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The role of STAT5B in Megakaryocyte Differentiation

Master thesis

University of Veterinary Medicine Vienna

submitted by

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Table of contents

1 Introduction

1.1 Hematopoiesis

The hematopoietic compartment, which is responsible for the formation of different blood cells, is one of the earliest to develop during gestation. One of the key functions of the blood is to deliver oxygen, nutrients and hormones to different parts of the body. This system relies on hematopoietic stem cells for the maintenance of blood homeostasis (Cumano and Godin 2007) that requires a precisely regulated balance between an active and inactive state of the stem cells. An imbalance of these states can have severe consequences and lead to hematopoietic deficiencies (Orkin and Zon 2008).

1.1.1 Early stem cell development

Hematopoiesis and hematopoietic stem cell (HSC) development occurs during embryogenesis and originates at many different anatomical sites (Mikkola and Orkin 2006). The first wave of hematopoiesis occurs in the embryonic yolk sac that leads to a large production of primitive erythroid cells (EryP) and endothelial cells. This led to the assumption that these two cell types arise from a common precursor named the hemangioblast (Moore and Metcalf 1970). EryP contain embryonic hemoglobin to transport oxygen and support the transition from the fast growing embryo to the fetus (Dzierzak and Speck 2008). Primitive hematopoiesis is transient and eventually replaced by definitive hematopoiesis. In the second wave of hematopoiesis the HSCs develop at the aorta-gonads-mesonephros region. The produced multipotent stem cells populate the liver, spleen and bone marrow and become the major sites of hematopoiesis (Fig. 1) (Mazo *et al.* 2011). Postnatally, HSCs reside in small numbers predominantly within the bone marrow and are responsible for the maintenance of hematopoiesis during adulthood (Morrison and Scadden 2014).

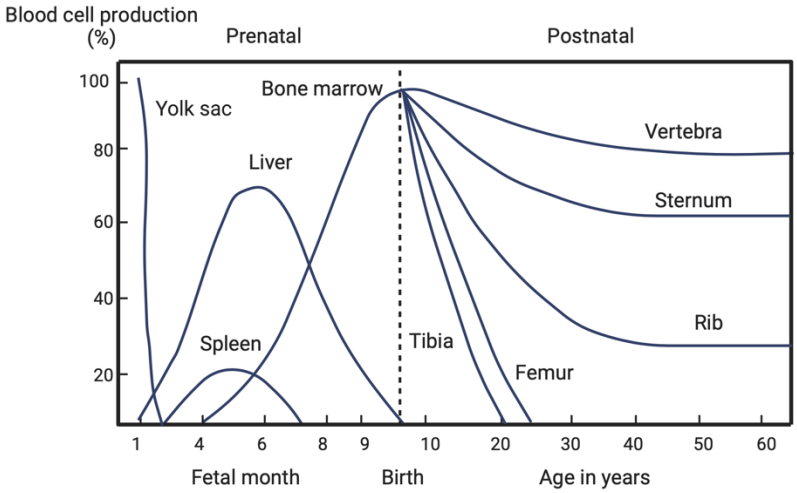


Figure 1: *Waves of hematopoiesis during human development.* Hematopoiesis occurs at different anatomical sites during human development. The yolk sac is the earliest hematopoietic site in early fetal development. HSCs then populate the spleen, liver and bone marrow to differentiate into all blood cell types. After birth, HSCs reside in the bone marrow (tibia, femur, vertebra, sternum, rib) to maintain hematopoiesis. (Image created using BioRender.com, adapted from Williams hematology)

1.1.2 Hierarchy of hematopoiesis

Hematopoiesis has classically been described to follow a step-wise hierarchical process with the rare multipotent HSCs at the top (Fig. 2). HSCs undergo self-renewal as well as a series of differentiation steps to give rise to the hematopoietic compartment (Fliedner *et al*. 1985). They consist of long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs). LT-HSCs reside in the bone marrow predominantly in a quiescence state – a state marked by the G0 cell cycle status. They can enter the G1 cell cycle phase upon activation for self-renewal and differentiation and give rise to ST-HSCs, capable of reconstituting the blood cell lineages (Nakamura-Ishizu *et al.* 2014). The classical differentiation leads to the commitment of the HSCs to either the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP). CLPs give rise to the lymphoid lineage, including natural killer (NK) cells, T-cells and B-cells, also known as lymphocytes. Lymphocytes play a crucial role in antibody production, the regulation of the immune response and the elimination of virus infected and tumor cells. CMPs differentiate further into either megakaryocyte/erythrocyte progenitors (MEPs) or granulocyte/macrophage progenitors (GMPs) (Machlus and Italiono 2019). The GMPs differentiate into eosinophils, basophils, neutrophils and monocytes that play a crucial role in the innate immune system. MEPs further develop into mature megakaryocytes (Mks) and erythrocytes (red blood cells). Erythrocytes undergo several maturation steps to enrich their hemoglobin and acquire a flat shape by losing their nuclei. After their release into the blood

circulation they become oxygen-carrying erythrocytes. The other cell type that arises from MEPs, Mks, are described in more detail in a later chapter.

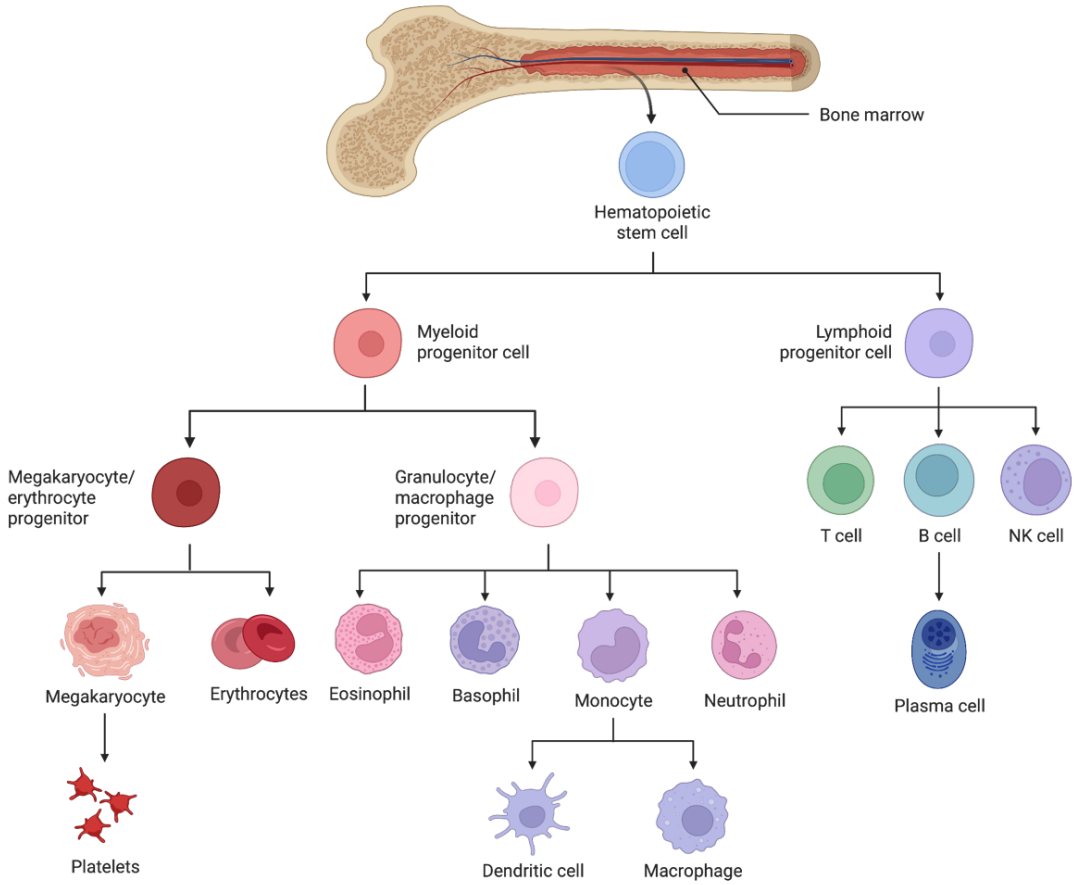


***Figure 2**: Hierarchy of hematopoiesis.* A representation of the bone marrow hematopoietic stem cell and the cell lineages that it gives rise to (Image created using BioRender.com).

1.1.1 The bone marrow niche

HSCs are localized in an interactive system within the bone marrow known as the bone marrow niche (Morrison and Scadden 2014). This microenvironment is made up of a heterogeneous population of stromal cells including fibroblasts, osteoblasts, endothelial cells, macrophages and Mks. These generate regulatory signals like cytokines and growth factors which regulate and support the multipotency of HSCs (Fig. 3) (Calvi and Link 2015). One of these regulatory signals is stem cell factor (SCF) that acts on HSCs, myeloid and lymphoid progenitors through binding to the surface receptor c-Kit (tyrosine kinase receptor; CD117) (Okada *et al*. 1992). It operates in synergy with various stimulatory cytokines including interleukin (IL)-3, IL-6 and

granulocyte-macrophage colony stimulating factor (GM-CSF) to ensure the maintenance but also differentiation of the myeloid lineage (Hoffbrand and Moss 2011). Another axis of HSC maintenance is mediated by Mks which can release chemokine C-X-C motif ligand 4 (CXCL4) which is responsible for regulating HSC quiescence (Bruns *et al*. 2014).

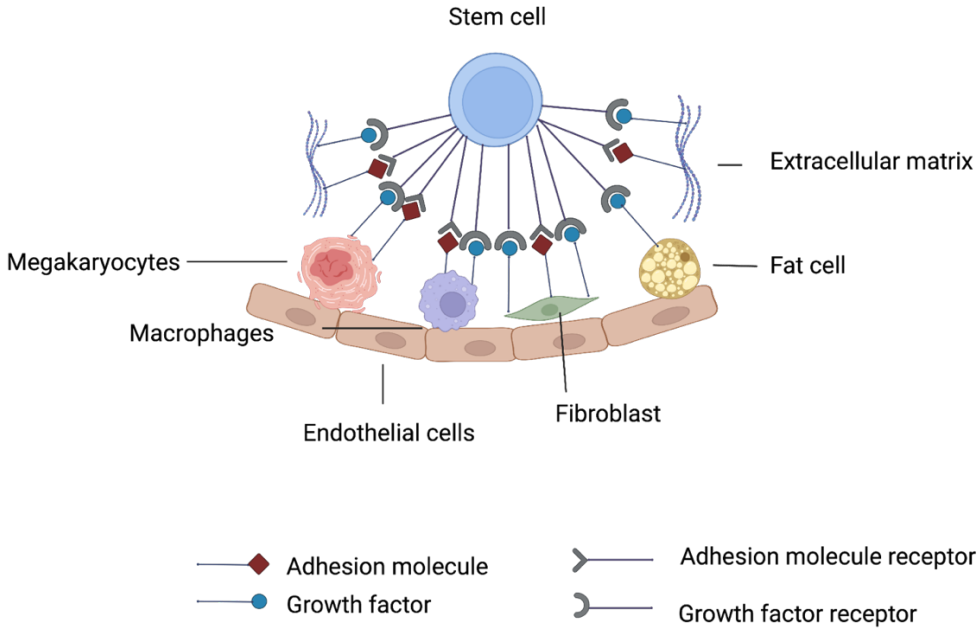


Figure 3: *The bone marrow niche.* Hematopoiesis occurs in an interactive system within the bone marrow named the bone marrow niche, where HSCs have the capability to self-renew and give rise to separate cell lineages. Within the microenvironment, various cell types release cytokines and growth factors to contribute to the regulation of blood homeostasis. Stromal cells like Mks, macrophages, endothelial cells, fibroblasts and fat cells contribute to the production of collagen and glycoproteins and the formation of the extracellular matrix. Another characteristic feature of stromal cells is the secretion of growth factors to regulate stem cell survival. (Image created using BioRender.com, adapted from Hoffbrand and Moss 2011).

1.1.2 Megakaryopoiesis

The red bone marrow is located in the cavities of flat bones and is the site where all types of blood cells are made (Malara *et al.* 2015). Mks are the only mature cell type that resides in the red bone marrow and are responsible for the production of platelets. The main role of platelets is to fulfill their function in blood clotting in response to vascular injury. Megakaryopoiesis is induced by the binding of the regulatory cytokine thrombopoietin (TPO) to its receptor c-Mpl (Park *et al*. 2016) which is expressed on hematopoietic stem cells, progenitor cells, Mks and platelets. The binding of TPO induces a differentiation pathway in HSCs that follows a hierarchical series of progenitor cells that lastly give rise to the mature Mks (Fig. 4). Interestingly, LT-HSCs have recently been described to also unilaterally differentiate into

mature Mks by bypassing the classical hierarchy and progenitor stages (Fig. 4). Megakaryopoiesis is dynamic and adaptive in response to the body`s need, marked by the intuitive differentiation of Mks directly from HSCs, essential to ensure bone marrow maintenance (Noetzli *et al.* 2019). The main regulator of Mk differentiation is TPO which is produced in the liver and kidney and released into the bloodstream where it ultimately reaches the HSCs to initiate differentiation. Circulating platelets sequester TPO through its binding to their c-Mpl receptor, thus lowering its concentration in the blood stream ultimately leading to decreased Mk differentiation (Li *et al*. 1999). Hence, the lower the number of platelets in the blood the higher the TPO concentration is, which in turn increases the stimulation of the differentiation program of platelet-producing Mks (Kaushansky 1998). Every day an adult human produces approximately 1 x 10^{11} platelets which can increase 10- to 20-fold upon elevated demand through the release of TPO. Platelets are then recruited to injured blood vessels to assist in tissue repair (Gremmel *et al.* 2013).

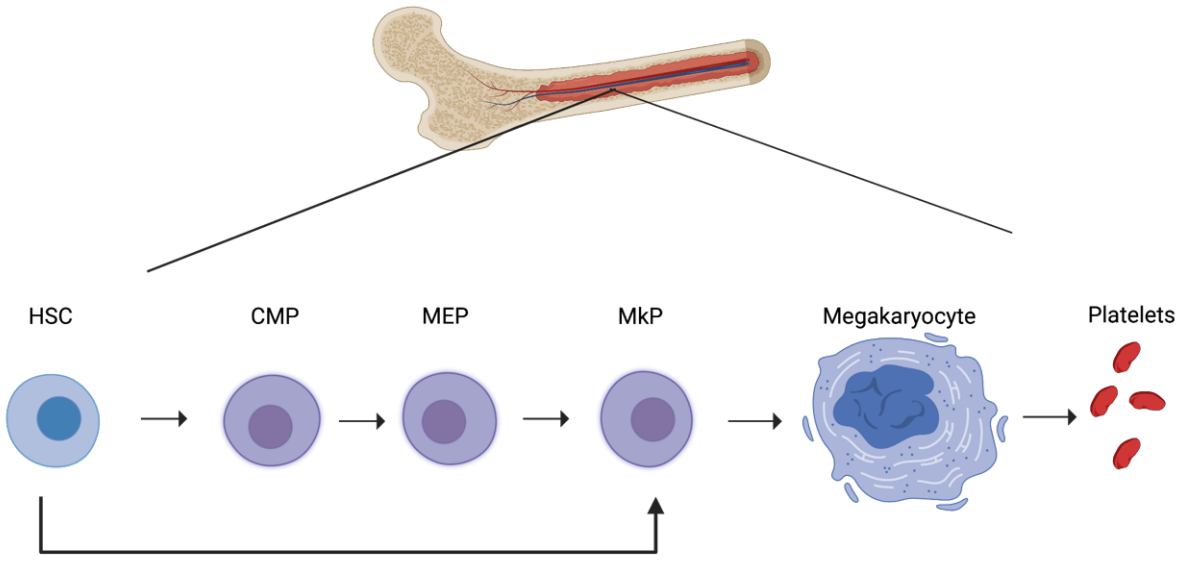


Figure 4: *Canonical and non-canonical differentiation of Mks*. The Mk differentiation process involves induction through the HSC followed by the CMP, MEP, megakaryocyte progenitor (MkP) and finally the Mk. The mature Mk undergoes polyploidization coinciding with an increase in size, DNA content and the shedding of platelets into the blood circulation. New findings propose the ability of LT-HSCs to differentiate directly into MkPs without following the classical pathway (Image created using BioRender.com, adapted from Noetzli *et al*.).

1.1.2.1 Endomitosis

Once Mks have reached a certain maturation level, they start to undergo endomitosis, a critical step between megakaryocyte progenitors (MkP) and the release of platelets. This process includes several rounds of DNA replication in the absence of nuclear or cytoplasmic division (Lordier *et al*. 2008). It increases their ploidy resulting in a highly polyploid nucleus with an average of 16N (nuclei) and up to 128N (Ravid *et al*. 2002). During Mk differentiation immature polynucleated Mks increase in size until they are big enough to start shedding platelets (Fig. 5) (Vainchenker and Raslova 2020).

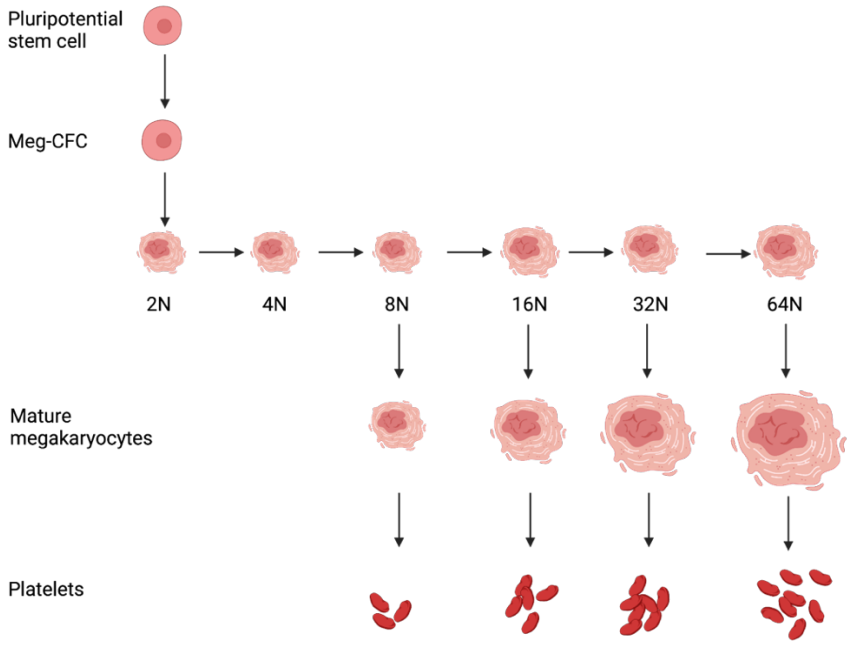


Figure 5: *Endomitosis.* Mks elevate their ploidy by a process called endomitosis. The pluripotent stem cell develops into megakaryocyte colony-forming cells (Meg-CFC). Once the Mks reach a certain maturation level, they undergo endomitosis, which includes several rounds of DNA replication without any nuclear or cytoplasmic division. After successful rounds of replication, the immature polynucleated Mks develop into giant, polynucleated cells that can produce platelets. (Image created using BioRender.com, adapted from Williams hematology)

Platelets are anucleated cells that play an essential role in thrombosis and hemostasis. Different models of platelet production have been proposed. The first model assumes the fragmentation of mature Mks (Kaluzhny and Ravid 2004). According to this model, platelets are generated from cytoplasmic extensions called proplatelets already equipped with platelet organelles that are then released into the blood by fragmentation (Italiano *et al*. 2021). These

protrusions reach into the bone marrow sinusoidal lumen driven by microtubules which are the major drivers of this platelet fragmentation. Scanning electron micrographs were able to show the existence of proplatelet extensions supporting the proplatelet model (Garraud and Cognassa 2015). The second model is the membrane budding model. In this model, prepackaged platelets are continuously released by fractionation of the megakaryocytic cytoplasm, before the Mks undergo apoptosis and are removed by macrophages (Machlus and Italiano 2019).

1.1.2.2 Extrinsic control of megakaryopoiesis

The primary regulators of differentiation into the blood cell lineages are cytokines. IL-3 and IL-6 stimulate cell growth of the myeloid lineage and can impact MkPs. As discussed above, Mks are descendent from HSCs and regulated by the growth factor TPO by binding to the megakaryocyte-specific receptor c-Mpl (Kuter 2013). TPO is the main, but not the only regulator of Mk differentiation. Later maturation stages and platelet production do not depend on the growth factor TPO (Fig 6). Mice with a c-Mpl and TPO knockout exhibited an 85% reduction of platelet and Mk numbers. The remaining 15% were functionally identical to WT platelets and led to the hypothesis that IL-3 and IL-11 are able to exert megakaryopoietic activities in the absence of c-Mpl and TPO (Bunting *et al.* 1997*).*

1.1.2.3 Intrinsic control of megakaryopoiesis

Intrinsically, Mk differentiation is determined by the activation and expression of megakaryocytic genes controlled by a variety of transcription factors (Fig. 6). One of them is GATA-2 that controls the proliferation and viability of progenitor cells in early stages of megakaryopoiesis. Another one is GATA-1 that associates with Friend of GATA-1 (FOG-1) and reinforces differentiation into the megakaryocytic lineage by binding to the *Itga2b* gene, leading to the expression of CD41 and CD42 (Doré and Crispino 2011). Other important proteins are CD61 and von Willebrand Factor (vWF) expressed at later stages of Mk maturation. Nuclear factor E2 (NF-E2), another transcription factor, regulates Mk maturation at the later stages of differentiation and the release of platelets. After their release from the Mks, the enucleated platelets are no longer under the control of transcription factors (Shivdasani *et al.* 1995). It has also been shown that estrogen receptor (ER) signaling is relevant in Mk maturation, which the next chapter will be focusing on.

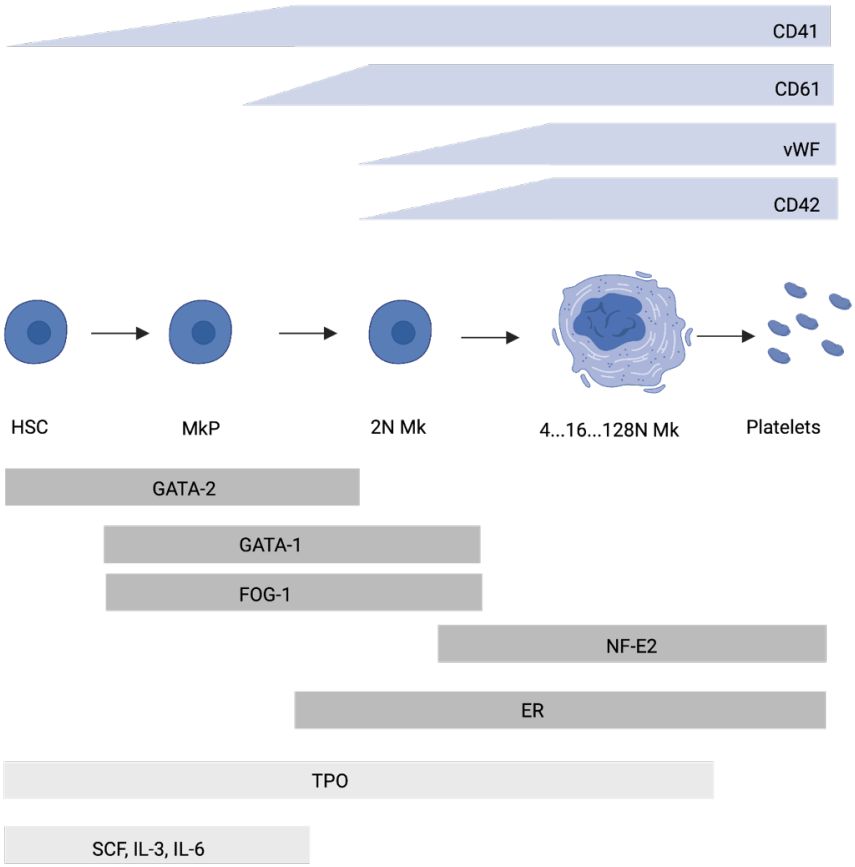


Figure 6: *Key transcription factors, surface proteins and cytokines of megakaryopoiesis*. Dark grey boxes: transcription factors vs light grey boxes: cytokines/growth factors. SCF acts in synergy with IL-3 and IL-6 to ensure the maintenance but also differentiation of the myeloid lineage. The main regulator of Mk differentiation is TPO. Later maturation stages and platelet production do not depend on TPO. GATA-2 controls viability and proliferation of HSCs and progenitors at early stages of megakaryopoiesis. GATA-1 and FOG-1 reinforce Mk differentiation leading to the expression of CD41 and CD42. Other important Mk proteins are CD61 and vWF. NF-E2 mediates polynucleation and platelet release. Estrogen receptor (ER) signaling is also relevant in Mk maturation. (Image created using BioRender.com, adapted from Hoffbrand and Moss 2011)

1.1.3 Megakaryocytes and estrogen signaling

The involvement of sex-specific differences in hematopoiesis has been known for a long time (Du *et al.* 2017). Estrogen is the primary female sex hormone, belonging to the class of steroid hormones, and is thus one of the prime suspects in the regulation of sex-specific differences in hematopoiesis. It can act on HSCs, myeloid and lymphoid progenitors to regulate proliferation and differentiation (Kovats 2015). The serum level of estrogen in females is 4 times higher compared to male levels, and reaches its maximum during pregnancy. The fat

soluble molecule migrates through the cell membrane to bind to the main estrogen receptors in the cytoplasm: ERα and ERβ (Du *et al.* 2017, Mooradian *et al. 1987*). Estrogen binding induces a conformational change of the receptors and subsequently their dimerization. The activated complex translocates to the nucleus where it regulates the transcription of its target genes (Fuentes and Silveyra 2019). Estrogen has been observed to have an effect on megakaryopoiesis. It has been demonstrated that elevated levels of estrogen lead to enhanced platelet counts, crucial in the development of thrombosis (Butkiewicz *et al.* 2006). Upon activation by estrogen, the nuclear ERβ binds to the promotor of *GATA1* and subsequently activates its transcription. Enhanced levels of GATA-1 upregulate NF-E2 to promote Mk polyploidization and extrusion of platelets (Du *et al.* 2017). The effect has been demonstrated in women treated with a hormone replacement therapy (HRT) to increase estrogen levels and to lower the symptoms of menopause. These changes lead to increased numbers of Mks accompanied with high platelet counts. Linked to platelets is the higher risk of thrombosis in women undergoing HRT (Dupuis *et al.* 2019; Bord *et al.* 2000; Wu 2005). However, sex-specific differences in disease susceptibility and therapeutic efficiency in hematopoietic malignancies remain under investigated.

1.1.4 Megakaryocyte pathology

Mks are a key cell type in health as well as disease. They have a crucial role in physiological processes including blood homeostasis, wound healing and in the defense against infections (Wen *et al*. 2016). Mks directly influence HSCs in the bone marrow niche to promote quiescence and HSC regeneration (Zhao *et al*. 2014). While Mk-derived chemokine CXCL4 and transforming-growth-factor β1 (TGF-β1) maintain HSCs in a quiescence state, the absence of Mks results in aberrant HSC activation and proliferation (Bruns *et al*. 2014). On the other hand, Mks are capable of enhancing HSC expansion through the release of fibroblast growth factor 1 (FGF1) (Zhao *et al.* 2014). Myeloproliferative Neoplasms (MPNs) are a group of blood cancers characterized by the expansion of HSCs and the overproduction of various mature myeloid cells, including Mks, which are especially important in disease pathology (Guo *et al.* 2017). The $JAK2^{V617F}$ mutation is a driver of MPN disease, it is present in a majority of MPN patients and is associated with an increased HSC expansion (Barbui *et al.* 2018). Even though dysregulated myelopoiesis in MPN is mainly associated with genetic alterations within HSCs, abnormalities in the bone marrow niche are also recognized to be an important factor. A mouse model expressing $JAK2^{V617F}$ restricted to the Mk lineage showed an MPN phenotype with increases in HSCs and mature myeloid cells. $JAK2^{V617F}$-positive Mks were found to interact with normal Mks and HSCs and contribute to the abnormal clonal expansion (Zhan *et al.* 2016). Since a curative treatment for MPN has not been found so far, it is important to better

understand the underlying processes regulating the differentiation of Mks and the complex interactions within the bone marrow microenvironment.

1.2 The JAK-STAT pathway

The Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is fundamental in hematopoiesis. Its role is to integrate extracellular signals and transmit them to the nucleus (Morris *et al,* 2018). It is activated through binding of cytokines, growth factors and hormones to cell membrane receptors and mediates differentiation, proliferation and survival of blood cells (Bousoik and Aliabadi 2018). A specific pathway, crucial for the development of various hematopoietic cells, is the JAK2-STAT5 pathway that is activated by the binding of TPO and erythropoietin (EPO) to their respective receptors. They represent the main cytokine signaling pathways leading to the production of Mks/platelets and erythrocytes (Morrison *et al. 2018)*. Since the JAK-STAT pathway is involved in fundamental cellular events, it needs to be tightly regulated. Its deregulation consequently leads to the development of hematological malignancies and it has been found to be hyper-activated in many forms of leukemia (Bousoik and Aliabadi 2018).

1.2.1 The signaling mechanism of the JAK-STAT pathway

The JAK-STAT pathway relays extracellular signals via JAK tyrosine kinases that are non-covalently attached to cell surface receptors (Fig. 7). Once the binding of various cytokine/growth factor ligands to their respective receptors has led to receptor activation and dimerization, the JAKs trans-phosphorylate one another as well as the C-terminal tails of the receptor (Bousoik and Aliabadi 2018*)*. This leads to the subsequent recruitment and activation of STAT anti-parallel dimers which bind to the phosphorylated receptor via their N-terminal domain. The STATs are then phosphorylated by JAKs at a key tyrosine residue within their SH2 domains, causing them to change into parallel dimers, become activated and translocate into the nucleus to act as transcription factors responsible for the activation of target genes (Villarino *et al.* 2016*)*. To preserve the functional homeostasis of cytokine signaling, suppressor of cytokine signaling (SOCS) proteins cause dephosphorylation of JAKs and STATs to repress the signaling pathway (Bousoik and Aliabadi 2018). This can happen via different mechanisms. The first one occurs by direct inhibition of JAK activation through competitive binding to the receptor. The second inhibits the action of the JAK proteins directly by a SOCS E3-ubiquitin ligase complex that induces their proteasomal degradation (Linossi *et al*. 2018). Additionally, protein inhibitors of activated STATs (PIAS) and protein tyrosine phosphatases (PTPs) belong to the negative regulators that terminate signaling of the JAK-STAT pathway. The PTPs

dephosphorylate tyrosine residues and consequently reverse the activity of the JAKs and STATs. The interaction of PIAS proteins and activated STAT dimers prohibit STAT-mediated transcription (Rawling *et al.* 2004).

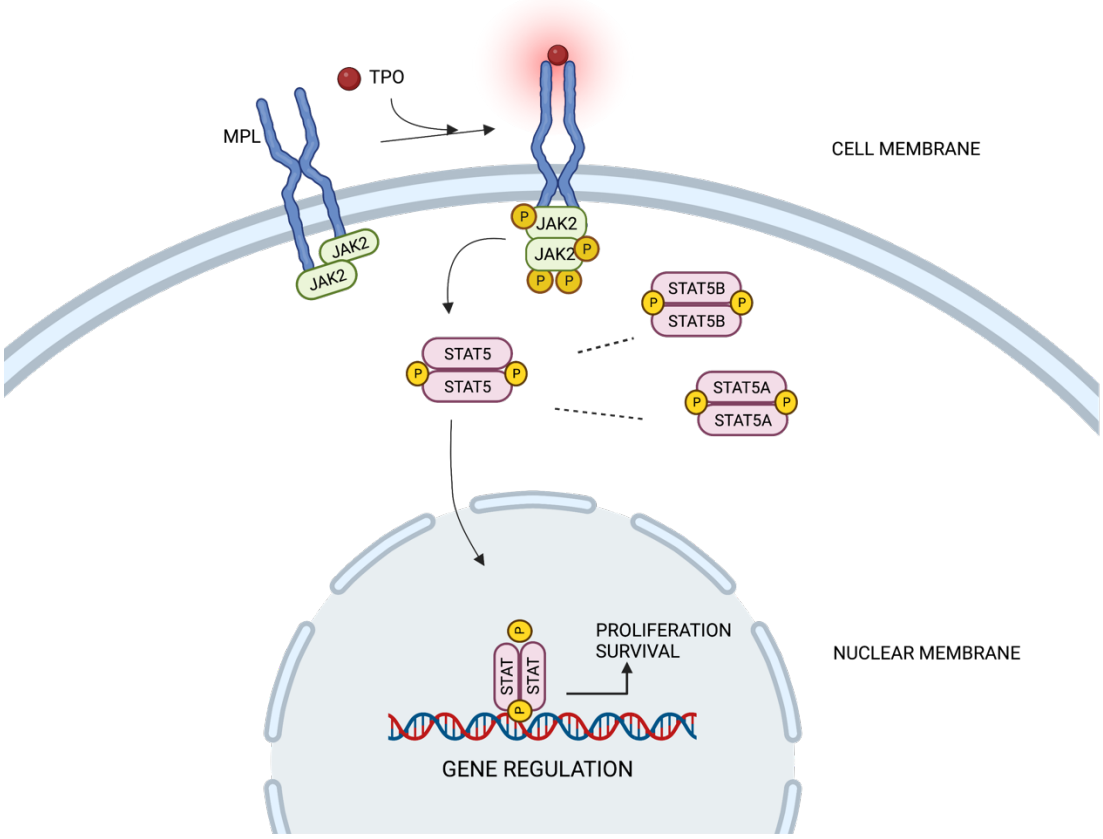


Figure 7: *The JAK-STAT signaling pathway.* The JAK2-STAT5 pathway relies on the binding of extracellular ligands, such as the growth factor TPO, to activate their surface receptors, like the TPO receptor c-Mpl. Once activated, the receptor-associated JAKs trans-phosphorylate one another as well as the receptor, which leads to the subsequent recruitment of STAT proteins. The STATs are then phosphorylated by the JAK kinases and translocate to the nucleus to activate their target genes, regulating important processes such as cell proliferation and survival. (Image created using BioRender.com)

1.2.2 The JAK family members

The JAK family consists of 4 members: JAK1, JAK2, JAK3 and TYK2 (Tyrosine kinase 2). The JAK proteins are made up of seven so-called JAK homology (JH) domains 1-7 (Fig. 8). The C-terminal JH1 domain is the tyrosine-kinase domain, responsible for the kinase activity of the JAKs. The JH1 domain is followed by the JH2 domain that has been classified as the pseudokinase domain. However, it has low-level functions of a serine/threonine-kinase (Min *et al.* 2015) and acts as negative regulator of the kinase activity of the JH1 domain. JH3 and 4 make up the Src homology 2 (SH2) domain that enables binding to phosphorylated tyrosine

residues. The four-point-one, ezrin, radixin, moesin (FERM) domain, formed out of the JH5-7 domains, is localized at the N-terminus and is required for the interaction with the receptor (Bousoik and Alibadi 2018).

Figure 8: *Structure of the JAK protein.* The JAK proteins are structured into seven JH domains: JH1 is the tyrosine-kinase domain and the JH2 domain (referred to as the pseudokinase domain) is a serine/threonine kinase domain that negatively regulates the JH1 domain. JH3 and 4 form the SH2 domain that binds to phosphorylated tyrosine residues. JH5-7 make up the FERM domain that enables the interaction with the receptor (Image created using BioRender.com).

1.2.3 The STAT family members

The members of the mammalian STAT family are STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. Their protein structure is made up of six conserved domains: the N-terminal domain, the coiled-coil domain, the DNA binding domain, the linker domain, the SH2 domain and the transactivation domain (TAD) at the C-terminus (Fig. 9). The N-domain is the oligomerization domain that mediates protein-protein interactions of STAT dimers to form tetramers (Mitchel and John 2005). The coiled-coil domain allows dimerization of anti-parallel unphosphorylated STATs. Dimerization of parallel STAT proteins takes place at the SH2 domain upon tyrosine-phosphorylation (pY). For STAT5, Y695/Y699 (STAT5A/STAT5B) is the position where the tyrosine-phosphorylation takes place, located between the SH2 domain and the TAD. The DNA binding domain is crucial for the action of the STATs in transcriptional regulation, and the TAD enables the interaction of the STATs with other proteins that participate in transcriptional regulation (Hennighausen and Robinson 2008).

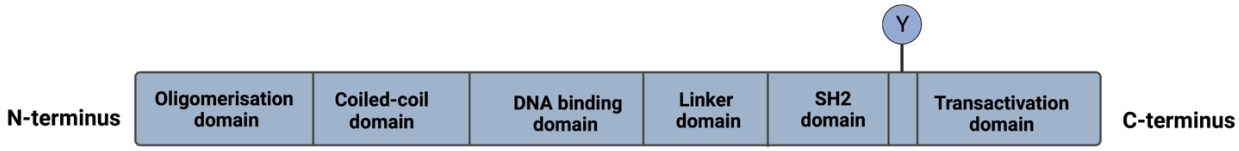


Figure 9: *Structure of the STAT proteins.* The STAT proteins contain six conserved domains: the N-terminal domain, the coiled-coil domain, the DNA binding domain, the linker domain, the Src homology 2 (SH2) domain and, at the C-terminus, the transactivation domain. The main, activating tyrosine (Y)-phosphorylation site induced by the JAKs occurs between the SH2 and transactivation domains (Image created using BioRender.com).

1.2.3.1 Role of STATs in megakaryocyte signaling

STAT1, STAT3 and STAT5 act as signal regulatory proteins in megakaryopoiesis and are activated through the binding of TPO to its receptor c-Mpl. It has been proposed that activated STAT1 acts downstream of GATA-1 and IFN-γ as a transcription factor involved in endomitosis, a later stage of megakaryopoiesis and the production of platelets (Huang *et al.* 2007). STAT3 is involved in early stages of megakaryopoiesis presumably through the effective expansion of Mk progenitor cells, dispensable for platelet production (Kirito *et al.* 2002). STAT5 plays an essential role in the regulation of survival and proliferation of HSCs. It is activated by early acting cytokines involved in hematopoiesis (Kirito and Kaushansky 2006).

1.2.3.2 STAT5A and STAT5B gene products

STAT5 refers to two different gene products, STAT5A and STAT5B. In humans the proteins are encoded on chromosome 17 in close proximity. They share 89% DNA sequence homology and the amino acid sequence shows 92% sequence identity. Due to their similar protein structure, STAT5A and STAT5B have been shown to have redundant functions in proliferation and survival induced by pY-STAT5 (Halim *et al*. 2020). The activated STAT5 proteins function as transcription factors by migrating to the nucleus where they regulate transcription of their target genes. STAT5-specific target genes include *BCL2L1* or *PIM-1* that regulate processes involved in cell survival. *Cyclin D1* or *c-MYC* contribute to cell proliferation (Alvarez and Frank 2008). Prior to activation, non-tyrosine phosphorylated STATs (uSTATs) have also been proposed to have transcriptional regulatory functions in the nucleus. This was demonstrated in a study where uSTAT5 binding to Mk affiliated genes led to a block in Mk differentiation. A STAT5B mutant (Y699F), that was used to increase uSTAT levels by preventing tyrosine-phosphorylation, showed diminished Mk differentiation after TPO treatment. It was concluded by the authors that activated STAT5 regulates Mk proliferation and survival but it was not considered a key regulator of megakaryopoiesis (Park *et al.* 2016).

1.2.3.3 Non-redundant roles of STAT5A and STAT5B

STAT5A and STAT5B have redundant functions due to their similar protein structure, but also show non-redundant functions in different tissues. The most significant differences in structure are found at the C-terminal TAD region where STAT5A has 20 and STAT5B 8 unique amino acids. This results in differences in protein-protein interactions, changes in post-translational modifications and target gene expression (Hennighausen and Robinson 2008; Able *et al.*

2017). Specific *Stat5a* and *Stat5b* knock-out mice demonstrate their non-redundant functions in distinct tissues. Impaired mammary gland development upon the absence of STAT5A showed the specific importance of STAT5A in lactogenic signaling (Lui *et al.* 1996). STAT5B is uniquely involved in growth hormone (GH) signaling, which has been demonstrated by the severe growth failure observed in *Stat5b* deficient mice (Udy *et al.* 1997). Humans with *STAT5B* deficiency show postnatal growth retardation, an impaired immune system and GH insensitivity syndrome (Kofeod *et al.* 2003). One case of *STAT5B* deficiency also showed indications of thrombocythemia associated with impaired platelet production, which indicates a potential involvement of STAT5B specifically in Mk differentiation (Hwa *et al.* 2011). Although STAT5B is preferentially activated upon TPO treatment (Kollmann *et al.* 2021), the differential effect of STAT5A or STAT5B on Mk differentiation remains unexplored. Additionally, STAT5B has a higher oncogenic potential than STAT5A. STAT5 mutations found in patients suffering from hematopoietic cancer predominantly appear in the SH2 domain of STAT5B (Maurer *et al.* 2019). The most common oncogenic STAT5B mutation is N642H and has been found in patients suffering from lymphoid neoplasia. The $STAT5B^{N642H}$ mutation expressed in the hematopoietic compartment in transgenic mice causes a highly aggressive $CD8^+$ T-cell leukemia (Pham *et al.* 2018). In comparison, similar expression levels of the $STAT5A^{S710F}$ activating mutation showed no disease phenotype in mice. Only strong overexpression of the STAT5A mutation was able to induce a T-cell malignancy in mice (Maurer *et al.* 2019). Therefore, in different tissues or contexts, STAT5A and STAT5B can have non-redundant functions despite being highly similar proteins.

1.3 Rationale of the master thesis

Our group has recently investigated a new aspect of STAT5B biology – its involvement in megakaryopoiesis. As mentioned in previous chapters, there are hints of this in the literature but no in depth analysis has been performed so far. Using a transgenic mouse model expressing human STAT5B (hSTAT5B) in the hematopoietic compartment, we observed that a 1.5-fold expression of hSTAT5B showed a significant increase in LT-HSCs (Pham *et al*. 2018) and MEPs (unpublished) in the mice (Fig. 10). We decided to do a more in-depth characterization of the myeloid lineage and observed significant changes only in the Mk lineage. Namely, the MkPs and mature Mks were increased in the hSTAT5B transgenic mice compared to wildtype littermate controls. However, this effect was only observed in female mice. On the other hand, other myeloid cell types such as erythrocytes or granulocytes were not changed (Fig. 10).

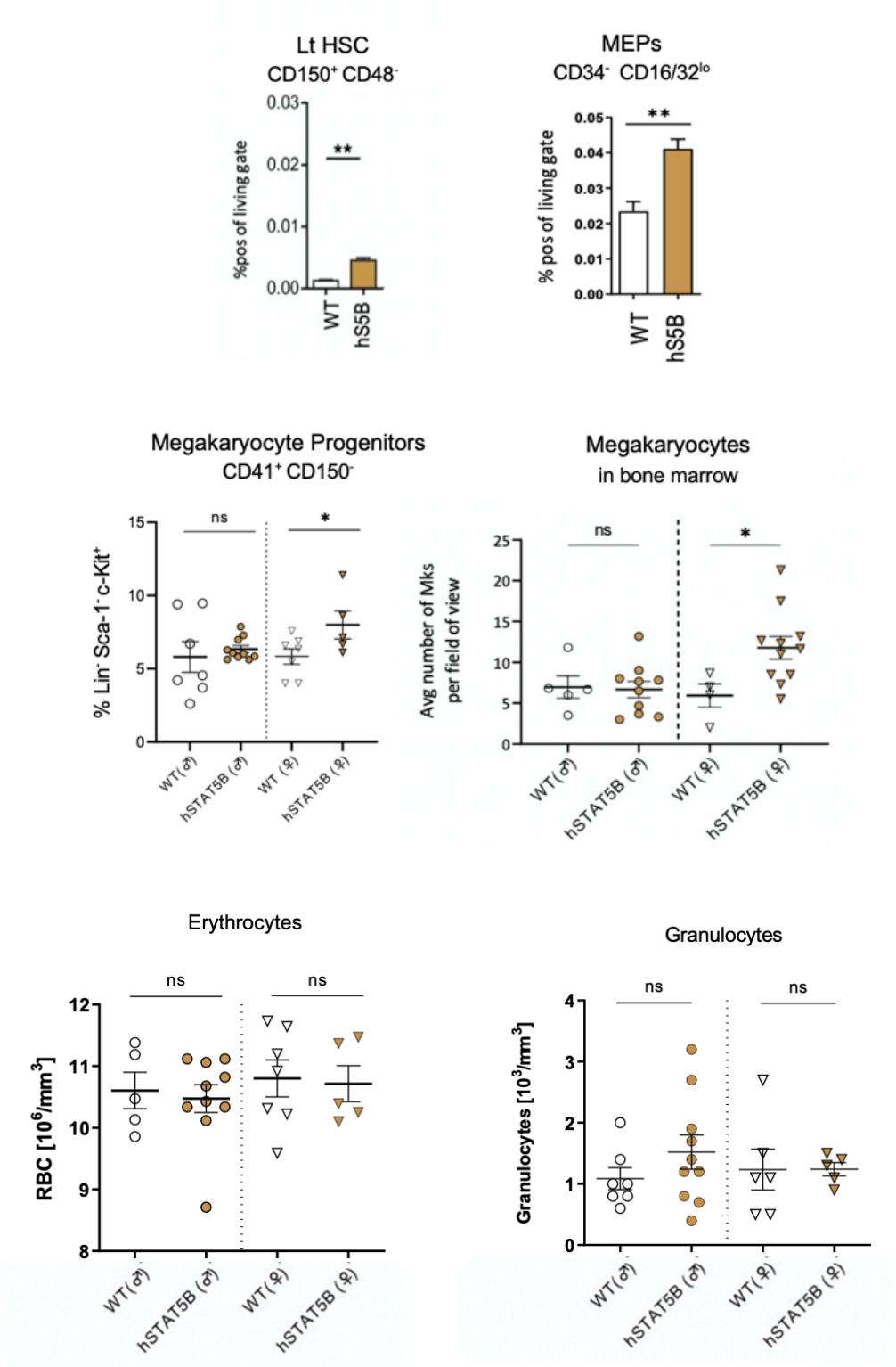


Figure 10: *Cells of the Mk lineage, but not other myeloid cell types, are increased in number in hSTAT5B transgenic mice.* LT-HSCs, MEPs and MkPs were quantified by FACS, Mks were quantified visually using H&E-stained histology sections, and erythrocytes and granulocytes were quantified using a VetABC hematocytometer. Error bars indicate the standard error of the mean. Statistics: unpaired Students t-test. ns = non-significant, * = $p < 0.05$, ** = $p < 0.01$ (LT-HSC data are from our previous publication, Pham *et al.* 2018, and the unpublished data are kindly provided by Marie Ploderer).

1.3.1 Aim of the master thesis

Based on the data above, we hypothesize that STAT5B promotes Mk differentiation in a sex-specific way. The aim of this study was to investigate the differential roles of STAT5A and STAT5B in megakaryopoiesis. Furthermore, we wanted to determine the effect of estrogen signaling on Mk differentiation mediated by STAT5B, since estrogen is the primary female sex hormone and we only saw a significant effect of increased STAT5B levels on cells of the Mk lineage in female mice.

For this we:

- investigated the effect of increased STAT5A and STAT5B levels on megakaryopoiesis using a murine hematopoietic stem cell line. Cell lines stably overexpressing either STAT5A or STAT5B were employed to perform Mk differentiation assays. To quantify the degree of differentiation, MkPs were quantified using flow cytometry. Additionally, the polynucleation of mature Mks was analyzed using propidium iodide and subsequent flow cytometry. Mature Mks were also visualized by cytospins stained with hematoxylin.
- studied the effect of the increased expression of either STAT5A or STAT5B on STAT1 and STAT3 expression, as well as on their activation level upon TPO stimulation, by performing western blot analyses on the overexpressor lines. We also studied the effect of TPO stimulation on the activation of STAT5A or STAT5B.
- investigated the sex-specific effect we observed in our mouse model by using the primary female sex hormone estrogen to stimulate the overexpressor lines during Mk differentiation.

2 Materials and Methods

2.1 Materials

2.1.1 Reagents and buffers used for qPCR analysis

RNA extraction

TRIzol™ LS Reagent (Thermo Fisher Scientific)
Trichloromethane/Chloroform (ROTH)
2-Propanol (ROTH)
3M Sodium acetate (Thermo Fisher Scientific)

cDNA synthesis kit

First strand cDNA Synthesis Kit (Thermo Fisher Scientific)

qPCR MasterMix

GoTaq qPCR MasterMix 2x (Promega)

qPCR primers

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

Name	Target	Species	Sequence	Origin
F2r fw	*F2r*	mouse	5' ATGAGCCAGCCAGAATCAGAG 3'	Designed by L. Schreiberhuber
F2r rev	*F2r*	mouse	5' CTCCAGCAGGACGCTTTCAT 3'	Designed by L. Schreiberhuber
vWF fw	*Vwf*	mouse	5' GGGACGACTTCATCAACACG 3'	Designed by L. Schreiberhuber
vWF rev	*Vwf*	mouse	5' CAGACAGGCTCATTCTCTTGC 3'	Designed by L. Schreiberhuber

MPL fw	*Mpl*	mouse	5’ GGCTCCTCTGGCTGTTTATT 3’	Designed by L. Schreiberhuber
MPL rev	*Mpl*	mouse	5’ TAGTCACAGCAGGCAGATTTC 3’	Designed by L. Schreiberhuber
GAPDH fw	*Gapdh*	mouse	5’ CAAGGTCATCCATGACAACTTTG 3’	Thermo Scientific cDNA synthesis kit
GAPDH rev	*Gapdh*	mouse	5’ GTCCACCACCCTGTTGCTGTAG 3’	Thermo Scientific cDNA synthesis kit

2.1.2 Reagents and solutions for Western blot

IP-buffer with inhibitors 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 (Sigma)
0.5% NP-40
1 mM Na_3VPO_4
1 mM NaF
10 µg/ml Leupeptin
10 µg/ml Aprotinin
1 mM PMSF
1x completeTM Protease Inhibitor Cocktail (Roche)

Loading buffer, 6x

1.6% SDS
20 mM Tris, pH 6.8
16% glycerol
0.24 g/ml bromophenol blue
0.04 g/ml dithiothreitol (DTT)
in ddH_2O

Stacking gel 5%

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

Running gel 8%

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

1x Tris/Glycine buffer
0.5% SDS

1x Transfer Buffer

1x Tris/Glycine buffer
20% Methanol

10x Tris buffered saline solution (TBS)

500 mM Tris, pH 7.9
1.5 M NaCl (Roth)

1x TBS-T

1x TBS
0.1% Tween-20

Blocking buffer

Odyssey® blocking buffer in PBS (Li-cor®)

Primary antibodies

***Table 2**: List of primary antibodies*

Antibody	Company	Cat. #	Dilution	Size in kDa	Host species
Actin (C-11)	Santa Cruz Biotechnology	B1411	1:10000	43	rabbit
STAT1	BD biosciences	610115	1:1000	84, 91	mouse
STAT3	BD biosciences	610189	1:1000	92	mouse
STAT5	BD biosciences	610192	1:1000	92	mouse
pYSTAT1	Cell Signaling Technology	9167L	1:1000	84, 91	rabbit
pYSTAT3	Cell Signaling Technology	9131S	1:1000	79, 86	rabbit
pYSTAT5	Cell Signaling Technology	9351S	1:1000	90	rabbit

Secondary antibodies

***Table 3**: List of secondary antibodies*

Antibody	Company	Target	Dilution	Host species
IRDye® 680RD Goat anti-Mouse IgG	LI-COR™	anti-mouse	1:10000	goat

IRDye® 800CW Goat anti-Rabbit IgG	LI-COR™	anti-rabbit	1:10000	goat

2.1.3 Reagents and solutions used for work with cell lines

Cultivation medium – HPC-7 cell lines

IMDM 1x (Gibco™)
10% fetal bovine serum, FBS (Gibco™)
1% penicillin-streptomycin solution (100x stock; Biowest)
5% SCF in media (kindly gifted by E. Heyes, Grebien group, Vetmeduni Vienna)
74.8 µM monothioglycerol (MTG; Sigma)

Cell proliferation assay

Solution prepared according to CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay protocol (Promega)

Megakaryocyte differentiation medium with Thrombopoietin (TPO)

StemMACS™ HSC Expansion Media, human
20 ng/ml recombinant mouse SCF (rmSCF; ImmunoTools)
100 ng/ml rmTPO (ImmunoTools)

Megakaryocyte differentiation medium with Estrogen

StemMACS™ HSC Expansion Media, human
20 ng/ml rmSCF (ImmunoTools)
100 ng/ml rmTPO (ImmunoTools)
10 nM beta-estradiol (Sigma-Aldrich)

2.1.4 Reagents used for FACS

FACS antibodies

***Table 4**: List of FACS antibodies*

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
CD19	Pacific Blue	eBio1D3 (1D3)	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	eBioMWReg30 (MWReg30)	mouse	ThermoFisher Scientific
CD150	APC	mShad150	mouse	ThermoFisher Scientific
CD16/CD32	-	93	mouse	eBioscience
Viability stain	APC-Cy7	-	-	eBioscience

2.1.5 Reagents used for Propidium Iodide staining

0.02 mg/ml Propidium Iodide Solution (BioLegend)
10 µg/ml RNase A Solution (Merck Millipore)
0.01% Triton-X in PBS

2.1.6 Reagents used for cytospins

Hematoxylin (Sigma-Aldrich)

2.2 Methods

2.2.1 Cultivation of mouse cell lines

Throughout the project, a SCF dependent hematopoietic stem cell-like murine cell line that was immortalized from embryonic stem cells (HPC-7) was used (Pinto et al. 1998), kindly gifted to us by Leif Carlsson (via the group of Veronika Sexl, Vetmeduni Vienna). They were regularly tested for mycoplasma with the MycoAlert™ mycoplasma detection kit (Lonza) and confirmed to be negative. Cells were maintained at a concentration of around $1x10^6$ cells/mL in cultivation media supplemented with monothioglycerol (MTG) and SCF, and incubated at 37°C with 5% CO_2 in a humidified environment. Cell viability was determined by taking an aliquot and mixing it 1:1 with sterile filtered trypan blue solution. A Neubauer chamber (Blaubrand) was filled with 10 µl of the stained suspension and the viable cells were counted visually using an Axiovert 35 Phase Contrast Microscope (Zeiss).

2.2.2 Harvesting of mouse cell lines

Cell harvesting for protein and RNA isolation was performed in the following way: Suspension cells were collected in a tube, centrifuged at 400x g for 5 min at 4°C, the media was removed and the cells were subsequently washed with ice-cold PBS. The spinning and washing steps were performed twice. Before snap-freezing the cell pellet in liquid nitrogen, as much PBS as possible was removed. The cells were then stored at -80°C until further analysis.

2.2.3 RNA extraction from mouse cell lines

3 x 10^6 suspension cells were used as starting material. 0.50 ml TRIzol™ LS Reagent was added to the cell pellet and incubated for 5 min at room temperature (RT). 0.1 ml of chloroform was added and the tube was then thoroughly mixed by shaking for 15 sec. The tubes were then incubated for 2-3 min at RT. Samples were centrifuged at 12000 x g at 4°C. The upper aqueous phase was transferred into a new tube without transferring any of the phenol or interphase. 0.25 ml of isopropanol was added to the aqueous phase. The solution was then incubated for 10 min at 4°C and centrifuged at 12000 x g at 4°C for 10 min. The supernatant

was removed and the RNA pellet was washed in 0.5 ml 75% EtOH and centrifuged at 7500 x g for 5 min at 4°C. The supernatant was discarded and the pellet dried at 55-60°C. 30 µL RNase-free water was used to dissolve the dry pellet. The tube was then incubated for another 10-15 min at 55-60°C. The RNA concentration was analyzed using a NanoDrop™ 2000 machine. For long-term storage the RNA was kept at -80°C.

2.2.4 Protein extraction from mouse cell lines

Cells were harvested and protein extraction performed by adding 50-200 µL of IP buffer to the harvested cell pellet and rotating it in a tube rotator STR4 (Cole-Parmer®) for 30 min at 4°C. The samples were then centrifuged at 16000 x g for 30 min at 4°C. The supernatant containing the soluble protein was then transferred into new vials, snap frozen in liquid nitrogen and stored at -80°C.

2.2.5 Bradford assay

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

2.2.6 Western blot analysis

The protein lysates were diluted in IP buffer and 6x loading buffer to allow the loading of 20 µg total protein per well. The samples were boiled at 95°C for 5 min to denature the proteins. The samples were then loaded onto the gel and the SDS-PAGE (sodium dodecyl-sulfate-polyacrylamide gel electrophoresis) was initially run for 10 min at 70 V and then an additional 2 h at 120 V. Each gel was loaded with a protein ladder (PageRuler™, Thermo Scientific). The proteins were blotted onto nitrocellulose by assembling a transfer sandwich in the following order: nitrocellulose membrane in the middle with Whatman® paper and fiber pads on top and underneath. All components were pre-soaked in transfer buffer. The sandwich was transferred into the transfer chamber together with ice cold transfer buffer and a magnetic stirrer. The blotting was conducted at 4°C and 340 mA for 1 h. After blotting, the membrane was incubated

in Odyssey® blocking buffer (LI-COR) for 1 h. Primary antibody was added in the appropriate dilutions (Table 2) and incubated for another 1 h at RT or overnight at 4°C on a shaker. The secondary antibody (Table 3) was used, after the membrane was washed three times for 5 min with TBS-T to reduce background signals, and incubated for 1 h at RT on a shaker. Detection was performed with the Odyssey® Imaging System and quantifications were done using the Image Studio™ Lite software (LI-COR).

2.2.7 qPCR analysis

0.5 - 1 µg of RNA was used to synthesize cDNA using the First strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. 2 µl of 1:5 diluted cDNA samples were pipetted into a 96-well plate and mixed with 8 µl of master mix (Table 5). The qPCR plate was analyzed using the CFX96 Touch Real-Time PCR Detection System with the program described in Table 6. The housekeeping gene *Gapdh* (Glyceraldehyde-3-phosphate-Dehydrogenase) was used to normalize the Ct values of each sample to the amount of cDNA employed.

***Table 5**: 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 6**: qPCR program*

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

2.2.8 Differentiation assay

HPC-7 cell lines were cultivated in StemMACS™ HSC Expansion Media at 37°C with 5% CO_2 at a concentration of 0.5 x 10^6 cells/ml. The cells were treated with SCF (20 ng/ml) and TPO (100 ng/ml) for the first three days, SCF was then removed and TPO treatment (100 ng/ml) was continued for an additional five days.

2.2.9 Differentiation assay with estrogen

HPC-7 cell lines were cultivated in StemMACS™ HSC Expansion Media at 37°C with 5% CO_2 at a concentration of 0.5 x 10^6 cells/ml. The cells were treated with TPO (100 ng/ml) for the first three days, TPO was then removed and cells were treated in 3 different ways for an additional five days: TPO treatment (100 ng/ml), estrogen treatment (10 nM), or no treatment.

2.2.9.1 Flow cytometry analysis

Cells from the differentiation assays were then harvested and stained with FACS antibodies (AB) in PBS at a 1:150 dilution listed in the MkP staining panel (Table 7). The samples were analyzed for MkPs, which are lineage negative (Lin^-), Sca-1^- and c-KIT^+ (LK) cells that are $CD41^+$ and $CD150^+$ positive, with a FACS Canto II flow cytometer. Lineage markers include: TER119 for erythroid cells, CD11b for macrophages and broad myeloid cell populations, CD3e for T-cells, CD19 for B-cells and Ly6G for monocytic and granulocytic cell lineages. The quantification was done with FlowJo software (Biosciences).

***Table 7**: MkP staining panel*

Antibody name	Fluorophore	Target species
TER-119	Pacific Blue	mouse
CD11b	Pacific Blue	mouse
CD3e	Pacific Blue	mouse
Ly6G	Pacific Blue	mouse
CD19	Pacific Blue	mouse

Sca-1	PE-Cy7	mouse
c-Kit	PerCP-Cy 5.5	mouse
CD41	PE	mouse
CD150	APC	mouse
CD16/32	-	mouse
Live-dead	APC-Cy7	mouse

2.2.9.2 Ploidy analysis

For the ploidy analysis, the harvested cells were washed with ice cold PBS and centrifuged at 300 x g at 4°C for 5 min. The cell pellet was resuspended in ice cold 70% ethanol to fix the cells. After an incubation time of 30 min, cells were incubated with propidium iodide (PI) staining solution (0.5 mg/ml; BD Bioscience) combined with RNase A solution (10 mg/ml) and 0.1% Triton X-100 for 45 min at 37°C. The stained cells were analyzed using a FACS Canto II flow cytometer and quantified with FlowJo software.

2.2.9.3 Cytospin

Cytospins of the cells were performed to visualize the differentiated Mks. The setup comprises of a slide clip to insert the slide and a filter card in position next to the cytofunnel. The assembled slide was placed into the Thermo Shandon Cytospin 3 Centrifuge and 400 µl of cell suspension (at a concentration of 0.5 x 10^5 cells/ml) was loaded into the cytofunnel. The cells were then centrifuged at 800 x g for 5 min. The slides were dried at RT for 2 h and later stained with hematoxylin.

2.2.10 MTS proliferation assay

Cells of each cell line were washed once with 1x PBS, counted and collected at 400 x g for 5 min. They were then seeded in triplicates into a 96-well plate in 100 µl per well of cultivation media at a density of 10,000 cells/ml. They were then cultivated under standard conditions for various lengths of time. Reagents for the MTS proliferation assay were prepared as described in the manufacturer's protocol (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation

Assay, Promega). At each different time point, 20 µl of the freshly prepared MTS/PMS solution was added per well. The cells were incubated for 2.5 h at 37°C and the absorbance was then measured on a plate reader at 490 nm.

2.2.11 Statistics

Where appropriate, one-way analysis of variance (ANOVA) or two-way ANOVA were performed with Bonferroni multiple comparison tests using the GraphPad Prism 5 software (Dotmatics).

3 Results

3.1 Characterization of model HPC-7 cell lines

As described above (Fig. 10), our unpublished data revealed that STAT5B could be involved in Mk differentiation. We thus wanted to further investigate how STAT5B influences Mk differentiation and if its effect is distinct from that of STAT5A. For this we chose the murine cell line HPC-7 as an *in vitro* model since it is known that it can undergo Mk differentiation in response to TPO stimulation (Park *et al*. 2016). Three HPC-7 lines were generated by Heidi Neubauer (Vetmeduni Vienna) before the start of this study (Table 8): one empty pMSCV vector control (EV), one overexpressing human STAT5A (hSTAT5A) and one overexpressing human STAT5B (hSTAT5B). All three lines also express GFP via an internal ribosome entry site (IRES). The increased STAT5A or STAT5B expression levels should allow for the study of how each variant contributes to the Mk differentiation program.

***Table 8**: Transduced HPC-7 cell lines used in this study*

Cell line	Cell type	Cytokine dependency
HPC-7 pMSCV_EV	HSC-like murine cell line expressing the EV	SCF dependent
HPC-7 pMSCV_hSTAT5A	HSC-like murine cell line overexpressing STAT5A	SCF dependent
HPC-7 pMSCV_hSTAT5B	HSC-like murine cell line overexpressing STAT5B	SCF dependent

3.1.1 Validation of the STAT5 variant expression levels

To make sure that the STAT5 variants stably expressed in the HPC-7 cell lines are expressed to a similar degree, the protein levels were assessed via Western blot. Additionally, the GFP expression was regularly measured by flow cytometric analysis. This procedure was repeated before and after every differentiation experiment to ensure a stable protein expression over time. Stable STAT5A and STAT5B protein expression levels between cell lines, as well as consistently high GFP expression levels (>95% of cells), were confirmed (Fig. 11 and Fig. 12). Under basal culturing conditions, without any additional cytokine stimulation, there is no pYSTAT5 signal (Fig. 11).

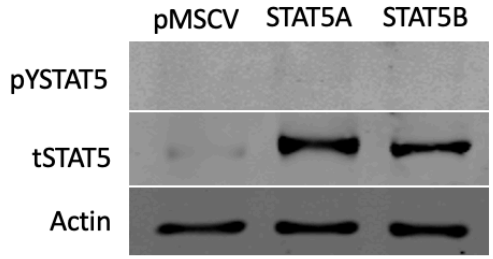


***Figure 11**: STAT5 protein expression levels of stably transduced HPC-7 cell lines*. Western blots from cell lysates of the transduced HPC-7 cell lines expressing STAT5A, STAT5B or empty vector pMSCV were performed using antibodies against pYSTAT5 and total STAT5. Actin was used as a loading control. Representative of two independent experiments (n=*2*).

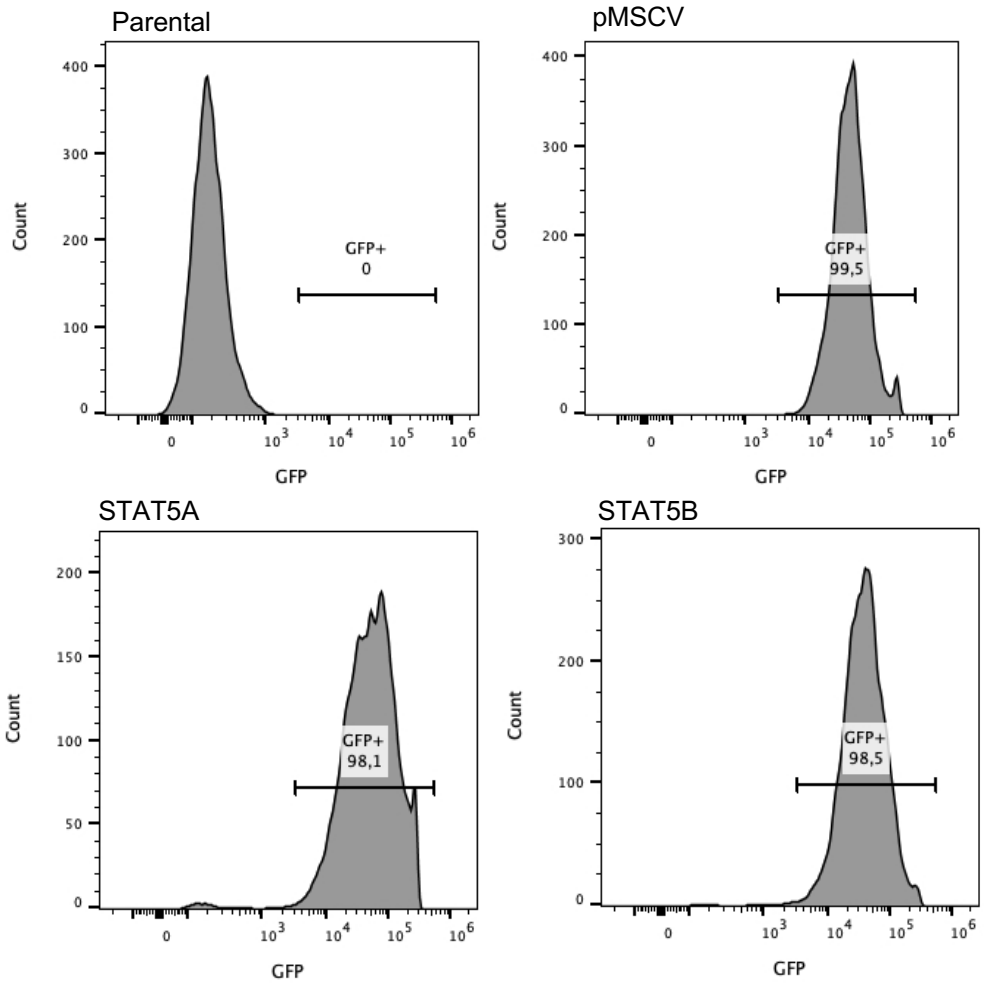


***Figure 12**: GFP expression of stably transduced HPC-7 cell lines*. HPC-7 cell lines stably expressing STAT5A, STAT5B or empty vector pMSCV, as well as parental HPC-7 cells, were analyzed for GFP expression (%) by flow cytometric analysis. Representative of two independent experiments (n=2).

3.1.2 Cell proliferation assay upon altered STAT5 levels

To further characterize the cell lines, we determined their proliferation rate using the MTS assay. Metabolically active cells reduce 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into formazan, that is visible as a purple color and linearly correlates with the cell number. The absorbance can then be recorded at 490 nm and the proliferation can thus be calculated as fold-change from day 0. The HPC-7 cell line with overexpressed STAT5B levels showed a significant proliferation advantage at day 4 in comparison to the lines overexpressing STAT5A or the empty vector control (Fig. 13). This demonstrates that STAT5B promotes proliferation of murine hematopoietic stem cells, whereas STAT5A seems to have the opposite effect. However, these results need to be validated by repeating the experiment.

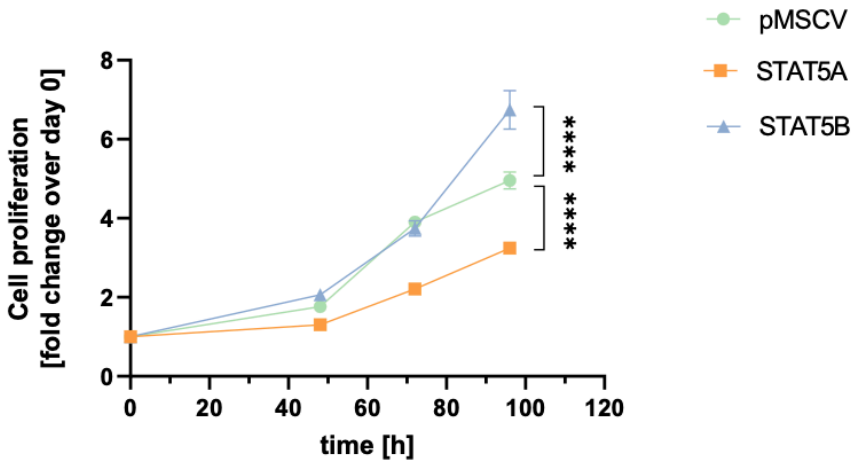


Figure 13: *MTS assay to monitor the proliferation of the HPC-7 cell lines with altered STAT5 expression levels.* The MTS assay was performed every 24 h for a time period of 96 h. Graph shows data of one experiment with four technical replicates, representative of one experiment (n=1). pMSCV – empty vector control, STAT5A – overexpression of hSTAT5A, STAT5B – overexpression of hSTAT5B. Error bars indicate the standard error of the mean. To determine the significance a two-way analysis of variance (ANOVA) with Bonferroni correction was performed. Shown are the p-values for pMSCV vs STAT5A and pMSCV vs STAT5B. **** = p-value < 0.0001.

3.2 Megakaryocyte differentiation

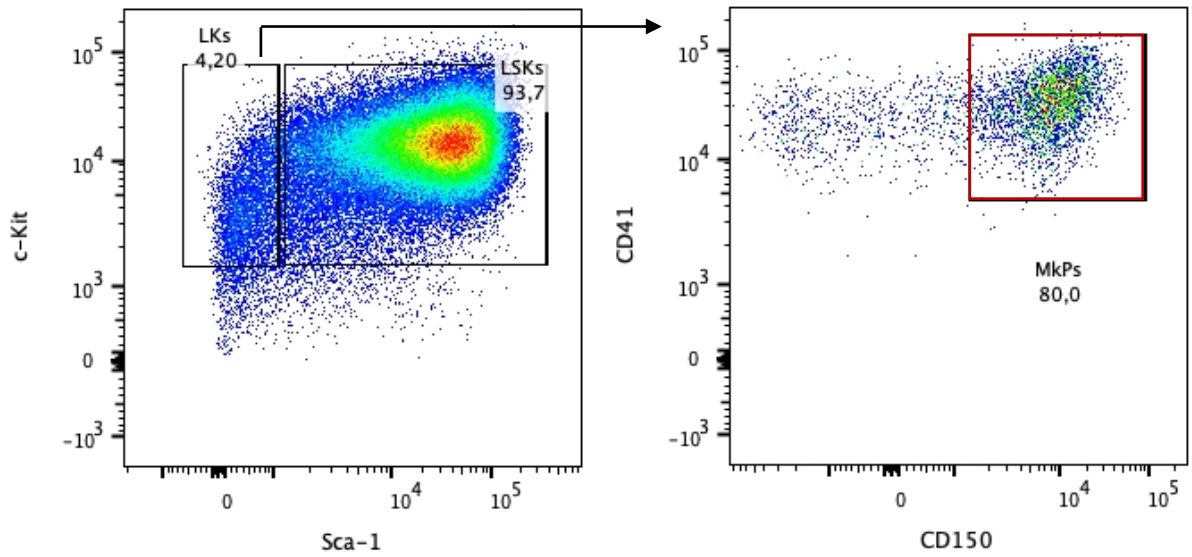
3.2.1 Megakaryocyte differentiation assay

Although STAT5B is preferentially activated by TPO stimulation compared to STAT5A (Kollmann *et al.* 2021), the different roles of STAT5A and STAT5B in Mk differentiation remain unexplored. To assess the impact of STAT5A or STAT5B on Mk differentiation, a differentiation assay was performed using the stably transfected HPC-7 cell lines. Before starting the experiments, several rounds of optimization to test different conditions were performed, to find the best possible culturing conditions for Mk differentiation from the HPC-7 cells. We achieved the best rate of Mk differentiation when treating the cells with TPO and SCF for 3 days and then removing the SCF, leaving the cells to differentiate for another 5 days in medium with only TPO. Mk differentiation was quantified by flow cytometric analysis of MkPs and mature Mks. Mature Mks were also visualized by stained cytospins of the treated cells. These analyses were performed at three different time points during the course of the differentiation assay (day 3, 6 and 8).

3.2.1.1 Quantification of MkPs

MkPs are a preliminary stage in the development of mature Mks. To identify them, specific cell surface markers can be used including CD41 and CD150 (Grisouard *et al*. 2015). Based on the literature we defined MkPs as Lin^-, Sca-1^- and c-KIT^+ (LK) cells that were $CD41^+$ and $CD150^+$. We found the highest percentage of MkPs in the STAT5B overexpressing cells. The STAT5A cells do not show the percentage of MkPs on the same level as in the STAT5B cells, but still higher compared to the pMSCV control (Fig. 14). This was the case at every time point we analyzed as well as at basal conditions. We infer from these data that both STAT5 variants can induce Mk differentiation, where STAT5B appears to promote megakaryopoiesis to a greater extent than STAT5A.

[A]



[B]

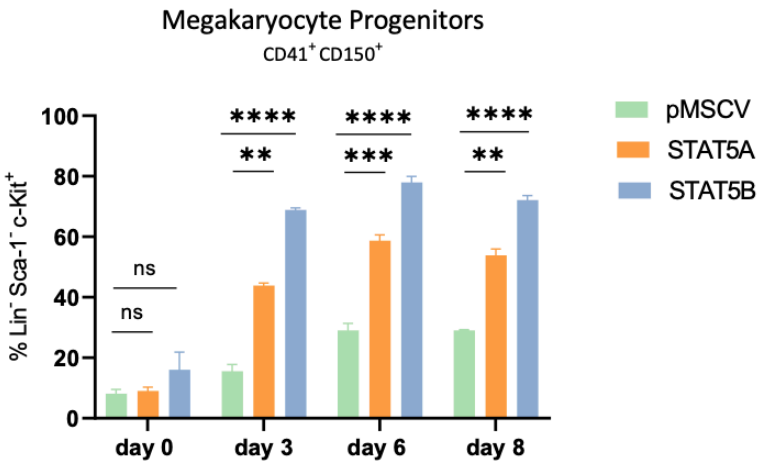


***Figure 14**: Flow cytometric analysis of MkPs differentiated from the HPC-7 cell lines with altered STAT5 expression levels.* Cells were treated with SCF (20 ng/ml) and TPO (100 ng/ml) for three days. SCF was removed and cells were left to further differentiate for another 5 days in the presence of TPO (100 ng/ml). [A] Gating strategy to identify the MkP population (red box) during the differentiation assay. The left plot was gated on Lin^- cells. [B] Percentage of MkPs in the stable HPC-7 cell lines assessed by flow cytometric analysis at three different time points of the differentiation assay. Graph shows quantification of one differentiation experiment with three technical triplicates and is representative of two independent experiments (n=2). Error bars indicate the standard error of the mean. To determine statistical significance a two-way ANOVA with Bonferroni comparison was performed. ns = non-significant, ** = p-value < 0.01, *** = p-value < 0.001, **** = p-value < 0.0001.

3.2.1.2 Ploidy analysis of polynucleated Mks

As mentioned above, Mks undergo polyploidization during their maturation process. The result is a highly polyploid nucleus with an average of 16N. Increased ploidy thus correlates with Mk maturation and its analysis can be used to determine the extent of Mk differentiation. The dye propidium iodide binds to DNA and emits fluorescence when bound, and the fluorescence intensity is proportional to the amount of DNA. We thus used propidium iodide to stain intracellular DNA and to determine the ploidy of the cells by flow cytometry during the differentiation experiments. Our analysis of cell ploidy showed a significant increase of mature 16N Mks in the overexpressed STAT5B cells over the course of the differentiation assay compared to the other cell lines (Fig. 15). No significant difference was observed between the STAT5A- and pMSCV-expressing cells. We thus conclude that STAT5B plays an important part in the final maturation stages of Mks.

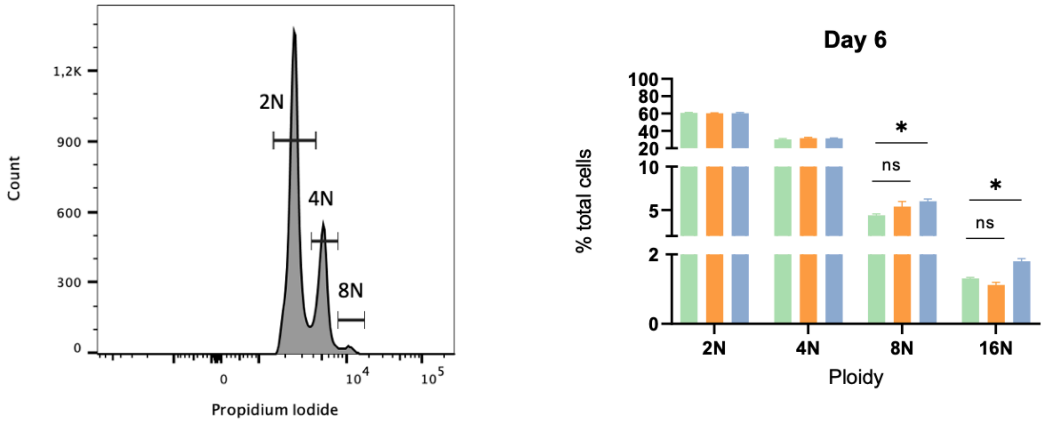


Figure 15: *Ploidy analysis of differentiated HPC-7 cell lines with altered STAT5 expression levels*. Cells were treated with SCF (20 ng/ml) and TPO (100 ng/ml) for three days. SCF was removed and cells were left to further differentiate for another five days in the presence of TPO (100 ng/ml). [A] Gating strategy of the ploidy of the cells stained with propidium iodide. [B] Ploidy of the cells was determined by flow cytometric analysis of the propidium iodide signal. Graph shows quantification of one differentiation experiment with three technical triplicates and is representative of two independent experiments (n=2). Error bars indicate the standard error of the mean. To determine statistical significance, a two-way ANOVA with Bonferroni comparison was performed. ns = non-significant, * = p-value < 0.05.

3.2.1.3 Visualization of Mks by cytospin analysis

We also wanted to directly visualize the mature Mks in the cell suspensions of the differentiation experiments, to support our ploidy analysis. For this, the treated cells were

attached to glass slides using a cytospin centrifuge and were stained with hematoxylin (Fig. 16). Due to their enlarged size and polynucleation, Mks can be easily distinguished from other cell types under a light microscope. From our images, we observed a trend of more mature Mks in the cytospins of the differentiated STAT5B cell line, compared to the STAT5A and pMSCV cells, fitting with our previous results.

All the data collected from the Mk differentiation assay thus suggests that STAT5B is involved in promoting Mk differentiation and that its role in this process is more prominent than that of STAT5A.

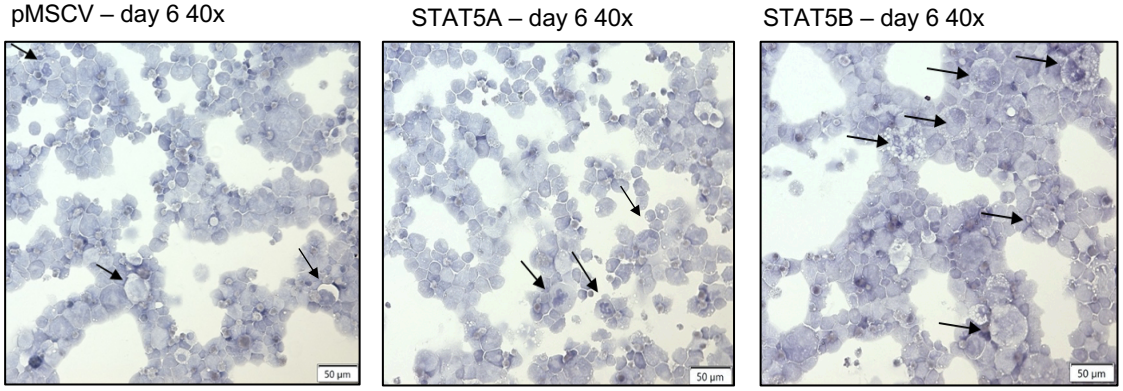


Figure 16: *Cytospin analysis of differentiated HPC-7 cell lines with altered STAT5 expression levels.* Cells were treated with SCF (20 ng/ml) and TPO (100 ng/ml) for three days. SCF was removed and cells were left to further differentiate for another five days in the presence of TPO (100 ng/ml). On day 6, cytospins were prepared for each cell line and stained with hematoxylin. Cells marked by an arrow are mature Mks identified by their increased size and ploidy. Representative of two independent experiments (n=2).

3.2.2 STAT signaling in the HPC-7 cell lines

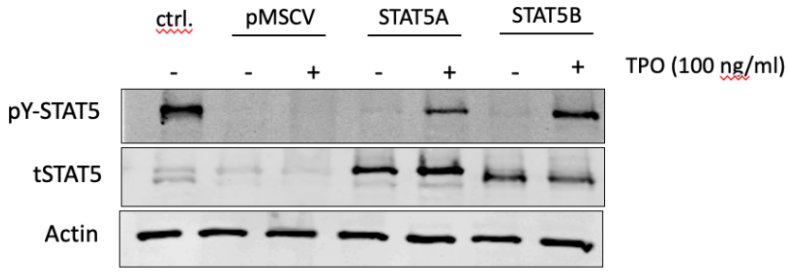
Given that TPO signals primarily through JAK2-STAT5, we wanted to examine the STAT5 activation status in the overexpressor lines upon TPO stimulation. Additionally, as other STATs, notably STAT1 and STAT3, have been shown to be involved in Mk differentiation (Huang *et al.* 2007), we also wanted to investigate if STAT5B indirectly impacts Mk differentiation by regulating the other STATs.

3.2.2.1 STAT5 activation upon TPO stimulation

It was important to examine the activation levels of STAT5A and STAT5B in the HPC-7 cell lines during the Mk differentiation assay. Western blot analysis was performed to evaluate the level of total STAT5 (tSTAT5) and pYSTAT5 in the stable STAT5A and STAT5B overexpressor

lines upon TPO stimulation. To determine the level of STAT5 activation, the pYSTAT5 signal was normalized to that of tSTAT5 for each line, in order to account for any differences in exogenous protein expression levels. We saw that, upon TPO stimulation, there is significantly stronger STAT5 activation in the STAT5B overexpressor line than in the STAT5A and EV control lines (Fig. 17). It appears that STAT5B is the dominant STAT5 protein activated downstream of TPO, confirming previous reports (Kollmann *et al.* 2021), which again supports our data that STAT5B has a key role in regulating megakaryopoiesis.

[A]



[B]

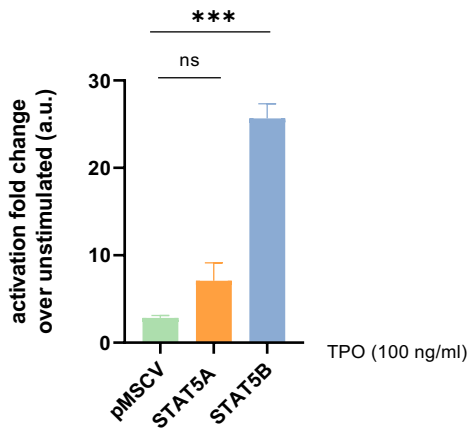


Figure 17: *STAT5 protein expression and activation upon TPO stimulation in the HPC-7 cell lines with altered STAT5 levels.* [A] Western blot analysis of the HPC-7 cell lines either unstimulated or TPO (100 ng/ml) stimulated for 30 min. Antibodies against total STAT5 or pYSTAT5 were employed. My-La cell lysates were used as a positive control for tSTAT5 and pYSTAT5. Actin was used as a loading control. [B] Quantification of the relative STAT5 activation of three independent experiments (n = 3). Error bars indicate the standard error of the mean. To determine statistical significance a one-way ANOVA with Bonferroni comparison was performed. ns = non-significant, *** = p-value < 0.001.

3.2.2.2 STAT1 and STAT3 expression and activation upon TPO stimulation

It is known that both STAT1 and STAT3 are also involved in the regulation of Mk differentiation. We thus evaluated whether higher STAT5 levels would impact STAT1 and STAT3 signaling.

We looked at both protein expression and activation by Western blot. Interestingly, pYSTAT3 could be detected in all samples, including in the absence of TPO (Fig. 18). Total STAT3 and pYSTAT3 levels, reflecting STAT3 activation, remained unchanged in the STAT5A and STAT5B cell lines (Fig. 18). We thus conclude that enhanced STAT5 levels do not alter STAT3 signaling.

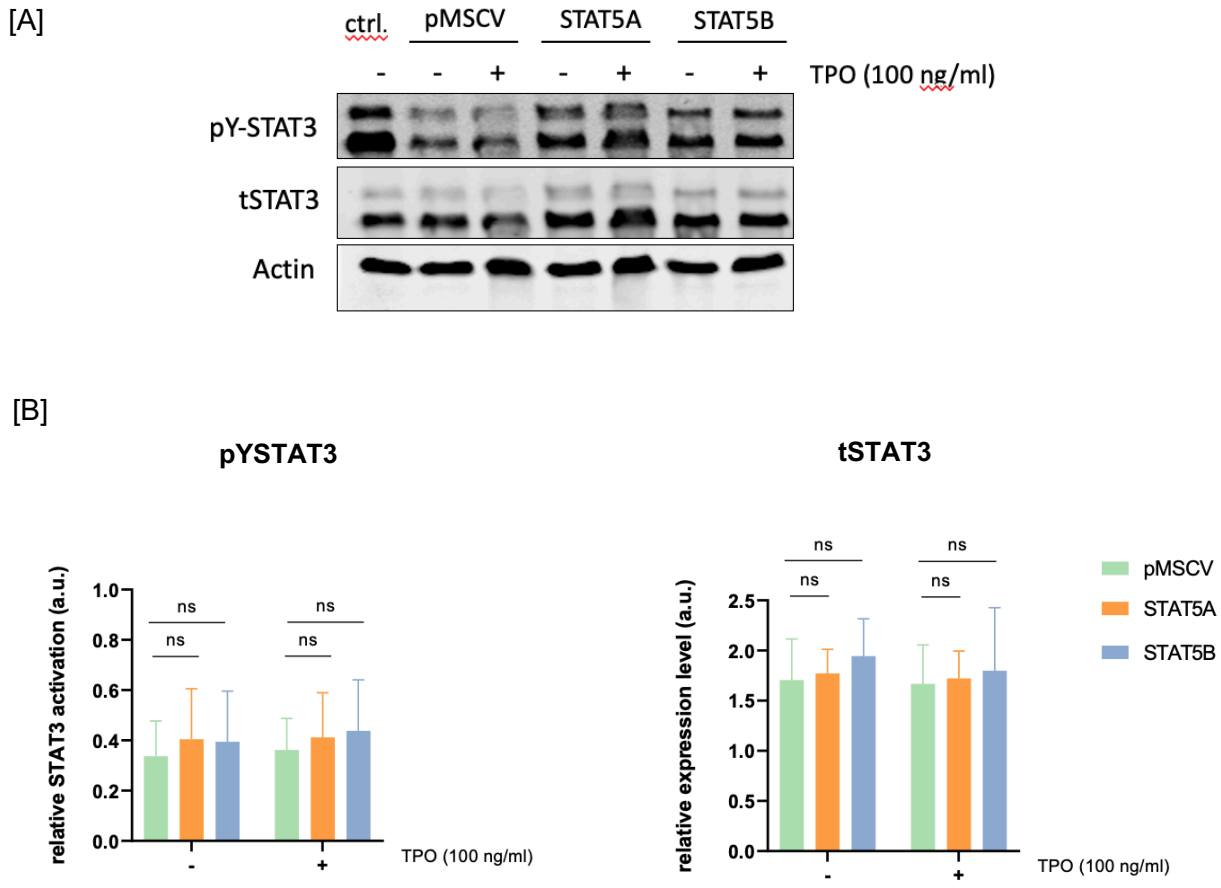


Figure 18: *STAT3 protein expression and activation in the HPC-7 cell lines with altered STAT5 levels.* [A] Western blot analysis of the HPC-7 cell lines from unstimulated and TPO (100 ng/ml) stimulated for 30 min. Antibodies against total STAT3 and pYSTAT3 were employed. My-La cell lysates were used as a positive control for tSTAT3 and pYSTAT3. Actin was used as a loading control. [B] Quantification of three independent experiments (n = 3) of total STAT3 levels as well as its relative activation. Error bars indicate the standard error of the mean. To determine statistical significance a one-way ANOVA with Bonferroni comparison was performed. ns = non-significant.

Even though STAT1 has been shown to be a key regulator in Mk differentiation, we could not detect any STAT1 phosphorylation after TPO treatment. The total protein levels of STAT1 remained unaltered by increased STAT5 expression (Fig. 19). We thus conclude that the effect that STAT5B has on megakaryopoiesis is not mediated indirectly by influencing STAT1 and STAT3 signaling.

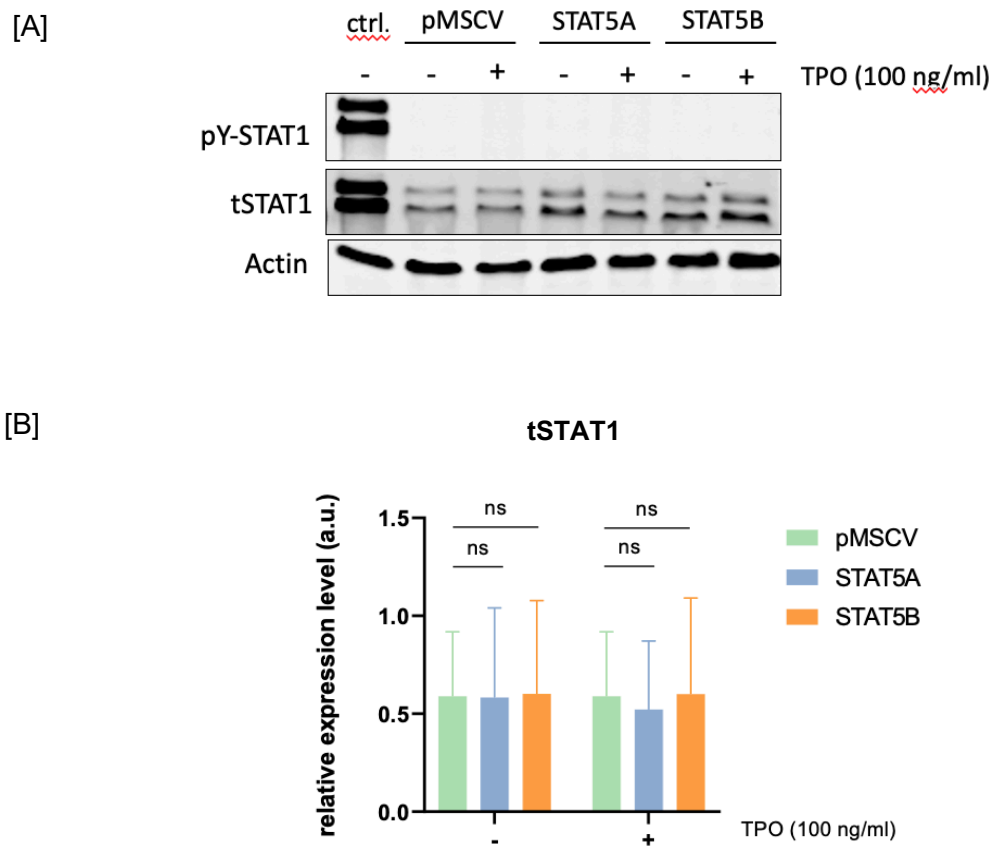


Figure 19: *STAT1 protein expression and activation in the HPC-7 cell lines with altered STAT5 level.* [A] Western blot analysis of the HPC-7 cell lines from unstimulated and TPO (100 ng/ml) stimulated for 30 min. Antibodies against total STAT1 and pYSTAT1 were employed. My-La cell lysates were used as a positive control for tSTAT1 and pYSTAT1. Actin was used as a loading control. [B] Quantification of three independent experiments (n = 3) of tSTAT1 levels. Error bars indicate the standard error of the mean. To determine statistical significance a one-way ANOVA with Bonferroni comparison was performed. ns = non-significant.

3.2.3 qPCR analysis of Mk specific genes

The collected data of the differentiation assay suggests that STAT5B has a more prominent role in Mk differentiation than STAT5A. We hypothesized that this would lead to a greater upregulation of genes characteristic of mature Mks in the STAT5B cell line. Therefore, the mRNA expression level of megakaryocyte genes, *F2r, Mpl* and *Vwf,* upon induced differentiation through TPO treatment was determined by qPCR analysis. F2r and vWF are associated with proplatelet-forming Mks and platelet function. c-Mpl expression on hematopoietic progenitors is the primary mechanism by which TPO can induce megakaryopoiesis. The c-Mpl receptor on Mks and platelets regulates the amount of TPO that is available to HSCs and progenitor populations (Tilburg *et al.* 2022).

3.2.3.1 Validation of qPCR conditions

First, the qPCR conditions and primers needed to be validated. The qPCR analysis was performed with three different primers pairs per gene. The primer efficiency was determined by running a standard curve of each primer pair. It is generally recommended that the efficiency of the primer pairs lies between 90-110%. All three primer pairs were found to be highly efficient and within the recommended range (*F2r* = 96,64%, *Mpl* = 97,37%, *Vwf* = 109.99%).

3.2.3.2 Results of qPCR analysis

The validated set of primers mentioned above were used to investigate the upregulation of genes in our cell models. Surprisingly, we found the lowest Mk affiliated gene expression of *F2r, Mpl* and *Vwf* in the STAT5B cell line after 6 days of differentiation, but the highest on day 0 (Fig. 20). The gene expression in the STAT5A cells was lower compared to the pMSCV control, even though STAT5B appears to promote Mk differentiation to a higher extent. This is not consistent with our results from the differentiation assay, where we saw an increase in Mk differentiation driven by STAT5B expression. The qPCR analysis was conducted once and needs to be repeated to validate the results.

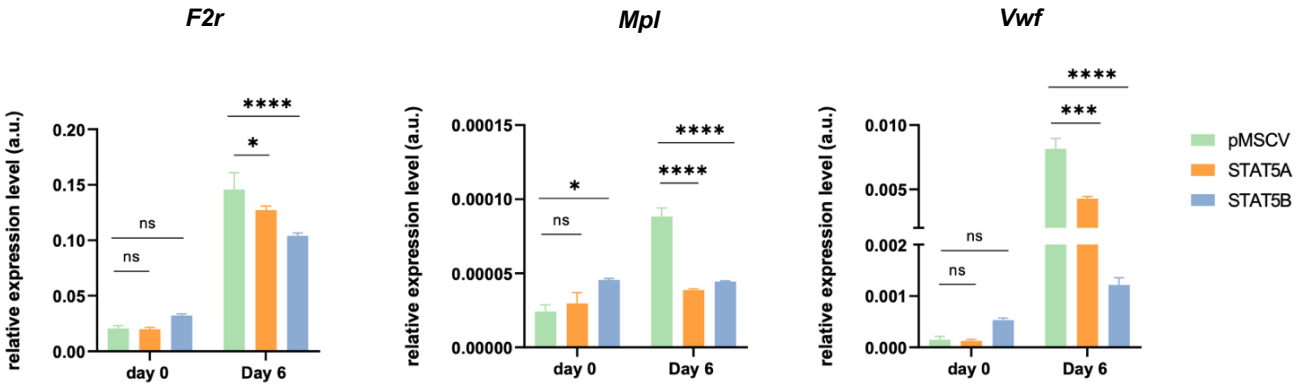


Figure 20: *Relative mRNA expression of Mk specific genes in the HPC-7 cell lines with altered STAT5 expression levels.* Before extracting the RNA, the cells were treated with SCF (20 ng/ml) and TPO (100 ng/ml) for three days. SCF was removed and the cells were left to further differentiate for another five days in the presence of TPO (100 ng/ml). mRNA expression was determined by qPCR analysis. *Gapdh* was used as a housekeeping gene for normalization. The ΔΔCt-Method was used to calculate the relative fold gene expression. The error bars indicate the standard error of the mean. To determine statistical significance a two-way ANOVA with Bonferroni comparison was performed. ns = non-significant, * = p-value < 0.05, *** = p-value < 0.001, **** = p-value < 0.0001.

3.3 Sex-specific effect in Mk differentiation

Preliminary data showed that a slight increase in hSTAT5B in a transgenic mouse model enhanced Mk differentiation in female mice, indicating a sex-specific effect. One instance in which estrogen has an effect on blood cells is in megakaryopoiesis (Du *et al.* 2017). This has been demonstrated in women treated with high doses of estradiol, which showed enhanced Mk differentiation and an elevated platelet count (Bord *et al.* 2000). However, the distinct role of estrogen and its regulatory function in megakaryopoiesis is largely unknown, and there are currently no reports of a sex-specific role for STAT5B in the hematopoietic system. To investigate the combined effect of STAT5B and estrogen on Mk differentiation, our cell lines were treated with estrogen during the Mk differentiation protocol. The previously described HPC-7 cell lines were cultivated with TPO for 3 days to initiate Mk differentiation. The cells were then subjected to three differentiation conditions for the remaining 5 days: TPO, estrogen or non-stimulated (TPO wash-out). These conditions were chosen to gain insight into the effect estrogen has on Mk differentiation and if it synergizes with STAT5B to promote Mk maturation. The condition where the cells were left without any stimulation after the initial TPO treatment was used as a negative control to reveal basal megakaryopoiesis, whereas the condition with continuous TPO exposure was the positive control of maximal Mk differentiation. The cells were harvested and analyzed via flow cytometry and ploidy analysis. We observed no difference between the cells treated with estrogen and the non-stimulated cells both in MkP percentage and ploidy of the cells (Fig. 21 and Fig. 22). This was the case for all three of the cell lines. As expected and previously observed, TPO induced strong Mk differentiation compared to the other conditions, where STAT5B induced the strongest Mk differentiation and STAT5A was also increased compared to the pMSCV control (Fig. 21 and Fig. 22).

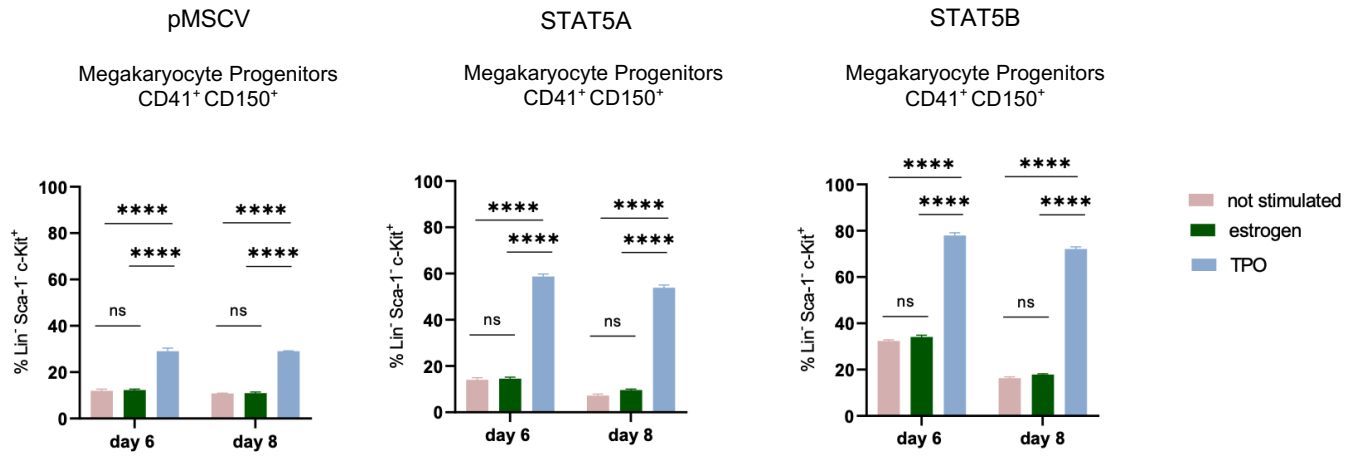


Figure 21: *Flow cytometric analysis of MkPs differentiated from HPC-7 cell lines with altered STAT5 expression levels to evaluate the effect of estrogen.* Cells were stimulated with TPO (100 ng/ml) for 3 days and subjected to three differentiation conditions for an additional 5 days: TPO (100 ng/ml), estrogen (10 nM) or non-stimulated. Percentage of MkPs treated with TPO, estrogen or non-stimulation was assessed by flow cytometry analysis. Graph shows quantification of one experiment with three technical triplicates and is representative of two independent experiments (n=2). Error bars indicate the standard error of the mean. To determine statistical significance a two-way ANOVA with Bonferroni comparison was performed. ns = non-significant, **** = p-value < 0.0001.

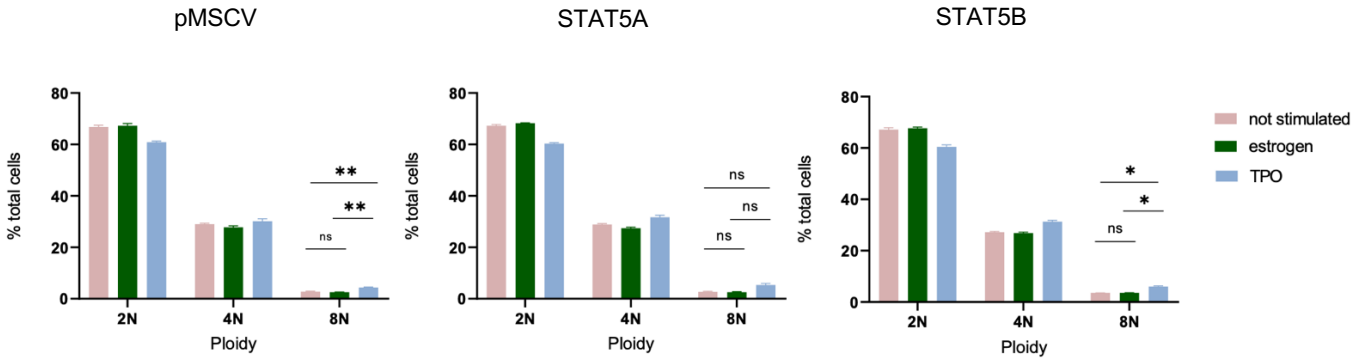
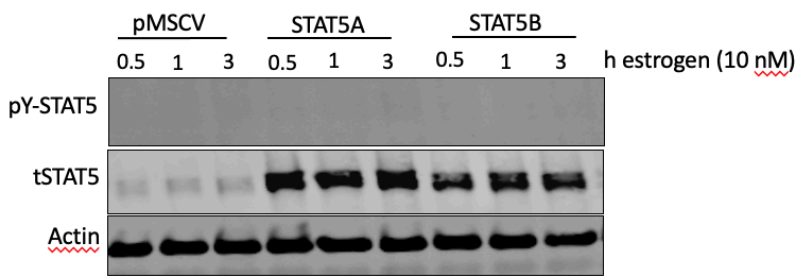


Figure 22*: Ploidy analysis of Mks differentiated from HPC-7 cell lines with altered STAT5 expression levels to evaluate the effect of estrogen.* Cells were stimulated with TPO (100 ng/ml) for 3 days and subjected to three differentiation conditions for an additional 5 days: TPO (100 ng/ml), estrogen (10 nM) or non-stimulated. Percentage of MkPs treated with TPO, estrogen or non-stimulation was assessed by flow cytometry analysis. Graph shows quantification of one experiment with three technical triplicates representative of two independent experiments (n=2). Error bars indicate the standard error of the mean. To determine statistical significance a two-way ANOVA with Bonferroni comparison was performed. ns = non-significant, * = p-value < 0.05, ** = p-value < 0.01.

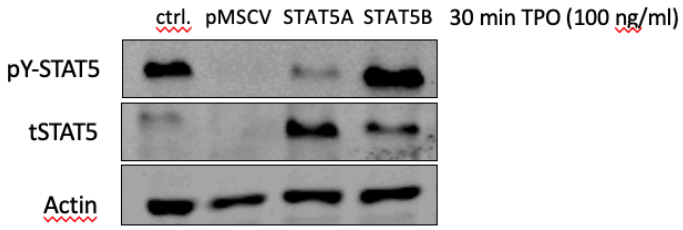
3.3.1 Western blot analysis upon estrogen stimulation

Since recent studies have reported that estrogen has distinct effects on polyploidization accompanied with higher platelet counts, our results from the Mk differentiation assay with estrogen surprised us. To determine if estrogen is having any effect on STAT5 signaling in our models, we performed a stimulation experiment with estrogen treatment, with and without pre-treatment with TPO, and looked at STAT5 activation by Western blot. We found that estrogen on its own does not activate STAT5 (Fig. 23A). As a control, the cells were stimulated with TPO alone, and as expected strong STAT5 activation was observed (Fig 23B). To replicate similar conditions to the previous differentiation experiments, where cells were exposed to TPO for three days before estrogen treatment, cells were pre-treated with TPO, then starved for several hours and then stimulated with estrogen. Samples were then collected at 0.5, 1 and 3 hours of estrogen treatment (combined treatment). As a negative control, cells were also harvested at the same time points after TPO starvation but without any addition of estrogen (starved condition). As expected, the pYSTAT5 signal decreased over time in the starved condition in both the STAT5A and STAT5B cell lines (Fig. 23C). Interestingly, in the combined treatment with estrogen, the pYSTAT5 signal and the tSTAT5B levels seemed to increase over time in the STAT5B but not the STAT5A cells (Fig 23D). This would suggest that estrogen can potentiate the effect of TPO on STAT5B and potentially stabilize its activation and total protein levels. The STAT5A signal did not increase but it did not go down either, which could also indicate a stabilizing effect. This effect was still observed when we normalized the pYSTAT5 signal of the starved condition and combined treatment to tSTAT5 with the original signal of the 30 min TPO stimulation (Fig 23E). So although we did not see an effect of estrogen on Mk differentiation, estrogen seems to influence STAT5 activation or total protein when combined with TPO stimulation. This stimulation experiment has been performed once so far and needs to be repeated to validate its results.

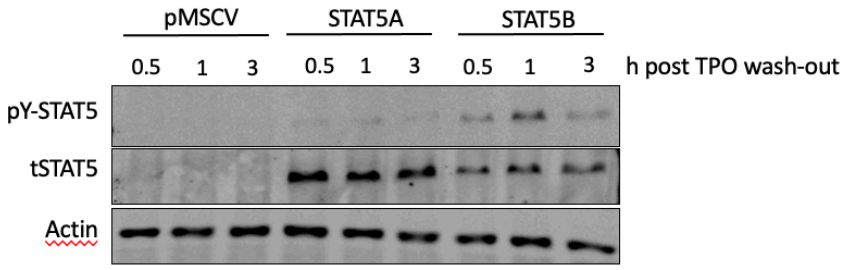
[A]



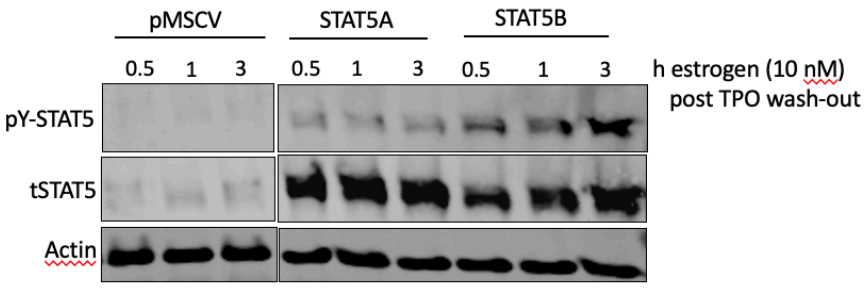
[B]



[C]



[D]



[E]

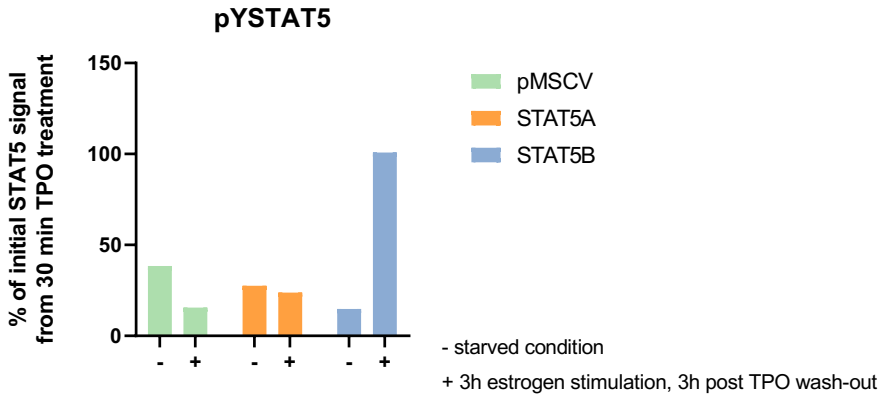


Figure 23: *STAT5 protein expression and activation in the HPC-7 cell lines with altered STAT5 levels.* Western blot analysis of the HPC-7 cell lines [A] treated with estrogen (10 nM), [B] treated with TPO (100 ng/ml), [C] treated with TPO (100 ng/ml) for 30 min, starved for 3 h and treated with estrogen (10 nM) for 3 h, or [D] treated with TPO (100 ng/ml) for 30 min then starved for 6 h. Antibodies against tSTAT5 or pYSTAT5 were used. My-La cell lysates were used as a positive control for tSTAT5 and pYSTAT5. Actin was used as loading control. [E] Quantification of western blots from one experiment (n = 1). To quantify the level of remaining pYSTAT5 after TPO removal, we normalized the starved condition to tSTAT5 [C] and combined treatment to tSTAT5 [D] signals, and then to the respective signal from the 30 min TPO stimulation.

In conclusion, the role of estrogen in regulating STAT5B-mediated Mk differentiation, to explain the sex-specific effects we observed in our transgenic mice, is inconclusive. However, estrogen may be able to regulate STAT5 activation levels or total protein levels. To confirm the role of estrogen in this process, further optimization and investigations in different models are needed.

4 Outlook and Discussion

Mks are a crucial cell type involved in blood homeostasis and they directly influence HSCs in the bone marrow (Zhao *et al*. 2014). Abnormalities in the bone marrow niche are recognized as a crucial factor in dysregulated megakaryopoiesis (Zhan *et al.* 2016). To further understand the complex interactions within the bone marrow niche, it is crucial to investigate Mk differentiation processes under hemostatic conditions. Our group's initial observations found that only a 1.5-fold increase in expression of STAT5B in the hematopoietic cells of transgenic mice enhanced the numbers of HSCs and MEPs (Fig.10). Further characterization revealed a significant increase in MkPs and Mks in female mice compared to the wildtype controls. Therefore, we hypothesized that STAT5B may act as a sex-specific regulator of megakaryopoiesis.

A immortalized SCF-dependent hematopoietic stem cell-like murine cell line (HPC-7) was used throughout the project. The cell line was generated from *LH2*-transduced embryonic stem cells of male mice. (Pinto *et al.* 1998). In the current project, stable HPC-7 cell lines were generated, containing the sequence of either hSTAT5A or hSTAT5B. These cell lines then expressed higher levels of either STAT5A or STAT5B, or an empty vector with GFP. As an initial characterization step of the cell lines, an MTS assay was performed to measure proliferation, which revealed that STAT5B overexpression conveys a proliferative advantage to the cells, contrary to STAT5A which may reduce the cell proliferation. Phosphorylated STAT5 dimers migrate to the nucleus and act on STAT5-regulated target genes involved in proliferation, including *Cyclin D1 and c-MYC (*Alvarez and Frank 2004). An upregulation of STAT5B thus leads to an increase of genes involved in proliferation, which has an effect on the number of cells.

Since TPO-activated STAT5 has been shown to promote proliferation and survival of the Mk lineage (Park *et al.* 2016), we wanted to address the question, if STAT5 just increases proliferation and survival rather than Mk differentiation itself. For this we studied the differentiation potential of the transfected HPC-7 cell lines by growing them in media with SCF and TPO to stimulate Mk differentiation. We included STAT5A-expressing cells because the specific roles of the two STAT5 variants in megakaryopoiesis have not been studied. The cells with exogenous STAT5B expression had a significantly higher rate of differentiation towards the megakaryocyte lineage than the STAT5A and empty vector control cells. Already at basal conditions, without any TPO stimulation, we observed increased levels of MkPs in the STAT5B

cell line. This supports the idea that STAT5B has a prominent role in influencing differentiation towards Mks and that STAT5A does not have this role to the same extent. These data also support our hypothesis that STAT5B does not just increase proliferation of the myeloid lineage, but directly influences Mk differentiation. However, it could still be the case that increases in STAT5B leads to increased Mk numbers by specifically promoting proliferation in the Mk lineage over the other myeloid lineages. This could be investigated in future studies by looking at gene expression of the pro-proliferative/survival target genes of STAT5B by qPCR analysis. To better understand the potential of STAT5B to regulate differentiation of Mks, we could perform a Mk-specific colony assay with defined numbers of murine HSCs from the STAT5B transgenic mice or wildtype littermate controls.

We also found that the cells with enhanced STAT5A expression did show an effect on the assay readouts, but not to the same extent as STAT5B in the proliferation and differentiation assay. It could therefore be proposed that STAT5A also plays a role in Mk differentiation even though we have to consider that the transfected HPC-7 cell lines have quite a high level of overexpression, different to the transgenic hSTAT5B mouse model. Overexpression can force effects that might otherwise not be physiological. The effects observed here of STAT5A and STAT5B on Mk differentiation, as a potential consequence of overexpression, could be further explored by sorting the cells for low-expression. Additionally, to further explore the role of STAT5A and STAT5B we could generate individual knock-outs using the HPC-7 cell line. CRISPR/Cas9 gene editing technology could be applied to generate the knock-outs.

MkPs show variabilities in their transcriptional program as they differentiate towards mature Mks, reflecting different Mk subsets (Tilburg *et al.* 2022). These subsets have been identified to be enriched for certain genes associated with their maturation level. 23 genes are known to be highly upregulated in Mks and platelets upon induction of differentiation (Park *et al.* 2016). To study the potential regulation of Mk differentiation genes by STAT5, we selected three of these important genes to quantify: *F2r, Vwf* and *Mpl.* Surprisingly, the cell line with elevated STAT5B levels did not show higher gene expression of genes involved in the process of Mk differentiation on day 6. STAT5A cells showed lower expression of Mk genes compared to the pMSCV control but still higher than in the STAT5B cells, even though STAT5B was shown to promote Mk differentiation to a higher extent. These observations are not consistent with the data of the Mk differentiation experiments where we saw a significant increase in MkPs and differentiated Mks driven by STAT5B. This could indicate that STAT5B is possibly involved in earlier stages of maturation where we would see more differences in gene expression. The

investigated genes (*F2r, Vwf, Mpl*) are defined as crucial regulators for platelet function and therefore associated with later stages of maturation. Mks and platelets express *Mpl* to regulate the amount of TPO present to HSCs and progenitor cells. To determine the effect of exogenous STAT5B on the Mk transcriptional program upon TPO treatment, we could investigate genes involved in earlier and later stages of Mk differentiation separately. There could possibly be a negative feedback signal at some point during differentiation to shut off the process and prevent continuous transcription of the same genes, suggesting that the results might differ at earlier time points. STAT5B could also have an effect on the housekeeping gene *Gapdh*, to which all of the gene expression data are normalized, which would skew the results. Therefore, another housekeeping gene will be tested in future experiments. qPCR analysis was conducted once and will need to be validated by further experiments to understand these contradicting results.

Differentiated HPC-7 cells analyzed by cytospin and hematoxylin staining showed mature Mks. The enlarged size and polynucleation of mature Mks can be easily distinguished from other cell types. Through the staining with hematoxylin we were able to qualitatively observe that cells with increased STAT5B levels had a higher differentiation potential towards Mks compared to STAT5A or pMSCV cells. *Vwf* gene expression is restricted to Mks and endothelial cells, which allows a vWF immunohistochemical staining to help in the quantification of Mks, which could be used in future studies. Further optimization steps for the cytospin experiments are necessary since an increased cell number in the STAT5B cell line was observed during the differentiation assay which could skew the results. Therefore, equal cell numbers should also be seeded for the cytospin experiments in the future.

The two STAT5 variants are downstream effectors of JAK2 and TPO signaling, which directly leads to STAT5 tyrosine phosphorylation. To assess if this signaling affects the activation of STAT5A and STAT5B differently, we performed a Western blot analysis of the transgenic cell lines stimulated with TPO. We found the strongest STAT5 activation in the cell line with enhanced STAT5B expression. This could be one explanation as to why STAT5B has a stronger effect on Mk differentiation than STAT5A. This is consistent with previous findings where TPO stimulation of a novel stem/progenitor cell line promotes primarily the presence of STAT5B, but not STAT5A, in the nucleus (Kollmann *et al.* 2021). Previously, STAT5 was shown to regulate Mk proliferation and survival (Park *et al.* 2016), but it has not been established as a key regulator of Mk differentiation. Interestingly, there was an absence of any pYSTAT5 in the pMSCV HPC-7 cells upon TPO stimulation. The tSTAT5 levels also seemed

to be quite low, even though STAT5 is fundamental for blood cells and should be activated upon TPO stimulation. The absence of pYSTAT5 in the pMSCV cells remains unclear and no other studies have seen this so far.

Additionally, since it is known that STAT1 and STAT3 contribute to megakaryopoiesis, we assessed whether the increased exogenous STAT5 levels affect STAT1 or STAT3 phosphorylation under basal conditions or upon short TPO stimulation, which could indirectly influence Mk differentiation. Our data however suggest that, in this cell model, enhanced STAT5 levels do not alter STAT1 and STAT3 signaling. Therefore, it is likely that the effects we observed on Mk differentiation were directly mediated by STAT5. It has been proposed that STAT1 acts downstream of GATA-1 as a transcription factor to promote the expression of target genes involved in endomitosis, a later stage of Mk differentiation (Huang *et al. 2007)*. Surprisingly, we did not see any phosphorylation of STAT1 after TPO treatment in the HPC-7 cells. To perform these Western blots, the HPC-7 cell lines were treated with TPO for 30 min. Since STAT1 is responsible for endomitosis and later stages of differentiation this short stimulation with TPO may not be long enough to see STAT1 activation during Mk differentiation. In order to see STAT1 phosphorylation, additional Western blot experiments could be performed with a prolonged TPO treatment. Interestingly, the HPC-7 cells had constitutive, strong pYSTAT3 signals. A potential explanation for the constitutive STAT3 activation in the HPC-7 cells could be that the cells were treated with SCF, alongside the TPO stimulation, which binds to the c-Kit receptor and induces STAT3 tyrosine phosphorylation important for transcriptional activation (Duarte and Frank 2000). To potentially avoid the constitutive activation of STAT3, the cells could be starved of SCF upon TPO treatment.

As mentioned above, in our transgenic mouse model already a slight overexpression of STAT5B increased megakaryopoiesis in female mice compared to male mice. Estrogen, which is the primary female sex hormone, has previously been shown to play a role in megakaryopoiesis. Women treated with high doses of estradiol showed elevated Mk numbers and platelet count (Bord *et al.* 2000, Dupuis *et al.* 2019). We thus hypothesized that increased STAT5B and estrogen might cooperate to increase megakaryopoiesis. We used our cell line models to investigate this by performing Mk differentiation experiments with estrogen. We were not able to replicate observations of previous studies where estrogen treatment increased the maturation of Mks (Dupuis *et al.* 2019), as we did not observe any impact of estrogen in our *in vitro* differentiation experiments. We did however find a potential effect of estrogen on pYSTAT5 levels in our STAT5B cell line. Estrogen treatment after TPO stimulation was able

to maintain and even increase pYSTAT5 levels in the STAT5B cell line compared to the condition without estrogen. Considering these results, different approaches could be taken to further investigate if estrogen has an effect on the differentiation process of Mks upon increased STAT5B levels. One of these would be to repeat the differentiation experiments but include TPO alongside estrogen, since estrogen on its own might not be able to push Mk differentiation in our cell model. Furthermore, since the high concentration of TPO used for these experiments could mask the added effect of estrogen, including a condition with lower TPO concentrations might be advisable. The HPC-7 cell line was immortalized from male embryonic stem cells in which the presence of the ER was demonstrated by qPCR analysis (unpublished data provided by Marie Ploderer). We tried to mimic the female environment by adding estrogen. Another approach could be to isolate LSKs from the hSTAT5B transgenic mouse model of both sexes and employ them in *ex vivo* differentiation experiments, as this might be a more physiological system. Our observation in the mouse model could also stem from cell intrinsic effects that would not be apparent in our *in vitro* model. To investigate this, we could transplant female bone marrow cells of our transgenic mouse model into male mice and quantify the Mk lineage. Considering our findings, it would be premature to rule out the role of estrogen and further experiments are needed to understand the sex-specific differences we observed in our hSTAT5B mouse model.

STAT5B is crucial for sex-dependent gene expression in the liver. Especially males require STAT5B, or STAT5B dependent factors, to induce sex-specific gene expression. In contrast, various female-predominant liver genes are downregulated in a STAT5B-dependent manner (Holloway *et al.* 2006; Park *et al.* 1999). *Stat5b* deficient males showed a loss of gene expression exclusively in the liver (Clodfelter *et al.* 2006). STAT5B has so far never been described to act as a sex-specific transcription factor in the hematopoietic system, but given its role in the liver, it is plausible that STAT5B could have a similar role in the blood system, which could be interesting to investigate further.

In summary, in our HPC-7 cell line model with elevated STAT5B levels had a significant impact on cell proliferation as well as Mk differentiation, whereas STAT5A does not appear to have these roles to the same extent. The observed effect on the Mk lineage specifically in female hSTAT5B transgenic mice requires further experiments in different models to study this sex-specific effect. One instance where understanding Mk differentiation and function is crucial is in myeloproliferative neoplasms (MPNs). MPNs are a group of blood cancers characterized by uncontrolled expansion of myeloid cells from mutated HSCs (Guo *et al.* 2017). In MPNs, Mks

are a key cell type for disease pathology and they directly act on HSCs in the bone marrow niche. Even though dysregulated hematopoiesis is mainly associated with alterations within HSCs, abnormalities in the bone marrow niche are recognized to be an important factor in MPN contributing to the disease (Zhan *et al.* 2016). Since a curative treatment for MPNs has not been found so far, it is important to better understand the underlying processes, such as the pathological differentiation of Mks and their complex interactions within the bone marrow microenvironment, to develop better treatment options. Elucidating the role of STAT5B in megakaryopoiesis could therefore have broader implications for these patients.

5 Bibliography

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6 Appendix

6.1 Abstract

The hematopoietic system relies on cytokine signaling to mediate differentiation, proliferation and survival of the various blood cells. One of these cytokines is thrombopoietin (TPO) that is responsible for the differentiation of megakaryocytes (Mks), the producers of platelets. TPO signals through the Janus kinase 2 (JAK2) - signal transducer and activator of transcription 5 (STAT5) pathway. Once TPO binds to the thrombopoietin receptor, the receptor associated JAKs trans-phosphorylate one another which leads to the subsequent recruitment and phosphorylation of STAT5. STAT5 then translocates to the nucleus where it acts as a transcription factor and activates the expression of its target genes. STAT5 comprises two different gene products, STAT5A and STAT5B, and although it has been shown that TPO signaling primarily activates STAT5B, it is not known if STAT5A and STAT5B are differentially involved in megakaryopoiesis.

To start addressing this question, our group investigated the myeloid lineage of a transgenic mouse model expressing 1.5-fold higher levels of STAT5B in the hematopoietic compartment. This moderate increase in expression of STAT5B already had an impact on the expansion of mature megakaryocytes but only in female mice. This led us to hypothesize that STAT5B plays a role in megakaryocyte differentiation in a sex-specific way.

The aim of my project was to further dissect the differential roles of STAT5A and STAT5B as well as the effect of estrogen, the primary female sex hormone, on megakaryopoiesis. For this, I used a murine hematopoietic stem cell line with altered STAT5 expression levels to perform megakaryocyte differentiation assays. Mk differentiation was assessed by quantification of the megakaryocyte progenitors using flow cytometry. The ploidy, a characteristic feature of mature megakaryocytes, was analyzed using propidium iodide staining. The mature megakaryocytes were also visualized by cytospin. We observed that the cells with increased STAT5B levels differentiated more strongly towards Mks than the ones with increased STAT5A levels or only expressing the empty vector.

This confirms what we previously observed in the transgenic mouse model. Estrogen, which was used to investigate the sex-specific differences observed in the mouse model, had no effect on megakaryocyte differentiation in this setting. To validate this lack of effect, further experiments in different models will be needed. To summarize, I was able to show that STAT5B has a more prominent role over STAT5A in regulating Mk differentiation in the cell line model.

6.2 Zusammenfassung

Das hämatopoetische System beruht auf Zytokinsignalen, um die Differenzierung, Proliferation und das Überleben der verschiedenen Blutzellen zu ermöglichen. Eines dieser Zytokine ist Thrombopoietin (TPO), das für die Differenzierung von Megakaryozyten (Mks), den Produzenten von Blutplättchen verantwortlich ist, nimmt Einfluss auf den JAK2-STAT5 Signalweg. Sobald TPO an den Thrombopoietinrezeptor bindet, transphosphorylieren die Rezeptor assoziierten JAKs einander, was zur anschließenden Rekrutierung und Phosphorylierung von STAT5 führt. STAT5 wandert dann in den Zellkern, wo es als Transkriptionsfaktor fungiert und die Expression seiner Zielgene aktiviert. STAT5 umfasst zwei verschiedene Genprodukte, STAT5A und STAT5B, und obwohl gezeigt wurde, dass die TPO-Signalgebung hauptsächlich STAT5B aktiviert, ist nicht bekannt, ob sie unterschiedlich an der Megakaryopoese beteiligt sind.

Um diese Frage zu beantworten, untersuchte unsere Gruppe die myeloische Abstammung eines transgenen Mausmodells, das ein 1,5-fach höheres STAT5B level im hämatopoetischen Kompartiment exprimiert. Dieser moderate Anstieg der Expression von STAT5B hatte bereits Auswirkungen auf die Expansion reifer Megakaryozyten, jedoch nur bei weiblichen Mäusen. Dies veranlasste uns zu der Hypothese, dass STAT5B auf geschlechtsspezifische Weise eine Rolle bei der Differenzierung von Megakaryozyten spielt.

Das Ziel meines Projekts war es, die unterschiedlichen Rollen von STAT5A und STAT5B sowie die Wirkung von Östrogen, dem primären weiblichen Sexualhormon, auf die Megakaryopoese weiter zu analysieren. Dazu habe ich eine murine hämatopoetische Stammzelllinie mit verändertem STAT5-Expressionsniveau verwendet, um Megakaryozyten-Differenzierungsassays durchzuführen. Die Mk Differenzierung wurde durch Quantifizierung der Megakaryozyten Vorläufer mittels Durchflusszytometrie durchgeführt. Die Ploidie, ein charakteristisches Merkmal reifer Megakaryozyten, wurde unter Verwendung von Propidiumiodid Färbung analysiert. Die reifen Megakaryozyten wurden auch durch Cytospin sichtbar gemacht. Wir beobachteten, dass die Zellen mit erhöhten STAT5B stärker in Richtung Mks differenzierten als die mit erhöhtem STAT5A Level.

Dies bestätigt, was wir zuvor im transgenen Mausmodell beobachtet haben. Östrogen, das verwendet wurde, um die im Mausmodell beobachteten geschlechtsspezifischen Unterschiede zu untersuchen, hatte in diesen Zellen keine Wirkung auf die Differenzierung von Megakaryozyten. Um diesen fehlenden Effekt zu validieren, sind weitere Experimente in

verschiedenen Modellen erforderlich. Zusammenfassend konnte ich zeigen, dass STAT5B eine wichtigere Rolle als STAT5A bei der Regulierung der Mk-Differenzierung im Zelllinienmodell spielt.