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„The Role of STAT5B in JAK2^{V617F}-Positive Myeloproliferative Neoplasms"

verfasst von / submitted by

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1. Introduction

1.1. Myeloproliferative Neoplasms

Myeloproliferative Neoplasms (MPNs) are a group of diseases marked by the clonal expansion of morphologically normal hematopoietic cells of the myeloid lineages. The WHO classification includes seven conditions: chronic myeloid leukaemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic neutrophilic leukaemia, chronic eosinophilic leukaemia-not otherwise specified and MPN-unclassifiable (MPN-U). (Barbui *et al.*, 2018) CML, PV, ET and PMF are often referred to as 'classical MPNs' with a further classification into Philadelphia chromosome positive (Ph^+; CML) and negative (Ph^-; PV, ET, PMF) MPNs. The Philadelphia chromosome results from a reciprocal translocation between chromosome 9 and 22 in humans; this results in the expression of the fusion protein BCR-ABL. BCR-ABL is a constitutively active tyrosine kinase that can be found in a majority of CML cases. (Luskin and DeAngelo, 2018) For simplification MPN will now only refer to the three Ph^- classical MPN subtypes (PV, ET and PMF) unless otherwise stated.

1.1.1. Clinical presentation, complications and risks of MPNs

The most common symptoms and complications found in all three MPNs include constitutional symptoms such as weight loss and night sweats (most likely the result of an inflammatory state caused by the release of proinflammatory cytokines), a heightened risk of both venous and arterial thrombosis, bleeding and progression to secondary acute myeloid leukaemia (AML). (Tefferi and Barbui, 2017) Elevated haematocrit and splenomegaly are present in many patients. Patients with PV have the highest risk of thrombosis and those with PMF are the most likely to develop secondary AML. (Zhou, Afzal and Oh, 2017; Spivak, 2018) 45.5% of patients with post-MPN AML have acquired mutations in the tumor suppressor gene *TP53,* which therefore likely drives this progression. The progression to secondary myelofibrosis has been observed in PV and ET patients and correlates with lowered survival. (Cerquozzi and Tefferi, 2015)

PV has also been found to be associated with atypical deep vein thrombosis and Budd-Chiari syndrome, with patients often being diagnosed with PV after such thrombotic events. (Cerquozzi and Tefferi, 2015) The median age at diagnosis for MPNs is around 60 years and life expectancy compared to the age and sex-matched population is decreased (see Figure 1), especially for people with PMF. Despite this, a majority of patients can survive for decades with the disease if treated properly. (Hultcrantz *et al.*, 2012; Cerquozzi and Tefferi, 2015) The presence of an MPN is often detected during routine complete blood cell count analysis, when increased levels of white blood cells (WBCs), red blood cells (RBCs), platelets or a combination of all three are found. (Barbui *et al.*, 2018; O'Sullivan and Mead, 2019) A study from the United States found the prevalence of PV to be 44-57 cases per 100 000 people and of ET 38-57 per

100 000. MF was found in about 1 in 100 000 people. The rates are likely to be similar in other countries. (Mehta *et al.*, 2014)

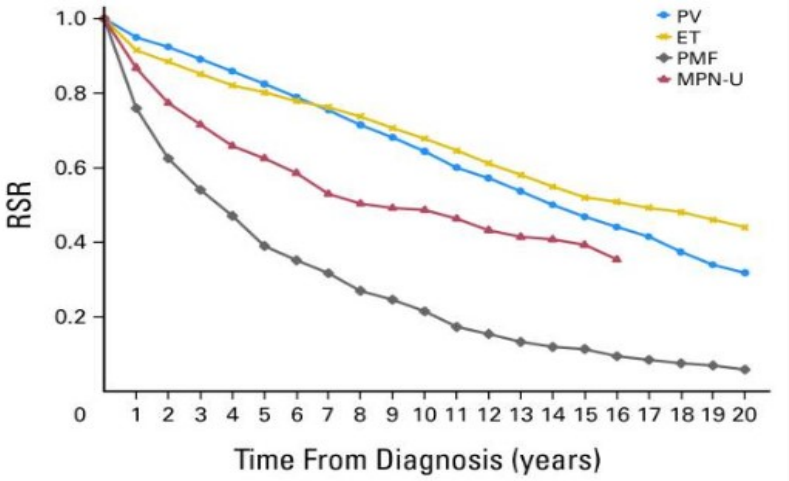

Figure 1: *Relative survival curve of patients diagnosed with PV, ET, PMF or MPN-U.* (Hultcrantz et al, Journal of Clinical Oncology, 2012)

1.1.2. Diagnosis of MPNs

Polycythemia vera, the most common of the three disorders, is characterized by an increase in the levels of red blood cells (erythrocytosis), white blood cells (leukocytosis) and platelets (thrombocytosis) or a combination of those. (Spivak, 2018) WHO diagnostic criteria for PV include an elevated haemoglobin level and elevated haematocrit as well as the presence of a mutation in the *JAK2* (janus kinase 2) gene. Additionally, a bone marrow biopsy shows increased proliferation of erythroid, granulocytic and megakaryocytic cells. (Barbui *et al.*, 2018) In contrast to PV, ET mainly involves the megakaryocytic lineage. The WHO diagnostic criteria for ET include an elevated platelet count and increased megakaryocytic proliferation in the bone marrow. The presence of *JAK2*, *CALR* (calreticulin gene) or *MPL* (thrombopoietin receptor gene) mutations as well as not fitting the criteria for other myeloproliferative diseases further establish the diagnosis of ET. (Barbui *et al.*, 2018) PMF is mainly distinguished from the other two MPNs through the presence of fibrotic tissue in the bone marrow. An increase in megakaryocytic proliferation accompanied by the presence of reticulin or collagen fibrosis in the bone marrow as well as the presence of a *JAK2*, *CALR* or *MPL* mutation are amongst the major diagnostic criteria defined by the WHO for PMF. Additionally, splenomegaly and anaemia are found in these patients, which are due to bone marrow failure induced by the replacement of healthy bone marrow with scar tissue. (Barbui *et al.*, 2018) PV and ET can both progress to myelofibrosis and ET can transition to PV, which is why some have suggested to treat them as spectrum of the same disease rather than separate entities. (Spivak, 2018) The fact that the same mutations can be found in all three disorders provides further evidence for this idea. But so far, they are still diagnosed and treated separately.

1.1.3. Driver mutations in MPNs: the importance of JAK2

MPNs are believed to arise from a single hematopoietic stem cell (HSC) that acquires mutations which lead to the expansion of mature cells of one or all the myeloid lineages. One of the first mutations to be identified and which has since been studied extensively, is the JAK2^{V617F} mutation. It is found in *exon 14* of the *JAK2* gene and was first discovered in 2005 by four different groups. It is present in 95% of PV and around 60% of ET and PMF patients. (Baxter *et al.*, 2005; James *et al.*, 2005; Jones *et al.*, 2005; Kralovics *et al.*, 2005) Before its discovery, diagnosis relied mainly on a patient's blood cell count and on histopathological analysis of the bone marrow. Since then, the presence of the *JAK2^{V617F}* mutation has been incorporated as one of the main diagnostic criteria for MPNs. (Barbui *et al.*, 2018) As mentioned above, PV patients almost exclusively harbour JAK2^{V617F}. Most of the remaining 5% of PV patients that are JAK2^{V617F} negative have activating mutations in *exon 12* of *JAK2*. ET and PMF patients can also have mutations in the *CALR* or *MPL* genes. Although some patients have been found to be triple-negative for these mutations, the JAK2 signaling pathway is hyperactive in all MPN patients, regardless of the driver. (Rampal *et al.*, 2014)

1.1.4. Thrombosis and MPNs

As mentioned previously, arterial and venous thrombosis are the major cause of morbidity and mortality in MPN patients with a majority of events being arterial. A thrombosis occurs when a blood clot obstructs a blood vessel, which prevents the proper oxygen supply to adjacent tissues. This can lead to a temporary or permanent impairment of the respective tissue's functions. If the blocked vessel is one that supplies the heart or the brain, thrombosis can be deadly. In healthy individuals such clots form during wound healing when the coagulation cascade is activated to prevent blood loss after injury. The molecular mechanisms for the increased incidence of thrombosis in MPN patients remains incompletely understood. Mutated *JAK2* has been shown to lead to structural and functional abnormalities in platelets, white blood cells and red blood cells as well as endothelial cells. These changes lead to increased cell aggregation and to binding and activation of the endothelium, causing increased risk of thrombosis. *JAK2^{V617F}* mutant-expressing endothelial cells can promote thrombosis through induction of endothelial P-selectin expression. P-selectin is a cell adhesion molecule expressed by both endothelial cells and platelets, which translocates to the surface of the cells during their coagulation related activation. (Arachchillage and Laffan, 2019) The expression of CD11b on the neutrophil surface allows the adhesion of neutrophils to endothelial cells and platelets. This surface protein as well as others have been found to be more highly expressed on cells of JAK2^{V617F} positive patients. (Coucelo *et al.*, 2014) An increase in neutrophilic extracellular trap formation by JAK2^{V617F}-mutated neutrophils has also been suggested as a possible cause for thrombosis. (Wolach *et al.*, 2018) It has also been suggested that biochemical changes in the cell membrane and content of red blood cells may impair blood flow through the formation of red cell aggregates.

Figure 2 shows an overview of the cellular mechanisms that are believed to be involved in the occurrence of thrombosis in MPN patients. In regard to thrombosis, patients are typically either put into the high risk or low risk category. The criteria for categorization into the high risk group are age (>60 years) and previous thromboembolic events. (Arachchillage and Laffan, 2019) A recent publication by experts in the field formulated the current unmet clinical needs in the management of MPN-related thrombosis. One of these was the need to improve the knowledge on the association between somatic mutations and risk factors for thrombosis in MPNs. (Barbui *et al.*, 2019) This highlights that molecular risk factors for thrombosis in MPNs are still incompletely understood and require further investigation.

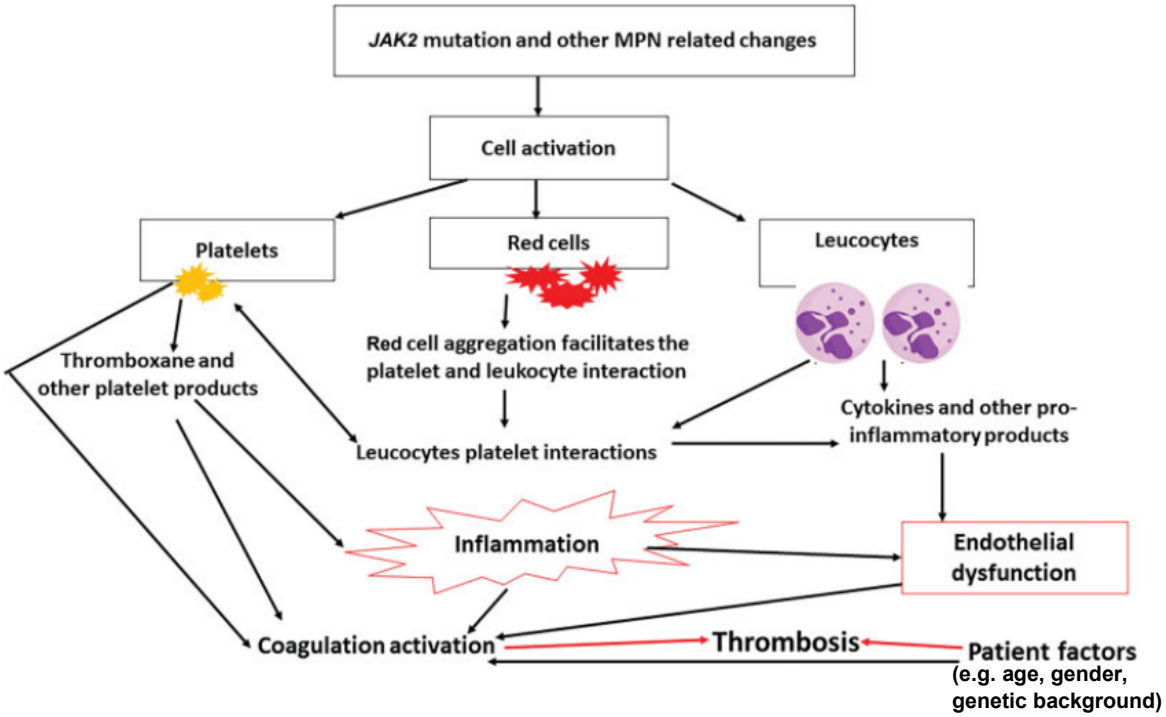

Figure 2: *Cellular mechanisms involved in the development of thrombosis in MPN patients.* (adapted from Arachchillage and Laffan, 2019)

1.1.5. MPN treatment strategies

Treatment of MPNs is highly dependent on the severity of the disease. As no curative treatment is available so far, the focus is placed onto reducing symptoms and increasing life expectancy. To facilitate the decision on appropriate treatment, patients are put in different risk categories. Low risk patients are typically those with lower cell and or platelet count and who are young. Patients who are above the age of 60 or have a history of thrombosis are referred to higher risk categories. The first line of treatment for low-risk PV patients is phlebotomy, which is simply the removal of excess blood to maintain a healthy haematocrit. Low-risk ET patients are treated with low doses of aspirin, to prevent excessive clot formation. Treatment of myelofibrosis is usually more complex and notably involves blood transfusions and splenectomy. When these measures are not enough, or for higher risk patients, a cytoreductive agent, mainly hydroxyurea, is employed. Interferon-α can also be used, but is more commonly prescribed for younger patients. (Aruch and Mascarenhas, 2016)

In light of these limited and non-curative treatment options, more targeted therapies for MPN patients have been developed. One of these is the JAK1 and JAK2-inhibitor ruxolitinib, which is primarily used in high-risk PV and PMF patients and has been shown to greatly improve symptoms. Unfortunately, it is not curative, contrarily to imatinib, a tyrosine-kinase inhibitor designed to target BCR-ABL, which is used to treat CML and which has led to complete remission in up to 80% of patients. (Sacha, 2014) Resistance to JAK2 inhibitors has been observed in MPN patients and seems to be mainly mediated by alternative activation of STAT5, e.g. through JAK-STAT activation. (Koppikar *et al.*, 2012) August 2019 saw a new JAK2-inhibitor called fedratinib in the clinic approved by the FDA for the treatment of "intermediate-2 or high-risk primary or secondary (post-PV or post-ET) myelofibrosis". It still remains to be seen, whether it will also be approved for the treatment of PV. (Pardanani *et al.*, 2011)

1.1.6. Heterogeneity in MPNs

MPNs are quite heterogenous disorders with some patients staying without symptoms for years and others progressing rapidly to AML. Environmental factors, age and gender play a role in the progression of MPNs. Differences have also been found between different driver mutations, for example *CALR*-mutated ET patients have increased survival compared to *JAK2*-mutated patients. (Rumi *et al.*, 2014) The $JAK2^{V617F}$ mutation in particular raises questions, since the $JAK2^{V617F}$ mutation can be found in PV, ET and PMF patients. How can one single mutation lead to such a wide variety of disorders? One explanation is gene dosage, which has been identified as playing a role in disease manifestation. A homozygous $JAK2^{V617F}$ mutation (allele burden >50%) is associated with PV and heterozygosity with ET. The homozygous state is the consequence of loss of heterozygosity through acquisition of uni-parental disomy in chromosome 9. This happens at a high frequency in MPN patients. (Levine, 2018) Since $JAK2^{V617F}$ has also been detected in around 0.18% of the healthy population, associated with clonal hematopoiesis of indeterminate potential (CHIP), additional factors must play a role in the development and manifestation of MPNs. (Jaiswal *et al.*, 2014) Another possible explanation is the presence of co-occurring somatic mutations that arise during the course of the disease. The most prevalent ones are found in genes of epigenetic regulators, notably enhancer of zeste homologue 2 (EZH2), ten-eleven translocation 2 (TET2) and DNA (cytosine-5)-methyltransferase 3A (DNMT3A). Interestingly, STAT5 interacts with both TET2 and EZH2 and DNMT3A is a direct target gene of STAT5. (Zuo *et al.*, 2019)

Still, these do not account for all differences between disease presentation in different patients. Alternative explanations for disease heterogeneity could be the differential activation or expression of other signaling molecules or pathways through mechanisms other than simply mutated proteins.

1.2. Hematopoiesis

As mentioned above, MPNs are a disease of the hematopoietic compartment and driver mutations involved occur very early on in hematopoiesis, in the HSCs. Thus, understanding hematopoiesis is important for understanding MPNs.

In the fetus the first sites of hematopoiesis are so-called blood islands in the yolk sack, where only erythropoiesis occurs. The HSCs, which give rise to all hematopoietic cells, migrate to the fetal liver and spleen where they continue to produce erythrocytes and certain granulocytes until shortly after birth. They then colonize the bone marrow where they are responsible for maintaining blood homeostasis throughout an individual's life. (Tavassoli, 1991) Hematopoiesis in healthy human adults starts in the bone marrow, where resident multipotent hematopoietic stem cells give rise to all cells of the blood. The only incidences where HSCs will leave the bone marrow are infections, anemia or similar events where additional hematopoiesis is required e.g. during high altitude mountaineering. The spleen and the liver then become sites of extra medullary hematopoiesis (EMH) to compensate for the increased need of blood cells. (Chiu *et al.*, 2015)

HSCs are multipotent stem cells that can give rise to all cells of the hematopoietic compartment. In human adults they reside in stem cell niches in the bone marrow where their multipotency is maintained through contact and interaction with stromal cells such as osteoblasts or mesenchymal cells. According to recent studies, there are two types of stem cell niches in the bone marrow, the arteriolar niche and the sinusoidal-megakaryocyte niche. The cells of these niches secrete different growth factors, like stem cell factor (SCF), to support the growth and maintenance of the HSCs. SCF is the ligand for the surface receptor c-KIT (also known as CD117) and it is an important growth factor for the cultivation of HSCs and multipotent hematopoietic progenitors (MPPs). Other important stimulating cytokines that are involved in myeloid cell maintenance but also differentiation, are IL-3, IL-6 and granulocyte-macrophage colony stimulating factor (GM-CSF). They are often used to support hematopoietic cell growth *in vitro*. (Oguro, Ding and Morrison, 2013; Calvi and Link, 2015)

Hematopoiesis is organized hierarchically with HSCs at the top. To become a mature blood cell, HSCs undergo a series of differentiation and cell division steps that are depicted in Figure 3. These differentiation steps are regulated by the expression of specific transcription factors whose expression is in turn partly regulated by cytokines. Through that process one cell can give rise to an exponential number of mature blood cells and only a small number of HSCs are required to maintain blood homeostasis. The first differentiation step gives rise to either the common lymphoid progenitor (CLP) cell or the common myeloid progenitor (CMP) cell. The CLP commits the cell to the lymphoid lineage and eventually gives rise to B- and T-cells. The CMP can differentiate into granulocyte/macrophage progenitors (GMPs) or megakaryocyte/erythroid progenitors (MEPs). The GMP goes on to form granulocytes and monocytes. The granulocytes are the basophil, the neutrophil and the eosinophil which all

play distinctive roles in the innate immune system. The neutrophil for example is responsible for the elimination of bacteria through phagocytosis or NET (neutrophilic extracellular trap) formation. (Ravindran, Khan and Palaniyar, 2019) Monocytes give rise to macrophages and myeloid dendritic cells. The MEP further differentiates into either an early megakaryocyte progenitor or an early erythroid progenitor (erythroblast). Each of these progenitors go through distinct stages of differentiation before reaching the final step and becoming a mature megakaryocyte or erythrocyte.

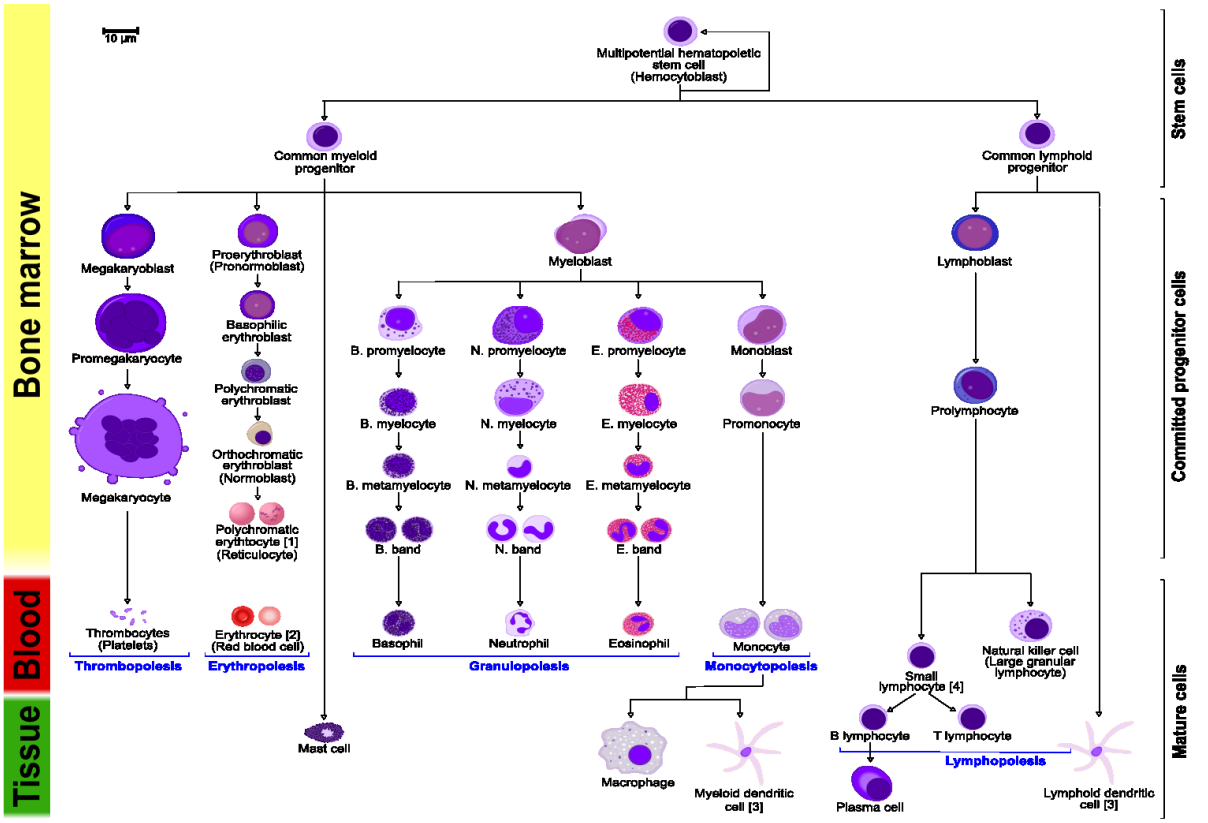

Figure 3: *Hierarchy of hematopoiesis.* (File:Hematopoiesis (human) diagram.svg - Wikimedia Commons, no date).

1.2.1. Megakaryopoiesis

Megakaryocytes (Mks) reside in the sinusoidal regions of the bone marrow where they increase drastically in size during differentiation and form protrusions that reach into the lumen of the blood sinus. Platelets then bud off from these protrusions and enter the blood stream where they can then fulfill their role in blood clotting. Mks are hence heavily involved in wound healing and thrombi formation. The factor responsible to initiate Mk differentiation is thrombopoietin (TPO), which is mainly secreted by the liver and signals through the JAK2-STAT5 pathway. (Kaushansky and Zhan, 2018) STAT5 has been found to play an important role in Mk differentiation, notably in its unphosphorylated form. (Park *et al.*, 2016) A transcription factor that is heavily involved in megakaryopoiesis is GATA-2, which when overexpressed in human leukemia cells, favors megakaryocytic over erythroid differentiation. (Doré and Crispino, 2011)

1.2.2. Erythropoiesis

Erythroblasts undergo a series of differentiation steps during which they enrich in hemoglobin and lose most of their cell organelles. During one of the final stages they lose their nuclei and take on their typical flat shape. They can then enter the blood stream to transport oxygen. The differentiation of erythroblasts is initiated through erythropoietin (EPO), which is mainly produced in the kidney upon anemic conditions or oxygen limitations such as high altitude. (Bunn, 2013) Recombinant EPO is widely but illegally used in the professional sport community to increase erythroid numbers and thus performance in athletes, leading to severe side effects and in some cases even death if combined with Insulin, growth hormone as well as testosterone. Similarly to TPO, EPO also uses the JAK2-STAT5 pathway to convey its signal. One of the target gene products of STAT5 is Bcl-X_L, an anti-apoptotic protein, which is important for the maintenance of erythroid cells as its absence leads to hemolytic anemia. (Ingley, Tilbrook and Klinken, 2004) Important transcription factors for erythroid differentiation are GATA-1 and its binding partner FOG-1. Their expression represses GATA-2 and thus megakaryocytic differentiation. GATA-1 is primarily expressed in mature cell types and knocking it out leads to severe anemia and *in utero* death in mice. (Doré and Crispino, 2011)

1.2.3. Cell-surface markers of hematopoietic cells

Different markers have been identified and associated with the different populations of cells that can be found in the blood, the spleen or the bone marrow. These markers combined with measurement of the size of the cells are being used in flow cytometry analysis to identify and quantify the cell types. HSCs and MPPs are comprised in the so-called LSK cell fraction. These are lineage negative cells, which also express c-KIT and Sca-1. Lineage markers usually include: TER119 for erythroid cells, CD11b (also MAC-1) for macrophages and broad myeloid cell populations, CD3e for T-cells, CD45R (also B220) for B-cells and Ly6G (also GR-1) for cells of the monocytic and granulocytic lineages. Using these markers during flow cytometric cell sorting, LSKs can be separated from the rest of the cells and their behavior can be studied *in vitro*. This fraction also contains early megakaryocytic progenitors which can be identified by using antibodies against the surface markers CD41 and CD150 during flow cytometry analysis. (Oguro, Ding and Morrison, 2013; Orfao *et al.*, 2019)

Hematopoiesis is a tightly controlled process in which the JAK2-STAT5 pathway plays a crucial role, especially in the erythroid and megakaryocytic lineages. As mentioned above, what remains unclear is the extent to which STAT5A and STAT5B are differentially or combinatorically involved in controlling blood homeostasis and clotting in health or in neoplastic situations.

1.3. The JAK-STAT pathway

Together with hormones and growth factors, cytokines are one of the most important signaling molecules. They mediate, amongst other things, cell differentiation, proliferation and apoptosis. They also play an important role for the proper activation of the immune system after infection. (Villarino *et al.*, 2015) Once a signal has reached the inside of the cell, different signaling pathways can be activated, depending on the cytokines involved. In a cancer setting many of these pathways can be up- or downregulated, thus leading to malignant proliferation of cells. One of these pathways is the JAK-STAT pathway. It is most often found to be deregulated through different mechanisms in hematopoietic cancers as well as solid cancers. (Bousoik and Montazeri Aliabadi, 2018) The JAKs and signal transducer and activator of transcription proteins (STATs) are the key molecules involved in this pathway. There are four JAK family members: TYK2 (Tyrosine kinase 2), JAK1, JAK2 and JAK3. The STAT family consists of seven members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. (Horvath, 2000)

1.3.1. Signaling mechanism of the JAK-STAT pathway

JAKs are receptor associated proteins that can be activated via phosphorylation of a Tyrosine residue. They associate with receptors that themselves do not have a kinase domain and could not otherwise relay the signal to the inside of the cell. These receptors are for example the Thrombopoietin (TPO) and the Erythropoietin (EPO) receptor: MPL and EPOR, respectively. These play a fundamental role in erythropoiesis and thrombopoiesis and are thus heavily involved in MPN pathogenesis. More generally, the receptors that interact with JAK family members are growth factor, interleukin (IL) or interferon receptors. (Bousoik and Montazeri Aliabadi, 2018)

Once a signaling molecule binds to the receptor (see Figure 4), the receptor dimerizes bringing two associated JAKs closely together, leading them to auto-phosphorylate each other through conformational changes. They then phosphorylate tyrosine residues on the cytoplasmic tail of the receptor. This allows anti-parallel STAT-dimers to bind, get phosphorylated by the JAKs and to rearrange to form parallel dimers. These activated STAT dimers then travel to the nucleus where they activate transcription of their target genes by binding to STAT response elements on the DNA to initiate transcription. These target genes mediate cell survival, proliferation and differentiation processes. (Villarino *et al.*, 2015) To switch off the signal, which is crucial to maintain a healthy homeostasis, several other proteins act on the JAKs and STATs and lead to their dephosphorylation. One class of proteins are the SOCS (Suppressor of Cytokine Signaling) proteins, that act in a negative feedback loop on the STATs. They achieve this by either marking it for proteasomal degradation or inhibiting JAK activity directly. Each SOCS protein targets a specific STAT protein ensuring the proper inactivation of the signaling

pathway involved. (Bousoik and Montazeri Aliabadi, 2018; Linossi, Calleja and Nicholson, 2018)

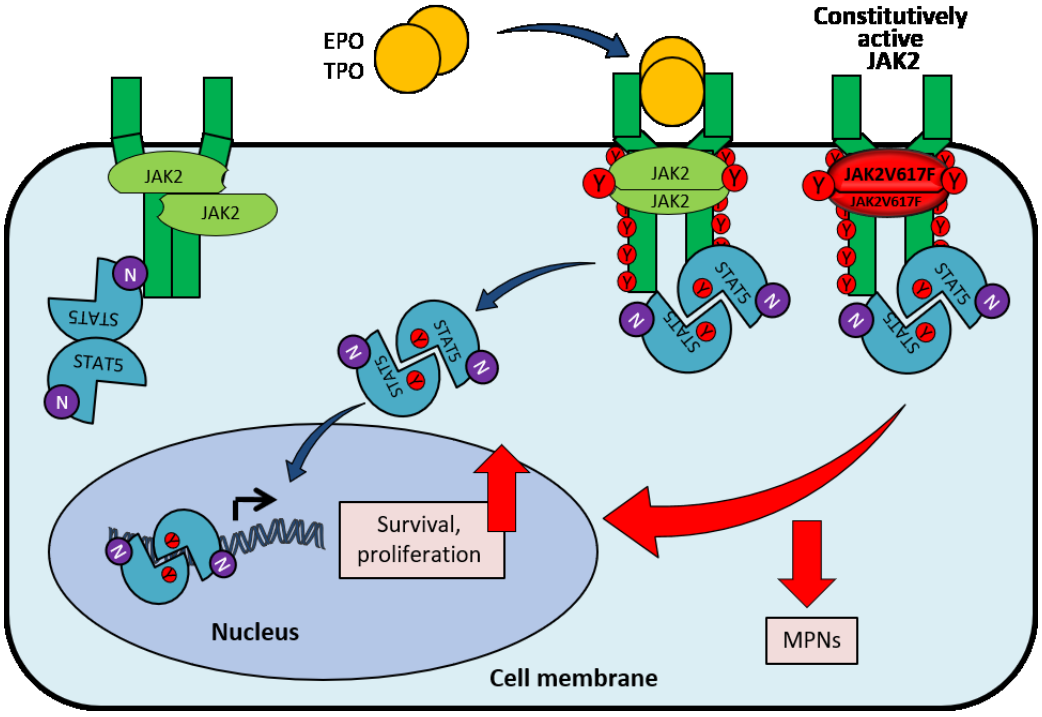

Figure 4: *The JAK2-STAT5 pathway in MPNs.* (adapted from Orlova A.)

1.3.2. The JAK family and its structure

Upon cytokine-binding, JAKs primarily form homo-dimers but can also form hetero-dimers. They are each activated by specific cytokines, although some overlaps exist. Upon loss of one of them, compensation by other JAKs has been observed. (Zouein, Duhé and Booz, 2011) Although their primary location is in the cytoplasm associated with cytokine receptors, JAK1 and JAK2 have also been found to localize to the nucleus. Nuclear JAKs play a role in gene expression and influence the epigenetic landscape. (Zouein, Duhé and Booz, 2011) The JAK proteins contain seven domains, termed JAK-homology (JH)-domains 1-7. The JH1 domain is the tyrosine-kinase domain, important for the activation of the JAK protein. The JH2 domain, which was long thought of as a pseudo-kinase, has been discovered to be a serine/threonine-kinase. It interacts closely with the JH1 domain and acts as a suppressor of phosphorylation. (Min *et al.*, 2015) JH3 and 4 make up the src-homology 2 (SH2) domain that enables the molecule to bind to phosphorylated tyrosine residues, such as those of another JAK molecule. The last domain is the FERM domain which is important for interaction with the receptors and is made up of JH5 – 7. As can be seen in Figure 5, the V617F mutation is located in the JH2, or kinase-like domain of JAK2. The replacement of Valine by Phenylalanine weakens the interaction between the JH1 and JH2 domains, preventing the proper inhibition of activation by JH2 and thus leading to a constitutively active JAK2 protein. (Sanz Sanz *et al.*, 2014)

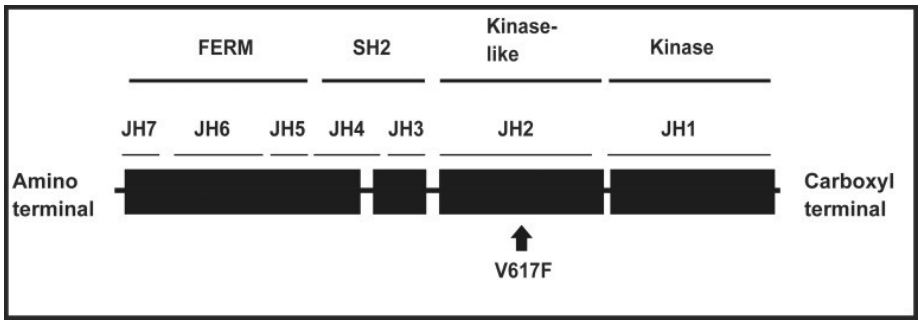

Figure 5: *JAK2 domain structure with the location of the V617F mutation (arrow).* JH - JAK homology domain, FERM - 4.1 protein Ezrin Radixin Moesin domain, SH2 - scr-homology 2 domain. (Barcelos and Santos-Silva, 2011)

1.3.3. The STAT family members

STATs act as transcription factors and can be activated by different JAKs. STATs have a highly conserved protein structure (Figure 6). They all contain the following domains in this order: an N-terminal domain, a coiled-coiled domain, a DNA-binding domain, a linker domain that connects the DNA-binding domain with the SH2 domain and finally the transactivation domain (TAD) at the C-terminus. Each domain has quite distinctive functions, such as the formation of higher-order structures like STAT-tetramers for the N-terminal domain. The coiled-coiled domain and the SH2 domain are both important for dimerization, with the former being important for forming anti-parallel unphosphorylated dimers and the latter for the STATs to form parallel dimers upon tyrosine-phosphorylation. The activating tyrosine-phosphorylation occurs at position Y695/Y699 (STAT5A/STAT5B), which is located in the linker domain between the SH2 and transactivation domains. The DNA-binding domain mediates the fundamental function of STATs in transcriptional regulation, and the TAD serves as a platform of interaction with other proteins that are involved in transcriptional regulation. (Hennighausen and Robinson, 2008)

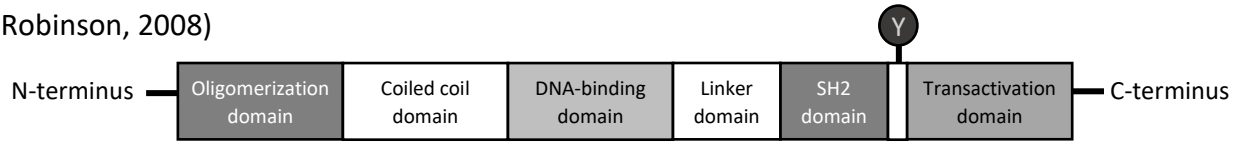

Figure 6: *Domain structure of STAT-proteins.* (adapted from Pham *et al.,* 2018)

For a long time unphosphorylated STATs (uSTATs) were believed to play no active role in gene regulation. Since then, uSTAT5A was found to interact with heterochromatin protein 1α (HP1α) and thus stabilize heterochromatin, which in turn suppresses tumor growth. (Hu *et al.*, 2013) Additionally, uSTAT5 was found to be involved in megakaryocyte differentiation. Nuclear uSTAT5 binds to and represses genes that are responsible for megakaryocyte differentiation and keeps the cells in an undifferentiated state until TPO signaling occurs, leading to STAT5 phosphorylation and activation. In one study they retrovirally expressed a Y699F STAT5B mutant, which cannot be tyrosine phosphorylated, into a murine hematopoietic cell line. This led to less megakaryocytic differentiation after TPO stimulation.

Although they did not show experiments with uSTAT5A, uSTAT5B might be more important in this context than uSTAT5A. (Park *et al.*, 2016)

The crucial pathway for disease development in MPNs, and especially PV, is the JAK2-STAT5-pathway. Even in patients with unknown drivers, STAT5 has been found to be highly phosphorylated, indicating that the activation of this protein is essential for disease development. The deletion of STAT5 in a JAK2^{V617F} knock-in mouse model, which develops PV-like symptoms, leads to normalization of blood parameters, highlighting again the importance of STAT5 in PV. (Yan, Hutchison and Mohi, 2012)

1.3.3.1. STAT5A and STAT5B gene products

STAT5 refers to two different gene products: STAT5A and STAT5B. In humans they have 89% DNA sequence homology and 92% amino acid sequence homology and are both encoded on chromosome 11 in mouse and 17 in human, in close proximity to each other. The main differences can be found in the C-terminal transactivation domain (TAD) where STAT5A has 20 and STAT5B 8 unique amino acids. They have many redundant functions, but also play distinct roles in different tissues. Their non-redundant functions might be explained by differences in the downregulation of their signaling, promoter specific transcription, post-translational modifications or their interaction with other proteins. (Hennighausen and Robinson, 2008; Able, Burrell and Stephens, 2017) STAT5B, for example, is more highly expressed in NK- and T-cell subsets when compared to STAT5A, indicating a more important role in these cells. Mouse models with specific *Stat5a* or *Stat5b* knock-out have distinct phenotypes, with *Stat5a* deficient mice showing impaired mammary gland development, demonstrating its dominant function in prolactin signaling. (Liu *et al.*, 1997) STAT5B mediates growth hormone signaling and thus *Stat5b* deficiency leads to growth retardation in mice. (Udy *et al.*, 1997) The latter has also been observed in patients with STAT5B loss-of-function mutations. These patients show severe postnatal growth failure, growth hormone insensitivity, IGF-1 deficiency (an important STAT5B target) and an impaired immune system. Some patients who lack STAT5B show impaired thrombocyte aggregation, highlighting its involvement in platelet function. (Hwa *et al.*, 2005, 2011)

1.3.3.2. Differential roles of STAT5A and STAT5B in hematopoietic cancer

An indication that STAT5B is the more oncogenic sibling of STAT5, is for example the fact that it has been found to be more frequently mutated in cancer patients than STAT5A. Also, when comparing an activating mutation in STAT5A with one in STAT5B, the expression of STAT5B^{N642H}, the STAT5B mutant, at physiological levels gave rise to a highly aggressive T-cell neoplasm in mice, whereas the STAT5A^{S710F} mutant when expressed at similar levels showed no disease phenotype. When more highly overexpressed, however, STAT5A^{S710F} also led to a T-cell malignancy in mice. (Pham *et al.*, 2018; Maurer *et al.*, 2019) In CML, STAT5 also plays an important role downstream of the driver BCR-ABL. Here again, STAT5B has recently been

found to be more leukemogenic than STAT5A in a mouse transplant model. (Kollmann *et al.*, 2019) Whether STAT5A and STAT5B play differential roles in PV is not known. They have not been found to be mutated in this disease, however different expression levels of these proteins might increase the risk for complications, since they are downstream of JAK2 and are so important in hematopoiesis.

1.4. JAK2^{V617F} mouse models

Different approaches have been used to express the JAK2^{V617F} mutation in mice in order to confirm its disease driving capacity and to recapitulate MPNs as observed in human patients. The first experiments were performed using retroviral transduction of JAK2^{V617F} into murine bone marrow cells, that were then transplanted into irradiated mice. The transplanted mice showed a PV-like phenotype with increased numbers of erythrocytes and leukocytes and splenomegaly. Secondary transplant experiments also resulted in a PV-like disease in the recipient mice, thus establishing JAK2^{V617F} as disease-initiating in mice. (Dunbar, Nazir and Levine, 2017) This was later confirmed using transgenic JAK2^{V617F} mouse models revealing a correlation between expression level and disease severity. Lower JAK2^{V617F}-expressing mice showed more of an ET-like disease whereas higher expression of JAK2^{V617F} led to a PV phenotype. This reflects the situation in humans as most PV patients are homozygous and ET patients are heterozygous for the JAK2^{V617F} mutation. (Dunbar, Nazir and Levine, 2017) To be able to study JAK2^{V617F} in a more physiological way, knock-in models were created where the mutant *JAK2* would be under the control of the wild-type (wt) *JAK2* promoter. Additionally, using tissue specific lox Cre recombinase systems, JAK2^{V617F} was expressed under the control of the *Vav1* promotor. Four different groups reported on conditional knock-in hematopoietic-specific mouse models around the same time and obtained similar results, with most of the mice showing PV-like diseases. (Dunbar, Nazir and Levine, 2017)

1.4.1. PV mouse model used in this study

The mouse model used for this master thesis was first published by Marty et. al in 2013. These mice express the JAK2^{V617F} mutation only in cells with an active *Vav1*-promotor and so primarily in hematopoietic cells. After recombination of the conditional allele, the resulting mice express JAK2^{V617F} at a ratio of 1:1 to wt JAK2. Contrarily to what might be expected from the previous studies, where heterozygosity led to ET, these mice develop a PV-like disease. At 12 weeks the mice show an increase in red blood cells, white blood cells and platelets as well as have an increased hematocrit. Myeloid progenitors were also found to be increased in the bone marrow and the spleen, as would be expected from an increase in mature blood cells. This observed phenotype resembles most closely tri-lineage PV in humans. (Hasan *et al.*, 2013; Marty *et al.*, 2013)

Taken together, these models represent valuable tools to study the molecular mechanisms that govern PV, and they have already been useful to deepen our understanding of this

disease. Despite these advances, we still lack knowledge on the mechanisms that govern the heterogeneity and increased risk of disease progression in patients. Thus, further studies are needed to shed light on the molecules (e.g. STAT5A and STAT5B) involved as well as their cooperation during disease development.

1.5. Rationale of the master thesis

The JAK2^{V617F} mutation is a potent driver of MPNs and has been detected in up to 98% of patients with different MPN subtypes. These patients suffer from increases in blood cell counts that require lifelong treatments, and carry the risk of developing secondary life-threatening complications such as thromboembolisms or AML. Functionally, the mutation enables constitutive activation of JAK2 and signaling via STAT5 transcription factors. However, it is currently unknown whether STAT5A or STAT5B is the major downstream effector of JAK2^{V617F} in MPNs. Additionally, heterogeneity in disease severity, progression and type in MPN patients has yet to be fully elucidated and could not be explained by additional mutations so far. Since one of the reasons for this heterogeneity might be different expression levels of the signaling molecules involved, we wanted to investigate the effect of different STAT5A or STAT5B levels in three MPN-like myeloid leukemia cell lines, two of which harbor the JAK2^{V617F} mutation.

A human STAT5B (hSTAT5B) transgenic mouse shows increases in myeloid progenitors

The hSTAT5B transgenic mouse published by the lab has low-level overexpression, which was not quantified so far, of hSTAT5B in the hematopoietic compartment. This mouse has no overt phenotype although a slight increase in platelets was observed in blood smears from the mice. (Pham *et al.*, 2018) Additionally, CMP and MEP counts in the bone marrow were increased compared to wt littermates (unpublished data). This indicates that a slightly higher expression level of wt hSTAT5B alone already has an impact in myeloid cell numbers, particularly in the megakaryocytic lineage.

JAK2^{V617F}/hSTAT5B compound mice have reduced survival compared to JAK2^{V617F} alone

Before starting this master thesis, the lab generated a new compound mouse by crossing the JAK2^{V617F} *Vav1*-Cre mouse model (Marty *et al.*, 2013) with the hSTAT5B transgenic mouse, and observed that these mice succumb to the PV-like disease earlier than JAK2^{V617F} alone mice (unpublished data, see Figure 7). An further increase in spleen size was also observed. This led us to the assumption that higher levels of STAT5B in patients might lead to a similar effect in human patients. More in depth characterization of this mouse model was required, as well as determining consequences of hSTAT5B expression in the context of the JAK2^{V617F} mutation, thus explaining the shorter life span of the JAK2^{V617F}/hSTAT5B compound mice.

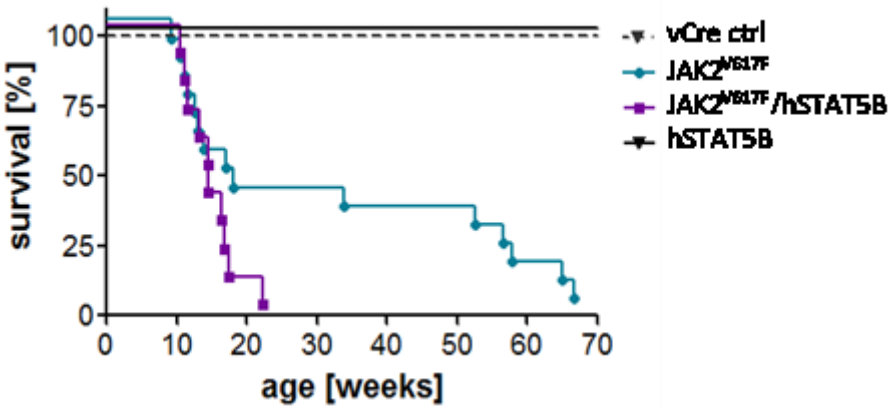

Figure 7: *Survival plot of mice of all four genotypes.* (adapted from B. Maurer, unpublished)

1.5.1. Aim of the master thesis

The aim of the master thesis was to investigate the role of STAT5B in JAK2^{V617F}-positive myeloproliferative neoplasms using two different model systems as well as patient samples, notably assessing the involvement of STAT5B in the development of thromboembolisms.

For this we:

- processed JAK2^{V617F}/STAT5B mice and the relevant control mice to collect data on organ weight and blood parameters. Organs were also collected and processed to allow for immunohistochemistry analysis as well as protein expression analysis. Stem cells from the bone marrow were cultivated and stimulated to compare differentiation capacities.
- analyzed the expression levels of STAT5A and STAT5B in seven different myeloid leukemia cell lines by Western blot and qPCR analysis.
- overexpressed STAT5A or STAT5B in three MPN-like myeloid leukemia cell lines, two of which harbor the JAK2^{V617F} mutation, and analyzed cell growth to characterize the effects of STAT5 overexpression compared to empty vector controls.
- collected and processed peripheral blood samples from MPN patients and determined their STAT5A and STAT5B expression levels by Western blot and qPCR analysis to be able to find correlations to disease severity.

The methods used in this study are standard as well as state-of-the-art molecular biology and cell culture techniques.

STAT5 is a crucial player in MPN disease mechanism but whether it is STAT5A or STAT5B that is more important remains unclear. It is also unknown if different levels of these proteins might have an influence in disease progression and morbidity, such as higher incidences of thromboembolisms, in patients. Since current MPN treatment options are non-curative it is important to further our molecular understanding of these diseases to facilitate the generation of new targeted molecules to find better treatment options.

2. Materials and Methods

2.1. Materials

2.1.1. Reagents and buffers used for qPCR analysis

RNA extraction kit
Allprep DNA/RNA MiniKit (Qiagen)

cDNA synthesis kit
First strand cDNA Synthesis Kit (Thermo Scientific)

qPCR MasterMix
GoTaq qPCR MasterMix 2x (Promega)

qPCR primers

Table 1: List of primer sequences used for qPCR analysis.

name	target	sequence	origin
hSTAT5A_1 RT fw	hSTAT5A	5' GGCTCCCTATAACATGTACCC 3'	(Warsch *et al.*, 2011)
hSTAT5A_1 RT rev	hSTAT5A	5' AAGACTGTCCATTGGTCGGCG 3'	(Warsch *et al.*, 2011)
hSTAT5A_2 RT fw	hSTAT5A	5' GAG AAC ACC CGC AAC GAG TG 3'	designed by me
hSTAT5A_2 RT rev	hSTAT5A	5' GTCAGCACGCTTGATCCTCTTC 3'	designed by me
hSTAT5B_1 RT fw	hSTAT5B	5' GATCAAGCAAGTGGTCCC 3'	(Warsch *et al.*, 2011)
hSTAT5B_1 RT rev	hSTAT5B	5' CCAGATCGAAGTCCCCATCGG 3'	(Warsch *et al.*, 2011)
hSTAT5B_2 RT fw	hSTAT5B	5' GCCGA GCG AGA TTG TAA ACC ATG 3'	designed by me
hSTAT5B_2 RT rev	hSTAT5B	5' CAT GCT TGG CTT TCA ATC CAC TGG 3'	designed by me
GAPDH fw	hGAPDH	5' CAAGGTCATCCATGACAACTTTG 3'	Thermo Scientific cDNA synthesis kit
GAPDH rev	hGAPDH	5' GTCCACCACCCTGTTGCTGTAG 3'	Thermo Scientific cDNA synthesis kit

2.1.2. Reagents and solutions for Western blot work

IP-buffer with inhibitors (added freshly) for protein extraction

25 mM HEPES pH 7.5
25 mM Tris/HCl pH 7.5
150 mM NaCl
10 mM EDTA
0.1% Tween-20
0.5% NP-40
1 mM Na_3VPO_4
1 mM NaF
10 µg/mL Leupeptin
10 µg/mL Aprotinin
1 mM PMSF
1x cOmplete™ Protease Inhibitor Cocktail (Roche)

Loading buffer, 6x
1.6 % SDS
20mM Tris, pH 6,8
16% glycerol
0.24 g/mL bromphenol blue
0.04 g/mL Dithiothreitol
in ddH_2O

Stacking gel 5%
5% acrylamide mix
130 mM Tris/HCl, pH 6.8
1% SDS
0.1% Ammonium persulfate (APS)
0.001% TEMED

Running gel 8%, for 5 mL
2.3 ml ddH2O
8% acrylamide mix
390 mM Tris/HCl, pH 8.8
1% SDS
0.1% APS
0.06% TEMED

10x Tris-Glycine Buffer
0.25 M Tris
1.92 M Glycine

1x Running buffer
1:10 dilution of 10x Tris/Glycine buffer
0.5% SDS

Transfer Buffer - Trans-Blot® Turbo™ Transfer System
Prepared according to manufacturer's instructions (Bio-Rad)

10x Tris buffered saline solution (TBS)
500 mM Tris, pH 7.9
1.5 M NaCl (Roth)
1% Tween® – 20 (Sigma)

1x TBS-T
1:10 dilution of 10x TBS
0.1% Tween® – 20 (Sigma)

Blocking buffer
Odyssey® Blocking Buffer in PBS (Li-cor®)

Table 2: List of primary Antibodies used in this study

Antibody	Company	Cat. #	Dilution	Size in kDa	Host species
HSC70	Santa Cruz Biotechnology	SC7298	1:10 000	70	mouse
STAT5A (C-6X)	Santa Cruz Biotechnology	SC271542X	1:5 000	90.6	mouse
STAT5B (G-2x) SCB	Santa Cruz Biotechnology	SC1656X	1:5 000	89.8	mouse
STAT5B 5077	In house #5077, C-terminal	-	1:1 000	89.8	rabbit
pYSTAT5 Y694	Cell Signaling Technology	C71E5 #9314	1:1 000	90	rabbit
STAT5	BD biosciences	#610191	1:1 000	90	mouse
Anti-FLAG M2	Sigma	F1804	1:5 000	-	mouse

Table 3: List of secondary Antibodies used in this study

Antibody	Company	Target	Dilution	Host species
IRDye® 680RD Goat anti-Mouse IgG	LI-COR™	anti-mouse	1:10 000	goat
IRDye® 800CW Goat anti-Mouse IgG	LI-COR™	anti-mouse	1:10 000	goat
IRDye® 800CW Goat anti-Rabbit IgG	LI-COR™	anti-rabbit	1:10 000	goat

2.1.3. Reagents and solutions used with cell lines

Cultivation medium - cell lines

Advanced RPMI 1640 Medium (Gibco™)
10% fetal bovine serum, FBS (Gibco™)
1% L-Glutamine (Gibco™)
1% Penicillin-Streptomycin Solution, 100x (Biowest)
+ 6 ng/mL hGM-CSF (ImmunoTools) for TF-1

Phosphate buffered saline solution (PBS)

PBS (Gibco™)
pH 7.4

Nucleofection buffer
5 mM KCl
15 mM $MgCl_2$
120 mM Na_2HPO_4/NaH_2PO_4
50 mM succinic acid
pH adjusted to 7.2 with HCl

MTS – cell proliferation assay
Solutions prepared according to CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay protocol (Promega)

2.1.4. Reagents and solutions used for work with primary mouse cells

Cultivation medium for mouse LSKs

GMP SCGM Stem Cell Growth Medium (CellGenix®)
5% SCF in media (kindly gifted by S. Kollmann, Sexl group, Vetmeduni Vienna)

ACK lysis buffer
150 mM NH_4Cl
10 mM $KHCO_3$
0.1 mM Na_2EDTA
pH 7.4

Megakaryocyte differentiation medium with TPO

GMP SCGM Stem Cell Growth Medium (CellGenix®)
5% SCF in media (kindly gifted by S. Kollmann)
100 ng/mL TPO (ImmunoTools)

Cytokines used for MethoCult™ colony assay

Table 4: Cytokine concentrations used for MethoCult colony assays

cytokine	Final concentration		origin
rm SCF	1	x	Gifted by S. Kollmann
rm IL-3	10	ng/mL	ImmunoTools
rh Il-6	10	ng/mL	ImmunoTools
rh EPO	20	ng/mL	ImmunoTools

2.1.5. Reagents and solutions for agarose gel electrophoresis

50x TAE buffer, 1L
2 M Tris
5.7% acetic acid
0.5 M EDTA; pH 8.0 (AppliChem)

Agarose gel
1% agarose
1x TAE
2 µl Atlas DNA stain

Loading dye, 6X
DNA Loading Dye (Thermo Fisher Scientific)

DNA ladder
Gene Ruler 100 bp Plus (Thermo Fisher Scientific)

2.1.6. Reagents and solutions for PCR analyses

Primers used for PCR amplification and sequencing

Table 5: Primer sequences used for PCR amplification and sequencing of the JAK2^{V617F} fragment

name	Sequence (5' → 3')	origin
h_JAK2^{V617F} fw	TGCTGAAAGTAGGAGAAAGTGCAT	(Krämer *et al.*, 2007)
h_JAK2^{V617F} rev	TCCTACAGTGTTTTCAGTTTCAA	(Krämer *et al.*, 2007)

MasterMix for PCR

Table 6: Reaction mix for PCR amplification of sequencing fragment

Reagent	1 reaction
10x Roche buffer	1x
$MgCl_2$ 100 mM	1.6 mM
dNTPs 10 mM	0.8 mM
Fw/rev primer 10µM	0.1 µM
Taq polymerase (Roche)	1.5 U
DNA	0.5 µg/µl

PCR purification kit

MiniElute PCR purification kit (Qiagen)

2.1.7. Plasmids used for nucleofection of cell lines

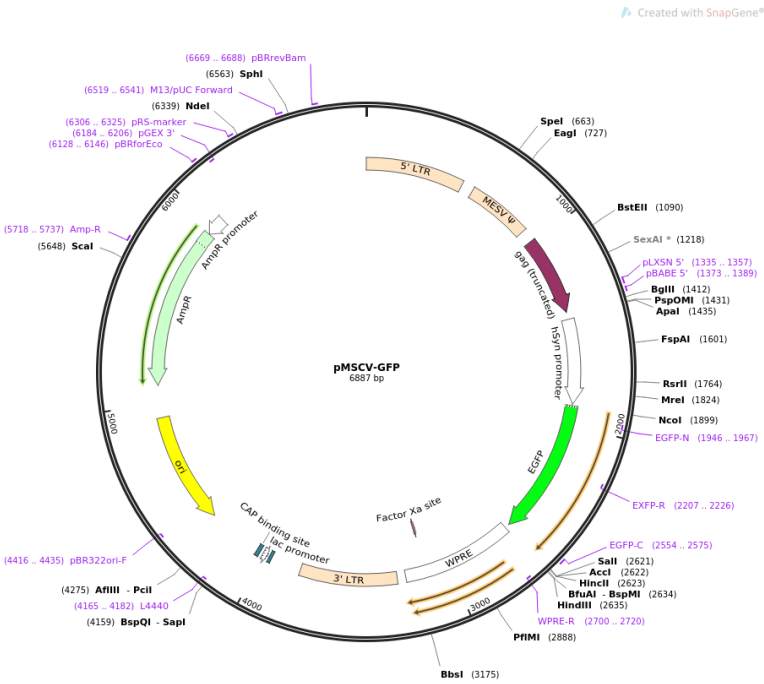

Figure 8: *pMSCV plasmid map*. (Addgene: pMSCV-GFP, no date)

Inserts:

hSTAT5A (NM_001288718, Gene ID:6776)

hSTAT5B (Gene ID:6777)

both with a C-terminal FLAG tag (DYKDDDDK)

2.1.8. Reagents for FACS analysis

Table 7: List of FACS antibodies used in this study

Marker	Fluorophore	Clone	Target species	Company
TER-119	Pacific Blue	TER-119	mouse	ThermoFisher Scientific
CD11b	Pacific Blue	M1/70	mouse	ThermoFisher Scientific
CD3e	Pacific Blue	eBio500A2	mouse	ThermoFisher Scientific
Ly6G (Gr-1)	Pacific Blue	RB6-8C5	mouse	ThermoFisher Scientific
CD45R (B220)	Pacific Blue	RA3-6B2	mouse	ThermoFisher Scientific
Ly6A/E (Sca-1)	FIT-C	D7	mouse	ThermoFisher Scientific
CD117 (c-Kit)	APC	2B8	mouse	ThermoFisher Scientific
Ly6A/E (Sca-1)	PE Cy7	D7	mouse	ThermoFisher Scientific
CD117 (c-Kit)	PerCP Cy 5.5	2B8	mouse	ThermoFisher Scientific
CD41	PE	HIP8	mouse	BioLegend
CD150	APC	mShad150	mouse	ThermoFisher Scientific
Viability stain	APC-Cy7	-	-	eBioscience

2.1.9. Reagents and solutions used to process patient samples

Dextran solution
1xPBS
5.5% dextran
1.5% EDTA
Sterile filtered

NaCl solution, physiologic
ddH20
0.9% NaCl

2.2. Methods

2.2.1. Cultivation of human cell lines

All seven cell lines (KU812, K562, MV4-11, MOLM-13, TF-1, HEL, SET-2) were brought up from frozen vials at the start of the Master thesis and tested for mycoplasma using a MycoAlert™ mycoplasma detection kit (Lonza). Cells were usually maintained at a concentration at or below $1x10^6$ cells/ml in cultivation media at 37°C with 5% CO_2 in a humidified environment. TF-1 cells were always grown in the presence of hGM-CSF.

2.2.2. Harvesting of human cell lines

For subsequent protein or RNA isolation cells were harvested, by washing them three times with ice-cold PBS and centrifugation at 400 x g at 4°C. As much supernatant as possible was removed before snap-freezing the pellets in liquid nitrogen. The pellets were stored at -80°C until further use.

2.2.3. RNA extraction from human cell lines and patient samples

The following procedure was employed with both the human cell line samples and the patient samples: RNA was extracted from the cell pellets using the Allprep DNA/RNA MiniKit (Qiagen) and following the manufacturer's instructions. RNA concentration was measured using the Tecani-control infinite 200 Pro by Nanodrop™.

2.2.4. Protein extraction from human cell lines and patient samples

The following procedure was employed with both the human cell line samples and the patient samples:
Protein was extracted by suspending the cell pellet in 50-200 µL ice-cold IP-buffer and rotating for 30 min at 4°C. The vials were then centrifuged at 17,000 x g for 20 min at 4°C and the supernatant containing the proteins was transferred into a fresh vial. The samples were snap frozen in liquid nitrogen and stored at -80°C until further use. Protein concentrations were measured by Bradford assay (described below).

2.2.5. Bradford assay

To generate a standard curve, a BSA (bovine serum albumin) solution with a concentration of 1 µg/ml was added to 1 ml of a 1:5 dilution of Bradford reagent (Protein Assay Dye Reagent Concentrate, Bio-Rad) in increasing amounts. The expected concentrations of the standards spanned from 1 µg/ml to 16 µg/ml. 1 µl of IP-buffer was added to each standard and additionally was used alone as a blank. 1 µl of sample was added to 1 ml of diluted Bradford reagent and the absorbance was measured with a BioPhotometer plus (Eppendorf) at 595 nm. The protein concentration was determined by using the linear equation of the BSA standard curve.

2.2.6. Patient samples

Peripheral blood samples of MPN patients or healthy controls collected in EDTA tubes were kindly provided by the Valent group of the AKH Vienna. The study was approved by the ethics review board of the Medical University of Vienna (1810/2018) and all patients gave informed consent for use of their tissue (1184/2014). Processing of the samples was performed on the same day as collection.

2.2.7. Mononuclear cell isolation – Ficoll density gradient centrifugation

All the centrifugation steps of the following protocol were performed at room temperature (RT). The peripheral blood sample was diluted 1:1 in physiologic NaCl solution. The diluted sample was layered over 3 parts Ficoll (Biocoll Seperating Solution, Biochrom) to 7 parts diluted sample in a 50 ml Falcon® tube. The tube was centrifuged at 1800 rpm without brakes for 30 min. The ring of mononuclear cells which formed at the interface of the two phases was removed and transferred into a fresh tube. The tube was then filled carefully with NaCl solution to 50 ml and centrifuged at 1920 rpm for 10 min. The supernatant was then removed and the cells vortexed and incubated in 5 ml ACK lysis buffer to remove erythrocytes for 10 min at RT. To stop the lysis, the tube was filled again with NaCl solution and centrifuged at 920 rpm for 15 min. The supernatant was removed, and the tube filled to 50 ml with NaCl solution and centrifuged at 1450 rpm for 5 min. After this last wash step, the supernatant was discarded, and the cell pellet resuspended in 2 ml NaCl and split into two Eppendorf tubes. These were centrifuged at 400xg for 5 min. After removal of the supernatant the cell pellets were snap frozen in liquid nitrogen and stored at -80°C until further use.

2.2.8. White blood cell isolation – Dextran sedimentation

All the centrifugation steps of the following protocol were performed at RT. The peripheral blood sample was mixed at a ratio of 1:1 in physiologic NaCl solution. The dextran solution was added to the diluted sample at the following ratio – blood : NaCl solution : dextran solution = 1:1:0.5. The thusly obtained solution was then split equally into two 14 ml Falcon® tubes with special care to avoid any bubble formation. After 1.5 h of gravity sedimentation the upper phase was transferred into fresh 50 ml Falcon® tubes. The tubes were filled to 50 ml with NaCl solution and centrifuged at 1920 rpm for 10 min. The supernatant was then discarded. This washing procedure was repeated once with a centrifugation speed of 980 rpm for 15 min and once at 1450 rpm for 5 min. The cell pellet which was obtained after these washes was resuspended in 2 ml of NaCl solution and split into two Eppendorf tubes. These were then centrifuged at 400 x g for 5 min. After removal of the supernatant the cell pellets were snap frozen in liquid nitrogen and stored at -80°C until further use.

2.2.9. Western blot

The protein extracts were mixed with loading buffer and boiled at 95°C for 5 min. The samples were then loaded onto the gel and the electrophoresis run for 1 h at 120 V. The blotting onto the nitrocellulose membrane was performed using the Trans-Blot Turbo Blot System by Bio-Rad. The membrane was then incubated in Odyssey® Blocking Buffer for 1 h at RT on a shaker. The primary antibody was then added in respective dilutions (Table 2: List of primary Antibodies used in this study and left to incubate for 1 h at RT on a shaker. Before adding the secondary antibody, the membrane was washed three times for 5 min with TBS-T. The membrane was then incubated with the secondary antibody for 1 h at RT, and subsequently washed.

Detection was performed with the Odyssey® Imaging System. Quantification was performed with the Image Studio™ Lite software.

2.2.10. qPCR analysis

cDNA was synthesized using 1 µg of RNA and the First strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. The cDNA solution was diluted 1:5 with nuclease-free water. 2 µl of cDNA sample was pipetted into a 96-well plate and 8 µl of Master mix (Table 8) to each well. The qPCR was performed using the Eppendorf RealPlexMastercycler© with the program listed in Table 9. GAPDH (Glyceraldehyde-3-phosphate-Dehydrogenase) was used as an internal control. For the normalization of the values the "ΔΔCt-Method" was employed, where the Ct value of each sample is normalized to the Ct value of GAPDH.

Table 8: Reaction mix for qPCR analysis

reagent	amount for 1 reaction [µl]
GoTaq qPCR MasterMix 2x	5
fw primer 10 µM	0.4
rev primer 10 µM	0.4
nuclease-free water	2.2

Table 9: qPCR program

Temperature [°C]	Time [s]	
95	120	
95	15	} 40 cycles
60	60	
melting curve: 65°C – 95 °C, 5 min		

2.2.11. Generation of stable over-expressor cell lines by nucleofection of human cells

$1x10^6$ cells were pelleted at 90 x g for 10 min and washed once with medium containing no antibiotics. After the second centrifugation step, all supernatant was removed, and the cells were resuspended in 100 µl nucleofection buffer. The cell suspension was transferred into an electroporation cuvette (2 mm) and 30 µg plasmid DNA added. The cells were then electroporated with the Amaxa I Nucleofector using the program X-05. 0.5 ml of pre-warmed medium with 20% FBS was added to the cuvette and the cells transferred to a 24-well plate containing 1 ml of medium with 20% FBS using a plastic Pasteur pipette. After 24 h the cells were expanded into a larger volume of media and analysed by FACS for their GFP expression.

To select for cells which incorporated the plasmid into their genomes, the cells were sorted three times for GFP expression over the course of several weeks. The increased expression of either STAT5A or STAT5B was confirmed by FLAG Western blot analysis.

2.2.12. MTS proliferation assay

Cells were seeded in triplicate into a 96-well plate in 100 µl per well at a density of 50,000 cells/ml for HEL and 62,500 cells/ml for TF-1 in normal culture media, and cultivated under standard conditions for various lengths of time. The reagents for the assay were prepared as described in the manufacturers protocol (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega). After the addition of 20 µl per well of the freshly prepared MTS/PMS solution, the cells were incubated for 2.5 h at 37°C in the dark. The absorbance was then measured on a plate reader at 490 nm.

2.2.13. Sequencing of *JAK2*^{V617F} loci of TF-1, HEL and SET-2 cells

gDNA isolation

Genomic DNA (gDNA) was extracted from the cell pellets using the Allprep DNA/RNA MiniKit (Qiagen) and following the manufacturer's instructions. DNA concentration was measured using the Tecani-control infinite 200 Pro by Nanodrop™.

PCR amplification of the *JAK2*^{V617F} fragment

25 ng gDNA were mixed in a 96-well plate with the reagents needed for the PCR according to Table 6. The reaction was performed with the protocol stated in Table 10 in an Eppendorf Master Cycler S. After the PCR, the amplified fragment was purified with the MiniElute PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

Table 10: Protocol for the PCR for *JAK2*^{V617F} loci sequencing

temperature [°C]	time [s]	
94 °C	120	
94 °C	30	35 cycles
52 °C	40	
72 °C	40	
72 °C	120	

Agarose gel electrophoresis

After purification, the samples were loaded onto an agarose gel (1x TAE, 1% agarose, 0.5 µg/ml Atlas DNA stain) and the electrophoresis was run for 40 min at 100 V. The gels were imaged by exposing them to UV light.

Sanger sequencing

2.5 ng/µl of the PCR product was mixed with either forward or reverse sequencing primers (Table 5) and sent to the Sanger sequencing facility at the Institute for Molecular Pathology (IMP), Vienna. The results were visualized and analysed using the ApE software.

2.2.14. Animals

Mouse colonies were kept at the University of Veterinary Medicine, Vienna under standardized, specific pathogen-free conditions. All experiments were carried out under ethical license protocols that were approved by the University of Veterinary Medicine and the Austrian Federal Ministry for Science and Research. For all experiments, C57BL/6N mice were used. Mice harboring a hematopoietic compartment specific knock-in of $JAK2^{V617F}$ (referred to as $JAK2^{V617F}$ mice) were generated previously by using the Cre/loxP system. (Marty *et al.*, 2013) Mice carrying a floxed $JAK2^{V617F}$ allele were crossed with mice expressing Cre recombinase under the *Vav1* promoter. hSTAT5B transgenic mice were generated previously by the Biomodels Austria platform using the *Vav1-hCD4* plasmid as previously described. (Pham *et al.*, 2018) hSTAT5B is hence also expressed under the *Vav1*-promotor.

To generate $JAK2^{V617F}$/hSTAT5B mice, mice with a floxed $JAK2^{V617F}$ allele were crossed to mice expressing both the *Vav1-Cre*-recombinase and hSTAT5B (all heterozygous). This resulted in mice of all four genotypes (Cre ctl, hSTAT5B, $JAK2^{V617F}$, $JAK2^{V617F}$/hSTAT5B). Unless stated otherwise, experiments were performed with 12-week-old mice.

2.2.15. Blood analysis from mice

Mice were anesthetized by intraperitoneal injection of ketamine:xylazine:PBS solution (3:3:4) before blood was collected via terminal heart puncture and transferred to EDTA-coated tubes. Blood parameters were determined with a scil Vet ABC™ Hematology Analyzer.

2.2.16. Harvesting of mouse LSK cells and differentiation assays

Mice were sacrificed and bone marrow cells were extracted by crushing tibia and femora with a mortar, washing them with ice-cold PBS and resuspending them as single cells. Using the MagniSort™ Mouse Hematopoietic Lineage Depletion Kit (Invitrogen), with only one round of purification, Lin^- cells were enriched. 2000 cells were stained using antibodies according to Table 11, and sorted by FACS. Lin^- $Sca1^+$ $cKit^+$ (LSK) cells were put into cultivation medium in 24-well plates either with or without SCF or into differentiation medium with TPO+SCF at 37°C with 5% CO_2 in a humidified environment. Cytokines were replenished every three to four days. After 9 days cells were stained using antibodies according to Table 12 and analyzed on

a FACS Canto II flow cytometer. On the same day the number of differentiated megakaryocytes per well was determined manually with an inverted light microscope.

Table 11: LSK staining panel

Antibody name/target	Fluorophore	Target species
TER-119	Pacific Blue	mouse
CD11b	Pacific Blue	mouse
CD3e	Pacific Blue	mouse
Ly6G	Pacific Blue	mouse
B220	Pacific Blue	mouse
Sca-1	FIT-C	mouse
c-Kit	APC	mouse
Live/dead	APC-Cy7	-

Table 12: Megakaryocyte progenitor staining panel

Antibody name/target	Fluorophore	Target species
TER-119	Pacific Blue	mouse
CD11b	Pacific Blue	mouse
CD3e	Pacific Blue	mouse
Ly6G	Pacific Blue	mouse
B220	Pacific Blue	mouse
Sca-1	PE Cy7	mouse
c-Kit	PerCP Cy 5.5	mouse
CD41	PE	mouse
CD150	APC	mouse
Live/dead	APC-Cy7	-

2.2.17. Colony assay

Mice were sacrificed and bone marrow cells were extracted by crushing tibia and femora with a mortar, washing them with ice-cold PBS and resuspending them as single cells. Spleens were extracted and smashed through a 70 µm strainer to obtain a single cell suspension. To lyse red blood cells (RBC), ACK buffer was applied for 10 min on ice. Lysis was stopped by topping up the tube with cold PBS. Cells were employed for colony assays using MethoCult™. The assay was performed according to the technical manual for Mouse Colony-Forming Unit (CFU) Assays Using MethoCult™ from Stemcell Technologies™. 25,000 bone marrow cells and 20,000 splenic cells per 3 cm plate were plated in triplicates in MethoCult™ (M3234) containing the cytokines stated in Table 4, and cultured at 37°C with 5% CO_2 in humidified conditions. Total colony numbers were determined after 9 days using an inverted light microscope.

2.2.18. Histology

Whole body perfusion with PBS was performed after CO_2 euthanasia of mice. The lungs were fixed in formaldehyde (4%) overnight at 4°C. They were then transferred to a 70% ethanol solution and later dehydrated and embedded in paraffin according to standard procedure. Sections were cut to a thickness of 3-4 µm. Hematoxylin & Eosin (H&E) staining was performed with the Raymond A. Lamb Stain Matestainer according to standard protocols.

2.2.19. Statistics

For statistical analysis (unless otherwise stated), one-way analyses of variance (ANOVA) were performed using the GraphPad Prism 5 software. ANOVAs were combined with Bonferroni's correction post-test.

3. Results

3.1. STAT5A and STAT5B in human myeloid leukaemia cell lines

We wanted to investigate if and how STAT5A and STAT5B levels might contribute differently to MPNs and if $JAK2^{V617F}$ directly affects their expression levels. For this we selected seven human myeloid leukaemia cell lines which all depend on STAT5 activation for survival. Three of these cell lines were established from MPN patients. Two of them are $JAK2^{V617F}$ positive (HEL, SET-2) and one cell line (TF-1) has an unknown driver. Two AML (MV 4-11, MOLM-13) and two CML (KU812, K562) cell lines were selected in order to compare $JAK2^{V617F}$ with other driver tyrosine kinases (Table 13).

Table 13: Human leukaemia cell lines used in this study (Lozzio *et al.*, 1981; Martin and Papayannopoulou, 1982; Chen, 1985; Kishi, 1985; Lange *et al.*, 1987; Kitamura *et al.*, 1989; Blom *et al.*, 1992; Matsuo *et al.*, 1997; Uozumi *et al.*, 2000)

cell line	disease	origin	mutations	cytokine dependency
TF-1	Erythroleukemia	heparinized bone marrow aspiration sample from a 35-year old Japanese male with severe pancytopenia	homogeneous chromosomal abnormality (54,X); truncated EpoR overexpressed	completely dependent on IL-3 or GM-CSF
HEL	PV/Erythroleukemia	established from the peripheral blood of a 30-year-old man	$JAK2^{V617F}$ (homozygous)	independent
SET-2	ET	megakaryoblastic cell line, from peripheral blood of a patient with leukemic transformation of ET	$JAK2^{V617F}$ (heterozygous)	independent
MV 4-11	AML/ B-myelomonocytic leukemia	blast cells of a 10-year-old male, peripheral blood	FLT3 ITD	independent
MOLM-13	AML/MLL	established from the peripheral blood of a 20-year-old man, blast cells	FLT3 ITD; CBL delta Exon 8, MLL-AF9 fusion mRNA, SETD2	independent
KU812	CML	peripheral blood of a patient in blast crisis	BCR-ABL	independent
K562	CML	pleural effusion of a 53-year-old female with chronic myelogenous leukemia in terminal blast crises	BCR-ABL; mutated TP53, MLH1, ASXL1 and BRCA1,	independent

3.1.1. Confirming the mutational status of the MPN cell lines

To ensure that our MPN cell lines harboured the JAK2^{V617F} mutation as reported in the literature, DNA was isolated from all three cell lines. PCR of the fragment containing the locus of the mutation was performed several times to increase purity. The samples were then ridded of any remaining reagents and submitted for sequencing. The mutation results in a nucleotide exchange of guanine to a thymine at the position 2343 and subsequently an exchange of valine for phenylalanine. We sequenced both the coding and non-coding strand and obtained the best results on the non-coding strand. Thus, we expected a signal for cytosine in case of a wild-type (wt) *JAK2*, a double signal for cytosine and adenine for heterozygous *JAK2*^{V617F} and a single adenine signal for homozygous *JAK2*^{V617F}. As can be seen in Figure 9, we were able to confirm the mutational status reported in the literature of all three cell lines: TF-1 had only wt *JAK2*, HEL was heterozygous for the mutation and SET-2 homozygous. (Quentmeier *et al.*, 2006)

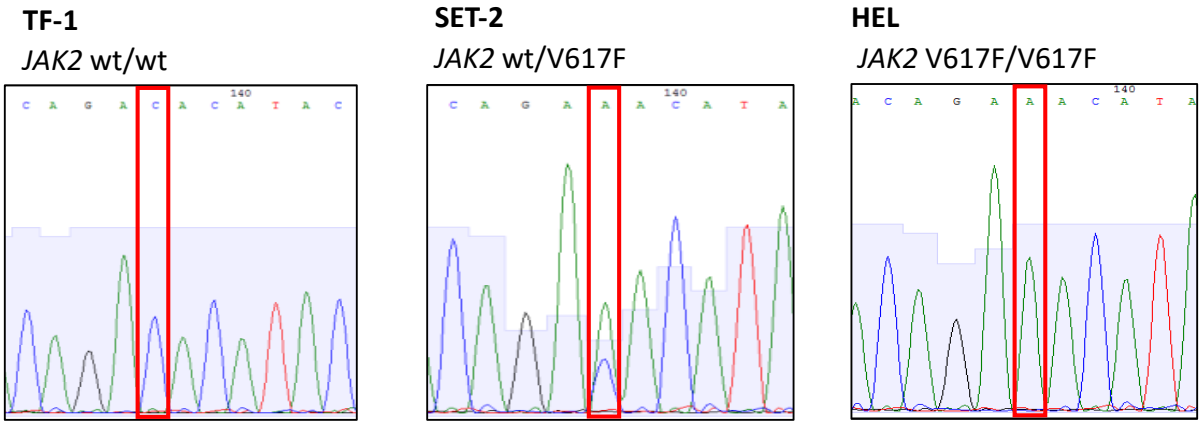

Figure 9: *Chromatograms generated by Sanger-sequencing of the three MPN-like cell lines at the JAK2*^{V617F} *locus.* PCR was performed to amplify the fragment containing the *JAK2*^{V617F} locus and the resulting DNA fragment sequenced by Sanger-sequencing. The nucleotide which is mutated in the case of JAK2^{V617F} is marked with a red box.

3.1.2. Endogenous STAT5A and STAT5B expression in seven myeloid leukaemia cell lines

To set up optimal conditions for the analysis of patient samples and to assess the basal levels of STAT5A and STAT5B in human myeloid cells at the mRNA and protein level, human myeloid leukaemia cell lines were used (Table 13). Their STAT5A and STAT5B expression was assessed via Western blot and qPCR analysis.

3.1.2.1. Validating the conditions for qPCR analysis

For the validation of our method for qPCR analysis of *STAT5A* and *STAT5B* mRNA levels, RNA was extracted from the seven human myeloid leukaemia cell lines (K562, KU812, HEL, SET-2, TF-1, MOLM-13, MV 4-11) and cDNA from these extracts generated. Initially, qPCR analysis was performed on three cell lines using two different sets of primers for each target mRNA: one with previously published sequences (Warsch *et al.*, 2011) and the other designed in this study. The primers were designed using basic online tools. Criteria for selecting a primer pair were: a GC content between 40% and 60%, a similar melting temperature for both and a low tendency to form secondary structures. The published primers yielded stronger signals (Figure 10) and were thus chosen for subsequent analyses. We then validated the specificity of the primers for each gene product by performing a qPCR analysis of RNA extracted from 32D cells, a myeloblast-like mouse cell line, that additionally expresses either hSTAT5A or hSTAT5B. As expected, the primers targeting STAT5A yielded a strong signal in hSTAT5A-expressing cells and the same was observed for hSTAT5B (Figure 11). The primers were found to be highly specific since the signal for their respective mRNA was massively increased and no considerable signal was detected for the other gene product mRNA.

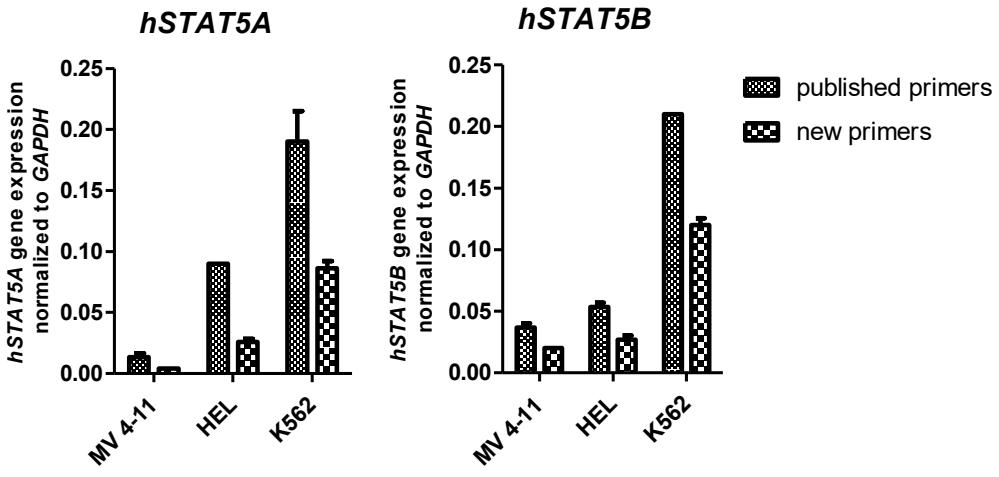

Figure 10: *Relative mRNA expression in three cell lines measured with two different primer pairs for each STAT5 variant.* Data shown is from three technical replicates. Gene expression was calculated relative to the housekeeping gene *GAPDH* and is displayed in its x-fold using the "ΔΔCt-Method". Error bars indicate the standard error of the mean.

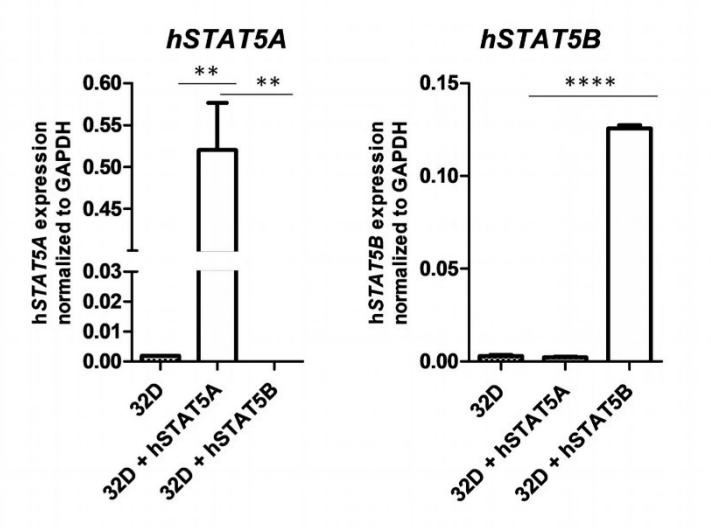

Figure 11: *mRNA expression of the human STAT5 variants in murine 32D cells with transgenic expression of either hSTAT5A or hSTAT5B relative to GAPDH.* Data shown is pooled from three technical replicates. Error bars indicate the standard error of the mean. ** = p-value < 0.01, **** = p-value < 0.0001.

3.1.2.2. Results of the qPCR analysis of all seven cell lines

The above-mentioned primers were then used to investigate the basal levels of STAT5A and STAT5B mRNAs in cell lines of MPN-like versus other leukemic diseases. The lowest levels were detected in the two AML cell lines (MV 4-11, MOLM-13) and the highest in the CML cell line K562 (Figure 12). Overall the qPCR analysis yielded good results with reasonable signal intensity and it was decided to keep the same conditions for the later analysis of patient samples. Interestingly, the trend of expression of STAT5A resembled the one of STAT5B in all cell lines, with the CML cell lines showing the highest and the AML cell lines lowest expression levels of both gene products. The other three cell lines (TF-1, HEL, SET-2), which should be representative of cells from MPN patients since they either carry the $JAK2^{V617F}$ mutation and were derived from MPN patients or were taken from patients with erythroleukemia, showed relatively intermediate expression levels. This could indicate that $JAK2^{V617F}$ does not necessarily drive the expression of higher levels of STAT5, which has been shown for BCR-ABL driven CML, since the $JAK2^{V617F}$-carrying erythroleukemia cell line (HEL) showed similar STAT5 levels as the erythroleukemia cell line which does not harbour $JAK2^{V617F}$ (TF-1). (Warsch *et al.*, 2011) The exception to this was the SET-2 cells, where STAT5 levels were higher and more similar to those in the CML cells (KU812, K562).

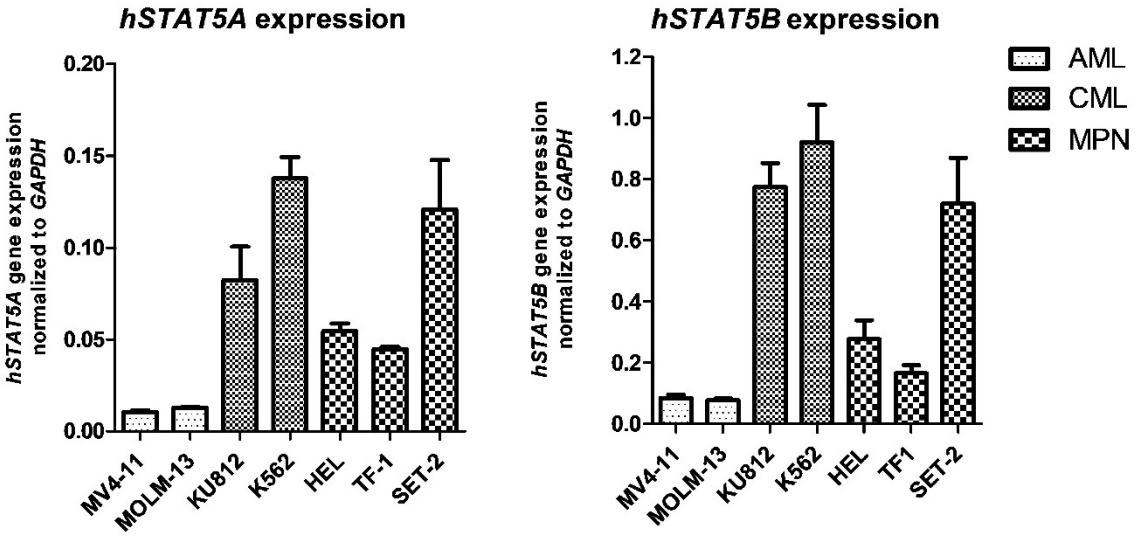

Figure 12: Relative mRNA expression of the human STAT5 variants in seven myeloid leukaemia cell lines. *Data shown is from three independent experiments. The gene expression was calculated relative to the housekeeping gene* GAPDH *and is displayed in its x-fold using the "ΔΔCt-Method". Error bars indicate the standard error of the mean.*

3.1.2.3. Validating the primary antibodies for Western blot analysis

To make sure that the primary antibodies against STAT5A and STAT5B used for the Western blot analysis were specific and sensitive enough for our purposes, protein extracts were made from MEF (mouse embryonic fibroblast) cells which had their endogenous *Stat5a/b* genes knocked out and additionally a stable overexpression of either mSTAT5A or mSTAT5B. Thus, only one STAT5 variant protein should be detectable in each extract. This was the case and the antibodies detected only their respective protein of interest (Figure 13).

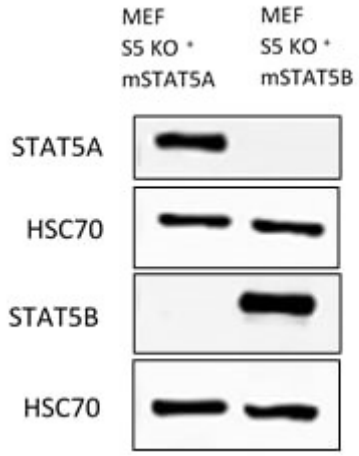

Figure 13: *STAT5A and STAT5B protein expression of MEF cells with different genotypes.* Western blot analysis of protein extracts from MEF cells which only express one of the STAT5 variants. HSC70 was used as loading control.

3.1.2.4. Results of Western blot analysis of all seven myeloid leukaemia cell lines

Western blot analysis was performed with all seven cell lines described above, and the levels of STAT5A and STAT5B protein were assessed and quantified (Figure 14). Although some differences between mRNA levels and protein expression can be anticipated, the correlation in expression from Western blots matched that from the qPCR results in nearly all cell lines. The CML cells expressed the highest level of STAT5A and STAT5B. Only very low amounts of STAT5A and STAT5B protein were seen in the two AML cell lines. In the three remaining MPN cell lines the signal was of similar intensity, again in line with what we saw at the mRNA level. Interestingly, the protein expression in the SET-2 cells was similar to that of the other two MPN cell lines contrarily to what was found at the mRNA level above. Post-transcriptional regulation might be involved in these cells. Again, this suggests that $JAK2^{V617F}$ does not affect STAT5 expression levels.

Tyrosine phosphorylation (pY) at residue Y694 (STAT5A) or Y699 (STAT5B) indicates activated STAT5, thus pYSTAT5 levels were also determined by Western blot analysis (Figure 15).

The strongest signal was seen in the cell lines which carry the $JAK2^{V617F}$ mutation (HEL, SET-2), confirming $JAK2^{V617F}$ as a strong driver for STAT5 activation. This is also supported by the presence of very low levels of activated STAT5 in the $JAK2^{V617F}$ negative cell line (TF-1). Considerable levels of activated STAT5 were found in one of the CML cell lines (KU812), which is not surprising as BCR-ABL, which is expressed in these cells, is a known driver for STAT5 activation.

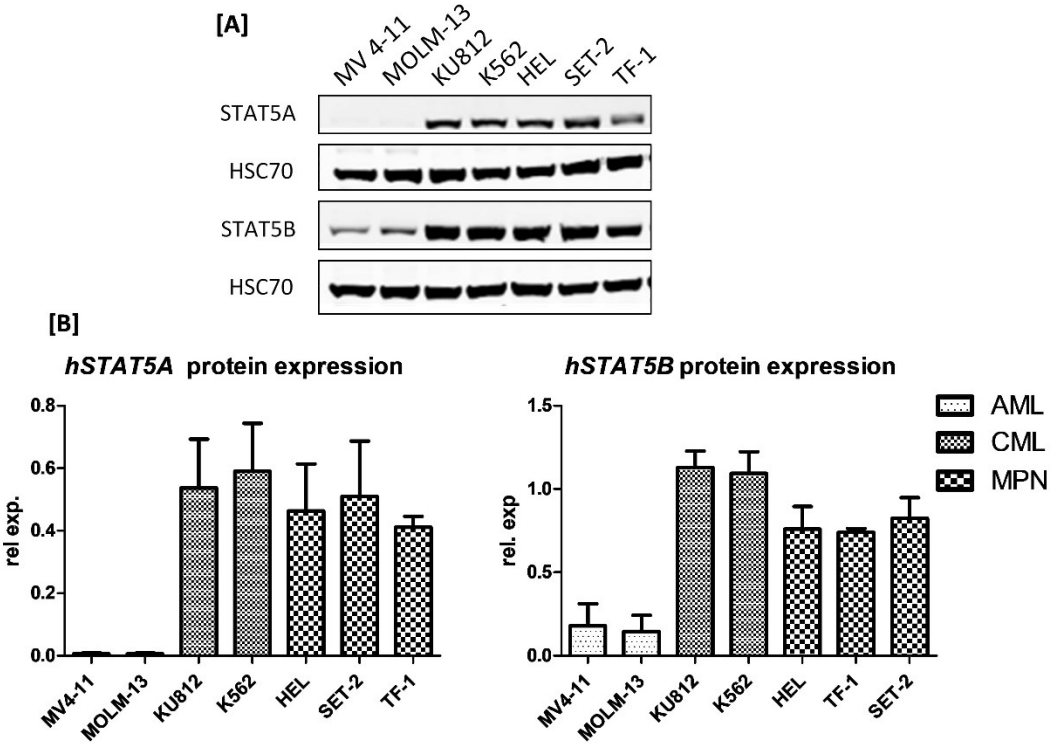

Figure 14: *Protein expression of seven myeloid leukaemia cell lines.* [A] Representative Western blot analysis of protein extracts from human myeloid leukaemia cell lines. HSC70 was used as loading control. [B] Quantification of three independent Western blot experiments with biological replicates. Error bars indicate the standard error of the mean.

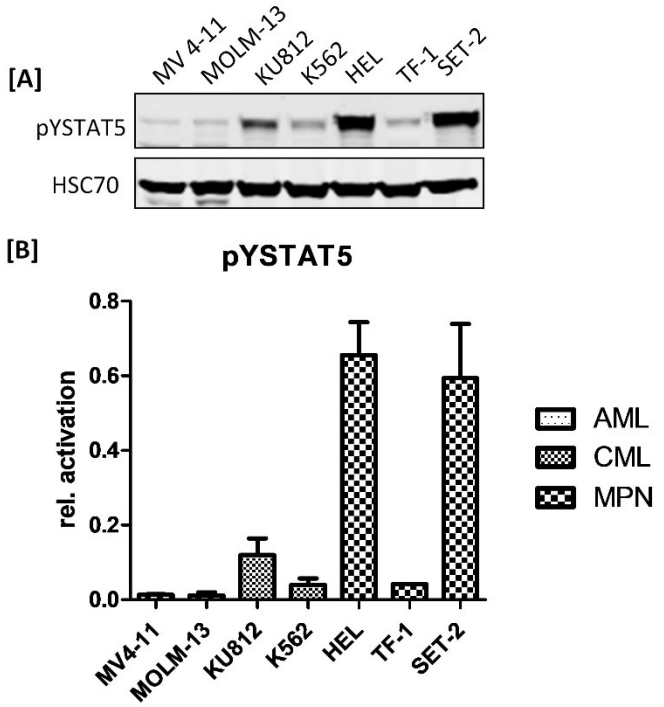

Figure 15: *STAT5 activation in seven myeloid leukaemia cell lines.* [A] pYSTAT5 Western blot analysis of protein extracts from human myeloid leukaemia cell lines. HSC70 was used as loading control. [B] Quantification of two independent Western blot experiments with biological replicates. Error bars indicate the standard error of the mean.

3.1.3. Effects of STAT5A or STAT5B overexpression on MPN cell lines

It is not known whether STAT5A or STAT5B is the major downstream effector of JAK2^{V617F}, but STAT5B is likely to be the more oncogenic sibling and its overexpression in our PV mouse model increased mortality of the mice. To investigate its effect in a human context we wanted to evaluate if the overexpression of one over the other would be of proliferative advantage to the JAK2^{V617F}+ MPN cells.

3.1.3.1. Nucleofection of the MPN cells and generation of stable over-expressor lines

For the generation of stable over-expressor lines, cells of each MPN cell line (SET-2, HEL and TF-1) were nucleofected with the pMSCV plasmid backbone containing the sequence for either hSTAT5A, hSTAT5B or only GFP as an empty vector control. The cells were then cultured in post-nucleofection medium for 24h and then analysed by FACS. Some optimization of the nucleofection conditions was necessary to achieve an efficiency of over 70% (Figure 16). Different buffers as well as different nucleofection conditions were tested. Additionally, the amount of DNA employed was varied until a satisfactory nucleofection efficiency was achieved. The cells were then cultivated and FACS-sorted on GFP positivity three times over the span of several weeks. The GFP expression was regularly monitored by FACS analysis to ensure stable expression (Figure 17). Since the SET-2 cells grew very slowly, any further analysis on them could not be performed during the time of the master study. This will be carried out in the lab in the future.

To ensure that STAT5A or STAT5B was indeed stably overexpressed, Western blot analysis of the FLAG tag or the total proteins was performed using protein extracts of these newly generated cell lines. As can be seen in Figure 18, low-level stable overexpression of the STAT5 proteins was achieved. These are physiologically-relevant levels of heightened expression that could be observed in human patients. These cell lines were then used to examine the effects of increased STAT5A or STAT5B expression in MPN cells.

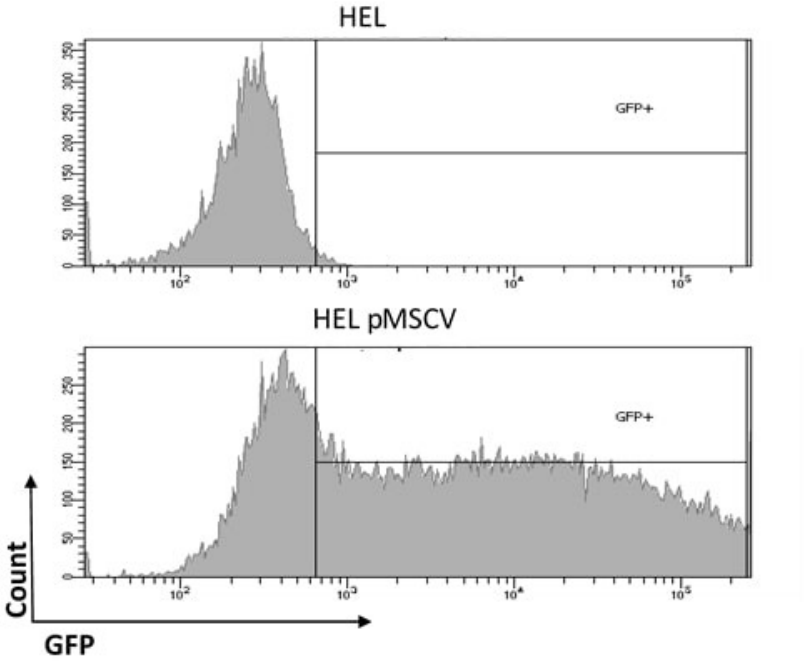

Figure 16: *GFP expression in HEL parental cells and pMSCV nucleofected HEL cells with optimized protocol.* HEL cells were nucleofected with a pMSCV vector and their GFP expression measured by FACS analysis. Representative chromatograms of cells 24h post-nucleofection.

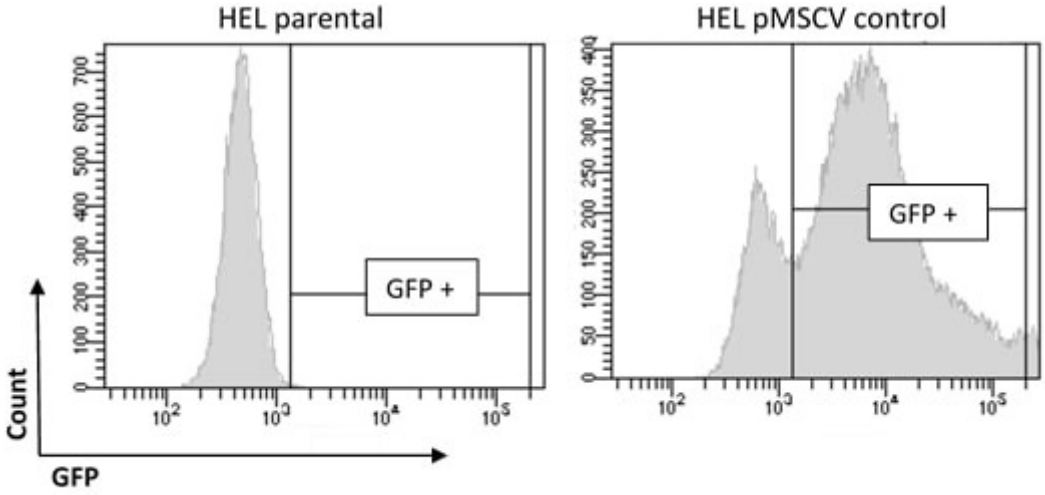

Figure 17: *GFP expression of stable cell lines.* HEL cells were nucleofected with a pMSCV vector and their GFP expression measured by FACS analysis after selecting for stably-expressing pools. Representative chromatograms of cells after three GFP sorts.

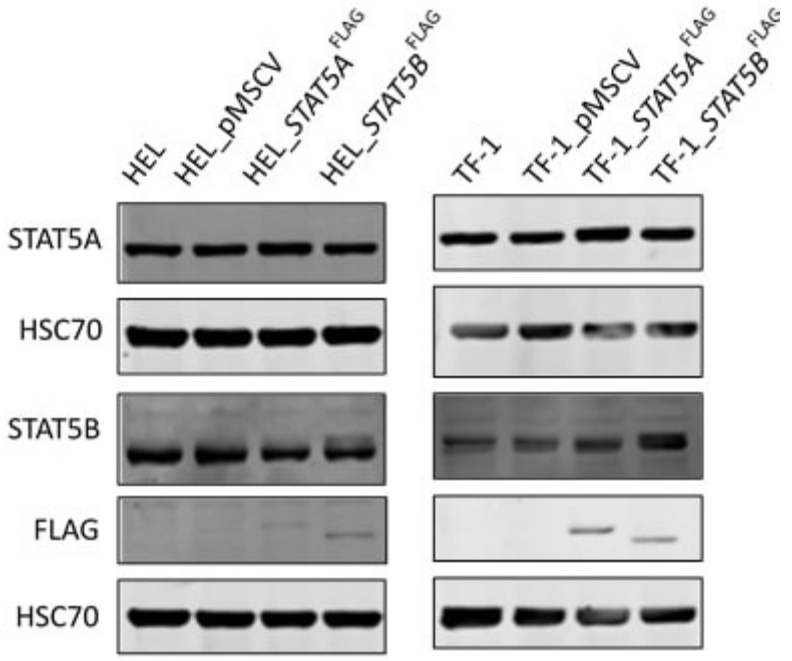

Figure 18: *Protein expression of the newly generated stable cell lines.* Western blot analysis of protein extracts from TF-1 and HEL cells of each genotype. HSC70 was used as loading control.

3.1.3.2. Proliferation assay of the stable over-expressor lines

The next step was to see if STAT5B would convey a proliferation advantage to cells over STAT5A. For this we performed an MTS assay which monitors the proliferation of the cells over several days (Figure 19). During this assay metabolically active cells turn MTS into soluble formazan. Its absorbance is then measured at 490 nm, which is directly proportional to the number of living cells in the culture. We observed a significant increase in proliferation of HEL cells with STAT5B overexpression compared to STAT5A, wt and empty vector control cell lines. This suggests that STAT5B plays a more important role in the proliferation of MPN cells compared to STAT5A. Since this was not the case for the TF-1 cell lines, which showed no differences in cell growth, it might be possible that this proliferation advantage driven by STAT5B requires the $JAK2^{V617F}$-positive background. Further analysis of the SET-2 cells would also help consolidate our hypothesis that STAT5B is the more important STAT5 variant in $JAK2^{V617F}$ positive MPNs.

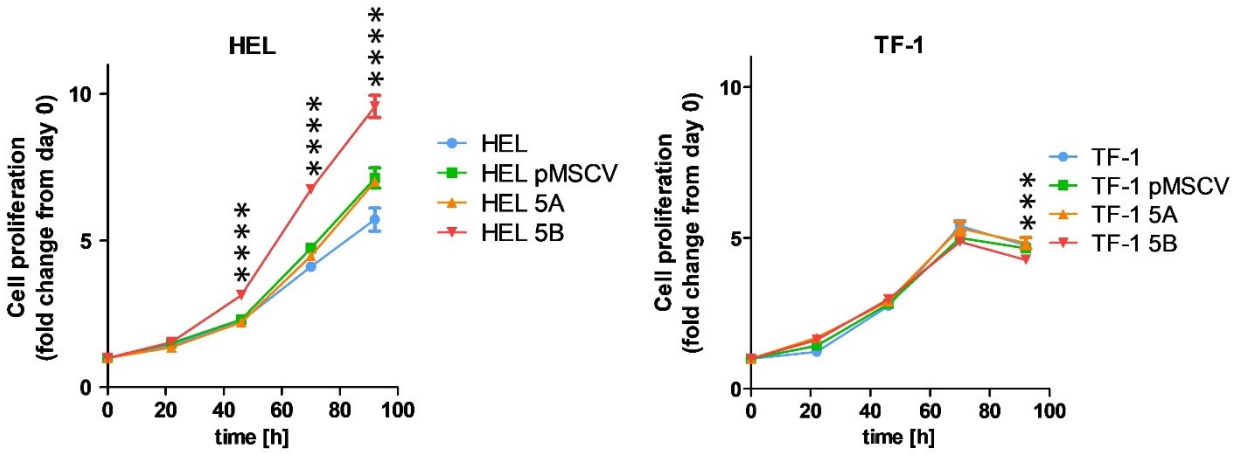

Figure 19: *Proliferation of TF-1 and HEL cell lines with overexpression of the STAT5 variants.* MTS proliferation assays were performed over 96h. Basal levels of absorption at 490 nm were measured at t=0. Graphs show data from one experiment with four technical replicates, which are representative of three independent experiments. pMSCV – empty vector control, 5A – overexpressing hSTAT5A, 5B – overexpressing hSTAT5B. Fold change was calculated relative to the basal absorption level on day 0. Error bars indicate the standard error of the mean. For statistical analysis a two-way ANOVA with Bonferroni comparisons was performed. The graphs show p-values for pMSCV vs 5B. *** = p-value < 0.001 ****=p-value < 0.0001.

3.2. Analysis of a JAK2^{V617F}/hSTAT5B compound mouse model

As mentioned above, previous members of the lab observed that mice carrying the JAK2^{V617F} mutation in their hematopoietic compartment with an additional approximatively 1.5-fold overexpression of hSTAT5B succumbed earlier to their PV-like disease compared to mice with JAK2^{V617F} alone. Part of this master thesis was to characterise this new mouse model and investigate the underlying causes for the reduced survival in these compound mice.

3.2.1. Overall characterisation of the JAK2^{V617F}/hSTAT5B compound mouse

To assess the extent to which symptoms in the compound mouse are worsened by the presence of additional hSTAT5B overexpression, weights of relevant organs from these mice as well as control mice were collected. Additionally, we recorded blood parameters, such as the haematocrit and cell counts that are relevant to the MPN disease.

The spleen to body weight ratio of the compound mice was significantly increased compared to the JAK2^{V617F} mice. No significant differences in haematocrit or red blood cell numbers were observed, but a clear trend towards higher white blood cell counts, lymphocyte, granulocyte and monocyte numbers could be detected in the compound mice (Figure 20). Platelet numbers could not be assessed so far, as the results obtained with the VetABC machine were

too variable and thus unreliable. Measurement of platelet numbers by FACS analysis will be conducted in the future.

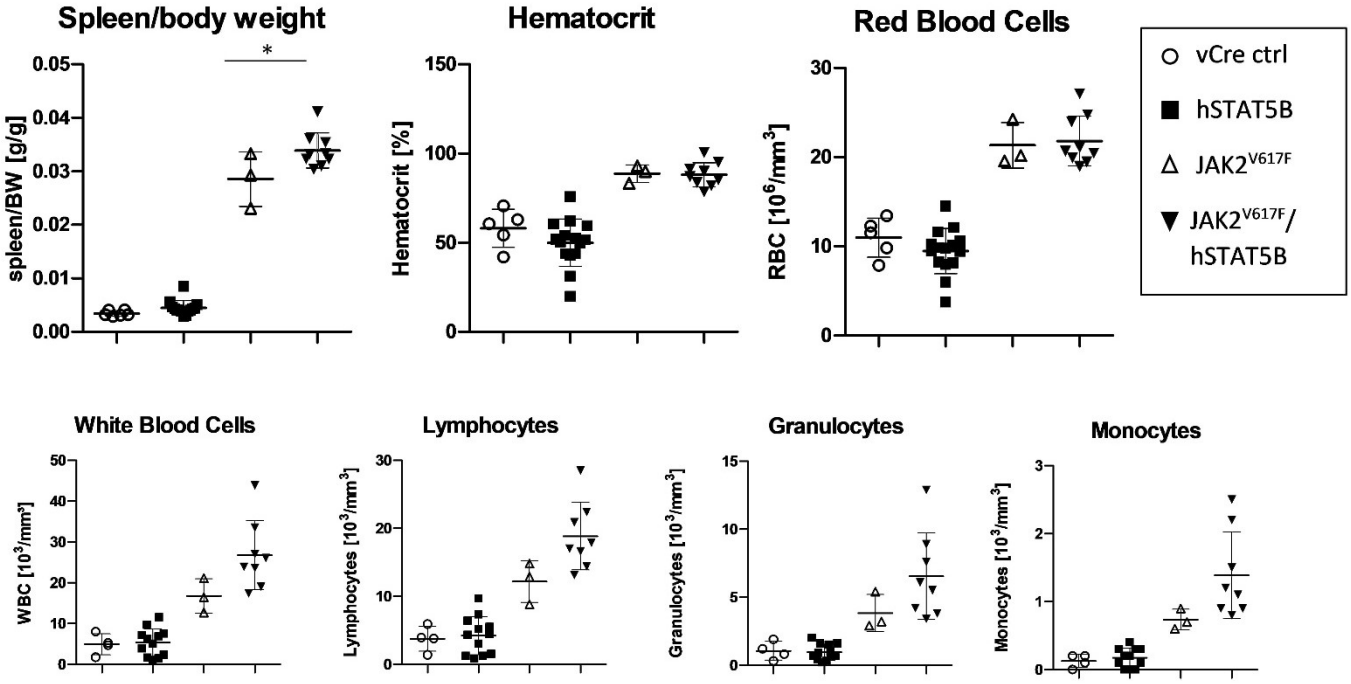

Figure 20: *Organ and blood parameters of compound mice and controls.* Blood parameters were determined with VetABC from vCre ctrl (n=5), hSTAT5B (n=13), JAK2^{V617F} (n=3), JAK2^{V617F}/hSTAT5B (n=8) mice. Error bars indicate the standard error of the mean. *= p-value < 0.05.

3.2.2. Colony assays with bone marrow cells and splenocytes

As MPNs are stem cell diseases we wanted to assess the capacity of the HSCs of our mice to form colonies as a readout of their disease-capacity. Generally, colony forming assays are used to assess mechanisms of partial or full transformation. They evaluate the potential of hematopoietic stem and progenitor cells to proliferate and differentiate into colonies of various myeloid lineages. These assays are performed in semi-solid media supplemented with several cytokines to ensure the survival of most types of progenitor cells.

To determine the colony forming capacity of bone marrow cells and splenocytes of the compound mice compared to the JAK2^{V617F} mice, we performed standard colony assays using a complete cytokine cocktail and we counted the total amount of colonies formed. We predicted that the cells from the compound mice would form the most colonies as we suspect them to either have more progenitor cells or a higher proliferation potential or a combination of both. As can be seen in Figure 21, we were able to confirm that hypothesis as we observed the highest number of colonies in the JAK2^{V617F}/hSTAT5B mice, both in the bone marrow and spleen samples. The JAK2^{V617F} mice only showed slightly higher numbers than the wt in the bone marrow, but significantly higher numbers in the spleen. For the hSTAT5B carrying mice, only a slight increase compared to wt was observed in the spleen samples. Together, this

indicates that even a slight increase of hSTAT5B has a positive impact on hematopoietic stem and progenitor cells' capacity to proliferate and/or differentiate.

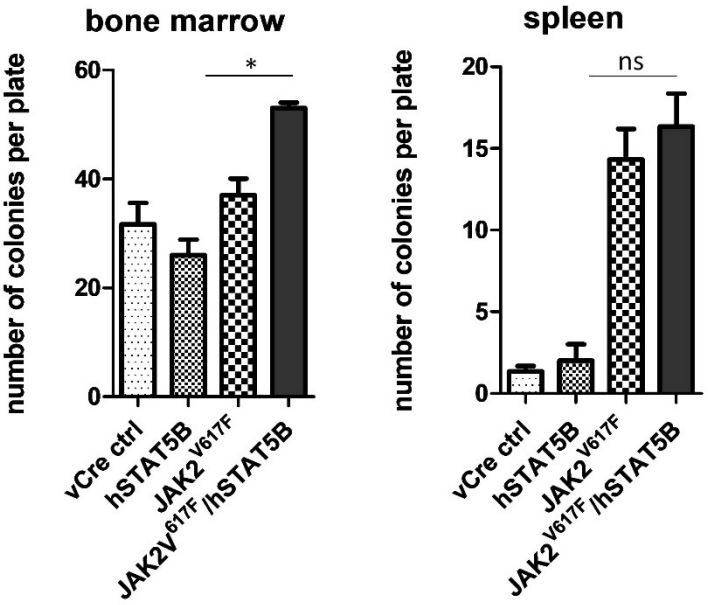

Figure 21: C*olony assay with cells isolated from spleen and bone marrow of mice.* Plates were incubated for 9 days and all colonies counted under a microscope. Plates were seeded in triplicate for each organ and mouse genotype (n=1). Error bars indicate the standard error of the mean. *= p-value < 0.05.

3.2.3. Differentiation assays with bone marrow cells

Since the hSTAT5B transgenic mice showed elevated platelet and MEP levels and the JAK2^{V617F}/hSTAT5B compound mice displayed a strong disadvantage in survival, we speculated that it might be due to an increase in megakaryocyte differentiation. To test this, we wanted to assess the differentiation-potential of their hematopoietic stem cells. For this, we isolated bone marrow cells and isolated lineage negative, Sca-1^{+} and c-KIT^{+} (LSK) cells by FACS sorting. We then cultivated them in media under different conditions, untreated and treated with TPO or SCF or both. Since megakaryocytes are easy to distinguish from all other cell types due to their significantly bigger size, we assessed their number under a light microscope after nine days of *in vitro* cultivation (see Figure 22). The highest number of megakaryocytes was present in the samples of the JAK2^{V617F}/hSTAT5B mice under both conditions (TPO and SCF or SCF alone), significantly higher than those of the JAK2^{V617F} carrying mice. Interestingly, even the mice overexpressing *hSTAT5B* alone had increased numbers compared to the wt control. Another important observation was that even without TPO stimulation, megakaryocytic-differentiation took place at higher rates in the JAK2^{V617F}/hSTAT5B mice, which suggests a partly autocrine mechanism contributing to enhanced megakaryopoiesis.

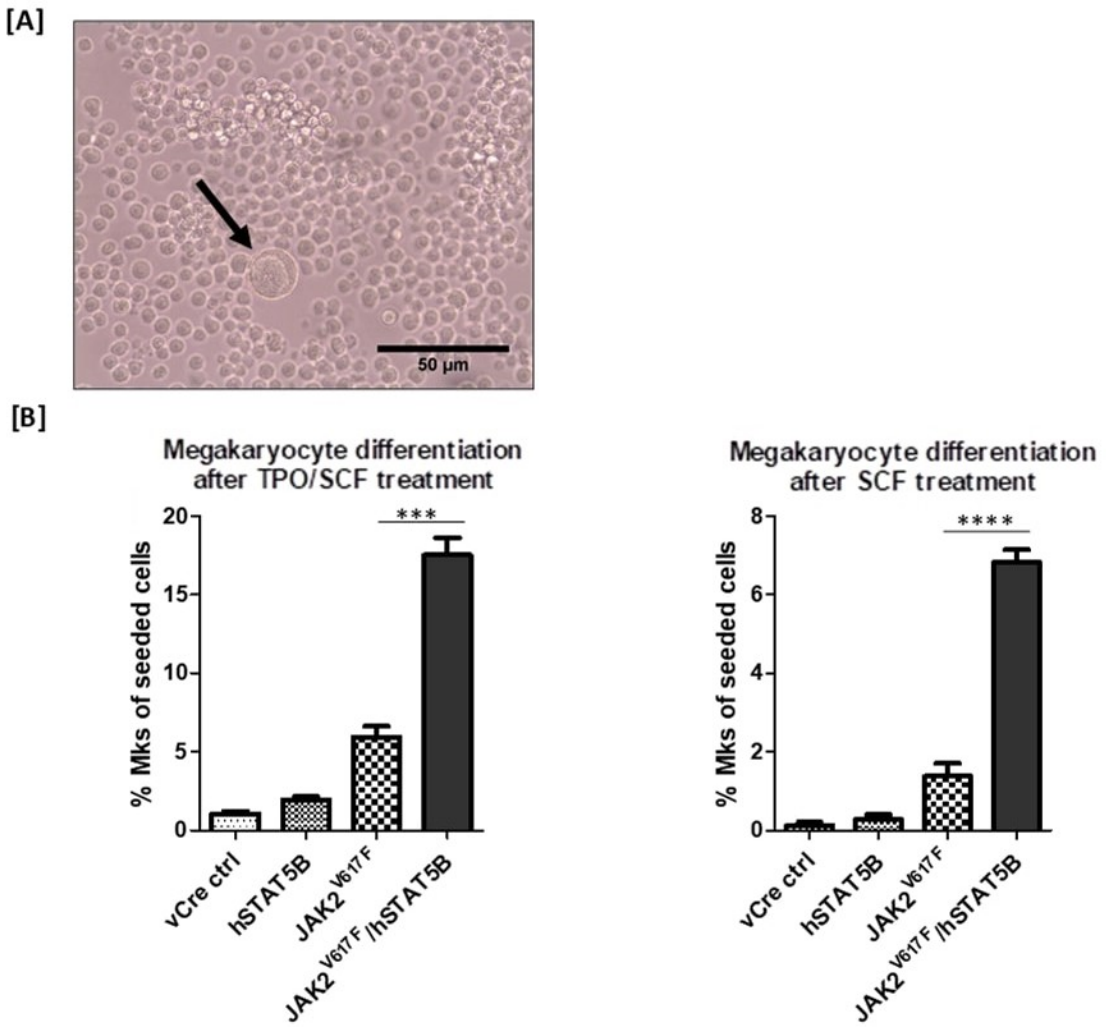

Figure 22: *Megakaryocyte differentiation of hematopoietic stem cells isolated from bone marrow.* Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$ cells were sorted into wells and grown in medium for 9 days with either TPO and SCF or only SCF. Cells with a clearly increased size compared to surrounding cells were counted as differentiated megakaryocytes. Their number was assessed under a light microscope. [A] Example of differentiated megakaryocyte (arrow). Magnification 40x. [B] Graphs show data of the quantification of differentiated megakaryocytes from one experiment with 2 technical replicates for vCre ctrl and 3 technical replicates for hSTAT5B, $JAK2^{V617F}$, $JAK2^{V617F}$/hSTAT5B mice and is representative for two experiments. (n=2) Graphs show data from Error bars indicate the standard error of the mean. *** = p-value < 0.001, **** = p-value < 0.0001.

We also FACS analysed the TPO/SCF or SCF-treated cells after 9-10 days and measured the percentage of Lin^-, $Sca\text{-}1^-$ and $c\text{-}KIT^+$ (LK) cells that were $CD41^+$ and $CD150^+$. These markers are used to detect the megakaryocytic progenitor cells (MkPs). (Grisouard *et al.*, 2015) Here, we got a similar picture, with the $JAK2^{V617F}$/hSTAT5B mice having the highest number of MkPs compared to the other genotypes (Figure 23). Numbers in the hSTAT5B mice were also slightly increased compared to wt. Increased Mk differentiation was also observed in the $JAK2^{V617F}$ and $JAK2^{V617F}$/hSTAT5B cells which were not stimulated with TPO. Due to a low number of available mice to analyse, these experiments will need to be repeated and confirmed in the future.

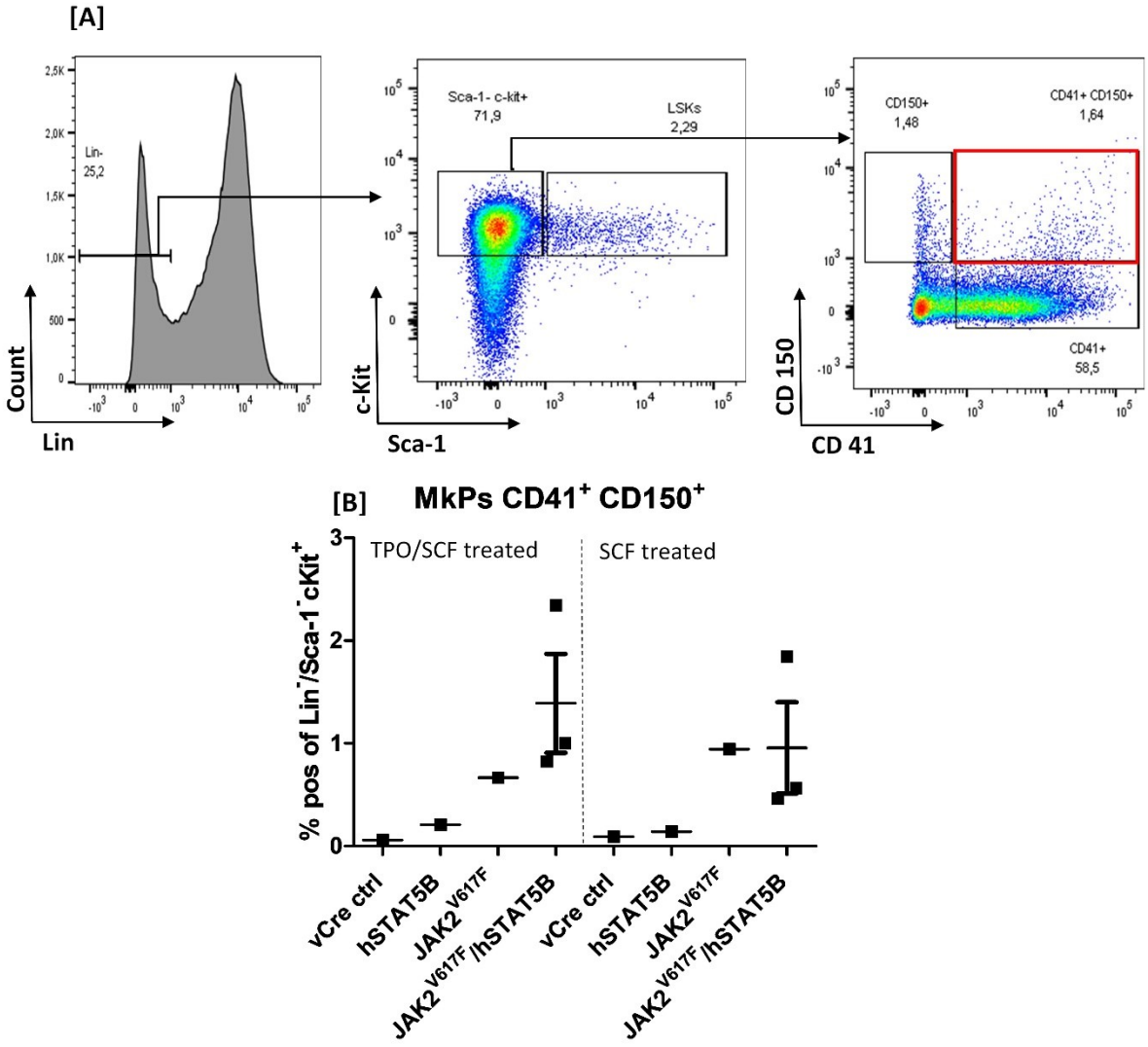

Figure 23: *Megakaryocyte progenitors differentiated from bone marrow stem cells under different conditions.* Lin$^-$ Sca-1$^+$ c-Kit$^+$ cells from bone marrow were sorted into wells and grown in medium for 9 days with either TPO and SCF or only SCF. [A] Representative FACS scatter plots showing the gating strategy for megakaryocyte progenitors (red box). [B] Quantification of the % of megakaryocyte progenitors in the Lin$^-$ Sca-1$^-$ c-KIT$^+$ population of the TPO/SCF and SCF treated cells. Data are graphed from one experiment for the *vCre ctrl*, *hSTAT5B* and *JAK2^{V617F}* genotypes (n=1) and from three biological replicates (n=3) for the JAK2^{V617F}/hSTAT5B genotype. Error bars indicate the standard error of the mean.

These results together would suggest that mice who have more STAT5B also have increased number of megakaryocytes and thus an increased production of platelets. This could then lead to a higher risk of thromboembolic events and thus could contribute to the decreased survival.

3.2.4. Histology of the mice

An important tool that is often used in diagnosis is immunohistological analysis of the relevant organs in patients as well as in mice. Bone marrow and spleen pathologies have been extensively characterised by immunohistological analysis in MPN mouse models. An organ that has received less attention in this context is the lung. Due to MPN patients suffering from clots and pulmonary-embolisms, its implication in the disease is well established. (Cerquozzi and Tefferi, 2015) A recent publication described clots in the lungs of mice from a MPN model. (Wolach *et al.*, 2018) Since we are interested in thromboembolisms and their potential increase in our compound mouse model, we wanted to see if we could find similar clots in the lungs of our mice. As such, we started a collaboration with a group from the Ludwig Boltzmann Institute for Lung Vascular Research in Graz who possess the expertise on lung sample preparation.

During this collaboration we were able to establish the methods to find and image clots in the lungs of our mice. The processing and imaging still need to be performed for all four genotypes and in more replicates, but our initial results are promising. As can be seen in Figure 24, the lung blood vessels (black arrows), as well as alveoli are clearly visible in all samples. The biggest difference between the lungs of hSTAT5B mice without and those who harbour the $JAK2^{V617F}$ mutation is the presence of blocked vessels (white arrows). Since lungs were perfused with PBS to remove blood before sample preparation, those blockages are solid clots and appear to be caused by the presence of the $JAK2^{V617F}$ mutation. Again, due to a low number of available mice of all genotypes, only preliminary results were obtained and the important $JAK2^{V617F}$ alone control mice could not be included in this experiment.

We want to use these techniques in the future to investigate if our compound mice have increased numbers of clots in their lungs compared to our controls. This would provide further evidence that higher numbers of thromboembolisms could indeed be a cause for the reduced survival we previously observed in these mice.

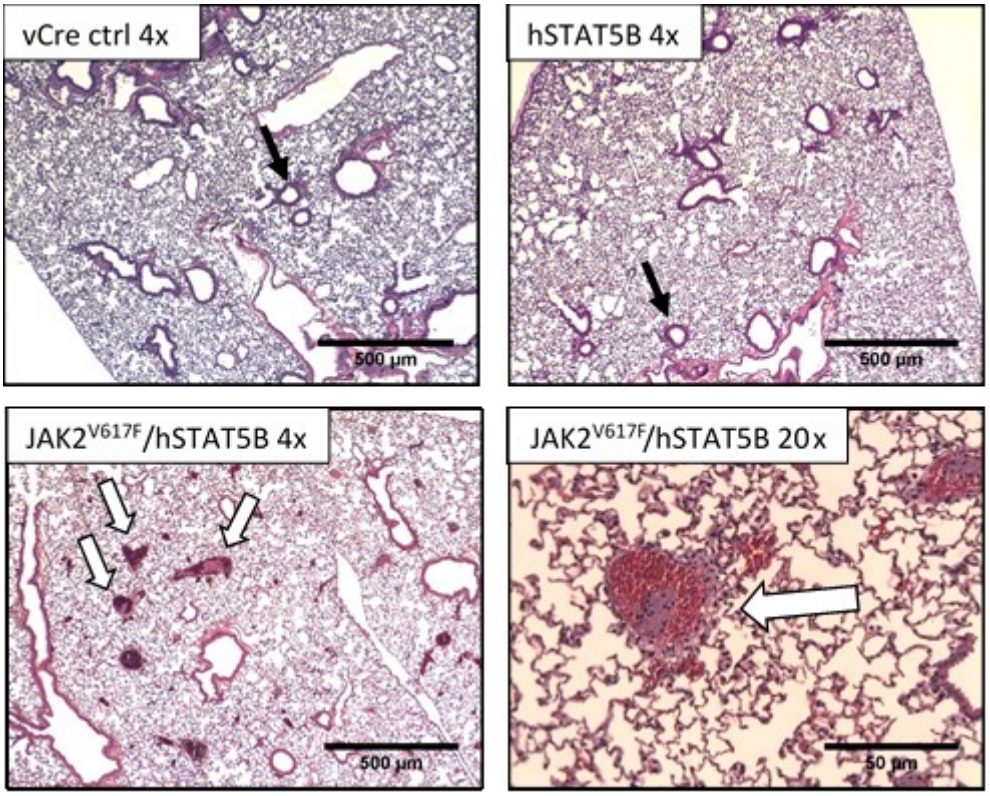

Figure 24: *H&E staining of lung sections of mice with different genotypes.* Perfused lungs were sectioned and stained with H&E. No clots could be identified in the vCre ctrl or hSTAT5B mice, all blood vessels are free (black arrows). Detectable clots were present in the smaller vessels of the JAK2^{V617F}/hSTAT5B compound mice (white arrows).

3.3. Analyses of patient samples

One of our goals was to assess whether higher expression of STAT5B in JAK2^{V617F}-positive MPN patients is correlated with a higher incident of thromboembolisms. In order to test this, we wanted to make sure that we were able to detect STAT5A and STAT5B expression in peripheral blood samples of patients. For this, we harvested peripheral blood mononuclear cells (PBMCs) from the samples using two different extraction methods that are commonly used: a Ficoll gradient and a Dextran extraction. We analysed healthy control samples by Western blot and qPCR, using the methods validated above, and decided to use the Ficoll samples for further analyses, since the signal they yielded was much stronger (data not shown). Two healthy control and four to five MPN patient samples were analysed, and we were able to detect hSTAT5A and hSTAT5B in nearly all seven samples both on the mRNA and protein level (Figure 25). The levels varied both among control samples as well as patient samples, with no clear differences between the two groups. This again indicates, in support of the cellular data, that JAK2^{V617F} does not seem to drive the expression of higher levels of STAT5. It is also interesting that the expression levels vary strongly from individual to individual. Results on the mRNA and protein levels correlated well, which consolidates that our methods are adequate for the analysis of patient samples.

Optimal conditions are now set for subsequent analyses of more MPN patient samples, which will then be cross-referenced with clinical data to investigate the correlation of STAT5B levels and thromboembolisms in patients. So far pYSTAT5 levels, which are crucial in MPN disease development, were not detectable in these patient samples by Western blot. Optimizations will be performed in the future to determine pYSTAT5 levels and assess their role in the incidence of thromboembolisms.

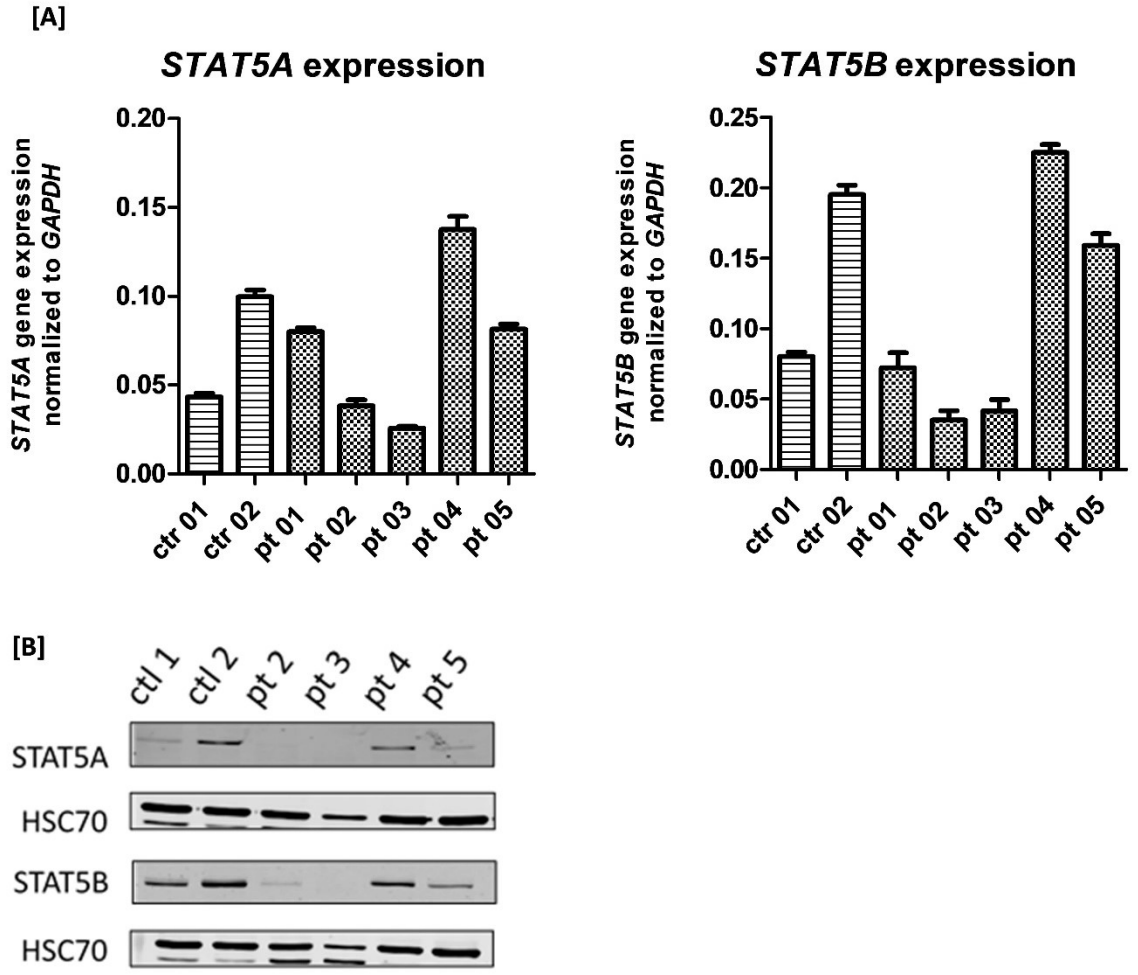

Figure 25: *Relative mRNA and protein expression in MPN patient samples.* [A] qPCR was performed to determine the mRNA expression of *hSTAT5A* and *hSTAT5B* relative to *GAPDH*. Data are graphed from three technical replicates. Error bars indicate the standard error of the mean. [B] Western blot analysis of protein extracts from PBMCs isolated from PV patient and healthy control samples showing STAT5A and STAT5B expression levels, HSC70 was used as loading control.

4. Outlook and Discussion

MPNs are a group of incurable diseases which are characterised by the expansion of mature myeloid cells. The leading cause of death for patients with PV and ET, two MPN subtypes, is thromboembolic events. The molecular mechanisms which are responsible for the increased risk for thromboembolisms in these patients are still incompletely understood.

The constitutively active *JAK2*^{V617F} mutation can be found in 95% of patients with PV and 50-60% of patients with ET. (Baxter *et al.*, 2005; James *et al.*, 2005; Jones *et al.*, 2005; Kralovics *et al.*, 2005) The expression of this mutant *JAK2* leads predominantly to the phosphorylation and thus activation of STAT5, but also STAT3. (O'Sullivan *et al.*, 2007) STAT5 has been shown to be the more oncogenic of the two in MPN, since the deletion of STAT3 in an MPN mouse model leads to decreased survival of the animals whereas a knock-out of STAT5 abrogated the disease development completely. (Yan, Hutchison and Mohi, 2012; Grisouard *et al.*, 2015) STAT5 comprises two gene products: STAT5A and STAT5B, but which of the two plays a more important role in MPNs is yet unclear. Since STAT5B has been found to be more frequently mutated in hematopoietic cancer patients than STAT5A, and was recently shown to play a key role in BCR-ABL^{+} CML, chances are it may also be the more important STAT5 protein acting downstream of JAK2^{V617F} in MPNs, but no studies have yet been done in this regard. (Shahmarvand *et al.*, 2018; Kollmann *et al.*, 2019)

We wanted to investigate this in a human setting and selected seven human myeloid leukaemia cell lines, which all depend on STAT5 signaling: MOLM-13 and MV4-11 (AML), K562 and KU812 (CML), TF-1 (JAK2^{V617F}-negative MPN), and HEL and SET-2 (JAK2^{V617F}-positive MPN). To confirm the mutational status of our three MPN cell lines, we sequenced the locus of the JAK2^{V617F} mutation and found the mutation to be present only in HEL and SET-2 as expected. To gain an overview of endogenous STAT5 expression and determine if different driver contexts affect STAT5 levels, we measured the mRNA levels of STAT5A and STAT5B by qPCR. We found the lowest levels of both STAT5A and STAT5B in the AML cell lines MOLM-13 and MV4-11. The highest levels were present in the CML lines K562 and KU812. The three MPN cell lines had intermediate levels, but with SET-2 approaching a similar level of STAT5A and STAT5B expression as the CML lines. At the protein level the expression of STAT5A and STAT5B was consistent with the mRNA levels, except for SET-2 which we found to have around the same protein expression as the other MPN cell lines, despite having higher mRNA levels of both gene products. This indicates that some post-transcriptional mechanisms are possibly active in the SET-2 cells and down regulate the STAT5 protein expression. These post-transcriptional regulatory mechanisms could impact the accessibility of the mRNA for the ribosomes or directly target the STAT5 protein and impact its stability.

We also measured STAT5 activation by pYSTAT5 Western blot analysis. We found the strongest activation in the cell lines harbouring the JAK2^{V617F} mutation (HEL and SET-2), confirming it as a strong driver for STAT5 phosphorylation. As mentioned previously, tyrosine phosphorylation of STAT5 leads to its translocation to the nucleus as a parallel dimer. There it acts as a transcription factor and activates genes involved in cell proliferation and survival. One of these genes is the *Bcl-xL* gene, an anti-apoptotic gene controlled by pYSTAT5 that is essential in erythrocyte proliferation. Other important target genes are *Bcl-2* and *Mcl1* which also have anti-apoptotic properties. (Silva *et al.*, 1998; Rhodes *et al.*, 2005; Malin, McManus and Busslinger, 2010) An increase in phosphorylated STAT5 thus leads to an increase in transcription of genes involved in proliferation and thus directly affects the number of cells. In patients, an increase in aberrant transcription of survival and proliferation genes in malignant cells leads to increased disease burden.

Together, these results suggest that JAK2^{V617F} is a very potent activator of STAT5 and that ultimately an increase in activated STAT5 molecules is important for the progression and severity of MPNs. The question still arises if a higher expression and thus higher availability for activation of STAT5 molecules has an impact on prognosis in patients, especially considering how strong the activator JAK2^{V617F} is. Importantly, our MPN cell line expression analyses suggest that the JAK2^{V617F} mutation itself does not drive the increased expression of STAT5A or STAT5B, as we saw similar mRNA and protein levels in the TF-1 cells compared with the HEL and SET-2 cells. Therefore, variations or increases in expression of these STAT5 gene products may occur independently of the JAK2^{V617F} mutation in MPN patients and may contribute to the heterogeneity in disease burden and outcomes. Additionally, increased STAT5B might have a bigger impact than STAT5A considering its higher oncogenic potential. (Shahmarvand *et al.*, 2018; Kollmann *et al.*, 2019)

To investigate the impact of increased levels of STAT5 in the presence of JAK2^{V617F} on cell proliferation, we overexpressed each of the two STAT5 variants in the three MPN cell lines (HEL, SET-2, TF-1), two of which harbour the JAK2^{V617F} mutation. For this we generated stable cell lines by nucleofecting the cells with a pMSCV vector containing the cDNAs of interest and sorting them three times for their GFP expression over the course of several weeks. We were able to produce these over-expressor variants for two cell lines - HEL and TF-1. The over-expression of STAT5A and STAT5B was confirmed by Western-blot analysis and was found to be low, close to physiological levels, which may mimic slight variations that could occur naturally between individuals. To determine the effect of this overexpression on cell proliferation we performed an MTS assay and found that STAT5B overexpression conveyed a proliferation advantage in the JAK2^{V617F}-positive cell line (HEL) but not in the JAK2^{V617F}-negative cell line (TF-1). STAT5A overexpression had no impact on proliferation in either cell line. We thus believe that STAT5B might indeed be more oncogenic than STAT5A in JAK2^{V617F}+ MPNs. This is consistent with previous findings, where STAT5B has been found to be more

frequently mutated in hematopoietic cancers than STAT5A. (Shahmarvand *et al.*, 2018) Additionally, this supports the idea that variations of STAT5B expression between patients might account for some of the heterogeneity in disease burden and progression.

The fact that no effect was seen with the JAK2^{V617F}-negative cell line could hint that the role of STAT5B is different when other drivers are present in the cell. It could also be possible that other drivers are not as strong in activating STAT5B. Thus slight variations in its expression would not have the same impact as when JAK2^{V617F} is present. This has to be confirmed with further experiments, since TF-1 cells are cytokine dependent and the high concentrations of GM-CSF in the growth medium could mask the effect that STAT5B might have. This masking effect might occur because the presence of the high GM-CSF concentration in the media already pushes cells to proliferate at their maximum capacity and thus additional signaling, which could positively affect proliferation, would not have any measurable impact. It is hence necessary to perform additional proliferation experiments with reduced cytokine concentrations.

To consolidate our observation in the HEL cells, we will also perform the same experiments with another JAK2^{V617F} positive cell line (SET-2). In another experiment we want to explore the importance of STAT5B over STAT5A in JAK2^{V617F}-positive MPNs, by the generating knock out cell lines. For this, we started preliminary CRISPR/Cas9 experiments (results not shown) and we plan to knock out STAT5A or STAT5B in each of the respective cell lines. Here, we speculate that the knock-out of STAT5B decreases proliferation more than that of STAT5A. Future experiments will test the heightened oncogenic character of STAT5B over STAT5A, specifically in the JAK2^{V617F}-positive MPN cells.

Additionally, to convey a proliferation advantage we suspect that higher STAT5B levels also have an impact on cell differentiation. An indication for this was previously found in the lab when a transgenic mouse with a 1.5-fold overexpression of wt hSTAT5B showed an increase in MEPs and platelets compared to wt mice. (unpublished) It has also been shown that STAT5, or more specifically uSTAT5 (unphosphorylated STAT5), is involved in megakaryocyte differentiation. (Park *et al.*, 2016) The question arose of the effect that increased levels of STAT5B would have in a JAK2^{V617F}+ background and if different levels of STAT5B could contribute to the heterogeneity of disease progression observed in patients, particularly involving risk of thromboembolic events. Shifting the population of diseased cells towards the megakaryocytic lineage and pushing proliferation of myeloid cells, thusly increasing platelet levels, led us to the hypothesis that patients with higher STAT5B expression levels may have a heightened risk of thromboembolisms.

To study this, transgenic hSTAT5B and JAK2^{V617F} knock-in mice were crossed by previous members of the lab. This crossing led to increased mortality of the compound mice (unpublished). To investigate what impact this increase in STAT5B expression had more precisely, we collected organ weight data and blood parameters. We found increased splenomegaly and a trend towards higher white blood cell numbers in the compound mice compared to JAK2^{V617F} mice. As mentioned above, we suspect that an increase in megakaryocytes could be a reason for this shift in mortality. We studied the differentiation of LSKs isolated from the bone marrow of the mice by growing them in media with only SCF, or with TPO and SCF to stimulate megakaryocyte differentiation. The cells from the compound mice showed a significantly higher tendency to differentiate towards the megakaryocyte lineage, which we observed in two different experiments. They also differentiated into megakaryocytes at a higher rate when they were not stimulated to differentiate with TPO. The presence of additional STAT5B in a JAK2^{V617F}+ background thus influences differentiation more strongly towards the megakaryocytic lineage.

Interestingly, the cells from the transgenic hSTAT5B mice had a tendency towards higher megakaryocyte differentiation compared to the wt control. It is also possible that STAT5B simply has an impact on cell proliferation and leads to a heightened cell division rate, as we saw in our cell lines. We were not able to confirm if this was true from this experiment, since determining the total cell number in the wells was not possible due to low cell numbers. We observed an increase in megakaryocyte progenitor cells by FACS analysis as an increase in percentage of the LK cell fraction. It is thus likely that differentiation is indeed biased towards the megakaryocyte lineage rather than the increase in megakaryocytes being only a side-effect of heightened proliferation. Since megakaryocytes have been shown to be critical for maintaining the stem cell niche in the bone marrow, and as MPN disease arises from transformation of the stem cells, which could not be eradicated by any treatment so far, targeting megakaryocytes directly could be a new approach to treat MPNs. (Bruns *et al.*, 2014; Zhan *et al.*, 2016) Considering our findings, STAT5B might be a potential target to decrease the numbers of megakaryocytes in MPN patients and thus alleviate their detrimental effects and potentially eradicate the stem cell clones.

Since one of the leading causes of death for MPN patients are thromboembolic events, we suspect that the same is true in our JAK2^{V617F} mice. We observed that these mice can succumb to the disease spontaneously with minimal prior observable symptoms, which could be due to a stroke or heart attack. The decrease of survival of the compound mice could be explained by an increase of megakaryocytes and thus platelets, which would lead to more blood clotting. It is still unclear by what mechanisms the presence of JAK2^{V617F} leads to thromboembolisms in patients. Research into activation and function of platelets in a JAK2^{V617F} background has shown that the mutated protein could lead to intrinsic changes of platelet activation. Whether these changes result in more or less aggregation of the platelets is not yet clear, as

controversial findings have been published. (Hobbs *et al.*, 2013; Lamrani *et al.*, 2014) Other studies showed that endothelial cells of MPN patients also express JAK2^{V617F} which leads to the expression of more cell adhesion proteins like P-selectin, which contributes to abnormal blood clotting. (Guadall *et al.*, 2018)

Another hypothesis for the formation of thromboses was proposed by a recent study which states that JAK2^{V617F} leads to the hyper-activation of neutrophils. (Wolach *et al.*, 2018) These neutrophils then form extra-cellular traps (NETs) which the authors of the study claim to lead to the formation of spontaneous clots in the lung of the mice. These NETs are a mechanism of the innate immune system to entrap pathogens and thus limit their mobility and spreading throughout the whole organism. Whether bacterial infections could be responsible for the formation of thrombi through triggering these hyperactive neutrophils, was not addressed in this study. In accordance with this, it has been shown that an increase in leukocytes correlates with an increase in thrombosis in MPN patients. (Barbui *et al.*, 2015) This could also be a plausible explanation for the heightened mortality in our compound mice, since we observed increased white blood cell numbers in these mice.

We also observed that cells from our compound mice showed increased numbers of colonies in a colony forming assay. Cells from spleen and bone marrow of the compound mice formed more total colonies than the ones of control mice. It was not assessed what kind of colonies formed but we believe them to be mostly from the granulocytic/monocytic lineage. Although these experiments need to be repeated for statistical purposes, it appears that STAT5B could also contribute to the heightened white blood cell numbers in mice.

As mentioned before, it has been observed that clots form in the lungs of a JAK2^{V617F} mouse model. (Wolach *et al.*, 2018) It is well established that MPN patients can suffer from pulmonary embolism since the lung is made up of very small vessels that generally are more easily clogged. (Cerquozzi and Tefferi, 2015) As we hypothesized that our compound mice suffer from increased thromboembolisms, which might be the result of increased clotting and are a major cause for mortality and morbidity in patients, we wanted to establish a method to detect clots that might form in our mice. We decided to analyse the lungs by immunohistochemistry since clots would be easy to identify in this organ under the microscope with appropriate staining. For this we collaborated with lung vasculature experts in Graz. For the correct preparation, the mice need to be perfused post-mortem and the lungs flushed with PBS. To fix them, they were inflated with and stored in formaldehyde for 24h. They could then be prepared with conventional methods for histological analyses. We had one litter of mice with one mouse per genotype (except JAK2^{V617F} alone) available for these analyses, and we were able to detect clots as described by Wolach et al. These clots were only present in the compound and not in the wt and *hSTAT5B* control mice. We were not able to

process any JAK2^{V617F} mice so far but will repeat these analyses with more mice from all genotypes in the future. We also want to establish a staining for collagen, since the presence of collagen would indicate that remodelling of the blockage has occurred and has likely been there for some time. Such a staining could be done with Masson's trichrome staining, which is routinely used to differentiate smooth muscle from collagen tissues, for example in tumors, staining the muscles red and the collagen green to blue. (Masson's Trichrome Stain - an overview | ScienceDirect Topics, no date)

Should we be able to find a significant increase in clots in the compound mice compared to the three control mouse genotypes, it would be a strong indicator that increased STAT5B expression indeed leads to increased thromboembolisms. If clots of the compound mice are found to contain more collagen than those of the JAK2^{V617F} mice it would show that STAT5B also influences disease progression, as reaching higher levels of platelets and leukocytes earlier would probably lead to the formation of clots earlier, since increased white blood cell numbers, which we observed in our compound mice, have also been shown to contribute to increased clot formation in patients. (Tefferi and Pardanani, 2015)

Since the results from our mouse experiments as well as those from our cell lines together indicate the strong oncogenic role of STAT5B in JAK2^{V617F}+ MPNs, the next step would be to look at the situation in patients. For this, we collected peripheral blood samples from JAK2^{V617F} positive MPN patients and subjected them to two different cell isolation protocols. We compared the results of both methods by qPCR and Western blot analysis and chose to employ the samples from the Ficoll gradient extraction for further analysis. We were able to detect STAT5A and STAT5B levels in our two control and five patient samples. Interestingly, expression levels both at the mRNA and protein level were quite heterogenous from sample to sample. The results from both qPCR and Western blot analysis correlated in all samples.

Despite a small sample size, it appeared that STAT5 levels were not elevated in the MPN patients compared to controls and the results were quite heterogeneous. This could indicate that the presence of JAK2^{V617F} alone does not drive high STAT5 expression, which is consistent with our previous findings that JAK2^{V617F} does not promote higher STAT5 expression in MPN cell lines. The heterogeneity surprised us but supports our hypothesis that the heterogeneity between patients in terms of the prevalence of thromboembolism might correlate with varying STAT5B levels. What we now suspect, but have not confirmed yet, is that those patients who express higher levels of STAT5B might have a higher risk for thromboembolisms. Additionally, we want to investigate if a faster disease progression and the requirement for cytoreductive treatment, indicating higher disease burden, could be correlated with high STAT5B levels. In order to correlate these parameters in the future, the patient's medical history information will be required, and we would need to sample more patients to make any

conclusions. Due to a limited number of MPN patient samples available to us, we were not able to define a cohort with thromboembolic complications versus those without, which will be critical for our analyses. However, the methods for quantifying STAT5 expression levels from patient samples were successfully optimized in these studies and can be used in the future.

In summary, even a slight increase in STAT5B expression had an observable impact on proliferation and differentiation in human JAK2^{V617F}postive MPN cell lines as well as in our compound JAK2^{V617F}/hSTAT5B mouse model. We now want to study this effect in human MPN patients to potentially establish STAT5B expression as a new prognostic marker for disease progression and risk assessment. Since there are no curative treatments for MPN patients to date, improving our understanding of the disease mechanisms will contribute to the development of better targeted therapies.

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6. Appendix

6.1. Abstract

Myeloproliferative neoplasms are a group of diseases marked by the malignant expansion of mature myeloid cells. One of these diseases is polycythemia vera, which is characterised by erythrocytosis, leucocytosis and thrombocytosis or a combination of those. Patients with polycythemia vera have an increased risk of developing thromboembolisms and of progression to secondary myelofibrosis and/or acute myeloid leukaemia. Thromboembolisms are the major cause of morbidity and mortality in these patients, although their incident rate varies quite broadly amongst patients. The underlying mechanisms that influence their development in these patients remain incompletely understood.

One of the major drivers in myeloproliferative neoplasms is the *JAK2*^{V617F} mutation, which can be found in around 95% of polycythemia vera patients. This constitutively active JAK2 variant signals through STAT5 and leads to the activation of survival and proliferation genes. STAT5 comprises two different gene products: STAT5A and STAT5B. They have largely redundant functions, although some differential functions have been identified. So far it is unclear which STAT5 variant is the more important one for the development of JAK2^{V617F} positive myeloproliferative neoplasms. We believe STAT5B to be the crucial STAT5 protein in this context, since it has been found to be more frequently mutated in cancer than STAT5A.

In this study, we used three different systems to evaluate the differential role STAT5A and STAT5B might play in JAK2^{V617F} positive myeloproliferative neoplasms. We assessed their expression levels in three human myeloproliferative neoplasm cell lines and, by comparing JAK2^{V617F} positive and JAK2^{V617F} negative cells, we determined that JAK2^{V617F} itself does not drive increased expression of STAT5B. We also generated stable myeloproliferative neoplasm cell lines which overexpress each of the STAT5 variants, and found that low-level STAT5B, but not STAT5A, overexpression conveys a proliferative advantage to a JAK2^{V617F} positive cell line.

We also studied megakaryocytic differentiation in a *JAK2*^{V617F} mouse model harbouring low-level hSTAT5B overexpression, since megakaryocytes are heavily involved in the development of thromboembolisms. We found that additional expression of STAT5B led to increased differentiation to megakaryocytes in these mice. These mice also had more bone marrow megakaryocyte progenitor cells compared to control mice, and their stem and progenitor cells had increased colony-forming capacity. We also studied the lungs of the mice and found that clots formed in the JAK2^{V617F}/ hSTAT5B carrying mice. Due to low mouse numbers, we would still need to confirm whether it is the expression of additional hSTAT5B that leads to increased clots, which would confirm our hypothesis that increased STAT5B is linked to increased thromboembolisms.

Lastly, we analysed peripheral blood samples of myeloproliferative neoplasm patients and assessed the expression levels of STAT5A and STAT5B. The expression levels were quite heterogeneous, and no clear differences were seen between patients and healthy control

samples. Therefore, natural variances in STAT5 expression exist in patients, and it will be of interest in the future to correlate higher STAT5B expression levels with increased disease severity or incidence of thrombotic events.

To summarize, experiments show that increased STAT5B expression promotes proliferation and increased differentiation into the megakaryocyte lineage, as well as more severe disease parameters in JAK2^{V617F} positive myeloproliferative neoplasm models. These data suggest that varying STAT5B levels, which are observed in patients, could influence disease progression and risk of thromboembolism in patients with JAK2^{V617F} positive myeloproliferative neoplasms.

6.2. Zusammenfassung

Myeloproliferative Neoplasien sind eine Gruppe von Krankheiten, die durch die maligne Expansion reifer myeloischer Zellen gekennzeichnet sind. Eine dieser Krankheiten ist die Polycythemia vera, die durch Erythrozytose, Leukozytose und Thrombozytose oder eine Kombination davon gekennzeichnet ist. Bei Patienten mit Polycythemia vera besteht ein erhöhtes Risiko für Thromboembolien und das Fortschreiten zu einer sekundären Myelofibrose und / oder einer akuten myeloischen Leukämie. Thromboembolien sind die Hauptursache für Morbidität und Mortalität bei diesen Patienten, obwohl ihre Inzidenzrate zwischen Patienten sehr stark variieren kann. Die zugrundeliegenden Mechanismen für diese Heterogenität bleiben bis jetzt unklar.

Einer der Hauptverantwortlichen von myeloproliferativen Neoplasien ist die JAK2^{V617F}-Mutation, die bei etwa 95% der Polycythemia vera-Patienten gefunden werden kann. Diese konstitutiv aktive JAK2-Variante aktiviert STAT5 und führt so zur Aktivierung von Überlebens- und Proliferationsgenen. STAT5 umfasst zwei verschiedene Genprodukte: STAT5A und STAT5B. Sie haben weitgehend gleiche Funktionen, obwohl auch spezielle Funktionen identifiziert wurden. Bisher ist unklar, welche STAT5-Variante für die Entwicklung von JAK2^{V617F} positive myeloproliferativen Neoplasien die wichtigere ist. Wir glauben, dass STAT5B das entscheidende STAT5-Protein ist, da es in Krebserkrankungen häufiger mutiert vorgefunden wurde als STAT5A.

In dieser Masterarbeit haben wir drei verschiedene Ansätze verwendet, um die unterschiedliche Rolle von STAT5A und STAT5B bei JAK2^{V617F} positive myeloproliferativen Neoplasien zu untersuchen. Wir haben ihre Expressionsniveaus in drei myeloproliferativen neoplastischen Zelllinien analysiert und stellten durch Vergleich von JAK2^{V617F} positiven und JAK2^{V617F} negativen Zellen fest, dass JAK2^{V617F} die Expression erhöhter STAT5B-Niveaus nicht beeinflusst. Wir erzeugten auch stabile myeloproliferative neoplastische Zelllinien, die eine der STAT5-Varianten über exprimieren, und stellten fest, dass STAT5B einer JAK2^{V617F} positiven Zelllinie einen proliferativen Vorteil verlieh.

Wir untersuchten auch die Megakaryozyten-Differenzierung in einem JAK2^{V617F}/hSTAT5B-Mausmodell, da Megakaryozyten stark an der Entwicklung von Thromboembolien beteiligt sind. Wir fanden heraus, dass eine zusätzliche Expression von STAT5B bei diesen Mäusen zu einer erhöhten Differenzierung von Blutstammzellen zu Megakaryozyten führt. Wir untersuchten auch die Lunge der Mäuse und stellten fest, dass sich in den JAK2^{V617F} tragenden Mäusen Gerinnsel in einigen Blutgefäßen bilden. Wir müssen noch bestätigen, ob die Expression von zusätzlichem hSTAT5B zu erhöhten Gerinnseln führt, was unsere Hypothese bestätigen würde, dass ein erhöhter STAT5B-Wert mit erhöhten Thromboembolien zusammenhängt.

Zuletzt analysierten wir periphere Blutproben von Patienten mit myeloproliferativem Neoplasien und ermittelten die Expressionsniveaus von STAT5A und STAT5B. Die Werte waren sehr heterogen und es wurden keine deutlichen Unterschiede zwischen Patienten- und Kontrollproben festgestellt.

Zusammenfassend zeigen unsere Experimente, dass eine erhöhte STAT5B-Expression die Proliferation und Differenzierung der Megakaryozyten fördert sowie Krankheitsparameter in JAK2^{V617F} positiven myeloproliferativen Neoplasmamodellen verschlimmert. Diese Ergebnisse legen nahe, dass unterschiedliche STAT5B-Spiegel, die bei Patienten beobachtet werden, das Fortschreiten der Erkrankung und das Risiko von Thromboembolien bei Patienten mit JAK2^{V617F} positiven myeloproliferativen Neoplasien beeinflussen können.