The Role of Pleckstrin Homology Domain-Interacting Protein (PHIP) in Triple-negative Breast Cancer Progression

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1 Abstract

Breast tumors are categorized into three main groups based on the expression of the most common markers (oestrogen and progesterone receptors and HER2)[1]. Tumors that do not express those markers are termed “triple-negative” breast cancers (TNBC), which to date lack any targeted therapies. Since the majority of triple-negative breast tumors is highly malignant[1], a better understanding of the molecular factors that promote the progression of TNBCs is crucial for the identification of novel treatments. Pleckstrin Homology Domain Interacting Protein (PHIP) has been shown to play a crucial role in metastasis of triple-negative melanomas. Evidence has been found that PHIP promotes tumor cell invasion, a role which is mediated by activating the glycolytic pathway, and angiogenesis in melanoma[2,3].

This thesis presents experiments with the triple-negative breast cancer cell lines MCF-7 and MDA-MB-436. ShRNA mediated suppression of PHIP expression resulted in significant inhibition of tumor cell proliferation and reduced cell invasion in those cell lines. Quantification of several markers of cell invasion provided evidence proinvasive role of PHIP in breast cancer might be mediated by activating TLN1 and AKT.

These results suggest that PHIP is involved in triple-negative breast cancer progression.

2 Introduction

2.1 Cancer in general

Cancer is today a disease for which people all over the world are looking for a cure. It was first described in the breast by an Egyptian circa 3000 BC; the ancient Greek physician Hippocrates reported several other types of cancer. The Egyptian Aretaeus (AD 81-138) was the first to comprehensively describe symptoms, signs, and treatment of cancer of the uterus. Treatments for cancer such as amputation of whole breasts, or polypectomy making use of a wire loop were introduced in the following centuries[4].

Today, both knowledge of cancer and its treatment are different. The US National Cancer Act of 1971 encouraged science and medicine to fight against cancer. Since then, our knowledge of the molecular mechanisms of cancer development and progression has profoundly grown. Nevertheless, cancer still remains a significant health concern and burden. This is proven by the fact that cancer is the leading cause of death in individuals younger than 65 years of age, and that one in four of all deaths in the United States are the result of cancer[5]. Still, due to greater knowledge, better treatments and increased awareness, overall cancer incidence and mortality rates have begun to decline over the last decade[5,6].

It is important to gain more knowledge about how normal cells acquire the six essential properties to become malignant cancer cells. These six hallmarks of cancer are: (i) grow autonomously; (ii) disregard cytostatic signals; (iii) ignore apoptotic signals; (iv) stimulate angiogenesis; (v) invade and metastasize; and (vi) become immortal[5].

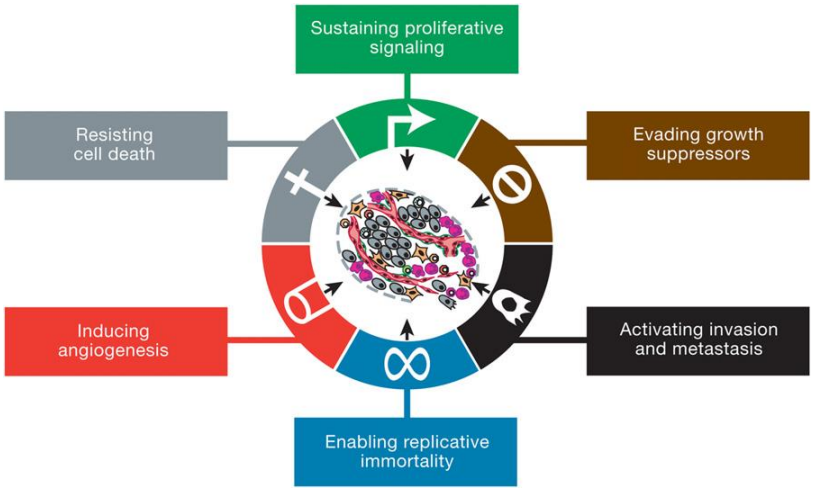

Fig.1: The six hallmarks of cancer, as suggested by Hanahan and Weinberg in 2000[7].

In addition to these six hallmarks of cancer described in 2000, Hanahan and Weinberg added four new emerging hallmarks of cancer to the list in their 2011 paper "Hallmarks of Cancer: The Next Generation"[8]. Those hallmarks include the deregulation of cellular energetics (vii), genome instability and mutation (viii), and the abilities of cancer cells to induce tumor promoting inflammation (ix) and avoid destruction by the immune system (x)[8].

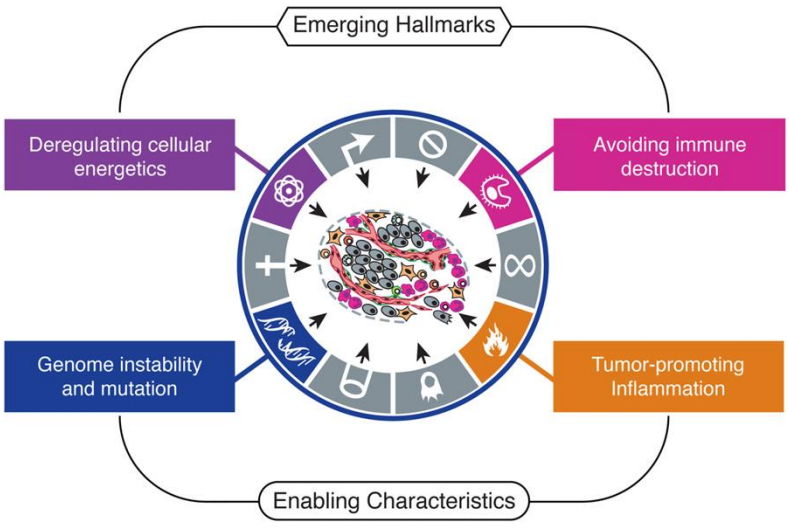

Fig.2: Four recently emerging hallmarks, as described by Hanahan and Weinberg in 2011[8].

2.1.1 Oncogenes

Oncogenes are genes that have the potential to cause cancer[9]. Their precursors are called proto-oncogenes. Oncogenes are the result of mutated or overexpressed proto-oncogenes[10].

Such mutations in proto-oncogenes are often gain-of-function mutations. The following three mechanisms can produce oncogenes from the corresponding proto-oncogenes[11]:

- Point mutations in a proto-oncogene resulting in a constitutively acting protein product
- Gene amplification of a segment of DNA that includes a proto-oncogene, thereby causing overexpression of the protein the amplified gene encodes for
- Chromosomal translocation: via translocation, a growth-regulatory gene can be brought under the control of a different promoter, resulting in inappropriate expression of the gene[11]

It is crucial that such gain-of-function mutations act dominantly. I.e., a mutation in only one of the two alleles (variants of a gene)[12] is sufficient for the rise of cancer[11].

When discovered, oncogenes gave rise to a lot of questions and discussions. How could oncoproteins, representing a single protein species, change so many different regulatory pathways in cells at the same time[10]?

Non-cancer cells receive external growth-signals, which are processed by the cell via complex mechanisms. The decision of the suitability of cell growth and division is then made. But the intracellular space is separated from the extracellular space by a lipid bilayer termed the plasma membrane, which allows only very small molecules to pass through[10]. Proteins encoded by proto-oncogenes are often part of the machinery that allows cells to “receive and process biochemical signals regulating cell proliferation” (Weinberg R. A., 2013) [10].

To contribute to the healing of wounds and defence against foreign substances, cells in a living tissue communicate via growth factors. These are proteins released by some cells that can make their way from one cell to another, carrying specific

biological messages. The cells that receive such signals then have to decide whether to grow and divide or to go into a quiescent state. Neighbouring cells provide help with this decision by either sending growth-stimulatory or growth-inhibiting signals[10].

In 1911, it was shown that viruses could cause cancer in studies conducted by Peyton Rous. He excised fibrosarcomas (connective tissue tumors) from chickens, and after filtration and isolation procedures, he injected the filtrate into chicks. Most of the injected animals developed sarcomas. The transforming agent eventually was shown to be a virus, and was named Rous sarcoma virus (RSV). In 1966, Rous was awarded the Nobel prize for his work[11,13,14].

It was later shown that RSV is a retrovirus whose RNA genome is converted into DNA via reverse transcription. The DNA is then incorporated into the host-cell genome. Oncogenic transforming viruses like RSV contain the v-src gene[11].

V-src is an important oncogene. Mammalian cells that have been transformed by the v-src oncogene exhibit a dramatically altered cell shape, are known to take up glucose much faster than normal cells, grow without anchorage to the extracellular matrix, and form tumors[10].

In the late 1970s, it was found that src acts as a protein kinase[15], an enzyme that phosphorylates other proteins[16]. This discovery was crucial since it meant that src can add phosphate groups to its countless substrate proteins, altering their structure and function. Hence, it became clearer how one protein could change the morphology of cells, namely by phosphorylating many other proteins, thereby creating a signalling cascade[10].

2.1.2 Growth factors

Growth factors play an important role in cancer. Epidermal growth factor (EGF), the first growth factor that has been discovered, was found to have mitogenic effects on epithelial cells[17]. Since several other cell types did not respond to EGF, it was suggested that a cell surface receptor (in this case EGF-R (epidermal growth factor receptor)), existed on certain cell types. Such cell surface receptors specifically

recognize EGF near the outer layer of the cell membrane. After binding of EGF to its receptor, a signal is conveyed into the interior of the cell[10].

It was found that EGF-R has a cytoplasmic domain containing a Src-like Kinase, which in turn phosphorylates tyrosines on its substrates, causing cell proliferation[10].

Mutations in genes encoding growth factor receptors are an important part of the hallmarks of cancer. They can cause ligand (in this case, ligands are growth factors)-independent firing by those mutated receptors (Figure 3). This means that, in contrast to normal cells, cancer cells with such mutations are not dependent on external growth signals any more, which represents a crucial part of uncontrolled proliferation[10].

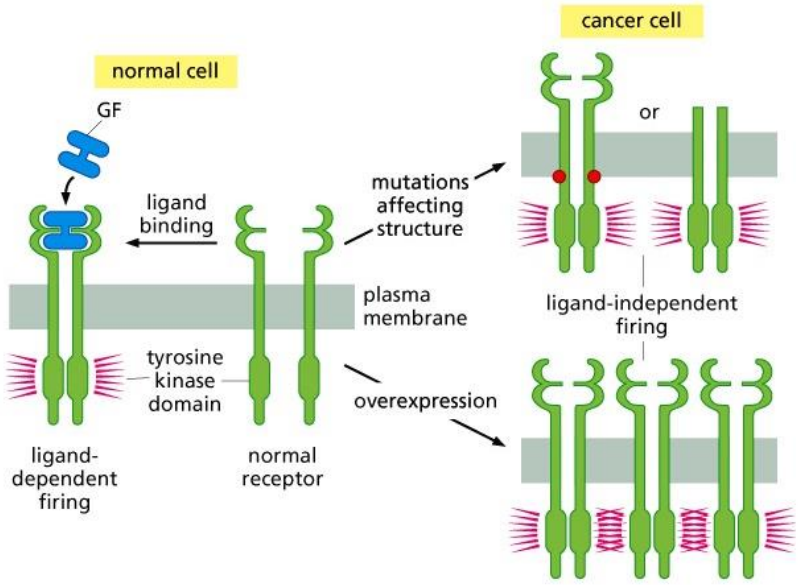

Fig.3: Mechanism of ligand-independent firing in cancer cells compared to ligand binding and receptor tyrosine kinase activation in normal cells[10].

Similarly to ligand-independent firing, receptor overexpression might make cells hyper-responsive to low concentrations of growth factors. Such overexpression is often the result of increased transcription of the receptor-encoding gene. However, in some cancers, the receptor genes are sometimes amplified. This elevated number of receptor genes results as well in an increased amount of receptor proteins[10].

Receptor molecules are sometimes only at the cell surface for a limited time. Then, the cell takes them up in a process called endocytosis[18]. The receptor protein is transported in vesicles to lysosomes, in which they might be degraded, or brought to

the surface once more. If this endocytosis is inhibited, the receptors will not be removed from the surface, resulting in a greater number of receptors displayed at the cell surface. This is another mechanism that enables proliferation of cells in cancer[10].

2.1.3 Integrin receptors and attachment of cells to the extracellular matrix

Normal cells can only proliferate when attached to a solid substrate, a meshwork of proteins termed the extracellular matrix (ECM)[19]. They will otherwise go into apoptosis (programmed cell death)[20]. Cancer cells, however, are able to grow in an anchorage-independent fashion, meaning they can proliferate without such attachment. To sense attachment to the matrix, cells express integrins, a special class of cell surface receptors[10].

Components of the extracellular matrix such as collagens, laminins, and fibronectin are the ligands of integrin receptors. The binding of those integrins to ECM components brings about mechanical stability in tissues[10].

So, through macromolecular clusters, cells form so-called “focal adhesions” with the extracellular matrix[21]. Due to the formation of focal adhesions, the cytoplasmic domains of integrins can activate signalling pathways with cellular responses such as cell migrations, survival and proliferation[10]. Integrins might also release anti-apoptotic signals, reducing the likelihood of anoikis, apoptosis induced by inadequate attachment to the extracellular matrix[10,22].

Integrins act as sensors to signal normal cells whether they are attached to the ECM or not. Other receptors like EGF or PDGF (platelet-derived growth factor[23]) sense soluble growth factors. Together, those receptors enable normal cells to determine whether the following preconditions for cell growth and division are fulfilled: a) adequate levels of mitogenic growth factors in the space surrounding the cell and b) adequate anchorage of the cell to matrix components[10].

If cells contain an activated ras oncogene[24], the most common oncogene in human cancers[25], those two requirements are usually abrogated[10]. Ras-transformed cells are therefore able to grow under conditions involving low concentrations of serum

and mitogenic growth factors, and can often proliferate regardless of attachment to the ECM. The Ras-oncoprotein seems to be able to mimic signals induced by activated growth factor receptors, as well as signals induced by integrins attached to ECM components[10].

2.1.4 Tumor suppressor genes

In the 1970's and early 1980's, suspicions rose due to experimental evidence which suggested that more, at that time unknown properties of oncogenes existed. It was thought that another type of growth controlling genes existed, a type that suppressed cell proliferation. Their loss or inactivation seemed to free cells from their growth-suppressing effects, resulting in an increase in cell proliferation[10].

As stated by Baltimore and Lodish in 2002, "tumor-suppressor genes generally encode proteins that in one way or another inhibit cell proliferation. Loss of one or more of these "brakes" contributes to the development of many cancers."[11] They described five classes of proteins that are generally recognized as being encoded by tumor-suppressor genes:

- Intracellular proteins that regulate or inhibit progression through a specific stage of the cell cycle
- Receptors for secreted hormones (e.g., tumor-derived growth factor β) that inhibit cell proliferation
- Proteins that control the checkpoints of the cell cycle and cause arrest of the cycle if DNA is damaged or chromosomes are abnormal
- Promoters of apoptosis
- Enzymes involved in repair of DNA[11]

Cells that have lost the ability to repair errors, gaps, or broken ends in DNA are prone to accumulate more mutations, including mutations in genes critical in controlling

growth and proliferation. Loss-of-function mutations in the genes encoding DNA-repair enzymes can thereby promote inactivation of other tumor-suppressor genes as well as activation of oncogenes[11].

Oncogenic loss-of-function mutations in tumor-suppressor genes act recessively: One copy of a tumor-suppressor gene is enough to control cell proliferation - both alleles of a tumor-suppressor gene must be lost or inactivated in order to promote tumor development. Tumor-suppressor genes in many cancers show deletions or point mutations, preventing the production of any protein or producing a non-functional protein[11].

2.1.5 Retinoblastoma protein (pRB)

The first tumor suppressor to be identified was the retinoblastoma gene (RB)[11]. It was stated by Murphree and Benedict in 1984 that “the loss or inactivation of both alleles of this gene appears to be a primary mechanism in the development of retinoblastoma”[26], a tumor of the human retina[11].

pRB turned out to play an important role in the cell cycle (Figure 4). The cell cycle clock is a network of proteins that interact with each other and, upon receiving signals from outside or inside the cell, processes such signals. It then decides whether to proliferate or to go into a quiescent state. After the generation of a cell through mitosis and cytokinesis, this decision has to be made soon[10].

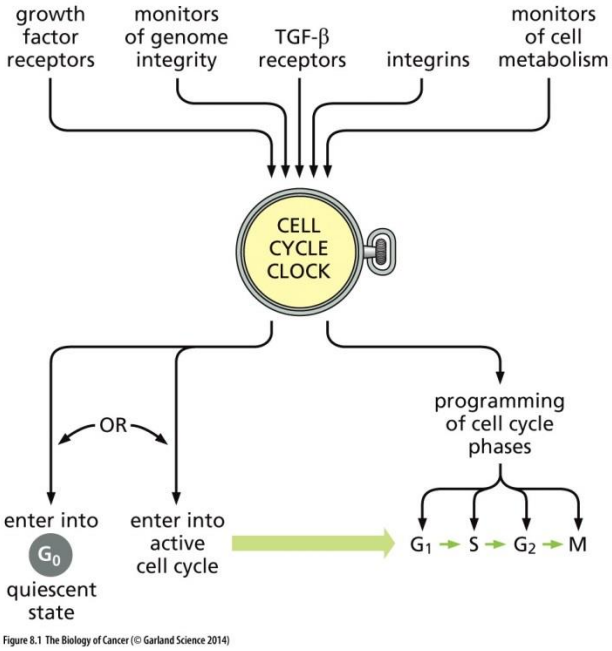

Fig.4: The cell cycle clock and its functions[10].

The quiescent state can be initiated both through the absence of mitogenic growth factors, and the presence of anti-mitogens like TGF-β. This quiescent state can often be reversed again in favour of proliferation due to the influence of growth factors; however, quiescence is sometimes irreversible, such cells are termed "post-mitotic"[10].

The cell cycle usually consists of four coordinated processes: cell growth (referring to the accumulation of cellular constituents such as macromolecules to ensure that after cell division, both daughter cells have enough of those constituents[10]), DNA replication, distribution of the duplicated chromosomes to both daughter cells, and cell division (mitosis and cytokinesis)[27].

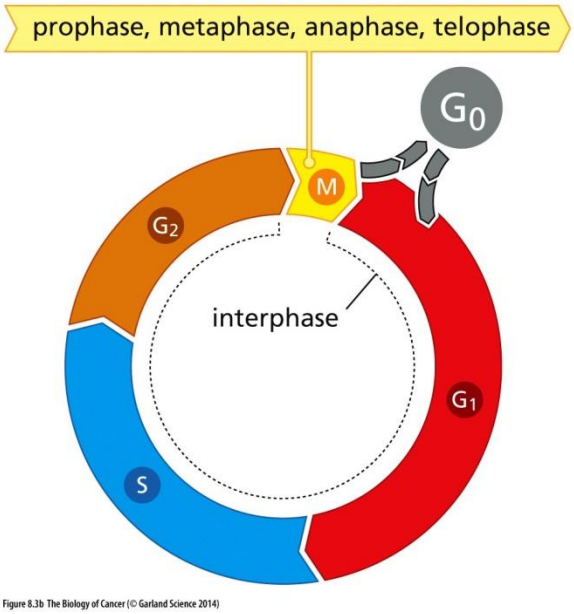

Fig.5: The phases of the cell cycle[10].

The cell cycle can be divided into two basic parts: mitosis and interphase (Figure 5). Mitosis (nuclear division) refers to the separation of daughter chromosomes ending with cytokinesis (cell division). However, mitosis and cytokinesis make up only about one hour of the approximately 24 hours of the eukaryotic cell cycle. Interphase makes up the rest of the cycle. During interphase, the chromosomes are decondensed, and both cell growth and DNA replication occur[27].

The cell cycle of eukaryotic cells consists of four discrete phases, separated by so-called checkpoints (Figure 6). The M phase refers to mitosis and cytokinesis. This phase is followed by the G1 phase (gap 1), the interval (gap) between mitosis and replication of the DNA in S Phase (synthesis). In G1, cell growth takes place. After S Phase and replication of DNA, another gap follows (G2 phase). Here, similar to G1, cell growth continues and proteins are synthesized to prepare the cell for mitosis[10,27].

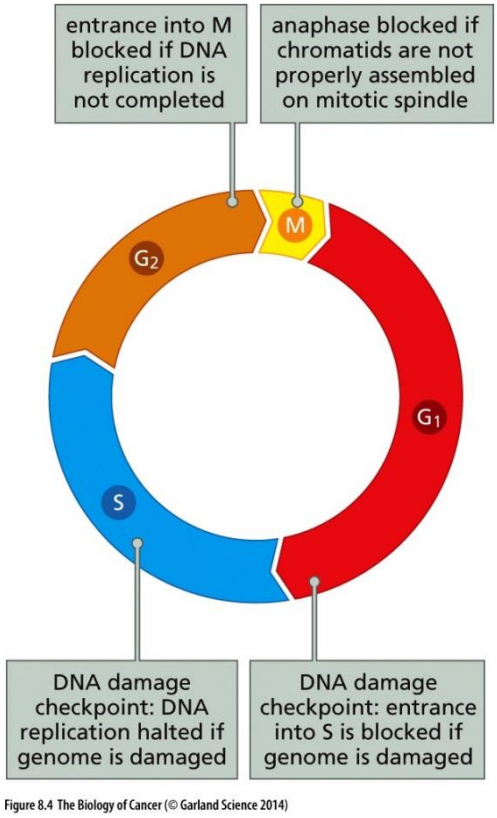

Fig.6: The cell cycle checkpoints[10].

A crucial point in the cell cycle is the restriction point (R point), found usually several hours before the G1/S phase transition. Until the R point, cells are sensitive to mitogenic growth factors and to TGF-β. Once the cells pass the R point, they will continue according to the cell cycle regardless of which growth signals are present[10].

2.1.6 Cyclins and cyclin-dependent kinases (CDKs)

The important enzymes that work in the cell cycle clock machinery are called CDKs. They depend on their regulatory subunits, the cyclins, with which they associate and which activate the catalytic activities of the CDKs[10].

In the G1 phase of the cycle, D-type cyclins associate with CDK4/6 and attach a few phosphate groups to pRB, retinoblastoma protein. This process of adding only a few phosphates leaves pRB in a “hypophosphorylated” state. In this state, pRB remains bound to E2F-family transcriptions factors, preventing them from transcription of genes(Figures 7 and 8)[10].

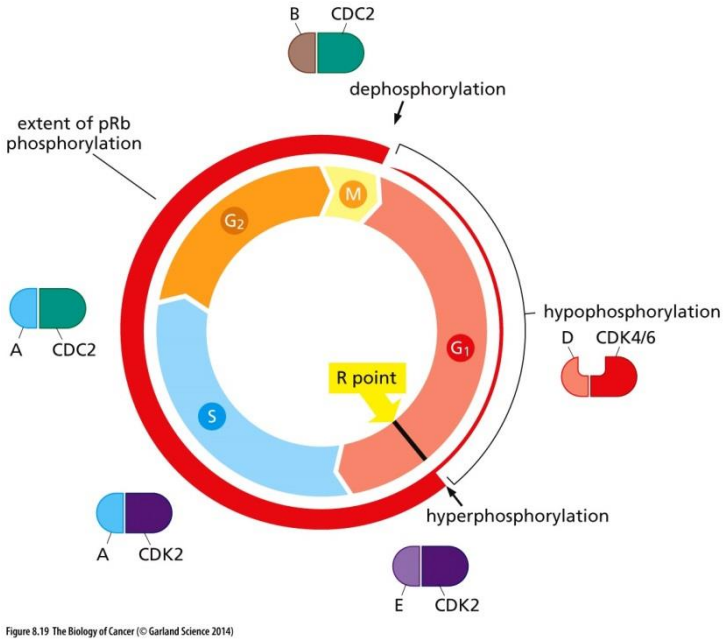

Fig.7: Level of phosphorylation of pRB during the cell cycle[10]

In late G1, however, cyclin E-CDK2 complexes attach many phosphate groups to pRB (“hyperphosphorylation”), causing retinoblastoma protein to dissociate from E2F-family transcription factors (Figure 8). The latter can now stimulate transcription of genes, producing proteins such as enzymes involved in replication that are crucial for the G1 to S phase transition. Throughout the rest of the cell cycle, the amount of phosphate groups attached to pRB remains constant, until cells enter M phase. PRB is dephosphorylated in M/G1 transition, and the cell cycle begins again (Figure 7)[10].

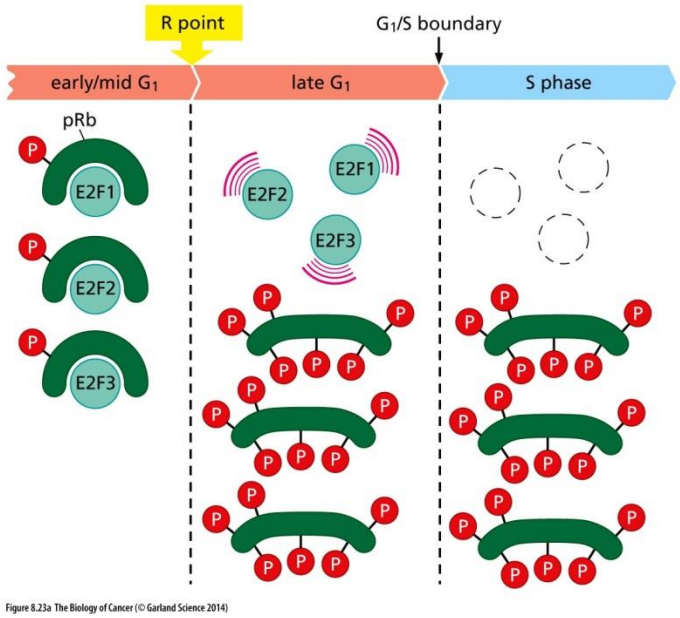

Fig.8: the binding and dissociation of pRB to EF2-family transcription factors according to its phosphorylation levels[10]

As mentioned above, TGF-β represents a growth-inhibitory signal. It does so by activating four INK4-proteins that inhibit cyclin dependent kinases(Figure 9)[10].

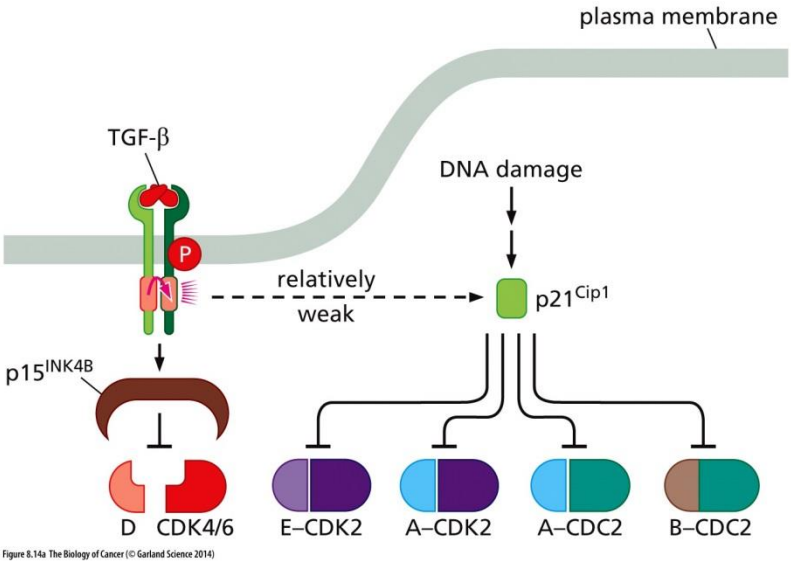

Fig.9: TGF-β can inhibit growth by activating proteins such as $p15^{INK4}$ and $p21^{Cip1}$ that inhibit cyclin dependent kinases [10].

This effect of TGF-β antagonises cell growth and proliferation and therefore also tumor progression. TGF-β can be considered an anticancer agent that prohibits the uncontrolled proliferation of epithelial, endothelial, and hematopoietic cells. TGF-β has this important tumor suppressor function at early stage of tumorigenesis. However, it acts as a positive modulator of tumor progression in late tumorigenesis[10,28]. Aberrations in its signalling pathway cause resistance to TGF-β-mediated growth arrest, and therefore give rise to human malignances[5,29–31]. As Tian M *et al.* wrote: “Paradoxically, these genetic and epigenetic aberrations conspire to convert TGF-β from a suppressor of tumor formation to a promoter of their growth, survival, and metastasis”[5].

2.1.7 Apoptosis – programmed cell death

The great number of cells in a multicellular organism is tightly regulated by controlling the rate of cell division and of cell death. When cells are no longer needed, they commit suicide by activating the intracellular death program termed apoptosis[32].

During embryonic development, massive cell death can occur. Cells die when the structure they form is no longer needed. The development of frogs is an example: When a tadpole undergoes the changes to become a frog, the cells in its tail die, and the tail thus disappears, since it is not needed in the frog[32].

In adult tissues, however, cell death is necessary to balance cell division. Tissues that would not be balanced would grow or shrink. In adult rats, for example, liver cell proliferation increases to make up the loss if a part of the liver is removed. On the other hand, a rat treated with a drug that stimulates liver cell division will show greatly increased apoptosis in the liver once the treatment is stopped. After about a week, the liver thus returns to its original size. The liver is thereby kept at a constant size due to the regulation of both the cell death rate and the cell birth rate[32].

In contrast to necrosis, when cells swell, burst and spill their contents all over their neighbors due to acute injuries, causing a potentially damaging inflammatory response, cells that undergo apoptosis die neatly, without damaging their neighbors. The cells shrink and condense. The nuclear envelope disassembles, the DNA breaks up into fragments ("DNA laddering" can be observed[33]) and the cytoskeleton collapses. Very importantly, the cell surface displays properties that cause the apoptotic cell to be rapidly taken up by a neighboring cell or by a macrophage, both of which thereby recycle the organic compounds of the cells taken up. This process also avoids the damaging consequences of cell necrosis[32].

2.1.7.1 The role of p53 in apoptosis

The protein p53 is a crucial player in apoptosis and also cancer in general. If p53 is activated due to a metabolic disorder or genetic damage within a cell, it can arrest cell cycle progression and facilitate the repair of the damage (see also figure 10). If this damage is too severe and cannot be repaired, p53 can emit signals initiating the apoptotic death program. This is crucial for the organism since the cell, if it continues to grow and divide, might pose a threat to the whole organism's health[10].

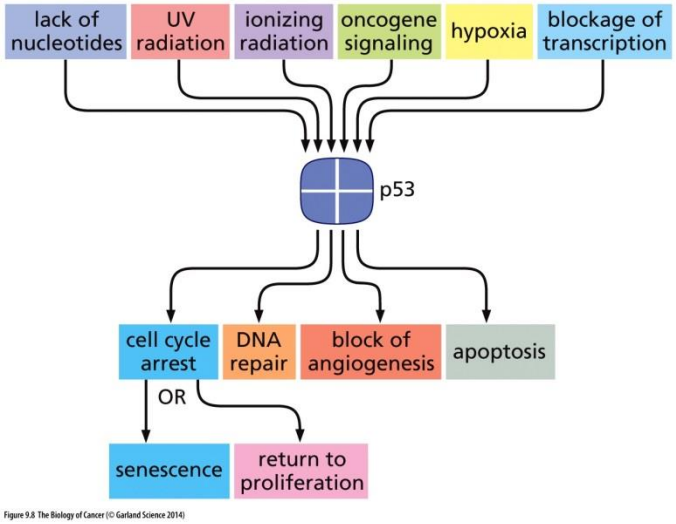

Fig.10: The activation of p53 and the resulting actions of the cell [10].

The mechanism by which p53 protects the cell and induces apoptosis works as follows: p53 is normally, i.e. in a situation without any physiological stress or damage, very unstable (it has a short lifetime and gets rapidly degraded) and thus cannot take action. However, signals of physiological stress, anoxia or DNA damage can block its degradation, activating its function as a transcription factor and inducing the transcription of genes involved in various cellular responses such as apoptosis. The initiation of apoptosis activates enzymes called caspases, which cleave many important proteins in the cell, resulting in its destruction and the apoptotic phenotype[10].

Mutations in p53 often allow cancer cells to inactivate the apoptotic machinery, helping them on their way to immortality by allowing them to evade cell death. By allowing DNA damage without p53 triggering apoptosis, cells can accumulate mutations, which paves the way for cancer[10].

2.1.8 Telomeres and immortalization

Evading apoptosis by deactivating the p53 damage response, however, is not enough for cells to be able to live forever. Eukaryotic cells have more ways to ensure that they die after an appropriate amount of time[32].

One of these ways has to do with the chromosomes themselves, with the ends of the chromosomes, called telomeres, to be more exact. Telomeres function as protective shields for the chromosomes, preventing end-to-end fusion of chromosomal DNA. Loss of functional telomeres is therefore disastrous for cells[10].

Telomeres are made up of thousands of repeats of the same six-base-pair sequence of DNA. In normal cells, telomeres shorten progressively during cycles of proliferation. At some point, they cannot longer protect the ends of chromosomes. Crisis occurs, chromosomes fuse, and, with intact p53 and other tumor suppressor proteins, apoptosis is triggered and the cell dies[10].

Alexey Olovnikov was the first to predict a compensatory mechanism for telomere shortening, what he then called “marginotomy”[34]. It was thought then that “after the exhaustion of telogenes the cells become aged and are eliminated ... Marginotomy is therefore responsible for the loss with age of various cell clones of the body...”(A.M. Olovnikov, 1973)[34].

The enzyme compensating for telomere shortening was discovered in 1985 by Greider and Blackburn[35] and is today known as Telomerase. In most human cells, except for germ line and some stem cells, the gene coding for the catalytic subunit of telomerase is not at all or only at very low levels expressed[32].

Most human cancer cells, however, express telomerase in higher levels, giving them the ability to potentially divide infinitely. Maintenance of telomeres and expression of telomerase is therefore a crucial hallmark of cancer[8,32].

2.1.9 Mutations

Mutations can occur in many different ways.

Enzymes involved in DNA replication, such as DNA-polymerase, sometimes incorporate the wrong bases into the new DNA strand. Other enzymes, involved in mismatch repair, however, keep the number of thusly created mutations low. Chemically reactive molecules can bring about generation of reactive oxygen species in the cell. X-rays can create double-stranded breaks in the DNA, which can also be caused by DNA breakage at replication forks. During Mitosis, when the

chromosomes are separated to the newly forming daughter cells, errors such as translocation of chromosome parts or unequal distribution of chromosomes can occur. Thereby created unusual numbers of chromosomes are common in cancer cell genomes and seem to help the cells in proliferation and survival[10].

The repair mechanisms in the cell that maintain the integrity of the genome, however, counteract such mutations and try to repair them. Without the breakdown of this complex machinery, mutation rates would very likely be too low to enable cells to "accumulate the ensemble of genetic changes required for tumor progression to reach completion in a human lifetime", as Robert A. Weinberg stated[10].

2.1.10 Inflammation and Angiogenesis

Newly created wounds in the body induce a burst of capillary growth nearby to satisfy the high metabolic requirements needed for repair. Also infections bring about the creation of new capillaries that usually regress and disappear once the inflammation has subsided[32].

Carcinoma cells exploit this effect and release certain agents that are usually part of an immune response against foreign substances. Such agents include cytokines, chemokines, and growth factors, recruiting cells that are part of the immune system such as neutrophils, macrophages, and lymphocytes. These cells then bring about an inflammatory response, which ultimately stimulates proliferation of nearby epithelial cells and also angiogenesis – the formation of blood vessels[10].

In this process, endothelial cells assemble to form the linings of the walls of capillaries as well as larger blood vessels and also lymphatic ducts. Assembled capillaries are crucial for growing neoplastic stroma as they provide oxygen and essential nutrients to the cells[10].

The invading endothelial cells respond to complex signals produced by the tissue which they invade. An important signal factor in angiogenesis is vascular endothelial growth factor (VEGF). The regulation of blood vessel growth depends on the control of VEGF production, regulated through changes in the stability of its mRNA and its transcription levels. A shortage of oxygen results in an increase in the intracellular

concentration of hypoxia-inducible factor 1 (HIF-1). HIF-1, a gene regulatory protein, stimulates transcription of, among other genes, the *VEGF* gene. Once secreted, VEGF diffuses through the tissue, and acts on nearby endothelial cells[32].

The endothelial cells will produce proteases which help them by digesting their way through the basal lamina of the parent capillary or venule. Then, the endothelial cells migrate toward the source of the signal and start to proliferate, subsequently differentiating and forming tubes. VEGF acts on endothelial cells selectively to stimulate these processes[32].

2.1.11 Invasion and Metastasis

In tumor progression, cancer cells multiply first at the site where the uncontrolled proliferation started. The result is a primary tumor mass, often consisting of billions of cells. Such a primary tumor can cause pain or discomfort when space is limited such as in brain tumors, but often remains unnoticed in expansible cavities. Sometimes, cells from the mass invade adjacent tissues and can compromise vital functions by, among others, obstructing passage of digestion products or body fluids[10].

However, 90% of cancer patients die because of cancerous growths discovered at sites far away from the primary tumor. They die due to so-called metastases, formed by cancer cells that have left the primary tumor, travelled through the blood stream or lymphatic vessels, and formed new colonies[10].

Carcinomas begin, by definition, on the epithelial side of the basement membrane. They are then termed “benign” if they remain on the epithelial side. If the cells are able to breach the basement membrane, they will invade the nearby stroma and are now considered to be malignant. The cells are now in close proximity to blood (and also lymphatic) vessels, allowing them to have relatively easy access to nutrients and oxygen. They can now invade the vessels (“intravasation”)[10,28].

The secretion of proteases is very important to remodel the extracellular matrix and thus generate space for the invading cancer cells. This invasiveness is stimulated by epidermal growth factor released by macrophages assembled to help cancer cells to invade[10].

Inside the vessels, the cancer cells can travel to other areas of the body. The cells remain without attachment, which poses a threat to them – they might die from anoikis, which is, as mentioned above, apoptosis induced by inadequate attachment to the extracellular matrix[22]. They might also depend on stromal support such as the supply of mitogenic factors. In smaller vessels, hydrodynamic shear forces can potentially tear the cancer cells apart[10].

Another obstacle for the cancer cells on their way to form metastases is the lung. They could get trapped in small capillaries, and form metastases in the lung. However, via mechanisms still poorly understood, the cancer cells can escape from the lung and travel to other parts of the body. The challenge then is to move from the lumina of the vessels into the surrounding tissue. This process of transmigration across the endothelial monolayer that enables successful establishment of a secondary metastasis is termed extravasation[36].

It has been suggested in the metastatic niche model that a suitably conducive microenvironment called premetastatic niche is necessary so that tumor cells can engraft and proliferate at secondary sites (micro- to macro- metastatic transition). Interaction of the cancer cells with extracellular matrix components such as collagen, laminin and fibronectin, is required[36,37].

Micrometastases are small clumps of disseminated cancer cells and are an important target of adjuvant chemotherapy[10,38]. The difficulty of forming macrometastases lies in the challenge of the foreign environment that does not provide the newly arrived cells with the familiar growth and survival factors. The rate of success of the formation of metastasis is very low and thus termed metastatic inefficiency[10,39].

2.1.12 Immune system versus cancer

Our immune system is designed to detect foreign agents such as fungi, bacteria and viruses. In recent years, evidence has been found that the immune system contributes to our defence against tumors[10].

The immune system identifies foreign infectious agents by recognizing specific molecular entities termed antigens. It then launches complex attacks against the

intruders. Antibodies are produced that bind to the specific antigens displayed by the infectious agents, and the immune cells can then neutralize the intruders[10].

It has been shown in mouse models that animals with certain immune deficiencies, such as deficiencies in function or development of T-cells (lymphocytes that develop in the thymus gland[32]) and natural killer (NK) cells (another type of lymphocyte that not online attacks foreign agents, but also infected body cells[40]), are much more susceptible to cancer development than the control group of mice. This suggests that both the innate (non-specific system that responds to all foreign agents in the same, unspecific way[32,41]) and adaptive (specific part of the immune system; involving antigen recognition and antibody production[32]) parts of the immune system are able to contribute significantly to immune surveillance and thus tumor eradication[8,42,43]. It has further been shown that patients with colon and ovarian cancer having been infiltrated by cytotoxic T cells and NK cells have a better prognosis than patients with tumors that contain fewer of those cells[8,44,45].

Hanahan and Weinberg described “immunoevasion as another emerging hallmark, whose generality as a core hallmark capability remains to be firmly established”[8].

2.1.13 Energy metabolism

Malignant cells that undergo uncontrolled proliferation need a lot of nutrients, most importantly glucose. Normal cells break down glucose under aerobic conditions through the process of glycolysis, yielding pyruvate. Pyruvate is then transported into the mitochondria, where it is further processed in the citric acid cycle. Under conditions with limited oxygen (hypoxic or anaerobic conditions), normal cells can only use glycolysis, and the generated pyruvate is reduced to lactate. This process yields only two molecules of ATP, as compared to 36 molecules of ATP per molecule of glucose yielded under aerobic conditions[10].

About 80% of cancer cells, however, use only glycolysis, even under aerobic conditions, as already discovered by Otto Warburg in 1924[46]. Due to this inefficient glucose metabolism, such cancer cells require the importing of huge amounts of glucose.

At first sight, this behaviour of cancer cells seems to be nonsensical. The so-called “Warburg effect” attributes this metabolic shift to mitochondrial dysfunction in cancer cells[47,48]. Studies conducted in the early 1970s found no evidence for a respiratory defect in cancer cells[48]. However, studies in respiratory chain activity on immortalized cancer cell lines showed that the increased glycolytic rate (glycolytic shift), which prompts a decrease in oxidative phosphorylation and mitochondrial density, increases growth rate of cancer cells[49]. Simonnet *et al*., concluded in 2002 that tumor cells with increased mitochondrial dysfunction show a more aggressive phenotype[48,50].

This goes along with the fact that glycolysis, apart from the generation of ATP, produces intermediates serving as precursors of several molecules that play a role in cell growth, i.e. the synthesis of lipids and nucleotides. This leads to accumulation of such intermediates, which is not found in normal cells that are not actively proliferating[10].

2.2 PHIP – Pleckstrin Homology Domain-Interacting Protein

PHIP has first been described in 2000 by Farhang-Fallah J. *et al.* as a protein that selectively binds to the pleckstrin homology (PH) domain of Insulin receptor substrate-1 (IRS-1) in pancreatic islets cells[51]. They suggested that PHIP, while it itself did not appear to be a substrate of the insulin receptor, represents a protein ligand of the IRS-1 PH domain that might link IRS-1 to the insulin receptor. Furthermore, evidence was found that these pancreatic cells used the pathway containing PHIP and IRS-1 was essential for promoting MAP kinase activation during insulin stimulation. They concluded that "PHIP may serve as an adaptor that integrates IRS-1-mediated signals with signals from other cellular effectors of the activated insulin receptor"[51].

In 2002, Farhang-Fallah, J *et al*. reported that overexpression of PHIP in fibroblasts enhanced insulin-induced transcriptional responses in a mitogen-activated protein kinase-dependent manner. They suggested that PHIP–IRS-1 PH domain interactions specifically promoted the proliferative actions of insulin and investigated the effect of PHIP on GLUT4 translocation in myoblasts[52]:

It was known that one of the main metabolic effects of insulin action on fat and muscle cells is the stimulation of glucose uptake. This involved the redistribution of the glucose transporter GLUT4 to the plasma membrane[53]. Farhang-Fallah, J *et al*. found that ectopic expression of dominant-negative-PHIP caused a nearly complete inhibition of insulin-dependent GLUT4myc (GLUT4 with a myc-tag) membrane translocation, suggesting that PHIP/IRS-1 complex formation plays a role in promoting the metabolic effects of insulin in muscle cells[52]. Furthermore, they found evidence that PHIP is involved in the regulation of processes promoting cytoskeletal remodelling and accompany incorporation of GLUT4 vesicles at the cell membrane surface[52].

Further research showed that overexpression of PHIP stimulates proliferation of pancreatic β-cells, both dependent and independent of insulin-like growth factor 1 (IGF-1)[54], a critical factor in β-cells function[55]. It was demonstrated that overexpression of PHIP1, a 206-kDa isoform of PHIP, localized exclusively in the nucleus, promotes the growth of INS-1 β-cells. This correlated with the accumulation

of cyclin D2 protein due to transactivation of its promoter. SiRNA-knockdown of PHIP1 was shown to inhibit IRS2-mediated DNA synthesis and cyclin D2 protein accumulation independent of phospho-AKT activation. PHIP1 overexpression also blocked apoptosis mediated via the activation of phospho-AKT and the inhibition of caspase-9 and caspase-3 activity. It was thereby shown that PHIP1 is a positive regulator of β-cells growth and survival[54].

In studies conducted by Li S *et al.*, mice lacking PHIP1 were born at normal size but suffered a 40% growth deficit by weaning. PHIP1 mutant mice developed hypoglycemia and had an average lifespan of only 4–5 weeks. Their observations suggested that PHIP1 regulates postnatal growth in an IGF-1/AKT pathway-independent manner[56]. These studies provided further evidence of the importance of PHIP in cell growth and survival.

In their paper published in 2012, De Semir *et al*. described the role of PHIP as a marker and mediator of melanoma metastasis[2]. Their research linked PHIP to cancer for the first time and showed that PHIP was activated in a subset of melanomas called “triple-negative”[2].

Triple-negativity refers to the fact that these melanomas are devoid of the three most common mutations that usually drive melanoma. These three mutations are v-Raf murine sarcoma viral oncogene homolog B1 (BRAF), neuroblastoma RAS viral (vras) oncogene homolog (NRAS), and phosphatase and tensin homolog (PTEN). De Semir D. *et al.* reported that in tumors that harbour wild-type versions of those three genes, PHIP is crucial in promoting metastasis[2].

PHIP has previously been identified as the gene most highly overexpressed in metastatic melanomas compared to primary tumors by cDNA microarray analysis[57]. De Semir D. *et al.* showed that when PHIP is targeted with siRNA or shRNA and thereby knocked down, it had profound effects on the knockdown cells versus the control cells. They observed that cells with PHIP knockdown showed significantly reduced their invasion into matrigel, and inoculation of cells with PHIP knockdown significantly prolonged the survival of B16 tumor-bearing mice compared to the control group[2]. Further investigations showed that when PHIP expression is supressed, also the expression of *Igf2* and *Tln1* was downregulated. De Semir D. *et al.* thus concluded that “PHIP can regulate the expression of upstream mediators of

the IGF axis and downstream mediators of tumor cell invasion"[2,58]. Overexpression of TLN1 or AKT in melanoma cells with PHIP knock-down resulted in significantly increased invasion into matrigel, suggesting that the proinvasive role of PHIP is mediated by activating TLN1 and AKT[2].

Additionally, immunohistochemical analysis of PHIP expression on a tissue microarray cohort of 345 patients with primary cutaneous melanoma showed that PHIP overexpression was significantly predictive of reduced distant metastasis-free survival and disease-specific survival[2].

In Homo sapiens, the PHIP gene is localised on the chromosome 6q14.1 locus. In melanoma, deletions of the 6q arm had been shown before[59] and had also been suggested as a possible diagnostic marker[60]. De Semir D. *et al.* performed interphase fluorescence in situ hybridization (FISH) analysis and observed that the PHIP locus was still present in all melanomas examined. They were also able to show that PHIP-overexpressing melanomas harbored increased PHIP copy number[2].

Furthermore, the group found that PHIP-overexpressing melanomas were predominately characterized either by a triple-negative genotype or by mutant BRAF with wild-type NRAS and PTEN and provided evidence that PHIP levels can be activated in a unique molecular subset of melanoma independent of those mutations[2].

In their very recent paper, published in 2013, the same group (Bezrookove V. *et al*., 2013) described the prognostic impact of PHIP copy number and its linkage to ulceration[3]. They stated that while tumor thickness seems to be regarded as the single most significant prognostic factor determining survival in melanoma, and while ulceration increases the risk of death within a given thickness range, additional factors are necessary to refine the prognostic assessment of patients with melanoma[3].

Analysing the prognostic impact of *PHIP* copy number in primary cutaneous melanoma, they found that 45.4% of patients with high copy number had distant metastasis, compared with 25.5% with low copy number. Of patients with high copy number 42.2% died of metastatic melanoma, when compared with 17.7% of patients with low PHIP copy number. While ulceration was only present in 28.8% of cases

with low PHIP copy number, it was present in 45.5% of cases with high copy number. This depicted the significant role of PHIP in the development of ulceration[3].

The group had reported in 2002 that ulceration was associated with increased tumor vascularity in the primary tumor[61]. Due to the fact that PHIP plays a role in the IGF1 receptor pathway which regulates glucose metabolism, Bezrookove V. *et al.* then determined if suppression of PHIP activity resulted in altered glycolytic activity of melanoma[3]. Sh-RNA mediated knockdown of PHIP resulted in decreased expression of lactate dehydrogenase 5 (LDH5), the enzyme that catalyses the conversion of pyruvate to lactate in the final step of glycolysis. The same cells with reduced PHIP expression also showed lower levels of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor 1 alpha subunit (HIF1A). These and further results led them to the conclusion that “the pro-invasive role of PHIP in melanoma is mediated, at least in part, by activating the glycolytic pathway”[3].

In vivo experiments showed that the group of mice containing tumors with PHIP-knockdown cells had large areas of necrosis, no or much less haemorrhage and much smaller vessels and significantly smaller microvessel density compared to the control group that showed large, abnormal blood vessels and haemorrhage. These results provide strong evidence that PHIP plays a crucial role in promoting angiogenesis in melanoma[3].

Given the impact of modulating PHIP expression in the progression of melanoma, it was decided to study the effects of PHIP activation in another aggressive cancer such as breast cancer.

2.3 Breast Cancer

Desantis C. *et al*. reported in 2011 that breast cancer is, apart from skin cancers, the most common malignancy among women. In the United States, breast cancers account for nearly 1 in 3 cancers diagnosed among women, and are the second leading cause of cancer death among women[62]. The American Cancer Society estimated that about 232,340 new cases of invasive breast cancer were diagnosed in 2013 and 39,620 women died from breast cancer[63].

While breast cancer occurs predominantly in women, it can also occur in men. It is, however, less common in men because their breast duct cells are less developed than those of women and because they normally have lower levels of female hormones that affect the growth of breast cells. Male breast cancer thus accounts for less than 1% of all breast cancer diagnoses worldwide. The American Cancer Society estimated that about 2,240 new cases of invasive breast cancer will be diagnosed for men in 2013[64,65].

2.3.1 Triple-negativity in Breast Cancer

Breast tumors can be categorized into three main groups based on the most common markers that are responsible for breast cancer: (a) expression of estrogen receptor (ER), (b) expression of the progesterone receptor and (c) the gene erbB2 amplified (HER2 = human epidermal growth factor receptor 2 positive) with and without ER/progesterone receptor expression. Triple-negative breast cancer (TNBC) is thus defined by the absence of ER/progesterone receptor expression and HER2 amplification[1].

Triple-negative breast cancers account for 12% to 24% of all breast cancers and are often found in younger women[66,67]. They have been associated with poor prognosis, due to aggressive tumor phenotypes, early metastasis to visceral organ or brain after chemotherapy and lack of clinically established targeted therapies[67].

Between 5% and 10% of all breast cancers are hereditary. Within this inherited group, 80-90% of cases are the result of germline mutations affecting two genes: BRCA1 and BRCA2[68]. These two genes are tumor suppressor genes, and mutations

in those genes can result breast and ovarian cancers. Both genes play a part in DNA repair and transcriptional regulation in response to DNA damage are required for maintenance of chromosomal stability and preventing genome damage. BRCA1 and BRCA2 also transcriptionally regulate some genes involved in DNA repair, the cell cycle, and apoptosis[69].

Germline mutations in the BRCA1 and BRCA2 genes have been associated with up to 15% of triple-negative breast cancers. TNBC accounts for 70% of breast tumors arising in BRCA1 mutation carriers and 16% to 23% of breast tumors in BRCA2 carriers[66].

3 Materials and Methods

3.1 RNA Extraction

RNA extraction was performed using the Qiagen RNeasy Mini Kit and according to the “RNeasy Mini Handbook”, Fourth Edition, September 2010, specifically according to the Protocol for the “Purification of total RNA from animal cells using spin technology”.

3.2 cDNA Synthesis

cDNA was synthesised from RNA using the Thermo Fisher Scientific “Maxima first strand cDNA synthesis kit for RT-PCR”.

Materials:

Tbl.1: Reagents and their amounts used for cDNA Synthesis.

Reagent	Amount
Reaction Buffer Mix	2 µl
Reverse Transcriptase Enzyme	1 µl
RNA	Volume corresponding to 500 ng of RNA
Nuclease-free water	Amount resulting in 10µl total Volume
	Total Volume = 10µl

Reaction steps:

Tbl.2: The steps of cDNA synthesis with temperatures and corresponding times.

Temperature [ºC]	Time [min]
25	10
50	15
85	5

3.3 TaqMan quantitative real-time PCR

TaqMan Analysis to determine the level of expression of certain mRNA was performed by using the Applied Biosystems 7500 Fast Real-Time PCR System.

A 1.7 µl probe assay was performed, filling triplicates of each sample into an optical reader 96-well plate.

Tbl.3: Reagents and their amounts used for TaqMan quantitative real-time PCR 1.7 µl assay. Total amount per well is 11 µl.

Reagent	Amount
Fast Universal PCR Master Mix (Applied Biosystems)	17.8 µl
dH_2O	10.7 µl
cDNA (previously diluted 1:5)	5.5 µl
TaqMan probe	1.7 µl

Tbl.4: The steps of TaqMan quantitative real-time PCR with temperatures and corresponding times.

Temperature [ºC]	Time [s]	
95	20	
95	3	40 cycles
60	30	

After the 40 cycles of amplification are done, the software gives, for each well, the number of cycles until the strength of the signal was above a certain threshold (the corresponding values are called C_t values).

Knockdown of cells infected with a lentivirus containing shRNA targeting PHIP was determined by comparing their level of expression of PHIP to that of a control cell line. Housekeeping genes such as HPRT1 were used to compensate for differences in the C_t values for PHIP between knockdown and control groups.

3.4 Quantitative Immunofluorescence

Cells were plated on coverslips in 6-well plates in their normal growth medium. After fixation with 4% formaldehyde in PBS, three washes with PBS followed. To permeabilize the membrane, a 0.1% solution of TritonX100 was applied for 10 minutes. After three more washes, 3% BSA in PBS was applied as a blocking buffer for 10 minutes. After washing to remove unbound blocking buffer, the primary antibody (diluted in DAKO antibody diluent) was applied to the cover slips, and the plate with the cells was incubated overnight at 4°C. Three washes of five minutes each to remove unbound antibody were conducted, secondary antibody (diluted in DAKO antibody diluent) was added to the wells, followed by incubation at room temperature for one hour. Subsequently, the cells were washed three times and then dehydrated with an ethanol series of 70%, 90% and 100%. The cover slips were mounted onto slides with Vectashield containing DAPI (from Vector Labs).

3.5 Lentiviral Infection

To achieve stable suppression of PHIP expression, cells were infected with a lentivirus containing shRNA (short hairpin RNA) targeting PHIP. Once the cells were infected, the viral DNA and RNA were integrated into the cell genome and the shRNA targeting PHIP (anti-PHIP shRNA) was expressed. Selection with puromycin was used to kill cells that were not infected.

The medium in the petri-dish containing cells was aspirated; the plate was washed with PBS. Subsequently, media containing 8µg/ml of polybrene and inactivated FBS were added to the plate. After adding the lentivirus containing the desired shRNA, the cells were incubated overnight. The next day, the medium containing the lentivirus was replaced by the regular growth medium. After approximately 72 hours, selection with puromycin was started.

As a control compared to cells with suppressed PHIP expression, other cells were infected with a virus containing shRNA targeting the luciferase gene (anti-luciferase shRNA). Since the cell line used does not express the luciferase gene, the targeting should have no effect.

3.6 Migration Assay

The Matrigel assay for tumor invasion was performed using a Biocoat 24-well plate (BD). For both MDA-MB-436 and MCF-7 cell lines, insert chambers were coated with 15μl matrigel at 5mg/ml protein. Hundred and fifty thousand cells of MDA-MB436 and two hundred thousand cells of MCF-7 were plated in the upper chamber in RPMI only medium without FBS. 30% FBS was used in the lower chamber to attract cells and promote invasion through matrigel overnight. Cells were fixed, stained with toluidine blue and counted under the microscope.

3.7 Colony Formation Assay

A relatively small number of cells was plated in a six-well plate. The cells were allowed to grow until colonies became visible. Then, the cells were stained with crystal violet, and the number of colonies in the wells was determined using ImageJ software.

3.8 Cell Proliferation Assay

Cells were plated in a 96-well plate in triplicate and were then allowed to grow for 4 days. The amount of cells in the different wells was determined by using a Cell Counting Kit from Dojindo Molecular Technologies, Inc.

3.9 Protein Extraction

Using the Thermo Fisher Scientific “HALT” Protease Inhibitor Cocktail” with RIPA Lysis Buffer from Santa Cruz Biotechnology, proteins were extracted from cell pellets. RIPA with the protease inhibitor was added to the pellets. After resuspending using insulin syringes, the tubes were left on ice for 30 minutes and then centrifuged for 10 minutes. The supernatant containing the protein was then collected.

3.10 Protein Quantification

Extracted proteins were quantified with a Bradford Assay using the BioRad Protein Assay Kit.

3.11 Western Blotting

The protein samples were mixed with SDS loading buffer and cooked at 95°C for 5 minutes before loading onto the gel.

In case the protein of interest was PHIP, to transfer the proteins from the gel onto a nitrocellulose membrane, a wet transfer at 75V for 2 hours was performed.

The membrane was blocked in 5% w/v nonfat dry milk in 1X TBST for 30 minutes. The primary antibody was added, in case of PHIP at a concentration of 1:500, and then the membrane was incubated at 4ºC overnight.

After washing the membrane with TBST, the secondary antibody is applied at a concentration of 1:1000. After incubation at room temperature for one hour, Luminol Reagent from Santa Cruz Biotechnology was added on the membrane for 1 minute. Then, the signal resulting from the proteins on the membranes are made visible on x-ray films.

3.12 Transfection of pcDNA3 and TLN1 plasmids

To overexpress TALIN-1 (TLN1) in MDA-MB-436 cells, a Turbofect-mediated transfection (9μL) of Addgene plasmid 26724 (3μg) or control pcDNA3 plasmid (3μg) (LifeTech) was carried out by following the manufacturer’s instructions (Thermo Fisher Scientific).

4 Results and Discussion

4.1 Suppression of PHIP expression

In order to determine and show the effect of PHIP *in vitro*, the effect of a different level of expression of PHIP in cells was investigated. Thus, MCF-7 and MDA-MB-436, two human breast cancer cell lines, were infected with a lentivirus containing shRNA targeting PHIP (anti-PHIP shRNA). Cells with suppressed expression of PHIP (also referred to as “127” cells) were used in a series of experiments.

First, however, the level of PHIP knockdown was determined by extracting RNA from cell pellets, generating cDNA and investigating the expression of PHIP on the mRNA level by TaqMan quantitative real-time PCR (Fig.11 and Fig.12).

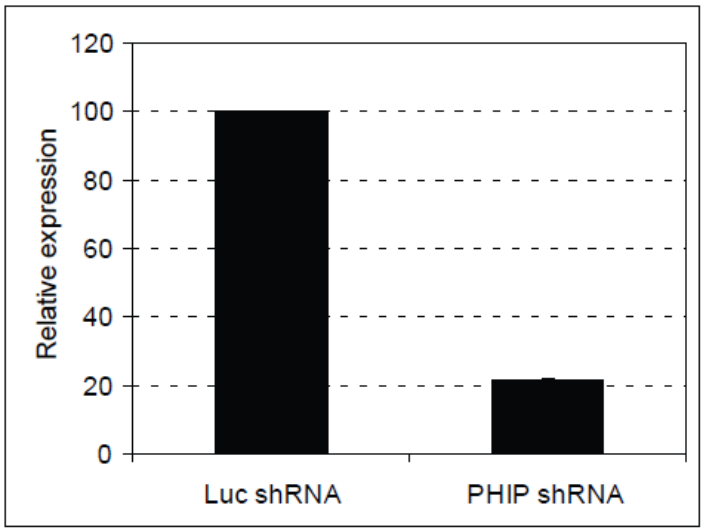

Fig.11: Relative Expression of PHIP in the luciferase-targeted control group vs the PHIP-targeted group of MCF-7 cells as determined by TaqMan Analysis.

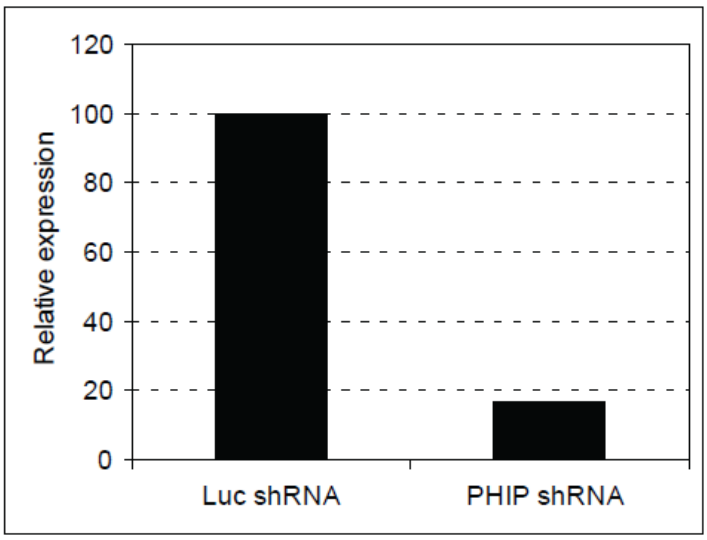

Fig.12: Relative Expression of PHIP in the luciferase-targeted control group vs the PHIP-targeted group of MDA-MB-436 cells as determined by TaqMan Analysis.

If the results showed sufficient knockdown (79% for MCF-7 and 83% for MDA-MB-436 in this case), the knockdown was investigated at the protein level by means of Western Blotting (Fig.13 and Fig.14). For TaqMan analysis, more than 70% of PHIP knockdown were considered to be sufficient.

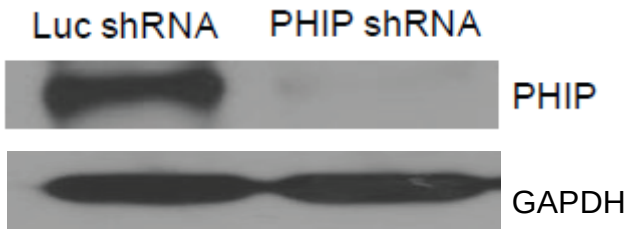

Fig.13: Relative Expression of PHIP in the luciferase-targeted control group vs the PHIP-targeted group of MCF-7 cells as determined by Western Blotting.

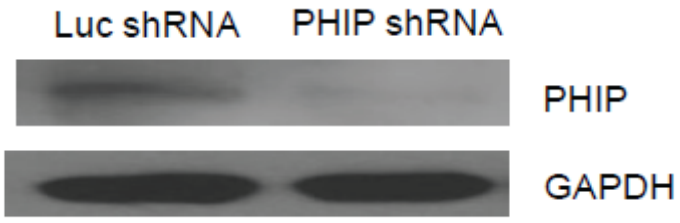

Fig.14: Relative Expression of PHIP in the luciferase-targeted control group vs the PHIP-targeted group in MDA-MB-436 as determined by Western Blotting.

Both cell lines showed significant knockdown of PHIP compared to control cells (figures 13 and 14).

In addition to quantitative real-time PCR and western blotting, quantitative immunofluorescence on MCF-7 was conducted to confirm suppressed expression of PHIP (see fig. 15).

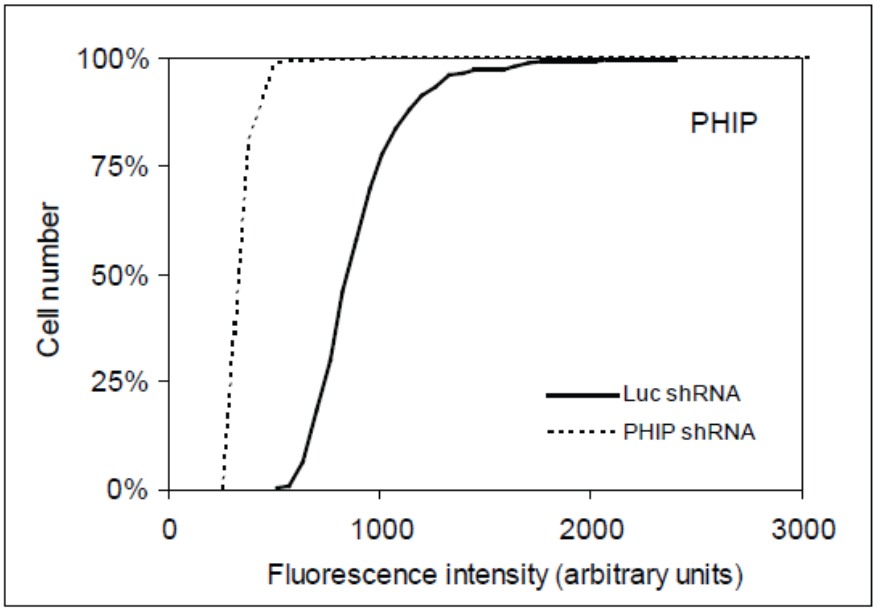

Fig.15: Relative Expression of PHIP in the luciferase-targeted control group vs the PHIP-targeted group of MCF-7 as determined by quantitative immunofluorescence.

4.2 Cell Proliferation

The activation of PHIP has been shown to promote melanoma progression and metastasis[2,3]. To investigated the role of PHIP in cell growth of breast cancer cells, MCF7 and MDA-MB-436 cells of both the control group (“luc” for anti-luciferase shRNA) and PHIP knockdown group (“127”, PHIP targeted) were plated in a 96 well plate at 2000 cells per well (wells contain normal cell growth medium).

The optical density was used as a measure to determine the number of cells after 24, 48 and 72 hours. The resulting growth curves are plotted in figures 16 and 17.

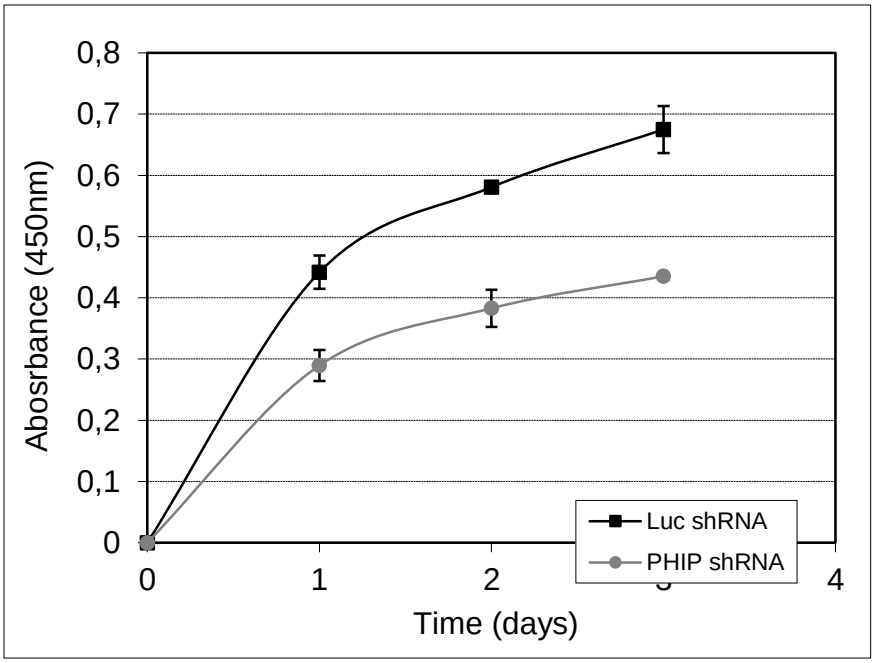

Fig.16: Cell growth of MCF-7 luc and MCF-7 127 (PHIP), with points of measurement after 24, 48 and 72 hours.

As can be seen in fig.16, the small difference between MCF-7 luc and 127 cells after 24 hours becomes greater steadily until the point of measurement after 72 hours, where the difference reaches its maximum. A t-test showed significance between luc and 127 groups for all points of measurement.

In MDA-MB-436, the difference in cell growth rate between the control group with anti-luciferase shRNA and the group with anti-PHIP shRNA is even more prominent (fig. 17).

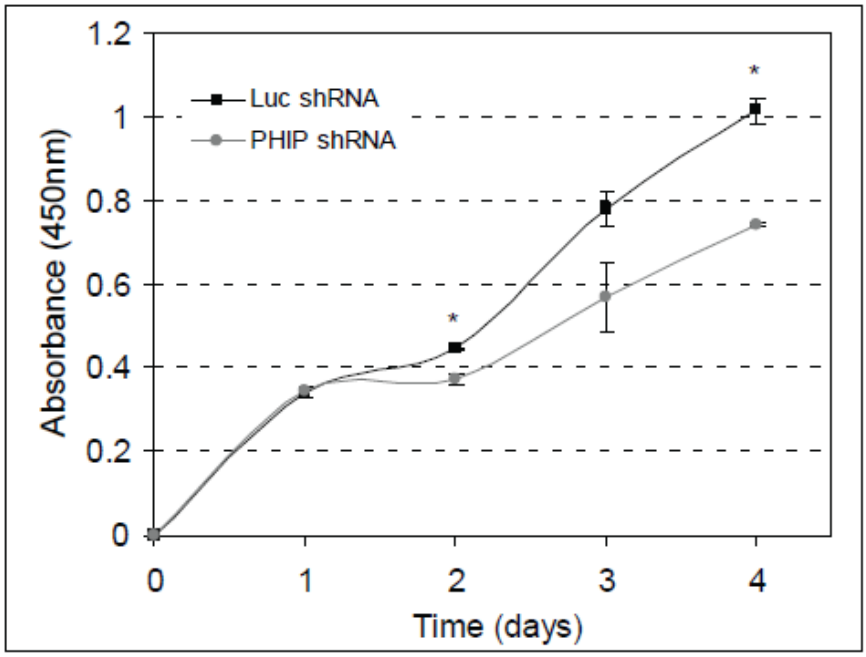

Fig.27: Cell growth of MDA-MB-436 luc and MDA-MB-436 (PHIP), with points of measurement after 24, 48 and 72 hours.

The growth curve shows that at the first point of measurement after 24 hours where no significant difference between MDA-MB-436 luc and 127 cells has been measured. Cells of the control group (MDA-MB-436 luc) grew faster till the second point of measurement after 48 hours, resulting in a slightly higher cell number than the group with suppressed expression of PHIP. The growth rate of the control group was increasingly greater than the growth of the 127 cells, resulting in a vast difference between the two groups at the last point of measurement after 72 hours. The graphs shows both in case of MCF-7 and MDA-MB-436 that the growth curve of the control group (luc) is steeper than the curve of 127 (PHIP knockdown), implying that shRNA-mediated targeting of PHIP in breast cancer cells slows cell growth to a certain extent. This provides evidence for the involvement of PHIP in breast cancer progression.

4.3 Colony Formation

The formation of colonies of MCF-7 and MDA-MB-436 cells was assessed by plating 1000 cells per well in a 6-well plate. After eight days of time for the cells to grow, the

number of colonies was determined by staining the cells with crystal violet; the number of colonies was determined with software.

Tbl.5: Number of colonies per well of the control and PHIP groups.

	well No1	well No2	well No3	mean	SEM	TTEST
MCF7 luc	125	145	134	134,7	5,8	
MCF7 127	60	50	55	55,0	2,9	0,00025

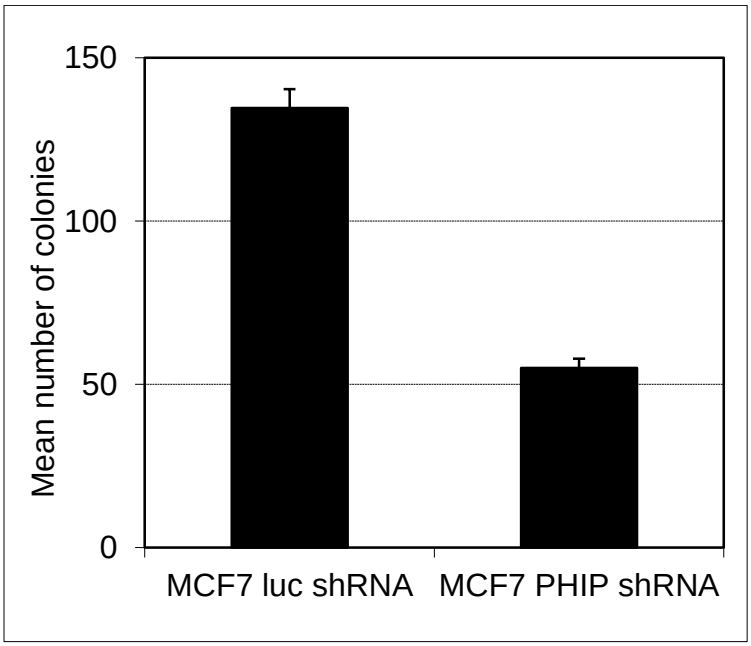

Fig.18: Mean number of colonies of control and PHIP knockdown groups of MCF-7 cells

As shown in table 5 and figure 18, there is a big difference in the number of colonies between the control cells with targeted luciferase and the 127 cells with suppressed expression of PHIP. The MCF-7 luc group shows a nearly 2.5 times greater number of colonies compared to the MCF-7 127. A t-test shows that the result is highly statistically significant.

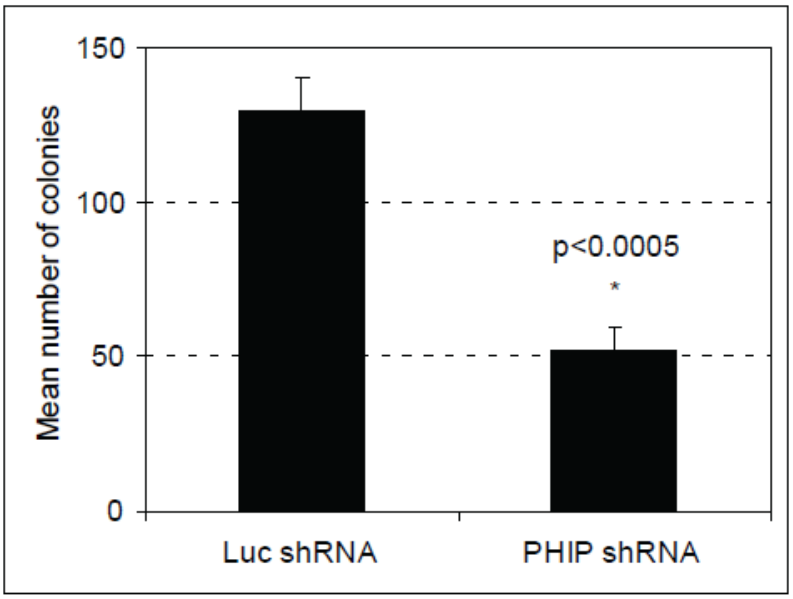

Fig.19: Mean number of colonies of MDA-MB-436 luc (control) and 127 (PHIP knockdown) groups

Figure 19 displays that MDA-MB-436 luc shows a more than 2.5 times greater number of colonies compared to the MDA-MB-436 127. A t-test ($p<0.0005$) shows that the result is highly statistically significant.

Stable suppression of PHIP in both cell lines proved to reduce the formation of colonies strongly, providing further evidence that PHIP is crucially involved in cell proliferation in triple-negative breast cancer.

4.4 Migration Assay

PHIP has been shown to promote cell invasion and the formation of metastases in melanoma[2]. To determine a possible role of PHIP in breast cancer cell invasion, a transwell migration assay with MCF-7 luc and 127 as well as with MDA-MB-436 luc and MDA-MB-436 127 cells respectively, was performed. The ability of those two groups to migrate through filter inserts in a 24-well plate was assessed. 200.000 cells per well were plated with 5mg/ml 15ul matrigel and 30% FBS in DMEM and incubated for 48 hours to allow them to migrate through the filters.

Tbl.6: Number of cells that migrated through the filter inserts of the control and PHIP groups.

	Mean number of migrated cells	SEM	TTEST
MCF7 luc shRNA	280	32,03	
MCF7 PHIP shRNA	170	10,63	0,015

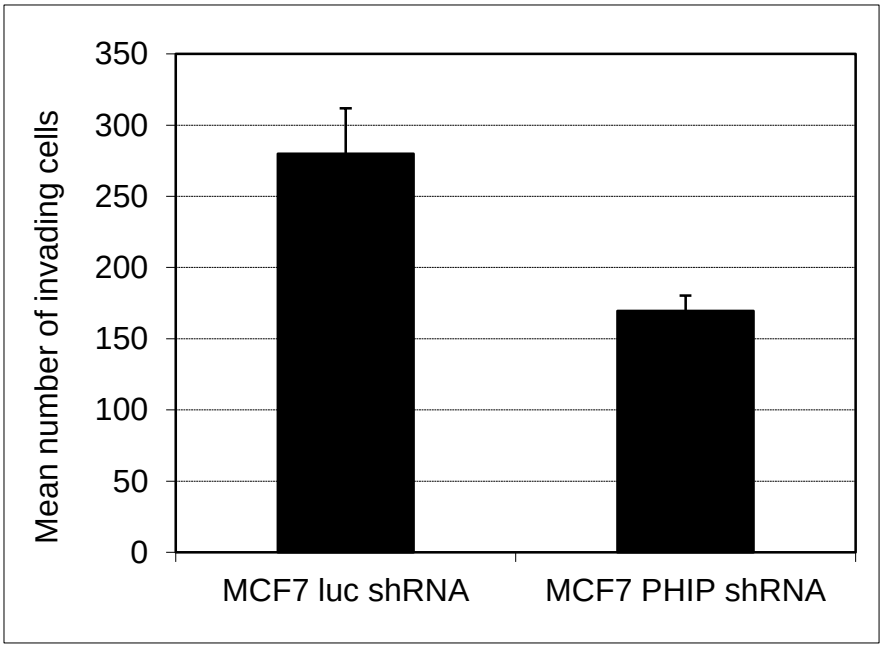

Fig.20: Mean number of migrating cells of MCF-7 luc and MCF-7 127.

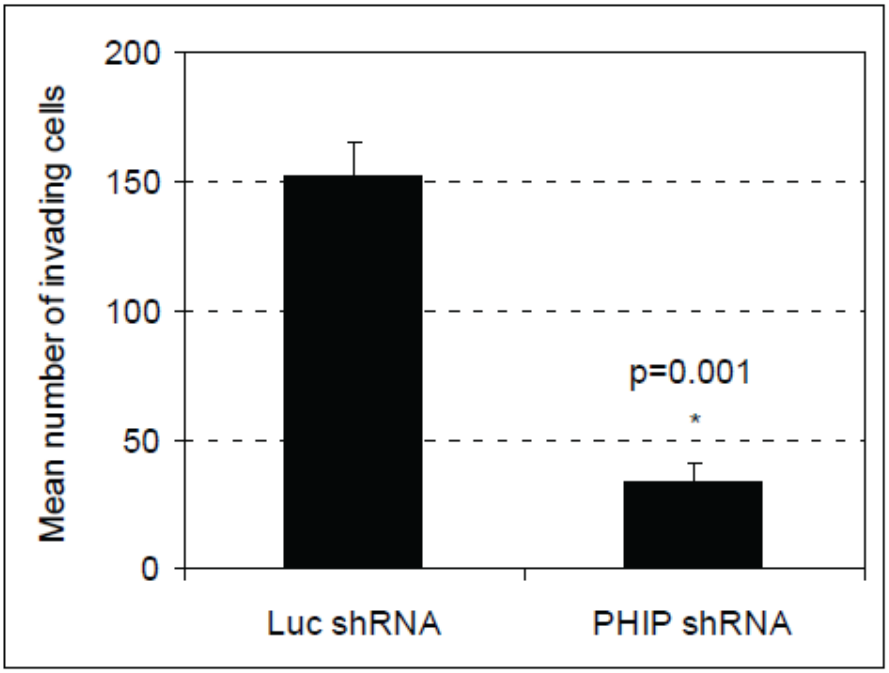

Fig.21: Mean number of migrating cells of MDA-MB-436 luc and MDA-MB-436 127.

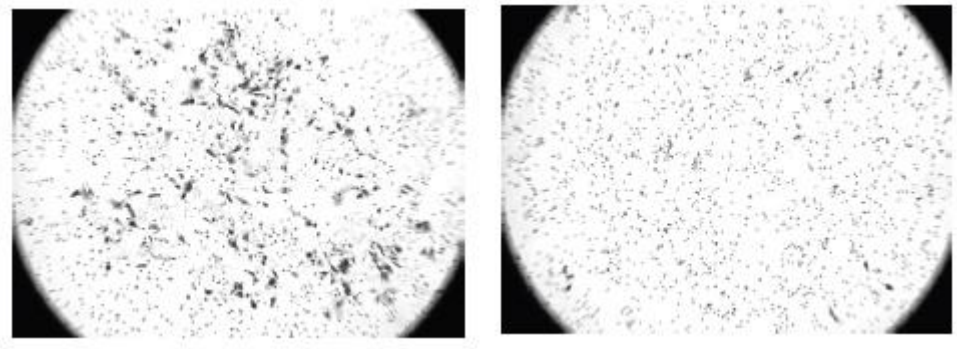

Fig.22: Image of the migrated cells of both groups taken after the transwell migration assay; MDA-MB-436 luc on the left, and MDA-MB-436 127 on the right.

Table 6 and figure 20 show knockdown of PHIP in MCF-7 cells reduces the ability of the cells to migrate through the filter inserts by nearly 40%.

Figures 21 and 22 show that the suppression of PHIP expression in MDA-MB-436 cells resulted in a migration potential reduced by 78% when compared to the control group.

These results suggest that PHIP is involved in invasion of breast cancer cells.

4.5 Talin-1

PHIP has been shown to play a proinvase role in triple-negative melanoma[2]. Several molecular markers of invasion were analysed in MCF-7 and MDA-MB-436 by quantitative immunofluorescence or western blotting.

The cytoskeletal protein Talin-1 (encoded by the gene TLN-1) has been shown to be a mediator for focal adhesion kinase (FAK) activation in integrin signalling[70], therefore playing a role in cell-cell and cell-matrix adhesion. Sakamoto *et al.* have shown that overexpression of Talin-1 enhances prostate cancer cell adhesion, migration and invasion by activating survival signals and allowing cell resistance to anoikis, mediated by AKT signalling[58].

De Semir *et al.* have conducted cDNA microarray analysis and identified Tln1 as a down-regulated gene after suppression of PHIP expression[2]. They have shown that overexpression of TLN1 into C8161.9 melanoma cells with suppressed expression of PHIP resulted in significantly increased invasion of those cells, suggesting that the proinvasive role of PHIP is mediated by activating TLN1 and AKT[2].

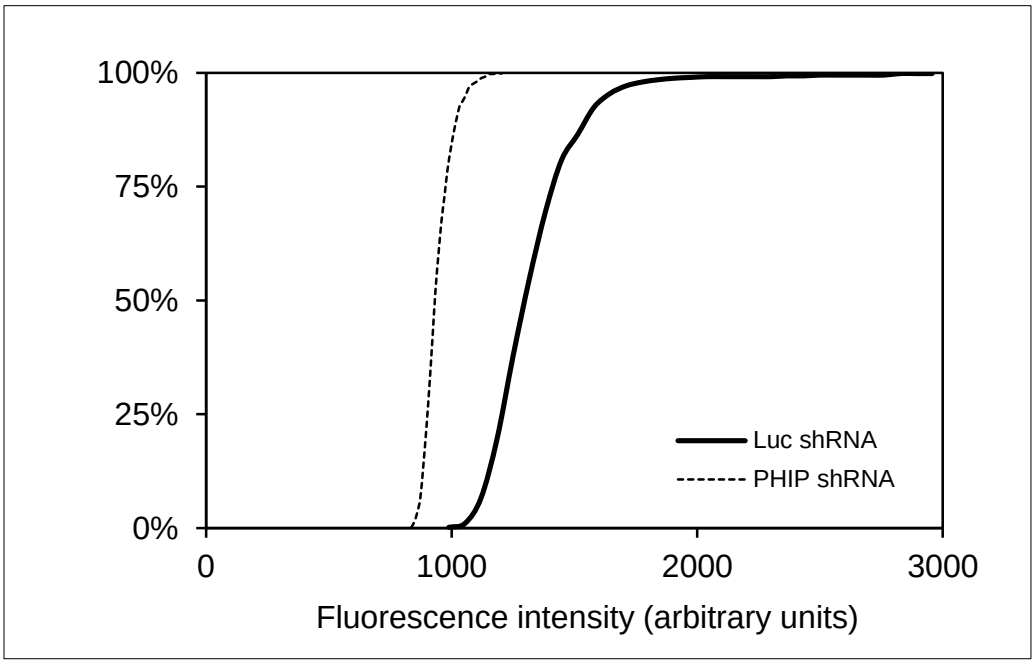

Fig.23: Level of expression of Talin-1 in MCF-7 luc and 127 (PHIP knockdown) as determined by quantitative immunofluorescence

The graph (Fig.23) shows targeting of PHIP with shRNA in MCF-7 significantly reduces the intracellular level of Talin-1, confirming that invasion in breast cancer could be regulated via PHIP, Talin-1 and AKT.

As suppression of PHIP expression results in decreased invasion of MDA-MB-436 cells, MDA-MB-436 cells overexpressing Talin-1 were plated to assess their ability to migrate through filter inserts (Fig.24).

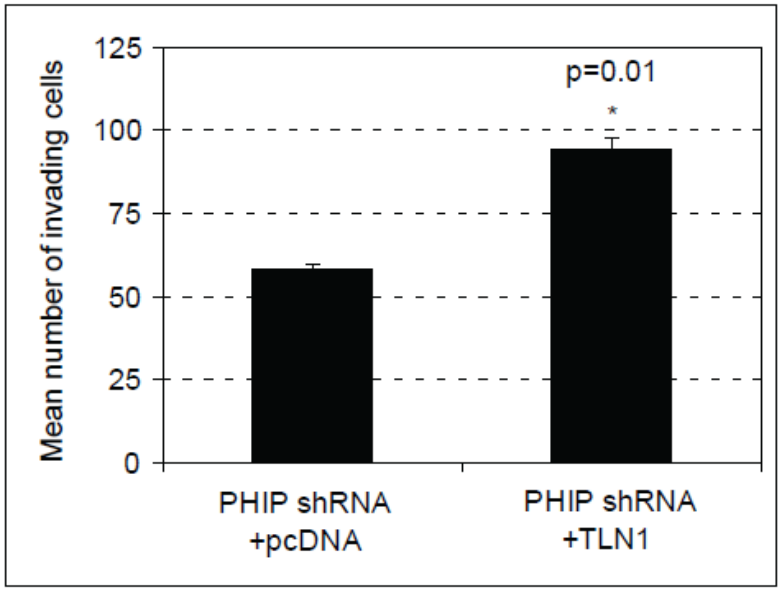

Fig.24: Mean number of migrating cells of MDA-MB-436 127 overexpressing pcDNA as a control and MDA-MB-436 127 overexpressing TLN-1.

The invasion assay showed that the overexpression of Tln-1 in MDA-MB-436 with PHIP knockdown resulted in a significantly greater number of cells that migrated through the filter inserts. This data represents further evidence that the role of PHIP in invasion is mediated via the Talin-1 and AKT axis, and shows that overexpression of Tln-1 can counteract lower levels of PHIP in breast cancer cells.

Suppression of PHIP expression in MDA-MB-436 also resulted in lower levels of the protein TLN-1 (Fig.25; 80% reduction), suggesting that the latter protein is downstream of PHIP and might even be regulated by it.

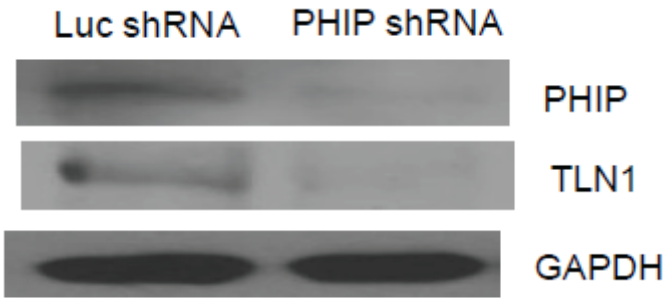

Fig.25: Expression of TLN-1 in MDA-MB-436 luc vs the MDA-MB-436 127 as determined by Western Blotting. GAPDH was used as a control.

4.6 Integrin β-1

Integrins are transmembrane proteins that are involved in adhesion of cells to the ECM and to each other[32].

Increased expression of Integrin β-1 (ITGB1) has been suggested to mediate AKT2 promoted invasion and has also been shown to correlate with increased metastasis in some cancers[71–73].

The level of Integrin β-1 as another marker for cell invasion was determined in MCF-7 cells by quantitative immunofluorescence (Fig.26).

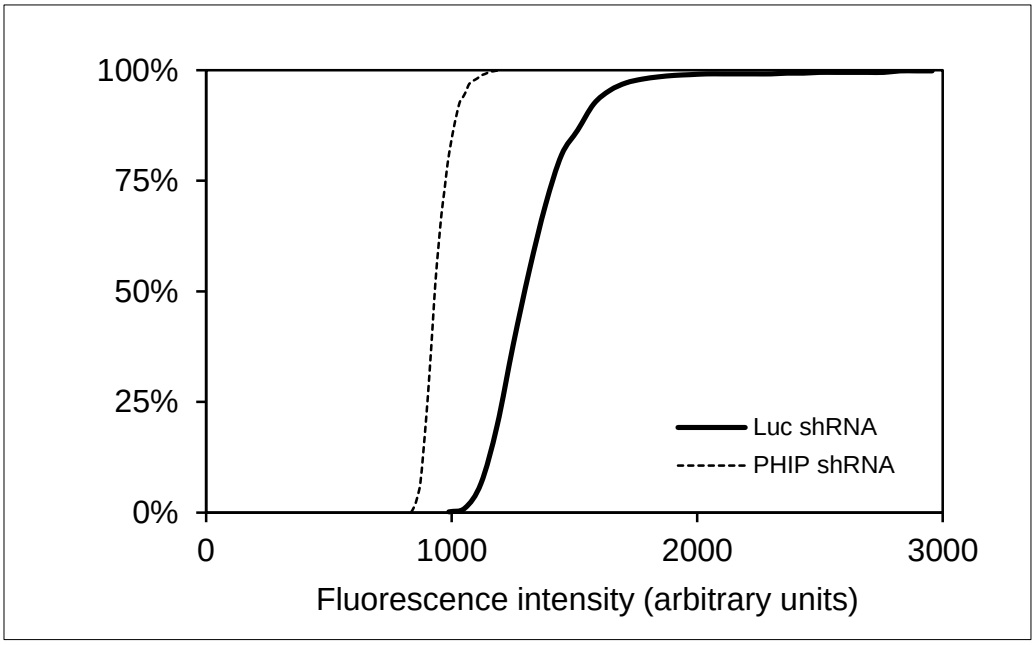

Fig.26: Level of expression of Integrin β-1 in MCF-7 luc and 127 cells

Significantly reduced expression of ITGB1 was found in MCF-7 cells with suppressed expression of *PHIP*. This shows that knocking down PHIP, which results in reduced invasion of breast cancer cells, also results in decreased intracellular levels of Integrin β-1, suggesting that ITGB1 is downstream of PHIP in signalling pathways.

4.7 Cyclin D1

D-type cyclins are active during G1 phase of the cell cycle. They associate with CDK4 or CDK6 to form a protein complex that is required for transition of the cell from G1 to S phase[10].

The level of intracellular cyclin D1 in MDA-MB-436 and MCF-7 luc and 127 was determined by western blotting (figures 27 and 28).

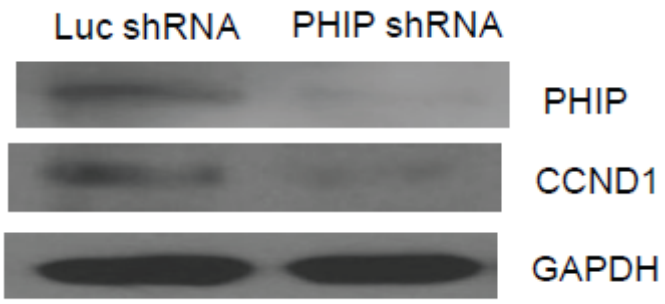

Fig.27: Level of expression of cyclin D1 in MDA-MB-436 luc and 127 cells as determined by western blotting.

The level of cyclin D1 (CCND1) showed to be substantially smaller in MDA-MB-436 127 than compared to MDA-MB-436 luc.

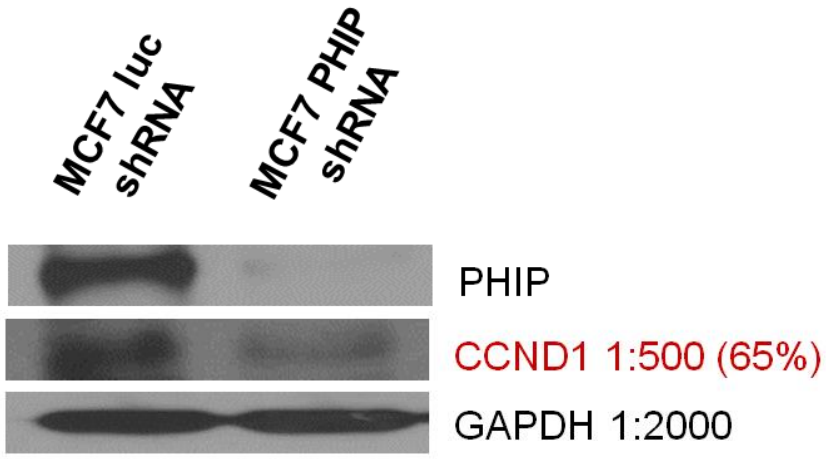

Fig.28: Level of expression of cyclin D1 in MCF-7 luc and 127 cells as determined by western blotting.

The cyclin D1 level was found to be 65% smaller in MCF-7 127 with a *PHIP* knockdown of 97% at a protein level when compared to MCF luc.

The results for both cell lines show significant smaller levels of cyclin D1 in cells with suppressed expression of *PHIP*, providing evidence that PHIP is involved in the progression of breast cancer cells through the cell cycle.

4.8 AKT

Efficient docking of Insulin Receptor Substrate (IRS) to insulin-like growth factor 1 receptor (IGF1R) is mediated via their pleckstrin homology domain. PHIP has been identified to interact with the pleckstrin homology domain of IRS proteins and has been shown to mediate transcriptional responses in pancreatic islet cells[54]. The activation of IRS results in PI3K recruitment and AKT activation[74].

AKT, also known as protein kinase B, functions as a critical regulator of cell survival and proliferation. It is crucially involved in the signalling pathways in response to growth factors and other extracellular stimuli, thereby regulating important cellular functions such as, cell growth, nutrient metabolism, apoptosis and survival[75]. Evidence suggests that components of the PI3K/AKT signalling pathway are frequently altered in human cancers[76].

De Semir *et al.* have suggested that the proinvasive and proliferative roles of PHIP are mediated by activating TLN1 and AKT[2]. Thus, the level of total AKT and phosphorylated AKT (AKT is activated via phosphorylation by the rictor-mTOR complex[77]) was determined by western blotting (figures 29 and 30).

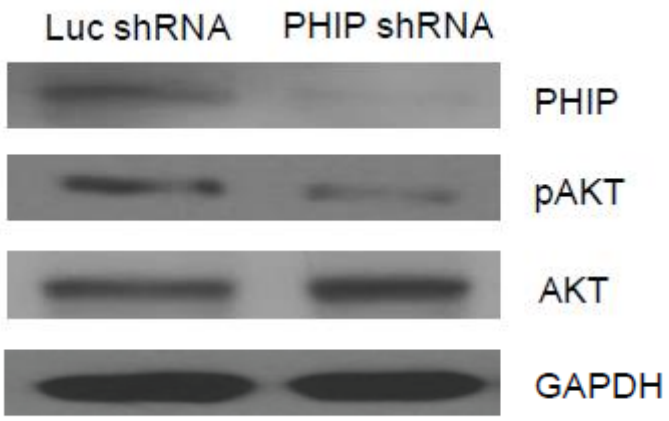

Fig.29: Level of phosphorylated (pAKT) and total AKT (tAKT) in MDA-MB-436 luc and 127 cells with GAPDH as a control.

The amount of the activated form of AKT (phosphorylated AKT) was significantly reduced in MDA-MB-436 with PHIP knockdown (52%).

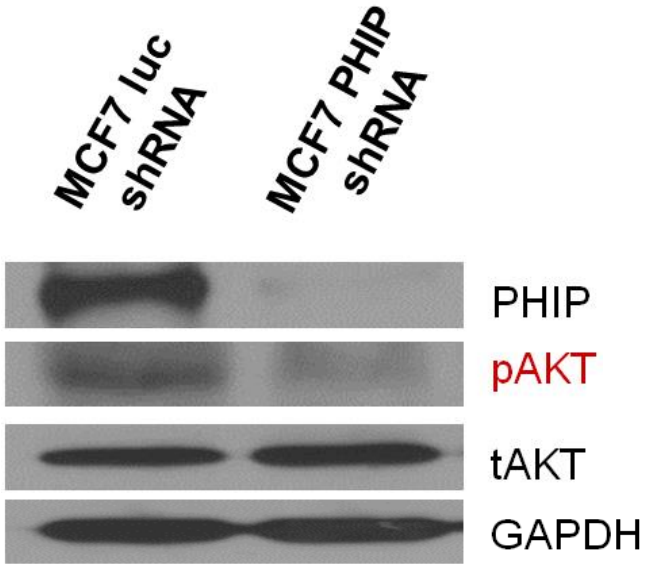

Fig.30: Level of phosphorylated (pAKT) and total AKT (tAKT) in MCF-7 luc and 127.

The level of phosphorylated AKT was found to be reduced by 38.7% in MCF-7 127 cells compared to the control (see fig.30). A similar result (52%) of pAKT knockdown was observed in MDA-MB-436 (see fig.29), suggesting that suppression of PHIP expression also reduces the proliferative and proinvasive effects of cells by suppressing the PI3K/AKT signalling pathway.

5 Conclusion

De Semir *et al.* have described the role of PHIP as a marker and mediator of melanoma metastasis. Their work showed that the protein plays a crucial role in triple-negative melanomas. The group has found evidence that PHIP regulates the expression of upstream mediators of the IGF axis and downstream mediators of tumor cell invasion, and therefore, that PHIP mediates its proinvasive role by activating the glycolytic pathway[2,3].

The research described in this thesis investigates the role of PHIP in breast cancer. Experiments conducted with triple-negative breast cancer cells showed significant effects of PHIP in the breast cancer cell line used. Cells with suppressed expression of PHIP were found to grow significantly slower, form less than half as many colonies, and exhibit a significantly reduced invasive potential when compared to a control group of cells.

Quantification of two markers of cell invasion, namely TLN-1 and AKT, has produced results leading to the conclusion that the proinvasive role of PHIP in breast cancer is mediated by activating TLN1 and AKT. Overexpression of TLN-1 in cells with suppressed expression of PHIP has resulted in a substantial increase of invasion of those cells. The pathway TLN1 and AKT are involved in has also been suggested to mediate the role of PHIP in melanoma cell invasion[2], suggesting that PHIP plays a similar role in different types of triple-negative cancers.

Furthermore, the level of expression of cyclin D1 was assessed by means of western blotting. This experiment showed a smaller amount of intracellular cyclin D1 in cells with suppressed expression of PHIP, providing evidence that PHIP is involved in the progression of breast cancer cells through regulation of the cell cycle.

References

1. Engebraaten, O., Vollan, H. K. M. & Børresen-Dale, A.-L. Triple-negative breast cancer and the need for new therapeutic targets. *Am. J. Pathol.* **183,** 1064–74 (2013).

2. 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.* **109,** 7067–72 (2012).

3. Bezrookove, V. *et al.* Prognostic Impact of PHIP Copy Number in Melanoma: Linkage to Ulceration. *J. Invest. Dermatol.* (2013). doi:10.1038/jid.2013.369

4. Hajdu, S. I. A note from history: landmarks in history of cancer, part 1. *Cancer* **117,** 1097–102 (2011).

5. Tian, M., Neil, J. R. & Schiemann, W. P. Transforming growth factor-β and the hallmarks of cancer. *Cell. Signal.* **23,** 951–62 (2011).

6. Jemal, A. *et al.* Cancer statistics, 2007. *CA. Cancer J. Clin.* **57,** 43–66

7. Hanahan, D. & Weinberg, R. A. The hallmarks of cancer. *Cell* **100,** 57–70 (2000).

8. Hanahan, D. & Weinberg, R. a. Hallmarks of cancer: the next generation. *Cell* **144,** 646–74 (2011).

9. Becker, W. M., Kleinsmith, L. J., Hardin, J. & Bertoni, G. P. *The World of the Cell, 7th Edition*. 912 (Benjamin Cummings, 2008). at <http://www.amazon.com/The-World-Cell-7th-Edition/dp/0805393935>

10. Weinberg, R. A. *The Biology of Cancer*. 960 (Garland Science, 2013). at <http://www.amazon.com/The-Biology-Cancer-Robert-Weinberg/dp/0815342209>

11. Baltimore, D. & Lodish, H. *Molecular Cell Biology, 4th Edition*. (W H Freeman & Co, 2002). at <http://www.amazon.com/Molecular-Cell-Biology-4th-Edition/dp/B002KNXJOO>

12. Feero, W. G., Guttmacher, A. E. & Collins, F. S. Genomic medicine--an updated primer. *N. Engl. J. Med.* **362,** 2001–11 (2010).

13. Rous, P. A TRANSMISSIBLE AVIAN NEOPLASM. (SARCOMA OF THE COMMON FOWL.). *J. Exp. Med.* **12,** 696–705 (1910).

14. Rous, P. A SARCOMA OF THE FOWL TRANSMISSIBLE BY AN AGENT SEPARABLE FROM THE TUMOR CELLS. *J. Exp. Med.* **13,** 397–411 (1911).

15. Collett, M. S. & Erikson, R. L. Protein kinase activity associated with the avian sarcoma virus src gene product. *Proc. Natl. Acad. Sci. U. S. A.* **75,** 2021–4 (1978).

16. Manning, G., Whyte, D. B., Martinez, R., Hunter, T. & Sudarsanam, S. The protein kinase complement of the human genome. *Science* **298,** 1912–34 (2002).

17. Carpenter, G. & Cohen, S. Epidermal growth factor. *J. Biol. Chem.* **265,** 7709–12 (1990).

18. Marsh, M. & McMahon, H. The Structural Era of Endocytosis. *Science (80-.).* **285,** 215–220 (1999).

19. Michel, G., Tonon, T., Scornet, D., Cock, J. M. & Kloareg, B. The cell wall polysaccharide metabolism of the brown alga Ectocarpus siliculosus. Insights into the evolution of extracellular matrix polysaccharides in Eukaryotes. *New Phytol.* **188,** 82–97 (2010).

20. Green, D. R. *Means to an End: Apoptosis and Other Cell Death Mechanisms*. 220 (Cold Spring Harbor Laboratory Press, 2011). at <http://books.google.com/books/about/Means_to_an_End.html?id=s8jBcQAACAAJ&pgis=1>

21. Chen, C. S., Alonso, J. L., Ostuni, E., Whitesides, G. M. & Ingber, D. E. Cell shape provides global control of focal adhesion assembly. *Biochem. Biophys. Res. Commun.* **307,** 355–61 (2003).

22. Frisch, S. M. & Screaton, R. A. Anoikis mechanisms. *Curr. Opin. Cell Biol.* **13,** 555–62 (2001).

23. Hannink, M. & Donoghue, D. J. Structure and function of platelet-derived growth factor (PDGF) and related proteins. *Biochim. Biophys. Acta* **989,** 1–10 (1989).

24. Goodsell, D. S. The molecular perspective: the ras oncogene. *Oncologist* **4,** 263–4 (1999).

25. Downward, J. Targeting RAS signalling pathways in cancer therapy. *Nat. Rev. Cancer* **3,** 11–22 (2003).

26. Murphree, A. L. & Benedict, W. F. Retinoblastoma: clues to human oncogenesis. *Science* **223,** 1028–33 (1984).

27. Cooper, G. *The Cell: A Molecular Approach - 2nd Edition*. 689 (Sinauer Associates Inc, 2000).

28. Tsuji, T., Ibaragi, S. & Hu, G. Epithelial-mesenchymal transition and cell cooperativity in metastasis. *Cancer Res.* **69,** 7135–9 (2009).

29. Blobe, G. C., Schiemann, W. P. & Lodish, H. F. Role of transforming growth factor beta in human disease. *N. Engl. J. Med.* **342,** 1350–8 (2000).

30. Galliher, A. J., Neil, J. R. & Schiemann, W. P. Role of transforming growth factor-beta in cancer progression. *Future Oncol.* **2,** 743–63 (2006).

31. Shi, Y. & Massagué, J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113,** 685–700 (2003).

32. Alberts, B. *et al. Molecular Biology of the Cell, Fourth Edition*. 1616 (Garland Science, 2002).

33. Wyllie, A. H. Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284,** 555–556 (1980).

34. Olovnikov, A. M. A theory of marginotomy. The incomplete copying of template margin in enzymic synthesis of polynucleotides and biological significance of the phenomenon. *J. Theor. Biol.* **41,** 181–90 (1973).

35. Greider, C. W. & Blackburn, E. H. Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* **43,** 405–13 (1985).

36. Jeon, J. S., Zervantonakis, I. K., Chung, S., Kamm, R. D. & Charest, J. L. In vitro model of tumor cell extravasation. *PLoS One* **8,** e56910 (2013).

37. Psaila, B. & Lyden, D. The metastatic niche: adapting the foreign soil. *Nat. Rev. Cancer* **9,** 285–93 (2009).

38. Pantel, K., Cote, R. J. & Fodstad, O. Detection and clinical importance of micrometastatic disease. *J. Natl. Cancer Inst.* **91,** 1113–24 (1999).

39. Wong, C. W. *et al.* Apoptosis: An Early Event in Metastatic Inefficiency. *Cancer Res.* **61,** 333–338 (2001).

40. Vivier, E. *et al.* Innate or adaptive immunity? The example of natural killer cells. *Science* **331,** 44–9 (2011).

41. Grasso, P., Gangolli, S. & Gaunt, I. *Essentials of Pathology for Toxicologists*. 166 (Taylor & Francis, 2002). at <http://books.google.com/books?id=QwPuKwriPHcC&pgis=1>

42. Teng, M. W. L., Swann, J. B., Koebel, C. M., Schreiber, R. D. & Smyth, M. J. Immune-mediated dormancy: an equilibrium with cancer. *J. Leukoc. Biol.* **84,** 988–93 (2008).

43. Kim, R., Emi, M. & Tanabe, K. Cancer immunoediting from immune surveillance to immune escape. *Immunology* **121,** 1–14 (2007).

44. Nelson, B. H. The impact of T-cell immunity on ovarian cancer outcomes. *Immunol. Rev.* **222,** 101–16 (2008).

45. Pagès, F. *et al.* Immune infiltration in human tumors: a prognostic factor that should not be ignored. *Oncogene* **29,** 1093–102 (2010).

46. Otto, W., Posener, K. & Negelein, E. Ueber den Stoffwechsel der Tumoren. *Biochem. Z.* **Vol. 152,** 319–344 (1924).

47. WARBURG, O. On the origin of cancer cells. *Science* **123,** 309–14 (1956).

48. Pavlides, S. *et al.* Warburg meets autophagy: cancer-associated fibroblasts accelerate tumor growth and metastasis via oxidative stress, mitophagy, and aerobic glycolysis. *Antioxid. Redox Signal.* **16,** 1264–84 (2012).

49. Rodríguez-Enríquez, S. & Moreno-Sánchez, R. Intermediary metabolism of fast-growth tumor cells. *Arch. Med. Res.* **29,** 1–12 (1998).

50. Simonnet, H. *et al.* Low mitochondrial respiratory chain content correlates with tumor aggressiveness in renal cell carcinoma. *Carcinogenesis* **23,** 759–68 (2002).

51. Farhang-Fallah, J., Yin, X., Trentin, G., Cheng, A. M. & Rozakis-Adcock, M. Cloning and characterization of PHIP, a novel insulin receptor substrate-1 pleckstrin homology domain interacting protein. *J. Biol. Chem.* **275,** 40492–7 (2000).

52. 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.* **22,** 7325–36 (2002).

53. Czech, M. P. & Corvera, S. Signaling mechanisms that regulate glucose transport. *J. Biol. Chem.* **274,** 1865–8 (1999).

54. 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.* **27,** 6484–96 (2007).

55. Kulkarni, R. N. New insights into the roles of insulin/IGF-I in the development and maintenance of beta-cell mass. *Rev. Endocr. Metab. Disord.* **6,** 199–210 (2005).

56. Li, S. *et al.* The full-length isoform of the mouse pleckstrin homology domain-interacting protein (PHIP) is required for postnatal growth. *FEBS Lett.* **584,** 4121–7 (2010).

57. Haqq, C. *et al.* The gene expression signatures of melanoma progression. *Proc. Natl. Acad. Sci. U. S. A.* **102,** 6092–7 (2005).

58. Sakamoto, S., McCann, R. O., Dhir, R. & Kyprianou, N. Talin1 promotes tumor invasion and metastasis via focal adhesion signaling and anoikis resistance. *Cancer Res.* **70,** 1885–95 (2010).

59. Bauer, J. & Bastian, B. C. Distinguishing melanocytic nevi from melanoma by DNA copy number changes: comparative genomic hybridization as a research and diagnostic tool. *Dermatol. Ther.* **19,** 40–9

60. Blokx, W. A. M., van Dijk, M. C. R. F. & Ruiter, D. J. Molecular cytogenetics of cutaneous melanocytic lesions - diagnostic, prognostic and therapeutic aspects. *Histopathology* **56,** 121–32 (2010).

61. Kashani-Sabet, M., Sagebiel, R. W., Ferreira, C. M. M., Nosrati, M. & Miller, J. R. Tumor vascularity in the prognostic assessment of primary cutaneous melanoma. *J. Clin. Oncol.* **20,** 1826–31 (2002).

62. DeSantis, C., Siegel, R., Bandi, P. & Jemal, A. Breast cancer statistics, 2011. *CA. Cancer J. Clin.* **61,** 409–18

63. www.cancer.org. at <http://www.cancer.org/cancer/breastcancer/detailedguide/breast-cancer-key-statistics>

64. Korde, L. A. *et al.* Multidisciplinary meeting on male breast cancer: summary and research recommendations. *J. Clin. Oncol.* **28,** 2114–22 (2010).

65. American Cancer Society. *Breast Cancer in Men - American Cancer Society*. at <http://www.google.at/url?sa=t&rct=j&q=&esrc=s&source=web&cd=6&ved=0CEcQFjAF&url=http://www.cancer.org/acs/groups/cid/documents/webcontent/003091-pdf.pdf&ei=YBTKUuPnLpPioATqwoDIDQ&usg=AFQjCNFOFa-xQ5tKLCaMF2j9vK7ZvMJdYQ&bvm=bv.58187178,d.cGU>

66. Stevens, K. N., Vachon, C. M. & Couch, F. J. Genetic susceptibility to triple-negative breast cancer. *Cancer Res.* **73,** 2025–30 (2013).

67. Mahamodhossen, Y. a, Liu, W. & Rong-Rong, Z. Triple-negative breast cancer: new perspectives for novel therapies. *Med. Oncol.* **30,** 653 (2013).

68. Duncan, J. A., Reeves, J. R. & Cooke, T. G. BRCA1 and BRCA2 proteins: roles in health and disease. *Mol. Pathol.* **51,** 237–47 (1998).

69. Yoshida, K. & Miki, Y. Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage. *Cancer Sci.* **95,** 866–71 (2004).

70. Chen, H. C. *et al.* Interaction of focal adhesion kinase with cytoskeletal protein talin. *J. Biol. Chem.* **270,** 16995–9 (1995).

71. Fujita, S., Watanabe, M., Kubota, T., Teramoto, T. & Kitajima, M. Alteration of expression in integrin β1-subunit correlates with invasion and metastasis in colorectal cancer. *Cancer Lett.* **91,** 145–149 (1995).

72. Morini, M. *et al.* The alpha 3 beta 1 integrin is associated with mammary carcinoma cell metastasis, invasion, and gelatinase B (MMP-9) activity. *Int. J. Cancer* **87,** 336–42 (2000).

73. Arboleda, M. J. *et al.* Overexpression of AKT2/protein kinase Bbeta leads to up-regulation of beta1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. *Cancer Res.* **63,** 196–206 (2003).

74. Manning, B. D. & Cantley, L. C. AKT/PKB signaling: navigating downstream. *Cell* **129,** 1261–74 (2007).

75. Song, G., Ouyang, G. & Bao, S. The activation of Akt/PKB signaling pathway and cell survival. *J. Cell. Mol. Med.* **9,** 59–71

76. Fresno Vara, J. A. *et al.* PI3K/Akt signalling pathway and cancer. *Cancer Treat. Rev.* **30,** 193–204 (2004).

77. Sarbassov, D. D., Guertin, D. A., Ali, S. M. & Sabatini, D. M. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307,** 1098–101 (2005).