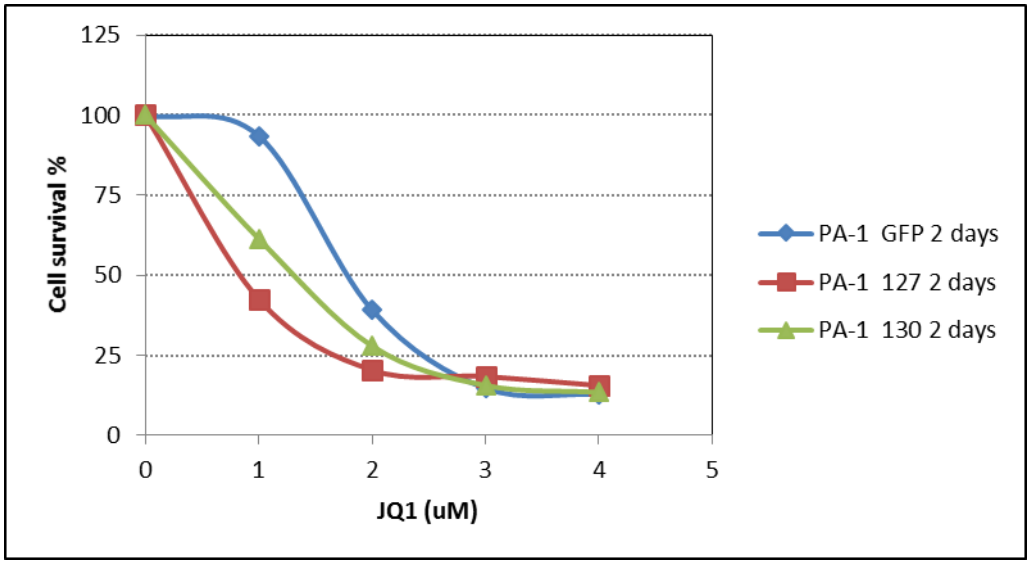


Figure 29: Sensitivity of PA-1 127, 130 and GFP cells to the selective bromodomain inhibitor JQ1

PA-1 127 and 130 cells are more sensitive to JQ1 concentrations of 1 and 2 µM than the control cell line GFP. ShRNA-mediated suppression of PHIP appears to lower the half maximal effective concentration (EC50) from 2 µM to 1 µM of JQ1.

6. Discussion

In 2000 Janet Farhang-Fallah et al. reported the Pleckstrin-Homology domain-interacting protein for the first time. Since then various papers showed its involvement in different cellular pathways like cytoskeletal organization or proliferation. One of them indicates that PHIP is significantly involved in invasion and progression of melanomas and that it is a potential target in cancer therapy (De Semir et al. 2012).

My work and this thesis investigate the unreported role of PHIP in ovarian cancer.

The research group, I was part of, published data that it is possible to introduce shRNA via lentiviruses interfering with the PHIP mRNA. This leads to a reduction of the final concentration of PHIP proteins in melanoma cells (De Semir et al. 2012). Therefore we assumed that this could be possible in ovarian cancer cells too. After the successful infection the stable PA-1 and SKOV-3 cells were successfully selected from those without shRNA genes by puromycin selection. A week after selection, a real time PCR was performed to determine the levels of PHIP mRNA knockdown. We achieved a significant knockdown in all cell lines. In Western Blots we showed that this mRNA knockdown correlated with lower levels of PHIP protein in PA-1 cells. We concluded that shRNA induced knockdown of the PHIP mRNA and protein is possible in ovarian cancer cells.

It has been shown that lower PHIP levels in melanoma can reduce tumor invasion. Additionally PHIP shows to coordinate melanoma cell invasion through TLN1 and AKT activation (De Semir et al. 2012). We assumed that this could be similar in our ovarian cancer cell lines PA-1 and SKOV-3. Immunofluorescence assays revealed that beside PHIP also pFAK, Integrin B1 and Talin 1 are significantly down regulated in PHIP knockdown cells. Talin 1 is involved in the assembly of actin filaments and therefore also in tumor invasion and metastasis (Sakamoto et al. 2010). Integrin B1 is reported to be involved in tumor progression (Koistinen and Heino 2000). The role of pFAK in cancer is controversial but there is evidence that abundance is associated with distant and lymph node metastases (Aust et al. 2014). The protein

AKT is reported to have interactions with PHIP and drives tumor progression in melanoma (De Semir et al. 2012). We performed Western Blots to analyze the amounts of AKT and phosphorylated AKT (pAKT). Unfortunately there is no difference in AKT or pAKT levels in knockdown and control cells. Therefore we can only report that PHIP knockdown reduces pFAK, Integrin b1 and Talin 1 proteins but not AKT or pAKT.

In melanoma cells reduced PHIP impairs the proliferation rate (De Semir et al. 2012). Therefore we decided to set up a colony formation assay. We assumed that shRNA-mediated suppression of PHIP may have an effect on ovarian cancer cell proliferation rates too. After conducting these experiments we observed that these PHIP knockdown cells have a much lower proliferation rate than the control cell lines. This gives us strong evidence that PHIP is able to regulate the cell cycle in ovarian cancers too. Lower levels of PHIP significantly impair their ability to divide.

After the analysis of our Immunofluorescence slides and the finding that several focal adhesion proteins like Talin 1 are down regulated, we decided to study their impact in the invasion potential of our PHIP knockdown cells. The PA-1 PHIP knockdown cells had a reduction of their invasiveness of more than 50%. The SKOV-3 knockdown cells had a 35% lower invasion potential. The same has been shown in melanoma cells (De Semir et al. 2012). This reveals that PHIP knockdown correlates with lower invasiveness in ovarian cancer cells.

To show that this reduced migration is induced by the focal adhesion protein Talin 1, we performed rescue assays. In these assays our shRNA-mediated PHIP suppressed ovarian cancer cell lines got transfected with plasmids containing the cDNA for Talin 1. After the successful transfection, an invasion assay was performed too. This time the cells transfected with Talin 1 cDNA plasmid showed a significantly higher invasiveness through the matrigel. This indicates that proteins like Talin 1 are probably regulated by PHIP in ovarian cancer too. Therefore a lower expression of PHIP by targeting it could lead to lower invasiveness and metastasis of tumors.

The two PHIP bromodomains are possible targets of selective bromodomain inhibitors (Muller, Filippakopoulos, and Knapp 2011). JQ1 is an inhibitor of bromodomains which causes cell cycle arrest in aggressive neuroendocrine tumors (Shao et al. 2014). We decided to treat our PHIP knockdown cells with this selective bromodomain inhibitor to investigate a possible cell cycle arrest. These experiments revealed that PHIP knockdown cells (PA-1 127 and 130) are more sensitive to this drug than our control cells (PA-1 GFP). This indicates that PHIP is a possible target of this inhibitor and reduced PHIP amounts amplify the impact of JQ1 on cell cycle arrest. The future goal is to reproduce these data in mouse models. JQ1 could be a candidate drug for targeting PHIP in tumors.

7. Conclusion

All together we can report that PHIP in ovarian cancer cells influences cell proliferation and invasion in matrigel by controlling the expression of several focal adhesion molecules like pFAK, Talin 1 and Integrin B1. We also report that reduced PHIP expression sensitizes to the selective bromodomain inhibitor JQ1.

These studies indicate that PHIP could be used as a target for therapy in ovarian cancer.

8. References

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