Production and observation of the STAT3 protein and its possible inhibitors for cancer treatment

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By

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Abstract

In 2016, about 1,685,210 new cases of cancer have been diagnosed in the US and approximately one third of the diseased did not survive.[1] In Austria 39,000 people develop a form of cancer per year.[2] The disease effects the diverse ethnic groups differently [1] and is diagnosed more frequently in older people. Due to the continuously increasing average lifespan, cancer treatment will become even more important in the near future.[2] When speaking of cancer, cells are meant which can grow and divide without the limitations of the ancestor cell type and can invade and colonize distant body parts due to mutations in their genome.[3] The many different types of cancer e.g. breast cancer, various leukemias, prostate cancer etc. create a great obstacle for cancer treatment. Though many types can be cured by different means like chemotherapy, radiation, surgery, drugs, etc., several types remain for which further treatments need to be developed. To develop anticancer drugs and therapies, the abnormalities, which are the natural features of cancer cells and render them so tough to treat, have to be observed and targeted if possible.[3] One abnormality that can be observed in several cancer types is the overexpression of Stat3.[4,5] The signal transducers and activators of transcription (Stats) are latent gene regulatory proteins, that are present in the cytosol and are activated by the Janus kinases (Jaks). After the phosphorylation by Jaks, which are associated with the cytosolic part of receptors, the Stats will dimerize and translocate into the nucleus, where they stimulate gene transcription together with other regulators. [3]

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List of Abbreviations

T-lymphocyte	thymus matured lymphocyte
B-lymphocyte	bone marrow matured lymphocyte
Stat	signal transducer and activator of transcription
Jak	Janus kinase
SH	Src homology
IFN	interferon
IL	interleukin
gp130	glycoprotein 130
JNK	c-Jun N-terminal kinases
Socs3	suppressor of cytokine signaling 3
CNTF	ciliary neutrophic factor
CDK5	cyclin dependent kinase
PKC-ε	protein kinase C-ε
CD45	cluster of differentiation 45
PTEN	Phosphatase and tensin homolog
Bcl-xl	B-cell lymphoma-extra large
Bcl-2	B-cell lymphoma 2
cIAP2	cellular inhibitor of apoptosis
PI_3K	phosphatidylinositol kinase
Akt	protein kinase B
MMP	matrix metalloproteinases
MUC1	mucin1

TIMP	tissue inhibitor of metalloproteinase.
TNF-α	tumor necrosis factor α
NK cells	natural killer cells
ATP	adenosine triphosphate
PTP	protein tyrosine phosphatase
SHP	SH2 domain-containing phosphatase
RTK	receptor tyrosine kinase
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
PBS	phosphate buffered saline
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
APS	ammonium persulfate
TEMED	tetramethylethylenediamine

1 Introduction

1.1 Cancer

Approximately one in five will develop and die of cancer and caused 8.8 million deaths in 2015. [3,6] The reason why the disease itself and hence, the treatments available are very complex is that cancer cells do not follow the basic principles of normal cell behavior in multicellular organisms. Due to mutations, they develop abilities not found in normal cells.[3] Metazoans are made out of cells that together form an ecosystem – their body. These cells reproduce via cell division and differentiate into various cell types forming tissues and organs. In contrast to free-living cells, these cells do not compete with each other but collaborate. The body of a multicellular organism is a society of cells that is used to self-sacrifices. All somatic cells within the body will eventually die, leaving no offspring, because their purpose is to support the germ cells, which are the only ones that can give rise to a new organism. This biological design requires highly controlled cooperation among the cells. Therefore, extracellular signals have to be sent, received and interpreted by the cells to dictate them how to behave. All possible actions of a cell including growth, differentiation, division, rest or death are carried out to support the wellbeing of the organism overall. If this controlled collaboration and signaling is impaired by e.g. mutations that cause a selfish cell behavior, the whole ecosystem will be effected. In the worst case, a cell might mutate in a way that is advantageous over the non-mutated cells and allows for rapid growth and division as well as for better survival mechanisms making it the possible source of a growing mutant clone. If the mutant cells are not cleared from the system, they undergo numerous rounds of mutation and natural selection over time, making them more and more abnormal. This is how the disease cancer arises. Therefore, one can say, that cancer is caused by an individual mutant cell that flourishes at the cost of its surrounding cells. [3]

The mutations taking place often cannot be observed via microscopy, since, they only effect individual genes or regions within that gene. However, many cancer cells

do not only have altered genes but even altered chromosomes. These chromosomes can easily be observed as they can have a different structure, be completely missing, be present in extra copies or fuse an arm with a part of another chromosome. The aneuploidy present in many cancer cells was first discovered in 1892 and majorly contributed to the rationale that cancer is a genetical deviation, hence, a mutation. [7]

Regardless whether the mutations can be observed on a chromosomal or genetic level, they ultimately lead to the two heritable characteristics of cancer – signaling-independent reproduction and invasion at distant locations. A benign tumor is one that lacks the ability to invade, hence, it is proliferating at an abnormal speed but does not colonize other locations than its origin. In contrast, a malignant tumor will have both properties with invasiveness being essential for breaking out of the tissue, entering the blood or lymphatic system and forming metastasis, new tumors in second generation at different locations. Metastasis are the major occurrence causing death in cancer patients. [3] However, also benign tumors may cause problems, as they can produce and release tremendously high amounts of hormones disturbing the physiological balance. An example are thyroid adenomas, which release growth hormones. [7]

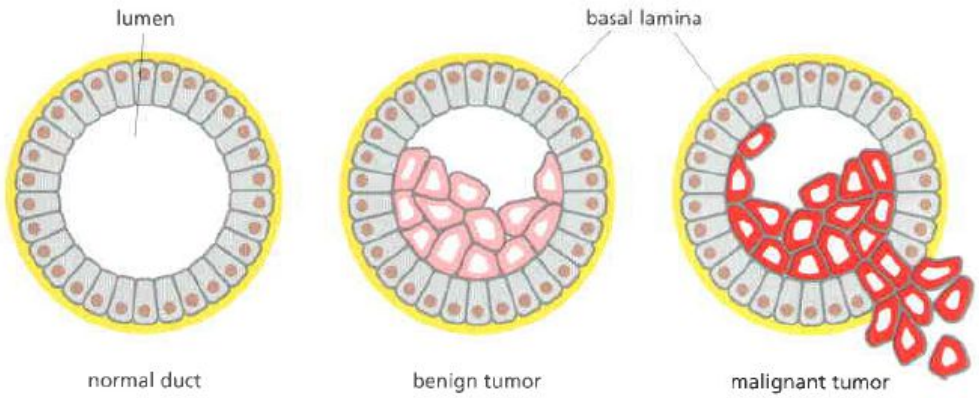

Figure 1: Benign vs. malignant tumor: On the far left the schematic depiction of a healthy duct is shown. In the center one can see cells accumulating on the inside forming a tumor but not growing into other tissue. In contrast, the picture on the far right shows invasion by the tumor cells.

As tumors can originate from nearly any tissue and often have according mutations, they require classification [3] Carcinomas are the most common cancers in humans (over 80 % of cancer-related deaths in the western hemisphere) and arise from epithelial cells. Epithelia are cell sheets that line cavities, canals or cover the body. A special type, the endothelial cells, lines the interior of capillaries and bigger vessels. There are different functions epithelial cells can have. On the one hand, they just line and protect the underlying tissue, on the other, they can secrete substances. If a tumor originates from the protective epithelial lining, it is called a squamous cell carcinoma. If it arises from the secreting cells, it is named adenocarcinoma. [7] Another type of cancer are sarcomas, which arise from connective tissue or muscle cells. [3] The third category originates from different cell types of the hematopoietic (blood-forming) tissues. These cells also include precursors of white blood cells, hence, immune cells like plasma cells, T- and B- lymphocytes, but also erythrocytes. Leukemia describes the cancer of freely circulating, non-pigmented blood cells. Lymphomas are developed from B- and T- lymphocytes and mostly form solid tumors at lymph nodes. The last major group are neuroectodermal tumors, which originate from various cells of the central and peripheral nervous system. The table below gives a brief overview of the different types mentioned. [7]

Table 1: The major tumor categories and the tissue of the founding cell

Tumor type	Original tissue
Carcinoma • Squamous cell carcinoma • Adenocarcinoma	Epithelia • Protective epithelial lining • Secreting epithelial cells
Sarcoma	Connective tissue or muscle cells
Leukemia and Lymphoma	Hematopoietic tissue
Neuroectodermal Tumor	Nervous System

1.1.1 Cancer progression

Mutations can happen in somatic cells as well as germ cells. This means that a transmission of the mutation effecting egg or sperm cells or their precursors to the offspring can occur. But a mutation in the somatic cells cannot be inherited. Inheriting a mutated cell often can be a predisposition to cancer.[7] Nearly all cancers develop from a primary tumor, which is formed by a single mutated cell. The daughter cells inherit the mutation and additionally will mutate rendering them more proliferate, faster growing and more resilient.[3] The resulting tumor is called monoclonal, as it developed from one single ancestor cell. Also possible, however less likely, is a tumor that originates from several malignant precursor cells. Therefore, this tumor is called polyclonal.[7]

A typical change in cancer progression happens in the cellular metabolism: the Warburg effect. Normal cells use glycolysis and then the Krebs cycle in the mitochondria to gain energy in an aerobic environment. If the conditions are anaerobic or hypoxic the cells will use glycolysis only, producing pyruvate that is metabolized to lactate and transported out of the cell. In contrast to them, cancer cells always use glycolysis solely, regardless whether oxygen is present. Nevertheless, cancer cells need a lot of energy to grow and reproduce at high speed, hence, they have to take up far more glucose than a normal cell would. Glycolysis yields only two ATPs whereas the complete oxidation by glycolysis and the Krebs cycle yields 36 ATPs. Therefore, the need for glucose is respectively enormous in tumours.[7]

But a single mutation is not enough to transform a normal cell into a malignant one. In a human approx. 10^{16} cell divisions will occur during the average lifespan. Also, if there would not be any influences from the outside present, mutations would occur 10^{-6} times per gene per division. Considering the many cell divisions happening, cancer would evolve far more often, if it was caused by a single mutation. However, as cancer needs several mutations to develop and therefore, various rounds of divisions and natural selections, it will take time to develop a malignant cell. This can

also be seen in the figure below - over time the probability to develop cancer rises as mutations accumulate, meaning it is more likely to develop cancer at a higher age.[3]

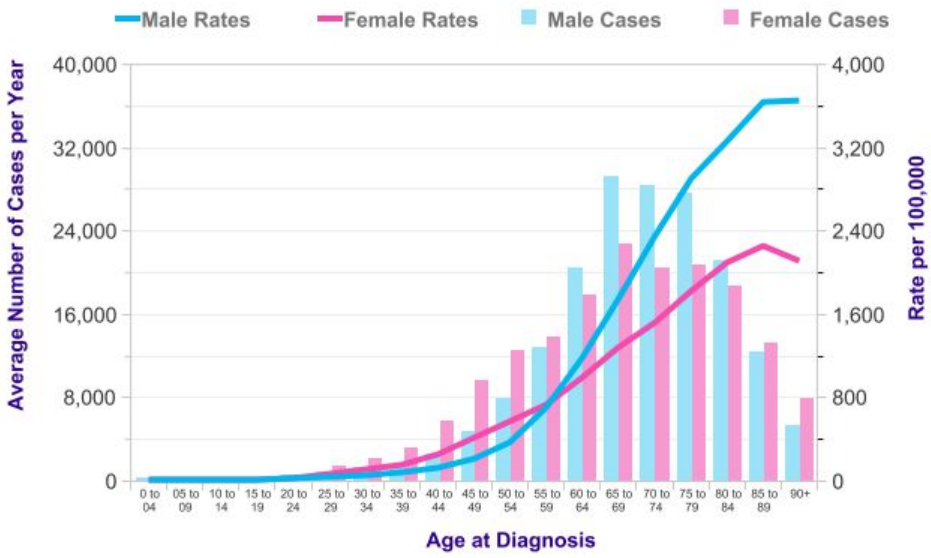

Figure 2: The number of cancer cases per year in the time span of 2012 - 2014 that have happened in the UK compared to the patients' age at the time of diagnosis. Since this figure shows absolut numbers, the drop of the bars at high ages is due to the smaller population at that age.

This gradual accumulation of mutations that cause cancer in the end is called tumor progression.[3] This event is also subjected to Darwinian selection, as the fastest growing cells will thrive and suppress also the less mutated ancestor cell. Furthermore, the DNA of malignant cells is unstable, favoring more and more mutations.[7] A mutated cell that can divide at an abnormal rate will lead to a condition called hyperplasia in which cells simply accumulate. The still normal looking cells will over time dedifferentiate and lose their normal appearance as well as their characteristic functions, causing dysplasia. The development of a single malignant cell into an invasive tumor is depicted in the figure below. Further excessive division, extremely abnormal structure and dedifferentiation at a high degree describe the in-situ cancer, which is not yet capable of leaving the site of origin. Invasive cancer will find its

way into the blood or lymphatic system and metastasize at other locations within the body.[8]

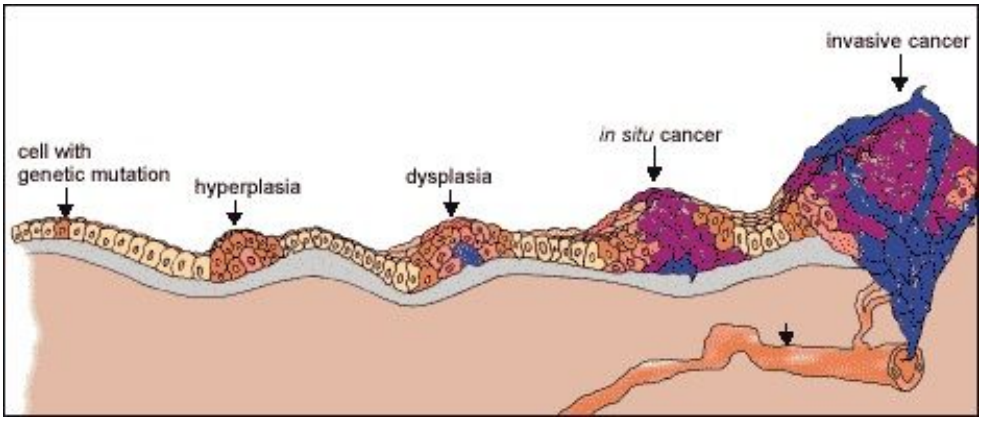

Figure 3: The transition of a single mutated cell into an invasive cancer can take decades and is subdivided in several stages during which the cells become more and more abnormal.[7]

The mutations that make cancer so resistant to treatments but also the normal cell-cycle arrest or apoptotic signals are summarized and grouped into the hallmarks of cancer, which are the following:[9]

- Sustaining signals for proliferation
- Evading growth suppressors
- Avoiding detection and destruction by the immune system
- Replicative immortality
    - The enzyme telomerase which prevents the shortening of chromosomes during repeated cycles of cell division is upregulated, protecting the telomers and supporting survival.
- Tumor promoting inflammations
- Invasion and Metastasis
- Angiogenesis
- Genome instability favoring mutations
- Avoiding cell death
- Altered metabolism and energetics[9]

The hallmarks are very much dependent on signaling pathways. Therefore, proteins that participate in sending, receiving or forwarding these signals deregulated in cancer are often targeted in cancer treatments.[9]

1.1.2 Carcinogens

Several chemicals as well as physical factors can lead to cancer. Entering the body chemicals e.g. benzene will impair tissues and cells. This can finally result in tumor formation. Many of these carcinogens where used or produced during wars or in industry in the 20th century. Also, it is perfectly known that cigarettes, further their contents are carcinogens. Furthermore, radiation can destroy DNA and a failed repair of the double strands can ultimately cause a cell to become malignant. Hence, X-rays and UV light are both mutagenic.[7]

Moreover, certain diets can favor tumorigenesis in certain organs. For example, a diet high in nitrates and salt but low in vegetables can lead to cancer in the stomach and the esophagus. A diet high in fat, low in fiber promotes cancers in the bowel, pancreas, prostate or breasts.[7]

However, cancer can also be the result of a viral infection. Viruses can cause numerous diseases in humans, as they enter cells, multiply inside of their host cells and then kill them by lysis to release a new generation of viruses which infect the neighboring cells. But viruses, as they e.g. integrate their viral DNA into the host's genome, can cause them to proliferate in an uncontrolled manner. These tumor viruses can alter the cells behavior to be malignant. Some of these viruses are pretty famous including the Hepatitis B virus, the Human papilloma virus, the Human herpesvirus and the Human adenovirus.[7]

1.1.3 Treatments

Cancer being one of the second leading cause of death in Austria, often is very challenging to treat and frequently not curable.[2] However, there is a variety of treatments available and to decide on one or more likely on a combination, one should first identify the subclass of the tumor detected and then think of what class the tumor can be assigned to. (I) Tumors that show low invasiveness, they will not metastasize not even during long periods of time. (II) Tumors which are highly prone to metastasis, will be very aggressive and probably have already invaded and metastasized other locations when detected. (III) Tumors which show an intermediate invasiveness and metastasizing potential, they can often be cytotoxically treated before they become lethal.[7] Usually it is recommended to only treat cancers of the third type, because treatments of tumors of class I often lead to more endeavor than benefit. Tumors of class III are incurable at present and treatments therefore, will not succeed and lead to even more anguish.[7] The main treatments are surgery, radiotherapy and chemotherapy. Although, these are very traditional approaches, they still show the best results. Surgery alone can already be highly beneficial, as the removal of primary rumors reduces the risk of recurrence. However, it is often combined with adjuvant chemotherapy or radiotherapy. In radiotherapy, the cancer cells are exposed to electromagnetic radiation, which leads to burn damage and in therapy ideally to necrosis. If tumor tissue cannot be completely removed by surgery or has spread, radioactive isotopes as drugs or coupled to monoclonal antibodies (so called radionuclides) are administered. Chemotherapeutics exert different means of killing cancer cells. Acylating agents, developed because of weapon mustard gas and its bone marrow-downsizing characteristics, can reduce lymphomas. Another example is paclitaxel or Taxol which blocks the breakdown of microtubules. Since, this is required in mitosis, they very much effect highly proliferating cells. But for many chemotherapeutic agents it is still not known, why they work primarily on cancer cells or their exact functions. Furthermore, there is one big issue, that requires the discovery and development of novel anti-cancer drugs: Cancer cells become resistant to chemotherapeutics over time.

1.2 Stat3

1.2.1 Stat proteins and their activation

Signal transducers and activators of transcription (Stat proteins) are latent gene regulatory proteins. They are present in the cytosol in an inactive state and only translocate into the nucleus to regulate gene expression upon activation.[3] The Stat protein family of mammals has seven members: Stat1, 2, 3, 4, 5a, 5b, and 6. Their structures share a similar basis consisting of six conserved domains. These domains are a N-terminal domain and a subsequent coiled-coil domain, a DNA-binding region, a linker domain, a SH2 domain and a transactivation domain. Phosphorylation hence activation due to cytokine signaling occurs on the tyrosine 705 residue near the C-terminus of the protein.[10] But also the NH_2 terminus contributes to transcriptional activation.[11]

The Stat proteins are activated by the Jak-Stat pathway, which is depicted bellow.[12]

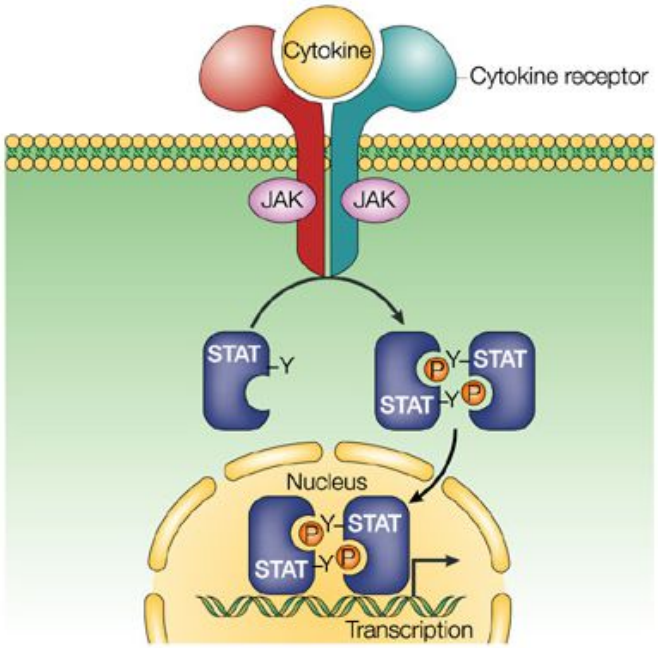

Figure 4: The Jak-Stat pathway is activated by cytokine signaling. The cross-linking of receptors brings the Jaks in close proximity. They trans-phosphorylate each other, then the

receptor, that can then attract the Stats. These are phosphorylated by the Jaks, dissociate, dimerize and translocate into the nucleus, where they bind to DNA and other gene regulators to activate transcription.[3]

Cytokine receptors are involved in numerous signaling pathways of local mediators, as well as growth hormones and prolactin, which is essential for mammary development.[3,12] Stably associated to these receptors are certain cytoplasmic tyrosine kinases shortly named Jaks for Janus Kinases. Upon receptor activation and cross-linking by cytokine signals the Jaks are able to cross-phosphorylate each other on tyrosines and then, in their active state, phosphorylate the receptors on tyrosines. This causes the formation of phosphotyrosine docking sites for the Stats or several other adaptor proteins. Again, phosphorylation takes place by the Jaks, this time on the receptor-associated Stat proteins, enabling them to dimerize using their SH2 domains and translocate into the nucleus. Inside, the dimers bind to DNA and other gene regulators to stimulate gene transcription. In contrast to the many other signaling pathways relaying signals from cell-surface receptors to the nucleus, the Jak-Stat pathway is a rather direct pathway. Besides the activation triggered by cytokine receptors and their associated Jaks, Stat proteins can also bind to RTKs via their SH2 domains, which can activate Stats directly. [3] Furthermore, for some Stats there is the possibility to form preformed dimers in the absence of signal. This dimerization usually depends on the N-terminal domain of the unphosphorylated protein, not the SH2 or phosphotyrosine domains.[10]

Stat3 was first discovered in the context of IL-6 cytokine signaling in hepatocytes. The signal is recognized by the gp130 receptor, which activates Stat3 via the associated Jak proteins. However, Stat3 can also be activated by many growth factors, oncogenes and IFNs.[10,12] In general, there are many activators of Stat3 including oncogenic proteins such as Src and Ras, but also various carcinogens such as tobacco smoke. Hence, activation can occur via receptor and nonreceptor protein kinases.[13]

Besides the already mentioned phosphorylation on tyrosine 705, Stat3 can also be activated by serine phosphorylation at position S727. Activation via serine phosphorylation can by carried out by several serine kinases, including Erk1 and 2, p38 JNK, mitogen-activated kinases and CDK5. Serine phosphorylation can have both, a positive and a negative effect on transcriptional activities.[12,13] PKC-ε is known to maximize the transcriptional activity of Stat3 by phosphorylation on positon S727.[13]

Furthermore, also histone acetyltransferase p300 exerts an effect on Stat3 by acetylating it on a lysine residue at position 685. This modification is reversible by type I histone deacetylase. The acetylation is essential for dimer formation, hence, also for the overall transcriptional activation.[13]

The most prominent activators of Stat3 are the previously mentioned IL-6 cytokines. Depending on the cell type the signal causes different responses. It can cause B lymphocytes to proliferate, activate differentiation or growth arrest in monocytes and the maintenance of pluripotency of embryonic stem cells.[12] Moreover, it is essential in embryonic development, as its deletion causes embryonic lethality, and in immunity and inflammation. Therefore, dysregulation of Stat3 or its activators can also be observed in many cancers and chronic inflammations. Stat3 can activate its own feedback loop e.g. by targeting Socs3, an inhibitor.[10] Up to now, eight different Socs proteins, sharing a similar structure, have been identified. Socs proteins exert their inhibitory function by binding as pseudosubstrates to the Jak activation loop, blocking the binding sites for Stat3. Socs3 for example interferes in the gp130 signal relay. Other inhibitors of Stat3 are protein inhibitors of activated Stat (PIAS), protein phosphatases such as CD45 or PTEN, and ubiquitin-dependent proteosomal degradation. PIAS are nuclear factors that can block transcription by interaction with pStat3.[13]

1.2.2 The functions of Stats

Gene experiments regarding the different Stat family members, have shown that they are highly specific in signaling pathways. Furthermore, deletion of one or more Stat genes causes the expression of a distinct phenotype, meaning that each Stat protein has a specific pathway.[12] Some responses triggered by Stats are for example the activation of macrophages, increased cell resistance to viral infections, stimulation of erythrocytes, milk proteins or production of growth factors.[3] However, Stat3 is an exception: Its deletion causes embryonic lethality and activation can be triggered by numerous cytokines. Hence, Stat3 is involved more often in a broad range of physiological processes.[12]

Studies on Stat3 ablation in late embryogenesis and beyond have shown that the absence of Stat3 leads to altered phenotypes in the tested mice. The lack of functional Stat3 in the skin, the epidermis leads to tremendously impaired wound healing as well as a compromised second hair cycle. Moreover, the absence of Stat3 can speed up the normal age-dependent hypoplasia in thymic epithelium or, as for younger mice, causes hypersensitivity to apoptosis-inducing agents. In T-cells Stat3 stimulates cell survival, although many T-cell cytokines activate other Stats. Because of its importance downstream of IL-6 and gp130, the T-cell survival triggered by IL-6 is impaired under the absence of Stat3. If Stat3 is missing in the myeloid linage, an increased likelihood for a endotoxic shock and/or a chronic enterocolitits can be observed. Stat5 is activated by growth hormones and prolactin, hence, is responsible for the mammary development. However, Stat3 is also involved in this process, as its activation happens simultaneously as the mammary gland involution. In its absence, the involution will be delayed. In the nervous system Stat3 is activated by signals in form of CNTF and leptin. Loss of the protein results in perinatal lethality, since the CNTF and LIF signal is required for the development of sensory neurons.[12] On the other hand inhibition of Stat3 can reduce brain damage in the disease hypoxic-ischemic encephalopathy. 40 % of the diseased infants die in an early stage and 30 % of the survivors will suffer from lifelong defects including epilepsy or cognitive disabilities. In the diseased tissue Stat3 is highly upregulated by

hypoxia ischemia and in is active form is often found with cell death-associated DNA degradation.[14] The absence of Stat3 in the liver leads to an enormous interference in the acute-phase response during inflammation, as it is mainly mediated by IL-6.[12]

1.2.3 Stat3 in cancer

Stat3 is classified as an oncogene and in numerous human cancers it has been observed to be continuously active. For example, this is the case in 30 – 60 % of primary breast cancers as well as for many other primary cancers, tumor cell lines but also oncogene-transformed cells. If Stat3 can be inhibited in any of these tissues or cells, for example proliferation can be suppressed.[13] Stat3 has been observed to regulate not only proliferation but also invasion, migration, and angiogenesis.[15]

1.2.3.1 Cellular Transformation and Stat3

Cells can undergo transformation by a broad range oncogenes. This event often is associated with Stat3 activation.[16] In more detail, Src protein kinase induced transformation depends on this activation.[17] The same is true for T-cells altered by the human T-cell lymphotropic virus I.[18] Moreover, transformation triggered by an infection with Hepatitis C virus happens via Stat3.[19] There are many more examples, such as transformation by the polyoma virus or by the growth- and scatter-factor Met.[13] Stat3 is classified as an oncogene due to the fact that constitutive activation of Stat3 is responsible for transformation, growth and tumor formation.[20]

1.2.3.2 Stat3 as a suppressor of apoptosis

As mentioned above, transformation to a high degree is linked to active Stat3. In most cells, the protein works as a suppressor of apoptosis, as it drives expression of survival signals including bcl-xl, bcl-2, survivin, Mcl-1 and cIAP2.[13,15] Moreover, it downregulates p53, the most abundant anti-proliferating and pro-apoptotic factor.[21]

On the other hand, Stat3 is able to act as a pro-apoptotic factor in mammary glands during postlactation.[22]

1.2.3.3 Stat3 as a mediator of cell proliferation

In addition to its effects on transformation and cell survival, Stat3 upregulates the expression of cyclin D1 and cMyc.[23,24] Since both are critical for cell cycle progression, this progression is accelerated by Stat3, which also induces the expression of other growth promoters.[13] But Stat3 does not only upregulate growth-promoting genes, it even downregulates proapoptotic factors such as Fas.[25] Moreover, there are findings suggesting an expression of the cell cycle inhibitor p21. Meaning Stat3 is also capable of inducing cell cycle arrest and blocking proliferation. In transformed cells however, p21 is usually blocked by the PI_3K-Akt pathway.[13]

1.2.3.4 Cellular invasion triggered by Stat3

Invasiveness is an essential milestone during tumorigenesis and various reports connote that Stat3 has a high impact on it.[13,15] It has shown that Stat3 regulates the expression of matrix metalloproteinases including MMP-1, MMP-2, MMP-7, and MMP-9. The matrix metalloproteinases in turn facilitate invasion and metastasis.[13,15,26,27] The MMP-2 gene is directly activated by the phosphorylated Stat3, which can bind to its promotor region. Similar mechanisms have been observed for other MMPs.[15] Further Stat3 positively regulates the MUC1 gene, that is also responsible for invasion. Since Stat3 often has contradicting functions, it is not too surprising that it can stimulate the expression of the MMP inhibitor TIMP.[13]

1.2.3.5 Stat3 and its role in migration and metastasis

Research has shown that Stat3 is involved in migration in healthy as well as in abnormal tissues. Stat3 has an important role in wound healing as well and is therefore, required in the skin and if absent leads to impaired healing and hair cycles.[28] Also Stat3 interacts with stathmin, which acts in microtubule depolymerization, and influences Rac1 activity enhancing directed migration.[15] Moreover, Stat3 upregulates the expression of the Gα-interacting vesicle-associated protein (Giv), which is required in wound healing, macrophage chemotaxis, tumor angiogenesis, vascular repair and cancer invasion as well as metastasis.[29] Metastasis requires circulation through the blood or lymph system, but not many cells survive their travel. The cells encounter mechanical stress, hemodynamic turbulence, loss of adhesion-induced signals, cytotoxic excretions etc. Stat3 offers a competent protection mechanism from the immune system during circulation. It leads to the release of inflammatory factors, that work as immunosuppressors, including TNF-α and suppresses the activity of NK cells.[15]

1.2.3.6 Stat3 induces angiogenesis

The formation of new blood vessels is an essential characteristic of cancer. Angiogenesis is mostly triggered by the vascular endothelial growth factor. The VEGF gene is a direct target of Stat3 and is significantly upregulated in the presence of constitutively active Stat3. This leads to increased angiogenesis.[30] Another important mediator of angiogenesis is the hypoxia-inducible factor1a (HIF1a), which is also upregulated by Stat3. Stat3 and HIF1a can interact with VEGF promoters simultaneously making it maximally active.[31]

1.2.3.7 Stat3 accounts for resistances in cancer treatments

The constitutive activity of Stat3 in many tumors renders them resistant to chemotherapeutics. This chemoresistance is facilitated by the Stat3 induced activation of

anti-apoptotic genes. Therefore, to overcome this resistance, Stat3 has to be down-regulated first.[13] There is also the possibility for cancer cells to become radioresistant. Experiments with Stat3-deleted B cells have shown that they are vulnerable to a high degree when treated with γ-radiation. IL-6 and also IL-10 combine in vivo with B-cell receptor ligands to induce B-1 cell radioresistance.[32,33]

1.3 Src protein-tyrosine kinase

The Src protein-tyrosine kinases belong to the overall class of cytoplasmic tyrosine kinases and constitute the biggest family of these proteins in mammals. The cytoplasmic tyrosine kinases are of high importance: Many cell surface receptors require for tyrosine phosphorylation for active signaling, but very often they do not have an own tyrosine kinase domain to exert this function. Therefore, they depend on other proteins that can interact with them and phosphorylate either the receptors' targets or the receptors themselves. These so-called tyrosine-kinase-associated receptors have many members e.g. antigen-, hormone or IL-receptors.[3] In humans, the Src family has 11 members named Blk, Brk, Fgr, Frk, Fyn, Hck, Lck, Lyn, Src, Srm, and Yes. Due to their nature, all of these are non-receptor protein-tyrosine kinases. The first studies on Src were carried out by observing the Rous sarcoma virus, a virus that contains viral Src and causes tumors in chickens. The physiological gene of Src, as it is expressed in animal cells, is the first of the proto-oncogenes.[34] Since Src relays a huge variety of signals, it is very ubiquitously expressed in vertebrates. Still, the highest levels of expression could be found in the brain, osteoclasts, and platelets. This high expression in neurons and platelets, 5 – 200x higher than regularly, suggests that Src has also other functions than those for cell division. In fibroblasts, Src is often bound to membranes including the perinuclear one, to the endosomes, secretory vessels, and the inner side of the plasma membrane. There it interacts with many proteins required in cell signaling such as growth factors, or G-protein-coupled receptors and is, therefore, an important signal-relay protein.[35]

1.3.1 The structure and activation of Src

In humans, Src is encoded by 536 amino acids. After the N-terminus including myristoylation and palmitoylations sites which are responsible for the cellular location as well as unique Src-family functions (SH4 domain), an SH3 domain that directly interacts with proline-rich structures, an SH2 domain which can interact with phosphotyrosines, an SH2-kinase linker domain that interacts with the SH3 region, a protein-tyrosine kinase domain called SH1 domain that carries out the phosphorylation as well as the association with substrates and contains an activation loop (A-loop) which includes the Tyr-416 autophosphorylation site, and a C-terminal regulatory domain that also contains a phosphorylation site at Tyr-527[34,36] (negatively-regulating the Src protein, Tyr-530 in humans[37]) and interacts with the SH2 domain.[34,36]

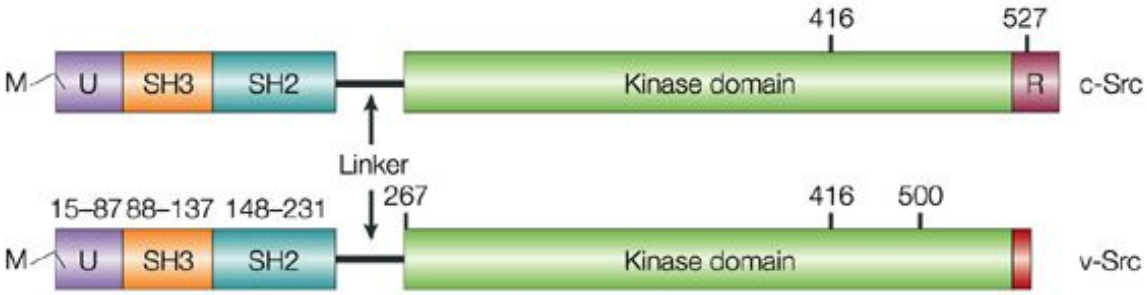

Figure 5 The Src protein is constituted out of a myristoyl- and palmitoyl-groups containing N-terminus, the subsequent unique domain, an SH3 domain, followed by an SH2 domain, an SH2-kinase linker domain, the kinase domain and a C-terminal regulatory domain. The physiological Src encoded by animal cells (c-Src) with its C-terminal regulatory domain is longer than the viral Src (v-Src) which is lacking seven residues within that region.[35]

Src consists of the bilobed protein kinase architecture. The small amino-terminal lobe from residue 267 – 337 is responsible for anchoring and the orientation of ATP and mainly consists of antiparallel β-sheets. A G-rich loop is important for nucleotide-phosphate binding. The larger C-terminal lobe mediates binding of the protein substrate and is involved in ATP-binding to a small degree. The large lobe primarily consists of α-helices. The catalytic cleft is located between the lobes, their movements open and close it. The open cleft allows ATP to bind and ADP-release which

results in an inactive state. The closed conformation is required for the catalytically active state. If a molecule can prevent this conformational change, it works as an inhibitor of Src. The tyrosine kinase activity depends on residues of both lobes, it can be supported or restrained by different orientations of the lobes.[34] Moreover, Mg^{2+} and Mn^{2+} support the catalytical activity of Src, as the coordinate the ATP phosphate groups β and γ. However, Mg^{2+} in cells is present to a higher degree and so physiologically more important.[35]

As mentioned, the Src activity is dependent on those structural changes of the lobes. These conformational changes are induced by phosphorylation and dephosphorylation of tyrosines and are, therefore, the major control mechanisms of its kinase activity. Not only phosphorylation on different sites is crucial, but also the SH2 and the SH3 domain are involved. In the inactive state of Src, the C-terminal Tyr-527 is phosphorylated and binds to the SH2 domain. Coincidently, the SH3 domain and the proline-rich motifs of the linker domain interact. The activation loop is rendered to a more compact structure that occludes the catalytic site. Hence, ATP- and substrate binding as well as phosphorylation on the autophosphorylating Tyr-416 is prohibited. The protein remains inactive. Naturally, activation of Src requires autophosphorylation on Tyr-416 which releases the bound SH2 or SH3 domain from other domains and phosphorylated Tyr-416 from the SH2 domain. Then substrates can interact with the Src protein.[36]

There are many possible ways by which Src can be activated or inactivated. For example Src-family kinases are controlled by receptor-protein kinases (e.g. RTKs), integrin receptors, G-protein coupled receptors, antigen- and Fc-coupled receptors, cytokine receptors, and steroid hormone receptors.[34] Also protein tyrosine phosphatases can activate Src, as they can dephosphorylate the Tyr-527 (Tyr 530 in humans) at the C-terminus, which is crucial for the inactive state. These phosphatases include PTPα, PTPγ, SHP1, SHP2, and PTP1B. Naturally, phosphorylation of the Tyr-527 inactivates Src, which is to a high degree carried out by Csk (c-Src kinase) and Csk-homologous kinase (Chk).[37]

1.3.2 The functions of Src

Protein kinases are key-layers in basically every aspect of cell functions. This includes metabolic control, transcription, cell division, cell movement, and programmed cell death. Furthermore, they have important roles during immune responses and in the nervous system. Due to their critical functions dysregulation is associated with many diseases such as cancer and inflammatory disorders.[38]

The Src kinase can act as an up- or downstream modulator of various receptors and also frequently works as a nonreceptor tyrosine kinase. These kinases are of high importance for functioning receptor tyrosine kinase (RTK) signaling pathways. Hence, Src is a signal transducer that conveys signals from cell surface receptors to its target substrates by phosphorylation.[37] Src significantly affects cell differentiation, proliferation and survival.[34] In mammalian cells, Src is plays a role in cell morphology, adhesion, migration, invasion, proliferation, differentiation and survival.[37] As for other kinases, Src has effects on a broad field of immunologic processes which includes but is not limited to immune cell development, chemotaxis, and phagocytosis. Src accelerates cell cycle progression of myeloid cells by causing increasing release of growth factors.[36] Generally, Src is activated during the G_2/M transition of the cell cycle. However, the fact that Src is also found in platelets and neurons also has contributed to the consensus that Src is involved in more cellular events than proliferation. Src knock-out mice or only viable for a few weeks. Src deficiency can cause osteopetrosis, increased bone density, in mice.[34]

1.4 Viruses

Viruses have evolved over a long time and basically are infectious entities which diameters range between 16 nm and 300 nm. Due to their small size, they can only be cleared out of a substance by ultrafiltration. Numerous viruses have developed

and have specific target organisms or cells. Virions which are the infectious virus particles consist out of proteins and can be enveloped by a lipid membrane in several species. A virion contains DNA or RNA exclusively. The nucleic acid encodes the genetic information of the structural components as well as regulatory proteins and enzymes, which can e.g. support the expression of viral proteins or the assembly of viral particles. Viruses can be considered intracellular parasites because of their replication principle. They do not increase their population by division, instead they infect living cells and use the machinery of this host cell to replicate. Inside the host, they exert their genomic activity to force the infected cell to produce the components the virus consists of. Hence, viruses neither can express their own proteins, nor can they generate energy via metabolism, but they can alter the course of cellular procedures to yield the best viral expression within the host.[39]

1.4.1 Viral lifecycle

The viral lifecycle starts with the infection of a host cell. To do so virus particles have to recognize specific receptors on the cell membrane of the host and have to interact and bind to these molecules. The process is called attachment and for enveloped viruses is mediated by proteins located in the envelope. The attachment can be very cell specific or can work with several cell types. If a non-enveloped virus attaches to a potential host cell, this interaction depends on surface structures of the capsid proteins and is more or less specific. The next step is called penetration: After the virus particle has successfully attached and interacts with the individual receptor, it will be translocated into the inside of the cell. This process often occurs via endocytosis. There are certain mechanisms viruses have developed to escape the vesicle after entering the cells to avoid degradation. During uncoating, which is the next step, the nucleic acid of the virus is released. With few exceptions, the viral DNA is transported into the nucleus, whereas the viral RNA remains as RNA-protein complex in the cytoplasm. After proper integration of the nucleic acid, viral reproduction starts which is due to its complexity different for all virus types. During this step of the replicative cycle the proteins encoded by the viral genome are expressed and sometimes certain proteins of a specific host cells are required. In the subsequent

morphogenesis, the viral particles are assembled from the previously expressed proteins. This phase is also called self-assembly, since usually only viral proteins and enzymes are involved. The last stage of the viral lifecycle is the release of the next generation of virus particles. This can occur by budding from the host cell, which produces enveloped virus particles. For non-enveloped viruses release is possible by cell lyses, which kills the host cell. Therefore, it is called lytic cycle. After the infectious particles are released they can infect neighboring cells and the viral lifecycle starts again.[39]

1.4.2 Bac-to-Bac® Baculovirus Expression System

The Bac-to-Bac® Baculovirus Expression System is an expression system first developed by researchers of Monsanto and is now merchandized by Invitrogen™. It is a system which allows its users to generate recombinant baculovirus for recombinant protein expression. In general, it is aimed to provide a rapid and efficient method to produce these recombinant viruses by site-specific transposition of an expression cassette into the bacmid. The bacmid works as a baculovirus shuttle vector and propagates in E.coli in this system.[40]

The Bac-to-Bac® Baculovirus Expression System has several crucial components listed below.

- A pFastBac™ donor plasmid is required to generate the expression construct which contains the gene of interest. Moreover, expression of the gene of interest is controlled via a baculovirus-specific promoter present in the donor plasmid.
- DH10Bac™ is an E.coli strain, further, in this case the host strain. It contains a baculovirus shuttle vector, which is the previously mentioned bacmid, and a helper plasmid. These components allow the generation of the recombinant bacmid which is dependent on the successful transposition of the pFastBac™

construct. The bacmid is present as a large plasmid that contains a kanamycin resistance gene and can be used for white-blue selection as it contains a lacZ gene.

- Moreover, an expression control plasmid is part of the system. This plasmid contains the Gus and/or CAT gene. When infecting insect cells with a recombinant baculovirus containing these genes, expression of β-glucuronidase and/or chloramphenicol acetyltransferase can be observed.[40]

The generation of recombinant baculovirus and the expression of the gene of interest follow the principle depicted below. After restriction enzyme digestion of the pFastBac™ donor plasmid the gene of interest is inserted and ligated. The expression construct is then transformed into the DH10Bac™ E.coli host. The subsequent transposition is facilitated by the helper plasmid present in the E.coli cell. This helper plasmid encodes a transposase and mediates the transposition of a mini-Tn7 element from the donor plasmid to the mini-attTn7 attachment site on the bacmid. After verified E.coli colonies have been identified they are incubated overnight and the recombinant bacmid DNA is isolated. Once the bacmid DNA is obtained, it can be used to transfect insect cells, which will then allow the production of the p1 viral stock.[40]

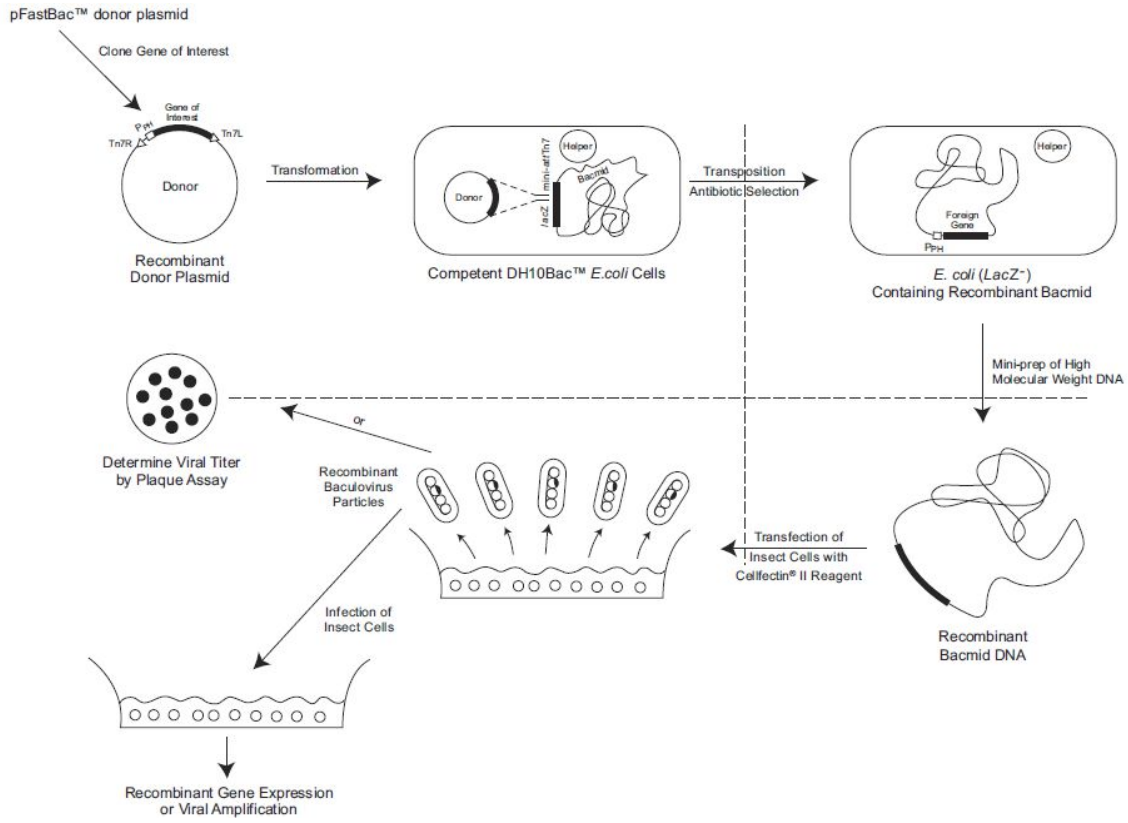

Figure 6 The principle outline for recombinant baculovirus expression and subsequent protein expression. The expression construct composed out of the pFastBac™ donor plasmid and the gene of interest is transformed into the E.coli host strain where transposition of the gene of interest into the bacmid is mediated by the helper plasmid.

This Expression system is commercially available and offers advantages over the traditional homologous-recombination method.

- The Bac-to-Bac® Baculovirus Expression System takes less than two weeks to successfully produce the recombinant baculovirus, whereas it takes up to six weeks by homologous recombination.
- Furthermore, there are far less rounds of plaque purification required. As the recombinant bacmid is harvested from selected colonies, there is usually no contamination by parental, non-recombinant virus.
- It can be performed rapidly as well as simultaneously for several recombinant baculoviruses and is suitable for the expression of different protein variants in structure and function studies.[40]

1.5 Insect cell lines Sf9 and Sf21

Over a long period of time insect cells have been used in research. The most important species is the fruitfly Drosophila melanogaster, which was used to proof the existence of chromosomes for the first time. More than 85 years later insect cells are still widely used among the different research disciplines.[3]

In this section, the very similar cell lines Sf21 and Sf9 will be discussed further. The Sf21 cell line was derived from Spodoptera frugiperda, the so-called fall armyworm. Further, it is a clonal isolate of the cell line IPLB-Sf-21-AE. The same is true for the Sf9 cells. Both of them can be adopted to grow in serum free, protein free medium. The cells can be grown in suspension or adherent cultures. However, the term adherent is not really suitable for insect cells in general, as they are not anchorage dependent but only attach firmly to surfaces. [41,42]

Figure 7 a) The Spodoptera frugiperda is also called fall armyworm and becomes a moth in its adult stage. b) In its early stage the fall armyworm is, as its name suggests, a caterpillar.

2 Materials and methods

2.1 Cell culture of Sf9 and Sf21

2.1.1 Thaw

The insect cells used were previously stored in liquid nitrogen. To obtain a viable culture from the frozen cells, the cryopreservation valve was shortly put into a water bath set to 37° C. Since insect cells should not be exposed to higher temperatures than 27°C, the vial had to be reobtained shortly before the hole frozen content has thawed. Naturally, working with insect cell culture was carried out under aseptic conditions. Usually three vials with a cell density of $1 – 2 \times 10^7$ cells/ml were thawed simultaneously and then put into a sterile 125 ml Erlenmeyer flask with approx. 30 ml of Sf-900™ II SFM (1X) Serum Free Medium Complete from gibco® by life technologies™ prewarmed to room temperature. The flasks were loosely covered with ethanol sprayed aluminum foil to allow gas exchange.

2.1.2 Culture conditions

As mentioned above, the insect cells were cultured as suspension culture in sterile 125 ml Erlenmeyer flasks or in 250 ml flasks loosely covered with ethanol sprayed aluminum foil. Both worked, however, a 125 ml flasks guarantees a better gas exchange due to the lower volumes of culture. The volumes of cell culture usually ranged from 50 – 100 ml per flask. The seeding densities ranged from $3 \times 10^6 – 6 \times 10^6$ cells/ml and were adjusted to the cells morphology and overall doubling time. The passage schedule used was a 3 – 4 days schedule. Throughout the expression system procedures Sf-900™ II SFM (1X) Serum Free Medium Complete from gibco® by life technologies™ prewarmed to room temperature was used as the culture medium.

However, to have a better and easier insight at the cells' morphology and conditions, insect cells were also culture in 25 ml cell culture flasks. Passaging of these cultures, requires vigorous shaking of the flask or pipetting medium over the cell monolayer. Simply shaking of the flask and tapping it on a surface, e.g. provides the easiest and fastest method to loosen the cells. Although insect cells due not adhere to surfaces as animal cells do, they still stick quite firmly to the bottom of the flask. The seeding density for adherent cell culture depends on the cells' morphology. However, they should not grow out of the monolayer and were usually seeded at $6 \times 10^4 – 7 \times 10^4$ cells/cm².

Regardless the type of culture, the cells were incubated at room temperature (approx. 23° C), which turned out to be the most favorable temperature. The cells kept in suspension culture naturally required for shaking, therefore they were placed on the shaker on a workbench at 125 rpm. Sf9 and Sf21 cells can be cultured the same way, however, after several passaging cycles we preferable used Sf21 as they grew better, which could be due to freeze and thaw cycles or the overall condition of the previously frozen culture.

2.2 Infection of the cells with recombinant baculovirus

Starting with this point only Sf21 cells were used due to their better growth rates and overall condition. In order to infect the precultured insect cells, the cell viability was observed and had to be >95 %. Furthermore, the cells' morphology, doubling time etc. had to be acceptable, because healthy cells which are in the log phase are crucial for the success of the infection. Therefore, the precultured cells were observed under a light microscope and to determine the cell density in the culture, samples were loaded on a counting chamber as a 1:1 dilution with trypan blue.

Seeding of the cells was performed in 6-well plates and 10^5 cells were loaded per well in 2 ml Sf-900™ II SFM (1X) Serum Free Medium Complete. Usually, two plates

were used: one which was used for the infection with Src gene containing virus and one with Stat3 gene containing virus. Per plate at least one control well was included. The infection steps were the following:

1. Seed 10^5 cells per well on a 6-well plate and incubate them for 24 h.
2. Aspirate the medium and add the appropriate volume of Src or Stat3 virus.
3. Put the plates on a rocking device for 1 h for gentle rocking to ensure proper distribution of the virus.
4. Incubate the cells for
    a. 48 h to express and harvest the recombinant protein or
    b. for 72 h to amplify the viral stock.

The volumes used for infection are subjected to the overall efficiency of the recombinant baculovirus and were emended for each infection procedure. The purpose of the infection, either virus amplification or protein expression, does not affect the volumes needed for infection. However, the volume required can be calculated by the following formula.

$$\text{Inoculum required}[\text{ml}] = \frac{MOI[pfu/cell] \times number\ of\ cells}{\text{titer of virus}[\text{pfu/ml}]}$$

MOI = multiplicity of infection. The MOI is the number of virus particles per cell. The titer of the viral stock can be assumed to range from $1 \times 10^6 – 1 \times 10^7$. Though, the calculation gives advice on how much volume to use, it mostly requires amendments.

The exact volumes used for procedures carried out according to the protocol stated above are listed below in µl per well. The volumes had to be changed because they did not yield favorable results

Table 2 The different volumes used for infection of Sf21 with p1 Src virus, p1 Stat3 virus, p2 Src virus or p2 Stat3 virus. Both p2 viruses used were the ones obtained from the amplification performed shortly before.

Procedure	Set-up	Volume Stat3	Volume Src	Date
Amplification of p1 virus	6 wells per virus, incl. 1 as control	60 µl of p1	60 µl of p1	21.11.2016
Expression of Stat3 and Src	6 wells per virus, incl. 1 as control	20 µl of p2	40µl of p2	28.11.2016
Expression of Stat3 and Src	6 wells per virus, incl. 1 as control	100 µl of p1	100 µl of p1	19.12.2016

Since there was no Src or Stat3 protein detected by electrophoresis and subsequent western blot (see 2.3 Electrophoresis, 2.4 Western Blot, 3.3 Electrophoresis and Western Blot), the procedure was slightly changed to the following:

1. Seed 10^5 cells per well on a 6-well plate and incubate them for 24 h.
2. Aspirate the medium and wash the cells with fresh prewarmed medium.
3. Add 600µl of fresh prewarmed medium.
4. Add the appropriate volume of Src or Stat3 virus.
5. Put the plates on a rocking device for 1 h for gentle rocking to ensure proper distribution of the virus.
6. Incubate the cells for
    a. 48 h to express and harvest the recombinant protein or
    b. for 72 h – 96 h to amplify the viral stock and check them daily under a light microscope.

The procedure for cell lyses stayed the same. However, for expression of a p2 virus of Src or Stat3 a filter step using a sterile 0.22 µm syringe-driven filter from Argos™ (Cat. No. FPV1322S) was included. Therefore, the medium was transferred from the wells excluding control into a 10 ml syringe from BD™ (Cat. No. 305482) under aseptic conditions after the incubation time. The filter was attached and the liquid

was pressed through the filter into a sterile falcon tube and then stored at 4° C protected from light. The volumes used per well during applying this procedure were the following.

Table 3 The different volumes used for infection of Sf21 with p1 Src virus, p1 Stat3 virus, p2 Src virus or p2 Stat3 virus for the protocol after trouble-shooting. Both p2 viruses used were the ones obtained from the amplification performed shortly before.

Procedure	Set-up	Volume Stat3	Volume Src	Date
Amplification of p1 virus	6 wells per virus, incl. 1 as control	100 µl of p1	100 µl of p1	28.12.2016
Expression of Stat3 and Src	6 wells per virus, incl. 1 as control	100 µl of p2	100 µl of p2	03.01.2016
Expression of Stat3 and Src	6 wells per virus, incl. 1 as control	60 µl of p1	60 µl of p1	09.01.2016

Again trouble-shooting was necessary as electrophoresis and western blot did not show the desired results (see 2.3 Electrophoresis, 2.4 Western Blot, 3.3 Electrophoresis and Western Blot). The procedure for infection was changed to the following set-up:

1. Seed 2 × 10^5 cells per well on a 6-well plate in 1900 µl prewarmed fresh medium.
2. Add 100µl of Src or Stat3 virus.
3. Centrifuge the plates at 23° C for 1 h at 800 g.
4. Wipe the outside of the plates carefully with ethanol.
5. Incubate the cells for
    a. 48 h to express and harvest the recombinant protein or
    b. for 72 h – 96 h to amplify the viral stock and check them daily under a light microscope.

Table 4 The different volumes used for infection of Sf21 with p1 Src virus, p1 Stat3 virus, p2 Src virus or p2 Stat3 virus for the protocol after trouble-shooting and changing the protocol. Both p2 viruses used were the ones obtained from the amplification performed shortly before. Since a lot of cells infected on the 23.01.2017 were dead 24 h later a new expression was performed additionally on the 24.01.2017.

Procedure	Set-up	Volume Stat3	Volume Src	Date
Amplification of p1 virus	3 wells per virus incl. 1 control	100 µl of p1	100 µl of p1	18.01.2017
Expression of Src and Stat3	3 wells per virus incl. 1 control	100 µl of p1	100 µl of p1	23.01.2017
Expression of Src and Stat3	3 wells per virus incl. 1 control	200 µl of p2	200 µl of p2	23.01.2017
Expression of Src and Stat3	3 wells per virus incl. 1 control	100 µl of p1	100 µl of p1	24.01.2017
Expression of Src and Stat3	3 wells per virus incl. 1 control	100 µl of p2	100 µl of p2	24.01.2017

2.3 Cell lysis

The harvest the proteins cell lysis was performed according to the steps below carried out at 0° - 4° C on ice.

1. Aspirate the medium and wash the cells twice with ice-cold PBS.
2. Add 100 µl of SDS-PAGE buffer (prepared from colleagues of doctor Fei's department) to the first well of the 6-well plate.
3. Detach the cells in the first well containing SDS-PAGE buffer using a cell scraper.
4. Transfer the cell-buffer mix of the first well to the next and add 100 µl of SDS-PAGE buffer to it.
5. Again, detach the cells using a cell scraper and transfer the mix to the next well.
6. Repeat step 5 until all wells excl. control have been treated.

7. Add 100 µl SDS-PAGE buffer to the first well and detach any remaining cells with the cell scraper.
8. Transfer the mix into the next well and detach the cells with the cell scraper.
9. Repeat step 8 until all wells excl. control have been treated.
10. Incubate the mix on ice for 20 min.
11. Transfer the lysate into a centrifugation tube and vortex it.
12. Boil the sample for at least 3 min.

2.4 Electrophoresis

The SDS-PAGE electrophoresis was carried out by using the system from Bio-Rad™. To prepare the gel the following recipe was used:

Table 5 Recipe of the SDS-PAGE gel in ml for the preparation of 1 – 2 gels.

Component	Separating Gel	Stacking Gel
Autoclaved water	4.6 ml	2.1 ml
30 % acrylamide mix	2.7 ml	0.5 ml
1.5 M Tris	2.5 ml	-
1 M Tris	-	0.38 ml
10 % SDS	0.1 ml	0.03 ml
10 % APS	0.1 ml	0.03 ml
TEMED	0.006 ml	0.003 ml

The loading pattern and the loading volumes differed among the various gels performed. However, the volume of the marker used was always 6 µl and the volume of the control was 8 µl when used. After loading the gel was then put into the chamber provided by Bio-Rad™ which was filled with 1×Tris/Glycine/SDS Buffer (from Bio-Rad™, Cat. No. 161-0772) and ran at 120 V for 1 h 10 min.

2.5 Western Blot

For the western blot analysis, the blotting sandwich was assembled, put into the transfer chamber containing 1×Tris/Glycine/ Buffer (from Bio-Rad™, Cat. No. 161-0771) and was run at 100 V for 1 h 15 min.

Ponceau staining was later included as a step before blocking the membrane to observe the overall presence of proteins on the nitrocellulose membrane. Therefore, the membrane is put in a small box and approx. 10 ml of Ponceau solution are poured onto it. The membrane can be incubated on a rocking device for up to 30 min, however, less incubation time is also possible. The stained membrane was usually obtained after 10 min and then washed with autoclaved water to remove any excess stain. The stained parts revealed the location of the proteins on the membrane (see 3.2 Ponceau Staining).

The western blot was then carried out as follows:

1. Blocking the membrane in a 5 % solution of Blotting Grade-Buffer, non-fat, dry milk, by Bio-Rad™ (Cat. No. 170-6404) in 1X TBS-T for 30 min
2. Wash it three times with 1X TBS-T.
3. Separate the membrane to obtain two pieces: Each of them contains half of the ladder of the marker and the loading site of either Stat3 or Src, this way less antibody is required for the next steps.
4. For 2 h the two pieces are incubated in small boxes containing 2 ml 1X TBS-T and depending on which piece contains the Src and the Stat3 protein with:
    a. 1µl of Src (36D10) Rabbit mAb (from Cell Signalling Technology, Cat. No. 2109S) or
    b. 1µl of Stat3 (124H6) Mouse mAb (from Cell Signalling Technology, Cat. No. 9139S)
5. Wash the membrane pieces three times with 1X TBS-T.

6. Incubate them overnight at 4° C or at room temperature for up to 2 h in small boxes containing 5 ml 1X TBS-T and depending on which piece contains the Src and the Stat3 protein with:
    a. 1 µl of Anti-rabbit IgG, HRP-linked Antibody (from Cell Signalling Technology Cat. No. 7074)
    b. 1 µl of Anti-mouse IgG, HRP-linked Antibody (from Cell Signalling Technology Cat. No. 7076)
7. Wash three times with 1X TBS-T.
8. To each piece add 400 µl of each Clarity™ Western ECL Substrate Luminol/enhancer solution and Clarity™ Western ECL Substrate peroxide solution from Bio-Rad™ and incubate for 1 min.
9. Observe the membrane pieces using chemiluminescence.

3 Results

3.1 Infection of cells

The infection of the used cells (here Sf21) was observed, every 24h. Incubation for 72h in order to obtain expressed proteins later, yields to following pictures under a light microscope and 20x magnification.

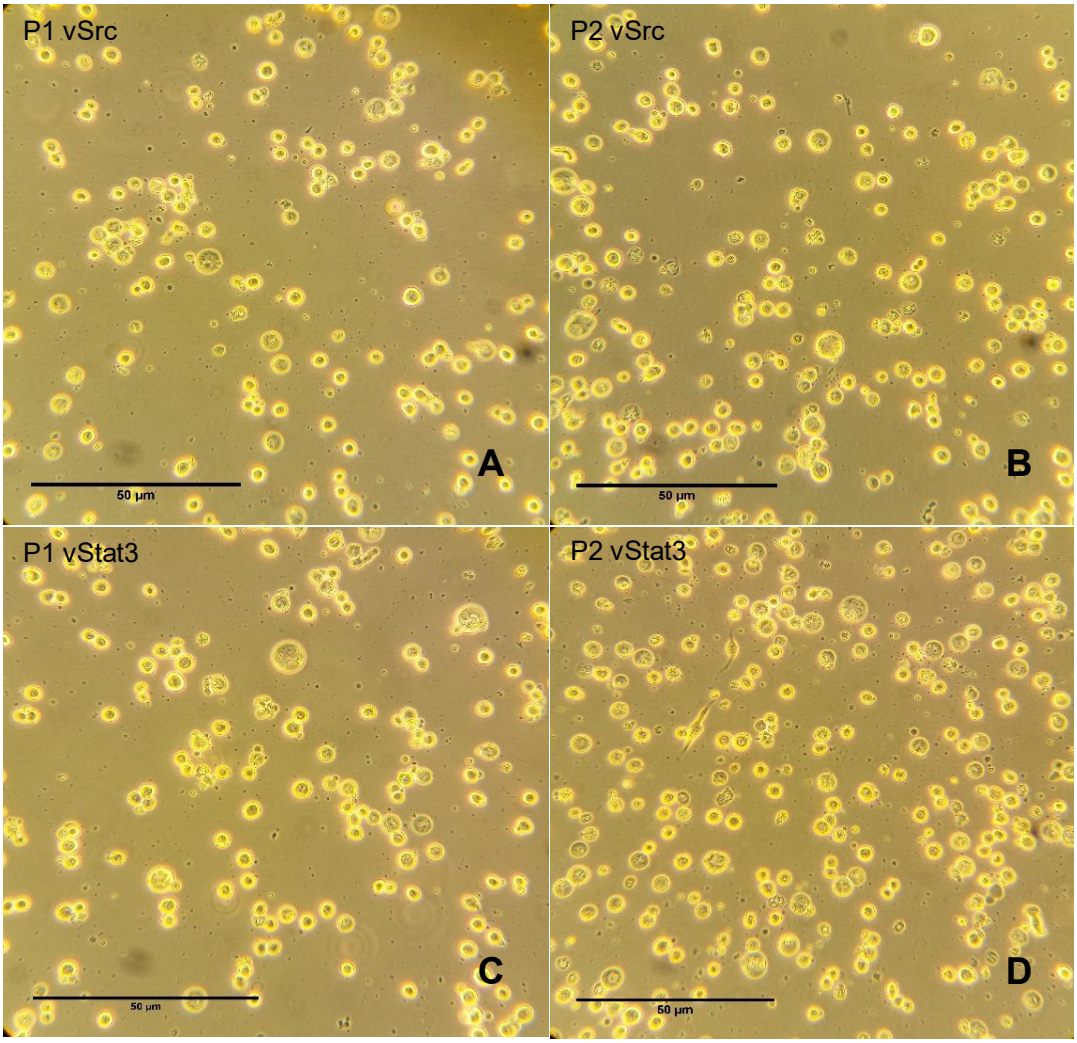

Figure 8 These pictures show Sf21 cells after 72h incubation time after viral infection under a light microscope at 20x magnification. A The insect cells were infected with p1 viral stock of the virus containing the Src gene. Several enlarged cells are visible, indicating viral infection. B The Sf21 cells were infected by p2 virus containing Src. The p2 sample was obtained by amplification of the viral stock p1 vSrc. Comparably better infection was achieved than with p1. C The infection was done by using p1 viral stock of Stat3 gene containing virus. Also, here the enlarged cells indicate infection. D The cells were infected

with p2 vStat3, which was obtained by previous amplification of p1 vStat3. Enlarged cells as well as lysed cells are visible.

3.2 Ponceau Staining

3.2.1 05.01.2017

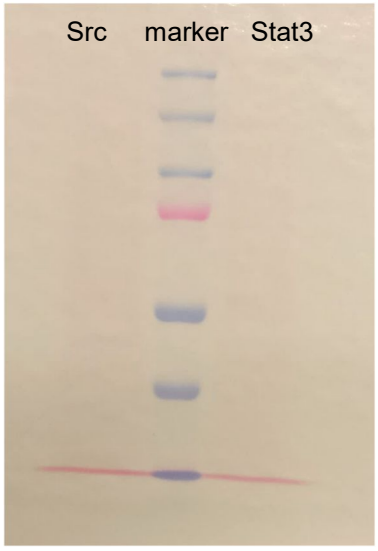

Figure 9: After the first western blot experiments did not show presence of Src or Stat3 (see 3.3 Electrophoresis and Western Blot), Ponceau Staining was introduced to check the overall presence of any proteins on the membrane. The figure shows the nitrocellulose membrane after electrophoresis and Ponceau staining. Only very poorly visible bands can be seen, indicating extremely low protein concentrations of the samples used. The volumes of the samples used were 60 µl for the marker it was 6 µl.

3.2.2 11.01.2017

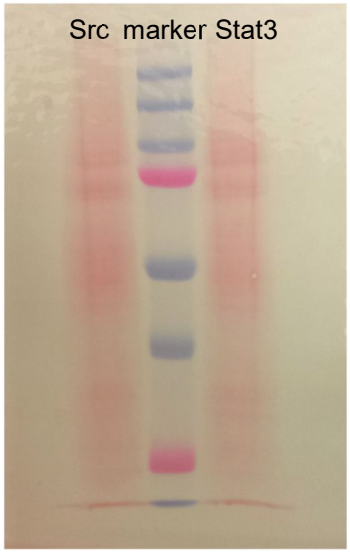

Figure 10 Since infections with both p2 viruses for Src and Stat3 did not yield the expected results, infection with p1 viral stocks for Src and for Stat3 was carried out. The two samples used for electrophoresis had volumes of 70 µl, the marker 6 µl. On the membrane, the pink bands typical for Ponceau staining are visible. However, protein expression in insect cells usually yields very high protein expression levels, which is cannot be observed here.

3.2.3 26.01.2017

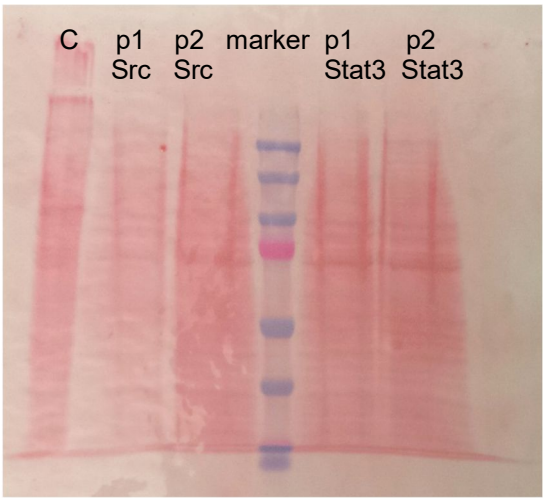

Figure 11 The membrane stained with Ponceau solution shows a comparingly high protein concentration in the samples used. The samples loaded from left to right were a control sample which contains both Src and Stat3, a sample obtained from infection with p1 virus for Src, a sample from infection with p2 virus for Src, the marker, a sample from infection with p1 viral stock for Stat3 and a sample obtained from infection with p2 virus for Stat3. The volumes used for the samples were 70 µl excluding control (8 µl) and marker (6 µl).

3.3 Electrophoresis and Western Blot

3.3.1 Western Blot results from 02.12.2016 and 05.12.2016

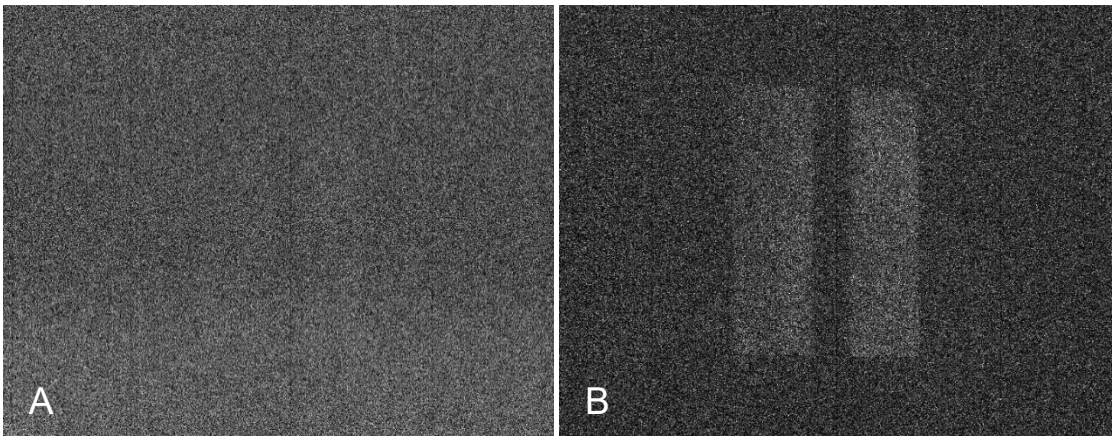

Figure 12: (A) The western blot experiment on 02.12.2016 shows no signal as well as (B) the one performed on 05.12.2016. Both western blots where carried out by using the same protein lysate produced with p2 viral stocks of Src or Stat3 according to the protocol described in the section "Materials and methods". Sample sizes used in (A) were 10 µl of Stat3 and 10 µl of Src and were increased for the electrophoresis and subsequent western blot of (B) to a volume of 50 µl each. However, the signal remained blank.

3.3.2 06.01.2017

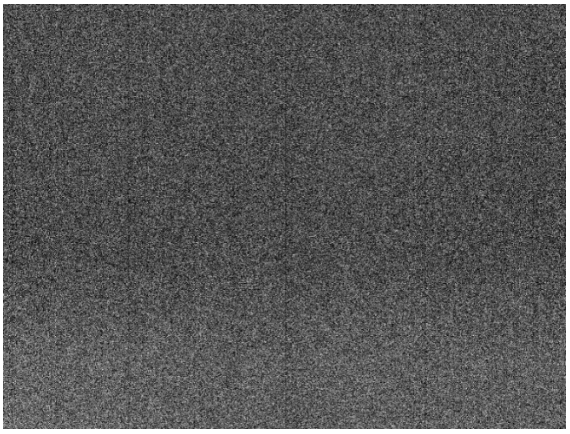

Figure 13: After expression of new p2 Src and Stat3 viruses, infection of insect cells by the two new p2 samples for protein expression was performed. For electrophoresis 50 µl of

total sample were used. Again, the final result of the western blot was blank, indicating there is no protein present.

3.3.3 12.01.2017

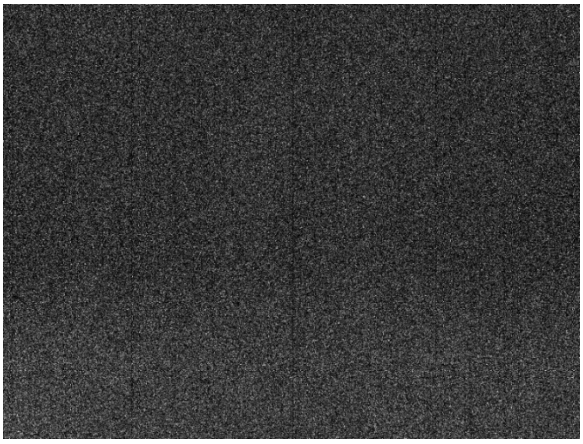

Figure 14 After the infections with Src and Stat3 p2 viruses failed, the Sf21 cells where infected using p1 viral stock of Src and Stat3. For electrophoresis and subsequent western blot 70 µl of Src and Stat3 were used. However, also for p1 produced samples there was no protein expression detected.

3.3.4 27.01.2017

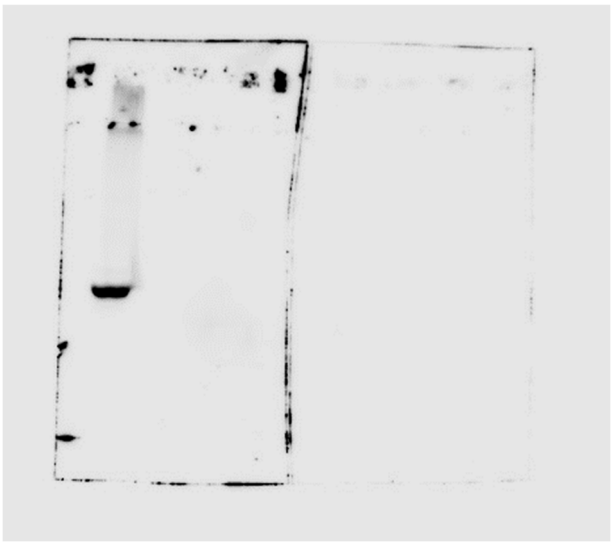

Figure 15 After the protocol for expression of p2 virus and infection for protein expression had been adopted, once more a new p2 virus for Src and one for Stat3 were produced according to the adopted protocol. The loading pattern of the electrophoresis was: a control

sample containing Src and Stat3, p1 produced Src, p2 produced Src, the marker, p1 produced Stat3, and p2 produced Stat3. For all samples loaded excluding the control and the marker, 60 µl were used as a volume. In this picture, clearly a signal is visible, however, this signal only is the control. The reason why there is only one band for the control although it contains Src and Stat3 is that the membrane usually is cut into two pieces (here Src on the left and Stat3 on the right) in order to decrease the volume of anti-bodies needed.

4 Discussion and Conclusion

Looking at the results created, one can conclude that the protein expression did not lead to the desired outcome. Since on the nitrocellulose stained proteins can be seen, after the membrane was stained with Ponceau solution, the SDS-PAGE gel electrophoresis and the subsequent western blot can be assumed to be carried out correctly. However, the question remains why a set-up like this failed to yield expression of the recombinant protein. In theory, there are several reasons which can negatively influence the outcome:

- One possibility is that a failure of the vector or an empty vector. Reasons for that are that the ligation of the pFastBac™ donor plasmids and the gene of interest did not work or the donor plasmid was not successfully digested by the enzymes used or the gene of interest was truncated/cut out at a wrong position.
- During production of the baculovirus a white and blue selection is carried out, when choosing the DH10Bac™ E.coli colony for extraction of the recombinant baculovirus. The colonies containing the donor plasmid with the gene of interest would not be able to degrade the blue dye, as their lacZ region would be interrupted.
- Also, decreased viral efficiency can occur after several infections. If the virus is frozen it will lower the efficiency to infect cells, since repeated freeze and thaw cycles are known to decrease it.
- Another possibility is the condition of the cells. Naturally, dead cells cannot express the protein desired, but usually dead cells are easy to detect when having a look at the cell culture. More likely is that the cells used are not growing in log phase, which logically effects the yield or overall expression of proteins.
- Also worth mentioning are the volumes used as well as the viral titers. If the volume or the viral titer of the virus used are too low, infection will not be successful, as it would take far longer for the viral particles to infect all or the majority of the cells as the cells would be able to live in the same medium without passaging.

- Another big issue was communication between the person producing the recombinant baculovirus and myself, whose task was to amplify the virus and produce high amounts of the proteins of interest. This colleague producing the baculovirus left his position and the state of Hawai'i before the amplification and protein expression were started. After several issues occurred, we managed to get back in touch with the former employee and learned that, although he had stated before that he followed the procedure written in the Bac-to-Bac® Baculovirus Expression System manual, he performed a very different infection method (see last section of 2.2 Infection of the cells with recombinant baculovirus). However, performing the changed method did not yield the desired results either.

All of the reasons above can prevent successful use of the expression system. However, some of them can be at least partially ruled out. First of all, the volumes of the virus used very varied frequently, hence, this should not be an issue. Furthermore, the viral samples were not frozen and thawed more than once. This can decrease the viral efficiency, but usually would not lead to such a dramatic decrease. The conditions of the cell can also be assumed to be a minor issue, as the cultures were maintained not longer than passage no. 20 also cell counts were performed frequently to observe the growth.

The generation of the recombinant expression construct containing the gene of interest as well as the above mentioned white and blue selection are among others crucial steps during production of the recombinant baculovirus. Unfortunately, these working steps were carried out by a colleague. As this colleague left his job and moved, only his statements about the matter are known, which indicate appropriate performance of the expression procedure.

In addition, one should mention why the expression of Src is required in this experiment that aims to produce functioning and dimerized Stat3 molecules. As

mentioned before (see 1.3 Src protein-tyrosine kinase), Src is a non-receptor protein-tyrosine kinase and exerts its enzymatic activity by phosphorylating proteins. Since Stat3 depends on phosphorylation on specific tyrosine residues (see 1.2.1 Stat proteins and their activation) and insect cells would not be able to activate the Stat3 proteins of animals, Src would need to be co-expressed to phosphorylate Stat3 and causing it to form dimers. To achieve this, a recombinant virus containing the Src gene was produced and after optimizing the method of infection used a double infection with the Src and the Stat3 containing baculovirus would have been performed. This would in the end yield phosphorylated and dimerized Stat3 molecules. The reason why insect cells were used in this expression system is that they are able to express proteins in dramatically high amounts as well as their ability to carry out post-translational modifications.

Another question remaining might be why SDS-PAGE gel electrophoresis was used as analyzing method and SDS-buffer as lysing buffer, although sodium dodecyl sulfate is known to denature proteins and add negative charges to them. SDS was used because up to the point of optimizing and establishing the procedures used, there was no need to change to another compound as the samples were only used for analyzing and not for further testing. After obtaining satisfactory amounts of protein the lysis buffer would have been changed to a more suitable one. The procedure of cell lysis (see 2.3 Cell lysis) itself was adopted in a way to use as low volumes of the lysis buffer as possible to not dilute the samples too much and to not miss any possible signals.

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Figures

Figure 1: [3]

Figure 2: adapted from Cancer Research UK, Accessed 08.03.2017, https://www.cancerresearchuk.org/sites/default/files/cstream-node/cases_crude_all_l14_0.pdf

Figure 3: [8]

Figure 4: [43]

Figure 5: adapted from Nature Reviews Molecular Cell Biology, Accessed: 24.03.2017, http://www.nature.com/nrm/journal/v3/n4/box/nrm779_BX1.html

Figure 6: [40]

Figure 7a: adapted from Maryland Biodiverstity Programme, Accessed: 20.03.2017, https://www.marylandbiodiversity.com/viewSpecies.php?species=7746

Figure 7b: adapted from Grain SA, Accessed: 20.03.2017 http://www.grainsa.co.za/pages/news/fall-armyworm-update

Figures 8 – 15: taken and adopted by the author