FH CAMPUS WIEN
UNIVERSITY OF APPLIED SCIENCES

Engineering Erythropoietin for Differential Receptor Binding Modes for Optimal Therapeutic Effects

Master Thesis

For the attainment of the academic degree

Master of Science

From the University of Applied Sciences Vienna

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Submitted on:
26. 03.2019

Abstract in English

Erythropoietin (Epo) is a cytokine that stimulates proliferation of red blood cells and is used to treat anemia patients. However, several clinical trials showed that patients receiving Epo treatment suffered from increased strokes, heart attacks, deep vein thrombosis and faster cancer progression.
A strategy to reduce the side effects of Epo has been developed in Dr. Pamela Silvers Laboratory. The protein engineering approach directs Epo activity to Erythropoietin receptor (EpoR) on red blood cell precursors and restrains binding to nonerythroid cells (Taylor *et al.*, 2010; Burrill *et al.*, 2016).
As a pleiotropic protein, Epo has beside its erythropoietic activity, also a neuroprotective activity.

The general aim of this study is, to optimize the therapeutic effects of Epo and reducing its side effects. However, the study is divided in three chapters. Each one is focusing on the interaction of Epo to one if its currently proposed receptors: EpoR, heteroreceptor complex ß-common receptor -EpoR (ßcR-EpoR) and most recently Ephrin receptor-B type 4 (EphB4).

Chapter one aims to increase the therapeutic window of the current targeted Epo fusion protein. This has been done by screening for an Epo mutation, with lower affinity to EpoR than the current mutation $Epo_{R150A,}$

Signaling of Epo through EphB4, has been identified to be key responsible for cancer relapse and progression. The aim of the second chapter was to engineer Epo to prevent EphB4 signaling in cancer patients. However, the binding of Epo to EphB4 could not be reproduced within this thesis study.

Beside its erythropoietic activity, Epo as a pleiotropic protein has also a neuroprotective activity. The third chapter proposes a rational design of an Epo fusion protein drug for the prevention and treatment of high-altitude cerebral edema.

This thesis provides an overview of the therapeutic potential of Epo and the interaction to its different receptors in order to optimize its therapeutic effects.

Abstract in German

Erythropoietin (Epo) ist ein Zytokin, das die Proliferation roter Blutkörperchen stimuliert und zur Behandlung von Anämie- Patienten verwendet wird. Klinische Studien zeigten jedoch, dass bei Patienten, die eine Epo-Behandlung erhielten, die Zahl der Schlaganfälle und Herzinfarkte stieg, es vermehrt zu tiefen Venenthrombosen kam und das Krebswachstum erhöht war.

In Dr. Pamela Silvers Labor wurde eine Strategie zur Verringerung der Nebenwirkungen von Epo durch ein Fusionsprotein entwickelt. Dieses verstärkt die Bindung von Epo zu dem Erythropoietin Rezeptor (EpoR) an myeloische Vorläuferzellen und verhindert die Bindung zu EpoR an nicht-erythroiden Zellen, wie zum Beispiel Thrombozyten (Taylor et al., 2010; Burrill et al., 2016).

Das Ziel dieser Studie ist es, die therapeutischen Wirkungen von Epo zu optimieren und die Nebenwirkungen zu reduzieren. Die Studie ist in drei Abschnitte unterteilt. Jedes Kapitel ist der Binding von Epo zu einem der drei folgenden Rezeptoren gewidmet: EpoR, dem ß-common Rezeptor- EpoR (ßcR-EpoR) und dem zuletzt identifizierten Ephrin Rezeptor-B Typ 4.

Im ersten Abschnitt wird nach einer Mutation in Epo, die eine geringere Affinität für den EpoR vorweist als die derzeitig im Fusionsprotein verwendete (Epo_{R150A}), gesucht. Das Ziel dieser Studie ist es, die therapeutische Breite des Epo Fusionsproteins zu vergrößern.

Die Bindung von Epo an EphB4 wurde von Pradeep und Kollegen (2015, Cell) als die Schlüsselfaktor für das raschere Fortschreiten von Krebserkrankungen bei Epo Therapie publiziert. Das Ziel des zweiten Kapitels ist es daher, die Nebenwirkungen zu reduzieren, durch das Hindern der Bindung von Epo an EphB4. Die Bindung von Epo an EphB4 konnte jedoch während dieser Masterstudie nicht nachvollzogen werden.

Als pleiotropes Protein besitzt Epo neben seiner erythropoetischen Aktivität auch eine neuroprotektive Aktivität. Beim Anstieg auf Höhen über 2000 Meter kann es zu hypoxischen Zuständen kommen die zu lebensbedrohlichen Hirnödemen (HACE) führen kann. Die Kombination aus dem neuroprotektiven Potential von Epo durch die Bindung an ßcR-EpoR und der erythropoietischen Aktivität durch die Bindung an EpoR, gezielt an rote Blutkörperchen, wird als potentielle Prävention von HACE erläutert.

Diese Arbeit gibt einen Überblick über das therapeutische Potenzial von Epo und die Wechselwirkung mit seinen verschiedenen Rezeptoren. Jeder der drei Abschnitte dient dem Ziel, die therapeutische Wirkung von Epo zu optimieren.

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Preface

The study of this Master thesis has been performed in the laboratory of Prof. Dr. Pamela Silver at Harvard Medical School under the Supervision of Dr. Jeffrey Way and the Co-Supervision of Prof. Dr. Ines Swoboda in order to receive the Master degree from the department of Molecular Biotechnology at University of Applied Life Sciences Vienna. Thankfully this thesis has been supported by the Marshall Plan Scholarship.

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List of Abbreviation`s

Abbreviation	*Full name*
AA	*Amino acid*
Amp	*Ampicillin*
BBB	*Blood brain barrier*
CKD	*Chronic kidney disease*
CNS	*Central nervous system*
DMSO	*Dimethyl sulfoxide*
E.coli	*Escherichia coli*
EphB4	*Ephrin receptor-B type 4*
ESA	*Erythropoietin stimulating agent*
Epo	*Erythropoietin*
EpoR	*Erythropoietin receptor*
FDA	*Food and Drug Administration*
FBS	*Fetal bovine serum*
GM-CSF	*Granulocyte-macrophage colony-stimulating factor*
HACE	*High-altitude cerebral edema*
HAPE	*High-altitude pulmonary edema*
LB	*Lysogeny broth media*
RBC	*Red blood cell*
scFv	*Single chain variable fragment*
VEGF	*Vascular endothelial growth factor*
WT	*Wild type*

1. Introduction

1.1. Overview of the problem statement of this thesis study

Patients suffering from anemia have a decreased amount of red blood cells (RBC) or total hemoglobin in the blood, resulting in a reduced quality of life due to symptoms such as shortness of breath, headache, fatigue, weakness, irregular heartbeats and dizziness (Beutler and Waalen, 2006).

Erythropoiesis stimulating agents (ESA) are used to treat cancer patients with chemotherapy-induced anemia and patients suffering from chronic renal failure related anemia. The recombinant version of human erythropoietin (EPO) has been extensively used in America and Europe as a treatment for anemia and led to reduction of blood transfusion in patients (Jain *et al.*, 2012).

However, controlled clinical trials have shown that chronic kidney disease patients receiving ESA to maintain target hemoglobin levels over 12 g/dl were more prone to serious adverse cardiovascular reactions (nonfatal myocardial infarction and heart failure), stroke, thrombosis and increased mortality than the control group. Additionally, treatment of cancer patients suffering from chemo-related anemia, experience risk of increased mortality and/or tumor progression (Lai *et al.*, 2013).

Since 2008 the evidence for severe side effects due to ESA treatment has accumulated resulting in a black box warning from the U.S. Food and Drug Administration (FDA) for ESA treatment.

The Laboratory of Dr. Pamela Silver has developed a strategy to reduce the side effects of ESA treatment by targeting Epo action to RBC precursors. (Burrill *et al.*, 2016). In this concept the Epo action on non-target cells that bear EpoRs is prevented by mutating Epo (activity element), such that its

receptor binding affinity is reduced. The Epo action on target cells is rescued by connecting mutated Epo via a linker to a targeting element, such that the avidity effect leads to Epo signaling on red blood cell precursors only.

1.2. Overview of aims and structure of the study

The overall aim of this study is to reduce the severe side effects of Erythropoietin while maintaining its full therapeutic potential. The aims of this thesis are divided into three parts (Figure 1), focusing on the interaction of Epo to each of its canonical and noncanonical receptors that have been found so far– EpoR homodimer, EphB4 (oligomeric state is unknown) and the heterodimeric complex of EpoR and common ß-chain receptor (ßcR-EpoR).

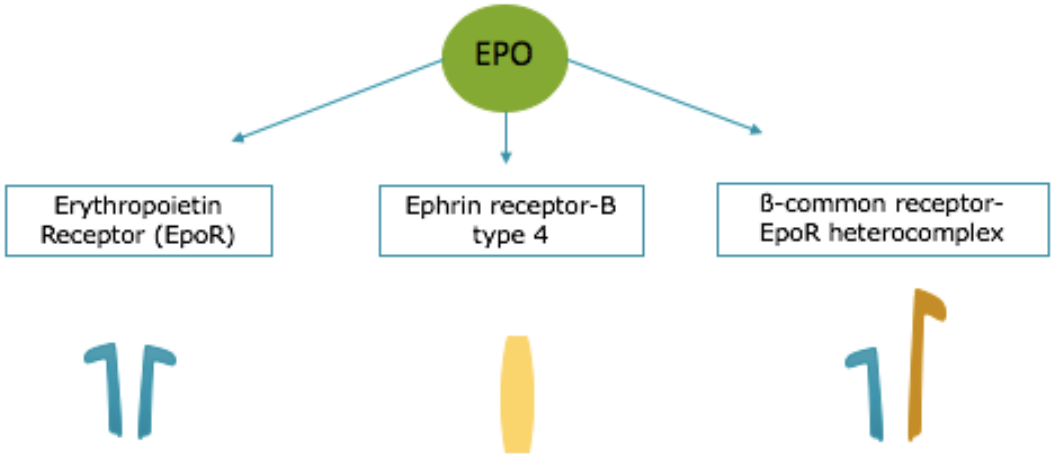

Figure 1 Schematic cartoon of Epo and its three identified receptors. Each of the three chapters in this thesis is focusing on the interaction and therapeutic optimization of Epo binding to one of the three depicted receptors. Starting from left to right.

While the fusion protein concept published by Burril et al. (2016, PNAS) stayed intact, the targeting molecule has subsequently been revised by the current PhD candidate Jungmin Lee, resulting in a higher targeting effect than the already identified molecule.

The first chapter of this thesis focuses on screening for a mutation possessing erythropoietic activity in the form of a fusion protein but has weaker affinity towards EpoR by itself, compared to the current mutation R150A. This stronger mutation helps to further reduce non-target cell receptor binding and diminishes side effects. Together with the new targeting element, this new mutation will further increase the therapeutic window of Erythropoietin.

The second aim of this study is based on published data by Pradeep et al (2015, Cell), (see Chapter 1.6.1 for more details), which suggests that EphB4 is a low affinity receptor for EPO. These findings propose that Epo-induced tumor growth and progression results from binding to EphB4 (Pradeep *et al.*, 2015). The objective of this study is to identify the binding site of EPO to EphB4 to reduce binding affinity, by introducing a mutation. The ultimate goal would further be to diminish the side effects by hindering the binding of EPO to EphB4 by introducing a mutation, while retaining erythropoietic potential targeted to RBC precursors.

Several studies show that Epo interacts with the heteroreceptor complex ßcR-EpoR (also known as CD131). Supported by several published data, this interaction leads to Epo-induced tissue- and neuroprotective activities (Brines *et al.*, 2004). Several attempts to use EPO for tissue-protective, but not erythropoietic effects, have been published but issues, such as side-effects, crossing the blood brain barrier, and high dose, need to be further addressed (Hernández *et al.*, 2017).

The third chapter presents a rational design of an EPO fusion protein drug, which is neuroprotective and has erythropoietic potential while reducing its pro-thrombotic side effects. This engineered Epo fusion protein could be a potential prevention and treatment for high-altitude cerebral edema, which is caused by hypoxia due to exposure in high-altitudes.

1.3. Erythropoietin

Erythropoietin (Epo) is a circulating hormone. Its gene is located on the human chromosome 7 (7q21-7q22) on the forward strand. It contains five exons and six introns. Downstream of the exon five lies the enhancer that is activated under hypoxic circumstances. In case of hypoxia, the two transcription factors HIF and HNF-4 bind to the transcriptional activator p300 in the Epo-producing cell and enhance the transcription. The kidney inducible element KIF is far upstream of the Epo gene and needed for up-regulation of mRNA in the kidney (Bunn, 2013).

Its molecular weight is 34 kDa, while about 40% of its molecular mass is due to its glycosylation in the Golgi apparat. Three chains are N-glycosylated (Asn-24, Asn 38 and Asn-83) while Ser-126 is O-glycosylated (Hernández *et al.*, 2017).

Lacking glycosylation patterns can lead to misfolding and reduction of the stability of erythropoietin circulation. Absence of N-linked glycosides have an effect on the in vivo bioactivity since it gets cleared more rapidly from the plasma by the liver (Jiang et al., 2014). A model of Epo, its glycosylation pattern and the two disulfide bridges can be found in Figure 2 (Kato, 2016).

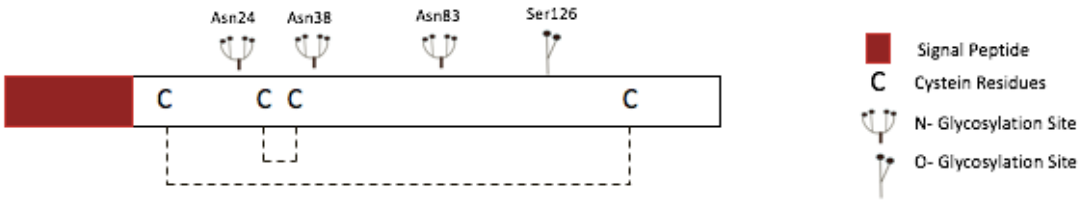

Figure 2 Human mature glycoprotein consisting of three N-glycosylation sites and one O-glycosylation site. Dashed lines indicate disulfide bridges. Model adopted from: Kato (2016, Handbook of Hormones).

The mature glycoprotein contains 165 amino acids. The amino acid (aa) sequence of Epo, containing the 27 aa signal peptide, which gets cleaved in the endoplasmic reticulum, and the c-terminal arginine, which gets cleaved after translation, can be found in Supplement S8.1. B (Debeljak, Solár and

Sytkowski, 2014). Figure 3 shows the structure of Epo with its four-antiparallel alpha-helical bundle structure (Elliott *et al.*, 1997).

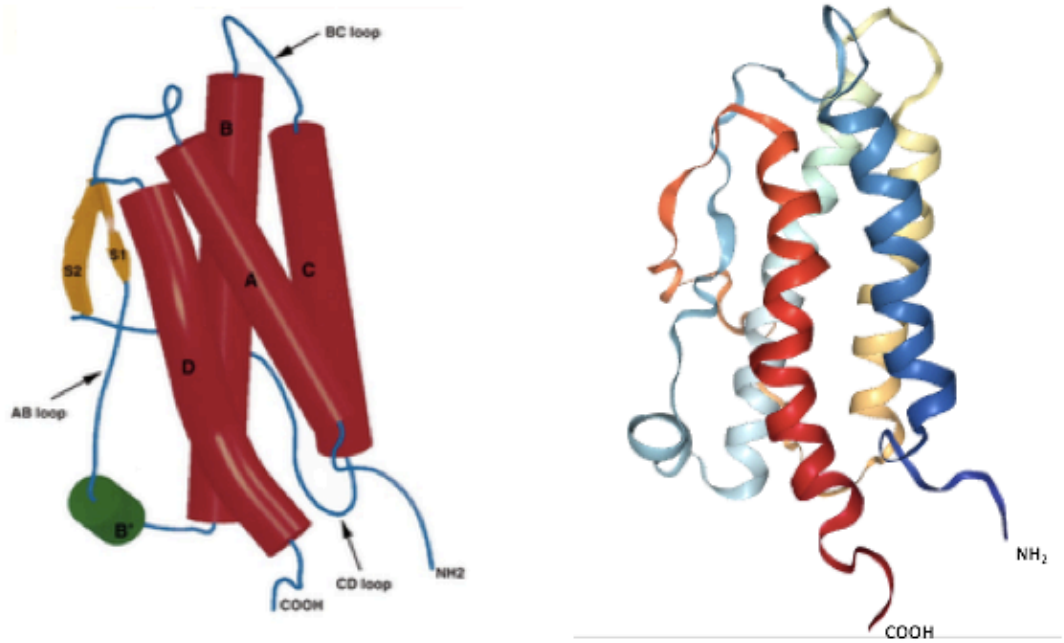

Figure 3 Structure of Epo. Cartoon on the left side is indicating the four alpha-helices A: Ser9 -Thr 26, B: Gly 57-Lys83, C: Leu91-Ala 111 and D: Phe138-Ala 160, the beta helix is indicated in green (Cheetham et al.*, 1998). On the right side the three-dimensional structure of Epo. Cartoon and Structure from Cheetham et al., (1998, Nature).*

1.3.1. History in Epo Research

The burst of erythropoiesis after the exposure of high-altitude hypoxia could be observed by Viault while traveling the mountain area of Morococha in Peru in 1890 (Bunn, 2013). The first ones to suggest a reliable factor for controlling the red blood cell production were Carnot and Deflandré at the University of Paris in 1906. (Jelkmann, 1986). While Carnot and Deflandré called their finding hematopoietic, Bonsdorff and Jalavisto first established the term Erythropoietin. Erythropoietin combines the greek words ērythrós meaning "red" and poieîn meaning "do".

Subsequently, the activity of Epo after hypoxia could be displayed in mammalian species (Jelkmann, 1986). 51 years after suggesting the

existence of a factor for erythropoiesis, Eugene Goldwasser and colleagues were able to isolate erythropoietin from Japanese patients with aplastic anemia (Bunn, 2013). The isolation and the ability of molecular cloning of Epo gen in 1985 opened a new chapter for research and future development of Epo as a therapeutic drug (Bunn, 2013).

1.3.2. Hematopoietic effect of Epo

Epo does mediate the regulation of RBC production. The circulating cytokine stimulates bone marrow progenitor cells to proliferate and inhibits apoptosis. The erythroid progenitors also differentiate into erythroid precursors leading to mature red blood cell production (Bunn, 2013). Epo does solely act in colony forming unit erythroid cells and not on pluripotent stem cells (Jain *et al.*, 2012).

Patients, suffering from anemia, show a reduced proliferation of erythropoietin which can be effectively corrected by administration of rhEpo. Therapeutic treatment with Epo, in order to increase RBCs, is therefore a valuable alternative to blood transfusions. Therefore, Epo is frequently used to treat patients suffering from cancer/chemotherapy related anemia and anemia due to CKD. For healthy patients, a hemoglobin value of about 14 g/dl is considered as normal, 12g/dl is considered as mild anemic and below 10 g/dl is heavily anemic (Wild and Piso, 2010).

A successful Epo treatment can be monitored by measuring the hemoglobin value.

Clinical trials have proven that the treatment of anemic patiets with Epo leads to reducing blood transfusion rates and included risks, such as viral or infectious diseases, allergic reactions and iron overload (Frille, 2012). Anemia is mostly accompanied with health-related issues, such as reduced quality of life due to fatigue and physical inability. The treatment of Anemia with Epo has been described as enhancing the quality of life (Jain *et al.*, 2012)

1.3.3. Non hematopoietic effect of Epo

Besides the expression of Epo on red blood cell precursors and hematopoietic cells, Epo is expressed in several tissue types including, but not limited to, liver, endometrium, pancreas, prostate, endothelium, heart, kidney, central nervous system and placenta.

Epo has shown cytoprotective (Leist *et al.*, 2004), angiogenic (Carlini, Reyes and Rothstein, 1995; Hugo H. Marti, 2004) and proliferative effects. On the one hand, ESA treatment is tested in pre-clinical and clinical trials for tissue protective effects for the heart and brain. While on the other hand, Epo treatment has additionally been linked to tumor growth, due to the indicated EpoR on several types of tumors, malignant cells and the reduced survival of ESA treated patients (Arcasoy, 2008).

About 30 years ago, mRNA expression of EpoR have been detected in human umbilicia vein (Anagnostou, 1994). Resulting from that, Epo has been established as enhancing cell migration and proliferation, being key factors to angiogenesis (Carlini, Reyes and Rothstein, 1995).

1.3.4. Clinical impact of Epo

In 1989, the first recombinant Epo alfa (Epogen/Procrit®) has been released to the American market. In 1994, Janssen Cilag released Erypo® in Austria, earlier than the EMEA for Europe, with exception of chemotherapeutic induced anemia and kidney failure. As one of the first "biotechnological drugs", Epo became one of the top-selling drugs in America and Europe in the following years. Since the early 2000s, Epo administration increased, frequently administered to patients before orthopedic or cardiological surgery where a significant blood loss was predicable (Wild and Piso, 2010).

1.3.5. Clinical trials and Black box warning

Awareness concerning erythropoiesis increased as not being the only side-effect of Epo based on the discovery of *in vitro* endothelial cell proliferation and the detection of EpoR on neuronal cells (Debeljak, Solár and Sytkowski, 2014).

Subsequently, the pleiotropic effects of Epo became public knowledge and pre-clinical and clinical trials found severe side effects accompanying ESA treatment (Jain *et al.*, 2012; Debeljak, Solár and Sytkowski, 2014). Results included increased risk of stroke, thrombosis and even higher fatal outcomes after treating patients suffering from CKD with ESA. The FDA issued the first black box warning in March 2007. A black box warning is a labeling that appears on the package of the drug, due to reasonable evidence that the administration of the drug has risks. By the end of 2008 the black box warning got more serious and suggested no further routine application of Epo (Wild and Piso, 2010).

1.3.6. Side effects of Erythropoietin stimulating agents

The medical journal *Lancet* Oncology reported about a clinical trial in 2003, where one half of 939 breast cancer patients with metastasize tumor were treated with Epo. 76% of the placebo group survive cancer and did not develop anemia, but only 70 % of the patients treated with Epo survived (Wild and Piso 2010).

A summary of all clinical trials conducted between 2009 and 2014, using erythropoietin stimulating agents (ESA) treatment, can be found in Debeljak, Solár and Sytkowski (2014, Frontiers in Immunology). The data analysis suggests the prothrombotic effects as the main reason for the negativ outcome. Moreover, a reduced overall survival has been deteced compared to ESA treated group (Debeljak, Solár and Sytkowski, 2014).

The expression of EpoR in tumor cells did rais awareness of a potential responsiveness of tumor cells stimulted by Epo (Cao, 2013). By activation of EpoR on cancer cells, Epo can enhance the proliferation rate, apoptosis resistence and invasion capacity. Additionally results show that Epo has an effect on the tumor microenvironment due to cytokine release and angiogenesis (Arcasoy, 2008).
Various preclinical studies confirmed the correlation of Epo induced apoptosis inhibition, cell migration and proliferation as well as chemoradiant resistance. However, sources also suggest that Epo could lead to chemosensitivity (Arcasoy, 2008).
In clinical studies, correlation between accelerated tumor growth and reduced survival (Debeljak, Solár and Sytkowski, 2014) during Epo treatment for cancer patients has been established. These findings go hand in hand with the effect of accelerated tumor progression, which could be asserted in an study of head- and neck- cancer patients (Cao, 2013). The activation of EpoR-EpoR homodimer by exogenous Epo could be detected to enhance tumor progression in breast cancer patients (Chan *et al.*, 2017).

1.4. Epo and its receptors

Overview of published interaction partner
The pleiotropic protein Epo has several binding partners. The most abundant (classical) receptor is the canonical Erythropoietin receptor (EpoR). The interaction of Epo to its binding partner EpoR homodimer does induce erythropoiesis, the most familiar biological activity of Epo. Additionally, tissue- and neuroprotective effects of Epo have been detected by binding to an EpoR with the ß-common receptor subunit (ßcR-EpoR) (Brines & Cerami, 2012). This heteroreceptor leads to rhEpo induced tissue protection (Miller *et al.*, 2015). Moreover, the transmembrane Ephrin type-B receptor 4 (EphB4), which belongs to the largest subgroup of the receptor tyrosine kinase family, has been identified as a low affinity receptor for Epo. This interaction has

been reported to promote carcinogenesis in human ovarian cancer (Pradeep *et al.*, 2015).

Three receptors described in this study were involved in experiments to improve treatment of Epo. This thesis is divided into three sections, each outlining the interaction with one of the three receptors: Erythropoietin receptor, Ephrin receptor-B type 4 and ß-common receptor –Erythropietin receptor heterodimer.

The following chapters (1.5, 1.6, 1.7) provide information to each of the three named receptors. A detailed problem statement and aim of Epo interaction with each receptor can be found at the end of each chapter.

1.5. Erythropoietin Receptor (EpoR)

In 1989, D´Andrea et al were the first ones to clone a cDNA from a mouse Erythropoietin receptor (EpoR) (Takahashi *et al.*, 1995). In humans, the EpoR gene is located on chromosome 19 (p13.3-p13.2) and has eight exons. The mature receptor consists of 251 Amino Acids and is a member of the type I cytokine receptor superfamily (Hernandez 2017).

The glycoprotein has a size of about 66kDa. While the highest receptor expression can be found in the bone marrow and thyroid, the receptor can be found in 22 other organ tissues such as kidney, spleen, brain, lung, and placenta (Y. Takei, H. Ando, and K Tsutsui 2016).

1.5.1. Structure of EpoR and its Epo binding sites

The extracellular part of the receptor consists of two immunoglobulin-like domains: the membrane distal domain (D1) and membrane proximal domain (D2). The domains are connected via a short hinge and are both important for the binding of erythropoietin (Howell *et al.*, 2006). Pelletier et al. proved the transmembrane domain being crucial to the orientation of the juxtamembrane domain and enabling full receptor activation.

Binding Epo to the EpoR-dimer leads to a conformational change where the distance between the two receptor monomers decreases from 73 angstrom to 39 angstrom (Livnah *et al.*, 1999). This change results in cross-phosphorylation of the Janus family tyrosine kinase 2 (Jak2) within the cytoplasmic domain of EpoR, which initiates the downstream signaling cascade (Bonnas, 2009; Bunn, 2013). Figure 4 shows a schematic representation of the EpoR structure and the conformational change upon binding of Epo to EpoR.

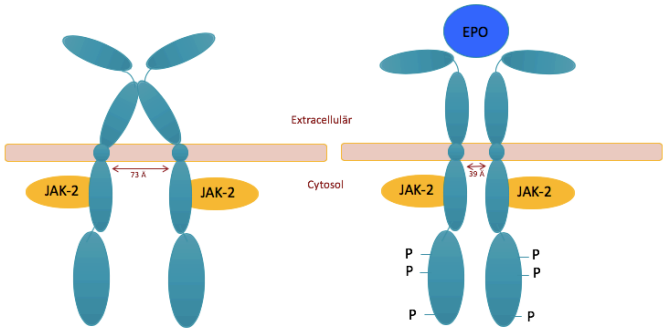

Figure 4 Schematic representation of transmembrane EpoR homodimer. Left side in unbound state. Upon binding on right side, Jak-2 signaling pathway gets activated. Cartoon adapted from Bunn (2013, Coldsrping Harbor perspectives in medicine).

Epo binds via two binding sites to the EpoR-dimer, which differ greatly in binding affinity. The high affinity binding site between the hydrophobic core of the receptor and the hydrophilic residues of Epo has a dissociation constant of about 1 nM (Elliott *et al.*, 1997). The second binding site (also known as low affinity binding site) between Epo and EpoR has an affinity of about 1uM, 1000 times less (Watowich, 2011). A cartoon of both binding sites can be seen in Figure 5.

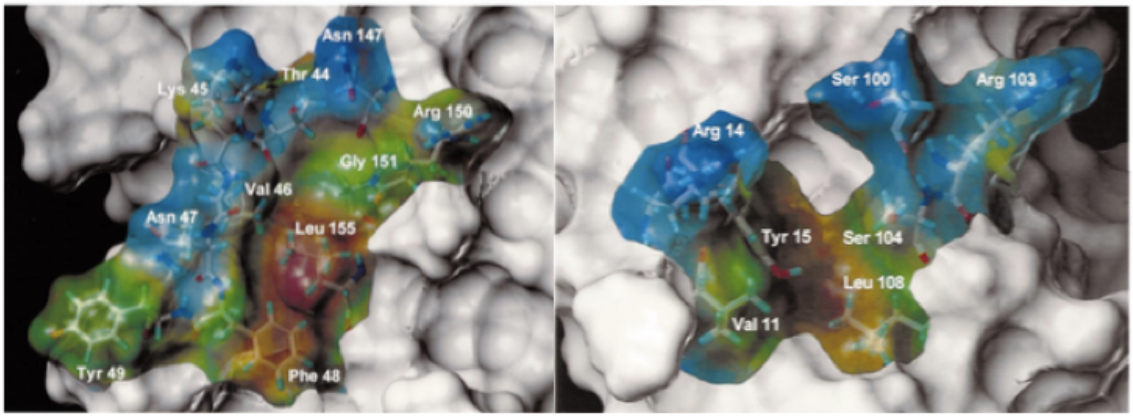

Figure 5 Surface involved in binding to homodimeric EpoR. Residues involved in EpoR -binding are highlighted. Left: High affinity binding site (Residues: Thr 44, Lys 45, Asn 47, Phe 48, Tyr 49, Asn 147, Arg 150). Right: low affinity binding site to EpoR. Residues critical for binding: Val 11, Arg 14, Tyr15, Ser 100, Arg 103, Ser104, Leu108). Cartoon from: Cheetham et al. (1998, Nature).

1.5.2. Targeting Epo to Red Blood Cell precursors

Naturally occurring proteins have been identified as important therapeutic agents. These proteins, such as growth factors or antibodies, lead to activation of intracellular signaling pathways upon binding to one or more receptors. Exogenous administration can lead to increased side effects due to binding of the effector molecule to receptors on non-target cells, for example cancer cells (Lienert *et al.*, 2014).

Protein engineering can aid in avoiding side effects of such multifunctional effector molecules by combination with so called "chimeric activators". This strategy involves two functional elements which are connected by a linker. One element targets the respective cell of interest (targeting element), while the other one is the effector molecule (activity element). By introducing a mutation in the activity element, the affinity to its receptor is lowered (Figure 6). Binding the target molecule to the respective cell, the local concentration of the mutated activity element increases which generates the binding process. The flexible peptide linker enables the binding of targeting element and mutated activity element simultaneously (Lienert *et al.*, 2014).

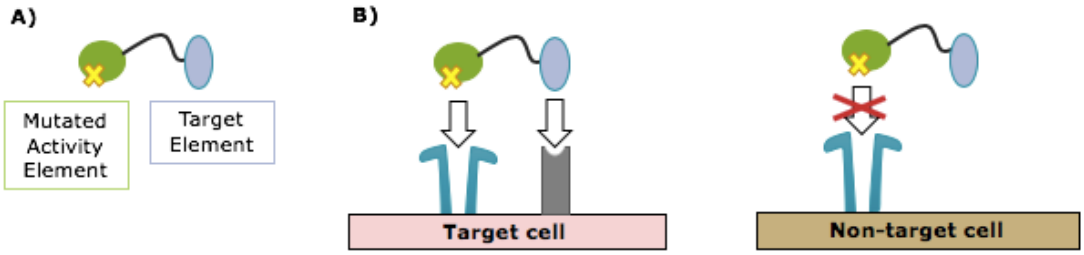

Figure 6 Cartoon of chimeric activator adapted from (Burrill et al.*, 2016). A) Mutated activity element linked to target element. B) Binding of target element to target cell results in increased binding frequency of activity element. Negligible affinity to non-target cell reduces the side effects.*

This chimeric activator approach has been used to target Epo selectively to RBCs and stimulate downstream signaling through EpoR (Burrill *et al.*, 2016). This approach focuses on disabling binding of Epo to nontarget cells, such as platelet precursors, and diminishing thrombotic side effects associated with exogenous rhEpo treatment.

The experimental design as described in the article by Burril et al. (2015, PNAS) involves the single-chain variable fragment (scFv) from the antibody 10F7 which directs binding to human glycophorin A (GYPA). This is a protein highly expressed on the human erythrocyte membrane. To reduce the binding affinity of Epo to EpoR, a mutation at position 150 from arginine to alanine has been introduced. This Epo_{R150A} mutation is at the strong affinity (binding?) site of Epo to EpoR and reduces the binding approximately by factor 12. The connective element of the mutated Epo to 10F7 is a 35-aa glycine/ serine linker (Burrill *et al.*, 2016).

However, further experiments have shown that the fusion protein 10F7-35aa-Epo_{R150A} is still thrombotic. The targeting antibody element 10F7 acted pro-inflammatory by stabilizing the glycophorin A band 3.

Subsequent research of Jungmin Lee and Jeffrey Way have led to the characterization of an antibody fragment, IH4v1, which does not lead to stabilization of band 3 and is not pro-inflammatory. Moreover, the new activity element IH4v1 is more potent than 10F7 due to its higher affinity for glycophorin A. The K_D of IH4v1-Epo R150A is 33nM in size, while the KD of 10F7-EpoR150A measures 100nM. Moreover, the length of the linker has been changed, from a 35 aa peptide linker to a 5 aa linker. Decreasing the

length implicates a decreasing chance of the bound Epo reaching another cell. This in return lowers the chance of vascular endothelial activation. The optimization led to a promising chimeric activator with an altered targeting element and linker length. This new candidate does have lower inflammatory signaling, higher therapeutic index and higher potency. However, the mutation within Epo still remained the same. A scheme of the optimization can be seen below in Figure 7.

Figure 7 Scheme of optimization of Epo. Adapted from Jungmin Lee (unpublished data).

1.5.3. EpoR: Problem statement and aim

1.5.3.1. Screening for a mutation in Epo with weaker affinity to EpoR, than the Epo mutation Arginine 150 to Alanine

Due to effective engineering optimization by Jungmin Lee, since the publication of the targeted Epo 10F7-35aa-Epo$_{R150A}$ (Burrill *et al.*, 2016) the targeting element and the linker length of the fusion protein have been changed. The current lead molecule, IH4v1-5aa-EPO$_{R150A}$, has a three times higher affinity for GYPA and the volume is five times smaller. Those features lead to a 15-fold higher effective molarity enabling a lower affinity of the Epo mutant to EpoR.

The goal of this study was to screen for a mutation in Epo with a weaker affinity to EpoR than the current mutation Epo$_{R150A}$, to further reduce non-target cell receptor binding.

It was also screened for a mutation on the high affinity binding site of Epo. In addition, the mutation was tested in a kinetic assay to compare the affinity of the new mutation to the current mutation Epo$_{R150A}$. The mutation had to

be fused to IH4v1 to see if the avidity for red blood cell precursors was still intact.

1.6. Ephrin receptor-B type 4 (EphB4)

1.6.1. EphB4 an EpoR?

In 2015, Pradeep and his colleagues identified EphB4 as a receptor for Erythropoietin. The binding of Epo to the tyrosine kinase receptor triggers signaling via STAT3 which induces cancer growth (Pradeep *et al.*, 2015). The binding of Epo to EphB4 has been examined using fluorescence microscale thermophoresis. The results are shown in Figure 8. Based on the published data by Pradeep et al. the K_D of Epo to EpoR is 880 nM, while the K_D of Epo-EphB4 is 28 nM. These measurements were made using Microscale thermophoresis experiments. This marks EphB4 as a low affinity receptor for Epo.

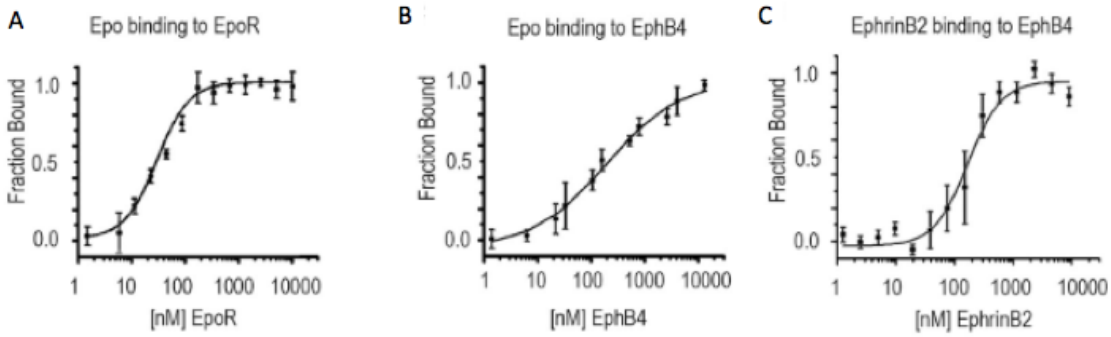

Figure 8 Determining affinity of binding interaction by Microscale thermophoresis experiment. Binding curves of A Epo binding to EpoR, B Epo binding to EphB4 and C EphrinB2 binding to EphB4. Graph from Pradeep et al. (2015, Cell).

Signaling pathways, triggered by stimulation with rhEpo in a time course of 0, 5, 15 minutes, were conducted using A2780 cells -shEpoR and -shEphB4 cells (Figure 9), leading to a proposal of a potential signaling pathway which can be viewed *in Figure 10.* The paper Pradeep et al. (2015, Cell) is presenting consistent evidence from patients suffering from ovarian cancer.

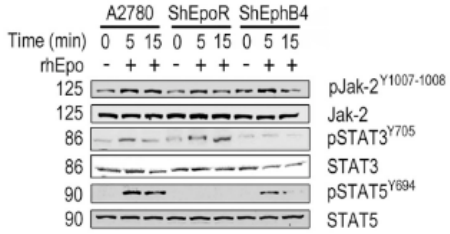

Figure 9 Signaling assay using western blot. Graphic from Pradeep et a. (2015, Cell). Stimulation of A2780, A2780-shEpoR, A2780-shEphB4 cells with Epo for 0, 5 and 15 min.

The survival rate after ESA based treatment decreases with EphB4-overexpressing tumors compared to patients with low EphB4 expression tumors. Summarizing this experimental data, Pradeep et al. concluded that EphB4 is a critical mediator in the process of Epo-induced cancer growth (Pradeep *et al.*, 2015).

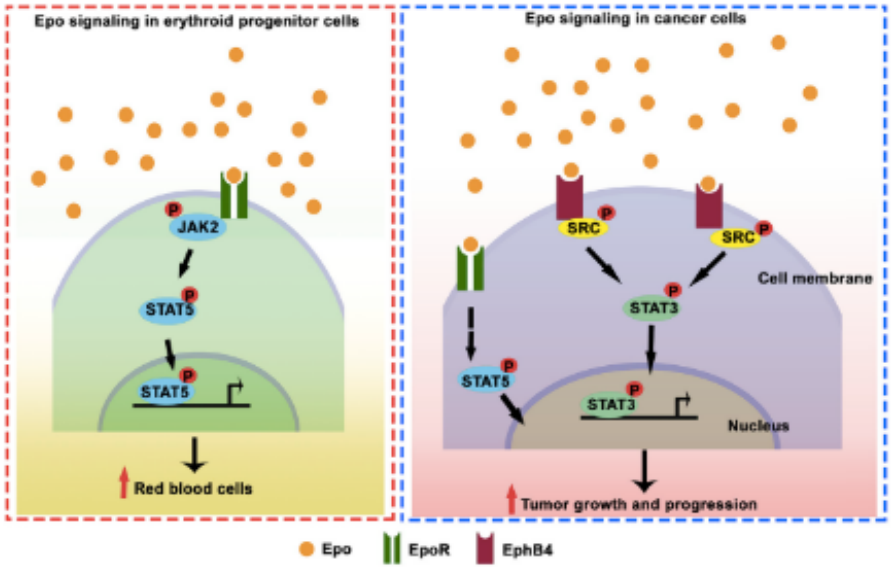

Figure 10 Proposed signaling model by Pradeep et al (2015, Science) of Epo induced stimulation of EphB4 and EpoR. Binding of Epo to EphB4 should lead to phosphorylation of SRC resulting in Stat3 pathway activation.

1.6.2. Eph-receptors and Ephrins

The intracellular communication in multicellular animals depends on the receptor tyrosine kinases (RTK). Already basal metazoans, such as Hydra and sponges, have a RTK repertoire. (Wheeler and Yarden, 2015) The largest subfamily of RTKs are Ephrin receptors (Eph receptor). In 1987, during the

research of Hirari and his colleagues on tyrosine kinases playing a role in the pathology of cancer, the first Eph receptor was identified and named. Eph was named after the erythropoietin-producing hepatocellular carcinoma cell line the cDNA was isolated from (Takahashi *et al.*, 1995; Murai, 2003). Since 1987, a total of 16 Eph receptors and eight of their ligands (ephrins) have been identified. A total of 15 out of the 16 receptors can be found in humans (Wheeler and Yarden, 2015).

All Eph receptors share the following structure: the extracellular domain consists of a globular ephrin ligand-binding domain, they contain a cysteine-rich region and two fibronectin type III domains. The extracellular domain is connected via a transmembrane domain to the cytoplasmic domain, consisting of a juxtamembrane region which is a highly conserved tyrosine kinase domain, adjacent to a sterile alpha motif (SAM) and a carboxy terminal tail (Himanen and Nikolov, 2003; Kania and Klein, 2016). Yet, there are ways to differentiate Eph receptors. They can be divided according to structure and sequence similarities into EphA and EphB. Ephrin-A ligands bind to EphA receptors which have a glycosylphosphatidylinositol linkage connecting them to the cell membrane, while EphB receptors bind to transmembrane ephrin-B ligands which allows cell-cell communication.

The single-pass transmembrane receptors have been functional versatile (Murai, 2003). Ephrin-Eph signaling is involved in many processes that need direct cell-cell communication, such as axon guidance in neurons, synapse formation and angiogenesis.
Important development processes, such as tissue and organ development, is mediated through Eph signaling via cell sorting, morphogenesis and pattern formation. In adulthood, the Ephrin-Eph signaling plays a critical role in homeostatic events, neuronal plasticity remodeling in vascular development and tumor angiogenesis and has also been brought into relation with tumor growth and metastasis (Himanen and Nikolov, 2003; Blits-Huizinga *et al.*, 2004; Kania and Klein, 2016).

An example of Eph receptors, dependent on short distance cell-cell interplay are ß cells located in the pancreas. Depending on glucose levels, EphA

receptors and ephrin-A ligand signaling leads to inhibition or promotion of insulin. EphA receptor inhibits insulin secretion via forward signaling upon low glucose levels. High Glucose levels lead to a decrease of EphA receptor downregulation via dephosphorylation and an increase of ephrin-A reverse signaling (Pasquale, 2008)

1.6.3. Eph Signaling

Activating the kinase domain in RTK receptors is typically called "forward signaling".

Forward signaling of Eph-receptors leads to the activation of the tyrosine kinase domain, subsequently the autophosphorylation and phosphorylation of other proteins. Even though many studies found out that the knockout of Eph receptors lead to decreased tumorgenicity, forward signaling in Eph-receptors does not necessarily lead to tumor promoting effects. Evidences have shown tumor suppressing activity through forward signaling of Eph receptor *in vivo* as well as *in vitro* of skin cancer, prostate and breast cancer (Pasquale, 2008).

Additionally, Eph receptors also have the possibility of bidirectional signaling, stimulated by ephrin-ligands.

Bidirectional signaling affects the receptor as well as the ephrin-expressing cells that are involved in the binding (Lisabeth, Falivelli and Pasquale, 2013) An example for bidirectional and forward signaling is the signaling of Ephrin-B2 to EphB4 which is involved in bone formation Figure 11

An upregulation of EphrinB2 osteoclasts gets triggered due to cytokine secretion from osteoblasts. The EphrinB2 ligands bind to the EphB receptors on osteoclasts which leads to bidirectional signaling that inhibits the differentiation of osteoclast and promotes bone formation in osteoblasts (Pasquale, 2008). Moreover, signaling of Eph receptors can even get activated without the involvement of ephrin ligands or kinase activations

(Lisabeth, Falivelli and Pasquale, 2013).

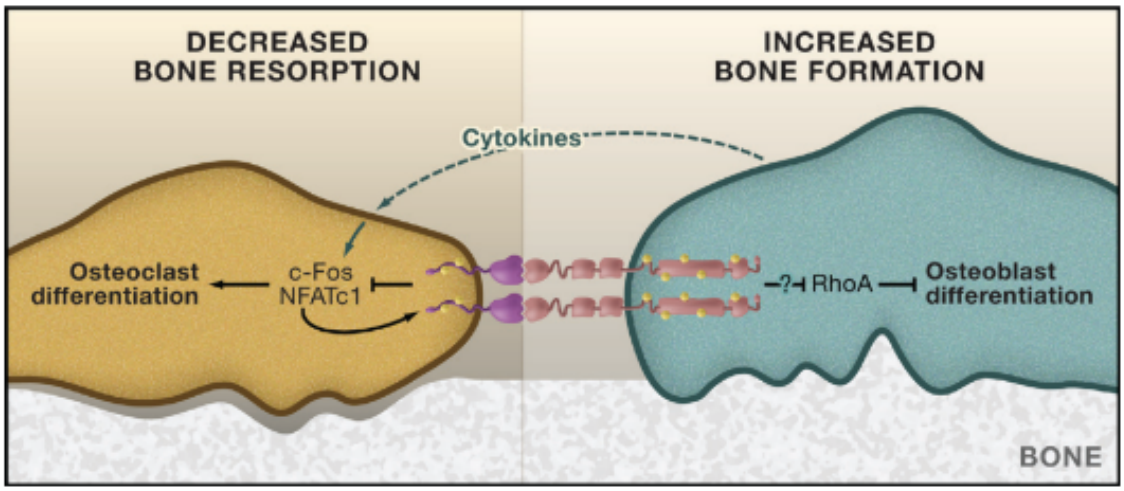

Figure 11 EphrinB2-EphB4 receptor activation leading to forward and bidirectional signaling in communication of osteoclasts with osteoblasts. Figure by Pasquale, (2008, Cell).

1.6.4. Ephrin type-B receptor 4 (EphB4)

EphB4 is a protein-coding gene, located on chromosome 7 (7q22.1) in humans. Its protein is expressed on the plasma membrane and is a transmembrane protein. The natural receptor for EphB4 is the Ephrin-ligand EphrinB2 which leads to bidirectional signaling into neighboring cells upon cell-cell contact (Kania and Klein, 2016). Previously thought of being mainly expressed by neuronal cells, EphB4 plays a major role in neuronal guidance and mediating molecules for tissue border. The Ephrin family is now known to have many cellular responses in the embryonal stage and in adults. Tissue expression of EphB4 is ubiquitously in the placenta, but can also be found in the kidney, lung, pancreas, skeletal muscle and heart.

EphrinB2-EphB4 signaling has been found as the master regulator for non-sprouting angiogenesis (intussusception), which leads to endothelial proliferation and establishment of new capillary networks due to the vascular endothelial growth factor (Groppa *et al.*, 2018). During embryonic development, targeted disruption of EphB4 gene leads to defects in

cardiovascular system, angiogenesis and results in lethality before embryonic day 11 (Blits-Huizinga *et al.*, 2004).

1.6.5. EphB4 and cancer

Eph signaling has an effect on the actin cytoskeleton, the integrins and the intracellular adhesion molecules resulting in influence of cell morphology, adhesion, migration and invasion. Various studies led to the conclusion that ephrin-Eph signaling results in tumor growth, metastasis and tumorgenicity. Within the ephrin-Eph family, EphrinB2-mediated activation of EphB4 has been repeatedly published to elevate proliferation, migration and tumor angiogenesis (Chrencik *et al.*, 2006; Alam *et al.*, 2008; Kania and Klein, 2016).
However, evidence of EphrinB2-EphB4 signaling has been found to be both, tumor promoting, as well as tumor suppressing. (Chen, Zhang and Zhang, 2017) For example while wildtype EphrinB2 signaling controls proliferation, mutations in EphrinB2 can be associated with prostate cancer which leads to conclusion that wildtype EphrinB2 can suppress tumor growth (Kania and Klein, 2016).

Attention has been drawn to the overexpression of EphB4 in cancer cells which has been discovered and studied in various types of cancer such as lung, colon, pancreatic, bladder, breast and prostate cancer (Zhu *et al.*, 2005; Udupa, 2006; Kumar *et al.*, 2007; Alam *et al.*, 2008; Pasquale, 2008; Kania and Klein, 2016; Chen, Zhang and Zhang, 2017) Interestingly enough, EphrinB2 and EphB4 mRNA levels and histochemical scores have been found elevated in ovarian cancer cells. Moreover, co-expression of EphB4 and EphrinB2 has been found ascending in clinical stages from I till IV (clinical I<II<III<IV, P<0.001) (Alam *et al.*, 2008).

Additionally, to the correlation of elevated EphB4 expression to advanced ovarian cancer stages, data shows that overexpression of EphB4 supported cancer cell migration and invasion. Furthermore, results of elevated caspase-

8 activity and reduced caspase-9 activity led to the conclusions that EphB4 protects the cell from extrinsic membrane-originating apoptotic pathways (Kumar *et al.*, 2007). Reports show that high levels of EphB4 expression correlated with malignant tumors while knock down of EphB4 expression led to disabled tumor growth (Murai, 2003; Pasquale, 2008; Chen, Zhang and Zhang, 2017).For an *in vivo* study of mice, xenografted tumors have been injected with either stable overexpressing EphB4, stable knockdown of EphB4 or transfecting with an empty vector. EphB4 overexpression compared to EphB4 knock down and empty vector xenografted tumors have been more invasive in vivo and resulted in bigger tumor size (Lv *et al.*, 2016).

Data from Zhu et al. support this assumption by stating that knockdown in *in vivo* murine tumor xenograft models reduced tumor proliferation for about 80%, leading to the conclusion that EphB4 provides a signal for cell survival (Zhu *et al.*, 2005).

Within all these studies, EphB4 plays a critical role in carcinogenesis and has therefore drawn worldwide attention to EphB4 as a potential therapeutic target. Tumor inhibiting compounds, targeting EphB4, are currently in laboratory, preclinical and clinical studies such as Dasatinib, Golvatinib, Quinazoline (EXEL-7647) and Phenylthiophene derivates (Chen, Zhang and Zhang, 2017).

1.6.6. EphB4: Problem statement and aim

1.6.6.1. Engineering Epo to prevent EphB4 signaling in cancer patients

In “Erythropoietin stimulates tumor growth via EphB4”, Pradeep et al. reveal EphB4 as a previously undiscovered receptor. The research results of this article lead to the conclusion that Epo signaling via this receptor is a critical mediator of Epo-induced cancer growth.
Based on the findings of Pradeep et al., Epo binds to EphB4 by using a

different binding surface as it does for EpoR.
With this in mind we hypothesized that by mutating Epo, the binding to EphB4 can be inhibited while maintaining the binding to EpoR and therefore its erythropoietic activity. This would lead to diminished Epo-induced side effects of cancer growth while Epo would still be erythropoietic.

Hence, this thesis' study has two main objectives.
The first objective of this study is to identify the binding site of Epo to EphB4. This will be done by a global and unbiased study for potential binding sites in Epo to its low affinity receptor EphB4. Mutations in Epo will be created and used in a binding assay of Epo to EphB4. The mutation, which inhibits binding in this kinetic assay, will then be tested for the binding affinity of EPO to its EpoR.

Identifying this mutation would facilitate the second objective of this study: creating a mutated Epo fusion protein, which has reduced binding of Epo to EpoR and eliminated binding to EphB4 and, therefore, reduces Epo-induced cancer growth. To maintain the erythropoietic activity of Epo, the mutation should only reduce the binding affinity to EpoR, which can further be maintained by targeting it to RBC precursors.

1.7. ß-common Receptor-Erythropoietin receptor Heterodimer (ßcR-EpoR)

1.7.1. Overview ßcR-EpoR (CD131)

Leist et al., (2004, Science) has published that the Epo mediated tissue protective- and erythropoietic activity could be separated and does depend on interaction with distinct receptors (Leist, 2004).
In an attempt to identify the receptor that induces Epo-mediated tissue protection, a common ß receptor (ßcR) subunit linked by a disulphide bond to a subunit of EpoR (ßcR-EpoR) has been detected (Brines *et al.*, 2004). This ßcR subunit is also commonly referred to as CD131 and is also a co-receptor for several cytokines such as granulocyte macrophage-colony-stimulating factors, Interleukine-3 and Interleukine-5 expression (Brines and Cerami, 2012).

1.7.2. ßcR-EpoR: gene and expression

EpoR and the heterodimer ßcR-EpoR, both have in common that they are single transmembrane receptors belonging to the type I cytokine receptor family (Bagley *et al.*, 1989). A scheme of the structure of both transmembrane receptors can be seen in Figure 12.

In humans the CD131 gene is located on chromosome 22 and contains 12 exons. The protein of the ßcR does consist of 897 amino acids with a predicted molecular weight of 97.3 kDa (Bagley *et al.*, 1989 and UniProt P32927).

The ßcR is in human tissue highly expressed in the bone marrow and the immune system. Expression has also been found in the lung, kidney, brain

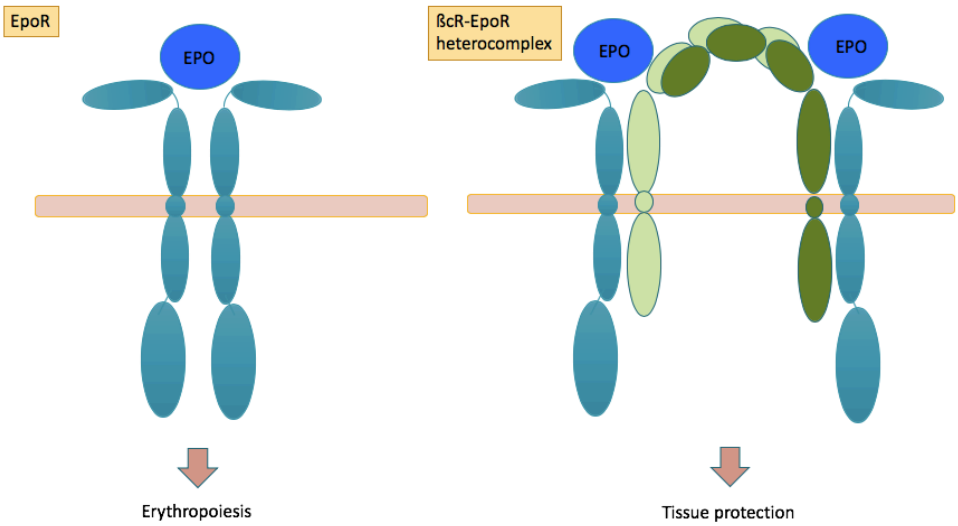

Figure 12 Scheme of the single transmembrane cytokine receptors: EpoR and ßcR-EpoR and their main therapeutic activity upon Epo binding. Scheme adapted from Hernandez et al., (2017, Neurogal Regeneration Research).

and on epithelium. However, the heterodimer of ßcR and EpoR has been demonstrated to complex with each other solely upon stimulation for example through an injury or Epo exposure (Collino *et al.*, 2015). Within stress conditions the expression of the ßcR-EpoR complex have been detected in the endothelium, central and peripheral nervous system, the heart, the brain, the kidney, macrophages and bone marrow derived mesenchymal cells (Brines *et al.*, 2004; Rabie and Marti, 2008)

1.7.3. Epo induced Neuroprotection

The high expression of Epo and its receptor within regions of the brain that are vulnerable to acute hypoxia, was one of the predictions of the neuroprotective role for Epo (Rabie and Marti, 2008).

1.7.3.1. Expression of Epo and EpoR within the brain

Epo plays an important role in fetal brain development, but the expression of EpoR is decreasing drastically in adult brains. In adult brains EpoR is expressed in neurons, astrocytes and microglia and on endothelial cells forming the blood brain barrier. (Tamura *et al.*, 2017; Sun *et al.*, 2019).

Intrinsic Epo has been found in the brain along with expression of EpoR, especially in regions of the brain that are sensitive for hypoxia (Rabie and Marti, 2008). Interestingly under hypoxic conditions Epo can be increased by a 100-fold in astrocytes (Sun *et al.*, 2019).

1.7.3.2. Neuroprotection

Even though the main clinical usage of Epo is based on its ability to stimulate RBC production, Epo is gaining accelerated attention for its neuro- and tissue protective activity. Attention arised even, when higher cognitive function had been observed in anemia patients, that have been treated with rhEpo due to CKD.
Accumulating data is suggesting that Epo has neuroprotective effects in many diseases of the CNS such as Alzheimer's disease, Amyotrophic Lateral Sclerosis and Parkinson's disease (Hernández *et al.*, 2017). The therapeutic potential of Epo has furthermore been reported for treatment of diseases such as schizophrenia, brain trauma, spinal cord injury and epilepsy (RABIE 2008, NOH 2014, Hernandez 2017).

A delayed onset of Amyotrophic Lateral Sclerosis symptoms could for example be archived by treating SoD1 transgenic mouse models with EPO. This treatment led to upregulation of anti-inflammatory cytokines and therefore delayed disease progression (Noh *et al.*, 2014).
The neuroprotective role of Epo has furthermore been supported in Alzheimer's disease model. In Alzheimer's disease an accumulation of the proteins Aß and tau can be found in hippocampus and cortex. Epo treatment can lead to reduction of the Aß protein along with reducing neuronal loss, and oxidative stress (Sun *et al.*, 2019).

1.7.3.3. Epo treatment for brain ischemia

Stroke is amongst the leading causes of disability and death worldwide. The sudden cerebrovascular disease of the brain caused by critical and immediate suppression of the blood supply to the brain may result in a prolonged loss of the central nervous system functions (Johnson *et al.*, 2016)**.** The cause of a stroke can be classified into subarachnoid hemorrhage, intracerebral hemorrhage or brain ischemia. While hemorrhagic stroke is caused by intracranial bleeding, brain ischemia occurs if the brain is not provided with enough oxygen. Since the brain does not have long term resources of stored energy, the levels of adenosine triphosphate drop. An ischemic cascade gets triggered which leads to activation of chemical processes involving, amongst others, the breakdown of mitochondria, leading to the release of toxins and apoptotic factors in the cell (Hernández *et al.*, 2017).
Studies showed that cerebral ischemia leads to upregulation of Epo and its receptor (Rabie and Marti, 2008). Epo treatment before the onset of strokes resulted in reduced infarct size in animal models, such as gerbils and mice (Rabie 2008).

1.7.3.4. High-Altitude Illness

People who are exposed to altitudes above 2500m or higher, are at risk to develop high-altitude illness up to 1-5 days after ascent. Acute altitude illness can be distinguished in three main forms: Acute Mountain Sickness, High-Altitude Cerebral Edema (HACE) and High-Altitude Pulmonary Edema (HAPE)
The body's response to hypoxia caused by altitude is hyperventilation, which can result in a noncardiac Pulmonary Edema. This illness is called High-Altitude Pulmonary Edema (HAPE) and is a severe form of the Altitude Illness, leading to a mortality rate up to 50% during expeditions to the Himalaya without treatment (Jensen and Vincent, 2019).
Acute mountain sickness is characterized by symptoms such as dizziness, nausea and headache (Luks, Swenson and Bärtsch, 2017) and can already

be experienced in altitudes starting from 1500 m. A severe form of altitude sickness, in contrast, is the High-Altitude Cerebral Edema. If this form does not get treated promptly it can lead to death within 24 hours. The exact pathophysiologic mechanism is not fully understood yet. However, one potential theory is that hypoxia stimulates the release of reactive cytokines, nitric oxide and free radicals as well as cerebral vasodilation resulting in intracranial hypertension. High capillary pressure and leakage is followed by disruption of the Brain Blood Barrier (BBB) and further cerebral edema. This illness can lead to coma and death within 24 hours of its onset (Hackett and Roach, 2001; Luks, Swenson and Bärtsch, 2017).

The response of the body to high altitudes includes increasing Epo production, which leads to elevated values of hematocrit and hemoglobin. Due to a higher red blood cell count, more oxygen can be delivered to the tissue (Paralikar and Paralikar, 2010). An elevated level of Epo can be detected in the blood within two hours after ascent, while it takes about 4-5 days to see an effect in increased mature red blood cell levels (Paralikar and Paralikar, 2010).

A clinical trial to examine the prophylactic effect of Epo on high altitude illness involved 20 healthy individuals receiving rhEpo treatment (for 1 month; 1x a week) and a control group of 19 healthy individuals. By ascending and descending the Annapurna base camp (4.130 m) in a defined time frame of 8 days, the group receiving Epo-treatment had an elevated Hemoglobin level and overall less symptoms of high-altitude illness (Heo *et al.*, 2014).

1.7.4. Challenges for Treatment of Epo induced Neuroprotection

1.7.4.1. Blood Brain Barrier (BBB)

The brain endothelial cells of the cerebral vasculature separate the central nervous system (CNS) from the peripheral tissue (Banks, 2012) These capillaries form the Blood Brain Barrier (BBB), which has selective tight

junctions and controls the permeability between circulating blood and the brain (Üzüm *et al.*, 2005).

Based on several studies, exogenous Epo treatment would lead to therapeutically benefit in a variety of brain injuries (Hugo H. Marti, 2004). Epo is not only produced in humans by the kidney but also in the brain itself. However, to be a successful neuroprotective therapeutic, Epo needs to cross the BBB. Several research studies proved that concentrations of endogenous Epo derived from the kidney is only increasing in the CNS if the BBB is disrupted (Hugo H. Marti, 2004; Miller *et al.*, 2015). Epo is above the BBB permeability, which disables passive mediated transport (Broadwell and Sofroniew, 1993). However, labeling Epo with Indium that was systematically administered in humans, it could be visualized that Epo penetrated the BBB of healthy individuals, as well as in individuals, suffering from schizophrenia (Rabie and Marti, 2008).

Additionally, it has been found that intravenously injected Epo crossed the intact BBB of mice at a similar rate as albumin (Banks *et al.*, 2004). About 0.05 to 0.1 % of the intravenously injected dose could be detected in the mouse brain (Banks *et al.*, 2004).

Another study led to the detection of Epo within the cerebrospinal fluid after administering systematically high doses of rhEpo. However, in this study only < 1% of the administered dose crossed the BBB (Hugo H. Marti, 2004). The exact molecular mechanism of how Epo does cross the BBB is yet unknown. However, these findings as well as the fact that EpoR are on the endothelial cells of the brain capillaries leads to a hypothesis that Epo binds to EpoR and can cross the BBB by transcytosis (Hugo H. Marti, 2004; Sun *et al.*, 2019).

However, crossing the BBB remains a major challenge in drug induced treatment for the brain. Establishing rhEpo levels in the CNS, which are of therapeutically relevance requires intracranial injection or high doses in cases of subcutaneous or intravenous administration. Administration of high doses however would increase the severe side effects associated with rhEpo treatment.

1.7.4.2. Required amount of Epo dosing

Dosage and its schedule depend on the different Epo derivates being used, such as Darbepoietin, Epo-alpha, or Epo-beta, as well on the respective patients and treatment options.
Despite the fact that EPO is a powerful neuroprotectant, the dosages required for an effective treatment of neurodegenerative diseases would have to exceed the secure administration level of Epo by far, most likely resulting in severe risks for the patients (Yu Gan, Ph.D.*, Juan Xing, M.D.*, Zheng Jing, Ph.D., R. Anne Stetler, Ph.D., Feng Zhang, M.D., Ph.D., Yumin Luo, M.D., Ph.D., Xunmin Ji, M.D., Yanqin Gao, M.D., and Guodong Cao, 2012)

Dependent on the therapeutic reason for Epo administration, different durations of treatment are implicated and need to be established for a therapeutic outcome. Epo used as a drug for neuropsychiatric illnesses, such as schizophrenia, would require long term Epo administration. This permanent administration of Epo results in increased blood viscosity and steady red blood cell proliferation (Tiwari *et al.*, 2019), besides the already discussed severe side effects of rhEpo treatment.

While in terms of exposure to altitude, the human body naturally responds by elevating erythropoietin levels. The results of Epo in increased RBC proliferation start to be seen within 4-5 days. Administering Epo before experiencing altitudes would lead to increased oxygen levels before ascending and, therefore, to a better oxygen delivery to the muscle. However, the black box warning of the FDA supports Epo administration only for people that are terminally ill, due to the side effects such as the possibility of strokes.

1.7.4.3. Potential interaction of Epo and other treatments

The risk factors resulting from erythropoietic treatment of stroke-patients with rhEpo can enhance occurrence of micro-coagulation and higher infarction risk. Moreover, a double-blind, placebo controlled, randomized clinical trial (n=522) has been conducted, using rhEpo to treat acute ischemic stroke administered 6, 24 and 48 hours after the incident. Over 60 % of patients received thrombolysis in form of recombinant tissue plasminogen, which was not intended at the start of the study. The clinical trial turned out negative and especially combining EPO treatment with recombinant tissue plasminogen activation led to higher death rate compared to the placebo control group. The mechanism behind this is unknown. One possible explanation could be the interactions between the two treatments applied simultaneously. Especially, the treatment of EPO led to side effects such as brain edema, intracerebral hemorrhage, thromboembolic events (Ehrenreich *et al.*, 2009).

1.7.4.4. Engineered Epo derivates having solely neuroprotective activity

To limit the side effects caused by Epo administration in cases of neuroprotective therapeutic potential, approaches were taken to create a mutated Epo with diminished abilities to induce erythropoiesis (Gan *et al.*, 2012). Asialoerythropoietin (Erbayraktar *et al.*, 2003), carbamylated Epo (Leist, 2004) and MEPO (Gan *et al.*, 2012) are derivates that have been generated with this ability. Moreover, these derivates has been found to be unable to bind to EpoR homodimer while still binding to ßcR-EpoR heterodimer. This interaction leads to signaling that induces neuroprotective potential of Epo in the brain (Tiwari *et al.*, 2019).

1.7.5. ßcr-EpoR: Problem statement and aim

1.7.5.1. Rational design of engineering Epo as a potential candidate for High-Altitude Illness

For a number of reasons (f. ex. Work, pleasure or athletic competitions), people all over the world are exposed to high altitudes (Luks, Swenson and Bärtsch, 2017). Exposure to high altitudes, for example while traveling rapidly to mountain destinations such as Aspen or Steamboat Springs in America without acclimatizing properly, can lead to milder symptoms of mountain sickness. However, climbers or mountain trekkers, ascending mountain tops of 2500 m or higher, can experience either only mild forms of high-altitude illness or severe forms, like High-altitude cerebral edema and High-altitude pulmonary edema (Klocke, Decker and Stepanek, 1998). High altitude cerebral edema (HACE) leads to symptoms such as progressive change of mental status, malaise and headache. People are experiencing problems in controlling muscle movement, speaking disabilities and abnormal eye movement. The underlying trigger is hypoxemia. The exact molecular mechanism still remains unknown but edemas in the brain can be identified on Magnet Resonance Imaging and can also be detected in the disruption of the BBB with hemosiderin deposition in the brain. Moreover, it is known that VEGF plays a crucial role in severe vasogenic edema, which can even lead to death.

In several *in vivo* and *in vitro* studies, it has been shown that EPO also possesses neuroprotective effects, beside its erythropoietic activity. Epo binding to EpoR homodimer is known to stimulate erythropoietic signaling. The neuroprotective potential has been reported to signal through the interaction of the hetero-receptor complex ßcR-EpoR (Ding *et al.*, 2017; Tiwari *et al.*, 2019).

In terms of HACE, administrating Epo can not only help elevating the hematocrit level, and therefore the oxygen supply to the tissue, but can also

enhance the protection of the cells during hypoxia due to the neuroprotective effect of Epo.
However, as indicated by the FDA with a black box warning, the ESA treatment is accompanied by severe side effects.
Another disadvantage is the need for relatively high dosages of Epo to detect a neuroprotective effect, since only a very limited percentage of circulating Epo accesses the brain.

The aim of this thesis' study is to design and evaluate an Epo mutation, possessing neuroprotective effects while keeping its erythropoietic activity solely targeted to RBC precursors. This would lead to direct benefits such as Epo protecting neurons or neuronal progenitor cells during hypoxemia, and indirect benefits such as higher oxygen delivery to the brain by increasing mature RBC production.
Additionally, exogenous Epo needs in general much higher dosages to exert neuroprotective effects than for inducing erythropoiesis, since it needs to cross the blood brain barrier. Targeting Epo to RBCs has another advantage of increasing its serum half-life and hinder renal clearance of Epo. This results in the advantage of reduced dosages of Epo.

This will be done by screening for a mutation that enables the binding of Epo to its hetero-receptor complex ßcR-EpoR, which is reported as key for its neuroprotective potential. The mutation, however, shall reduce the affinity of Epo to EpoR homodimer, which is well known to stimulate erythropoiesis but is also associated with severe side effects. By targeting the mutated Epo to EpoR on RBC precursors via the chimeric activator established by the Silver laboratory of Dr. Pamela Silver, the side effects should eventually be diminished.

2. Material and Methods

2.1. Cell culture

Table 1 Cell lines cultured and used within the study

Name	Abbre-vation	Origin	Tissue	Culture Media	Assay Media
Tissue Fibroblast 1	TF1	Human	Bone marrow	RPMI-1640 + 2 ng/ml recombinant human GM-CSF and 10%FBS.	RPMI-1640 + 10%FBS
A2780		Human	Ovary	RPMI-1640 + 2mM Glutamine + 10% FBS.	RPMI-1640 + 10%FBS
Cos-1	Cos-1	Cercopithecus aethiops	Kidney	DMEM +10%FBS	
MCF-7		Human	Mammalian gland derived from metastatic site	EMEM + 0.01% Insulin + 10% FBS	
Henrietta Lachs	HeLa	Human	Cervix	EMEM +10%FBS	
Freestyle HEK293	HEK-293 F	Human	Embryonic kidney	FreeStyle 293 Expression Medium	

2.1.1. Maintaining cells in culture

All cells, unless stated differently, were cultured in the incubator with the settings: 5% CO_2, 95% air and at 37°C.

Cell lines already were in the possession of Dr. Pamela Silvers laboratory or have been purchased from ATCC. Upon cell line arrival on dry ice, the vial

thawed in a 37°C water-bath for about 2 min. The cryoprotective tube was quickly dried and decontaminated with Ethanol. A prepared sterile centrifugation tube containing 9 ml of pre-warmed culture medium was centrifuged for 10 min at 125g. The supernatant was carefully aspirated and resuspended in 5 ml of fresh pre-warmed medium. The cells got seeded into a T25 culture flask and incubated under standard conditions for 24 hours before getting split into a T75 culture flask. While some of the cells either were kept alive most of the cells were frozen to create stocks of $1*10^6$ cells/ml.

2.1.2. Stimulation with rhEpo and EphrinB2 in A2780 and TF-1 cells

In order to evaluate the pSTAT3, pSTAT5 and pSRC levels followed by rhEpo and EphrinB2 treatment in A2780 cells and TF-1 cells, the cells have been seeded 12 hours prior to treatment with both proteins into 6 well plates. At about 70-100% cell confluency, the cells were incubated with rhEpo (100U/ml) in steps of 15, 10, 5 min, with EphB2 (2 µg/ml) for 10 and 5 min, and with PBS only. The stimulation was stopped by aspirating the medium and rinsing with ice cold PBS. The process was repeated three times. Ice cold RIPA lysis buffer was mixed 1:100 with a Halt protease/phosphatase inhibitor cocktail to prevent protein degradation and dephosphorylation. The cells were scraped using a cell scraper and transferred into a new Eppendorf tube. After 30 minutes of periodic mixing at 4 °C, the lysate was spun down at 13000g for 10 min at 4 °C. The cell lysate supernatant was transferred to a new ice-cold Eppendorf tube and the protein concentration was determined using the BCA assay by Pierce. 50 µg per sample were loaded on the SDS-PAGE gel and analyzed by using Western blotting.

2.1.3. Lysates from cell culture

The cells were being washed three times by aspirating the medium and rinsing with PBS while kept on crushed ice in the hood. The ice-cold Lysis buffer RIPA and the Halt protease Inhibitor cocktail were mixed at a 1:100 ratio. The mixture was pipetted on to the cells and the adherent cells were scraped from the cell culture dish using a cell scraper. The cell suspension got pipetted into an ice-cold micro centrifuge tube and kept in constant agitation for 30min to 1 hour at 4 °C. The cell debris got pelleted at 13000 g for 10 min at 4 °C, and the lysates got transferred to new microcentrifugation tubes. Prior to further analysis, the whole protein concentration got determined by BCA protein assay using the Pierce assay kit.

2.2. Cloning

The expression vector pSecTag2A of about 5.17 kb was used for the cloning process and the expression of Erythropoietin mutants and Erythropoietin Fusion proteins. Figure 13 shows a schematic representation of the mammalian expression vector. (A more detailed version is added in Appendix S.8.2). The genes under investigation were cloned within the frame of the initiation ATG of the N-terminus IgK-chain leader sequence and the C-terminal myc epitope/poly-histidine tag. The murine Ig K-chain leader sequence is effective in secreting the investigated protein into the supernatant.
Plasmids were amplified in NEB 5-alpha Competent *E.coli* cells. All Epo and Epo-Fusion constructs carried a His tag at the C terminus for further purification.

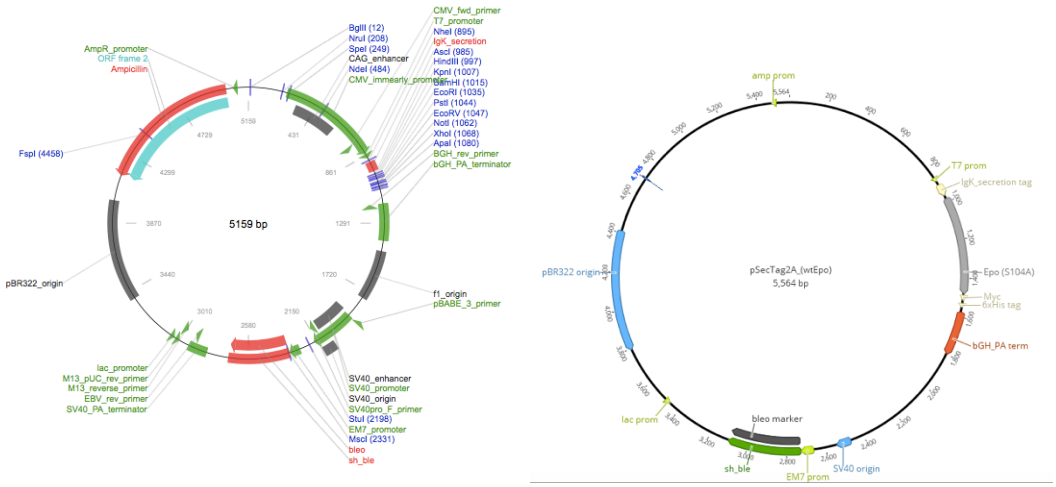

Figure 13 The pSecTag2 A is a mammalian expression vector which is nonviral. It contains a secretion tag and an ampicillin resistance. The size of the plasmid itself is 5159 bp. Including Epo without a fusion element it contains 5564 bp. Arrows indicating the orientation of the marked elements.

HEK293F cells were used for protein expression. Those cells are a common mammalian expression vector, derived from a primary embryonic human kidney cell line sheared with adenovirus 5 DNA. Twelve different variants of HEK293 cells are available. HEK293F is cloned from HEK293 cell line and got adapted to the commercially available medium Freestyle.

2.3. Graphic Mutations using PyMol

Mutations within the binding site of Epo to EpoR or EphB4 have been described using the molecular visualization software PyMol: The PDB file of interest was loaded into PyMol. Under the Wizard menu the function Mutagenesis was chosen. The residue of interest was chosen in the PyMol viewer window and the chosen mutation was applied.

2.4. Site-directed mutagenesis

In order to induce mutations into EPO, primers containing a single amino acid (aa) mutation were specifically designed. Out of the 64 possible nucleotide

triplet combinations, the choice of codons for the aa mutation was influenced by the codon usage tables revealing the codon usage frequency. (Athey, J 2017) The primers have been checked for specificity using Primer BLAST from NCBI.

2.5. Polymerase Chain Reaction

Polymerase chain reaction (PCR) is an enzyme based *in vitro* method, allowing the amplification of samples under examination. In this thesis' study, the master mix 2x Phusion was used. A 20 µl PCR mixture was prepared to amplify DNA for subsequent cloning purposes. The total of 20 µl PCR mixture was prepared using 10 µl 2x Phusion master mix, 1 µl DNA template to reach a final concentration of 5ng DNA template, 0.6 µl DMSO, 1 µl of each primer (forward and reverse) to reach a final concentration of 0.5 µM per primer and 6.4 µl H_2O. The organosulfur compound DMSO can bind at cytosine residues, helping to denature GC rich templates, which can further lead to lowering the primer melting points. Table 2 shows the actual thermocycler conditions used in this specific setting. By using a gel electrophoresis with a 0.7% agarose gel, the PCR product and the correct length was verified.

1st

Table 2 Thermocycler conditions using Phusion master mix.

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	4 min	1x
Denaturation	98 °C	15 sec	32x
Annealing	Depending on length and base composition of primer	30 sec	
Elongation	72 °C	$\frac{Xbp}{1000bp * 1min}$	
Final Elongation	72 °C	5 min	1x
Store	4 °C	Forever	

2.6. Restriction Digest

The methylation sensitive restriction enzyme DpnI was also used in this study. It is a fast digest enzyme and works best at a temperature of 37 °C. The DpnI treatment has therefore been executed at a constant level of 37°C for 15-30 min with 0.5 µl of DpnI and 2 µl of FD buffer (10x) to each 20 µl PCR reaction.

2.7. Gibson Assembly

he Master Mix used to assemble the DNA fragments contains three different enzymes: a 5´exonuclease, a DNA polymerase and a DNA ligase.
Up to three DNA fragments were joined using the Gibson assembly method. The PCR amplified DNA inserts exhibit 15-30 bp overlapping ends that are complementary to the fragments to be joined. 5 µl of the 2x Gibson Master Mix was incubated with 1 µl of the Insert-PCR products and the Vector-PCR product, and substituted with H2O up to 10 µl for 50 °C for 15 min.

2.8. Transformation into Competent *E.coli*

Artificially induced competence in bacteria transforms the organism, such as *E.coli*, to a convenient host for introducing foreign DNA for purposes such as protein expression.
In this study, plasmid DNA has been introduced into the *E.coli* strain NEB 5-alpha via heat shock. The principle of this transformation method is a sudden temperature change which leads to the disruption of the plasma membrane enabling the DNA to enter the host.
For this purpose, Eppendorf tubes were pre-cooled on ice and a mixture of 40 µl competent NEB 5-alpha cells and 1.7 µl plasmid were incubated on ice for 30 min. Exposing the mixture to 42 °C for 30 seconds in a water bath led

to the heat shock which was abruptly stopped by putting the mixture back on ice for 5 minutes. After the sudden temperature change, 950 µl of SOC medium were added to the Eppendorf tube and left shaking at 37°C in the warm room for 1 hour. 200 µl of the cell suspension was plated on pre-warmed selective agar plates and incubated for about 16 hours at 37°C. All plasmids contained Ampicillin (Amp) and, therefore, Amp-plates were mainly used as selective agar plates. However, since pSecTag2 originally carries the Amp and Zeocine resistance gene, both could be used for selection of transformants in *E.coli*.

Single colonies were picked and inoculated in 3 mL LB. To amplify the plasmid, the single cell colonies were left shaking for about 16-20 hours at 37°C. The plasmid DNA was purified following the protocol from the commercially available Plasmid Miniprep Kit from Qiagen. The DNA was eluted from the column using 50 µl of ddH2O. The concentration was measured using Nanodrop.

2.9. Sanger Sequencing

To determine if the obtained result after a cloning step or transformation has the correct DNA sequence, the purified plasmid was sent to Genewiz for Sanger Sequencing. The method to determine the nucleotide sequence of DNA was developed by Frederick Sanger and his colleagues.

Additional to the 12 µl plasmid DNA, the specific primer was also sent to Genewiz for sequencing. The results were aligned to the reference plasmid which has been designed previously using Geneious.

2.10. Preparation of Glycerol stocks

The bacterial cultures carrying the correct plasmid, verified by sequencing, were stored at -80 °C as glycerol stocks, in order to protect the bacteria. To

0.5 ml of bacterial LB culture, 0.5% of glycerol were pipetted into a cryo-protective tube, gently mixed and frozen at -80 °C.

2.11. Transfection into HEK293F cells and Purification of the Secreted Protein

HEK293F cells were grown in Freestyle HEK293 medium as suspension cultures at 37°C and 8% $C0_2$ in an orbital shaker rotating at 125 rpm. Cells were sub-cultured every 3 to 4 days in disposable Erlenmeyer flasks. In order to transfect the suspension cell line with the correct plasmid, the cationic lipid-based reagent 293fectin was used according to the protocol provided by the manufacturer ThermoFisher.

2.12. Purification using Cobalt Affinity Resin and Concentration of Purified Protein

About 4 to 5 days after transfection, the proteins were secreted to the supernatant and were ready to be harvested.
The 30 ml of transfection culture was transferred to a 50 ml falcon tube and centrifuged for 15 min at 500g. The supernatant was then transferred to a new 50 ml falcon tube and sterile-filtered using a 0.22 µm vacuum filter. To concentrate the secreted proteins a 10 kDa, a cut off concentrator has been used and spun down at 3000-5000g for 20 min until it reached a final volume of 5 ml.
Purification of the his-tagged Epo proteins was achieved by using gravity-flow purification TALON Metal Affinity Resin (takarabio). The resin (tetradentate chelator) was charged with cobalt which is bound to the resin at four sites hindering metal-ion phase out. The binding of cobalt to his-tagged proteins is more specific than nickel to his-tag resulting in a higher purity. All Buffers used were from the HisTALON Buffer Set, only the washing buffer was a ratio of 1:13 Equilibration to Elution Buffer. The gravity column was prepared using 1.5 ml of the TALON resin. After draining the buffer, the

column was equilibrated with the provided equilibration buffer. The sample was added to the column and incubated with the resin for minimum 30 min rotating at 4°C. The column was filled and drained in the following order: collecting the flow-through (PB), 10 ml Equilibration buffer (PW1), 10 ml Equilibration buffer (PW2), 5 ml Wash Buffer (PW3), 3 ml Elution buffer (PE1), 3 ml Elution buffer (PE2). All fractions were collected in labeled falcon tubes, stored immediately at 4°C. To detect which fraction contains the eluded protein, the collected fractions were loaded on an SDS Page gel (as further described in Methods SDS PAGE gel) and analyzed by Coomassie staining as shown in *Figure 14*. As can be seen on the gel, in most cases the purest protein has been found in PE1. To further concentrate and exchange the buffer, the fractions containing the target protein have been concentrated down to 0.5-1 ml using the 10kDa cut-off concentrator while subsequently adding 4-5 ml of PBD for 3 times

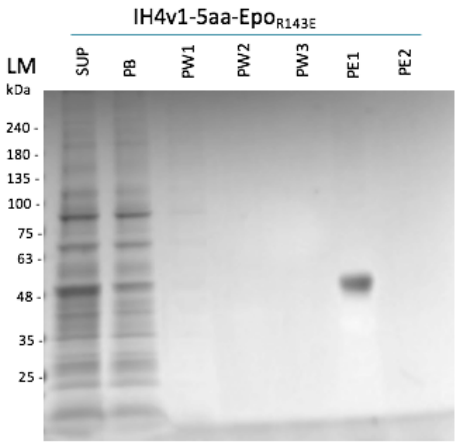

Figure 14 Fractions of gravity protein purification using TALON metal affinity resin visualized by SDS-PAGE followed by Coomassie staining-
As in most purifications within this study the protein has been found in PE1 (post elution 1).

The protein concentration has been measured using Pierce BCA Protein Assay Kit according to the manufactures protocol. The purified protein has further been snap frozen using LN2 and stored at -80 °C in small aliquots for further experiments.

2.13. SDS Page

To denature, linearize and reduce the disulphide bonds, about 10-20 µl of the sample was combined with 2.5 µl DTT and 12.5 µl 2x SDS loading dye and boiled for 2 min at 100 °C. To visualize protein migration during the SDS-

Page electrophoresis, the sample was combined with 2 µl of the pre-stained ladder. The protein gel box was prepared with a Novel 1-mm 15 well 4-20% Tris-Glycine gel and 1x Tris Glycine SDS buffer and ran for 1hour 25 min at 125 V.

Gels were further either stained with Coomassie, imaged using GelDoc™EZ (Bio-Rad) digitalized and archived, or the unstained gels were directly used for western blotting.

2.14. Western Blot

The analytical technique was invented in 1979 by Robert Nowinski and, simultaneously, by George R. Stark. Western blots are used to visualize proteins on a blot membrane that have been separated by gel electrophoresis. In this study NuPage 4-12% Bis-Tris Protein gels were used. The sample containing proteins were collected and treated according to the specific need.

The prepared samples were run on an SDS Page gel according to the description "SDS Page gel". The transfer from the protein on the gel to the PVDF membrane happened using an iBlot2 system. After transferring, the membrane was blocked for 1 hour at room temperature or overnight at 4°C using TBS-T and milk powder. If phosphorylated proteins had to be visualized, sterile filtered TBS-T with 5% BSA was used.

After blocking, the membrane was incubated for two hours at room temperature with the appropriate dilutions of primary antibody in the blocking buffer. The membrane was then washed three times with TBS-T. As a next step, the membrane was incubated for one hour at room temperature with the recommended dilution of horseradish peroxidase conjugated with secondary antibodies in the blocking buffer. After three additional rounds of washing with TBS-T, the membrane was washed in 1:1 stable peroxide solution and luminol/enhancer solution from SuperSignal West Dura

Extended Duration Substrate. The blot was visualized using the machine GelDoc™XRS+ (Bio-Rad). For a numeric comparison of the band intensities, the program ImageJ was used.

2.15. Protein quantification

To determine the protein concentration, the BCA Pierce Assay was used according to the provided protocol and the absorbance was measured at 560 nm.

2.16. TF-1 proliferation assay

The Erythroleukemic cell line TF-1, derived from human blood cells, was cultured in a RPMI 1640 medium (2 ng/ml recombinant human GM-CSF and 10% fetal bovine serum). To determine the stimulation of TF-1 cell proliferation by screened proteins, the culture medium was exchanged with a RPMI 1640 medium containing 10% FBS but lacking GM-CSF. The assay was performed in a 96 well microtiter plate, incubating about $1,5*10^4$ cells per well. Cells were seeded about 16 hours before the proteins were added in serial dilution, decreasing from highest to lowest concentration (10^{-7} to 10^{-11} M). Each condition was performed in triplets and a representation of a 96 well plate experiment is presented in Figure 15. The plate was kept in the incubator with standard conditions (5% CO_2, 95% air at 37°C) for 72 hours. 20 ul/well of MTS reagent (CellTiter 96® AQ_{ueous} One Solution Reagent containing a tetrazolium compound Promega) was added and after an additional incubation period of 4 hours, the absorbance was measured at 490 nm using a Synergy Neo2 Hybrid Multi-mode Reader. The results were analyzed and the data was plotted using Prism GraphPad with fitted nonlinear regression.

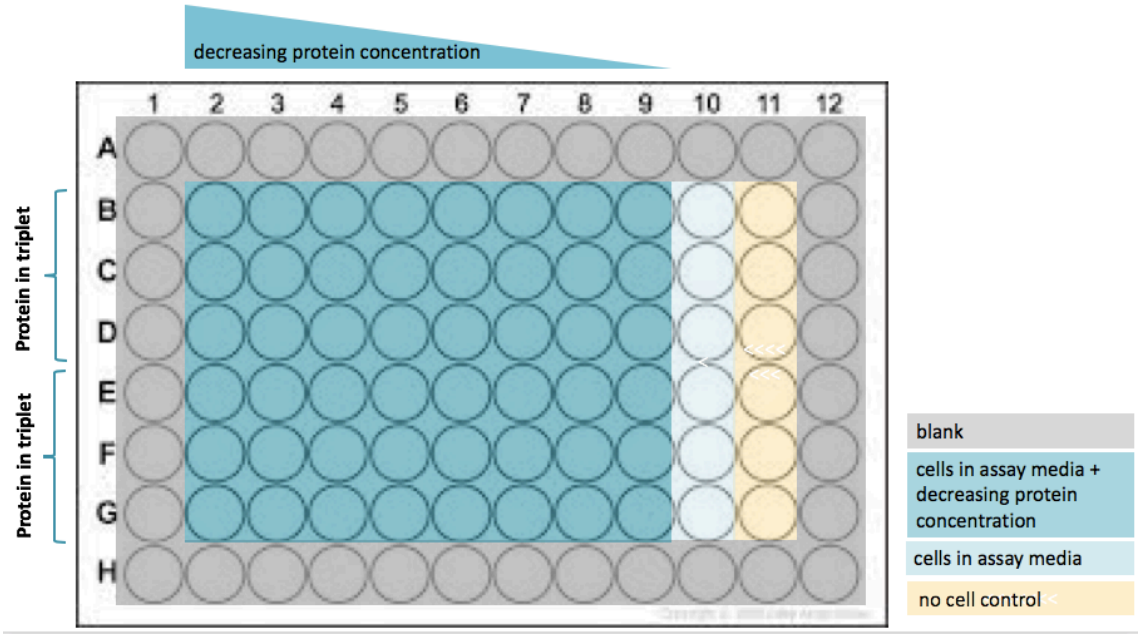

Figure 15 Layout of the 96 well plate for the TF-1 proliferation assay. Each protein concentration has been tested in triplet.

2.17. Kinetic analysis of protein-protein interaction

Bio-layer Interferometry (BLI) is used to measure molecular interactions in real time. The optical technique enables label-free experiments, used to determine kinetic and affinity of molecular interactions. The highly sensitive optic sensor has two interfaces separated by a thin layer at the tip (called optical layer). White light gets reflected from each of the two layers, the respective beams interfere constructively and destructively at different wavelengths in the spectrum. (Renee Tobias and Sriram Kumaraswamy ForteBio "Biomolecular Binding Kinetics Assay on the Octet Platform"). In this study, Octet RED384 and BLItz Systems from ForteBio were used for measuring macromolecular interactions. The sensors in both machines have to be pre-soaked at least for 10 minutes in a buffer solution (PBS +0.02 Tween20 and 0.1% BSA). For this study, biosensors of the type Protein A and Streptavidin have been used. Protein A biosensors do bind with high affinity to the Fc region of human IgGs. EpoR has been fused to an Fc region. However, other proteins such as EphB4 had to be biotin-tagged in order to be immobilized to the streptavidin-coated biosensor.

2.17.1.Octet RED384 System (ForteBio)

Immobilized protein: EpoR-Fc Analyte: Epogen, Epo_{R143E}, and Epo_{R150A} proteins per sample used in five concentration stages (10x, 5x, 1x, 0.5x, 0.1x) using a 384 well plate. See Table 3 below for further details. Sensor used: Protein A.

Table 3 Settings for Biolayer Interferometry experiments on Octet (ForteBio).

Octet	Step type	Duration (s)	Flow (RPM)	Buffer/Protein
Step 1	Baseline	60	1000	Buffer
Step 2	Loading	120	1000	Immobilized Binding partner
Step 3	Baseline	60	1000	Buffer
Step 4	Association	300	1000	Analyte
Step 5	Dissociation	600	1000	Buffer

2.17.2.BLItz (ForteBio)

Protein immobilized: EpoR-Fc, EphB4, Epo wt EphrinB2. Analyte: Epo wt, EphrinB2, EphB4. Sensors used: Streptavidin Sensors and Protein A sensors. Settings of the experiment can be found in the table below.

Table 4 Settings of Biolayer Interferometry experiments on BLItz (ForteBio).

BLItz	Step type	Duration (s)	Buffer/Protein	Volume (ul)
Step 1	Baseline	30	Buffer	250
Step 2	Loading	240	Immobilized Binding partner	4

Step 3	Baseline	30	Buffer	250
Step 4	Association	240	Analyte	4
Step 5	Dissociation	240	Buffer	250

2.17.3. Biotinylating of proteins using EZ-link NHS-PEG4-Biotin

The Protein sample was dissolved, or buffer was exchanged in the respective ratio: buffer 0.1 M phosphate, 0.15 M sodium chloride, pH 7.2 + 0.02% Tween20. 170 µl ddH2o were added to 2 mg of NHS-PEG4-Biotin to prepare a 20 mM of stock solution.
50 µl of 20 mM NHS-PEG4-Biotin stock solution was diluted in 950 µl of ddH2O to prepare a 1 mM working solution. The appropriate volume of NHS-PEG4-Biotin stock solution was calculated and added to the protein sample. After an incubation period of 30 minutes the unbound NHS-PEG4–Biotin was removed via buffer exchange.

2.18. Flow Cytometry based binding assay

To detect Epo binding to EpoR and EphB4 a flow cytometry analysis of Cos-1 and TF-1 cells has been done.
Cells have been cultured and seeded in a V-bottom 96-well plate at 40000 cells/well. The cells have been pelleted by centrifugation and the supernatant has been gently removed. The cells have been resuspended in PB. Epo protein dilutions from 10^{11}-10^{7} have been added to the cells and incubated on Ice for an hour. The cells have further been spun down at 300 g for 5 min and washed two times with 200ul of FACS buffer. The 4 used antibodies can be found in Material list. 2 µl of each antibody has been added with 98µl of FACS

buffer/well. After half an hour of incubation the cells have been washed again twice with FACS buffer (300g for 5 min) and analyzed via Flow Cytometry.

2.19. Materials

List of Enzymes, ladders and proteins used in this study	
PRODUCT	VENDOR
1Kb plus DNA ladder	*Invitrogen*
293fectin Transfection reagent	*Invitrogen*
2xMM Gibson Assembly	*New England BioLabs*
FastDigest DpnI	*Thermo Fischer Scientific*
FD buffer (10x)	*New England BioLabs*
Phusion 2x Master Mix	*Invtrogen*
293 Transfection Reagent	*Thermo Fisher Scientific*
Dimethyl sulfoxide (DMSO)	*Sigma-Aldrich*
EZ-Link TM NHS-PEG4-Biotin	*Thermo Fisher Scientific*
Epogen (epoietin alpha)	*Amgen*
EphB4	*RnD Systems (#P54760)*
EphrinB2	*Sino Biologicals (#10881- HCCH-100)*

List of Buffers, Solution and Kits used in this study	
BUFFER	INGREDIENTS or VENDOR
Biolayer Interferometry Buffer	PBS+0.02% Tween20, 0.1%BSA, 0.05% sodium azide
HisTALON Equilibration Buffer	Takarabio
HisTALON Elution Buffer	Takarabio
Tris glycine running buffer	Tris 50 mM, Glycine 190 mM, SDS 1 g/L
Coomassie staining solution	Acetic acid 10 % (v/v), methanol 40% (v/v), Coomassie brilliant blue R250 0.25 % (w/v)
Glycerol	Glycerol 50%
RIPA Lysis and Extraction Buffer	Thermo Fisher Scientific
CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS)	Promega
Pierce BCA Protein Assay Kit	Thermo Fisher Scientific
Miniprep Kit	Qiagen

List of Biosensors for Biolayer Interferometry, Gels, purification Resins and competent cells	
Streptavidin biosensor	Pall corporation (#18-5019)
Protein A biosensor	Pall corporation (#18-5010)
NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.0 mm, 15-well	Invitrogen
TALON Metal Affinity Resin charged with cobalt by Takarabio	Takarabio
NEB 5-alpha competent *E.coli*	New England BioLabs

List of all antibodies used in this study	
ANTIBODY	VENDOR (KATALOG #)
EphB4	Invitrogen (37-1800) Abcam (ab66336) and Cell Signaling (14960)
EphrinB2	Abcam (ab96264)
EpoR	Santa Cruz (sc-697)
Stat3	Cell Signaling Technology (4904)
pStat3	Cell Signaling Technology (9145)
Stat5	Cell Signaling Technology (9363)
pStat5	Cell Signaling Technology (4322)
Src	Cell Signaling Technology (2108 and 2110)
pSrc	Cell Signaling Technology (2105)
ß-actin	Cell Signaling Technology (4970)
6x His-tag Antibody HRP	Abcam (ab232492)

3. Results

3.1. EpoR: Screening for a mutation in Epo, with weaker affinity to $EpoR_2$ than the Epo mutation Arginine 150 to Alanine

In order to find a mutation in Epo that weakens the affinity for EpoR-EpoR dimer even further than Epo_{R150A}, a library of single amino acid mutations within Epo was established.

Based on well-known binding interactions between Epo and EpoR, structural modelling using PyMol and a literature research, the following amino acid residues were included in the library: T44, K45, R143 and L155 (Table 5). All surface residues included in the library were chosen on the high affinity site of Epo to EpoR, as indicated in Figure 16

In order to pick the most effective mutations for each amino acid without disrupting proper protein folding, the published data from Elliot et al. (1997) on how mutations affect RBC proliferative activity and folding of Epo have been taken into consideration (Elliott *et al.*, 1997). Additionally, to recreate the potential changes in structure and binding, surface residues have been visualized using PyMol.

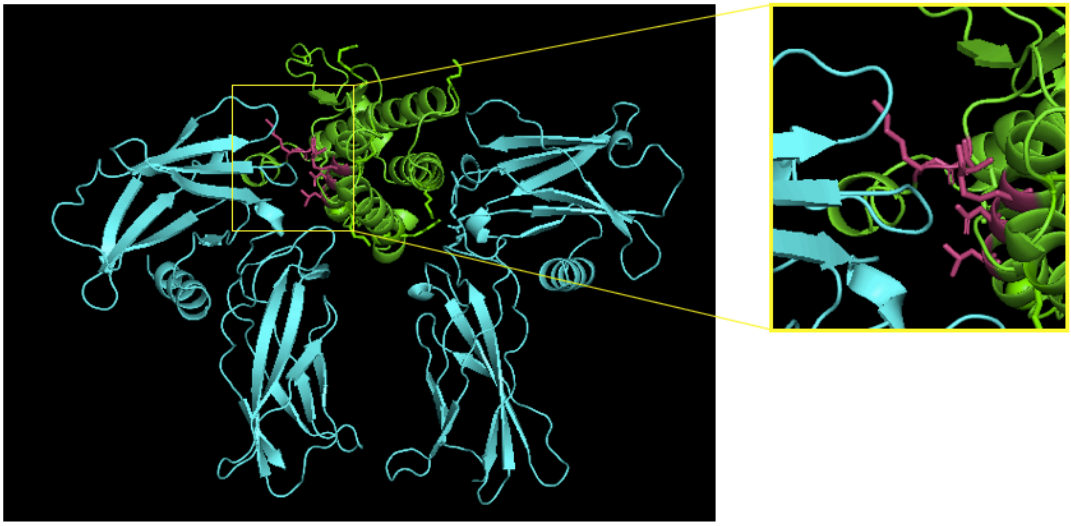

Figure 16 The four Epo surface residues of the library (T44, K45, R143 and L155) are depicted in red, are within the high affinity binding site of Epo (green) to EpoR (blue).

Table 5 Library of Epo residues and mutated DNA codons to reduce affinity for EpoR.

Epo Residues	Mutation to
T44	Isoleucine
K45	Aspargine
R143	Glutamic acid
L155	Alanine

Next, the established library was cloned into the mammalian expression vector, and the expressed protein was purified (detailed description can be found in "Purification using Cobalt Affinity Resin" in section Methods). To measure the *in vitro* activity of the purified mutated proteins, the erythroleukemic cell line TF-1 was treated with Epo wt and Epo mutants. After 72 hours, the proliferation was measured (for a detailed description see chapter "TF-1 proliferation assay" in Methods). The bioactivity of each Epo mutant was compared to Epo_{R150A} and the wildtype Epo, Epogen. The cell proliferation data was plotted against the protein concentration and fitted using nonlinear regression. The results of the cell proliferation assay are illustrated in Figure 17.

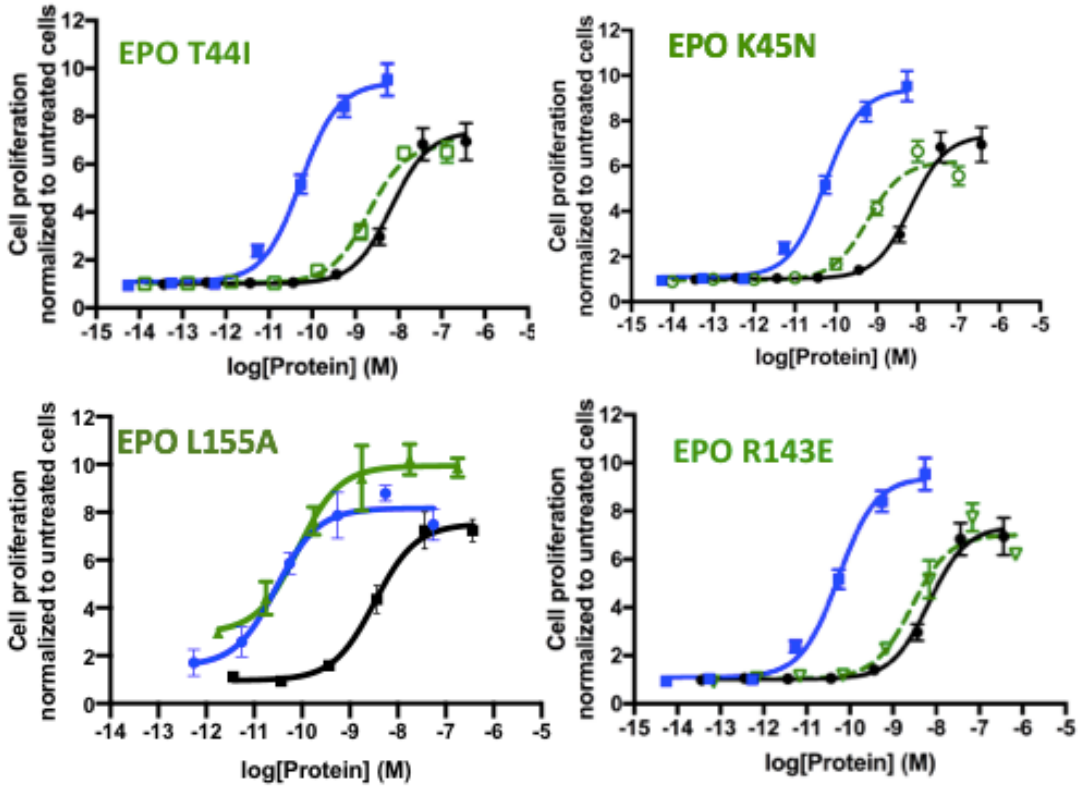

Figure 17 In vitro cell proliferation assay testing the bioactivity of the Epo mutants. The graph shows the relation of Epo wt (blue), Epo_{R150A} (black) and Epo mutants Epo_{T44I}, Epo_{K45N}, Epo_{L155A}, Epo_{R143E} (green).

To further verify if the mutation is a potential candidate, the Log (EC50) of each new mutation and of Epo_{R150A} was calculated from the nonlinear regression fitted plots. Normalizing the Log (EC50) of the screened mutations to the Log (EC50) of Epo_{R150A} presented Epo_{R143E} as the most promising candidate (Table 6). The second most promising mutation is K45N, while T44I and L155A have an even higher Log (EC50) and therefore are less effective in weakening the bioactivity compared to R150A.

Table 6 Comparison of Log (EC50) from mutations of the screening library to Log (EC50) of established mutation EPO_{R150A}. Results of normalization: if value <1, mutation is stronger in terms of binding affinity to EpoR than Epo_{R150A}. If values >1, mutation is weaker than Epo_{R150A}, and if value =1, mutation is as effective as Epo_{R150A}. Log (EC50) values obtained from proliferation data fitted using nonlinear regression by GraphPad Prism.

Mutation	Log (EC50) from TF-1 proliferation	Log (EC50) Epo_{R150A} from the same assay	Log (EC50) normalized to Epo_{R150A}
T44I	-9.227	-8.166	1.130
K45N	-8.698	-8.166	1.065
R143E	-8.566	-8.166	1.049
L155A	-9.799	-8.319	1.177

To inhibit non-target cell receptor binding, the binding affinity to EpoR needs to be reduced tremendously. However, in order to have a functional therapeutic protein, the binding to EpoR on target cells (erythroid cells) has to be preserved. This can be achieved by targeting the fusion protein through an antibody fragment to glycophorin A, which is highly expressed and unique to the target cells.

Therefore, fusion protein IH4v1-5aa-Epo_{R143E} has been cloned to verify if the reduced activity of Epo to its canonical receptor can be preserved by the antibody element IH4v1 connected with a 5-aa linker. The purified fusion protein has been tested in the cell proliferation assay. As depicted in graph Figure 19, the fusion protein showed *in vitro* activity relatively similar to EPO wt.

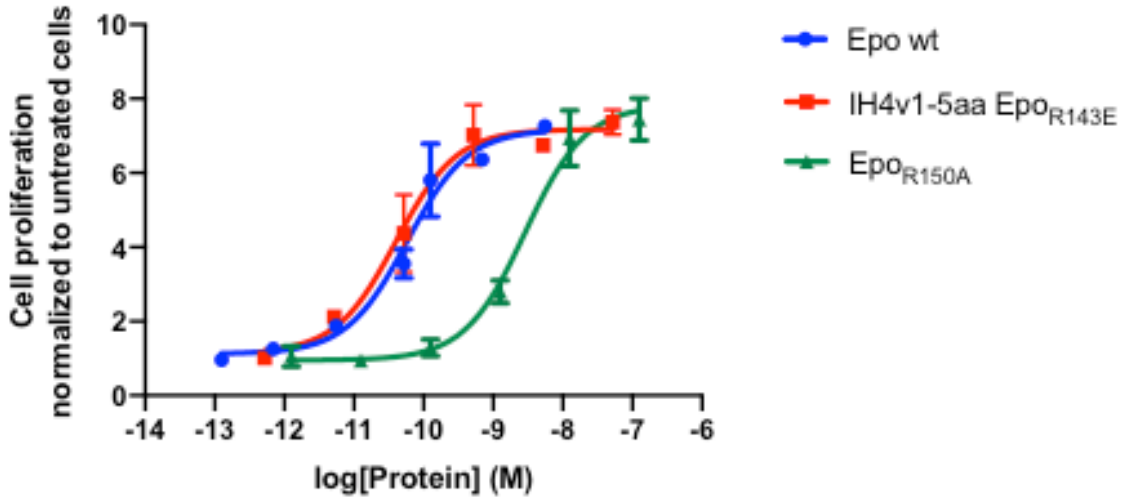

Figure 18 In vitro cell proliferation using TF-1 cells. Comparing the fusion protein (IH4v1-5aa-Epo$_{R143E)}$ with Epo wt and Epo$_{R150A}$.

The weakened receptor affinity and resultant lowered *in vitro* activity of the Epo$_{R143E}$ mutant was rescued by combining it with IH4v1, to be as strong as the *in vitro* activity of Epo wt.

The in vitro kinetic analysis of the most promising Epo mutation candidate

To quantify the difference between the *in vitro* interaction of the Epo mutations R150A, R143E and Epo wild-type to EpoR, a kinetic analysis using Biolayer Interferometry was performed (Figure 19). For this analysis the Fc fused EpoR was immobilized to a Protein A Biosensor and, subsequently, different concentrations of each analyte (Epo) were measured by using the Octet System (ForteBio). Further details can be found in chapter Octet "Methods".

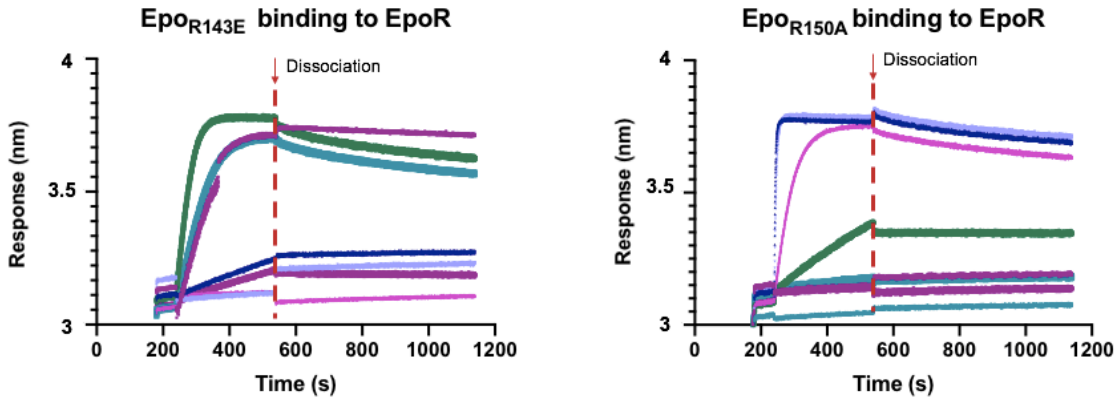

Figure 19 Biolayer Interferometry analysis of A) Epo$_{R143E}$ and B) Epo$_{R150A}$ binding to EpoR. Association and Dissociation Kinetics using Octet System (ForteBio).

While the off-rates of Epo wt and the Epo mutants are similar ($1.47*10^{-4}$ s^{-1}, $4.4*10^{-4}$ (R150A) and $3.3*10^{-4}$ (R143E)), the association rates of Epo wt, Epo$_{R150A}$ and Epo$_{R143E}$ to EpoR resulted in $2.84*10^{5}$ $M^{-1}s^{-1}$, $2.65*10^{4}$ $M^{-1}s^{-1}$ and $2.03*10^{5}$ $M^{-1}s^{-1}$, respectively. The affinity of Epo wt to EpoR and Epo$_{R150A}$ to EpoR differs by 32-fold, while the affinity for Epo wt to EpoR and Epo$_{R143E}$ differs by factor of 3.16. A summary is depicted in Table 7.

Table 7 In vitro kinetic analysis using Octet systems. Results of interaction between EpoR and Epo wt, Epo$_{R150A}$, Epo$_{R143E}$

Protein	K_D(M)	Ka(1/Ms)	Kdis (1/s)
Epo $_{WT}$	$5.18*10^{-10}$	$2.84*10^{5}$	$1.47*10^{-4}$
EPO $_{R150A}$	$1.66*10^{-8}$	$2.65*10^{4}$	$4.4*10^{-4}$
EPO $_{R143E}$	$1.63*10^{-9}$	$2.03*10^{5}$	$3.3*10^{-4}$

A structural model of the mutated amino acid Arginine 143 to Glutamic acid using PyMol (PDB: 1EER) is depicted in Figure 20.

Original Epo Surface Residue:
Arginine 143

Mutation in Epo:
Glutamic Acid 143

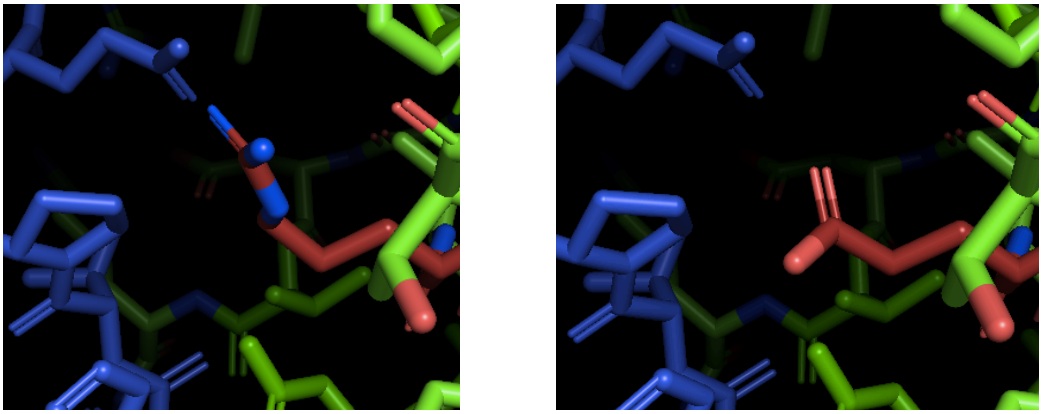

Figure 20 Structural model of Epo mutation using PyMol (PDB: 1EER). Epo (green) binding on EpoR (blue). The *surface* residue of Epo 143 is depicted on the left side in its original state and mutated to decrease the binding affinity on the right side.

3.2. EphB4: Engineering Epo to prevent EphB4 signaling in cancer patients

The objective of this study is to design and create an Epo treatment for renal and cancer patients suffering from anemia without the well-known thrombotic or tumor angiogenic side effects based on the evidence that Epo stimulates tumor growth via EphB4.

3.2.1. Identifying the potential binding site of Epo to EphB4

To identify the potential binding sites of Epo to EphB4, a library of potential residues involved in the binding was established. The following paragraph discusses various aspects considered in order to identify these potential Epo residues.

Pradeep et al., 2015, indicated that the binding pocket of Epo to EphB4 is near the ephrinB2-EphB4 ligand binding pocket. The receptor binding sites of Epo are highly conserved. It was hypothesized that the highly conserved

regions in Epo that are not involved in EpoR interaction might be involved in EphB4 interaction. With these regions of Epo as

possible binding sites, an alignment of Epo from 10 mammals with human Epo has been generated using BLAST (Way *et al.*, 2005). Moreover, a PhD candidate has established a Rosetta protein-protein docking to

model Epo-EphB4 complex (data not shown). However, since this precise knowledge relies on a limited set of published data, a less biased global search for potential binding sites has been carried out.

This global search aims for an unbiased way of detecting the positions within Epo that enable binding to EphB4. General steric rules, glycosylation sites, hydrophobic packing, and electrostatic interactions have been taken into account. Based on the crystal structure of Epo (PDB: 1EER), a global screen for Epo residues that are facing outward to mediate interaction with other proteins and not interfering with the glycosylation sites has been conducted to identify potential amino acid residues.

The global screening for surface amino acid residues led to a library of twenty Epo amino acids (Figure 21) potentially being involved in interacting with EphB4, all outer residues considered. Mutations hindering the interaction between Epo and EphB4 have been identified for each of the twenty amino acids.

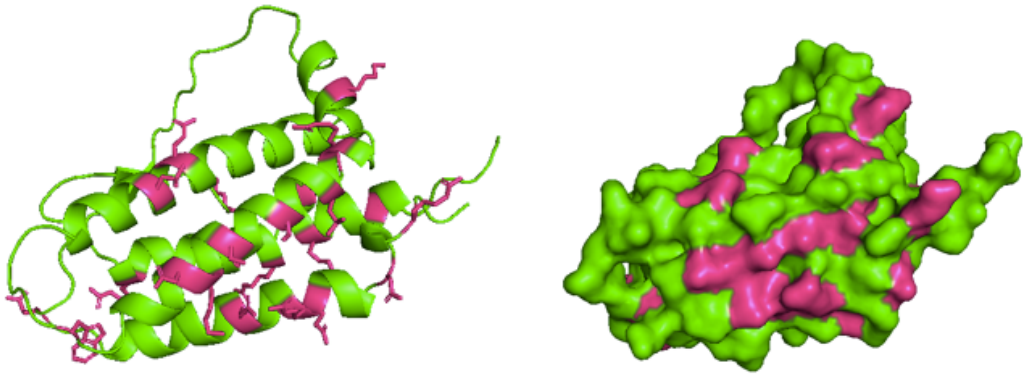

Figure 21 Epo structure. Amino acids from library marked in red displayed in cartoon (left) and surface (right) using PyMol (PDB: 1EER).

This library was established and would further be screened for binding interactions (the table can be found in supplemental S.8.). The screening of mutated residues requires Biolayer Interferometry and Flow Cytometry to identify the amino acids involved in Epo-EphB4 binding. The mutations reducing or, in the best case, abolish this binding affinity could subsequently be tested in the TF-1 proliferation assay. This is an effective way to test the erythropoietic potential of Epo and its mutations. Finally, the erythropoietic activity of Epo would be maintained by establishing a fusion protein, which would anchor the mutated Epo to a target cell, enabling the interaction of Epo to EpoR solely on targeted cells.

3.2.2. Kinetic Binding Assay using Biolayer Interferometry

The data of Epo to EphB4 binding (Pradeep et al., 2015) has to be reproduced before the screening for the interaction of mutated Epos (from the library Table 5) and EphB4. Reproduction of the kinetic assay was done via Biolayer Interferometry using the Blitz System (ForteBio). The interactions of Epo to EpoR and EphrinB2 to EphB4 were used as positive controls. Epo binding to EphB4 was tested using the same method.

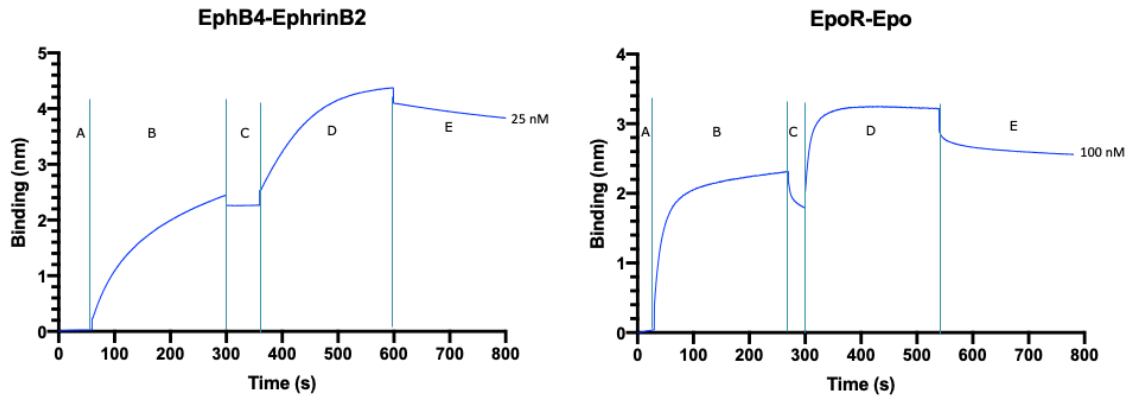

Figure 22 Binding assays of the two positive controls (EphrinB2 binding to EphB4 and Epo binding to EpoR) using BLItz sytem (ForteBio). EphB4 immobilized on Protein A Biosensor with analyte recombinant human EphrinB2. Fc-EpoR immobilized on Streptavidin Biosensor with analyte recombinant human Epo. Segmentation: A) initial buffer background, B) association

of EphB4 or EpoR to the sensor tip, C) Washing step D) Association (binding of Analyte (EphrinB2 or Epo)) E) Dissociation.

In both positive controls (Epo to EpoR and EphrinB2 to EphB4), the association and dissociation of the ligand

receptor pair was successfully detected. However, association could not be reproduced successfully for Epo-EphB4. Although, loading EphB4 to the sensor was successful, no association with EphrinB2 was observed. Since Epo binding to EpoR could be reproduced as well as EphrinB2 binding to EphB4, but Epo binding to EphB4 could not be reproduced.

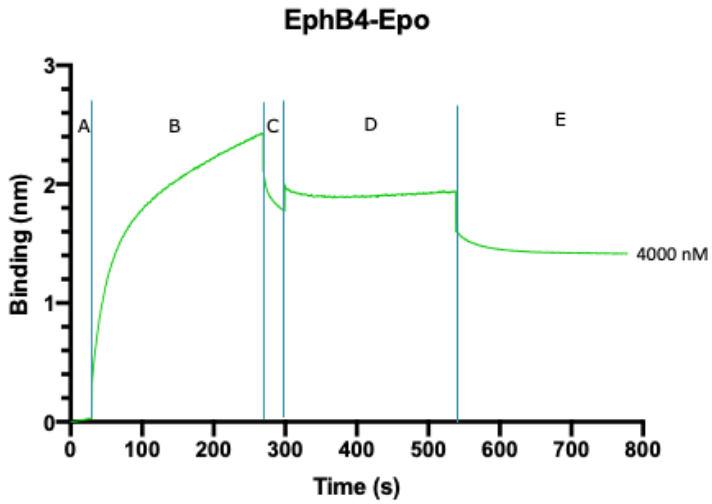

Figure 23 Binding assays of Epo to EphB4 using BLItz sytem (ForteBio). Set up remained the same as in Figure 18. A) initial buffer background, B) association of EphB4 or EpoR to the sensor tip, C) Washing step D) Association (binding of Analyte (EphrinB2 or Epo)) E) Dissociation.

The core facility manager of the Harvard Medical School was then consulted. The manager suggested to test higher concentrations. The concentration of EphB4 as analyte in later tests ranged from 0.1x Kd (400 nM) to 10x Kd (40000 nM). Additionally, biotinylated EphB4 was tested as the immobilized protein, with Epo as analyte. The known binding interactions of Epo to EpoR and EphrinB2 to EphB4 could be reproduced. Regardless of the efforts, the binding of Epo to EphB4 could not be reproduced.

3.2.3. Cell line with EphB4 expression

Another line of experiments investigated the expression level of EphB4 receptors in cell lines. The expression level of EphB4 and EpoR plays an important role in reproducing the signaling assay presented in Pradeep et al., 2015, suggesting that Epo triggers downstream signaling of EphB4 via STAT3 (Pradeep *et al.*, 2015). Therefore, the expression of EphB4 and EpoR in three human cancer cell lines, MCF-7, HeLa, and TF-1, and the non-human primate cell line, Cos-1, have been analyzed. The cell lines were cultured according to standard culture conditions, lysed and analyzed via Western Blot. ß-actin functioned as loading control in order to interpret the data provided by the Western Blot, and to determine the protein expression accurately (Figure 24)
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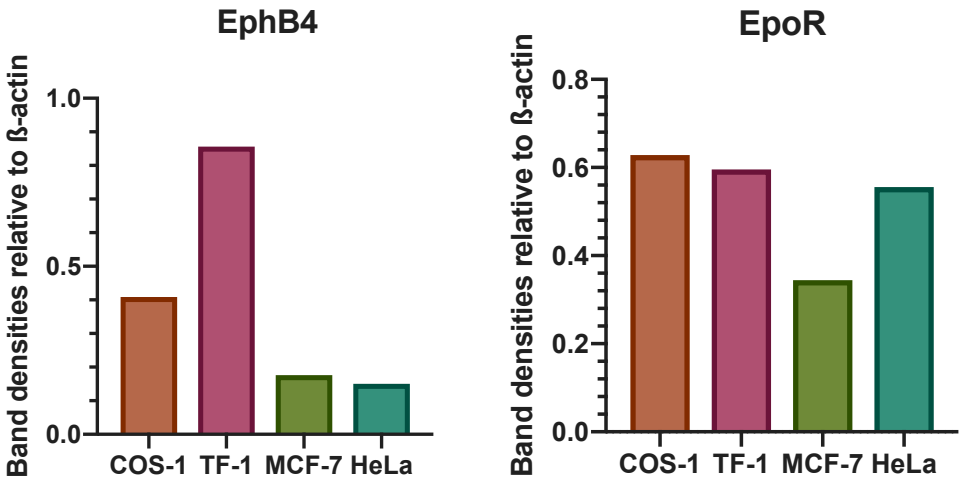

Figure 24 Band densities of Western Blot relative to ß-actin. Four different cell lines have been screened for expression levels of EphB4 and EpoR.

While EphB4 expression showed a strong band in TF-1 cells, only light bands could be detected in MCF7 and HeLa cells. Normalizing the band intensity of EphB4 to ß-Actin using ImageJ verified the highest EphB4 expression in TF-1 and Cos-1 cells and low expression in MCF-7 and HeLa cells.

The expression of EpoR normalized to ß-actin showed that TF-1, Cos-1 and HeLa cells have similar expression levels, while MCF-7 has the least. However, both receptors are expressed in all four cell lines.

3.2.4. Cell Binding Assay using Flow Cytometry

In order to evaluate Epo wt binding to EphB4 via another assay, a flow cytometry based binding assay was done. The aim was to test the interaction of Epo with the surface receptors EpoR and EphB4.

Based on the results from the Western Blot, both TF-1 and Cos-1 cells express EphB4 and EpoR while Cos-1 cells have a lower EphB4 expression level compared to EpoR. For the flow cytometry assay TF-1 and Cos-1 cells were used to test for the binding of wt Epo. Cells were treated with Epo proteins containing C-terminal His6 tag and the bound Epo was detected using anti-His6-PE antibody. For background subtraction, the binding was compared to cells+PBS+antiHis6-PE antibody. Epo binding could be detected in untransfected Cos-1 and TF-1 cells. The results can be seen in Figure 25 .

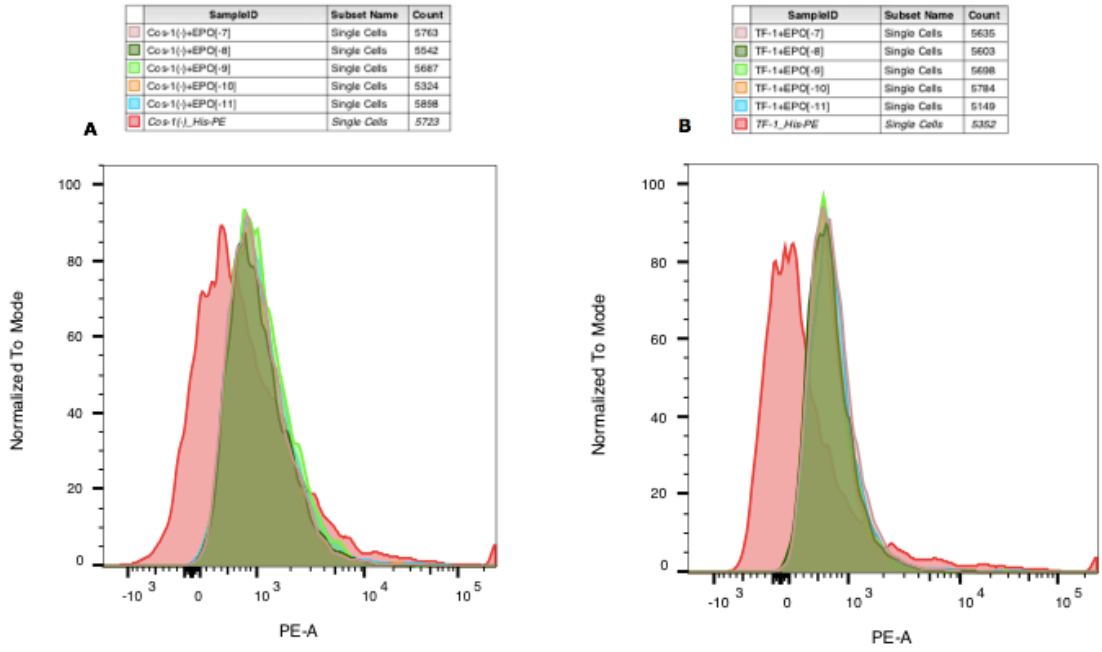

Figure 25 Cell binding assay using Flow cytometry. The Epo concentration varied from 10^{-11} to 10^{-7} and the bound Epo was detected using anti-His6-PE antibody. A) Analysing Cos-1 cella and B) TF-1 cells.

3.2.5. Epo induced Signaling Assay

The human ovarian carcinoma cell line, A2780, used as cell line for the signaling assay in the paper Pradeep et al., 2015, expresses both receptors, EpoR and EphB4.

Pradeep et al., 2015 reported rhEpo mediated activation of Jak2/Stat5 signaling in A2780-shEphB4 cells, but not in A2780-shEpoR cells. The Jak2/Stat5 pathway gets activated via signaling through EpoR. According to this paper, Epo binding to EphB4 leads to the activation of the Src/Stat3 signaling pathway which enhances tumor growth. (A scheme of this proposed signaling pathway can be viewed in Figure8). Elevated pStat3 levels have been reported after treatment with rhEpo for subsequently 0, 5 and 15 minutes using A2780-shEpoR cells. However, faint bands could be observed in A2780 cells and A2780-shEphB4.

In an attempt to investigate the signaling pathway, TF-1 cells were treated with rhEPO and EphrinB2 for 5, 10 and 15 minutes. The amount of protein in the lysates was measured. The same amount (about 50 ng) of total protein was loaded on an SDS-PAGE gel, and pStat3, pStat5 and Stat5 levels were measured by Western Blot. As can be viewed in Figure 26 the treatment of TF-1 cells led to phosphorylation of pStat5 solely upon stimulation with rhEpo. However, the pStat3 levels are similar upon stimulation of rhEpo or EphrinB2.

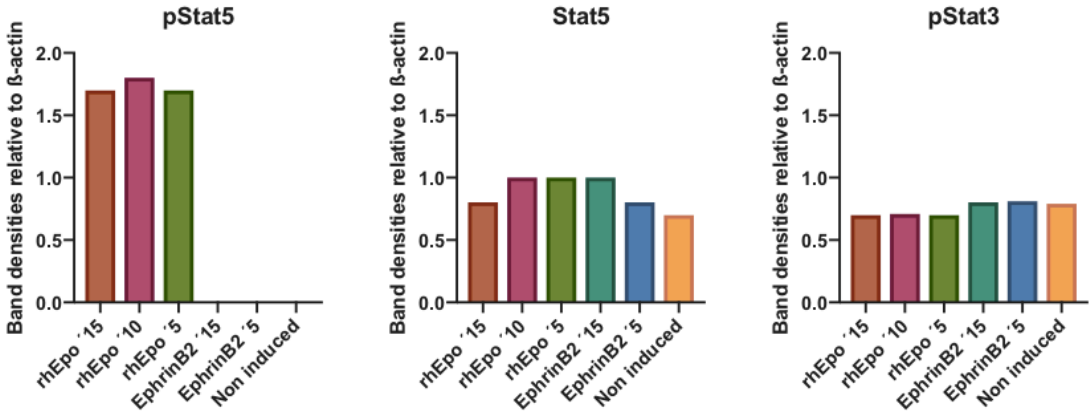

Figure 26 Epo and EphrinB2 induced signaling assay using TF-1 cells displaying levels of pStat5, Stat5 and pStat3 normalized to ß-actin levels. X-axis indicating used proteins for stimulation and time frame of stimulation.

To reproduce the experiment successfully, the cell line A2780 and the antibodies have been ordered from the same vendor as used in the paper mentioned above. The normalized blots can be viewed in Figure 27.

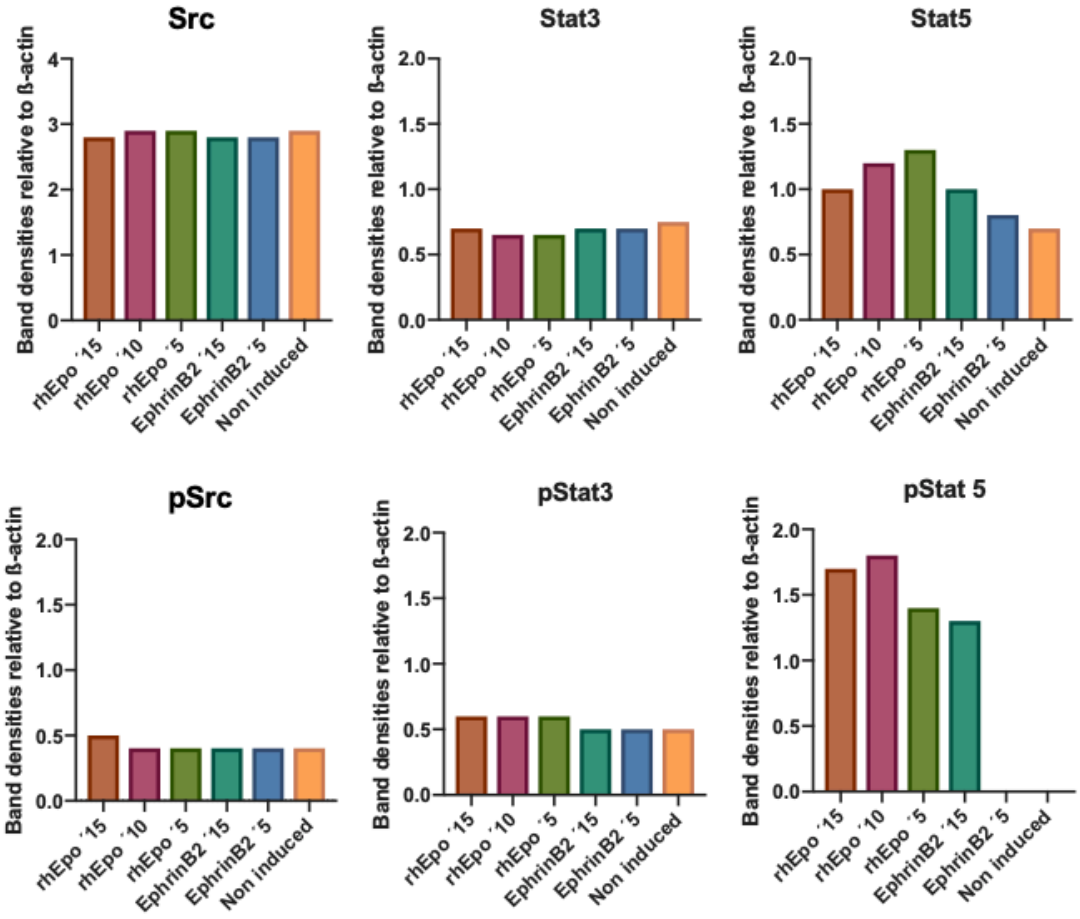

Figure 27 Western Blot analysis result of signaling proteins involved in Epo-EpoR and EphrinB2-EphB4 signaling in A2780 cells. Band intensity of signaling proteins are normalized to ß-actin. X-axis indicates stimulation of either rhEpo or EprinB2 for indicated time (0, 5, 10 or 15 min).

Exogenous Epo and EphrinB2 treatment failed to stimulate downstream signaling in A2780 cells for all proteins except for pStat5. However, in the case of pStat5 the treatment of rhEpo led to elevated expression in A2780 cells and in TF1 cells. However, in A2780 cells, upregulated pStat5 could be detected after 15 min stimulation with EphrinB2. The results for pStat3 levels are very similar between TF-1 cells and A2780 cells.

A summary of the Western Blots can be found in the appendix S.8.5.

3.3. CD131: Engineering Epo as a potential candidate for treating High-Altitude Illness

This thesis' study aims at presenting a reasonable design of an EPO fusion protein drug, with neuroprotective and erythropoietic characteristics while at the same time reducing its thrombotic side effects (Figure 28).

The engineered Epo derivate can be a potential candidate for treating/preventing the hypoxia induced high altitude illness. Especially as a prevention for its severe form high-altitude cerebral edema (HACE) which can be fatal due to brain herniation (Hackett and Roach, 2001).

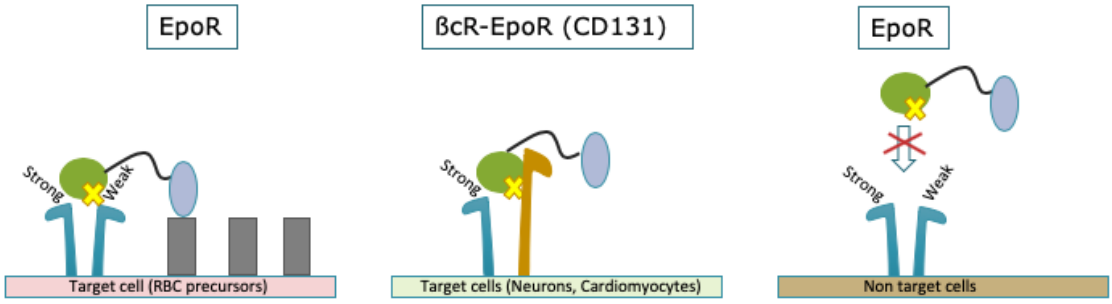

Figure 28 Cartoon displaying the design of the engineered Epo version. The mutation on the weak affinity binding site of Epo (green) reduces the affinity to EpoR drastically. This leads to disabled binding and signaling activation via EpoR homodimers. The binding of a targeting element to a target cell-specific marker (grey) on RBC precursors increases the local concentration of Epo, helps overcome the receptor binding deficit, and enables signal activation. However, the mutation does not interfere with the interaction with CD131 and therefore enables neuroprotective signaling on target cells such as neurons.

Various studies showed that mutations within the erythropoietic motif lead to nonerythropoietic Epo while maintaining its neuroprotective features.
Gan et al., (2012, Stroke), validated these findings with the mutation Epo_{S104I} which lacks erythropoietic activity. However, the mutant provided protection of neurons *in vitro,* challenged by a neurotoxic substance. Additionally, neuroprotection by Epo_{S104I} was shown in a mouse model of focal ischemia. The administration led further to decreased infarct volume similar to the efficacy of Epo wt (Gan et al., 2012).

3.3.1. Screening for nonerythropoietic Epo mutations still enabling binding to ßcR-EpoR heterodimer

To find a mutation presenting a strongly reduced binding activity to EpoR-EpoR homodimer and therefore reduced erythropoietic activity, Epo was screened for mutations at its low affinity receptor binding site. The surface residues indicated in the surface representation in chapter "Introduction" Figure 5, are especially interesting for this approach (Val 11, Arg14, Tyr15, Ser100, Arg 103, Ser 104 and Leu108). The central region consisting of Ser 104 and Tyr 15 is hydrophilic and surrounded by Arg 103 and Arg 14 (Cheetham *et al.*, 1998). These residues are either within the amino terminal (aa 1-17) or the internal motif (aa 99-109). These residues were mutated based on data dealing with mutation studies published by Gan et al., (Stroke 2012) and protein folding by Elliott et al., (1997, Blood). The established library can be seen below including the codon mutation.

Epo Residues	Mutation to	DNA codons
R14	Glutamic acid	R: AGA E: GAA
R14	Aspargine	R: AGA N: GAA
Y15	Isoleucine	Y: TAC I: ATC
S100	Alanine	S: TCT A: GCC:
R103	Lysine	R: AGA K: AAA
R103	Glutamine	R: AGA Q: CAG

As a first step, the Epo mutations were generated by site-directed mutagenesis and were expressed using HEK293F cells.
In order to test if the mutations in the surface residues lead to inhibition of the erythropoietic activity, the purified protein was tested in an *in vitro* cell proliferation assay. The Epo mutations were tested along with positive controls, Epo wt and the mutation Epo_{R150A} as reference (Figure 29).

The erythroleukemic cell line TF-1 is dependent on the cytokine Epo in order to proliferate and survive. While Epo and Epo_{R150A} had the expected proliferative effect on TF-1 cells, the mutations (R14E, Y15I, S100A, R103Q

and R103K) lacked bioactivity, as anticipated. However, the bioactivity of the mutation Epo_{R14N} seems to be diminished but not completely disabled.

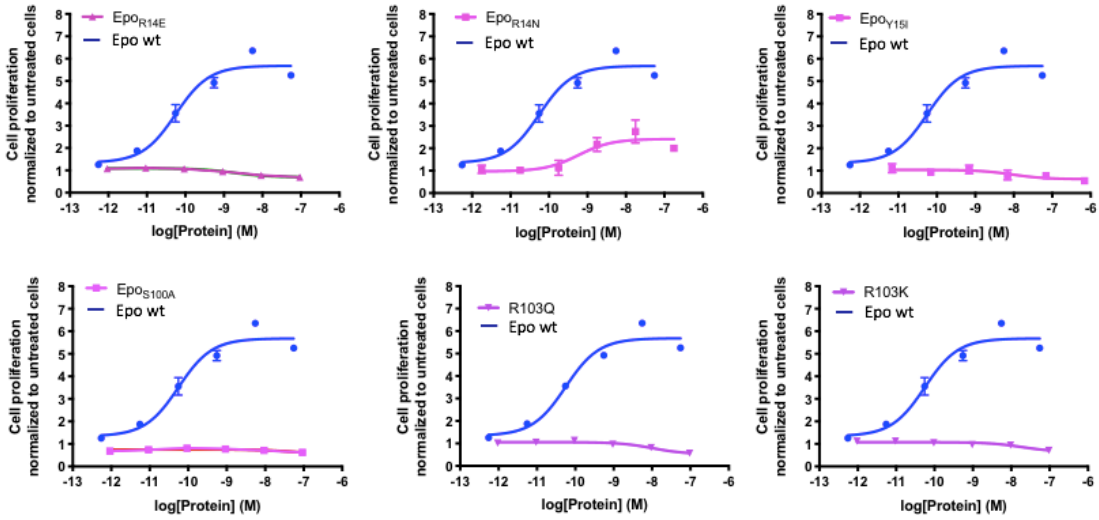

Figure 29 In vitro cell proliferation assay using TF-1cells to test erythropoietic activity of Epo mutations (violet) from the library compared to Epo wt (blue). Epo mutations starting from left top: Epo_{R14E}, Epo, Epo_{R14n}, Epo_{Y15I}, Epo_{S100A}, Epo_{R103Q} and Epo_{R103K}.

3.3.2. Restoring erythropoietic activity by targeting Epo to Red Blood Cell precursors

To prevent HACE, the engineered Epo should have erythropoietic activity aiming at RBCs. This might activate the positive effect of elevated RBC and therefore facilitates higher oxygen delivery to the tissue without thrombotic effects due to elevated platelet count, platelet activity and undesired signaling in vascular endothelial cells.

However, the generated Epo mutations do have a highly reduced ability to bind to the low affinity receptor binding-site which is important to activate erythropoietic signaling through EpoR homodimer. In order to restore binding to EpoR homodimer solely on RBC precursors, the mutation gets fused to the antibody IH4v1 with a linker (detailed information about the fusion protein in Introduction 1.5.2). The fusion proteins of the mutations Epo_{R103K}, Epo_{R103Q}, and Epo_{R14Q} were cloned and measured by TF-1 proliferation. The results are shown in the following diagrams below.

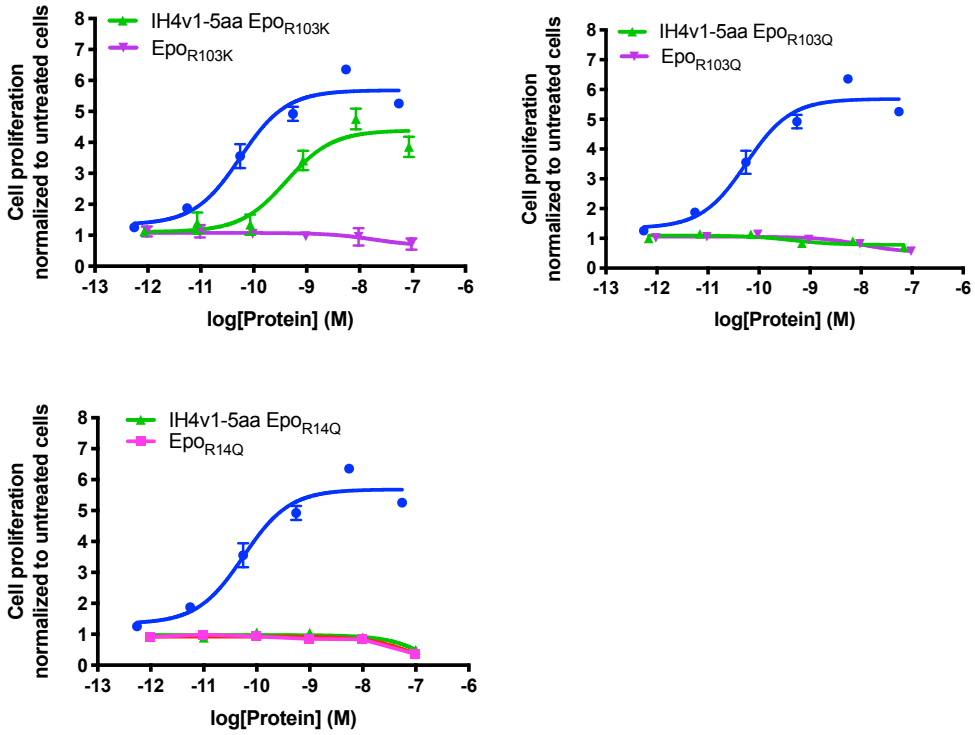

Figure 30 Results of TF-1 cell proliferation assay of unfused Epo mutations and Epo mutations, fused with a short linker and a targeting element (IH4v1) to glycophorin A on RBC precursors. Cell proliferation assay for each protein concentration was done in triplicate. Epo wt (blue), mutated Epo (violet), Fusion protein of same Epo mutation (green).

The result of the proliferation assay indicated that the fusion of IH4v1-5aa to the mutation Epo$_{R103K}$ could rescue the erythropoietic activity. The other mutations (Epo$_{R103Q}$ and Epo$_{R14Q}$) could not be rescued by fusion to IH4v1. Moreover, the Log (EC50) of Epo wt (-10.25) could be elevated to a Log (EC50) of -9.198 for IH4v1-5aa Epo$_{R103K}$.

To confirm the neuroprotective effects of IH4v1-5aa Epo$_{R103K}$, additional investigations, including *in vitro* and *in vivo* experiments need to be done. Unfortunately, conducting that line of experiments would have exceed the given time frame of my thesis. As this provides a promising research area, chapter 4.3. "Discussion and Conclusion" discussed and evaluates further approaches.

4. Discussion / Outlook

4.1. EpoR: Screening for a mutation in Epo, with weaker affinity to EpoR than the Epo mutation Arginine 150 to Alanine

The new lead molecule IH4v1-5aa-Epo$_{R150A}$ engineered by PhD candidate Jungmin Lee exhibits high targeting effect and therefore can accommodate a mutation in Epo, which further weakens the affinity to EpoR in comparison to the current mutation R150A. Further decreasing the binding affinity to EpoR on non-erythroid cells will widen the therapeutic window of Epo fusion protein.

Within the established library of five surface amino acids, which are on the strong binding site of Epo to EpoR, the mutation of Epo$_{R143E}$ can be found within the proliferation assay to weaken the bioactivity the most.
By targeting Epo$_{R143E}$ to red blood cell precursors via the antibody fragment IH4v1, the bioactivity can be preserved, compared to the bioactivity of Epo wt.

The results of the kinetic analysis confirm that the mutation in Epo of arginine 143 to glutamic acid weakened the interaction with EpoR. The *in vitro* binding affinity is reduced by factor 3.16 compared to wt Epo. However, by mutating the surface residue Arginine 150 to Alanine, the affinity compared to Epo wt is weakened by factor 32.

This leads to the conclusion that, out of the established library, the selected surface residues based on literature and structural analysis did not lead to a stronger reduction of receptor binding activity. However, it was detected that Epo$_{R143E}$ is a potential mutation candidate, reducing the binding affinity with EpoR compared to Epo wt by factor 3.16. Additionally, Epo$_{R143E}$ established

an *in vitro* biological activity compared to Epo wt after tethering it via a 5 amino acid long glycine-serine linker to the IH4v1 element.

The screening for a mutation in Epo to weaken its affinity for the EpoR did not lead to a mutation that is stronger than Epo_{R150A}. However, it is possible that changing the residues chosen for the screening library can weaken the binding affinity further by selecting another mutation. Possibly further experiments can be conducted by changing the selected mutations for the selected residues or different mutations within the strong affinity site of Epo to EpoR in order to increase the therapeutic window of Epo.

4.2. EphB4: Engineering Epo to prevent EphB4 signaling in cancer patients

Initially the data published in „Erythropoietin stimulates Tumor Growth via Initially the data published in „Erythropoietin stimulates Tumor Growth via EphB4", by Pradeep et al., 2015 in Cancer Cell appeared convincing. Especially considering the fact that it involves *in vivo* and *in vitro* data, showing a correlation between rhEpo and EphB4. However, taking a closer look at the published binding curve, using Microscale Thermophoresis Analysis, the interpretation of the binding data seemed less promising. Extensive research was done until the 30th of January 2019, but only led to finding papers citing or referencing the existing, and already mentioned, paper. A patent filed by co-authors of the paper has been filed about the interaction of Epo and EphB4 in 2009 (Jackson and Stein, 2006). The general knowledge about EphB4 being a direct receptor for Epo is therefore based on a very narrow data set from scarce sources.

The reproduction method for binding assay has been asserted as a useful tool. The binding of Epo to EpoR and EphrinB2 to EphB4 was successfully measured. Nevertheless, it was not possible to infer the binding of Epo to EphB4. Even in cooperation with experts, it was not possible to yield the results presented in the paper by Pradeep et al., 2015.

One possible explanation is that the binding of Epo to EphB4 most likely needs an Epo-EphB4 tetrameric complex for a stable binding. This assumption is largely based on published data saying that Ephrin receptor-ligand clusters are important for a stable signaling between EphrinB2 and EphB4 (Kania & Klein 2016, Himanen et al. 2010 and Chrencik et al 2006). Yet, the method used by Pradeep et al. did not take any special Epo-EphB4 complex into consideration.

Additional attempts to reproduce the binding of Epo to EphB4 were made by using a flow cytometry-based binding assay. For this purpose, the cell lines Cos-1 and TF-1 have been used. Both cell lines express EphB4 and EpoR.

However, in comparison to TF-1 cells, Cos-1 cells do have less EphB4 expression. The untransfected TF-1 cells showed a binding of Epo to the cell membrane receptors, as did Cos-1 cells. This result demonstrated that Epo does bind to both cells. However, it cannot be determined if Epo binds through EpoR and/or EphB4 on to these cells. For further experiments cell lines with knock out of EphB4 and EpoR should be established. It would be of high interest to repeat the Flow cytometry-based cell binding assay, as well as the signaling assay.

Although the Pradeep et al., 2015's results that EphB4 is an alternative receptor for Epo could not be reproduced in this study, it can be argued that the paper features numerous more data for the binding of Epo to EphB4 that has not been attempted to reproduce yet. This question can be elaborated on as an objective for future research in this field.

In an attempt to reproduce the signaling assay of Pradeep et al, the exact same cell line and antibodies have been purchased and used for experiments. The set-up of the signaling assay in this study involved stimulation of A2780 cell line with rhEpo as well as EphrinB2. However, to reproduce the data successfully, a cell line with knockdown of EpoR and one with knockdown of EphB4 need to be established. The analysis of Paragh et al., in contrast to Pradeep et al, has found no effect of exogenous Epo on EpoR signaling in A2780 carcinoma cells. Moreover, in the study of Paragraph et al. the effect of exogenous Epo was examined in a proliferation assay showing no effect of rhEpo on proliferation of A2780. In a matrigel invasion assay no effect on the invasiveness of A2780 cells was found (Paragh *et al.*, 2009). This leads to the conclusion of Paragraph et al. that A2780 cells are insensitive to exogenous Epo and do not signal through autocrine Epo-EpoR pathways. Although the reason for these different results is currently unclear, they may result from clonal differences in cell lines due to different sources or culture conditions (Paragh *et al.*, 2009).

Even though a direct correlation in binding could not reproduced, an interplay between Epo and EphB4 is very likely. Publications such as from Pasqual 2008 show that EphrinB2-EphB4 is involved in maintaining oxygen homeostasis in

the blood, due to hypoxia induced EphrinB2 upregulation in endothelial cells and in bone marrow stromal cells. This upregulation of EphrinB2 should further stimulate EphB4 signaling in hematopoietic progenitor cells leading to red blood cell formation. (Pasquale, 2008) However, it is well known that hypoxia does also lead to upregulation of Epo.

Publications have also indicated an interaction between EphB4 and Epo, without direct binding but through upregulation of transcription. Recently published results by Zhang et al., state that Epo leads to higher expression of EphB4 and EphrinB2, facilitating post-ischemic angiogenesis (Zhang *et al.*, 2019).

Given that the findings in this study are based on a limited set of data, the results have to be treated with considerable caution. Attempts to reproduce the published data by setting up new experiments to investigate the interaction of EphB4 with Epo had been commenced during this study but had to be stopped due to time limitations.

4.3. CD131: Rational design of engineering Epo as a potential candidate for High-Altitude Illness prevention

To engineer an Epo derivate, which could be a potential drug for treatment of High-altitude illness and HACE prevention, the Epo mutation R103K was chosen in this study as the most promising one.
The mutation Epo_{R103K} is within the internal motif of Epo. Publications in this research area show that mutations within this motif results in loss of erythropoietic activity (Gan *et al.*, 2012).
By fusing the mutated Epo to the antibody fragment IH4v1, targeting glycophorin A on RBC precursors, the erythropoietic activity could be maintained. This facilitates erythropoietic activity by linkage to EpoR on RBC precursors and not other cell types, such as platelets. Detailed information to this approach can be found in Introduction 1.4.2. Chimeric activator.

Widespread concerns surrounding Epo administration are the severe side effects that have been associated with Epo treatment.
Based on the concept published by Burrill et al. (2016, PNAS), and subsequent optimization by Jungmin Lee and Jeffrey Way (publication in process), prothrombotic side effects due to enhanced platelet production and activity can be abolished. The efficacy of IH4v1-5aa Epo_{R143E} still needs to be tested *in vitro* as well as *in vivo*, to ensure a diminished prothrombotic effect of this mutation with the already tested chimeric activator.

Asialoerythropoietin (Erbayraktar *et al.*, 2003), carbamylated Epo (Leist, 2004) and MEPO (Gan *et al.*, 2012) have one characteristic in common: they are derivates which do not possess erythropoietic activity but have neuroprotective effects.
Gan et al., (2012, Stroke) found that for stroke-treatment, multiple administrations of Epo are most likely necessary since Epo induced signaling pathways are only detectable for 24-40 hours after Epo exposure (Gan *et al.*, 2012). While the plasma half-life of Epo derivates such as Darbepoetin alfa is

about 7 hours in rats, the plasma half-life period of Asialoerythropoietin is 1.4 minutes due to renal clearance.
However, the binding of Epo to glycophorin A on RBCs as well as increasing the molecular weight, by fusing it to IH4v1, enables an increase of plasma half-life to 28 hours in mice (Burrill et al., 2016). This increases the clinical applicability of IH4v1-5aa Epo_{R143E}, since it lowers the administration frequency.

Another challenge for the clinical application of Epo as a successful neuroprotective drug is crossing the BBB. Except of leakage due to ruptures, Epo has limited permeability across the BBB. As already discussed in the introduction 1.7.4.1, less than 1 % of the exogenous Epo arrives in the brain. The mechanism of Epo crossing the BBB is not entirely understood. One possible explanation is that Epo binds to EpoR on vascular endothelial cells as well as on the luminal side of the brain capillaries via the high affinity binding site. The transition is then mediated by transcytosis (Sun *et al.*, 2019).
Since the mutation Epo_{R103K} is within the weak affinity site, the binding to EpoR via the high affinity receptor binding site would still be possible. A mutation on the weak face does prevent receptor dimerization and could lead to enhanced endogenous receptor mediated transcytosis.
This theory still needs to be tested experimentally. Additionally, it is well known that due to rupture or brain ischemia enhances BBB permeability (Sun *et al.*, 2019) due to hypoxia-induced biochemical alterations (Hackett 2001). This will further enable crossing of the BBB by Epo.
Furthermore, it has been published that the swelling of the brain in the clinical course of HACE also originates from the increased permeability of the BBB due to rupture (Luks, Swenson and Bärtsch, 2017). However studies have shown that Epo administration prior to the rupture of the BBB leads to a BBB protection and confined leakage (Üzüm *et al.*, 2005) increasing its potential as a treatment for HACE.

The percentage of the proposed Epo derivate IHv1-5aa Epo_{R143E} crossing the BBB has to be tested experimentally to enable correct dosing. In case the transcytosis mediated through the high affinity binding site of EpoR is not sufficient, strategies that target the protein to an additional endogenous receptor could be one possible approach to tackle that question. Sun et al., (2019, NeuroMolecular Medicine) reported for example the following approach: Crossing the BBB was enabled by joining of a chimeric monoclonal antibody to the endogenous transferrin receptor in the BBB (Sun *et al.*, 2019).

By administering Epo at least 4-5 days prior to exposure of high altitudes, the mature RBC will already be circulating. This enhances the preventative benefit of Epo in connection with HACE due to an elevated hematocrit, supporting higher oxygen delivery to the brain and tissue in case of severe hypoxia.
Additionally, shown during *in vivo* experiments with rats, Epo can be a sensible therapeutic after HACE due to a rise of VEGF and brain-derived neurotrophic factor (BDNF). These growth factors are involved in angiogenesis as well as in neurogenesis which can aid the functional recovery (Ding *et al.*, 2015).
Overall, as already specified in the introduction, the neuroprotective activity of Epo did prove in studies to protect neurons from damage caused by an oxygen shortage (Rabie and Marti, 2008).

To test the neuroprotective effects of Epo_{R143E}, *in vitro* assays with neurons or a neuroblastoma cell line can be used. A potential assay would be adding Epo about eight hours prior, challenging the neurons with neurotoxic substance, such as NMDA. Then the media needs to be changed and the neurons are again treated with Epo. Cell death can be measured and compared to Epo wt, and cells not having received an Epo treatment. Additionally, to ensure neuroprotective activity and to exclude unexpected side effects of IH4v1-5aa Epo_{R143E}, *in vivo* model systems need to be part of further investigations.

The results of this study, being part of this master thesis, indicate that IH4v1-5aa Epo_{R143E} possesses erythropoietic activity avoiding thrombotic side effects and increasing the plasma half-life period. Even though neuroprotective activity of the molecule can be expected, since the binding site to ßcR-EpoR heterodimer is not mutated, the actual neuroprotective effect has to be tested and compared to Epo wt.

This rational design of IH4v1-5aa Epo_{R103E} allows erythropoietic activity and neuroprotective activity to prevent and treat HACE.

5. References

- Alam, S. M. *et al.* (2008) 'Coexpression of EphB4 and ephrinB2 in tumour advancement of ovarian cancers', *British Journal of Cancer*, 98(4), pp. 845–851. doi: 10.1038/sj.bjc.6604216.
- Anagnostou, A. (1994) 'Erythropoietin receptor mRNA expression in human', 91(April), pp. 3974–3978.
- Arcasoy, M. O. (2008) 'Erythropoiesis-stimulating agent use in cancer: Preclinical and clinical perspectives', *Clinical Cancer Research*, 14(15), pp. 4685–4690. doi: 10.1158/1078-0432.CCR-08-0264.
- Bagley, C. *et al.* (1989) 'The Structural and Functional Basis of Cytokine Receptor Activation: Lessons From the Common b Subunit of the Granulocyte-Macrophage Colony-Stimulating Factor, Interleukin-3 (IL-3), and IL-5 Receptors', *Blood*, 89(5), pp. 1471–1482.
- Banks, W. A. *et al.* (2004) 'Passage of erythropoietic agents across the blood-brain barrier: A comparison of human and murine erythropoietin and the analog darbepoetin alfa', *European Journal of Pharmacology*, 505(1–3), pp. 93–101. doi: 10.1016/j.ejphar.2004.10.035.
- Banks, W. A. (2012) 'Brain meets body: The blood-brain barrier as an endocrine interface', *Endocrinology*, 153(9), pp. 4111–4119. doi: 10.1210/en.2012-1435.
- Beutler, E. and Waalen, J. (2006) '16189263.Pdf', 107(5), pp. 1747–1750. doi: 10.1182/blood-2005-07-3046.BLOOD.
- Blits-Huizinga, C. T. *et al.* (2004) 'Ephrins and their receptors: Binding versus biology', *IUBMB Life*, 56(5), pp. 257–265. doi: 10.1080/15216540412331270076.
- Bonnas, C. B. (2009) 'Identification of erythropoietin isoforms and evaluation of their biological importance.', *PhD Charité Berlin*, p. 150. doi: http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000009488.
- Brines, M. *et al.* (2004) 'Erythropoietin mediates tissue protection through an erythropoietin and common -subunit heteroreceptor',

Proceedings of the National Academy of Sciences, 101(41), pp. 14907–14912. doi: 10.1073/pnas.0406491101.

- Brines, M. and Cerami, A. (2012) '<Title/>', *Molecular Medicine*, 18(3), p. 1. doi: 10.2119/molmed.2011.00414.
- Broadwell, R. and Sofroniew, M. (1993) 'Serum Proteins Bypass the Blood-Brain Fluid Barriers for Extracellular Entry to the Central Nervous System', *Experimental Neurology*, pp. 245–263.
- Bunn, H. F. (2013) 'Erythropoietin', *Cold Spring Harbour perspectives in medicine*, 3(3), pp. 1–20.
- Burrill, D. R. *et al.* (2016) 'Targeted erythropoietin selectively stimulates red blood cell expansion in vivo', *Proceedings of the National Academy of Sciences*, 113(19), pp. 5245–5250. doi: 10.1073/pnas.1525388113.
- Cao, Y. (2013) 'Erythropoietin in cancer: A dilemma in risk therapy', *Trends in Endocrinology and Metabolism*. Elsevier Ltd, 24(4), pp. 190–199. doi: 10.1016/j.tem.2012.10.007.
- Carlini, R. G., Reyes, A. A. and Rothstein, M. (1995) 'Recombinant human erythropoietin stimulates angiogenesis in vitro', *Kidney International*, 47(3), pp. 740–745. doi: 10.1038/ki.1995.113.
- Chan, K. K. *et al.* (2017) 'Erythropoietin drives breast cancer progression by activation of its receptor EPOR', *Oncotarget*, 8(24), pp. 38251–38263. doi: 10.18632/oncotarget.16368.
- Cheetham, J. C. *et al.* (1998) 'NMR structure of human erythropoietin and a comparison with its receptor bound conformation', *Nature Structural Biology*, 5(10), pp. 861–866. doi: 10.1038/2302.
- Chen, Y., Zhang, H. and Zhang, Y. (2017) 'Targeting receptor tyrosine kinase EphB4 in cancer therapy', *Seminars in Cancer Biology*. Elsevier, (October), pp. 0–1. doi: 10.1016/j.semcancer.2017.10.002.
- Chrencik, J. E. *et al.* (2006) 'Structural and biophysical characterization of the EphB4·EphrinB2 protein-protein interaction and receptor specificity', *Journal of Biological Chemistry*, 281(38), pp. 28185–28192. doi: 10.1074/jbc.M605766200.
- Collino, M. *et al.* (2015) 'Flipping the molecular switch for innate

protection and repair of tissues: Long-lasting effects of a non-erythropoietic small peptide engineered from erythropoietin', *Pharmacology and Therapeutics*. Elsevier B.V., 151, pp. 32–40. doi: 10.1016/j.pharmthera.2015.02.005.

- Debeljak, N., Solár, P. and Sytkowski, A. J. (2014) 'Erythropoietin and cancer: The unintended consequences of anemia correction', *Frontiers in Immunology*, 5(NOV), pp. 1–14. doi: 10.3389/fimmu.2014.00563.
- Ding, J. *et al.* (2015) 'The lack of CD131 and the inhibition of Neuro-2a growth by carbamylated erythropoietin', *Cell Biology and Toxicology*, 31(1), pp. 29–38. doi: 10.1007/s10565-015-9292-y.
- Ding, J. *et al.* (2017) 'Neuroprotection and CD131/GDNF/AKT Pathway of Carbamylated Erythropoietin in Hypoxic Neurons', *Molecular Neurobiology*. Molecular Neurobiology, 54(7), pp. 5051–5060. doi: 10.1007/s12035-016-0022-0.
- Ehrenreich, H. *et al.* (2009) 'Recombinant Human Erythropoietin in the Treatment of Acute Ischemic Stroke', *Stroke*, 40(12), pp. 647–657. doi: 10.1161/strokeaha.109.564872.
- Elliott, S. *et al.* (1997) 'Mapping of the active site of recombinant human erythropoietin', *Blood*, 89(2), pp. 493–502.
- Erbayraktar, S. *et al.* (2003) 'Asialoerythropoietin is a nonerythropoietic cytokine with broad neuroprotective activity in vivo', *Proceedings of the National Academy of Sciences*, 100(11), pp. 6741–6746. doi: 10.1073/pnas.1031753100.
- Frille, A. (2012) 'The Role of Erythropoietin and its Receptor in Growth and Survival of non-small cell Lung Cancer cells submitted by'.
- Gan, Y. *et al.* (2012) 'Mutant Erythropoietin without Erythropoietic Activity is Neuroprotective against Ischemic Brain Injury', *Stroke*, 286(43), pp. 3071–3077. doi: 10.1161/STROKEAHA.112.663120.
- Groppa, E. *et al.* (2018) 'EphrinB2/EphB4 signaling regulates non-sprouting angiogenesis by VEGF', *EMBO reports*, p. e45054. doi: 10.15252/embr.201745054.
- Hackett, P. and Roach, R. (2001) 'High -Altitude Illness', *The New England Journal of Medicine*, 345(2), pp. 107–114.

- Heo, K. *et al.* (2014) 'Prophylactic Effect of Erythropoietin Injection to Prevent Acute Mountain Sickness: An Open-Label Randomized Controlled Trial', *J Korean Med Sci*. The Korean Academy of Medical Sciences, 29(3), pp. 416–422. Available at: http://synapse.koreamed.org/DOIx.php?id=10.3346%2Fjkms.2014.29.3.416.
- Hernández, C. C. *et al.* (2017) 'Neuroprotective effects of erythropoietin on neurodegenerative and ischemic brain diseases: The role of erythropoietin receptor', *Neural Regeneration Research*, 12(9), pp. 1381–1389. doi: 10.4103/1673-5374.215240.
- Himanen, J. P. and Nikolov, D. B. (2003) 'Eph receptors and ephrins', *International Journal of Biochemistry and Cell Biology*, 35(2), pp. 130–134. doi: 10.1016/S1357-2725(02)00096-1.
- Howell, S. *et al.* (2006) 'Two Domains of the Erythropoietin Receptor Are Sufficient for Jak2 Binding/Activation and Function', *Molecular and Cellular Biology*, 26(22), pp. 8527–8538. doi: 10.1128/mcb.01035-06.
- Hugo H. Marti (2004) 'Erythropoietin and the hypoxic brain', *The Journal of Experimental Biology*, 207, pp. 3233–3242. doi: 10.1242/jeb.01049.
- Jackson, D. B. and Stein, M. (2006) '(12) United States Patent', 2(12). doi: 10.1038/incomms1464.
- Jain, K. *et al.* (2012) 'Erythropoietin use and abuse', *Indian Journal of Endocrinology and Metabolism*, 16(2), p. 220. doi: 10.4103/2230-8210.93739.
- Jelkmann, W. (1986) 'The experiment by Carnot and Deflandre', *Respiration Physiology*, 63(November 1985), pp. 257–266.
- Jensen, J. D. and Vincent, A. L. (2019) *High Altitude Pulmonary Edema (HAPE)*, *StatPearls*. StatPearls Publishing. Available at: http://www.ncbi.nlm.nih.gov/pubmed/28613569 (Accessed: 17 March 2019).
- Jiang, J. *et al.* (2014) 'Site-specific qualitative and quantitative analysis of the N- and O-glycoforms in recombinant human erythropoietin', *Analytical and Bioanalytical Chemistry*, 406(25), pp. 6265–6274. doi:

10.1007/s00216-014-8037-8.

- Johnson, W. *et al.* (2016) 'Stroke: a global response is needed', *Bulletin of the World Health Organization*, 94(9), p. 634–634A. doi: 10.2471/blt.16.181636.
- Kania, A. and Klein, R. (2016) 'Mechanisms of ephrin–Eph signalling in development, physiology and disease', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 17(4), pp. 240–256. doi: 10.1038/nrm.2015.16.
- Kato, T. (2016) *Erythropoietin*, *Handbook of Hormones*. Elsevier Inc. doi: 10.1016/B978-0-12-801028-0.00385-8.
- Klocke, D., Decker, W. and Stepanek, J. (1998) 'Altitude-related illnesses', *Mayo Clinic Proceedings*. Mayo Foundation for Medical Education and Research, 73(10), pp. 988–993. doi: 10.4065/73.10.988.
- Kumar, S. R. *et al.* (2007) 'The receptor tyrosine kinase EphB4 is overexpressed in ovarian cancer, provides survival signals and predicts poor outcome', *British Journal of Cancer*, 96(7), pp. 1083–1091. doi: 10.1038/sj.bjc.6603642.
- Lai, D. *et al.* (2013) 'Influence of safety warnings on ESA prescribing among dialysis patients using an interrupted time series', *BMC Nephrology*. BMC Nephrology, 14(1), p. 1. doi: 10.1186/1471-2369-14-172.
- Leist, M. (2004) 'Derivatives of Erythropoietin That Are Tissue Protective But Not Erythropoietic', *Science*, 305(5681), pp. 239–242. doi: 10.1126/science.1098313.
- Leist, M. *et al.* (2004) 'Derivatives of erythropoitein that are tissue protective but not erythropoietic', *Science*, 305(5681), pp. 239–242. doi: 10.1126/science.1098313.
- Lienert, F. *et al.* (2014) 'Synthetic Biology in Mammalian Cells', *Nat Rev Mol Cell Biol*, 15(2), pp. 95–107. doi: 10.1038/nrm3738.Synthetic.
- Lisabeth, E., Falivelli, G. and Pasquale, E. (2013) 'Eph Receptor Signaling and Ephrins', *Cold Spring Harbor Perspectives in Biology*, 5.
- Livnah, O. *et al.* (1999) 'Crystallographic evidence for preformed

dimers of erythropoietin receptor before ligand activation', *Science*, 283(5404), pp. 987–990. doi: 10.1126/science.283.5404.987.

- Luks, A. M., Swenson, E. R. and Bärtsch, P. (2017) 'Acute high-altitude sickness', *European Respiratory Review*, 26(143), pp. 1–14. doi: 10.1183/16000617.0096-2016.
- Lv, J. *et al.* (2016) 'EphB4 promotes the proliferation, invasion, and angiogenesis of human colorectal cancer', *Experimental and Molecular Pathology*. Elsevier Inc., 100(3), pp. 402–408. doi: 10.1016/j.yexmp.2016.03.011.
- Miller, J. L. *et al.* (2015) 'Discovery and Characterization of Nonpeptidyl Agonists of the Tissue-Protective Erythropoietin Receptor', *Molecular Pharmacology*, 88(2), pp. 357–367. doi: 10.1124/mol.115.098400.
- Murai, K. K. (2003) ``Eph'ective signaling: forward, reverse and crosstalk', *Journal of Cell Science*, 116(14), pp. 2823–2832. doi: 10.1242/jcs.00625.
- Noh, M. Y. *et al.* (2014) 'Erythropoietin modulates the immune-inflammatory response of a SOD1G93A transgenic mouse model of amyotrophic lateral sclerosis (ALS)', *Neuroscience Letters*. Elsevier Ireland Ltd, 574, pp. 53–58. doi: 10.1016/j.neulet.2014.05.001.
- Paragh, G. *et al.* (2009) 'RNA interference-mediated inhibition of erythropoietin receptor expression suppresses tumor growth and invasiveness in A2780 human ovarian carcinoma cells', *American Journal of Pathology*. American Society for Investigative Pathology, 174(4), pp. 1504–1514. doi: 10.2353/ajpath.2009.080592.
- Paralikar, S. J. and Paralikar, J. H. (2010) 'High-altitude medicine.', *Indian journal of occupational and environmental medicine*. Wolters Kluwer -- Medknow Publications, 14(1), pp. 6–12. doi: 10.4103/0019-5278.64608.
- Pasquale, E. B. (2008) 'Eph-Ephrin Bidirectional Signaling in Physiology and Disease', *Cell*, 133(1), pp. 38–52. doi: 10.1016/j.cell.2008.03.011.
- Pradeep, S. *et al.* (2015) 'Erythropoietin Stimulates Tumor Growth via EphB4', *Cancer Cell*, 28(5), pp. 610–622. doi:

10.1016/j.ccell.2015.09.008.

- Rabie, T. and Marti, H. H. (2008) 'REVIEWS Brain Protection by Erythropoietin ':, *Physiology*, 23, pp. 263–274.
- Sun, J. *et al.* (2019) 'The Promises and Challenges of Erythropoietin for Treatment of Alzheimer's Disease', *NeuroMolecular Medicine*. Springer US, 0(0), p. 0. doi: 10.1007/s12017-019-08524-y.
- Takahashi, T. *et al.* (1995) 'Characterization of three erythropoietin (Epo)-binding proteins in various human Epo-responsive cell lines and in cells transfected with human Epo-receptor cDNA [see comments]', *Blood*, 85(1), pp. 106–114. doi: 10.1017/S002211200300733X.
- Tamura, T. *et al.* (2017) 'Neuroprotective erythropoietin attenuates microglial activation, including morphological changes, phagocytosis, and cytokine production', *Brain Research*, 1662, pp. 65–74. doi: 10.1016/j.brainres.2017.02.023.
- Taylor, N. D. *et al.* (2010) 'Anti-glycophorin single-chain Fv fusion to low-affinity mutant erythropoietin improves red blood cell-lineage specificity', *Protein Engineering, Design and Selection*, 23(4), pp. 251–260. doi: 10.1093/protein/gzp085.
- Tiwari, N. K. *et al.* (2019) 'Carbamoylated erythropoietin induces a neurotrophic gene profile in neuronal cells', *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. Elsevier, 88(February 2018), pp. 132–141. doi: 10.1016/j.pnpbp.2018.07.011.
- Udupa, K. B. (2006) 'Functional significance of erythropoietin receptor on tumor cells', *World Journal of Gastroenterology*, 12(46), pp. 7460–7462. doi: 10.3748/wjg.v12.i46.7460.
- Üzüm, G. *et al.* (2005) 'Erythropoietin prevents the increase in blood-brain barrier permeability during pentylentetrazol induced seizures', *Life Sciences*, 78(22), pp. 2571–2576. doi: 10.1016/j.lfs.2005.10.027.
- Watowich, S. S. (2011) 'The erythropoietin receptor: molecular structure and hematopoietic signaling pathways', *J Investig Med*, 59(7), pp. 1067–1072. doi: 10.2310/JIM.0b013e31820fb28c.
- Way, J. C. *et al.* (2005) 'Improvement of Fc-erythropoietin structure and pharmacokinetics by modification at a disulfide bond', *Protein*

Engineering, Design and Selection, 18(3), pp. 111–118. doi: 10.1093/protein/gzi021.

- Wheeler, D. L. and Yarden, Y. (2015) 'Receptor tyrosine kinases: Structure, functions and role in human disease', *Receptor Tyrosine Kinases: Structure, Functions and Role in Human Disease*, pp. 1–440. doi: 10.1007/978-1-4939-2053-2.
- Yu Gan, Ph.D.*, Juan Xing, M.D.*, Zheng Jing, Ph.D., R. Anne Stetler, Ph.D., Feng Zhang, M.D., Ph.D., Yumin Luo, M.D., Ph.D., Xunmin Ji, M.D., Yanqin Gao, M.D., and Guodong Cao, P. D. (2012) 'NIH Public Access', *Stroke*, 85(43), pp. 3071–3077. doi: 10.1016/j.neuroimage.2013.08.045.The.
- Zhang, S. J. *et al.* (2019) 'MEPO promotes neurogenesis and angiogenesis but suppresses gliogenesis in mice with acute ischemic stroke', *European Journal of Pharmacology*. Elsevier B.V., 849, pp. 1–10. doi: 10.1016/j.ejphar.2019.01.066.
- Zhu, S. *et al.* (2005) 'EphB4 receptor tyrosine kinase is expressed in bladder cancer and provides signals for cell survival', *Oncogene*, 25(5), pp. 769–780. doi: 10.1038/sj.onc.1209108.
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6. Acknowledgment

I would like to acknowledge and thank the people who made this project possible. I would like to thank Jungmin for being the most kind, organized and supportive advisor. Thank you for your thoughts, scientific input from being in the lab up to reading this thesis. I would like to thank Jeff for being a motivated and inspiring supervisor and Dr. Pam Silver for hosting me in her laboratory. I have learned so much in the Silver lab and will remember my time there fondly. I would also like to thank all the Silver lab members for the scientific discourse, making me feel welcome and enriching the experience. In particular I want to thank the Wysslings: Yuki, Jungmin, Katie, Tim, Sarabeth, Finn and Michael for all the creative evenings, fun coffee and chocolate experiences and most importantly for being such amazing people. I would like to thank the Marshall Plan for my scholarship. Lastly, I would like to thank Stefan, Tobias and my parents for their support. I know how happy I am to have you.

7. Statuary declaration

Erklärung:

Ich erkläre, dass die vorliegende Diplomarbeit/Masterarbeit von mir selbst verfasst wurde und ich keine anderen als die angeführten Behelfe verwendet bzw. mich auch sonst keiner unerlaubter Hilfe bedient habe.

Ich versichere, dass ich diese Diplomarbeit/Masterarbeit bisher weder im In- noch im Ausland (einer Beurteilerin/einem Beurteiler zur Begutachtung) in irgendeiner Form als Prüfungsarbeit vorgelegt habe.

Weiters versichere ich, dass die von mir eingereichten Exemplare (ausgedruckt und elektronisch) identisch sind.

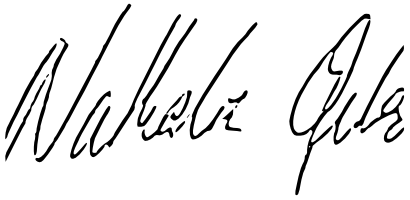
Declaration:

I hereby declare that the submitted Master thesis was written by myself and that I did not use any aids other than those indicated, none of which are unauthorised.

I assure that I have not previously submitted this Master thesis or its contents in any form for assessment as part of an examination either in Austria or abroad.

Furthermore, I assure that all copies submitted by myself (electronic and printed) are identical.

date: 26.3.2019... signature:



8. Appendix

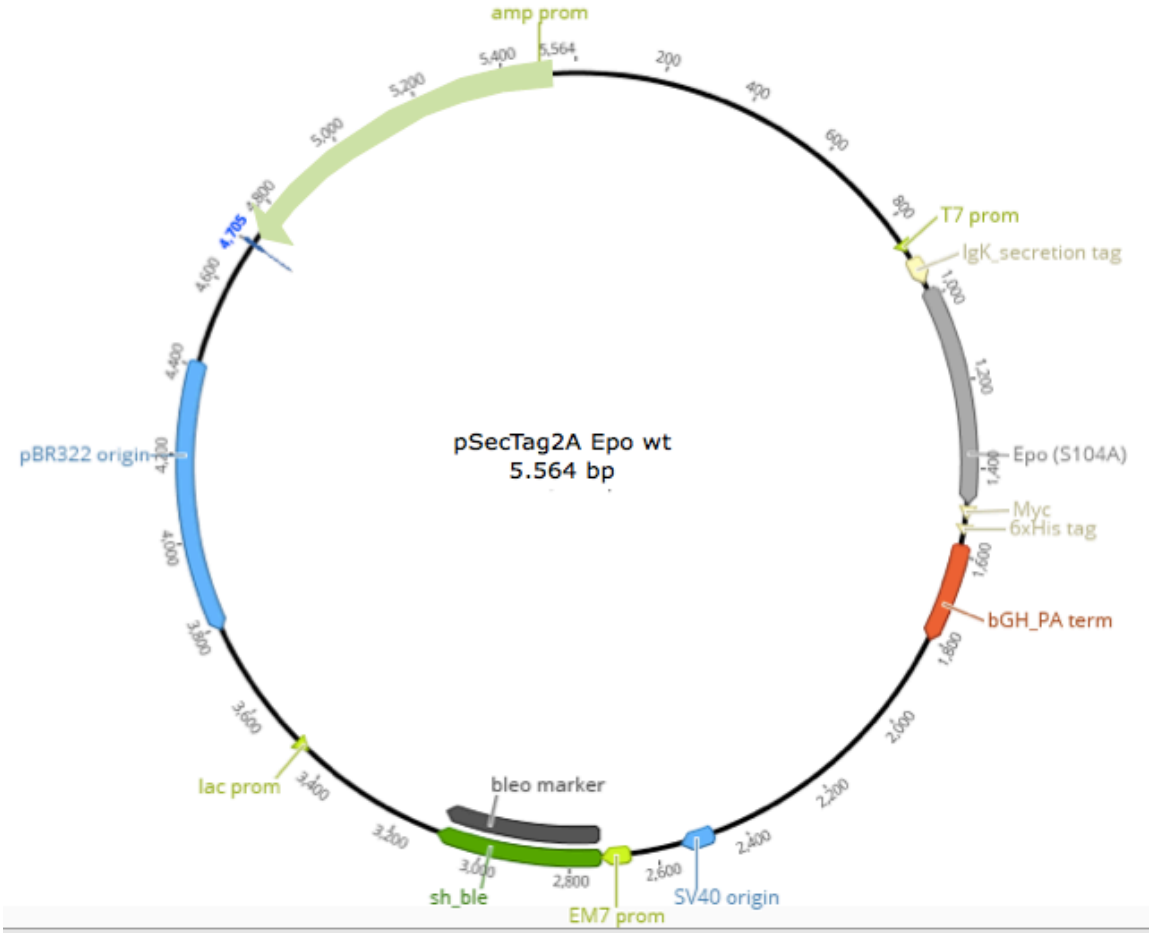
S.8.1 Erythropoietin amino acid sequence:

MGVHECPAWLWLLLSLLSLPLGLPVLGAPPRLICDSRVLERYLLEAKEAENITTGCAEHC
SLNENITVPDTKVNFYAWKRMEVGQQAVEVWQGLALLSEAVLRGQALLVNSSQPWEPLQL
HVDKAVSGLRSLTTLLRALGAQKEAISPPDAASAAPLRTITADTFRKLFRVYSNFLRGKL
KLYTGEACRTGD**R**

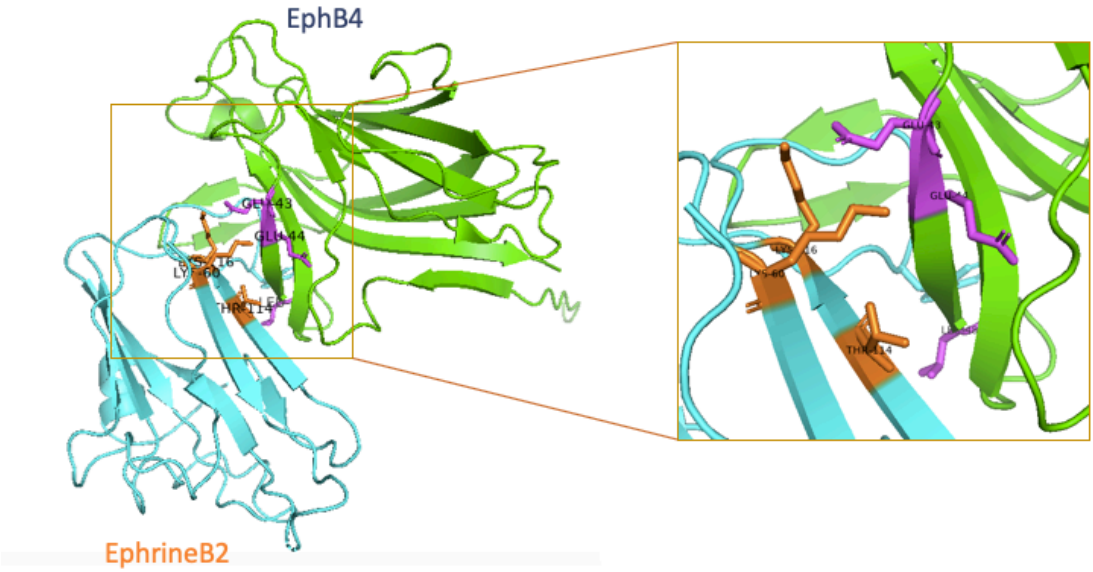
In bold red: N-terminal Signalpeptide

In bold green: C-terminal Argininrest

S.8.2 A scheme of the mammalian expression vector used within this study.



S.8.3 Binding of EphrinB2 to EphB4



S.8.4 Library of Epo mutations, identified to be possible involved in binding of Epo to EphB4

- EPO K97S
- EPO Y49S
- EPO N147K
- EPO S104A
- EPO S104I
- EPO L108A
- EPO S100A
- EPO K116E
- EPO R14N
- EPO E18S
- EPO E13A
- EPO D8S
- EPO L108A
- EPO L75S
- EPO R4N
- EPO W88F
- EPO E31I
- EPO R53A
- EPO E72A
- EPO R150E
- EPO K45D
- EPO K154S
- EPO E159S

S.8.5 Western Blot evaluating pStat3, pSrc and PStat5 levels in A2780 cells and TF1 cells after treatment with rhEpo and rhEphrinB2**.**