universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

ANALYSIS OF THE EFFECTS OF siRNA-MEDIATED KNOCK-DOWN OF GLUCOCORTICOID TARGET GENES IN PRIMARY ERYTHROID PROGENITOR CELLS

Verfasserin

MICHELA PARTH

angestrebter akademischer Grad

Magistra der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A 441
Studienrichtung lt. Studienblatt:	Diplomstudium Genetik – Mikrobiologe (Stzw)
Betreuerin / Betreuer:	Ao. Univ.-Prof. Mag. Dr. Ernst Müllner

Für meine Eltern, die mir alles
in meinem Leben ermöglicht haben

TABLE OF CONTENT

Zusammenfassung

Hämatopoetische Stammzellen haben die Fähigkeit zur Selbsterneuerung und können zu den unterschiedlichen adulten Blutzellentypen differenzieren. Unter Erythropoese versteht man den Prozess, in dem myeolide Vorläuferstammzellen zu kernlosen hämoglobinisiereten Erythrocyten differenzieren. Hematopoese und Erythropoese sind komplexe Abläufe, welche einer strengen Kontrolle unterliegen müssen, da eine Störung des Gleichgewichts zwischen Selbsterneuerung, Differenzierung und Zellteilung zu ernsten Krankheitsbildern führen kann.

Steroidhormone, wie z.B. Glukokortikoide, spielen in der Kontrolle über die Entscheidung der Selbsterneuerung und Differenzierung zu erythroiden Vorläuferzellen eine essentielle Rolle. Glukokortikoide sind außerdem noch in verschiedensten Abläufen im Körper involviert, so regulieren sie z.B. den Fett- und Eiweißmetabolismus, und sind beteiligt in der Kontrolle bestimmter Funktionen des zentralen Nervensystems, sowie des Immunsystems. Glukokortikoide entfalten ihre Wirkung durch Bindung an den nukleären Glukokortikoid-Rezeptor (GR). Dieser agiert nach erfolgter Aktivierung als Transkriptionsfaktor und moduliert auf diese Weise direkt oder indirekt die Gentranskription verschiedenster Zielgene.

In meiner Diplomarbeit hab ich die Funktion folgender drei GR-Zielgene analysiert: Raf-1, Tis11d, FKBP51. Alle Experimente wurden in einem primären erythroiden Zellkultur-System durchgeführt, wobei die erythroiden Vorläuferzellen aus humanem Nabelschurblut isoliert wurden. Ziel meiner Diplomarbeit war es eine verlässliche und reproduzierbare Transfektions-Methode zu etablieren, mit deren Hilfe ich die Expression aller drei GR-Zielgene durch Elektroporation mit siRNAs erfolgreich vermindern konnte. Ziel der Runterregulierung war es, dadurch die Funktion derselben Gene herauszufinden. Dafür wurden morphologische Veränderungen in den Zellen, Effekte auf deren Viabilität und auf andere Signalwege analysiert. Im Laufe meiner Arbeit konnte ich zeigen, dass eine verminderete Expression von Tis11d und FKBP51 eine erhöhte Apoptose zur Folge hatte. Daraus konnte geschlossen werden, dass Tis11d und FKBP51 womöglich in anti-apoptotischen Mechanismen in erythroiden Vorläuferzellen involviert sind. Doch die genauen molekularbiologischen Hintergründe, sowie die möglich involvierten Signalwege müssen noch ausführlicher untersucht werden.

Abstract

Hematopoietic stem cells (HSCs) are able to self-renew and to differentiate into all mature blood cell types, during erythropoiesis early erythroid progenitor cells differentiate into enucleated red blood cells (RBC). Hematopoiesis and erythropoiesis are complex processes, which have to be under strict control, disturbance of the balance between self-renewal, proliferation and differentiation results in severe diseases.

Steroid hormones, such as glucocorticoids, have been shown to be one of the key regulators of the decision between self-renewal and differentiation into erythroid progenitor cells. Glucocorticoids have been demonstrated to be involved in the regulation of numerous other physiological and developmental processes throughout the body, such as regulation of lipid and protein metabolism, regulation of central nervous system functions and modulation of the immune system. Glucocortiocids bind to their nuclear receptor (GR), which then functions as a transcription factor and induces expression or inhibition of various target genes. Glucocorticoid Receptor functions are essential for survival, since mice lacking the GR die at birth and show defects on several organs (Cole et al., 1995).

In my diploma thesis I studied the function of the three different glucocorticoid target genes: Raf-1, Tis11d and FKBP51. All of the experiments were performed in a primary erythroid cell culture system, derived from umbilical cord blood.

During my diploma thesis I established a reliable and reproducible method for transfection of human EPCs by electroporation. With this method I could successfully knock down all three target genes by electroporation with complementary siRNAs. Downregulation of Raf-1, Tis11d and FKBP51 gave us insights into their function and the mechanisms in which they are involved during erythroid cell proliferation and spontaneous differentiation. Morphological changes, effects on the cell viability and changes in downstream signaling after the knock-down were analysed. While there were no remarkable effects after the Raf-1 knock-down, we could show that downregulation of Tis11d and FKBP51 led to an increase of apoptosis. This indicates that both GR-target genes are involved in anti-apoptotic mechanisms within erythroid progenitor cells. The exact regulation of the survival pathway needs further investigation.

1. Introduction

1.1 Human Hematopoiesis

Hematopoiesis is the process where all types of blood cells of the hematopoietic system, the white leuko- and lymphocytes and the red blood cells, are formed from **hematopoietic stem cells (HSCs)** (see figure 1 and 2) (Tsiftsoglou et al., 2009).
The red colour of the blood is the result of the accumulated hemoglobin in the erythrocytes. Erythrocytes are responsible for the oxygen transport through the body. As oxygen transport is a stressful process and is causing damage to the erythrocytes, the lifespan of erythrocytes is limited to approximately 90 to 120 days. Therefore it is essential for the organism to produce new red blood cells throughout the whole life of an individual. This is achieved by differentiation of hematopoietic stem cells into progenitor cells and finally into mature enucleated erythrocytes (Orkin and Zon, 2008).

HSCs are able to perform **self-renewal**, which means that a cell can divide into two daughter cells, where one cell is able to differente and the other cell is maintained in the undifferentiated stem cell-like state. Due to its ability of self-renew, one single hematopoietic stem cell could generate an entire hematopoietic system and thereby maintain hematopoiesis for the lifetime of one individual (Orkin and Zon, 2008; Renstrom et al., 2009).
The processes of self renewal and differentiation have to be strictly and tightly controlled, and a failure in the regulation of HSCs can lead to a variety of diseases, such as leukaemia or anaemia (Carlesso and Cardoso, 2010).

Primitive and definitive haematopoiesis

Human hematopoiesis can be divided into primitive and definitive hematopoiesis. From week 3-6 of gestation, hematopoiesis takes place in the yolk sac **(primitive erythropoiesis),** whereas from week 6-22 the fetal liver functions as the primary site of hematopoiesis; after this period the bone marrow becomes the predominant

and lifelong site of blood-cell- production (**definitive haematopoiesis,** see figure 1) (Bonifer et al., 1998; Palis and Yoder, 2001).

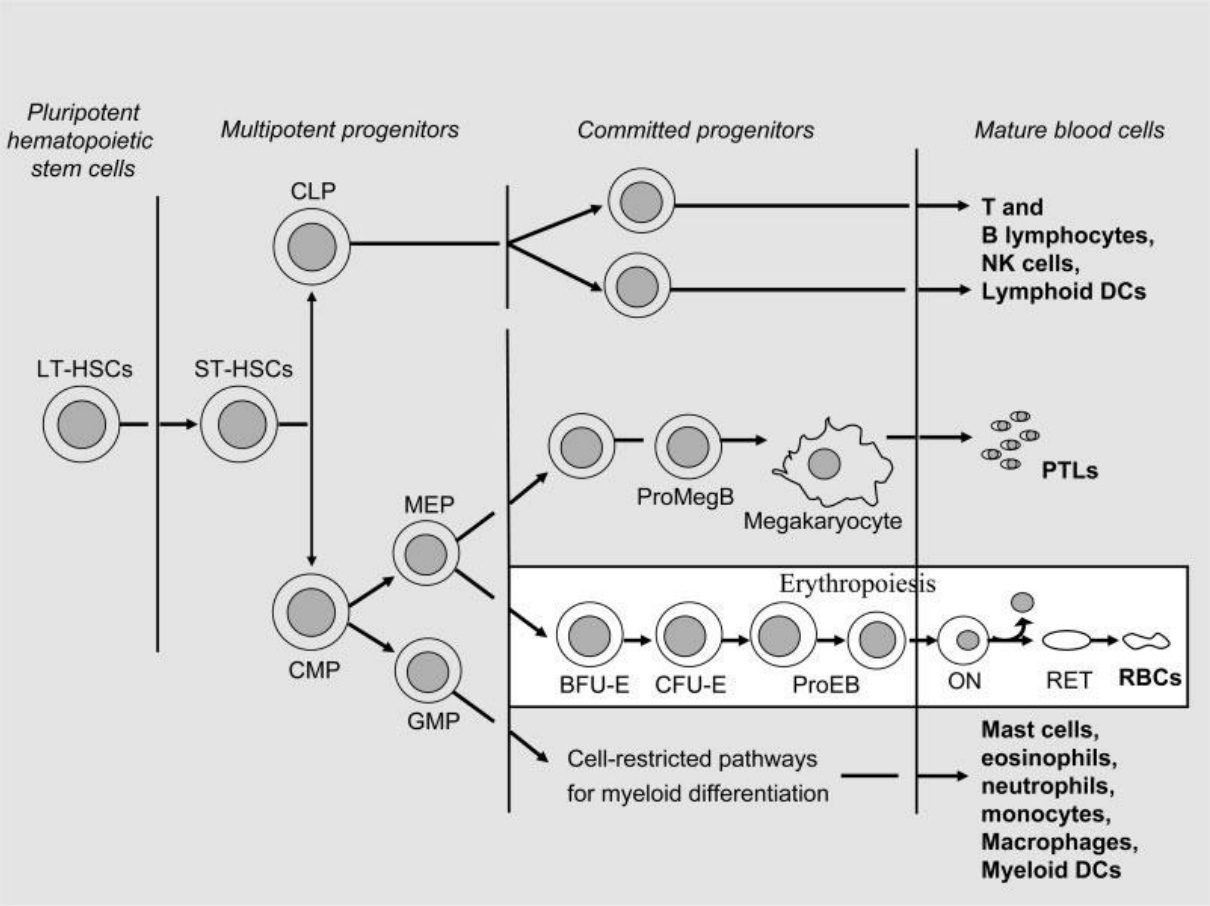


Figure 1: Human hematopoiesis

Hematopoiesis begins in the bone marrow, where a pluripotent long-term HSC gives rise to a short-term HSC, which turns into a ***multipotent hematopoietic progenitor cell.*** *Such multipotent hematopoietic progenitor cells then undergo several steps of differentiaton and generate the* ***common myeloid progenitor (CMP)*** *and the* ***common lymphoid progenitor (CLP)****, which can differentiate into T- and B- lymphocytes, natural killer cells and dendritic cells.*

The common myeloid progenitor cells turn into ***granulocyte-myeloid progenitors (GMP)*** *and into* ***megakaryocytic/erythroid progenitors (MEP)****, which can differentiate into* ***erythroid burst forming unit-erythroid (BFU-E)*** *and* ***colony forming unit-erythroid (CFU-E)****. The CFU-Es then mature into enucleated erythrocytes (red blood cells, RBCs); (picture taken from Tsiftsoglou et al., 2009).*

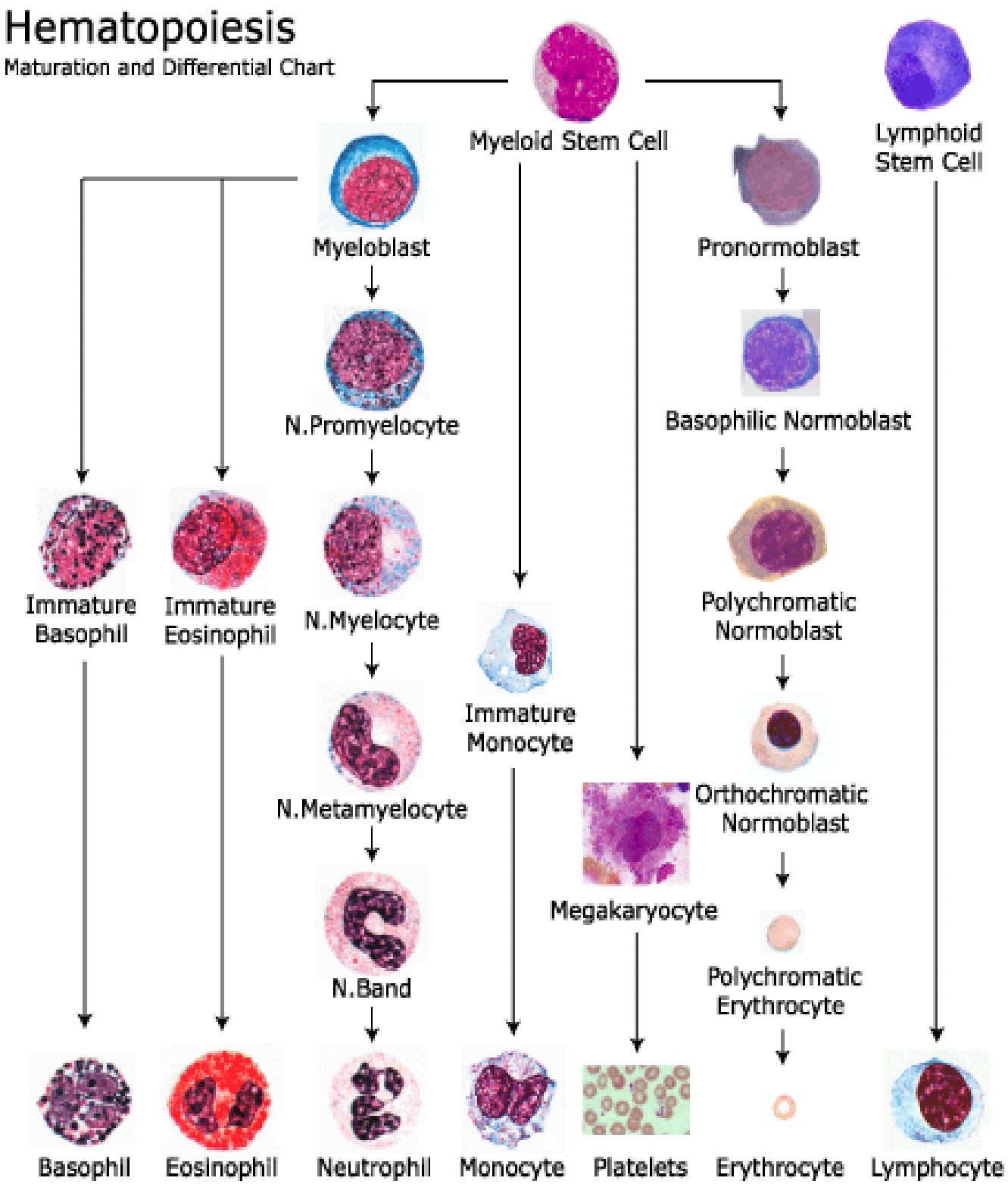


Figure 2: Maturation and differentiation in human hematopoiesis
Hematopoietic stem cells (HSC) give rise to all types of blood cells of the hematopoietic system. Blood cells can be classified into two main groups: ***lymphoid cells*** *(T, B, and natural killer cells) and* ***myeloid cells*** *(granulocytes, monocytes, erythrocytes and megakaryocytes).*
(picture taken from: Alexander Fleming, Biomedical Sciences Research Center: www.fleming.gr/en/investigators/Strouboulis/index.htm)

1.1.2 Hematopoietic stem cells and their niches

HSCs are able to self-renew and to differentiate into blood cells of multiple lineages (see figure 1 and figure 2). The fate of stem cells is controlled by a specific microenvironment, **the stem cell niche** (Renstrom et al., 2009). In adults the niche of HSC is the bone marrow microenvironment, which provides intrinsic and extrinsic factors crucial for the maintainance the stem cell properties of HSCs. The stem cell niche is a highly organized **three-dimensional microenvironment**, which consists of different cell types (the HSCs and other niche cells), soluble factors derived from the niche cells (Lin, 2002) and the extracellular matrix, formed mainly by fibronectin and collagen (Arai et al., 2005).
Within the stem cell niche **cell-cell-interactions** and interactions between the HSCs and the extracellular matrix take place. Such interactions are essential for the balance between self-renewal, differentiation and cell-cycle quiescence (see figure 4) (Wilson and Trumpp, 2006).

The HSC niche consists of two parts: the **vascular niche** and the **osteoblastic niche** (Yin and Li, 2006). The vascular niche supports the differentiation into hematopoietic progenitor cells, whereas the osteoblastic niche maintains HSC in a **quiescent state** with slow cycling or in G0-state. By keeping HSC in a quiescent state it is ensured that their potential for long-term hematopoiesis is maintained and premature exhaustion of HSCs is prevented (Shiozawa et al., 2008). In the osteoblastic and vascular niche cytokine signaling pathways such as **Ang-1/Tie2, THPO/Mpl** (see figure 3) and **SCF/cKIT** play an important role in the regulation and maintainance of HSCs (Arai et al., 2009).

Angiopoietin-1 (Ang-1) is a ligand for the Tie2 receptor on the surface of HSCs. By binding to its receptor, Angiopoetin-1 activates Tie2 and thereby promotes the interaction of HSCs with the extracellular matrix of the niche. Thereby it induces adhesion of HSCs to fibronectin and collagen, which keeps the HSCs within the niche. The Tie2/Ang-1 signaling promotes quiescence, apoptosis protection as well as tight adhesion. By preventing cell division of HSCs, Ang-1 maintains the long-term blood-repopulating activity (Arai et al., 2005).

Thrombopoetin (THPO) is produced in the osteoblastic island, THPO and its receptor Mpl maintain HSCs quiescence. It has been demonstrated that inhibiton of the Trombopoetin/Mpl signaling-pathway reduces the interactions of HSCs to the niche and this susequentely results in reduction of the quiescent HSC population (Arai et al., 2009).

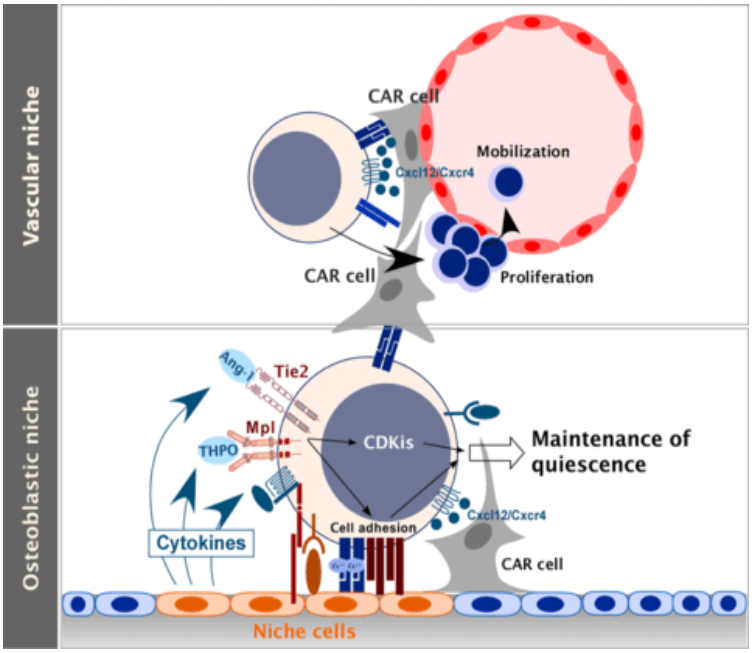


Figure 3: The HSC niche

*Cytokine signaling pathways such as **THPO/Mpl and Ang-1/Tie2** between HSCs and osteblastic niche cells promote cell adhesion, which contributes to HSC quiescence.*

(taken from: Arai et al., 2009)

Stem cell factor (SCF) is one of the most important hematopoietic growth factors. It is produced by fibroblasts and endothelial cells within the niche and it stimulates the proliferation of primitive hematopoietic cells. SCF binds to its receptor c-kit, which is expressed on the surface of HSCs. The **SCF/c-kit signaling** plays an important role in migration, survival, proliferation and differentiation of HSC in the bone marrow niche (Nakamura et al., 2004). It has been reported that loss of SCF leads to hematopoietic failure (Bernstein et al., 1991) and that SCF might therefore have an essential role in the control of HSC activation and the mobilization of HSCs from the niche into the blood stream (Heissig et al., 2002; Yin and Li, 2006).

Under stress and injury, the c-kit ligand is converted into **soluble Kit ligand (s-kit),** it can bind to SCF with the same affinity as c-kit. Thereby s-kit inhibits the ability of SCF to bind to the HSC. This inhibition stimulates the entry of HSCs into the cell

cycle, the mobilization to the vascular niche and the differentiation. The Mechanisms that regulate the HSCs mobilization are not fully understood up to now (Nakamura et al., 2004).

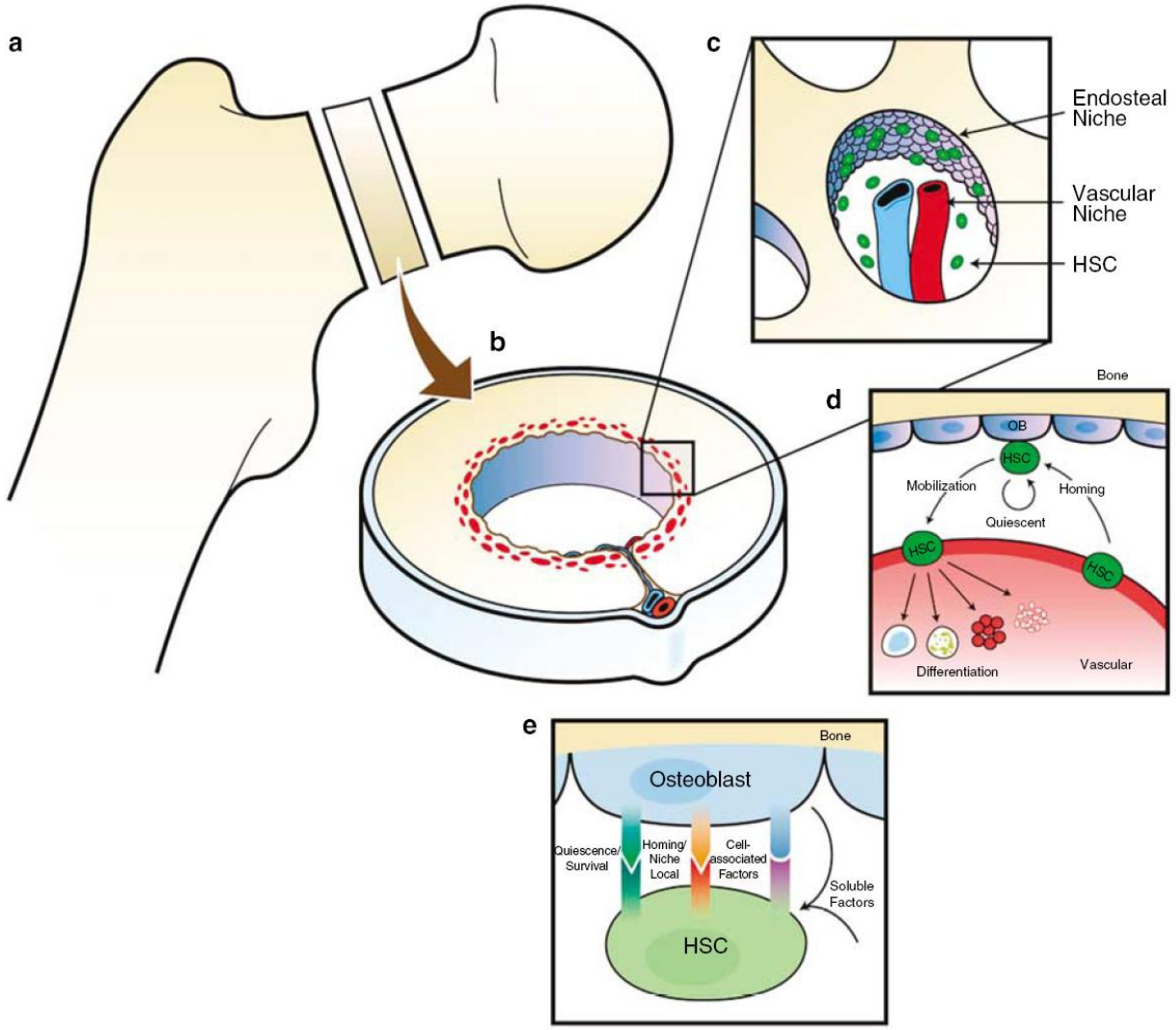


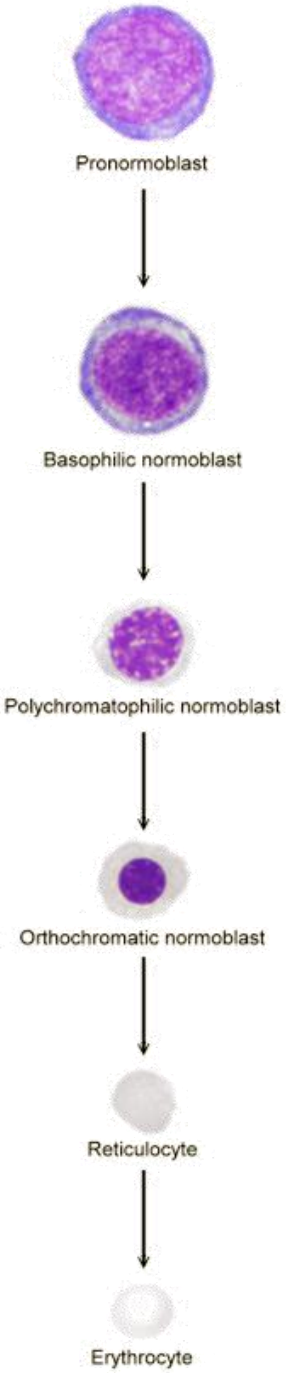
Figure 4: Niches for HSCs in adult bone marrow

*The niche in the bone marrow **(a,b)** can be divided into two parts: the **osteoblastic niche** and the **vascular niche (c)**. While the vascular niche supports the differentiation into hematopoietic progenitor cells and the mobilization into the blood stream **(d)**, the osteoblastic niche keeps the balance between self-renewal and cell-cycle quiescence. **(e)** In the osteoblatic niche the HSCs adhere to the niche cells through the adhesion molecules and through factors produced by osteoblasts so that the HSCs are kept in a quiescence state. Picture taken from: (Shiozawa et al., 2008)*

1.2 Human erythropoiesis

During erythropoesis, early erythroid progenitor cells differentiate into enucleated red blood cells (RBC) (Palis, 2008). Eryhtropoiesis is a process of multiple stages and depends on the correct balance between cell proliferation, differentiation and apoptosis (Orkin and Zon, 2008; Weissman, 2000). The disruption of this equilibrium/balance leads to haematological diseases like aplastic anaemia and thalassemia (Testa, 2004).

Figure 5 a):



Erythropoiesis is a process of multiple differentiation steps , see figure 5 a) and figure 5 b):

The myeolid stem cell gives rise to the pronormoblast. When the cytoplasm becomes more basophilic due to the presence of ribosomes, **the pronormoblast** has turned into a **basophilic normoblast.**

During the ongoing development the nucleus shrinks, the cell becomes smaller and begins to produce hemoglobin. At this stage the cell is called **polychromatophilic normoblast**.

When the nucleus moves to the periphery of the cell and the cytoplasm becomes more eosinophilic, the cell converts into an **orthochromatic normoblast**.

When the orthochromatic normoblast extrudes its nucleus, it enters the blood circulation as a **reticulocyte.** Reticulocytes were named after the characteristic reticular networks of polyribosomes which they contain. After 1-2 days reticulocytes loose their polyribosomes and become mature red blood cells **(erythrocytes).**

Mature erytrhocytes are biconcave disks without nucleus, mitochondria and other organelles and their main function is to bind oxygen through the accumulated hemoglobin (Tsiftsoglou et al., 2009).

Picture taken and modified from:
http://sakurako.iza.ne.jp/blog/entry/1362003/

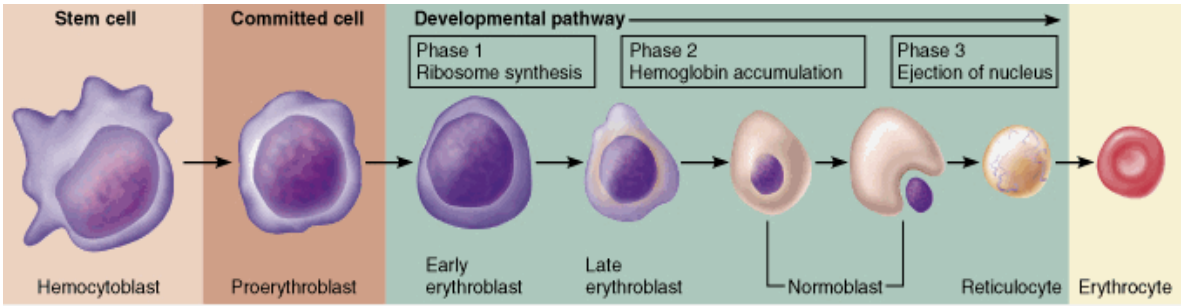


Figure 5 b): The differentiation steps in erythropoiesis
Hematopoietic stem cell becomes commited (pronormoblat) and differentiates into early basophilic normoblast, which contains ribosomes (phase 1), then the the basophilic normoblast (or early erythroblast) accumulates iron and hemoglobin (phase 2) and finally ejects organelles and the nucleus (phase 3) and is released into blood stream as a reticulocyte, where it differentiates into a mature erythrocyte. Picture from taken from: http://legacy.owensboro.kctcs.edu/gcaplan/anat2/notes/Notes6%20Blood%20Cells.htm

When erythrocytes differentiate starting from HSC, the cells undergo characteristic changes: The nucleus of hematopoietic stem cells is big and contains open chromatin. When HSC mature into red blood cells, the chromatin of the nucleus condenses and the cytoplasmic matrix increases in amount, the cytoplasm turns from basophilic to acidophilic due to the decrease in the amount of RNA and DNA, the nucleus decreases in size and is finally extruded. In the process of maturation a basophilic pronormoblast is converted from a cell with a large nucleus and a volume of 900 fL to an enucleated disc with a volume of 95 fL (Testa, 2004).

1.2.1 Erythropoiesis takes place in erythroblastic islands

Erythropoiesis takes place in the bone marrow in the so called **erythroblastic islands** (Allen and Dexter, 1982). Erythroblastic islands are microenvironmental compartments, where erythroblasts proliferate and differentiate into mature reticulocytes (Bessis, 1958). Those islands consist of a **central macrophage** surrounded by developing erythroblasts of various differentiation stages (see figure 6) (Bessis, 1958; Tsiftsoglou et al., 2009).
The number of erythroblasts surrounding the macrophages can vary from 5 to 30 erythroblasts per island. Erythroblastic islands are distributed over the entire bone marrow and are not localized to specific regions (Chasis, 2006).

The central macrophage has been shown to function as a **"nurse" cell**, as it is providing the iron for the hemoglobin synthesis and other nutrients needed for erythropoiesis (Bessis and Breton-Gorius, 1962). It gives proliferative and survival signals to the erythroblasts and takes up the nuclei from the enucleated orthochromatic normoblasts by phagocytosis (Chasis, 2006; Skutelsky and Danon, 1972). There is also evidence that macrophages are promoting the enucleation itself. The cultivation of erythroblasts in the absence of macrophages leads to decreased erythroid cell proliferation, maturation and enucleation as well as increased apoptosis (Chasis and Mohandas, 2008).

Macrophages secrete several cytokines, which stimulate the differentiation and proliferation, like for example **insulinlike growth factor-1** (**IGF-1)**, that has been shown to stimulate growth of erythroblasts (Kurtz et al., 1982).
In macrophages also the mRNA of **erythropoetin (EPO)** has been detected (Rich et al., 1988), which suggests that they could provide EPO to the surrounding erythroblasts and thereby stimulate differentiation and inhibit apoptosis.

Within the erythroblastic islands erythropoesis is regulated by positive and negative feedback loops, which occur by soluble factors. Such soluble factors are for example **SCF, EPO and VEGF**, which activate erythropoiesis via positive feedback mechanisms, whereas **IL-6, TGF-ß, TNF-α and IFN-γ** are inhibiting erythropoiesis and promoting cell death of erythroblasts. IL-6, for example, is inhibiting the iron release from the central macrophage in the erythroblastic island (Tsiftsoglou et al., 2009).

Also the cell-cell interactions between the erythroblasts and the central macrophages trigger intracellular signaling pathways, which then lead to changes in differentiation-, proliferation- and apoptosis- related processes (Chasis and Mohandas, 2008). And components of the extracellular matrix, like **fibronectin and laminin** influence terminal differentiation and migration of erythroid cells by interacting with fibronectin receptors on the surface of erythroblasts (Chasis and Mohandas, 2008).

Erythroblastic islands

a)

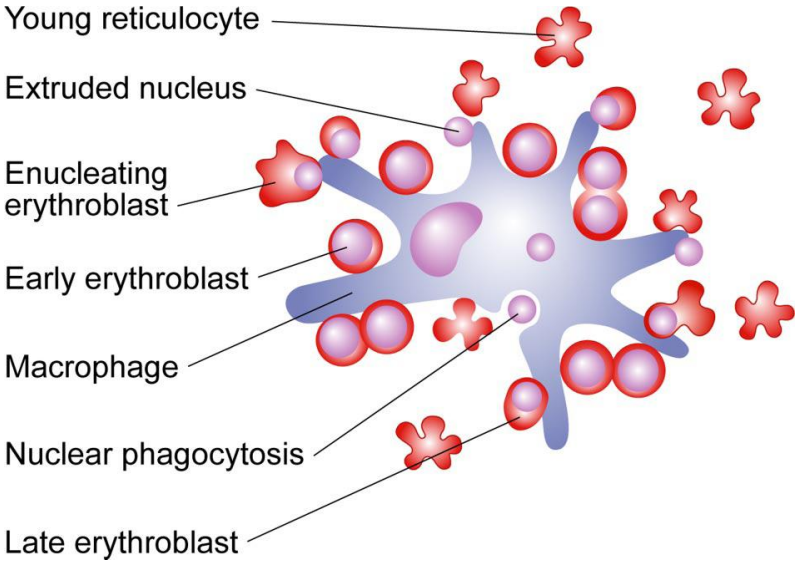
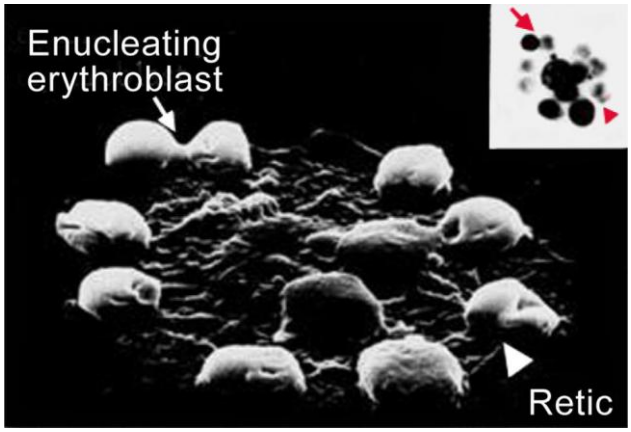


Figure 6:
Erythropoiesis occures in erythroblastic islands

a) Developing erythroblasts of different stages of maturation surround a central macrophage, which is providing iron for the hemoglobin synthesis, takes up extruded nuclei from differentiating normoblasts and thereby stimulates the differentiation into reticulocytes.
(Chasis and Mohandas, 2008)

b)



b) Scanning electron micrograph of an isolated erythroblastic islands.
Arrow shows enucleating erythroblast.
(Chasis and Mohandas, 2008)

1.3 Clinical use of HSCs

During postnatal life HSCs populate a very small compartment, less than 0,05% up to 0,5% of the cells in the bone marrow consist of HSCs (Gunsilius et al., 2001), it is estimated that 1 in 10,000 bone marrow cells and 1 in every 100.000 blood cells are hematopoietic stem cells. HSCs can be used to treat bone marrow failure and aplastic anaemia by transplantation of HLA-identical allogeneic hematopoietic progenitors (Congdon, 1957; Kondo et al., 2003). The source of HSCs for transplantation can be the bone marrow, but also autologous HSCs isolated from peripheral blood can be used for restoration of hematopoiesis in patients with malignant diseases after chemotherapy (Gunsilius et al., 2001). Another clinical use in the future could be the use of genetically modified HSC to cure metabolic defects even at embryonal stage, but this method is still under investigation and is still far away from clinical use. After transplantation of allogeneic or autologous HSCs, hematopoiesis can be restored and maintained by a small number of clones. Besides bone marrow and mobilised peripheral blood, in the last years umbilical cord blood was found to be a good source of hematopoietic stem cells.

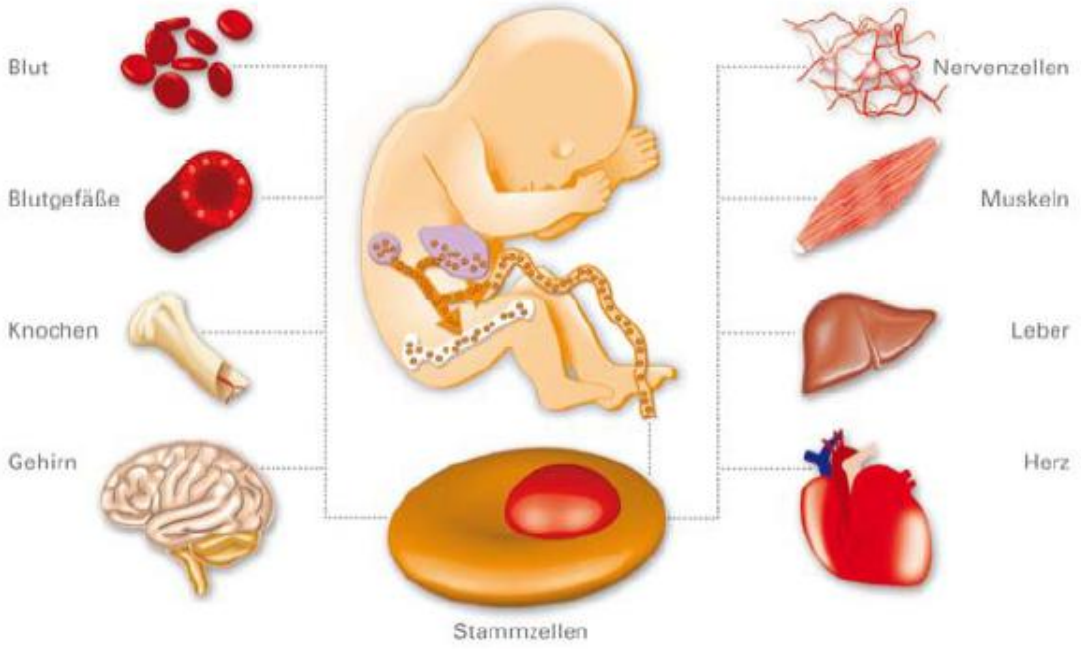


Figure 7: Clinical use of HSCs isolated from umbilical cord blood
(picture taken from: www.vivocell.org)

Compared to the bone marrow, the use of mobilized blood stem cells from the peripheral blood or the cord blood has the advantage of a more rapid hematopoietic recovery, therefore they are slowly replacing the bone marrow as alternative source of stem cells. The umbilical cord blood is richer in stem cells with a higher proliferative potential than bone marrow or peripheral blood (Gunsilius et al. 2001). But the drawback is that just a limited number of HSCs can be isolated from a single umbilical cord blood sample. The required amount of HSC for transplantation is $2x10^6$ CD34+ cells/kg body weight. In umbilical cord blood, the percentage of CD34 + stem cells is about 1% of the mononucleated cells. Therefore, the minimum cell dose required for restoration of haematopoiesis in adult patients can usually not be reached using HSCs from a single umbilical cord blood sample. One solution for the problem of too low HSC numbers isolated from one cord blood could be the in vitro expansion of HSCs leading to cell numbers that are high enough for the transplantation in adults. Therefore CD34+ cells could be cultivated for seven to 14 days in a liquid culture system with a combination of various cytokines (e.g. SCF, IL-3, IL-6, EPO) and then re-infused to the patients. But it has been reported that expansion of HSC in vitro led also to differentiation of the cells, whereby the stem cell potential and the life-long self-renewal capacity of the expanded cells got lost (Gunsilius et al., 2001).

1.4 Erythroid cells in culture

Although up to know the expansion of HSCs is not optimised yet, the cultivation of primary erythroid progenitor cells isolated from human cord blood is a valuable model system for the study of hematopoiesis *in vitro* or *ex vivo*. Mononuclear cells can be isolated from human cord blood samples, expanded in serum free medium to **human erythroid progenitor cells (hEPCs)** and subsequentlty induced for terminal erythroid differentiation (Dolznig et al., 2005; Leberbauer et al., 2005).

The so expanded EPCs show renewal and differentiation characteristics, which are similar to the *in vivo* situation. In comparison to other primary model systems, which often suffer from insufficient cell numbers or from heterogeneity of cell populations, the EPCs can be expanded to high numbers keeping a homogeneous cell population.

Steady state erythropoiesis involves just a limitied number of differentiation divisions and is controlled mainly by Erythropoietin (EPO). **Stress erythropoiesis** *in vivo* is induced by hypoxia or anaemia and is co-regulated by erythropoietin, stem cell factor and glucocorticoids. *Ex vivo* expanded erythroid progenitor cells mimic the process of stress erythropoiesis, as they are cultivated in serum free medium, which is supplementd with those factors, which are active in stress erythropoiesis: low EPO, SCF and DEX (Dolznig et al., 2005; Kolbus et al., 2003). In this model system, cells retain the proerythroblast phenotype, but can also be induced to terminally differentiate, if the renewal factors are replaced by differentiation factors: high EPO, Insulin (Dolznig et al., 2005).

This *in vitro* expansion system can be used as a model to study molecular and cellular events of hematopoiesis and additionally used to find the best conditions to enable an *in vitro* expansion of hematopoietic stem cells to high numbers of progenitor cells, which then could be used for transfusion in patients suffering from hematological disorders.

1.4.1 Growth factors for in vitro proliferation

For *in vitro* expansion of human erythroid progenitor cells, the mononuclear cells isolated from cord blood samples can be cultivated in serum free medium, supplemented with a factor mix of **EPO, SCF, DEX, IGF-1** and **Lipids.**

EPO- Erythropoietin

Epo is one of the most important factors in the regulation of erythropoiesis, it is produced mainly in the fetal liver and the adult kidney. EPO binds to its receptor (EpoR), which is a member of the hematopoietic cytokine receptor superfamily, whereby the receptor undergoes a conformational change and activates several pathways. Signal transduction by EPO is mediated through the EpoR/Jak2/Stat signalling cascade (Miura et al., 1994; Witthuhn et al., 1993). It has been reported that EPO protects erythroid progenitor cells from apoptosis by activating antiapoptotic proteins like Bcl-X(L) (Dolznig et al., 2002) and that it is essential for terminal differentiation by stimulating hemoglobin synthesis (Dorn et al., 2008). EPO-deficient mice or mice lacking the EpoR die at the embryonic stage of abscence of definitive erythrocytes (Lin et al., 1996).

SCF- stem cell factor

SCF is an important proliferation factor that plays an essential role in hematopoiesis: it promotes proliferation of erythroid progenitor cells by stimulating DNA synthesis (Dorn et al., 2008). SCF is a member of the type III superfamily of receptor tyrosine kinases. It is expressed mainly in hematopoietic progenitor cells, B- and T- cell progenitors, mast cells and germ cells (Tsiftsoglou et al., 2009).
In mice deficient for the receptor of SCF, **c-Kit**, erythrocytes are generated, but these KO-mice die at birth because of severe anaemia and their bone marrow contains a reduced number of erythroid progenitors (Broxmeyer et al., 1991; Nocka et al., 1990).

SCF and EPO are both required for the proliferation and survival of erythroid progenitor cells *in vitro*. During terminal erythroid differentiation, the stem cell receptor c-kit is downregulated, whereas EPO becomes the essential survival factor. If EPO and SCF were added simultaneously to an *in vitro* culture of hEPCs,

it has been shown that the proliferative effects were additive, which suggests that SCF and EPO might cooperate with each other. Importantly, when EPO was added alone and in higher concentrations, differentiation was induced (Muta et al., 1995; Muta et al., 1994).

DEX- Dexamethasone

Addition of glucocorticoids, like **Dexamethasone (DEX),** is required to ensure optimal proliferation rates of *in vitro* cultures with low spontaneous differentiation. Dexamethasone regulates a variety of developmental processes by binding to the **glucocorticoid receptor (GR)** and thereby activating the glucocorticoid receptor pathway. Leberbauer et al. could demonstrate that dexamethasone stimulates proliferation and blocks spontaneous differentiation of erythroid progenitors (Leberbauer et al., 2005). *In vivo* the GR is required for stress erythropoiesis. It has been reported that GR-deficient- mice are viable, show normal erythropoiesis, but show no stress-induced erythrpoiesis in spleen, which means they could not respond to hypoxia or blood loss with increased erythroblast renewal (Bauer et al., 1999). In chicken and mouse erythroblasts, the activated GR cooperates with the activated EpoR and c-kit (SCF receptor). This cooperation keeps erythroid progenitor cells in an immature state and induces long-term proliferation (Wessely et al., 1997). A similar effect of glucocorticoids has been described in human erythroblasts (von Lindern et al., 1999).

IGF-1- Interleukin-growth factor-1

Panzenböck et al. demonstrated that addition of **Interleukin-growth-factor-1 (IGF-1)** to the serum-free cultures also stimulates proliferation and inhibits differentiation of hEPCs. IGF-1 appears not to be a crucial growth factor, but it does further improve the proliferation conditions (Panzenbock et al., 1998).

Lipids

In addition, **cholesterol-rich Lipids** were found to be essential for the extended proliferation of human EPCs. Addition of Lipids extended the proliferation periode from 30 to 45 days (Leberbauer et al., 2005).

Addition of **fetal bovine serum (FBS)** was shown to limit the expansion of erythroid progenitor cells compared to serum-free medium, even when the crucial factors like EPO, SCF and DEX were provided (Leberbauer et al., 2005).
In summary, a combination of EPO, SCF, IGF-1, DEX and Lipids in serum free media are the best culture conditions to reach optimal proliferation rates of human erythroid progenitor cells.

1.5 Glucocorticoid Receptor (GR)

Glucocorticoids have an important function in multiple physiological and developmental processes. By binding to its receptor (GR), glucocorticoids modulate the activity of various target genes, which then influence the differentiation and proliferation of e. g. hematopoietic stem cells.
The GR cooperates with the activated **EpoR** (EPO Receptor)**,** and **c-Kit** (SCF Receptor) to maintain erythroid cells in an immature state, inhibit terminal differentiation and to maintain the long-term proliferation potential of these cells (Dolznig et al., 2006; Srivastava et al., 2006).

The Glucocorticoid Receptor is a **steroid hormone receptor** that belongs to the nuclear receptor superfamily of transcription factors (Revollo and Cidlowski, 2009). Glucocorticoids are the most widely prescribed pharmaceuticals worldwide and key drugs for treating disesases like hematological cancers (leukemia, inflammatory states). The GR has a DNA binding domain (DBD) with which it can interact directly with target genes that contain a glucocorticoid response element **(GREs)** or a negative glucocorticoid response element **(nGREs)** in their promotor region, see figure 8 (Wessely et al., 1997).

The Glucocorticoid Receptor is a ligand-regulated transcription factor, which in abscence of glucocorticoids is situated in the cytoplasm in an inactive form, where it is associated with the heat shock protein 90 chaperone complex, that keeps the receptor inactive (Paakinaho et al.).

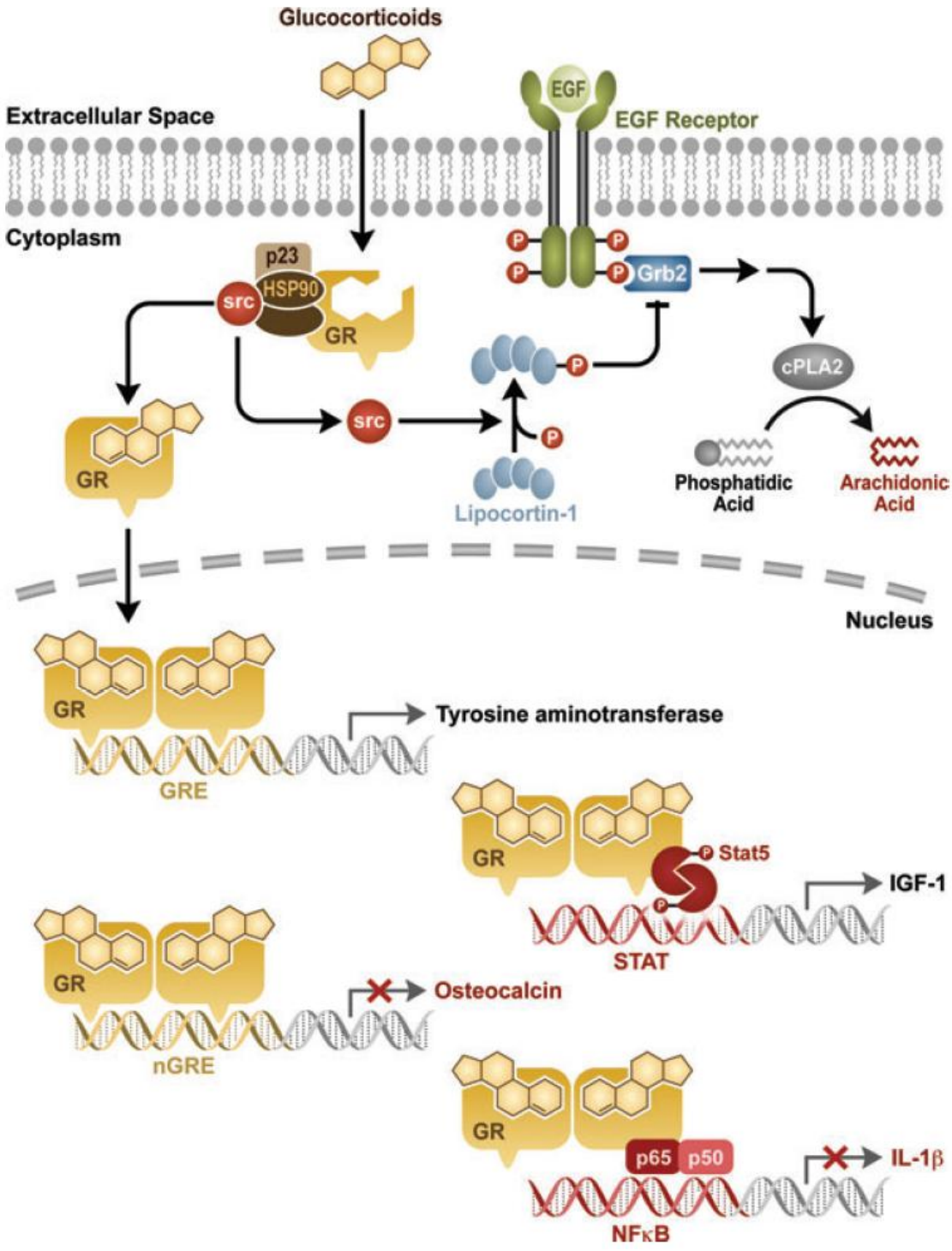


Figure 8: The Glucocorticoid Signaling
Glucocorticoid signaling induces changes in gene expression. When Glucocorticoids bind to the GR, the ligand-bound-GR-complex dissociates and translocates into the nucleus, where it can ***activate or inhibit gene expression*** *by protein-protein interaction with Transcription factors, like STAT5 or NFkB or by directly binding to DNA with their DNA binding domain via GRE or nGREs (GRE=Glucocorticoid Response Elements) (nGREs=negative Glucocorticoid Response Elements); (picture taken from: (Revollo and Cidlowski, 2009).*

1.5.1 Glucocorticoid signaling

When the ligand (different glucocorticoids) binds to its receptor (GR), the receptor is activated, and dissociation of the Ligand-bound-GR-complex is induced (Amaral et al., 2009; Stellacci et al., 2009). The activated receptor then translocates to the nucleus, where it acts as a transcription factor and influences gene expression in two ways:

It can either activate or inactivate gene expression:
→ by **protein-protein interaction**: it associates directly with transcription factors, like STAT5 or NF-kB, and thereby activates or inhibits the transcription of specific target genes.
→ by **direct DNA binding:** it can directly bind to target genes on DNA level by associating with high affinity to short DNA sequences (DNA binding sites: via GREs and nGREs) (Revollo and Cidlowski, 2009).

1.5.2 Glucocorticoid Receptor and erythropoiesis

The Glucocorticoid Receptor is a key regulator of the decision between self renewal and differentiation of erythroid progenitors.
Dexamethasone in combination with SCF and EPO, has been shown to down-regulate **GATA-3** expression and to up-regulate the expression of **GATA-1** (Srivastava et al., 2006).
GATA-1 and GATA-3 are members of the GATA gene family. Members of this family are expressed at very early stages of hematopoiesis and are key regulators of proliferation and survival of early hematopoietic cells under the influence of factors such as glucocorticoids, SCF and EPO (Srivastava et al., 2006). In media lacking a GR ligand or containing a GR antagonist, erythroid progenitors faile to self-renew (Wessely et al., 1997). In avians glucocorticoids were shown to stimulate the formation of erythroid colonies *in vitro*. In the presence of glucocorticoids lower concentrations of erythropoietin are required to induce maximal erythroid cell proliferation (Udupa et al., 1986).

1.5.3 Glucocorticoid Receptor target genes

In a microarray screen performed with RNA from EPCs stimulated with Dexamethasone (alone or in combination with different factors), several target genes of the GR have been identified, as they were either upregulated or downregulated in the prescence of DEX (Kolbus et al., 2003; Rubiolo et al., 2006) and data not shown). The most prominent upregulated GR-targets genes were: Raf-1, Tis11d and FKBP51.

- **Raf-1**

Raf-1 is a 74 kDa **serine/threonine kinase** that is located in the cell cytoplasm and is activated by phosphorylation (Muszynski et al., 1997). The Raf kinases are key signal transducers that are activated by mitogens or oncogenes.
During differentiation of primary mouse erythroblasts Raf-1 has been demonstrated to be downregulated (Kolbus et al., 2002).
Raf-1 deficient mouse embryos are anaemic, and die at midgestation with anomalies in the placenta and fetal liver, which contained lower numbers of erythroid progenitors than the wild type livers (Mikula et al., 2001). The *in vivo* and *in vitro* studies with genetically modified fibroblasts and hematopoietic cells (Kolbus et al., 2002; Mikula et al., 2001), revealed that in summary in the mouse system the main functions of Raf-1 are:

→ the inhibition of apoptosis
→ the stimulation of proliferation and
→ the regulation of erythroid maturation by delaying erythroid differentiation through inhibition of caspase activation.

- **Tis11d**

Also **Tis11d**, a **zinc-finger-like protein** and member of the TTP protein family, has been shown to be a GR- target gene in erythroid progenitor cells. Members of the Tis11-family of proteins contain two tandemly repeated zinc finger motifs, by which they can bind to adenine-uridine rich elements in different mRNAs. They

have been reported to induce degradation of a variety of different mRNAs, containing AU-rich elements in the 3' untranslated region (Baou et al., 2009).
Since many mRNA transcripts contain AREs, it is possible that glucocorticoid signaling regulates the expression of many genes in this manner (Revollo and Cidlowski, 2009). Little is known about the function of Tis11d in humans and generally in erythropoiesis. Glucocorticoids have been reported to induce TIS11 mRNA and protein in lung epithelial cells and this induction may be important for the glucocorticoid mediated control of inflammatory gene expression (Smoak and Cidlowski, 2006). It has been demonstrated that fetal liver cells isolated from Zfp36l2 knock-out mice (Zfp36l2 is the synonym for Tis11d in the mouse system) were unable to reconstitute the hematopoietic systems in lethally irradiated WT-mice, which suggests that Zfp36l2 might be required for self-renewal and maintenance of adult HSCs (Stumpo et al., 2009). These studies demonstrate that Zfp36l2 plays an important role in definitive hematopoiesis during mouse development, but little is known about the specific function of Tis11d in human erythropoiesis.

- **FKBP51**

FKBP51 (FK506-binding protein 51) is a 51 kDA co-chaperone protein, which belongs to the immunophilin protein family and is a component of the heat shock protein 90 chaperone complex (HSP90 complex). This immunophilin has isomerase activity, which performs important biological functions in the cell. The immunophilin family is a highly homologous multigene family, which is containing at least 5 other proteins, that can bind to the protein FK506 (Park et al., 2007; Romano et al.). In a recent genome-wide profiling of human lung biopsies, FKBP51 has been identified as the most highly glucocorticoid-induced gene (Woodruff et al., 2007). FKBP51 is expressed in various human cell tissues, mainly in brain, muscle, liver and thymus (Vermeer et al., 2003). Overexpression of FKBP51 limits the Glucocorticoid Receptor signaling. In human A549 lung cancer cells, glucocorticoid (Dex) exposure led to a rapid accumulation of FKBP51 mRNA (Paakinaho et al.). The specific function of FKBP51 in hematopoietic cells is not fully understood up to now, but it is estimated that it may play an important role in cell survival, proliferation and differentiation (Giraudier et al., 2002).

1.6 Apoptotic pathways

Apoptosis or programmed cell death is a highly regulated process, which allows a cell to **self-degrade**. Cell death is a fundamental cellular response that plays an important role in development and homeostasis of multicellular organisms.

As shown in figure 9 below, apoptosis involves specific morphological and biochemical changes within the cell, such as cytoplasm condensation, nucleus shrinkage, nuclear chromatin condensation, loss of mitochondral membrane potential and DNA fragmentation. These processes are then followed by breakage of the cells into membrane-bound apoptotic bodies, that contain a variety of cytoplasmic organelles and nuclear fragments, which finally are engulfed by surrounding cells (Testa, 2004; Van Herreweghe et al., 2010). The apoptotic process can be divided into three phases: **initiation, integration/decision** and **degradation**. The initiation stage is heterogenous and depends on the type of apoptosis-inducing stimulus. During the integration phase caspases and mitochondrial effectors are activated, while in the execution stage the cell is broken into cell fragments.

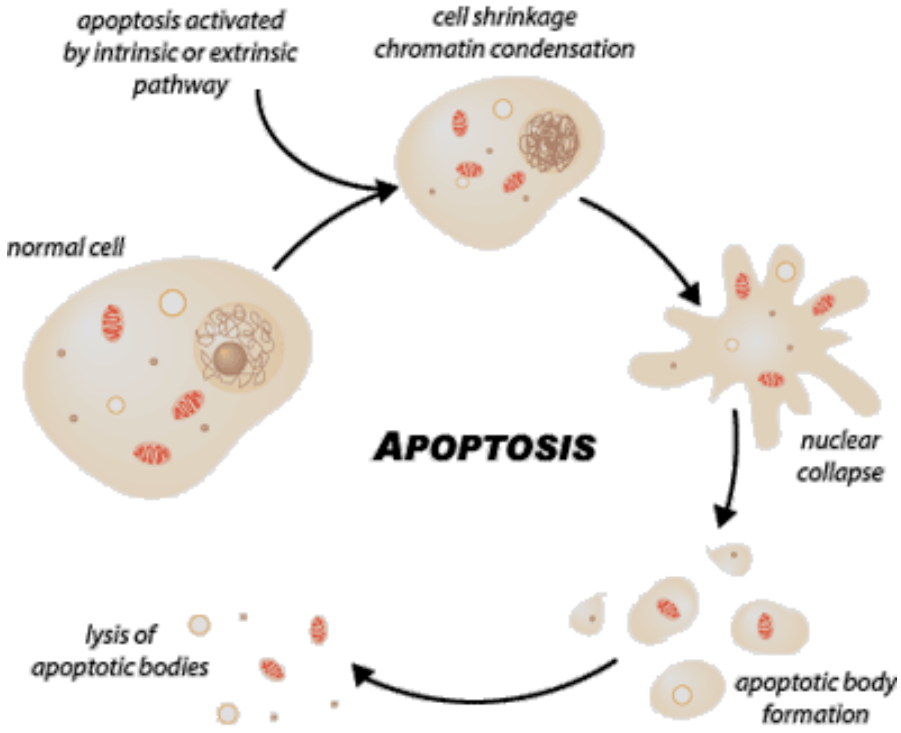


Figure 9: Morphological changes within the cell during the apoptotic process
(picture taken from: www.scq.ubc.ca/apoptosis)

The **cysteinyl aspartate proteinases**, called **Caspases** play a central role in the regulation and control of apoptosis. They are a group of evolutionarily conserved proteases, which can be found in nearly all animal cells. In recent years it has been shown that Caspases are not only involved in apoptosis, but also in other cellular processes. High levels of Caspase activation lead to apoptosis, whereas low levels of activated Caspases have been shown to play a role in cell proliferation and differentiation of erythroid cells and macrophages (Droin et al., 2008). Caspases are synthesized within a cell as inactive percursors: the **Procaspases**. The inactive percursors can be activated by cleavage into a big and a small subunit. Caspases can be classified into **Initiator Caspases** (Caspase-2, -8, -9, -10) and **Effector Caspases** (Caspase-3, -4, -5, -6, -7, -11, -12, -13). Initiator Caspases function as activators of Effector Caspases. Caspase-8 for example promotes cleavage and thereby activation of various downstream Caspases, such as Caspase-3, -6 and -7. Activated Effector Caspases in turn induce degradation of regulatory proteins within the cell (Amaral et al., 2009; Van Herreweghe et al., 2010).

When cells undergo apoptosis several pathways can be involved. In mammalian cells the apoptotic response is mediated either by an instrinsic or extrinsic pathway. Both pathways are Caspase-dependent, but different Caspases are involved.

The extrinsic pathway is initiated through the stimulation of transmembrane death receptors, such as **TNF-Receptor1** (TNF-R1) or **Fas**, which are located on the surface of the cell membrane, wheareas the intrinsic pathway is initiated through the release of signalling factors by mitochondria within the cell.

1.6.1 The extrinsic pathway

In the extrinsic pathway (see figure 10 and 11), soluble ligands, such as FasL or TNF-α, bind to their **transmembrane death receptors**. For example TNF-α binds to the TNF-R1 and FasL binds to the Fas-Receptor. The binding of TNF-α to the TNF-R1 induces a conformational change of the receptor. This conformational change leads to recruitement of **RIP1** (protein serine-threonine kinase 1) and binding of the adaptor protein **TRADD** (TNF-receptor associated via death domain) to the death domain (DD) of the receptor on the cytoplasmic side (see figure 10).

Subsequently, TRADD recruites **FADD** (Fas-associated via death domain), another adaptor protein, which binds to the TRADD/RIP-complex on the death domain of the receptor. FADD then induces recruitement of the **Procaspase-8** (an Effector Caspase) to form the death-inducing signal complex **(DISC)**.

At a high concentration, the Procaspase-8 induces its autoproteolytic activation and is further activated by dimerisation into the active Caspase-8 form. This activation is sufficient to induce apoptosis. In the extrinsic pathway, the activated Caspase-8 directly activates the Effector Caspase Caspase-3, which initiates the degradation of the cell and thereby induces apoptosis (Amaral et al., 2009; Van Herreweghe et al., 2010).

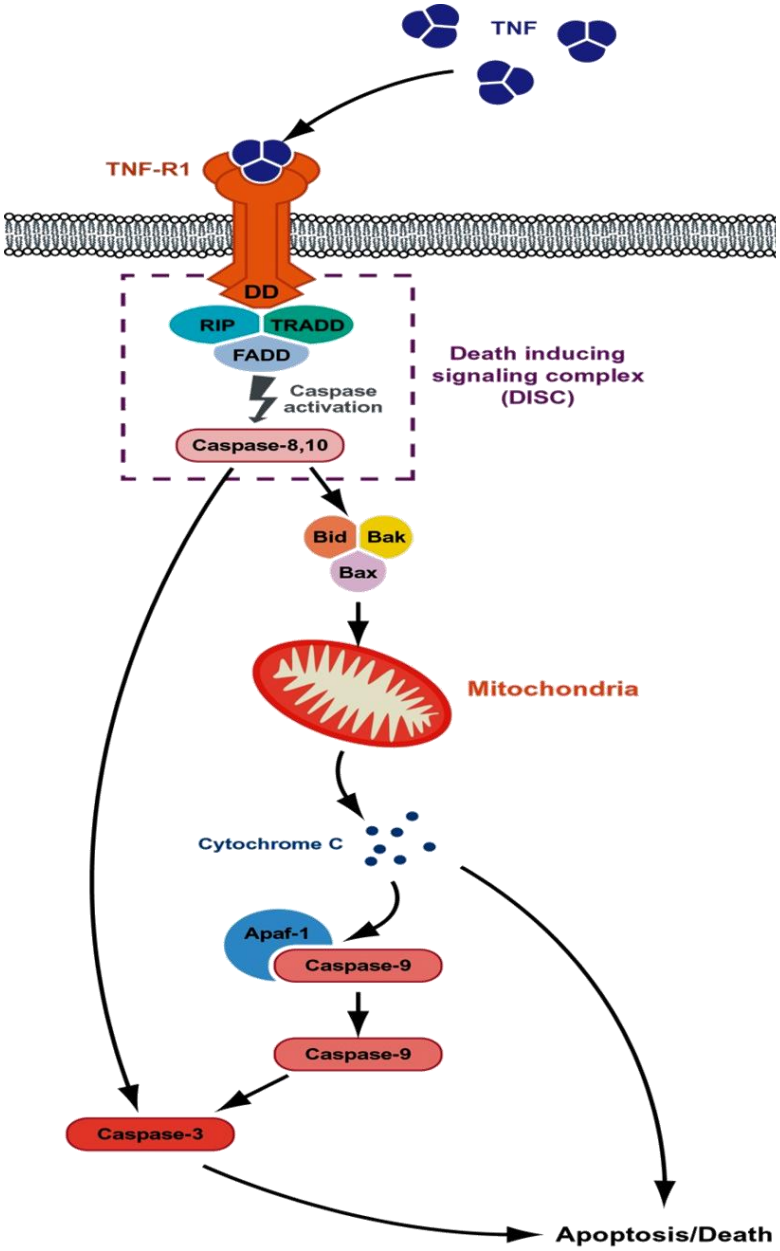


Figure 10: The extrinsic and instrinsic pathway

If TNF-α binds to its receptor TNF-R, a conformational change of the receptor is induced and the RIP1/TRADD/FADD complex is build. By binding to this complex the Caspase-8 is activated and apoptosis is induced either through the extrinsic or intrinsic pathway. In the extrinsic pathway Caspase-8 induces direct Caspase-3 activation, where-as in the intrinsic pathway Caspase-3 activation is induced after activation of proteins of the BCL-2-family. Picture taken and modified from:

(Rahman and McFadden, 2006).

1.6.2 The intrinsic pathway

The intrinsic pathway (see figure 11) is regulated by proteins of the **BCL-2-family**, such as **BAX** (Bcl-2-associated X protein) and **BAK** (Bcl-2- antagonist/killer), which are activated by **BID** (BH3 interacting domain death agonist). These pro-apoptotic proteins are crucial in regulating a wide range of apoptotic stimuli. The antiapoptotic members of the BCL-2-family like Bcl-2, are mainly localized in the outer mitochondrial membrane, wheareas proapoptotic members, such as BAX and BAK are situated in the cytosol as inactive percursors. After induction of apoptosis, BAK and BAX undergo conformational changes, oligomerize and translocate from the cytosol to the outer mitochondrial membrane.

The intrinsic pathway is triggered by cellular stress or activated Caspase-8. Active Caspase-8 can cleave BID to **tBID**, which in turn activates BAX and BAK. Active BAX binds to its receptors in the outer membrane of the mitochondria. The insertion of BAX in the mitochondrial outer membrane is required for the pore formation, which results in mitochondrial outer mebrane permeability **(MOMP)**. This permeability induces the release of **cytochrome c** and other mitochondrial factors. Cytochrom c then binds to the subunit of **APAF-1** (apoptotic peptidase activating factor 1), which induces conformational change of APAF-1 and thereby its activation through hydrolysis of ATP.

A complex of seven activated APAF-1-molecules is forming the so called **apoptosome**, which binds to the Procaspase-9 (Initiator Caspase) and induces its activation. The so activated Caspase-9 is interacting with the apoptosome, and thereby inducing the activation of Procaspase-3 or -7 by proteolytic cleavage. Activated Caspase-3 and -7 are then inducing cell degradation and apoptosis by cleavage of many proteins from different cellular compartments, leading finally to the cellular disintegration (Amaral et al., 2009; Van Herreweghe et al., 2010).

1.6.3 NF-kB activation inhibits apoptosis

Binding of TNF-α to its receptor, can either induce apoptosis through the extrinsic or intrinsic pathway, or activate **NF-kB** (nuclear factor kappa B) (see figure 11). The transcription factor NF-kB plays a critical role in inflammation, control of cell death pathways and cell proliferation. There are 5 known members of the NF-kB family: NF-kB1 (p50/p105), NF-kB2 (p52/p100), c-Rel, RelB and RelA (p65). All different members are distinguished by the Rel homology domain, the part of the protein that controles the DNA binding. They form various homodimers and heterodimers bound to DNA (Adli et al., ; Bouwmeester et al., 2004).

The transcriptional activity of NF-kB is controlled by its intracellular localization. NF-kB is located as an inactive form in the cytoplasm, where it is associated with the **inhibitor proteins IkBs**. In most mammalian cells, IkBα and IkB-β represent the two major forms of the IkB family. The phosphorylation of the serine residues of the IkB proteins leads to the proteolysis by the 26S proteasome, which results in the liberation of NF-kB and its translocation into the nucleus.

It has been demonstrated that the NF-kB pathway can be induced by TNF-α (Bouwmeester et al., 2004). Thus, RIP-1, which together with TRADD is bound to the death domain of the activated TNF-R1, leads to direct association of **TRAF2**. The binding of TRAF2 to the RIP-1/TRADD/TNF-R1 complex is crucial for the activation of the NF-kB pathway. TRAF2 recruits the multicomponent protein kinase **IKKαβ**, which is then activated and phosphorylated by RIP1. IKK can be activated by phosphorylation in response to a variety of stimuli not only by the TNF-R (see chapter 4.3 of this study). pIKKαβ in turn phosphorylates **IkB-α**, which leads to degradation of IkBα and NF-kB release.

NF-kB translocates to the nucleus and thereby accumulates and mediates the transcription of anti-apoptotic factors and of a variety of proteins, which are involved in cell survival and proliferation (Komura et al., 2005; Merkhofer et al.).

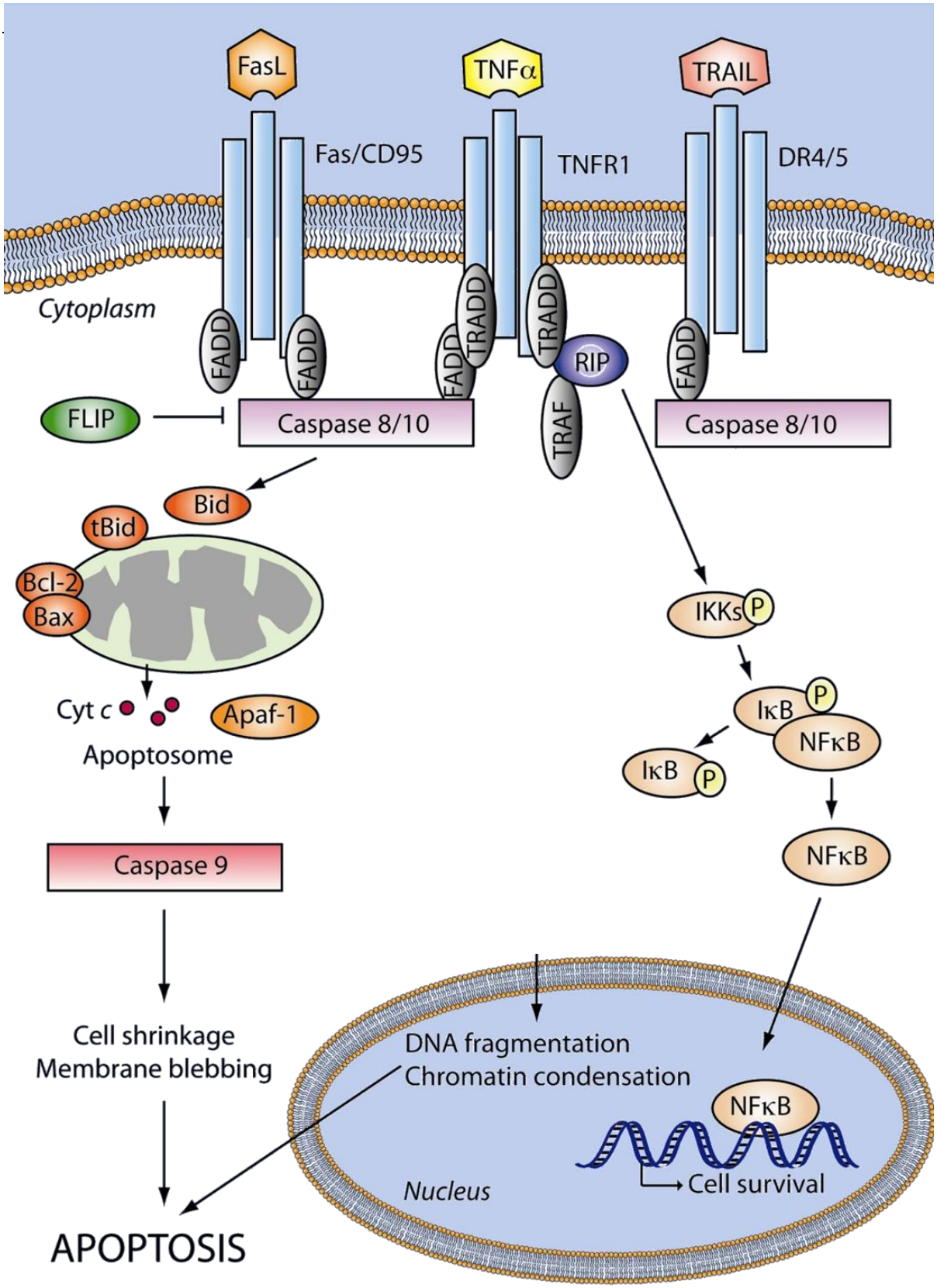


Figure 11: TNF-α mediated pathways

If TNF-α binds to its receptor TNF-R1, either apoptosis can be induced or NF-kB is activated. After building of the RIP1/TRADD/FADD complex either Caspase-8 is activated and apoptosis is induced through the extrinsic or intrinsic pathway, or NF-kB is activated, through phosphorilation of IKK, which in turn phosphorilates IKB and induces release of NF-kB into the nucleus, where it can stimulate the expression of different pro-apoptotic genes. (Picture taken and modified from: Krakstad and Chekenya, 2010).

1.7 Cell-surface markers for human erythroid progenitor cells

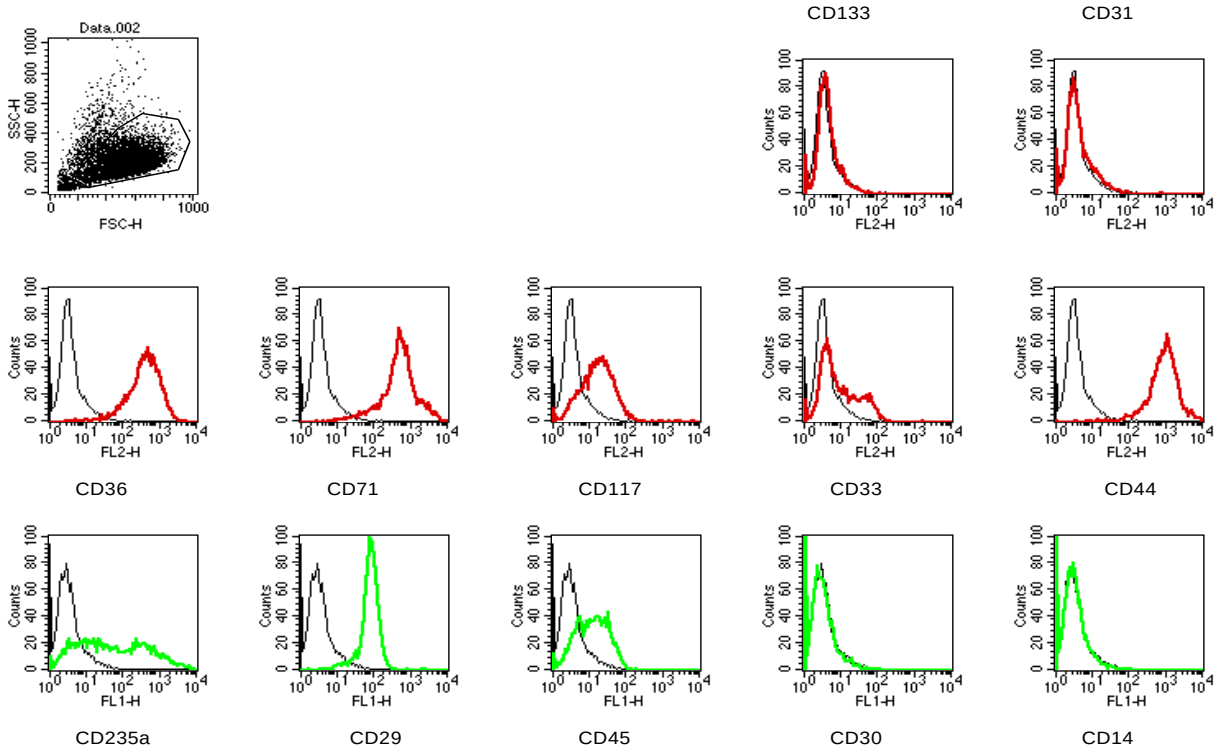


Figure 12: Characterization of hEPCs (d14) regarding their surface markers by FACS analysis (*picture taken with the consent of Dr. Mario Mairhofer)*

Erythroid progenitor cells can be distinguished by their **surface markers:** depending on their stage of differentiation they express different types of markers. In humans CD34 was described as a marker for hematopoietic stem cells (Gangenahalli et al., 2006). CD34 expression has been found in immature stem cells and committed progenitors. One to three percent of cells in the bone marrow express CD34, whereas in the peripheral blood CD34+ cells are extremely rare (less than 0,1%). In umbilical cord blood around 1% of the mononuclear cells express CD34. With the increase of differentiation the expression level of CD34 decreases (*Baum, Christopher 2009, Genetic Modification of Hematopoietic Stem Cells. Methods in Molecular Biology, volume 506, Springer Protocols, page 13.).*

In the present study, cultures of hEPCs isolated from umbilical coord blood are used. These cells are negative for CD34, but express high levels of CD71, CD36, CD29, CD44, CD117 and CD235a.

- **CD71** (the transferrin receptor) is expressed on many proliferating cells, but the very strong, homogeneous expression is characteristic for erythroid cells. The transferrin receptor CD71 is needed for the iron transport into proliferating cells, and it is lost when hEPCs differentiate into mature erythrocytes (Leberbauer et al., 2005).

- **CD36** is a receptor for thrombospondin and is a very early marker for erythroid maturation, it is found on the surface of erythroid progenitors, but also on monocytes and platelets, but not on lymphocytes and granulocytes.

- **CD29**, also known as integrin-ß-1, is a transmembrane glycoprotein, which forms heterodimers with integrin alpha subunits. It can mediate a variety of cellular responses including adhesion, proliferation and differentiation.

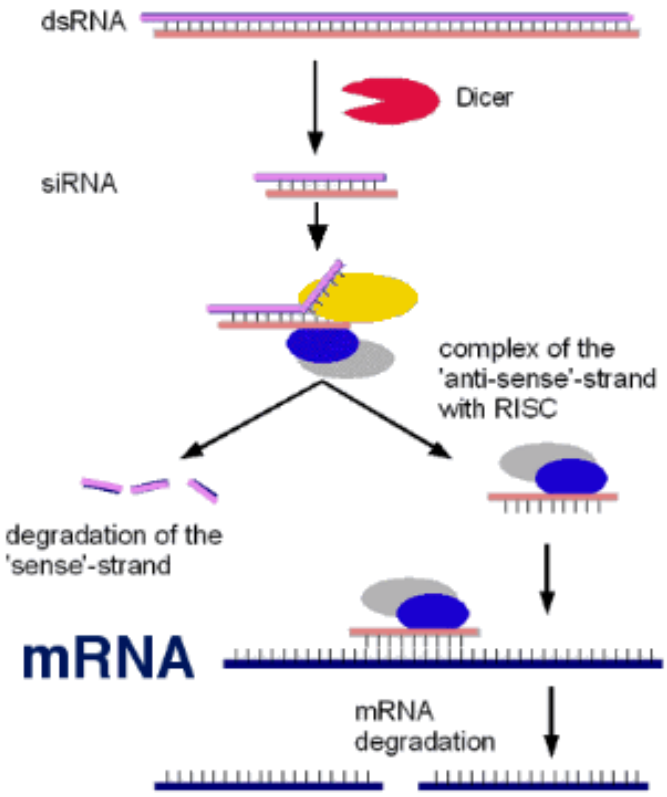
- **CD44** is it is a cell adhesion receptor that binds to components of the extracellular matrix and is widley expressed on hematopoietic and non-hematopoietic cells. Bone marrow derived progenitor cells, which are expressing CD44, have been shown to be able to repopulate the thymus (Rajasagi et al., 2009).

- **CD117**, the c-KIT receptor, is a tyrosine kinase receptor. High CD117 expression is typical for erythroid progenitors. As already mentioned SCF and its receptor play an essential role in the proliferation and differentiation of HSCs and hEPCs. Leberbauer et al. reported that freshly isolated mononuclear cells from human cord blood expressed very low CD117 levels, while around d14 of the cell culture almost 100% of the cells were CD117 positive (Leberbauer et al., 2005).

- **CD235a**, also known as Glycophorin A, is a transmembrane glycoprotein that is expressed on mature erythrocytes and erythroid precursors (Auffray et al., 2001). It is a marker for erythroid differentiation, the more differentiated the cells are, the higher Glycophorin A expression is.

1.8 RNA interference (RNAi) pathway

In this study the Glucocorticoid Receptor target genes were downregulated by a knock-down approach via electroporation with siRNA- oligonucleotides. Silencing of a target mRNA by siRNAs is a powerful tool to study gene function. siRNAs are double-stranded RNA (dsRNA) molecules of 21-23 nucleotides with characteristic 2-nucleotide overhanging 3' ends (Tulac et al., 2004). Through the electroporation-method target genes can be silenced post- transcriptionally by sequence-specific mRNA degradation. Normally siRNAs act as intermediates in the **RNA interference pathway (RNAi)** (see figure 13), which is active in the cells to protect mammalian cells from infection by a variety of viruses (Zamore and Aronin, 2003). Therefore the ribonuclease Dicer generates siRNAs by cleaving long dsRNAs. In the RNA-interference pathway siRNA strands associate with another set of proteins to form a protein-enzyme complex, called **RNA-induced silencing complex (RISC)**.



RISC directs the protein-enzyme complex to mRNAs which are complementary to the siRNA strand. After binding to the mRNA, RISC induces mRNA-degradation by cutting it once. After cleavage the RISC-complex binds to the next complementary mRNA and begins with a new cycle of mRNA-cleavage. With this mechanism siRNAs are able to induce degradation of complementary mRNAs, which has made siRNAs to an important tool for knock-down induction of various target genes (Zamore and Aronin, 2003).

Figure 13: RNA- interference pathway
(picture taken from: http://hades1.bioquant.uni-heidelberg.de/rnai.html)

1.8.1 The electroporation method

siRNAs can be introduced into cells by electroporation. In the 1970s it has been demonstrated for the first time that applying electrical pulses to cells enabeles increased uptake of biological materials into cells. Nowadays, electroporation has become an important tool for nucleic acid delivery into various cells.

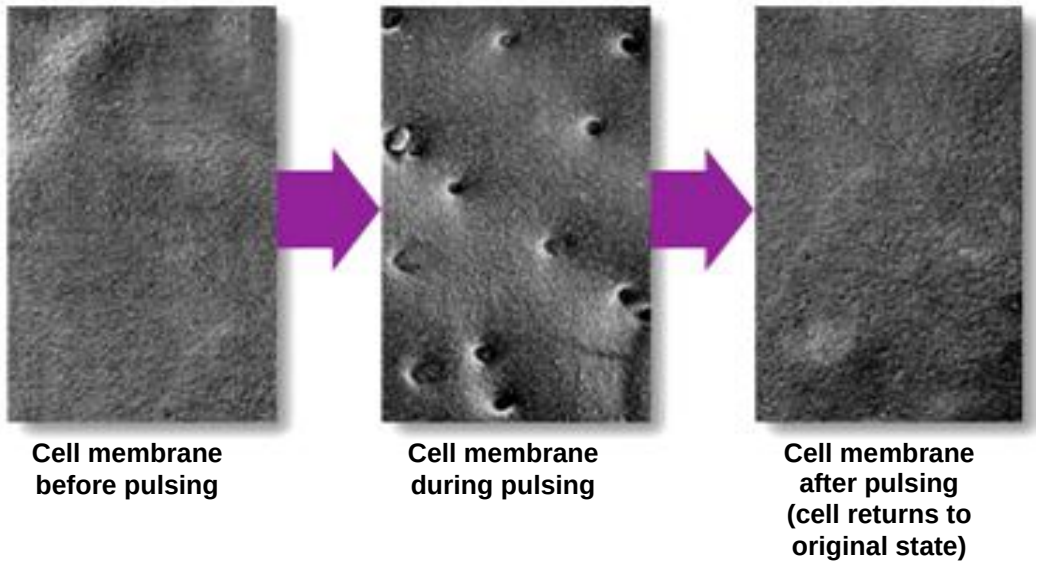


Figure 14: The phenomenon of electroporation
By applying an electrical pulse on cells, the cell membrane becomes porouse for a short periode of time. Thereby DNA-fragments or siRNAs can be introduced into cells. (picture taken from: www.iovio.com/technology/howelectroporationworks.htm)

By the application of a brief, controlled electrical pulse on the cells, a temporarily enhanced permeability of pores in the cell membrane is created (see figure 14). During this short periode of time (some milisecoconds), in which these pores exist, a significant quantitiy of the biomolecules of the sorrounding medium is taken up into the cells. After some miliseconds the pores in the membrane reseal and the cell membrane returns to its original state. Introducing siRNAs (small interfering RNAs) into cells by electroporation has become an important and powerful tool to study gene function.

(For more information about electroporation: www.iovio.com/technology/howelectroporationworks.htm)

Aim of this work:

Human erythroid progenitor cells (hEPCs) undergo renewal in response to erythropoietin (EPO), stem cell factor (SCF) and glucocorticoids.
Glucocorticoids are a class of steroid hormones that regulate a variety of essential biological functions. They have anti-inflammatory and immunosuppressive properties and have been shown to mediate apoptosis. Glucocorticoids repress or activate gene expression by binding to their nuclear receptor, the Glucocorticoid Receptor. Interaction of glucocorticoids with their receptor leads to activation and translocation of GR into the nucleus, where it acts as a transcription factor and modulates the activity of different target genes by binding to the DNA regulatory DNA binding sites. Thereby the GR can influence several developmental and biological processes.

Aim of this work is to study the function of different glucocorticoid target genes. In previous work with mouse EPCs and in a microarray screen where EPCs were stimulated with DEX alone or in combination with EPO, SCF and GR inhibitors, Raf-1, Tis11d and FKBP51, among others, have been identified to be glucocorticoid target genes (Kolbus et al., 2003; Rubiolo et al., 2006) and data not shown.
We chose to analyse the functions of these three GR-target genes in human primary erythroid progenitor cells by a transient, siRNA-mediated knock-down approach. During my diploma thesis I established a reliable and reproducible method for transfection of human EPCs by electroporation. With this method I could successfully downregulate all three target genes by siRNA electroporation. Thus I studied the effects of the knock-downs on proliferation, spontaneous differentiation and cell viability in primary hEPCs from different cord blood donors. The obtained results give us insights into the function of these specific GR-target genes and the mechanisms in which they are involved during erythroid cell proliferation and differntiation.

2. Materials and Methods

2.1 Working with human erythroid progenitor cells (hEPCs)

2.1.1 Isolation of mononuclear blood cells via a Ficoll-gradient

Mononuclear cells were isolated from human umbilical cord blood by a Ficoll-gradient. Cord blood was obtained in heparin-vials from General Hospital Vienna, Medical University of Vienna, Department of Obstetrics and Gynaecology after signing of an informed cosent, that has been approved by the local Ethical Comission.

- Approximatley 15mL of human cord blood were transfered into a sterile 50mL-tube and mixed well with 30mL PBS (blood-PBS-ratio: 1:2).
- Then 15ml of a Ficoll-paque solution (Biocoll, Biochrom AG) was overlayed carefully with 15mL of the diluted blood in a 50mL- tube
- The step gradient was centrifuged for 20 minutes at 1660rpm (300xg) at room temperature (important: without break).
- Note: Ficoll induces aggregation of erythrocytes by which they completely sediment through the Ficoll solution and are therefore located at the bottom of the 50mL-tube. While Granulocytes were situated in the layer immediatley above the erythrocytes, the mononuclear white blood cells (erythroid progenitors, lymphocytes, platelets and monocytes) were localized in the interface between the plasma and the Ficoll due to their lower density.
- This interface was collected in a preferably small volume, mixed with PBS (1:2) and centrifuged for 5 minutes with 1200rpm (250xg). (This time with break).
- The pellet was then resuspended in approximatley 2,5mL of PBS, filled up with 1x erylysis-Buffer 1/25 (v/v) to 50mL
- The mixture was incubated for 15-20 minutes at room temperature to remove also the rest of the red blood cells by erylysis. *(10x Erylysis Buffer: 89,9g Annoniumchlorid, 10g KHCO3 (Kaliumhydrogencarbonat), 0,37g EDTA, add A.d. up to 1000mL (pH 7,3), sterile filtratated)*

- After incubation, the cell suspension was centrifuged at 1200rpm (250xg) for 5minutes and the cell pellet was resuspended in 10mL PBS.
- After counting the cells by a CASY cell analyzer, the suspesion was centrifuged at 1200rpm for 5minutes
- The cell pellet then was either frozen in $10x10^6$ cell- aliquotes or was resuspended in an appropriate amount of 1x proliferation-medium and kept in culture (at a cell density of approximatly $8x10^6$ cells/mL up to $1x10^7$ cells/mL).

2.1.2 Isolation of mononuclear blood cells without a Ficoll-gradient

Mononuclear cells could also be isolated from human cord blood without Ficoll-gradient:

- Therefore 2,5 to 3mL of cord blood were transferred into a sterile 50mL-tube and filled up with 1x erylysis-Buffer 1/25 (v/v) to 50mL
- The tubes were incubated for 15-20 minutes at room temperature. During this time lysis of red blood cells takes place and therefore the suspension loses its viscosity and its dark red colour turns into light red.
- Then the cell suspension was centrifuged for 10 minutes at 1200rpm (250xg)
- The supernatant was removed and the different cell pellets of one cord blood were first resuspended in 10mL PBS, transferred into a 50mL-tube and filled up with PBS to 50mL.
- After mixing well, the cell-suspension was washed 2 times with 50mL PBS by centrifuging at 1200rpm (250xg) for 5 minutes.
- Then the cell pellet was resuspended in 10mL PBS and 50µl of the cell suspension were taken out for cell counting by the electronic cell counter (CASY-1, Schärfe-System).

After having established the amount of isolated mononuclear cells, the suspension was centrifuged again for 5 minutes at 1200rpm (250xg) and the cell pellet was then either frozen in $10x10^6$ cell- aliquotes or resuspended in the appropiate amount of 1x proliferation media and kept in culture (at a cell density of approximatly $8x10^6$ cells/mL up to $1x10^7$ cells/mL).

10x Erylysis Buffer:	89,9g Ammoniumchlorid
	10g KHCO3 (Kaliumhydrogencarbonat)
	0,37g EDTA, add A.d. up to 100mL (pH 7,3)
	→ sterile filtered

2.1.3 Determination of cell number by CASY

To monitor number and size of cultured cells, they were characterized every day with a CASY cell analyzer (Schärfe-System, Reutlingen, Germany).

- Therefore the cell suspension was gently resuspended and 50µl of cell suspension were diluted 1:100 with CASYton (Schärfe-System Reutlingen, Germany) and an aliquot of 200µl of the mixture was then measured by the CASY cell analyzer.
- The measurement was performed with a 60µm capillary, since the evaluated cells had a diameter between 5 and 15µm, depending on their differtiation stage.
- The CASY- counts and -profliles are used as quality control to compare different preparations of EPCs.
- With the obtained data the proliferation rates were calculated, moreover also the spontaneous differentiation or apoptosis could be detected and overall the outgrowth of hEPCs out of mononuclear cells was monitored.

2.1.4 Outgrowth of hEPCs from cord blood mononuclear cells

After isolation from human cord blood samples, mononuclear cells were cultivated for ten days at 37°C, 5% CO_2 and 100% humidity in serum free StemSpan medium (StemCellTechnologies) supplemented with the following factors:

•	**Epo** (Erythropoietin)	2U/mL	Jannsen-Cilag
•	**DEX** (Dexamethasone)	1µM/mL	Sigma-Aldrich
•	**IGF-1** (Interleukin-growth-factor-1)	40ng/mL	Sigma-Aldrich
•	**SCF** (Stem cell factor)	100ng/mL	Peprotech/Sigma
•	**Cholesterol-rich lipid mix**	20µg/mL	Sigma-Aldrich

This 1x proliferation-medium was also used for long-term cultivation, after the cultures have reached homogeneity (>95% erythroid cells) by day 6-10. Partial medium changes were performed every 24h to ensure optimal factor supply. After outgrowth of EPCs, the cell counts were adjusted to $1x10^6$ cells/mL daily.

After isolation from human cord blood the cell suspension was heterogenous, as besides the rare erythroid and multipotent progenitor cells, it was mainly containing other types of mononuclear cells (granulocytes, B- and T-leukocytes, monocytes).
During the first six days in culture those other cell types died off and around day 6 erythroid progenitor cells began to accumulate.

Cells were counted every day by the CASY-cell-analyzer and as soon as the plate contained more than $2x10^6$ cells, the culture was kept at a cell density of $1x10^6$ cells/mL.

Around d10 cell aliquots of $10x10^6$ hEPCs were frozen in cryotubes and stored in liquid nitrogen or cells were kept in culture till around d12 to d14, when they were ready for experiments.

2.1.5 Freezing erythroid progenitor cells

- The cell-suspension was centrifuged in a sterile plastic centrifuge-tube at 1200rpm (250g) for 5minutes
- The cell pellet was then resuspended in freezing medium (either FCS and 10% of DMSO or FILOCETH medium (Fischer Scientifics). DMSO protects the cell membranes during the freezing process.
- Preferentially aliquots of $10x10^6$cells were frozen in cryotubes in 1mL freezing medium.
- The cells were cooled down slowly to -80°C, either in an isopropanol box (Mr. Frosty), which provides a cooling rate of -1°C/ minute to prevent cell damage by a too quick freezing process.
- Cell-aliquots were transfered into a liquid N2 tank on the next day.

2.1.6 Thawing erythroid progenitor cells

- Cell aliquots were thawed quickly in a 37°C wather bath (approximatly 3-5minutes)
- Then the cell suspension was transferred into a sterile plastic centrifuge-tube, where 10ml of pre-warmed StemSpan medium (without proliferation factors) were added drop by drop, while gently shaking the tube.
- The cell suspension was centrifuged for 5minutes at 1200rpm (250g) to remove all of the DMSO, which is toxic for the cells at high concentrations.
- The supernatant was therefore removed and the cell pellet was resuspended in pre-warmed serum free StemSpan media supplemented with 1x proliferation factors and transfered into a culture-dish at a cell density of $1x10^6$ cells/mL.

2.1.7 Histological staining of erythroid cells with benzidine

50-100µl of the cell suspension were cyto-centrifuged onto a glass-slide (150xg for 7minutes) and stained according the following protocol. The glass slides were stained as described in the protocol below:
The methanol is fixing and permeabilizing the cells on the slide, the benzidine is reacting with the accumulated haemoglobin in the cytoplasm. H_2O_2 is then interacting with the bound benzidine, which leads to a brown staining of haemoglobinized cells. The Diff. Quick RED solution is staining the cytoplasm and the Diff. Quick BLUE solution the nuclei.

Working steps	Time
Incubation in methanol	4min
Incubation in 1% benzidine solution (2g O-Diamisidine dissolved in 200mL MetOH)	2min
Incubation in H_2O_2 solution (fresh!)	1,5 min
Washing in milliQ water	0,5 min
Stain with Diff. Quick RED (I)	4min
Stain with Diff. Quick BLUE (II)	35 sec
Washing in milliQ water	40sec
Dry by air	/

The H_2O_2 solution was always prepaired fresh (75mL Ethanol, 75mL H_2O, 2,5mL hydrogen peroxide. The dried slides were mounted with Entellan (Merck) and a cover slip and analysed under the microscope.

2.2 Flow Cytometry (FACS)

FACS (Fluorescence Activated Cell Sorting) is a powerful method to study and purify cells and has wide applications in immunology, cell biology and in other branches of biology. Cells can be stained with specific fluorescently labeled antibodies or other proteins (Annexin-V). During meassurement the cells pass through one or more laser beams and cells, to which specific fluorescently labeled antibodies or proteins have bound, are stimulated by the laser light and thereby they emit light at various frequencies. Photomultiplier tubes convert this light to electrical signals and cell data is collected. Thereby cells can for example be sorted and counted according to specific surface markers, or proteins expressed on their surface (like Annexin-V).

2.2.1 Propidium-Iodide staining

Propidiumiodide (PI) is a standard flow cytometric viability probe. PI can be used to distinguish viable from non viable cells. Viable cells with intact membranes exclude PI, whereas the membrane of dead and damaged cells is permeable to PI.

PI-Staining:

- 100.000 cells were transferred into FACS tubes
- The suspension was washed two times with 1x PBS (250xg, for 5minutes)
- The cells then were resuspended in 500µl PBS+1%FCS
- 5µl of PI were added immediatly before the meassurment
- The cells were vortexed and meassured by FACS

2.2.2 Propidium-Iodide and Annexin-V staining

When cells undergo apoptosis they show certain morphological chages, like for example loss of plasma membrane asymmetry, condensation of the cytoplasm and nucleus. Loss of plasma membrane asymmetry is one of the earliest features. In apoptotic cells the phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane and the cells are therefore

exposing PS to the cellular environment. Annexin-V is a 35-36kDa phospholipid-binding protein and in the analyses, this Annexin-V protein was flourescently labelled, can bind to the PS positive cells and can be used for flow cytometric analysis. Therefore Annexin-V positive cells can be identified as the cells undergoing apoptosis at an early stage.

Annexin-V- Staining:

- 100.000 cells were transfered into FACS-tubes
- The cell suspenstion was washed twice with 1mL 1xPBS (1250xg, 5minutes)
- the cells were resuspended in 100µl 1x Annexin-V binding buffer (BD Bioscience)
- Then 5µl of Annexin-V solution were added
- The mixture was incubated for 15minutes at room temperature (in the dark)
- Then 400µl of 1x Annexin-V binding buffer were added
- 5µl of Propidiumiodide (PI) were added (directly before the meassurment)
- vortexed and meassured by FACS

Informations about AnnexinV- and PI- staining: datasheet and homepage of BD Bioscience.

2.2.3 Fixing cells for FACS-analysis

If meassurement by FACS analysis couldn't be performed immediately, cells were fixed as following:

- $1x10^5$ to $2x10^6$ cells/mL were transferred into FACS tubes
- centrifuged at 1200 rpm (250xg) for 5 minutes
- cell pellet was resuspended in 2mL 1x PBS
- centrifuged at 1200 rpm (250xg) for 5 minutes
- cell pellet was resuspended in 4% Paraformaldehyd (PFA= 500µl PBS mixed with 500µl 8% PFA)
- cell suspension was incubated for 15minutes at room temperature
- stored at 4°C (note: fixed cells can be stored at 4°C for approximatley 2weeks)

2.2.4 Surface marker staining

To characterize the hEPCs in culture, the expression of specific surface markers was analysed. The different markers can be stained with specific antibodies and subsequentely analyzed by FACS-measurements.

- For each meassurement approximatley 500.000 cells are needed, therefore the appropriate amount of the cell suspension is resuspended and transferred into a sterile 50mL plastic tube.
- Mixed with 10mL of 1x PBS and centrifuged for 5minutes at 1200rpm (250xg)
- The cell pellet is resuspended with the appropriate amount of 1x PBS+1%FCS (50mL PBS and 500µl FCS). (NOTE: 500.000 cells are resususpended in 100µl of PBS+1%FCS, if you had 20 different approaches, the pellet was resuspended in 2mL)
- 100µl of the cell suspenstion (approximatley 500.000 cells) was transfered into a FACS-tube
- 10µl of the specific antibody was added to each tube (2µl of the antibody to 100.000 cells as recommended by the manufacturer)
- after short vortexing the mixture was incubated for 30 minutes at 4°C
- then it was centrifuged for 5 minutes at 1200rpm (250xg) and the cell pellet was resuspended in 500µl of PBS+1%FCS
- the samples were ready to be measured by FACS

2.3 Electroporation

The electroporation was performed as mentioned below:

After thawing, the cells were first kept in culture for 2-3days to enable the cells to recover from the stressful freezing- and thawing-process. We mainly performed the electroporation with hEPCs cultured for 12-14 days.
Per approach, always $2x10^6$ cells were electroporated in a volume of 200µl of Opti-MEM$^{(R)}$ medium with the optimized settings 300V and 150µF. (Note: Opti-MEM$^{(R)}$ is a low-salt medium Transfection Reagent from Invitrogen). The cells first had to be washed and prepared for the electroporation.

- The cell suspension was resuspended and counted with the CASY cell analyzer
- Per approach always $2x10^6$ cells were needed, depending on the cell-concentration the amount of the cell suspension needed for one experiment was calculated (for example: For 6 approaches $12x10^6$ cells ($2x10^6$cells x 6) were needed, if cell density was $1x10^6$ cells/ml, 12mL of cell suspension was used)
- The appropriate amount of the suspension was transferred into a sterile 50mL tube. (Note: As through the washing steps some cells will get lost, instead of 12mL→ 13 to 13,5mL were taken).
- The cell suspension was filled up to 50mL with ice cooled Opti-MEM$^{(R)}$
- Centrifuged at 1200rpm (250xg) for 5minutes
- The cell pellet was resuspended in 50mL of ice cooled Opti-MEM$^{(R)}$ and centrifuged again for 5minutes at 1200rpm (250xg).
- In the meanwhile 1x proliferation media was prepared and prewarmed at 37°C in the waterbath
- The sterile electroporation cuvettes were labeled and placed on ice (the temperature of the cuvettes influences the time-constant)
- Then the cell pellet was resuspended in the appropriate amount of Opti-MEM$^{(R)}$, 200µl per approach.

- (Note: $2x10^6$ cells per approach were electroporated in a volume of 200µl of Opti-MEM(R), therefore if you had 6 approaches ($12x10^6$ cells), the pellet was resuspended in 1200µl Opti-MEM(R) (6 x 200µl), but to counteract the pipetting errors, 220µl per approach were used)
- 200µl of the cell suspension was transferred into a sterile 15mL tube and mixed with the appropriate amount of siRNA (for example: if final concentration was 400nM, 4µl of siRNA (20µM stock) were added to 200µl of Opti-MEM(R))
- The mixture was gently vortexed and transferred into an ice cold electroporation cuvette
- Each approach was electroporated with 300V and 150µF
- The actual Volts and the timeconstant (msec) was noted after each impulse
- Immediately after electroporation the cells were mixed with 2mL prewarmed 1x proliferation medium and transferred to a 6well plate ($1x10^6$ cells/mL)
- 24h after the electroporation a total media change was performed

2.3.1 Different siRNAs used for electroporation

To ensure that the experimental outcome is true and reproducible, it is recommended to use at least 3 types of control samples in every electroporation experiment:: a positive control, a negative control and an untreated control. Positive controls show the efficiency of siRNA delivery into the cells and negative controls distinguish sequence-specific silencing from non-specific effects. Untreated samples determine the level of cell viability.

Positive controls: Lamin A/C, GAPDH, FITC and Cell Death siRNA

- **Lamin A/C:** (siGLO Lamin A/C Control siRNA, Dharmacon(R))

Lamin A/C- and GAPDH- siRNAs are positive silencing controls for human cells. Lamins are nuclear filament-type proteins, which are involved in the nuclear stability and chromatin structure (Tulac, Dosiou et al. 2004).

- **GAPDH:** (Stealth™ RNAi GAPDH Positive Control, Invitrogen)

GAPDH (Glyceraldehyde 3-phosohate dehydrogenase) is an enzyme involved in glycolysis and serves to break down glucose for energy and carbon molecules.
As Lamin A/C and GAPDH are non-essential in the cells, the knock-down of Lamin A/C or GAPDH does not induce any effects in the cells and their knock-down should therefore not affect cell viability or result in any other phenotype. This makes the Lamin AC- siRNA and the GAPDH- siRNA to a good positive control in experiments with siRNA-mediated knock-down.

- **FITC:** (BLOCK-IT™ Fluorescent Oligo, Invitrogen)

The BLOCK-iT TM Fluorescent Oligo is a fluorescein-labeled double-stranded RNA duplex with the same length and charge as a standard siRNA. The sequence of the FITC siRNA is not homologous to any known gene and is therefore a non-silencing oligonucelotide, which is not inducing any knock-down in the cells. It can be used as a control for the transfection efficiency, because due to the fluorescence signal of FITC, the transfection efficiency can be analyzed by flow cytometry (FACS). The fluorescent signal is localized in the nucleus and is therefore a signal for efficient uptake. The higher the fluorescent signal is, the higher also the transfection efficiency.

- **Cell Death control: (**AllStars Hs Cell Death Control siRNA, QIAGEN)

AllStars Hs Cell Death Control siRNA is a mix of siRNAs targeting human genes that are essential for cell survival. Knock-down of these genes induces cell death. Therefore this siRNA mix can be used in every electroporation as a positive control and as a control for transfection efficiency. 24h to 96h after electroporation the transfected cells undergo apoptosis, therefore the transfection efficiency can be estimated by simply observing the cells under the microscope or in the CASY cell analyzer, without to make time-consuming FACS analysis.

Negative controls: Low, med and high GC-siRNAs

- **Low-, med- and high- GC:** (Stealth™ RNAi Negative Control, Invitrogen)

Stealth™ RNAi Negative Control siRNAs serve as a negative control for the RNAi response. For each electroporation a control siRNA with a similar GC content as the target siRNA was used. Thereby it could be shown that the effects of the knock-downs were specific and not just induced by the uptake of an unspecific siRNA oligo.

Low GC: (Stealth™ RNAi Negative Control, Invitrogen) → 36% GC-content
Med GC: (Stealth™ RNAi Negative Control, Invitrogen) → 48% GC-content
High GC: (Stealth™ RNAi Negative Control, Invitrogen) → 68% GC-content

siRNAs for the Glucocorticoid-Receptor targets:

- **RAF-1: (Invitrogen, validated)**

Raf-1-1	GGA GUA ACA UCA GAC AAC UCU UAU U
Raf-1-2	AAU AAG AGU UGU CUG AUG UUA CUC C

- **Tis11d: (Invitrogen, non validated)**

Tis11d-1:	forward:	5’ CCG CCU UCU ACG AUG UCG ACU UCU U 3’
	reverse:	5’ AAG AAG UCG ACA UCG UAG AAG GCG G 3’
Tis11d-2:	forward:	5’ ACC UGA ACA ACA UGC UGG ACA AGA A 3’
	reverse:	5’ UUC UUG UCC AGC AUG UUG UUC AGG U 3’
Tis11d-3	forward:	5’ CGC ACC UUU CAU ACC AUC GGC UUC U 3’
	reverse:	5’ AGA AGC CGA UGG UAU GAA AGG UGC G 3’

- **Fkbp51 : (Invitrogen, non validated)**

Fkbp51-1:	forward:	5’ ACC AAA GCU GUU GAA UGC UGU GAC A 3’
	reverse:	5’ UGU CAC AGC AUU CAA CAG CUU UGG U 3’
Fkbp51-2:	forward:	5’ CCA GUC AUG AUA GAA AUG AAC CAU U 3’
	reverse:	5’ AAU GGU UCA UUU CUA UCA UGA CUG G 3’
Fkbp51-3:	forward:	5’ GGA UGC CAA GGA AGA GGC CAA UAA A 3’
	reverse:	5’ UUU AUU GGC CUC UUC CUU GGC AUC C3’

2.4 Cell Isolation Kits

2.4.1 Dead Cell Removal

After electroporation, dead cells can be romoved from the cell suspension by the "*MACS (R) Dead Cell Removal Kit" (Miltenyi Biotec)*. Dead Cell Removal MicroBeads recognize Annexin-V in the plasma membrane of apoptotic and dead cells. Using antibodies against Annexin-V, dead and early apoptoic cells can be romoved from the cell suspension.

- 1x Binding Buffer was prepared by dilution of 20x Binding Buffer Stock solution (250µl of 20x Binding Buffer was diluted with 4,75mL of sterile H_2O)
- Cell suspension was collected and centrifuged for 5minutes at 300xg
- Supernatant was removed completely and cell pellet was resuspended in 100µl of Dead Cell Removal MicroBeads per approximately 10^7 cells (for fewer cells same volume is used)
- Mixed well and incubated for 15minutes at room temperature (Note: During this incubation time the magnetically labeled Dead Cell Removal MicroBeads bind to the dead and apoptotic cells)
- The Column was placed in magnetic field and prepared by rinsing with 500µl of 1x Binding Buffer
- The Cell suspension was applied onto the column (500-1000µl)
- Rinse the column 4 x with 500µl of 1x Binding Buffer
- The magnetically labeled dead cells are retained in the column, while the unlabeled living cells pass through and are collected in a sterile 15mL plastic tube.
- 50µl of collected cell suspension was taken for cell count by the CASY cell analyzer
- Centrifuged 1200 rpm (250xg) for 5minutes
- Cell pellet was resuspended in appropriate 1x proliferation factor medium to reach a cell density of $1x10^6$ cells/mL.
- Note: Approximately half of the cells are lost during this process

2.4.2 CD34+ Isolation

Out of mononuclear cells the CD34+ cells can be isolated by the *"CD34 MicroBead Kit" (MACS Miltenyi Biotec).* The CD34 antigen is a transmembrane glycoprotein expressed on human hematopoietic stem and progenitor cells. The CD34 MicroBead Kit contains MicroBeads, which are directly conjugated to CD34 antibodies. Thereby CD34-expressing hematopoietic cells can be magnetically labelled and isolated by applying them on a column within a magnetic field, whereby the magnetically labeled CD34+ cells are retained. When column is placed out of the magnetic field, the bound CD34+ cells can be eluted:

- Mononuclear cells are isolated or cell aliquots are thawed and resuspended in 10mL RPMI-medium containing 2mM EDTA
- Cell suspension is centrifuged for 5 minutes with 250 x g (1200 rpm)
- Supernatant is removed completely and cell pellet is washed with 10mL PBS+ 2mM EDTA
- Centrifuge for 5 minutes with 250 x g (1200rpm)
- Supernatant is removed completely and cell pellet is resuspended in 300µl of buffer (per 10^8 cells) (BUFFER: PBS+0,5% bobine serum albumin (BSA) and 2mM EDTA)
- Add 100 µl of FcR Blocking Reagent for up to 10^8 total cells
- 100µl of CD34 MicroBeads (for up to 10^8 total cells) are added, mixed well and incubated for 30minutes at 4°C (Note: CD34+ cells are bound to the magnetically labeled MicroBeads)
- Cells were washed by adding 5-10mL of buffer and centrifuged at 300xg for 10minutes, the cell pellet was resuspended in 500µl of buffer for 10^8 total cells
- The column was placed in the magnetic field and prepared by rising with 500µl of buffer
- Cell suspension was applied on the column, and the column was washed 3 x with 500µl of buffer. The flow-through can be collected (Note: the magnetically labeled Lin+ cells (CD34+) are retained within the column while the unlabeled Lin- cells pass through)

- Column is removed from the magnetic field and placed on a sterile 15mL plastic tube
- 1mL of buffer was pipetted onto the column and the the magnetically labeled CD34+ cells (now not longer bound within the magnetic field) are eluted by pushing the plunger into the column
- 10mL of PBS are added, cell suspension was counted and centrifuged 1200 rpm (250xg) for 5minutes**,** cell pellet was resuspended in appropriate 1x proliferation factor medium to reach a cell density of $1x10^6$ cells/mL.
- By FACS analysis the purity of CD34+ cells can be measured

2.5 Working with Proteins

To analyse changes on the protein level, Western blot analysis was performed.

- between $5x10^5$ to $1x10^6$ cells were transferred into an Eppendorf tube
- centrifuged for 5minutes at 250xg
- the supernatant was completely removed
- the cell pellet was washed once with 1ml PBS
- centrifuged for 5min at 250xg
- The washed cell pellet was shock-frozen in liquid nitrogen and stored at -80°C.

2.5.1 Protein Isolation

The pellet was lysed with **110µl to 130µl** Tx100-Lysisbuffer depending on it's size. The lysis steps were as follows:

→ mixing well by vortexing (for ca. 30sec)
→ incubating on ice for 10minutes
→ mixing well by vortexing (for ca. 30sec)
→ incubating on ice for 10minutes

Tx100 Lysisbuffer: 150mM NaCl
10mM Tris pH 7,4
1% Triton X-100
0,1%SDS
1mM EDTA

Before use to 10mL of the Tx100 Lysisbuffer there was added:

- 1 pastille of PhosSTOP Phosphatase inhibitor cocktail (ROCHE)
- 1 pastille of cOmplete Mini, EDTA free, Protease inhibitor coctail (ROCHE)
- 5µl of Okadaic Acid (1:2000) (stock: 100µM)

After shaking 1mL aliquots of the Lysisbuffer were stored at -80°C.

Protein concentration was measured by the *Pierce(R) BCA Protein Assay Kit* (Thermo Scientific).

- Therefore the BCA protein-assay-reagent A was diluted 1:50 with BCA protein-assay-reagent B.
- Six dilutions of BSA were measured together with the samples to determine the standard curve (2mg/ml, 1mg/ml, 0,5mg/ml, 0,2mg/ml, 0,1mg/ml and 0,05mg/ml).
- 200µl of the BCA-Protein-Assay-Reagent-mix were added to 10µl of each of the standard-concentrations (in triplicates) on a 96-well plate.

This mixture was incubated at 37°C for 30minutes, where its colour turned from green to dark- or light- violett depending on the protein-concentration. The absorbance was measured photometrically at 650nm. The protein-concentrations of the samples were computed with the help of the BSA standard curve.

The isolated proteins were mixed with 5x protein loading buffer and incubated at 95°C for 5minutes to denature the proteins further sonificated at the ultrasonic-bath for 20minutes to shear the DNA in total cell lysates.

10µg to 20µg of the isolated proteins were loaded on a polyacrylamid gel for Western Blot analysis.

2.5.2 Western Blot analysis

Western Blot analysis is a standard method to determine the expression of various proteins by using specific antibodies; the method consists basically of the following steps:

- After protein-isolation the proteins were seperated by denaturing polyacrylamide gel electrophoresis (SDS-PAGE)
- The separated proteins were transferred from the polyacrylamide gel to a PVDF-membrane (Millipore)
- The proteins bound to the membrane were analyzed with specific antibodies

SDS gel electrophoresis

SDS PAGE is a classical method for separation of proteins in an electric field according to the molecular weight. SDS-polyacrylamide gel consists of two parts: a lower part (separating gel), where the proteins are seperated, and an upper part (stacking gel), where the proteins are accumulated before entering the separating gel.

Solutions for SDS-PAGE

5x sample buffer:	0,1M Tris-Cl 5% SDS 17% Glycerol 5% mercaptoethanol 1/100 volume of a 0,5% bromophenolblue solution
Separating Gel-buffer:	36,34 g Tris 4mL 20% SDS 150mL A.d. pH 8,8 add A.d. to reach a volume of 200mL
Stacking Gel-buffer:	6,08 g Tris 2mL 20% SDS 50mL A.d. pH 6,8 add A.d. to reach a volume of 100mL
10x Running Buffer for 1000mL:	1,9 M Glycin (142,6g) 250 mM Tris-Base (30,3g) 1% SDS (50mL)
1x Running Buffer for 1000mL :	100mL 10x Running Buffer 900mL A.d

10x Blotting Buffer for 1000mL:	1,9 M Glycin (142,6g) 250 mM Tris-Base (30,3g) 10% Methanol (100mL)
1x Blotting Buffer for 1000mL:	100mL 10x Blotting Buffer 200mL Methanol 700mL A.d.
10x TBS Buffer for 1000mL:	1,5 M NaCl 100mM Tris, pH 7,4 or 8
1x TBS-T Buffer for 100mL:	100mL 10x TBS Buffer 900mL A.d. 0,05 % Tween 20 (500µl)
Stripping Buffer:	2% SDS 62,5 mM Tris, pH 6,8 to 50mL Stripping Buffer add 390µl ß-Mercaptoethanol (prepare always fresh)

Two 10% polyacrylamid-separating gels were prepared as follows:

milliQ water	8,97 mL
Polyacrylamid (30%)	7,53 mL
Separating Gel-buffer	5,7 mL
APS (10%) (Ammonium-peroxidisulfate)	336µl
TEMED (Tetramethylethylendiamine)	15µl

Two stacking gels were prepared as follows:

milliQ water	3 mL
Polyacrylamid (30%)	0,9 mL
Stacking Gel-buffer	1,33 mL
APS (10%)	52,5 µl
TEMED	7,5 µl

First, the components of the running gel were mixed carefully, then poured and Isopropanol was added on the top to eliminate air-bubbles.
After the gel polymerized, the isopropanol was removed and the stacking gel mixture was poured on the running gel (a comb was placed immediately between the two glass plates).
Depending on the size of the respecitve proteins, the concentration of acrylamide of the running gel can be decreased or increased. For proteins between 40kD and 80kD like for example Lamin A/C, Gapdh, Raf-1, Tis11d, FKBP51 a 10% gel were used and if we wanted to analyse smaller protein-fragments of for example Caspase-3, we used a 12% gel.
Depending on the amount of isolated proteins 10µg of protein and 5µl of the marker (PageRuler prestained protein ladder, Fermentas) were loaded. The electrophoresis was performed in the 1x PAGE-Running buffer and the proteins were separated with:

50Volt, 100mA, 50W: till proteins run through the stacking in the separating gel
70Volt, 100mA, 50W: till the proteins run to the middle of the separating gel
100-130V, 100mA, 50W: till the proteins run to the end of the separating gel

Western transfer

The proteins were transferred from the polyacrylamid gel to the PVDF-membrane (Millipore), which is highly hydrophobic and therefore first had to be activated by incubation in methanol for 10minutes.

The transfer was performed in blotting buffer for:

→ 1,5hour, 400mA, 100V, 50W, on ice

→ 15-16hours (over night), 90mA, 30-35V, 50W, at +4°C

Antibody incubation

- The membrane was first stained for 5minutes with Ponceau S, which is an unspecific protein stain, thereby blotting-problems (like airbubbles) or unequal loading can be assessed immediately.
- The dye was washed away at first with water and then with TBS-T buffer until the membrane was completely clear from the Ponceau staining.
- The membrane was blocked for 1h at room temperature with 5% dry low fat milk powder in 1xTBS-T to block unspecific antibody binding to the PVDF-membrane.
- Then the membrane was washed 3times with TBS-T for 5minutes.
- The first antibody was diluted to the appropriate concentration and depending on the antibody it was either incubated over night at 4°C or for 1hour at room temperature with the membrane.
- After the first antibody-incubation the membrane was washed again 3times with 10mL TBS-T for 10-15 minutes each and then incubated with the appropriate secundary antibody diluted in TBS-T for 1hour at room temperature.
- This was followed by another three washing steps with 1x TBS-T.
- The membrane was transferred into a transparent plastic foil and 400-800µl of equal amounts of the chemoluminescence ECL solution 1 and 2 (Thermo Scientific) was distributed eaqually over the membrane.
- A light sensitive X-ray film (GE Healthcare) was put in an exposure box together with the membrane, exposed for appropriate time (from 20sec-20minutes, depending on the intensity of the signal) and then developed.

In the presented work the following antibodies (in the indicated dilutions) were used:

First antibody	Company	Second antibody
Lamin A/C (1:200, in TBS-T)	Santa Cruz Biotech.	Mouse (1:25.000, in TBS-T)
Tis11d (1:200, 1%BSA in TBS-T)	Santa Cruz Biotech.	Rabbit (1:20.000, in TBS-T)
Fkbp51 (1:1000, 1%BSA in TBS-T)	Santa Cruz Biotech.	Goat (1:30.000, in TBS-T)
Gapdh (1:1000, in TBS-T)	Cell Signalling	Rabbit (1:20.000, in TBS-T)
Raf-1 (1:1000,1%BSA in TBS-T)	Cell Signalling	Mouse (1:25.000, in TBS-T)
Caspase3 (1:1000,5%milk in TBS-T)	Cell Signalling	Rabbit (1:20.000, in TBS-T)
pERK (1:1000, 1%BSA in TBS-T)	Cell Signalling	Rabbit (1:20.000, in TBS-T)
panERK (1:5000, 1%BSA in TBS-T)	BD Biosciences	Mouse (1:25.000, in TBS-T)
Actin (1:10.000 in TBS-T)	Cell Signalling	Mouse (1:25.000, in TBS-T)
pIKK α/ß (1:1000, 1%BSA in TBS-T)	Cell Signalling (NF-kappa B Pathway sampler Kit	Rabbit (1:2000 in TBS-T,of KIT)
pNFKB (1:1000, 1%BSA in TBS-T)	Cell Signalling (NF-kappa B Pathway sampler Kit	Rabbit (1:2000 in TBS-T,of KIT)
NFKB (1:1000, 1%BSA in TBS-T)	Cell Signalling (NF-kappa B Pathway sampler Kit	Rabbit (1:2000 in TBS-T,of KIT)
pIKB-α (1:1000, 1%BSA in TBS-T)	Cell Signalling (NF-kappa B Pathway sampler Kit	Rabbit(1:2000 in TBS-T,of KIT)
IKB-α (1:800, 1%BSA in TBS-T)	Cell Signalling (NF-kappa B Pathway sampler Kit	Mouse(1:2000 in TBS-T,of KIT)

All of the membranes were blocked always with 5% dry low fat milk and washed with TBS-T.

2.6 Working with RNA

To analyse the knock-down efficiency on mRNA-level we performed Q-PCR, therefore first the RNA was isolated, reverse transcribed into cDNA and then a Real-Time PCR was done.

2.6.1 RNA Isolation

The RNA extraction was performed with the "RNA-Easy Mini Kit 250" (Qiagen), the extraction of the RNA was performed according to the protocol provided with the kit. The amount of isolated RNA was analysed with the nanodrop-method photometer.

2.6.2 cDNA synthesis

The cDNA synthesis was performed with the "First Strand cDNA Synthesis Kit" (Fermentas), for each cDNA synthesis 500ng of total RNA were reverse transcribed into cDNA as described:

Total RNA	500ng (1 to 11µl depending on the RNA concentation)
Random hexamer primer	1µl
DEPC-treated water	to 12µl
Total volume	12µl

After adding all of the components, the mixture was vortexed, spinned down and incubated for 5minutes at 65°C and then on ice. To this mixture 8µl of the Mastermix were added to reach a total volume of 20µl.

Mastermix:

5x reaction buffer	4µl
RiboLock™ RNase Inhibitor (20 U/µl)	1µl
10mM dNTP mix	2µl
M-MuLV Reverse Transcriptase (20 U/µl)	1µl
Total Volume	20µl

The mixture then was incubated for 5minutes at 25°C and then at 42°C for 60minutes. To terminate the reaction the mixture was heated at 70°C for 5minutes.

2.6.3 Real Time PCR (Q-PCR)

The amplified cDNA was diluted 1:1 with RNAse free water (20µl cDNA+20µl RNAse free water). The Real Time PCR was done in triplicates, the PCR mixture included:

Diluted cDNA (1:1)	2 µl
2x Power SYBR Green Master Mix*	5 µl
forward primer (10pmol)	0,5 µl
reverse primer (10pmol)	0,5 µl
RNAse free water	2 µl
Total volume	10 µl

*(Applied Biosystems)

To determine the optimal Annealing Temperature**: (G+C) x 4°C + (A+T) x 2°C – 5°C** (calculate the mean value of the 2 primers).

RT-PCR conditions:

Stages	Temperature	Time
Stage 1	50°C	2 minutes
Stage 2	95°C	10 minutes
Stage 3 (40 cycles)	95°C	15 sec
End	60°C	1 minute

In the present study following primer pairs were used for the Q-PCR :

- **LAMIN A/C: (SIGMA)**

LAMIN A/C:	forward:	5’ GAC TCA GTA GCC AAG GAG CG 3’
	reverse:	5’ GCA GCT ATC AGG TCA CCC TC3’

- **GAPDH : (SIGMA)**

GAPDH:	forward:	5’ CCT GTT CGA CAG TCA GCC G 3’
	reverse:	5’ CGA CCA AAT CCG TTG ACT CC3’

- **Raf-1 : (SIGMA)**

Raf-1:	forward:	5’ CTG GGA CTC CAC TAT CAC CA 3’
	reverse:	5’ GCA CTG TAG CAC CAA AGT AC 3’

- **Tis11d : (SIGMA)**

Tis11d:	forward:	5’ TGT GCA AGA CAG AGA GAA ATC CC 3’
	reverse:	5’ GAG TGC CGT CGG AGG AAT C 3’

- **Fkbp51 : (SIGMA)**

Fkbp51:	forward:	5’ CCG CAA GTT CTA CTC CAT CC 3’
	reverse:	5’ CCT TGG CTG ACT CAA ACT CG3’

- **hCSNK1d : (SIGMA)** (houskeeper)

hCSNK1d:	forward:	5’ AGG AGA AGA GGT TGC CAT CAA G 3’
	reverse:	5’ TCC ATC ACC ATG ACG TTG TAG TC 3’

- **ß-2-Microglobulin (B2M): (SIGMA)** (housekeeper)

B2M:	forward:	5’ GGG TTT CAT CCA TCC GAC ATT G 3’
	reverse:	5’ TGG TTC ACA CGG CAG GCA TAC C3’

3. RESULTS

3.1 Isolation and outgrowth of mononuclear cells from cord blood

All of our experiments were performed with human erythroid progenitor cells. Therefore mononuclear cells were isolated from human umbilical cord blood according the protocols in Materials and Methods and as demonstrated in figure 15 below:

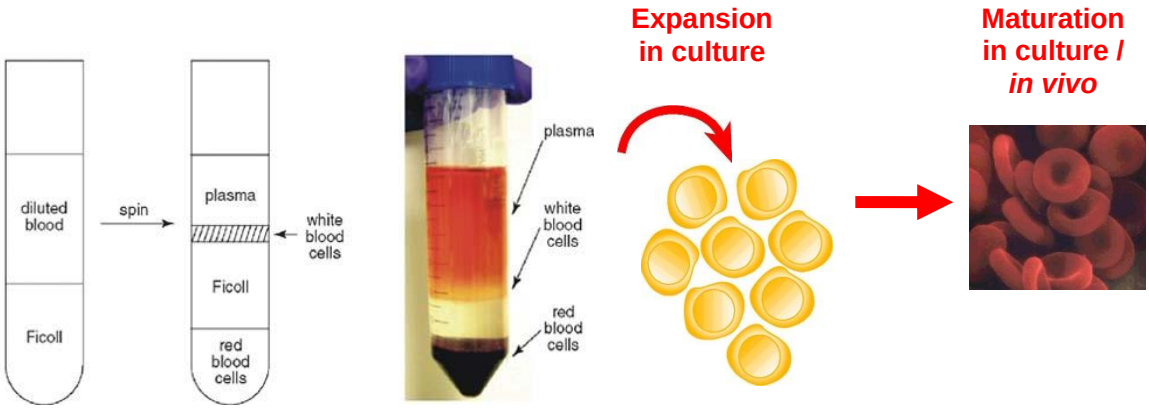


Figure 15: Isolation of mononuclear cells from human cord blood through a Ficoll-gradient *(picture taken from: Ernst Müllner)*

After isolation, mononuclear cells were expanded under defined culture conditions to erythroid progenitor cells (hEPCs). Figure 16 shows the outgrowth of EPCs from mononuclear cells. Therefore daily cell aliquots of mononuclear cells were cytocentrifuged onto glass slides and stained by histological stainings (as described in Materials and Methods), also cell counts were performed every day by the CASY-cell-analyzer.

At d0 the cytospins showed mainly a heterogeneous cell suspension with different types of mononuclear cells, whereas at d10 the cell suspension was more homogeneous due to the accumulated progenitor cells. Also the CASY-profiles, shown in the right panel of figure 16, changed, they are giving you informations of the cell-count and the cell-size. While at d0 the peak was around 7µm, at d10 a

clear shift towards bigger size (around 10µm) was observed, which indicates that the smaller already differentiated cells (erythrocytes, granulocytes, macrophages) died off and the undifferentiated bigger EPCs accumulated. Therefore we could show that around d10 out of the heterogeneous culture at d0, a homogeneous culture of erythroid progenitors (hEPCs) grew out. The cells were kept in culture till around d12 to d14, when they were ready for experiments.

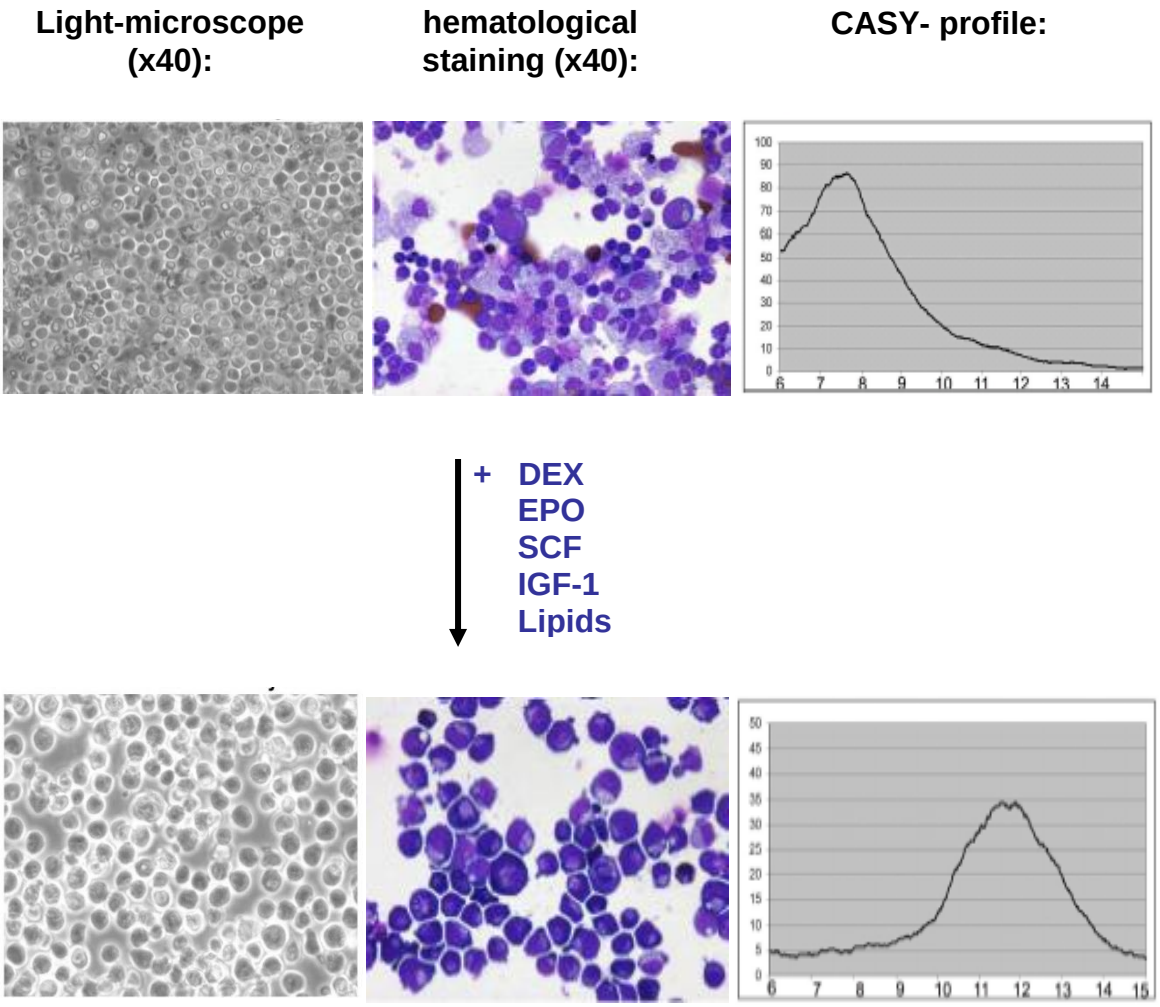


Figure 16: Outgrowth of hEPCs from mononuclear cells:
After isolation from human umbilical cord blood, mononuclear cells are expanded under defined culture conditions to erythroid progenitor cells (hEPC's) in serum free StemSpan medium supplemented with Epo (2U/mL), SCF (100ng/mL), DEX (10-6M), IGF-1 (40ng/mL) and a cholesterol-rich lipid mix (20µg/mL).
Here are shown pictures of the cell suspension at d0, immediately after isolation of the mononuclear cells and at d10 after cultivation: the picture on the left is showing an exposure with the light-microscope (x40), in the picture in the middle are demonstrated the cytospins(x40) and the picture on the right panel shows the Casy-profiles.

3.2 Optimization of the electroporation method

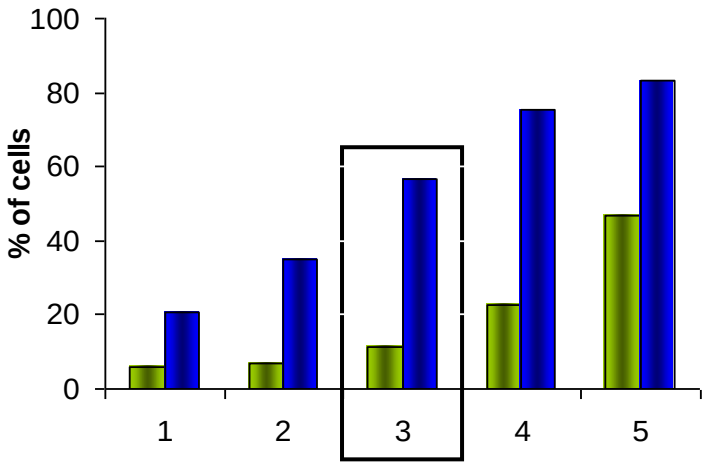
In order to be able to downregulate specific glucocorticoid target genes, the settings for transfection of siRNAs into the cells by electroporation had to be optimized. Several variables can be changed in order to determine the ideal electroporation conditions. Every cell type requires different electroporation settings, which have to be determined empirically. The pulse length, temperature, cell density, voltage- and capacity- settings and the siRNA- concentration are all critical parameteres, which have to be optimized at the beginning to determine the best conditions to achieve a high knock-down efficiency, a high cell viability and a high transfection efficiency at the same time.

3.2.1 Best Voltage and Capacity settings

The voltage- and capacity- settings critically determine cell survival and transfection rate. If the voltage-settings (field strength: V/cm) are high enough, it induces permeabilization of the membrane and allows an uptake of molecules from the surrounding media (Jordan et al., 2008). This event is depending on several factors, like cell diameter and temperature. In general smaller cells require higher voltages than larger cells. But higher voltage-settings can also promote cell damage. Therefore it is essential to determine the best settings, by which a high knock-down efficiency and also a high cell viability are achieved at the same time.

For this purpose $2x10^6$ cells were electroporated with a non- silencing fluoresently labeled siRNA (FITC) with different voltage- and capacity- settings. The transfection efficiency and cell viability was then analysed by Flow Cytometry. The results of the obtained FACS-data are shown in figure 17. Our data revealed that the higher the voltage- and capacity settings were, the higher the transfection-efficiency was. When cells were electroporated for example with 950µF or with 400V, over 90% of the hEPCs were transfected, but just 20% of the cells survived the procedure. And at the same time when the settings were below 300V or 150µF, cell viability was over 95%, but just 25% of the cells were transfected.

Best Voltage settings:



Applied Voltage settings:

1: 200V, 150µF

2: 250V, 150µF

3: 300V, 150µF

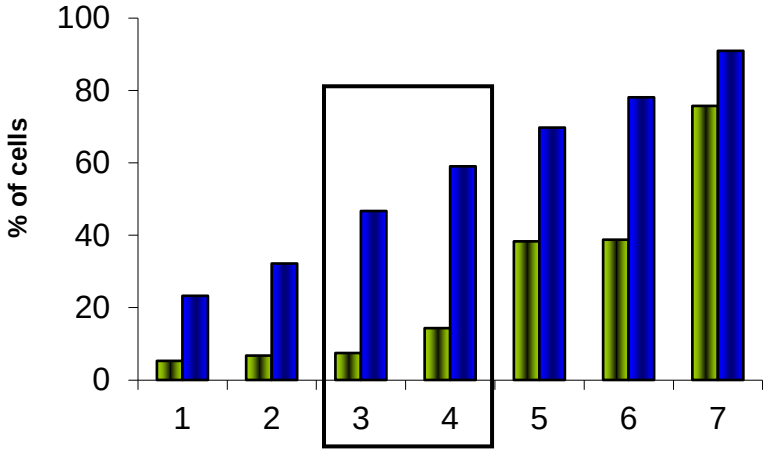
4: 350V, 150µF

5: 400V, 150µF

dead cells

transfected cells

Best Capacity settings:



Applied Capacity settings:

1: 25µF, 300V

2: 50µF, 300V

3:100µF, 300V

4: 200µF, 300V

5: 400µF, 300V

6: 600µF, 300V

7: 950µF, 300V

Figure 17: Best voltage and capacity settings
$2x10^6$ cells were electroporated with a non- silencing fluoresently labeled siRNA (FITC) with different voltage- and capacity- settings. The transfection efficiency and cell viability was then analyzed by Flow Cytometry (FACS).

From this data we concluded that 300V and 150µF resulted to be the best settings to achieve a high transfection efficiency (over 60%) and also a high cell viability (over 90%) at the same time. Note: Transfection efficiency is slightly under-estimated by flow cytometric analysis because the signal intensity for the FITC-labeled oligo is very low, and therefore, the peaks of control and transfected cells always showed an overlap. When the cells were transfected with FITC-siRNA by electroporation, transfection efficiency was always higher than 75%.

3.2.2 Best siRNA- concentration

Transfection of siRNA can result in off-target effects, in which siRNAs do not just induce the degradation of homologous mRNAs, but they can affect also the expression of partially homologous or even non homologous genes. Those off-target effects can be avoided by using lowest effective siRNA concentrations. But on the other side, if siRNA-concentration is too low, knock-down may be undetectable. To find out the lowest siRNA- concentration, by which off-target effects are reduced and at the same time a high a knock-down efficiency and a low cytotoxicity is still guaranteed, hEPCs (d15) were electroprated with different amounts of LAMIN A/C- siRNA (siRNA concentration varies from 100nM to 1,6µM), one approach electroporated without siRNA and one approach not electroporated at all served as a control.

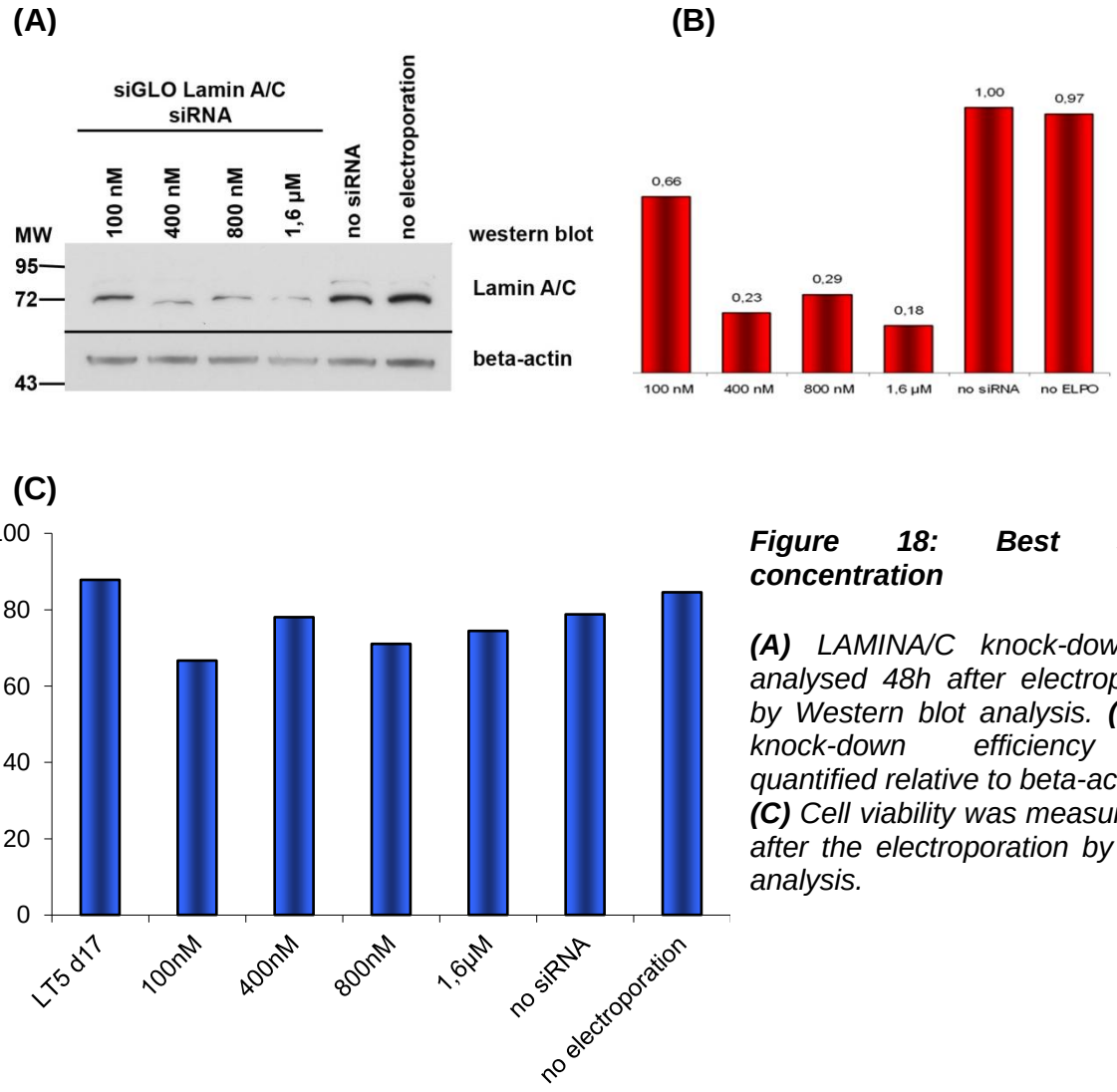


Figure 18: Best siRNA-concentration

(A) *LAMINA/C knock-down was analysed 48h after electroporation by Western blot analysis.* ***(B)*** *The knock-down efficiency was quantified relative to beta-actin.*
(C) *Cell viability was measured 48h after the electroporation by FACS-analysis.*

As shown in figure 18 the knock-down efficiency was analysed 48h after the electroporation by Westernblotting and quantified relative to beta-actin. The cell viability was measured by FACS-analysis. Our data revealed that a siRNA-concentration of 400nM seemed to be sufficient to achieve a knock-down efficiency of 75% and a cell viability of approximately 80% (48h after the electroporation).

3.2.3 Stability and time-course of the knock-down

Electroporation with siRNAs is resulting in a transient not stable knock-down. If cells proliferate the amount of the uptaken siRNA in the cytoplasm decreases, which leads again to an increase of the expression level after a certain periode of time (72h to 96h). To determine the stability of the knock-down after the electroporation, $2x10^6$ cells (d15) were electroporated with 2 different siRNAs: LAMIN A/C- and GAPDH-siRNA (siRNA- concentration: 100nM and 400nM). Knock-down efficiency and cell viability was analysed 24h to 96h after the electroporation (by Western Blotting, Q-PCR and FACS analysis). One approach electroporated without siRNA and one approach electroporated with the non-coding FITC-siRNA served as a control.

Cell viability 24h to 96h after electroporation

As shown in the upper panel of figure 19 the cell viability seemed not to be affected by the siRNA- concentration or the type of siRNA, as cell viability didn't differ between the approaches electroporated with a concentration of 100nM or 400nM, nor we could detect differences in cell viability, when cells were electroporated with different types of siRNAs. Cell viability was always around 90% in all of the appraoches, even 96h after the electroporation.

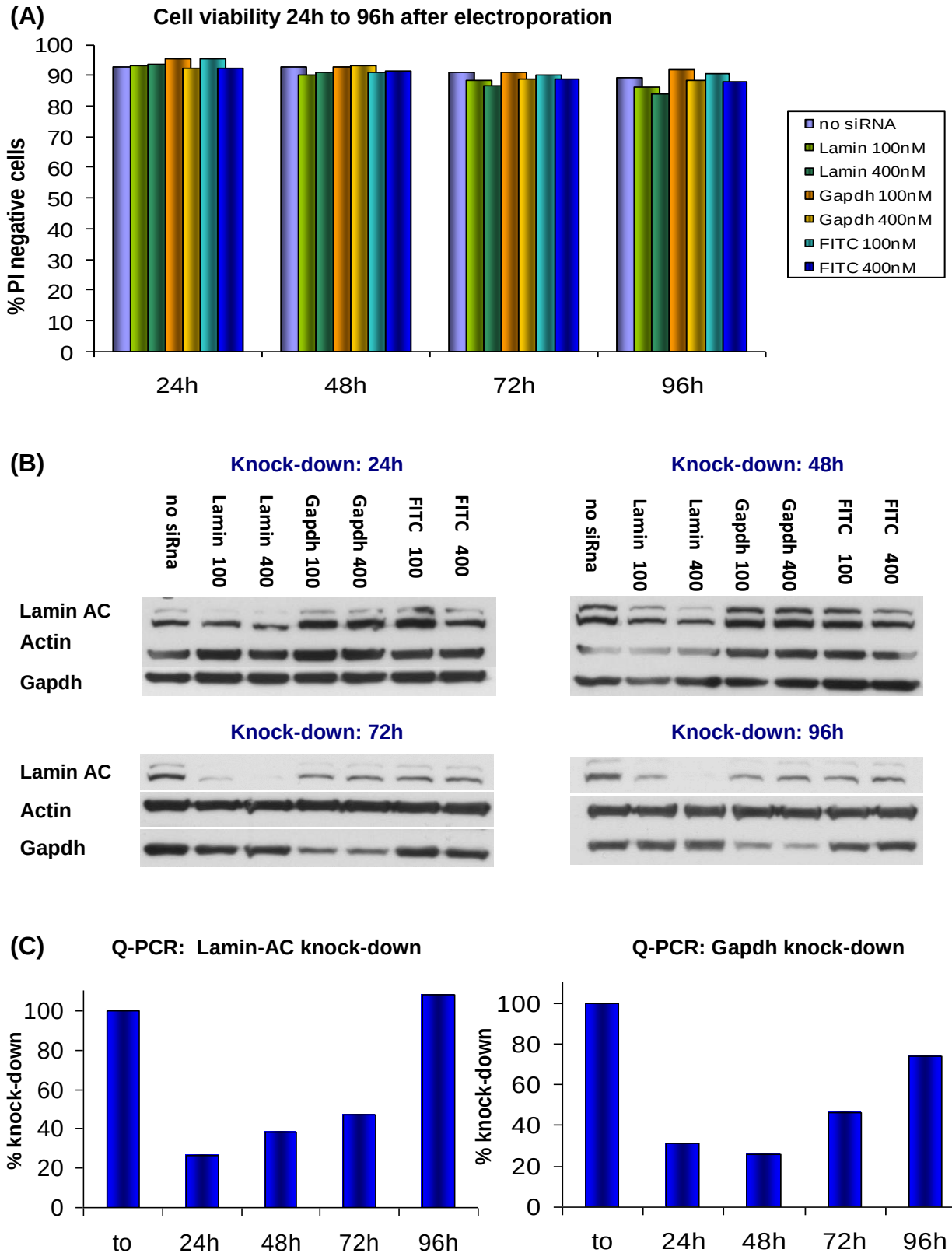


Figure 19: Time-course of the knock-down

2x10^6 cells (d15) were electroporated with 2 different siRNAs: LAMIN A/C- and GAPDH-siRNA at a concentration of 100nM and 400nM. One approach electroporated without siRNA and one approach electroporated with the non-coding FITC-siRNA served as a control. ***(A)*** *24h to 96h after electroporation the cell viability was analysed by flow cytometry, whereas the knock-down was analysed on protein-level by Westernblot-analysis* ***(B)*** *and on mRNA-level by Q-PCR 24h to 96h* ***(C)****.*

LAMIN A/C knock-down

The knock-down efficiency on protein level was analysed by Westernblotting and on mRNA-level by Q-PCR 24h to 96h after the electroporation. We could show that when the siRNA- concentration was 400nM, the LAMIN A/C- knock-down on protein-level was stable until 96h after electroporation, whereas at a concentration of 100nM, the expression increased again after 72h (see figure 19 B) and C)).

This data reveal that the used siRNA- concentration might influence the knock-down stability. The higher the siRNA- concentration, the higher the stability of the knock-down might be.The highest down-regulation of LAMIN A/C on protein-level could be achieved 72h after electroporation and on mRNA-level after 24h.

GAPDH- knock-down

In this first experiment the GAPDH- levels couldn't be reduced as much as the Lamin- A/C levels, because the highest knock-down on protein-level was achieved just 96h after the electroporation, while on mRNA level the highest down-regulation seemed to be achieved after 48h.

The GAPDH-siRNA was used in all of our following electroporations as a positive control and as shown in figure 20 with the optimized settings we were able to reduce the GAPDH- expression on mRNA- level by over 80% after 48h (data of 4 independent experiments and 3 different hEPC cultures isolated from 3 different cord-blood samples) and on protein- level up to 80% after 72h.

(A) Knock-down on mRNA level (48h): (B) Knock-down on protein-level (72h):

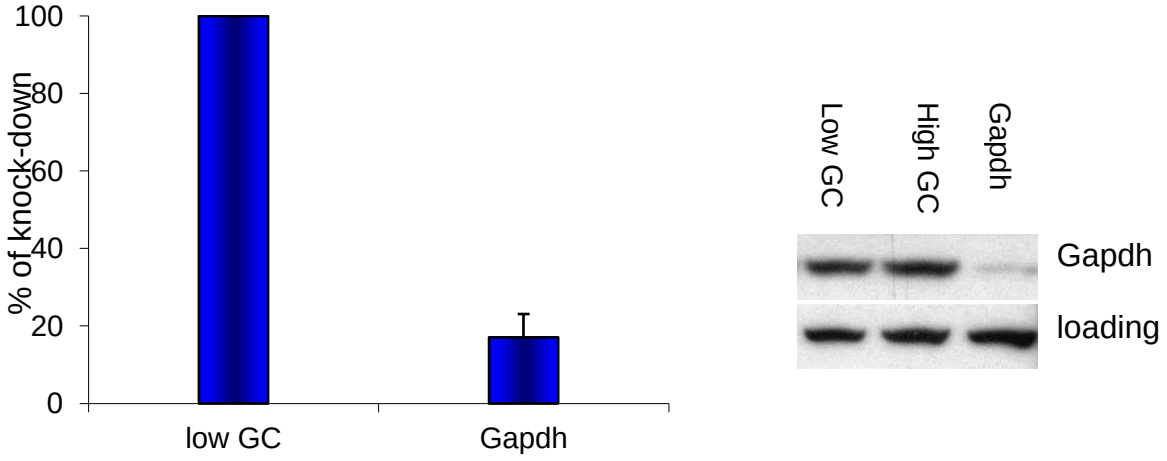


Figure 20: Gapdh knock-down 48h after electroporation

(A) 48h after electroporation the GAPDH- expression on mRNA-level could be reduced by over 80% in 4 independent experiments (analysed by Q-PCR) and on protein level (B) up to 80% 72h after electroporation (analysed by Westernblotting).

3.2.4 Best time constant and electroporation volume

Other important paramenters which have to be considered for a successful electroporation are pulse length and temperature. The pulse applied to the cells can be a square or an exponential wave (Jordan et al., 2008). The time constant is defined as the duration of the decay of the exponential wave, it is the product of the capacitance settings and the resistance of the sample (resistance is dependend on the ionic composition of the electroporation buffer). Therefore the higher the capacitance settings, the higher also the time-constant and the lower the cell viability was.

In our experiments, the cuvettes were always placed on ice before the electroporation, as we detected that the lower the temperature of the electroporation cuvettes was, the higher also the time-constant was. We observed that a time-constant around 10 msec to 13 msec was optimal to achieve a high viability and a high knock-down efficiency.

We always electroporated $2x10^6$ cells in a volume of 200µl and we found that also the electroporated volume seems to be a crucial factor for the knock-down success. If volume was higher than 200µl (for example 600µl) the time-constant decreased dramatically from 12msec to 3msec. This short pulse was definitely too short to allow the siRNA to enter the cells, therefore also no knock-down could be achieved (data not shown).

In summary the best electroporation settings for hEPCs were:

- Voltage- and capacity settings: 300V and 150µF
- Cell density: $2x10^6$ cells
- sufficient siRNA concentration: 400nM
- electroporation volume: 200µl (to ensure a time-constant around 12msec)
- precooled electroporation cuvettes
- low salt transfection medium (Opti-MEM $^{(R)}$)

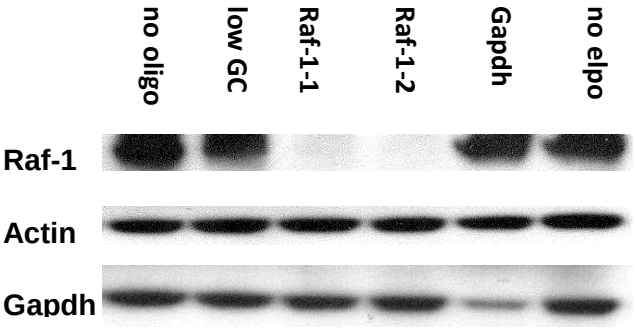
3.3 Raf-1: serine/threonine kinase 1

To study the function of Raf-1 in human EPCs, downregulation of Raf-1 was performed with two validated Raf-1- siRNAs (Raf-1-1 and Raf-1-2). For this experiment three different negative controls were used: One approach not electroprated (no elpo), one approach electroporated without siRNA (no oligo) and one approach electroporated with the non-coding siRNA (low GC-siRNA). To control the electroporation efficiency, one approach was electroporated with the Gapdh-siRNA under the same conditions. The Gapdh-expression on protein-level could be reduced by over 80% (analysed by Western-Blotting 48h after electroporation, shown in figure 21).

48h afer the electroporation the down-regulation of Raf-1 on protein-level was analysed by Western-Blotting. The Raf-1 protein- level could be reduced by over 90% with both Raf-1 siRNAs (Raf-1-1 nd Raf-1-2), as demonstrated in figure 21 (A). This result was obtained with both used siRNAs.

The Raf-1 levels could be reduced not only on protein-level, but also on mRNA-level, which was analysed by Q-PCR (shown in figure 21 (B)).

A) Raf-1 knock-down on protein-level (48h)



B) Raf-1 knock-down on mRNA-level

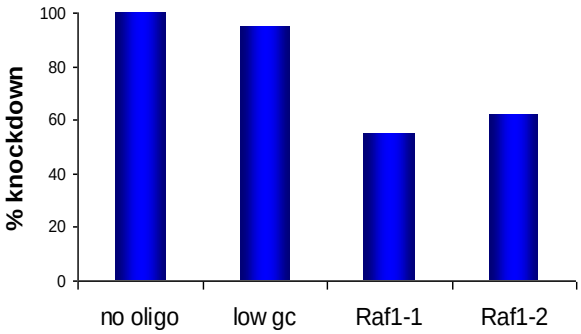


Figure 21: Raf-1 knock-down
*$2x10^6$ cells per approach were electroporated with 2 different Raf-1 siRNAs (Raf-1-1 and Raf-1-2). As a positive control the Gapdh-siRNA was used; the non-coding low GC-siRNA and two approaches either not electroporated (no elpo) or electroporated without siRNA (no oligo) served as negative controls. 48h after electroporation the downregulation was analysed on protein-level by Westernblot-analysis **(A)** and on mRNA-level by Q-PCR **(B)**.*

Effects of the Raf-1 knock-down

As it has has been reported that Raf-1-deficient mouse erythroblasts differentiate much faster than the wild-type erythroblasts (Kolbus et al., 2002), we expected more spontaneous differentiation and a lower proliferation-rate of human EPCs after reduction of Raf-1. In the second round of experiments, the Raf-1 protein levels could be reduced by over 65% and the mRNA-levels by over 60% 48h after the electroporation, as demonstrated in figure 22 (B).

The Gapdh-siRNA was used as a positive control; the non-coding low GC-siRNA and one approach electroporated without siRNA (no oligo) served as negative controls. The Gapdh-expression on protein-level could be reduced by over 85% (analysed by Western-Blotting after 48h, shown in figure 22 (A)).

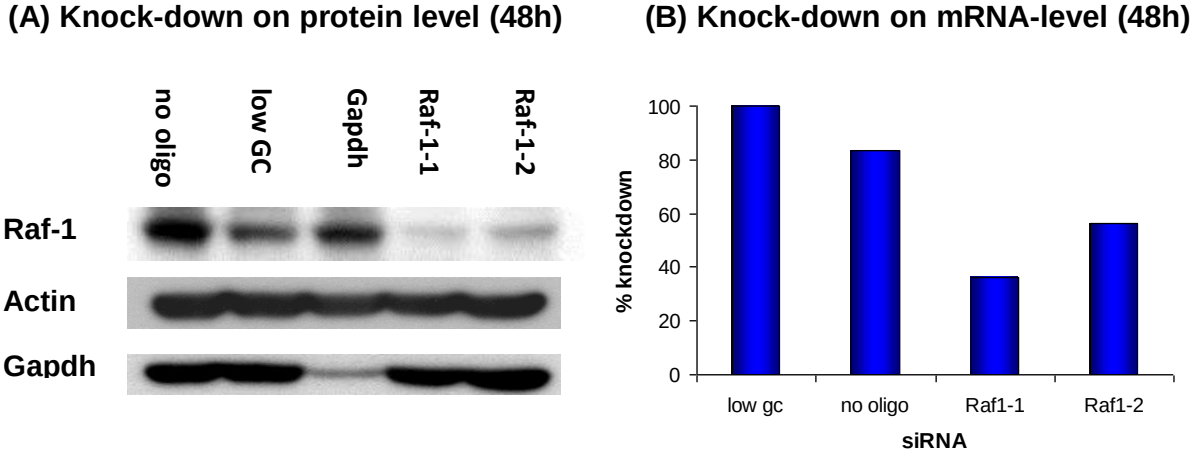


Figure 22: Raf-1 knock-down on protein- and on mRNA-level
*$2x10^6$ cells per approach were electroporated with 2 different Raf-1 siRNAs (Raf-1-1 and Raf-1-2). The Gapdh-siRNA was used as a positive control; the non-coding low GC-siRNA and one approach electroporated without siRNA (no oligo) served as negative controls. Knock-down has been analysed on protein level by **(A)** Western Blot- and on mRNA level by **(B)** Q-PCR- analysis 48h after electroporation.*

When compared to mouse EPCs, human EPCs show more spontaneous differentiation. Additionally the spontaneous differentiation was also increased when the hEPCs were cultivated at densities higher than $1x10^6$ cells/ml.

Thus we tried to enhance the expected effects of the Raf-1 knock-down by keeping the Raf-1 deficient cells at higher cell-densities.

As the data of our first Raf-1 knock-down experiment revealed that the highest knock-down of Raf-1 could be achieved 48h after the electroporation, this timepoint was used for seeding the electroporated cells in three different concentrations (in triplicates of 100µl each), the cumulative cell number was analysed from 48 to 120 hours after the electroporation. The three different used concentrations were:

→ $1x10^6$ cells/ml

→ $2x10^6$ cells/ml

→ $4x10^6$ cells/ml

The cytospins, the Casy-counts and -profiles of different timepoints (72h to 120h) gave us insights of the effects of the Raf-1 downregulation.

3.3.1 Effects of the Raf-1 knock-down on proliferation rate

While we couldn't detect significant differences in the proliferation rate at a cell density of $1x10^6$ and $2x10^6$ cells/ml, we could demonstrate that Raf-1 deficient cells proliferated slower compared to the cells electroporated with the lowGC-control, when they were cultivated at a cell-density of $4x10^6$ cells/ml.

As demonstrated in figure 23 the highest difference between cells with reduced Raf-1 levels and the controls according the cumulative cell numbers, was at the timepoint of 96h after the electroporation. At later time points, e.g. 120hours after the electroporation the cell numbers of the Raf-1 approaches were again similar to those of the control. We didn't analyse wheater the Raf-1 knock-down was still present 120h after the electroporation, so it is possible that at this timepoint the Raf-1 protein levels were increased again and thereby the inhibitory effects on the proliferation got lost.

Imortantly, it should be noticed that the different approaches were not adjusted daily to their specific cell-concentration ($1x10^6$, $2x10^6$ and $4x10^6$ cells/mL), as the sample volume of 100µl was too small. Therefore all of the approaches (for each siRNA in three different concentrations in triplicates) were fed every day by simply adding 100µl of fresh proliferation medium.

Effects of the Raf-1 knock-down on proliferation rate

Proliferation-rate after Raf-1 knock-down (with three different cell densities)

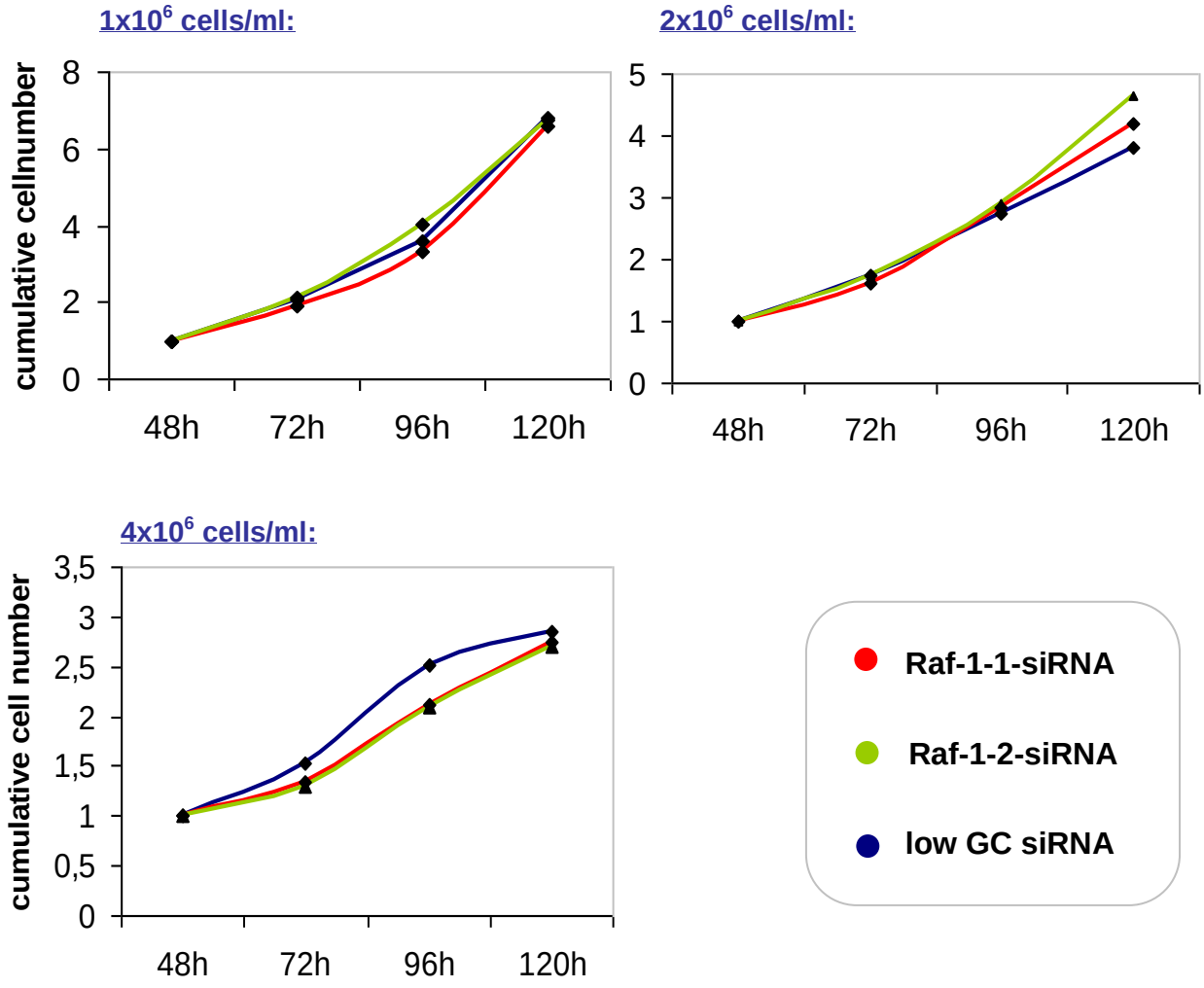


Figure 23: Effects of the Raf-1 knock-down on proliferation
48h after electroporation with 2 different Raf-1-siRNAs (Raf-1-1 and Raf-1-2) and lowGC-siRNA as a control, the cells were seeded at the 3 different indicated concentrations (1x10^6 cells/ml, 2x10^6 cells/ml, 4x10^6 cells/ml) and the proliferation rate was analysed by Casy-counts at 3 different timepoints: 72h, 96h and 120h after electroporation.

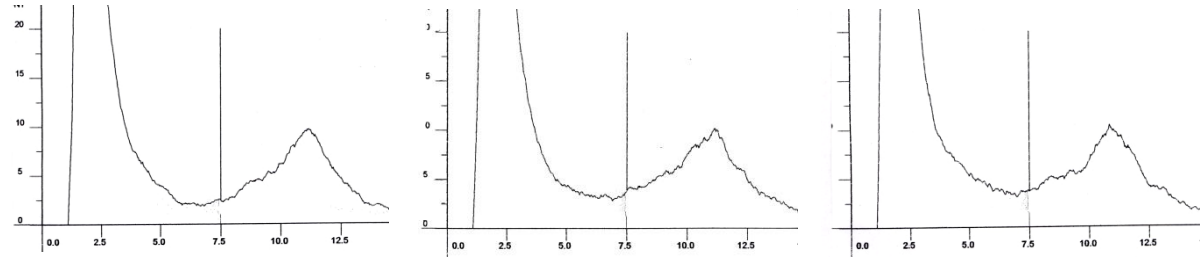
Thereby the cell-concentrations were diluted every day step by step and 120h after electroporoation the cell density might have been much lower than 4x10^6 cells/ml. This could also explain why the effects on the proliferation were diminished or reverted 120h after electroporation.

3.3.2 Effects of the Raf-1 knock-down on the Casy profiles

The cell suspensions were analysed daily by the CASY-cell analyser starting from 24 to 120 hours after electroporation. We could detect a difference between the CASY-profiles (cell size distribution) of the cells with reduced Raf-1 levels and the control cells. Figure 24 shows the CASY-profiles of the different cells at the indicated time points. 24h after electroporation, cells electroporated with the Raf-1-siRNAs did not show remarkable differences compared to the control cells. Importantly, 120 hours after electroporation a clear shift towards smaller cell size could be observed and also a defined peak of differentiated cells could be noticed in the profiles of Raf-1 deficient cells. The decrease in size and is one hallmark of differentiation (see figure 24).

24h after electroporation:

low GC: **Raf-1-2:** **Raf-1-1:**



120h after electroporation:

low GC: **Raf-1-2:** **Raf-1-1:**

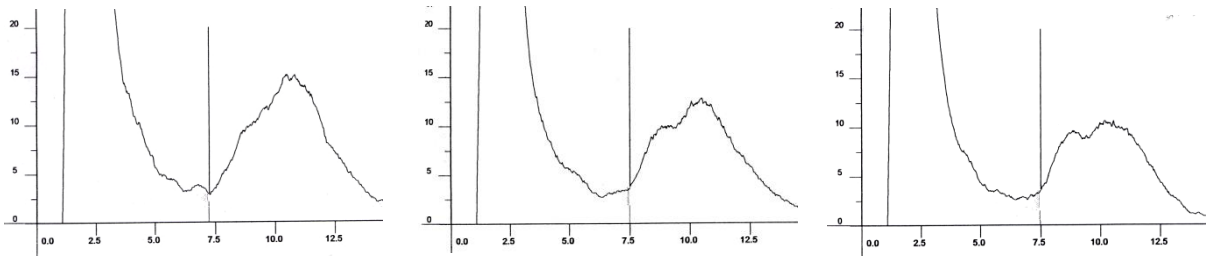
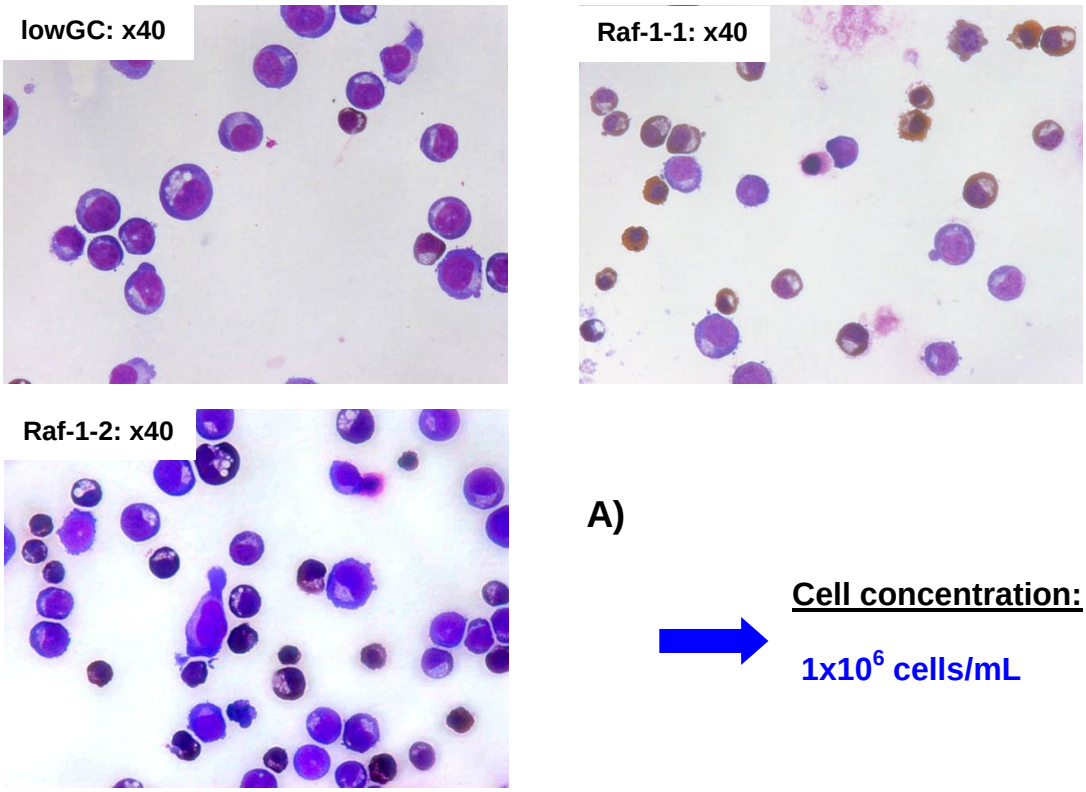


Figure 24: Casy Profiles 24h and 120h after electroporation
After electroporation with 2 different Raf-1-siRNAs (Raf-1-1 and Raf-1-2) and lowGC-siRNA as a control, cell numbers were analysed daily (24h to 120h after the electroporation) by the CASY-cell analyser. CASY-profiles show cell-size distribution.

3.3.3 Effects of the Raf-1 knock-down on the cell morphology

The cell morphology of the different cells was analysed via cytocentrifugation onto glass slides and subsequent staining with benzidine and hematological dyes (cytospins). Such cytospins were perfomed and analysed with each approach in the accordant cell concentration.

We found that the cytospins of Raf-1 deficient cells showed more differentiated cells relative to the control cells; this effect was noticed at low cell densities ($1x10^6$ cells/mL) (see figure 25 (A)). Against the effects detected on the proliferation rate, the cytospins of Raf-1 deficient cells seeded at higher concentrations showed no significant difference in differentiation compared to the control cells (low-GC). The shown experiment was performed with EPC culture (isolated from cord blood), with an innately high spontaneous differentiation. Thus, at higher concentrations ($2x10^6$ cells/mL and $4x10^6$ cells/mL) the levels of differentiating cells were also increased in the control cells (see figure 25 (B) and (C)).



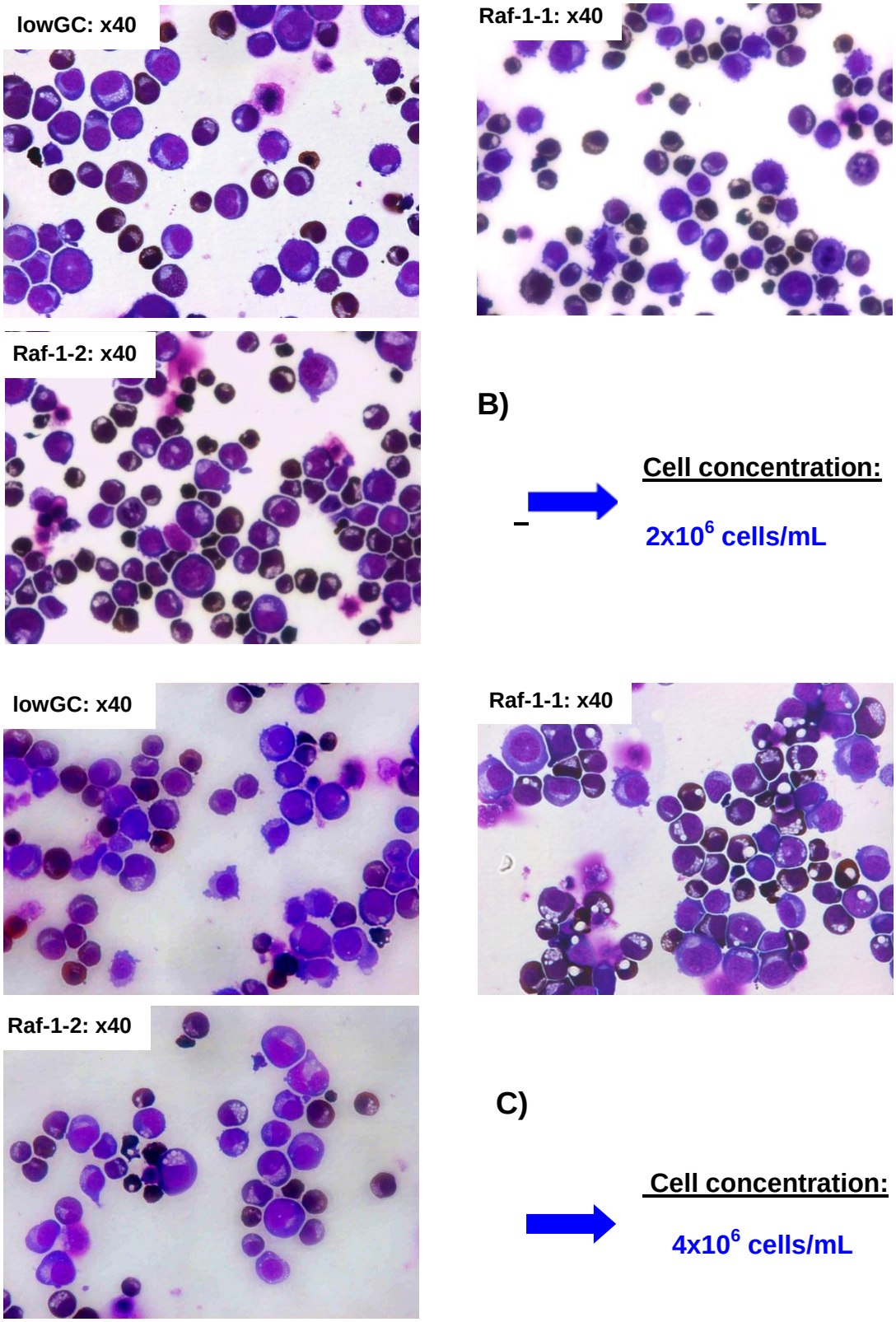


Figure 25: Raf-1 deficient cells show higher spontaneous differentiation
*48h after electroporation with 2 different Raf-1-siRNAs (Raf-1-1 and Raf-1-2) and lowGC-siRNA as a control, the cells were seeded at 3 different concentrations **(A)** 1x10^6 cells/ml, **(B)** 2x10^6 cells/ml, **(C)** 4x10^6 cells/ml). 24h to 120h after the electroporation cell aliquotes were cyto-centrifuged onto glass slides and stained. Here are shown the cytospins of the time-point 120h after the electroproation.*

Nevertheless, we could show that the reduction of Raf-1 resulted in an increase of spontaneously differentiated cells when seeded at a concentration $1x10^6$cells/mL. The cells with lower Raf-1 protein levels show a decrease in size and an increased hemoglobin accumulation, two parameters which are typical signs for terminal differention (see figure 25 (A)).

3.3.4 Effects of Raf-1 knock-down on pERK- and Caspase-3 levels

In the mouse erythroid cells Raf-1 has been demonstrated to stimulate ERK activation and thereby to inhibit Caspase activation, which is involved in the differentiation processes (Rubiolo et al., 2006).

To analyse whether Raf-1 has a similar function in human cells, we analysed the phosphorylation of ERK- and the cleavage of Procaspase-3 on protein level in the electroporated cell. Electroporation was performed in 2 different EPC- cultures isolated from 2 different cord blood donors (LT11 and CB14). Cells were electroprated with 2 different Raf-1 siRNAs (Raf-1-1- and Raf-1-2- siRNA); and the non coding lowGC-siRNA and one approach electroporated without siRNA served as negative controls. The knock-down efficiency and the pERK- and Procaspase-3- levels were analysed by Westernblotting 72h or 96h after electroporation (figure 26).

As demonstrated on the left panel of figure 26, when Raf-1 knock-down was performed in cord blood LT11, 72h after electroporation, the pERK levels were slightly reduced (in the approach electroporated with Raf-1-2-siRNA), whereas the Procaspase-3 levels remained unchanged. Furthermore, in the approach electroporated with the Raf-1-1 siRNA, where the knock-down was even stronger compared to the Raf-1-2 oligo, the pERK levels were not reduced, but Caspase-3 (which is correlating with reduced Procaspase-3) was slightly activated.
On the other hand in the Raf-1 knock-down performed in cord blood CB14 (see right panel of figure 26), the pERK-levels were slightly reduced compared to the lowGC-control and the Caspase-3 was activated (shown is the reduction of Procaspase-3), especially in the approach electroporated with Raf-1-2 –siRNA.

Effects of Raf-1 knock-down on pERK- and Caspase-3 levels

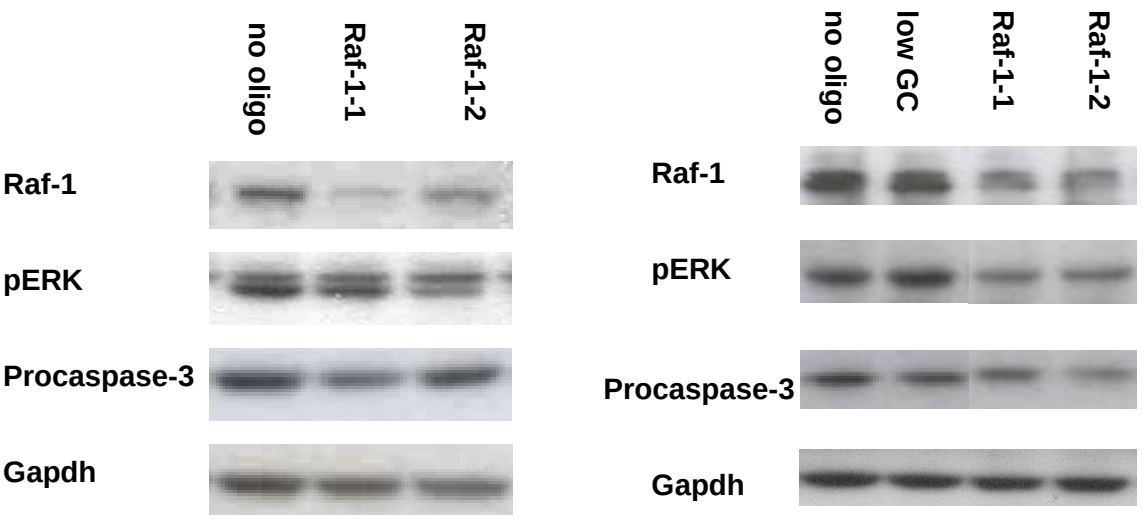


Figure 26: Effect of Raf-1 knock-down on pERK and Caspase-3 levels
Electroporation was performed in 2 different EPC cultures from 2 different cord blood donors (LT11 and CB14). Cells were electroporated with 2 different Raf-1 siRNAs (Raf-1-1- and Raf-1-2- siRNA); the non coding lowGC-siRNA and one approach electroporated without siRNA (no oligo) served as negative controls. The knock-down efficiency and the effects on the pERK- and Procaspase-3- levels were analysed by Westernblotting 72h or 96h after electroporation.

Thus, we could detect in the human system similar effects as reported in the mouse system. Unfortunately, when the Raf-1 knock-down was performed in other EPC cultures from a different donor, the effects could not be reproduced: there were no effects on the phosphorylation of ERK or on the cleavage of Procaspase-3.

3.4 Tis11d: Zinc Finger Protein 36 like 2

In a microarray screen Tis11d has been identified as a glucocorticoid target gene. Tis11d is a zinc-finger-like protein and member of the Tis11-family. The Tis11-family consists of four mammalian members: TTP, Tis11b, Tis11d and Zfp36L3 (expressed only in rodents) (Baou et al., 2009).

We analysed the expression levels of all of the three Tis11-like proteins in human EPCs on mRNA-level by Q-PCR and as shown in figure 27, we found that Tis11d was the highest expressed zinc-finger-like protein in erythroid cells. While the Tis11b-expression was extremly low, TTP showed intermediate expression levels.

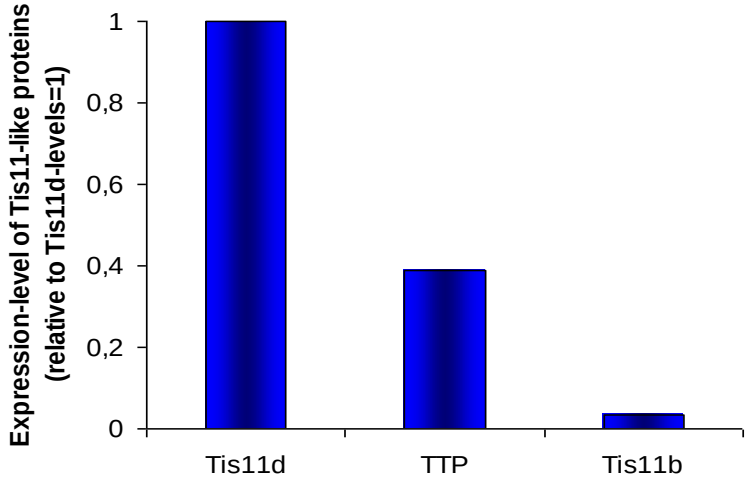


Figure 27: Expression levels of Tis11-like proteins in human EPCs

The levels of Tis11d, TTP and Tis11b were analysed by Q-PCR in hEPCs (d14).

To study the function of Tis11d in hEPCs we performed a Tis11d knock-down with three different Tis11d-siRNAs (Tis11d-1, -2, -3).

The two non-coding low GC- and high-GC-siRNAs and one approach without siRNA (no oligo) served as negative controls. 72h after the electroporation we showed an over 80% decrease of the Gapdh protein level when using Gapdh-siRNA as a positive control (figure 28). With the siRNA Tis11d-2, the Tis11d protein-levels could be reduced by over 70% on protein-level 72 hours after electroporation as shown in figure 28 (A). Importantly also the mRNA-levels were reduced by over 60% after 72h in the samples treated with the siRNA Tis11d-2, as demonstrated in figure 28 (B).

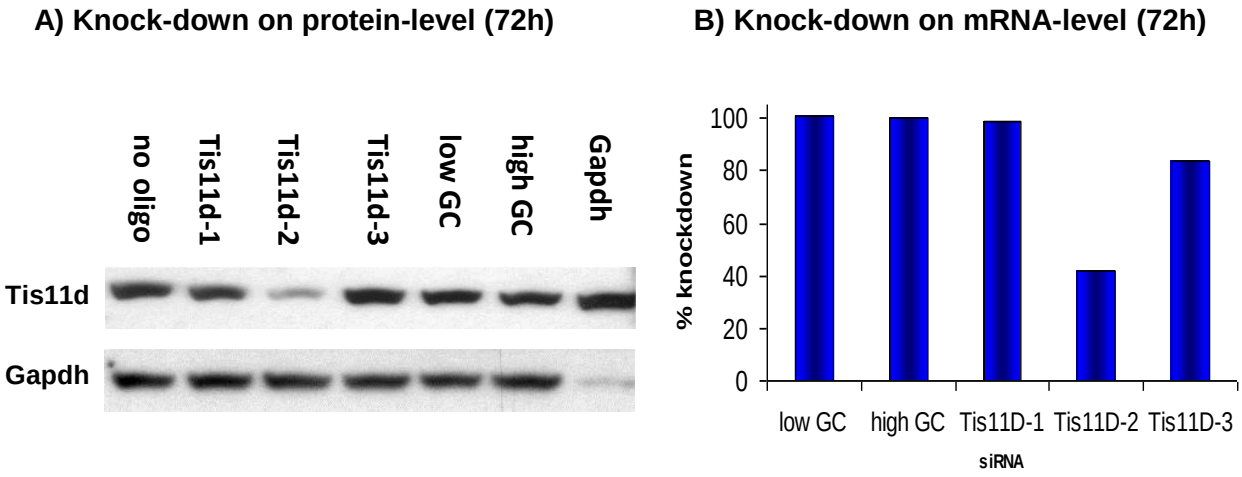
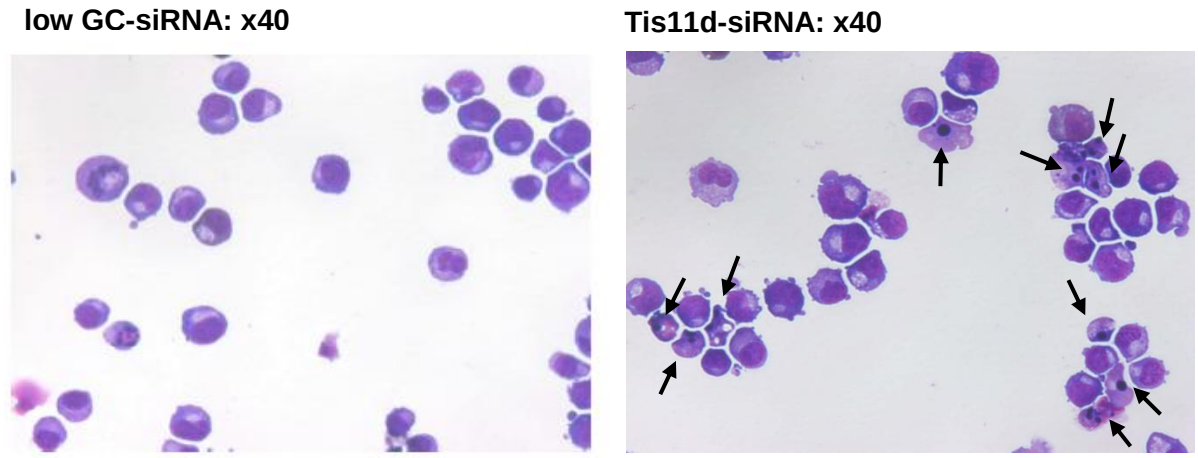


Figure 28: Tis11d knock-down 72h after electroporation
*$2x10^6$ cells per approach were electroporated with 3 different Tis11d- siRNAs (Tis11d-1, -2, -3). The Gapdh-siRNA was used as a positive control; the non-coding low GC-, and high GC-siRNAs and one approach electroporated without siRNA (no oligo) served as negative controls. 72h after electroporation the knock-down was analysed on protein-level by Westernblot-analysis (**A**) and on mRNA-level by Q-PCR (**B**).*

3.4.1 Effect of the Tis11d knock-down on cell morphology

In the cell samples in which Tis11d- levels were reduced, we noticed an increase of apoptotic cells in cytospin preparations 96h after the electroporation. As mentioned above, when cells undergo apoptosis specific morphological changes within the cell can be observed: cytoplasm condensation, nucleus shrinkage, nuclear chromatin condensation, breakage of the cells into membrane-bound apoptotic bodies, which are containing a variety of cytoplasmic organelles and nuclear fragments. As shown in figure 29, in the cytospins of the Tis11d-deficient cells exactly these morphological changes could be observed: When Tis11d was knocked down, more cells with condensed and fragmented nuclei and apoptotic body formations could be detected, those cells are pointed out in the cytospins shown in the figures below by black arrows. The dead and apoptotic cells were counted and are shown as % of total cells (figure 29 (B)). In the approaches where Tis11d has been knocked down, an increase of approximatley 16% of dead and apoptotic cells relative to the low-GC control was determined.

A)



B)

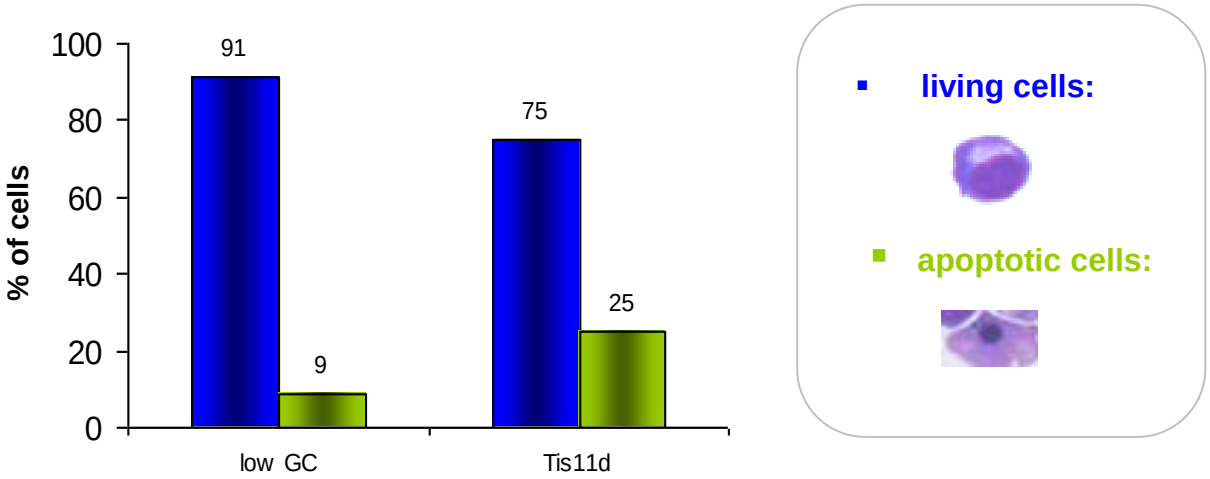
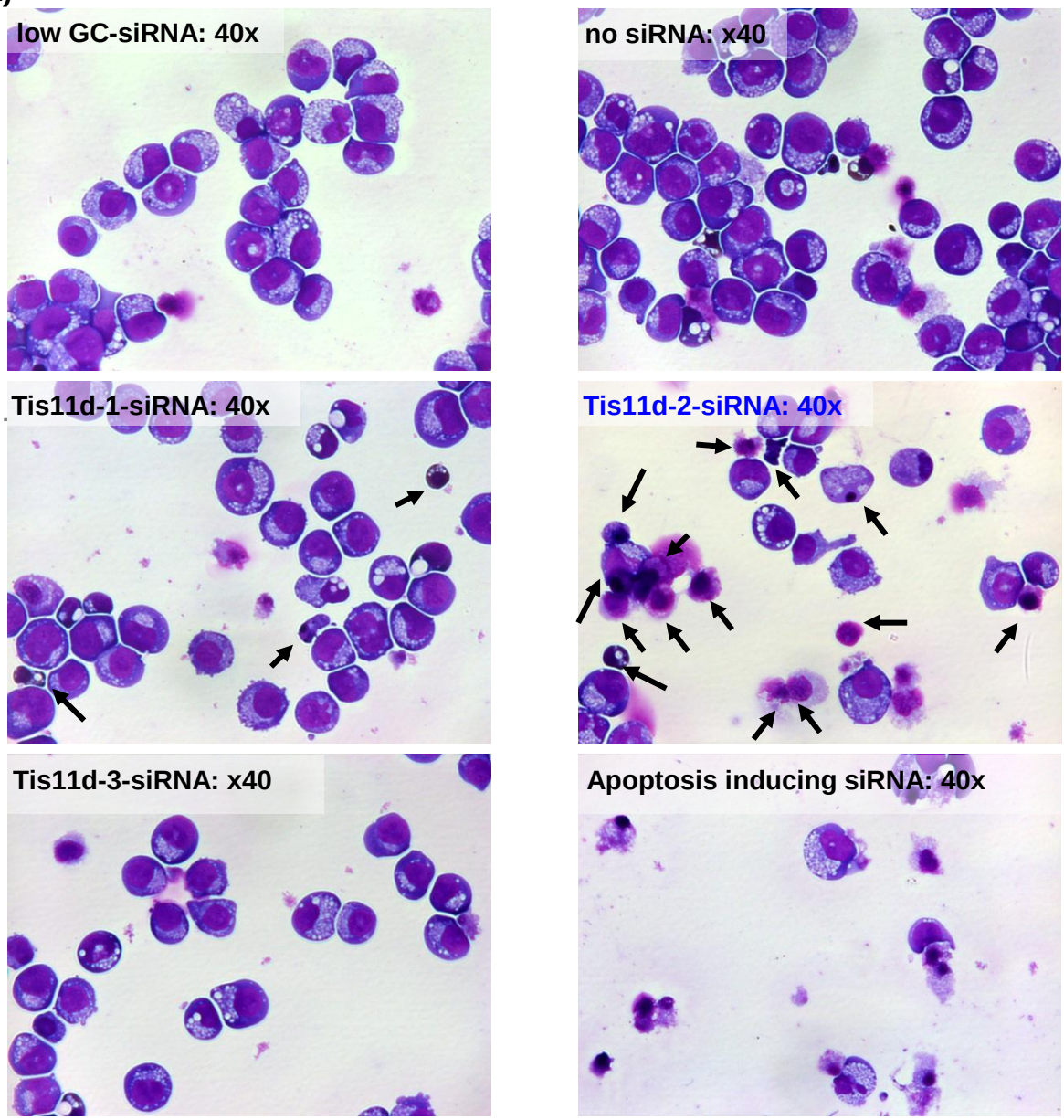


Figure 29: Increase of apoptotic cells 96h after Tis11d-knock-down
(A) *96h after electroporation cell aliquots were cyto-centrifuged onto glass-slides and stained as described (microscope: x40). Black arrows point out the cells with condensed and fragmented nuclei and apoptotic body formations during apoptosis.* ***(B)*** *Dead and living cells were counted and are shown as % of total cells.*

The experiment was repeated with another batch of EPCs from a different donor, and we observed a reproducible increase in apoptotic cells only in the approach, where Tis11d was effectively downregulated. Electroporation was performed with 3 different Tis11d- siRNAs (Tis11d-1, -2, -3). The non-coding low-GC-siRNA, the apoptosis-inducing siRNA and one approach electroporated without siRNA (no oligo) served as negative controls. Figure 30 (A) shows the cytospins of the different approaches, in which the black arrows point out the cells with the condensed and fragmented nuclei. Dead and apoptotic cells were counted and compared to the total cell number and shown as a % of cells in figure 30 (B).

A)



B)

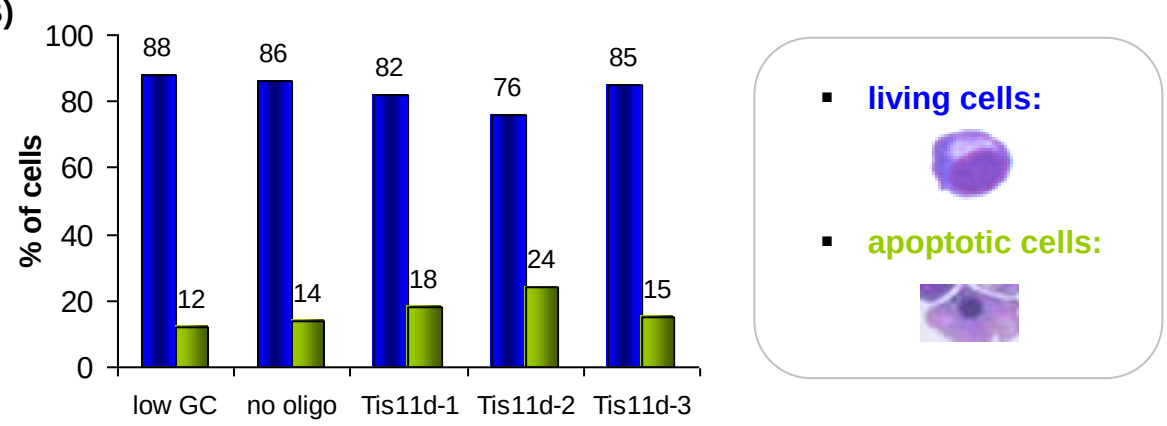


Figure 30: Increase of apoptotic cells 72h after Tis11d-knock-down
(A) *72h after electroporation with 3 different Tis11d-siRNAs and lowGC-siRNA and apoptosis-inducing siRNA as controls, cell aliquots were cyto-centrifuged onto glass-slides and stained (microscope: x40). The knock-down was achieved with the Tis11d-2 siRNA. In this approach were more dead and apoptotic cells were detectable, pointed out by black arrows.* ***(B)*** *Dead and living cells were counted and are shown as % of total cells.*

The most efficient downregulation of Tis11d was achieved with the Tis11d-2-siRNA and as shown in figure 30 exactly in this approach approximately 24% apoptotic cells could be determined, whereas in the low GC control sample only 12 % of the cells were apoptotic. The cells electroporated with the low GC- control or with the ineffective Tis11d-siRNAs, which didn't reduce the expression of Tis11d, showed mainly big cells with a large nucleus and a high amount of cytoplasm, whereas the cells with reduced Tis11d protein levels showed an increased amount of dead cells with small, dark and fragmented nuclei and a condensed cytoplasm (evaluated in figure 30 (A)).

3.4.2 Effect of the Tis11d knock-down on cell volume and cell size

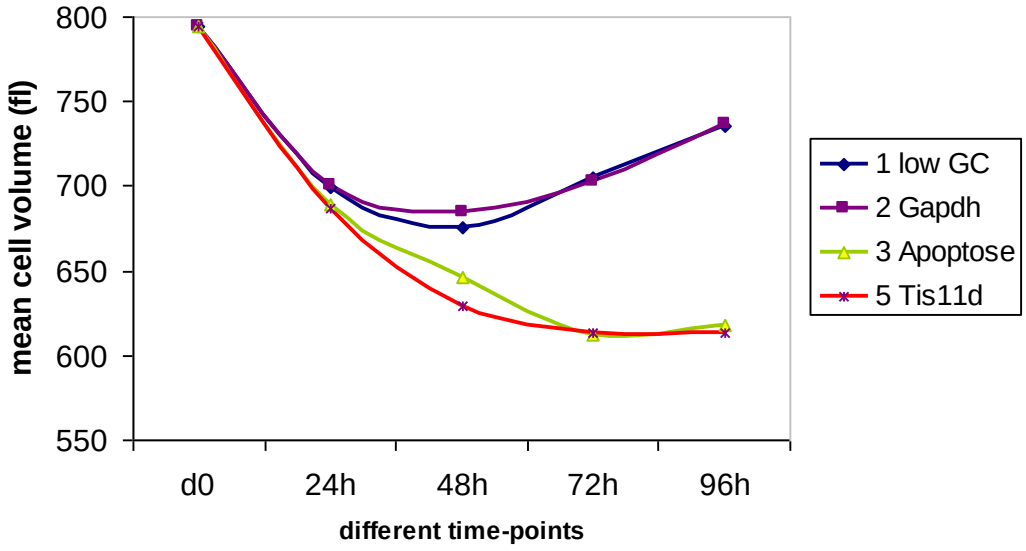
When cells undergo apoptosis a condensation of the cytoplasm occurs, before the nucleus becomes defragmented. To analyse this morphological changes, an additional electroporation was performed and the mean cell volume and the mean cell diameter of the different approaches were analysed 24h to 96h after electroporation by the CASY Cell Analyser. The Casy-measurements give information about the cell count and the cell size.

The electroporation was performed with the Tis11d-2 siRNA. Gapdh-siRNA and an apoptosis-inducing siRNA were used as positive controls, and the non-coding low GC-siRNA served as a negative control.

We observed that cells with lower Tis11d levels show a decrease in the mean cell volume and in the mean cell diameter after the electroporation, as shown in figure 31. The decrease of cell size is as high as in the approach electroproated with the apoptosis-inducing-siRNA, where over 80% of the cells died 96h after the electroporation.

Effect of Tis11d knock-down on mean cell volume and mean cell diameter:

A) Decrease in mean cell volume 24h-96h after the electroporation



B) Decrease in mean cell diameter 24h-96h after the electroporation

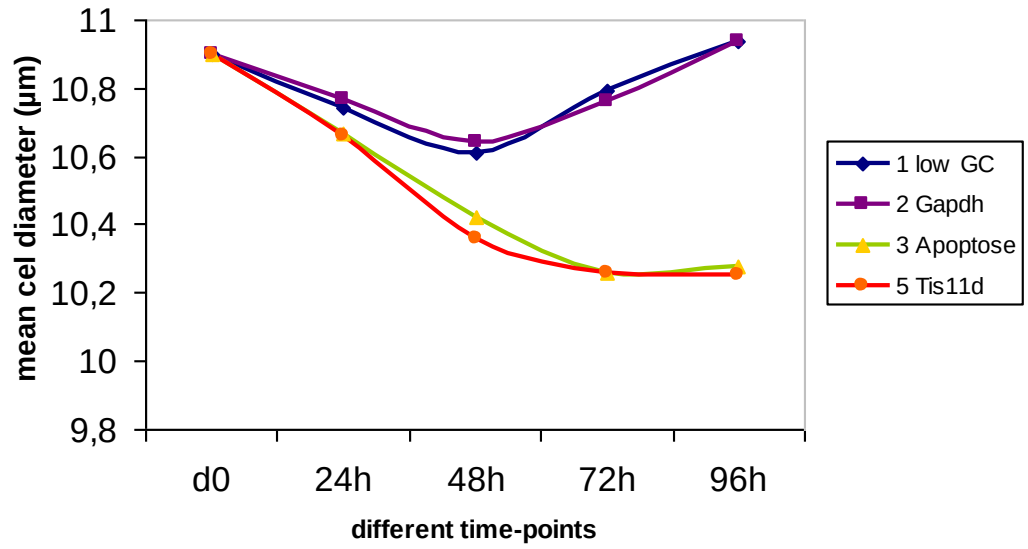


Figure 31: Decrease of mean volume (fl) and mean diameter (µm)
*24h to 96h after the electroporation with Tis11d-2-, lowGC-, GAPDH-, and apoptosis inducing –siRNA, the cells were counted daily by the CASY-cell analyzer. The Casy-profile gives you the cell count and as well the cell size (the mean cell volume **(A)** and **(B)** mean cell diameter).*

3.4.3 Effect of the Tis11d knock-down on cell viability

By comparing the cells treated with the positive control for apoptosis with the effects obtained with the reduction of Tis11d, we could prove that downregulation of Tis11d induces apoptosis.

To verify the apoptosis-inducing function, we performed further Tis11d knock-downs with three different hEPC- cultures and analysed the cell viability by FACS analysis. Thus, mononuclear cells were isolated from three different cord bloods (CB14, LT8 and LT9) and cultivation of EPC was performed as described in Materials and Methods until day 14, when the electroporation was performed with two Tis11d- siRNAs (Tis11d-1 and Tis11d-2). The non-coding low GC-siRNA served as a negative- and the Gapdh-siRNA as a positive- control. The downregulation on protein-level was analysed 96h after the electroporation by Western-Blotting.

The knock-down efficiency of Tis11d and Gapdh on protein-level varied depending on the used cord blood sample. As shown on the left panel of figure 32 the highest Tis11d knock-down could be achieved in EPCs isolated from the cord blood CB14, in which 96h after the electroporation the Tis11d levels could be reduced by over 80%, whereas in EPCs isolated from the cord blood LT8 and LT9 the knock-down efficiency was lower. 96h after the electroporation the cell viability of each approach was measured by an Annexin-V-staining by FACS-analysis, and the results are shown in the right panel of Figure 32.

As shown in figure 32, we found that a reduction of Tis11d-protein levels resulted in an increase of dead and apoptotic cells, measured as Annexin-V-positive cells in all of the three different hEPC cultures.
In the approaches where Tis11d has been down-regulated efficiently (depending on the cord blood, either with the Tis11d-1- or Tis11d-2- siRNA), nearly a two- fold higher amount of dead and apoptotic cells was measured.

Effect of Tis11d knock-down on cell viability

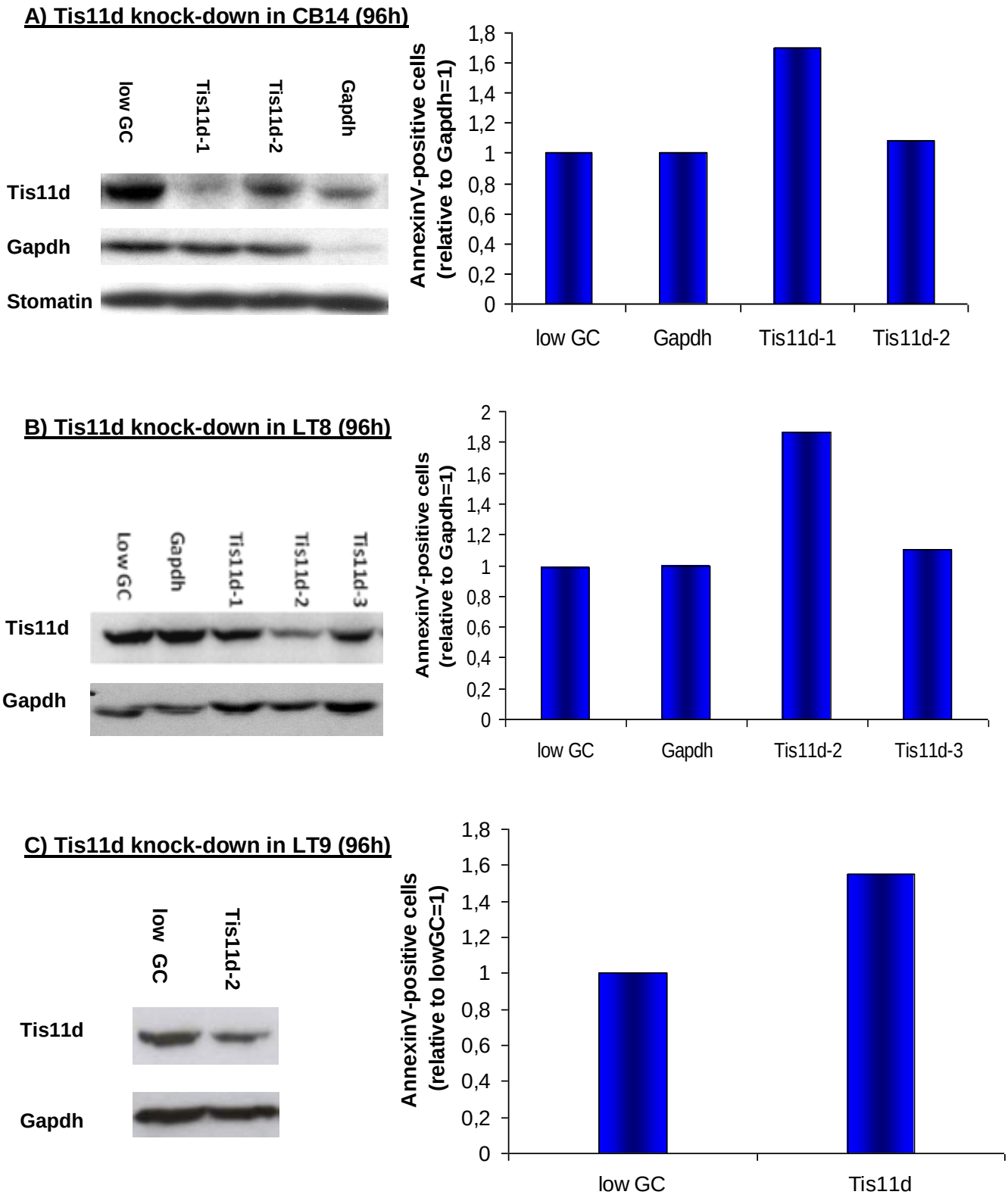


Figure 32: Tis11d knock-down induces apoptosis
*Tis11d knock-down was performed in 3 different EPC cultures isolated from 3 different cord blood samples **(A)** CB14, **(B)** LT8, **(C)** LT9. The non-coding lowGC-siRNA and Gapdh-siRNA were used as controls. 96h after the electroporation the knock-down was analysed by Western Blotting, as shown on the left panel of the figure. In each approach the cell viability was measured by an Annexin-V-staining by FACS analysis and is shown on the right panel of the figure.*

In figure 33 the cell viability data of those 3 different experiments are taken together and illustrated with the according standard deviation. In summary the downregulation of Tis11d led to an 1,7 fold increase of Annexin-V-positive cells, when compared to the cells transduced with lowGC-siRNA (relative to lowGC=1).
A performed student's t-test showed that the differences are statistically significant (the p-value was 0,027).

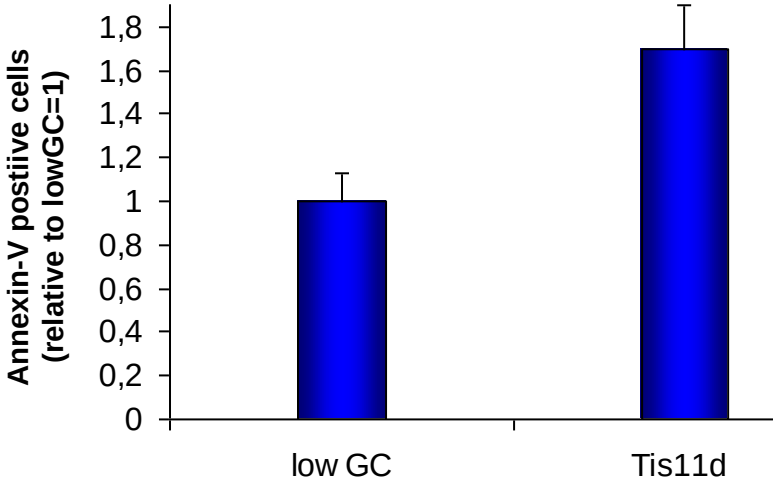


Figure 33: Tis11d knock-down induces apoptosis
Tis11d knock-down led to an increase of Annexin-V-positive cells (relative to lowGC=1). Data of three independent experiments, performed with 3 different EPC-cultures, isolated from 3 different cord blood samples.

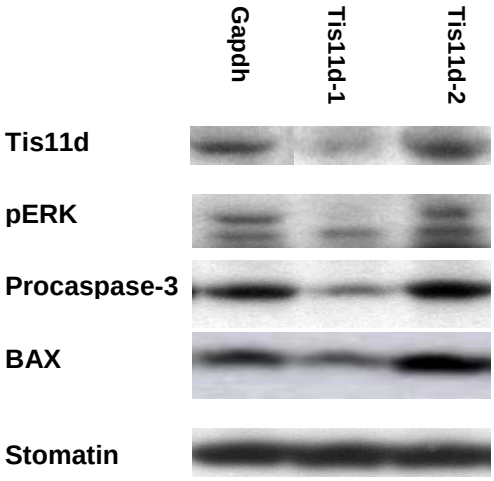
3.4.4 Effect of the Tis11d knock-down on Caspase-3 levels

When cells undergo apoptosis several pathways can be involved. To analyse if the apoptotic effect, seen after the Tis11d knock-down (shown on the left panel of figure 32), is mediated either by intrinsic or extrinsic pathways, the levels of Procaspase-3, -8 and BAX were analysed by Westernblotting. (Results are shown in figure 34).

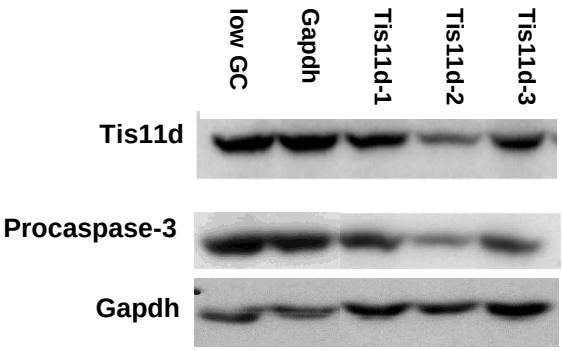
When Tis11d levels are reduced, we saw that Caspase-3 is activated, while the pERK-levels are reduced. We analysed the levels of the Procaspase-3 (figure 34), whose downregulation correleates with Caspase-3 activation.

Effect of the Tis11d knock-down on Caspase-3 levels

A) First Tis11d knock-down in CB14 (96h)



B) Tis11d knock-down in LT8 (96h)



C) Second Tis11d knock-down in CB14 (72h)

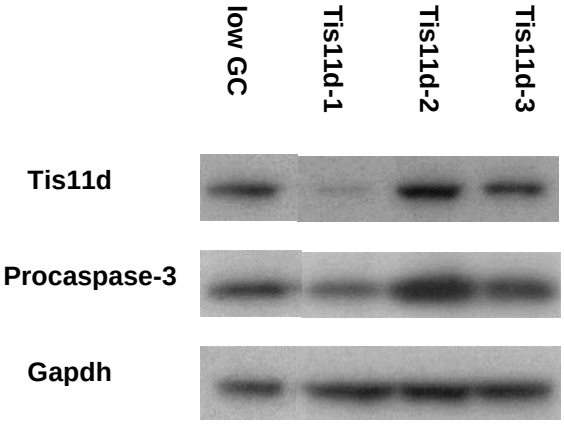


Figure 34: Downstream effects of the Tis11d-knock-down
Tis11d knock-down was performed in 3 independent experiments, with two different hEPC-cultures (isolated from two different cord blood samples CB14 and LT8). 96h and 72h after the electroporation the knock-down efficiency of Tis11d was analysed by Westernblotting. Here are shown the downstream effects of the Tis11d downregulation on the pERK-, Procaspase-3- and Bax-levels. Downregulation of Procaspase-3, correlates with activation of Caspase-3.

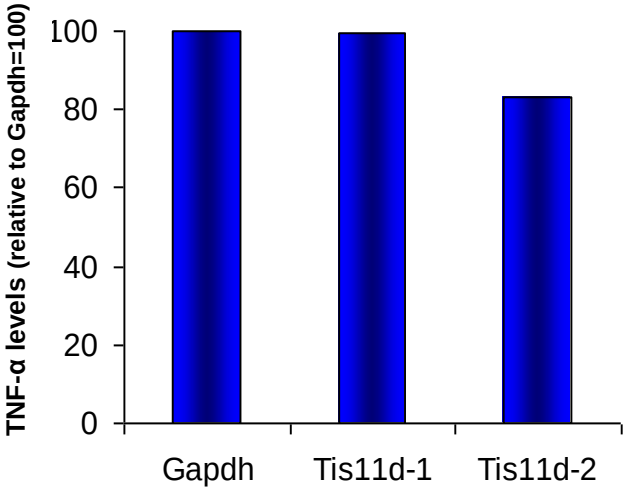
Surprisingly the BAX-levels were also down-regulated after reduction of Tis11d (figure 34 A). The Westernblot results concerning the caspase-8 are not conclusiv, since the used antibody gave too high background levels (data not shown).

3.4.5 Effect of the Tis11d knock-down on TNFα-levels

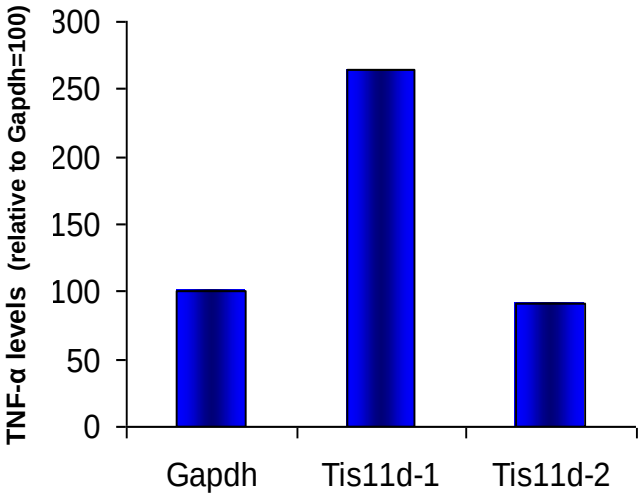
Members of the Tis11-protein-family have been reported to be able to bind to ARE- containing mRNAs and subsequently induce their degradation (Lai et al., 1999). Tristetraprolin (TTP) is the most studied member of this family and it has been shown in several publications that TTP can bind directly to the AU-rich element in the TNF-α mRNA and thereby induce its degradation (Carballo et al., 1998; Lai et al., 1999; Lai et al., 2000; Stumpo et al., 2009).

To analyse whether downregulation of Tis11d affects expression of TNF-α, we measured the mRNA-level of TNF-α by real-time PCR (Q-PCR) in cells with reduced Tis11d levels (see figure 35).

(A) normalised to the housekeeper



(B) not normalised to the housekeeper



(C) Tis11d knock-down (96h)

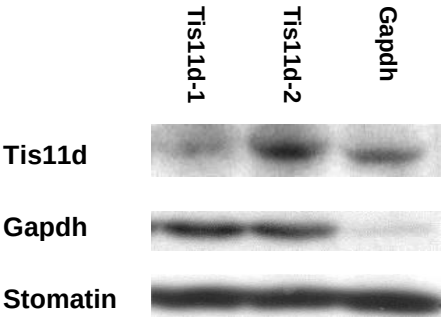


Figure 35: TNF-α levels after Tis11d-knock-down

*Electroporation was performed with 2 different Tis11d-siRNAs. Gapdh-siRNA was used as a positive control. 96h after the electroproatin the Tis11d knock-down was analysed by Westernblotting (**C**). The TNF-α mRNA-levels in the approaches electroproated with the 2 different Tis11d-siRNAs, and the Gapdh-siRNA were analysed by Q-PCR (A) and (B).*

***(A)** On the left panel of the figure are shown the TNF-alpha mRNA-levels when normalised to the housekeeper CSNK1d and on the right panel of the figure are shown **(B)** the Q-PCR results of the TNF-alpha levels evaluated according the dCT-values.*

When the Q-PCR-data were evaluated relative to the housekeeper CSNK1d (Casein kinase I isoform delta) (see figure 35 (A)), we could not detect a difference of TNF-α mRNA expression after the Tis11d knock-down (figure 35 (C)).
But if the TNF- α levels were determined only according the dCT-values we could demonstrate a nearly 3-fold increase of TNF-α mRNA in the absence of Tis11d, as shown on the right panel of Figure 35 (figure 35 (B and C)).

As secreted TNF-α protein could also be responsible for the increased apoptosis; we tried to measure the amount of TNF- α in the supernatant of the erythroid cells. Therefore the supernatant of the different approaches of the Tis11d knock-down was collected and the TNF- α levels were analysed by ELISA (Enzyme- linked immunosorbent assay).
Unfortunately we could not detect TNF-α in the analysed samples. Probably the TNF-α levels of the tested samples were below the limit of detection of the assay, which was in the range of 10 pmol TNF-α (data not shown). Although we could show that the mRNA for TNF-α is present in these cells, we couldn’t measure TNF-α in our samples. Additionally, we can not exclude that soluble TNF-α is not produced in hEPCs.

3.5 Fkbp51: FK506-binding protein 51

To study the function of FKBP51 in primary hEPCs we performed a knock-down with three different Fkbp51-siRNAs (Fkbp51-1, Fkbp51-2, and Fkbp51-3). The non-coding low GC-, med- and high GC-siRNAs were used as negative controls. The knock-down efficiency was analysed 72h after the electroporation by Westernblotting, as shown in figure 36 (A). With all three siRNAs the expression of FKBP51 could be reduced by over 90% on protein-level (data of the FKBP51 knock-down induced by the Fkbp51-1-siRNA are not shown). Importantly also the mRNA-levels were reduced by over 60% to 75% with all three siRNAs 72h after electroporation, as shown in Figure 36 B).

(A) Knock-down on protein-level (72h) **(B) Knock-down on mRNA-level (72h)**

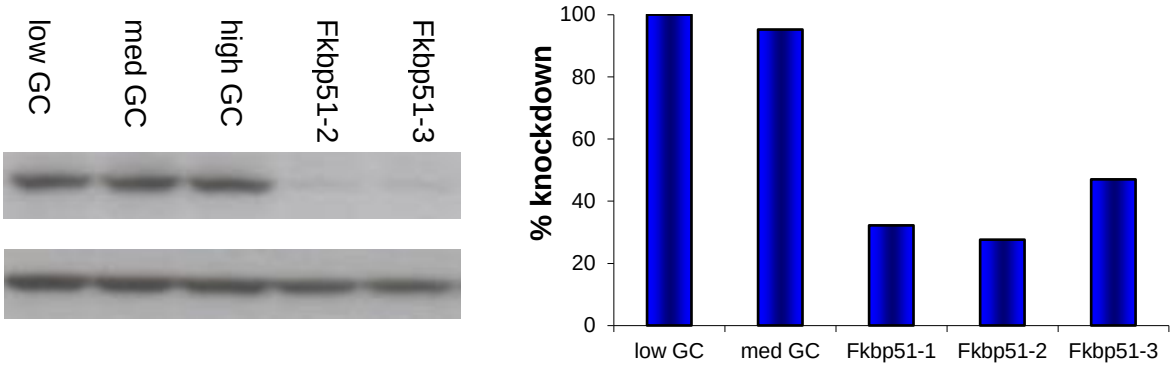


Figure 36: FKBP51 knock-down 72h after electroporation
$2x10^6$ cells per approach were electroporated with 3 different FKBP51- siRNAs (FKBP51-1, -2, -3). The non-coding low GC-, and high GC- siRNAs were used as negative controls. 72h after electroporation the knock-down was analysed on protein-level by Westernblot-analysis ***(A)*** *and on mRNA-level by Q-PCR* ***(B)****.*

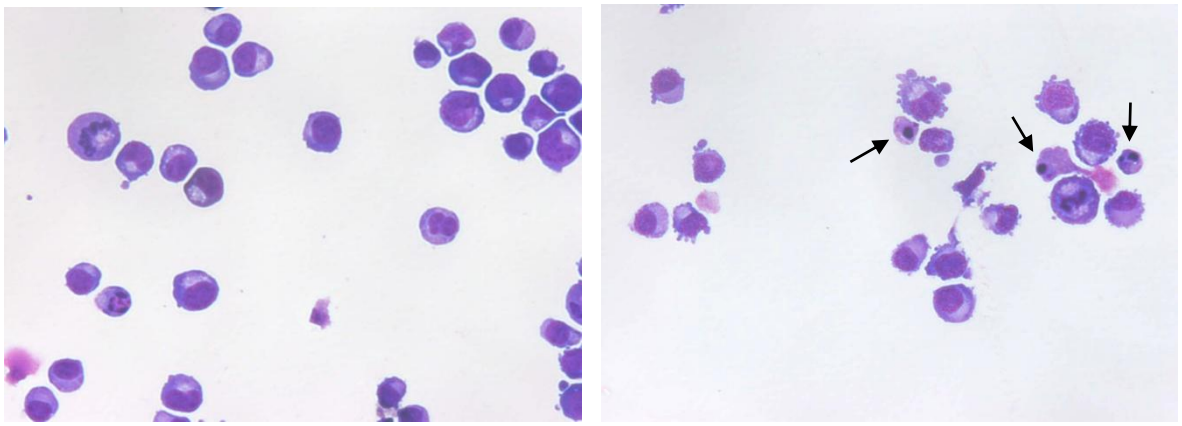
3.5.1 Effect of FKBP51 knock-down on cell morphology

When FKBP51- levels were reduced we again observed an increase of apoptotic cells. This effect was further analysed using cytospins. In the appraoches where FKBP51 has been knocked down, a two fold increase of apoptotic cells relative to the low GC control could be detected, as shown in figure 37. The cytospins of the cells with reduced FKBP51-levels showed more cells with a condensed nucleus and cytoplasm. The apoptotic cells are indicated by black arrows.

Increase of apoptotic cells with reduced FKBP51 levels

(A)

Low GC-siRNA: x40 **Fkbp51-siRNA: x40**



(B)

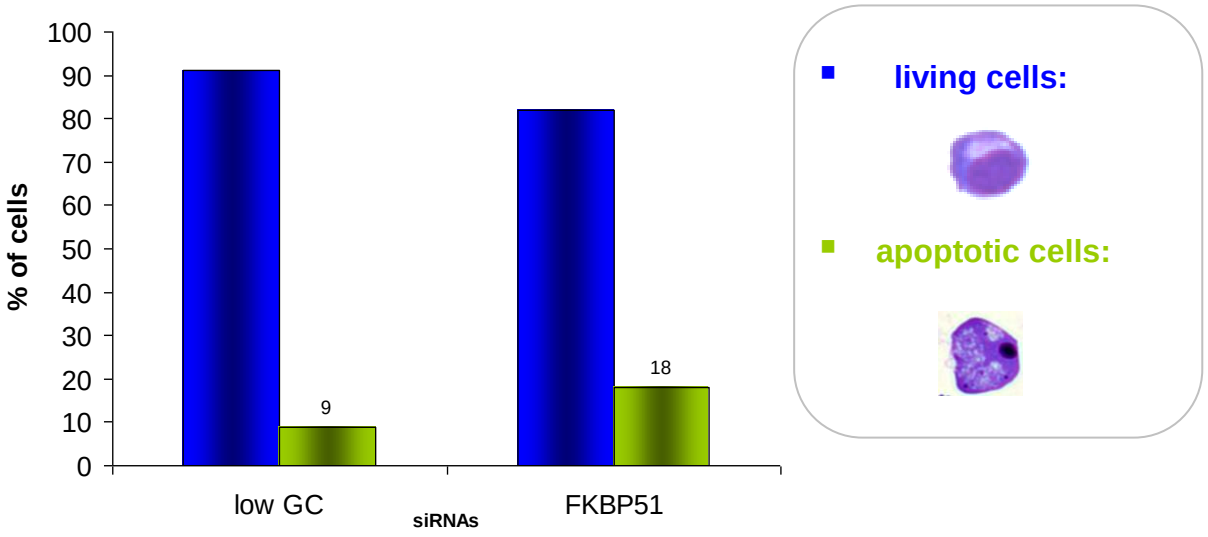


Figure 37: Increase of apoptotic cells 96h after FKBP51-knock-down
(A) *96h after electroporation cell aliquots were cyto-centrifuged onto glass-slides and stained (microscope: x40. Black arrows point out the cells with condensed and fragmented nuclei and apoptotic body formations after downregulation of the FKBP51-levels.* ***(B)*** *Dead and living cells were counted and are here shown as a % of cells.*

The experiment was repeated with another batch of EPCs from a different cord blood sample (LT11), and we observed a reproducible increase in apoptotic cells in both approaches, where FKBP51 was effectively knocked down (data not shown).

Electroporation was performed with 2 different FKBP51- siRNAs (FKBP51-1, -2). The non-coding low GC-siRNA and one approach electroporated without siRNA (no oligo) served as negative controls. Knock-down efficiency was analysed by Westernblotting 72h after the electroporation and the results are shown in figure 38. With both used FKBP51-siRNAs we could significantly reduce the protein levels of FKBP51.

FKBP51 knock-down in LT11 (72h):

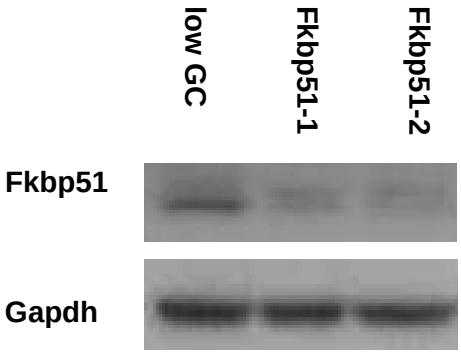


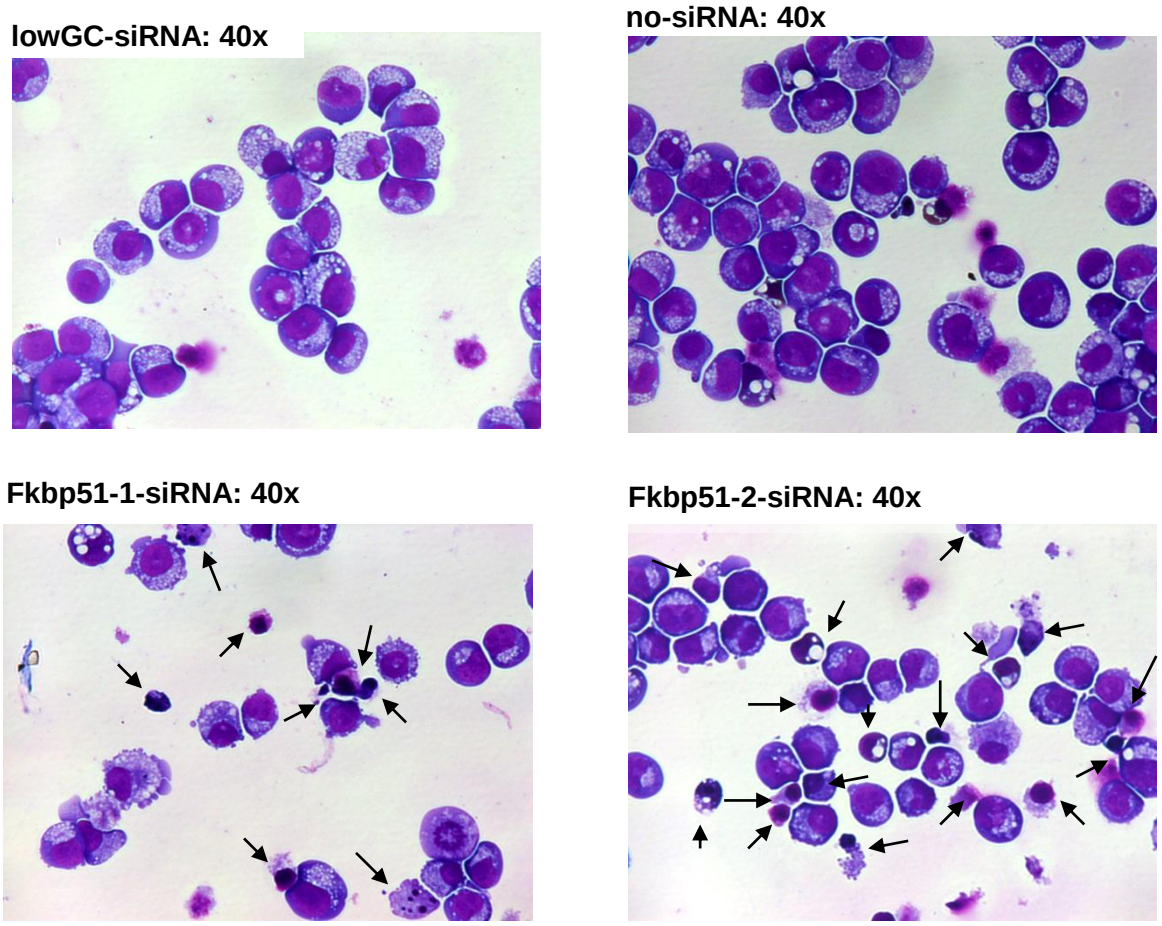
Figure 38: FKBP51- knock-down in LT11
FKBP51 knock-down was performed in EPCs of a different cord blood sample (LT11) with two different FKBP51-siRNAs (FKBP51-1 and FKBP51-2), the non-coding low GC-siRNA was used as a control. Knock-down efficiency was analysed by Westernblotting 72h after the electroporation.

Figure 39 shows the morphological analysis of the different approaches. The cells electroporated with the low GC- control showed mainly big cells with a large nucleus and a high amount of cytoplasm, whereas the cells with reduced FKBP51 protein showed an increased amount of cells with small, dark and fragmented nuclei and a condensed cytoplasm, all hallmarks of apoptosis. The dead cells are pointed out by black arrows (figure 39 (A)).

Dead and apoptotic cells were counted and are shown as a % of cells in figure 39 (B). In both approaches, where FKBP51 levels were reduced, approximately 24% of apoptotic cells could be determined, whereas in the low GC control sample only 12% of the cells were apoptotic. Thus, reduced FKBP51 leads to a two-fold increase of apoptosis in erythroid cells.

Increase of apoptotic cells with reduced FKBP51 levels

(A)



(B)

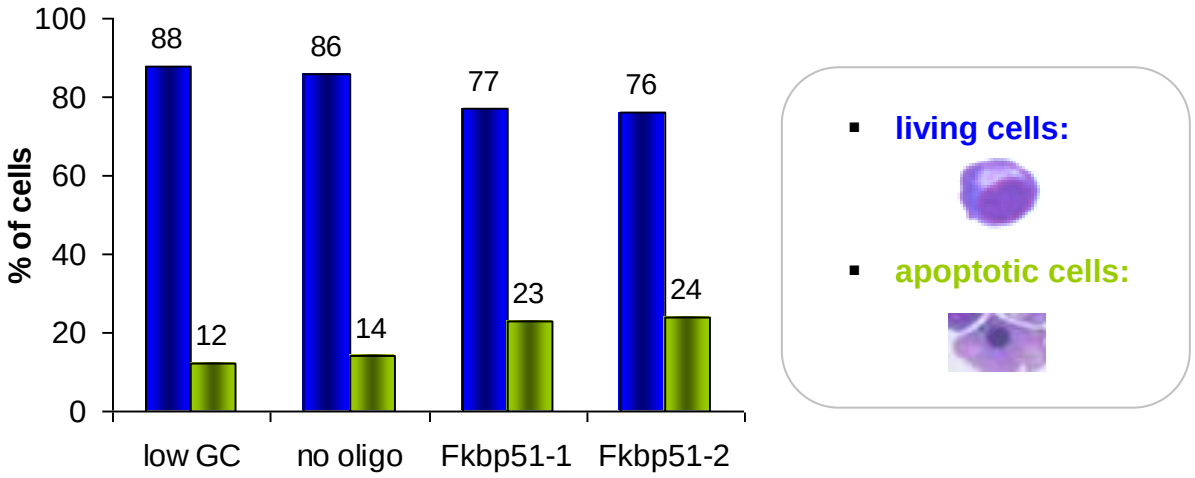
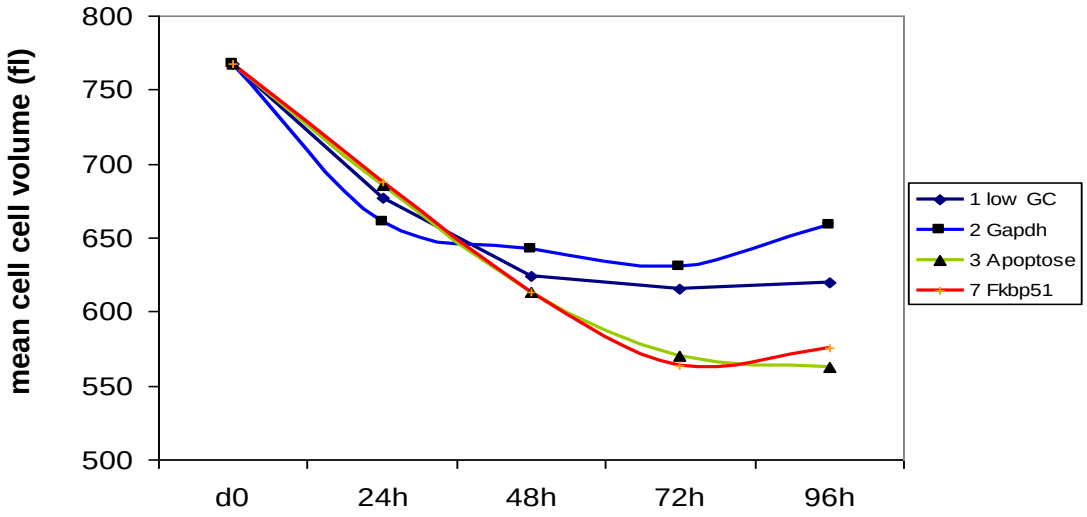


Figure 39: Increase of apoptotic cells in FKBP51 deficient cells
*72h after electroporation with 2 different Fkbp51-siRNAs, cell aliquots were cyto-centrifuged onto glass-slides and stained (microscope: x40). LowGC-siRNA and one approach electroporated without siRNA served as negative controls. (**A**) In the cytospins of FKBP51-deficient cells more dead and apoptotic cells were detectable. Dead cells are pointed out by black arrows. **(B)** Dead and living cells were counted and are shown as % of total cells.*

3.5.2 Effect of FKBP51 knock-down on cell volume and cell size

The mean cell volume and the mean cell diameter) of the cells were analysed 24h to 96h after the electroporation of the different approaches by the CASY-cell counter. We found that the cells with reduced FKBP51 showed a decrease in the mean volume and in the mean diameter at the analysed time points shown in figure 40 (A) and (B). Condensation of nuclei and cytoplasm during apoptosis is leading to reduced cell volume (measured by mean volume and diameter; see figure 40). Thus we found again that the reduction of the FKBP51 protein leads to induced apoptosis in hEPCs.

A) Decrease in mean cell volume 24h-96h after the electroporation



B) Decrease in mean cell diameter 24h-96h after the electroporation

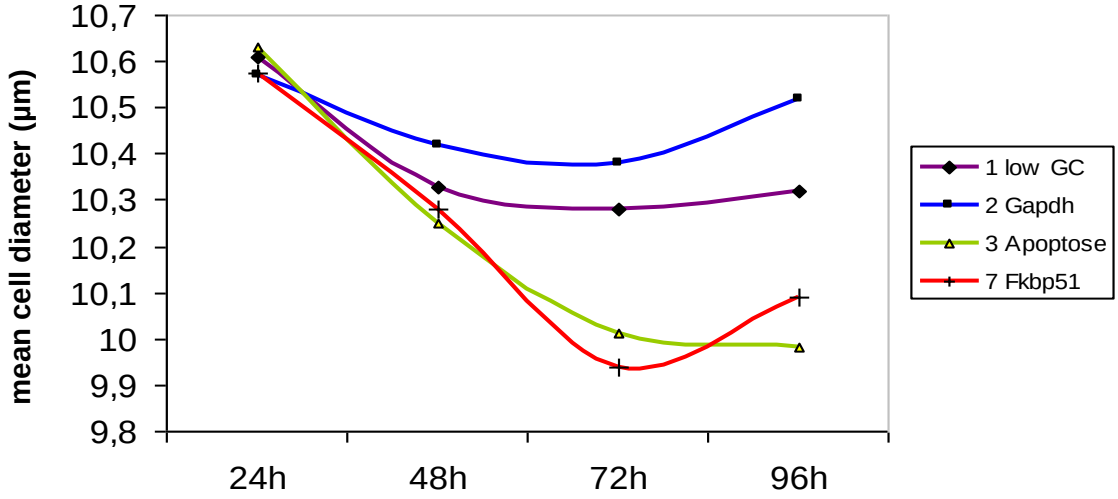
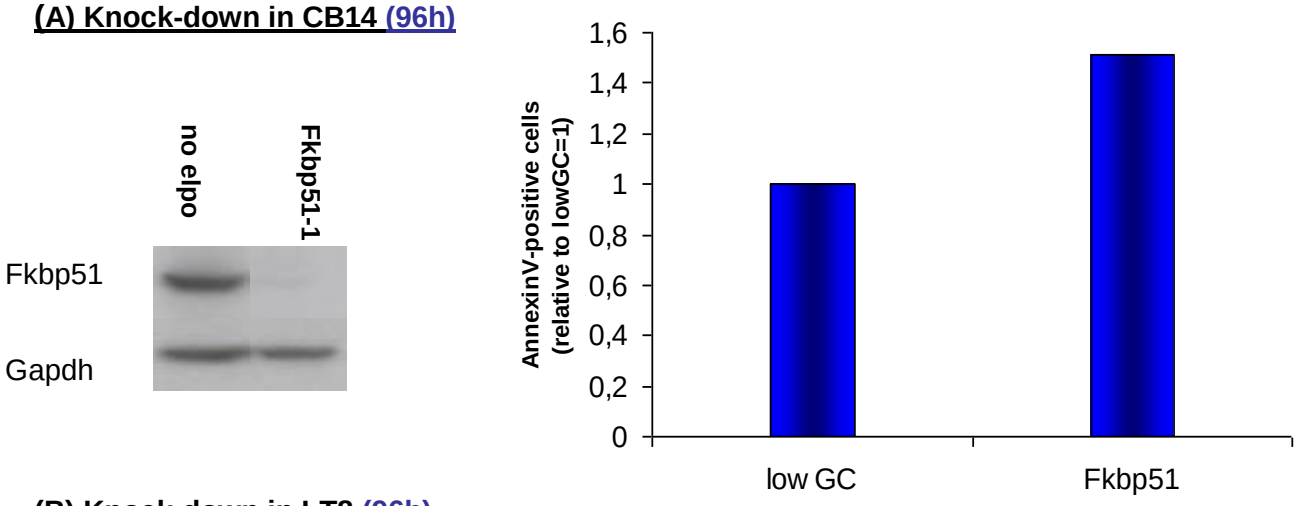


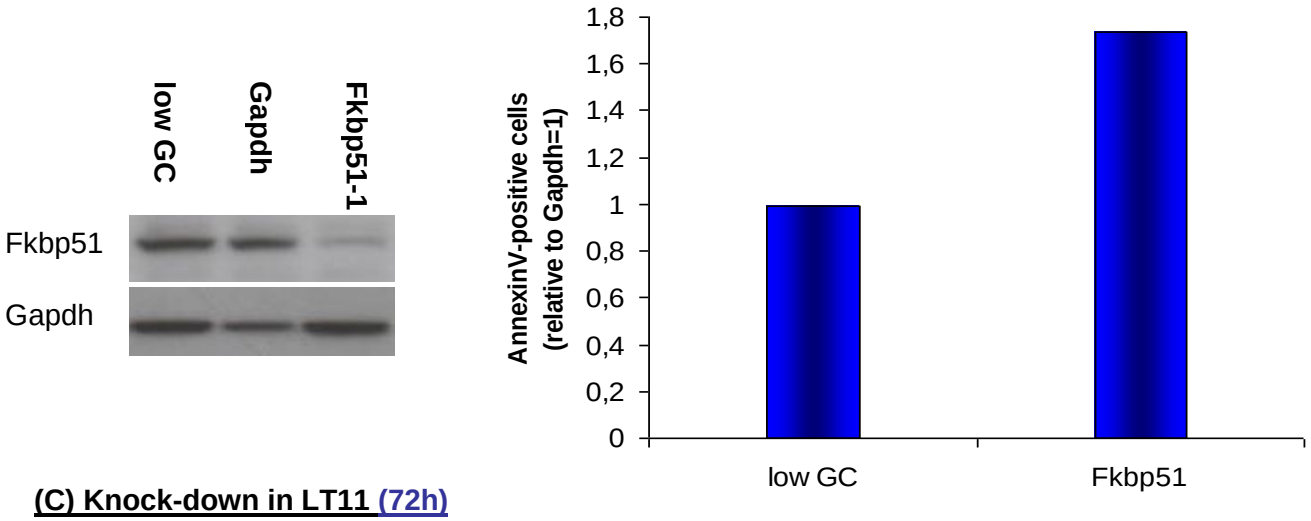
Figure 40: Decrease of mean volume (fl) and mean diameter (µm)
*24h to 96h after the electroporation with FKBP51-1-siRNA (lowGC-siRNA, Gapdh-siRNA and apoptosis-inducing siRNA were used as controls), the different approaches were counted and the mean volume (**A**) and the mean diameter (**B**) were analysed by the CASY-cell counter. The casy profiles show cell count and cell size.*

Effect of FKBP51- knock-down on cell viability

(A) Knock-down in CB14 (96h)



(B) Knock-down in LT8 (96h)



(C) Knock-down in LT11 (72h)

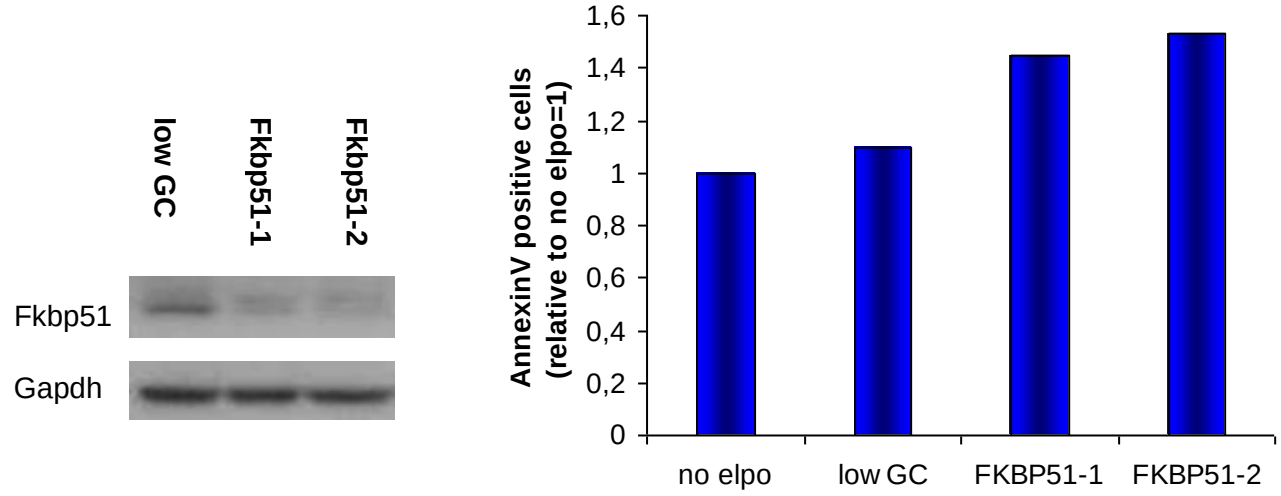


Figure 41: FKBP51 knock-down induces apoptosis
*FKBP51 knock-down was performed in 3 different EPC cultures isolated from 3 different cord blood samples (**(A)** CB14, **(B)** LT8, **(C)** LT11). The non-coding lowGC-siRNA was used as a control. 96h or 72h after the electroporation the knock-down was analysed by Western Blotting, as shown on the left panel of the figure. Cell viability was measured by FACS analysis (Annexin-V-staining) and is shown on the right panel of the figure.*

3.5.3 Effect of FKBP51 knock-down on cell viability

To analyse whether the reduction of FKBP51 is inducing apoptosis, further FKBP51 knock-downs were performed with three different hEPC- cultures isolated from 3 different cord blood samples (CB14, LT8, LT11) and cell viability was analysed by flow cytometry.

Electroporation was performed with two FKBP51- siRNAs (FKBP51-1 and FKBP51-2). The non-coding lowGC-siRNA and one approach electroporated without siRNA (no oligo) served as negative- controls. The downregulation on protein-level was analysed 96h and 72h after the electroporation by Western-Blotting (shown in left panel of figure 41). The knock-down efficiency of FKBP51 and Gapdh on protein-level varied depending on the cord blood sample and the analysed time-point (72h or 96h). We saw that a reduction of FKBP51-expression also resulted in an increase of AnnexinV-positive cells (right panel of figure 41). In the approach with the maximal FKBP51 reduction, an increase of apoptotic and death cells of up to 1,6 (relative to lowGC=1) could be measured (see right panel of figure 41).

In figure 42 the cell viability data of those 3 different experiments were taken together and are illustrated with the according standard deviation. In summary the downregulation of FKBP51 led to an 1,55 fold increase of Annexin-V-positive cells, when compared to the cells transduced with lowGC-RNA (relative to lowGC=1).

A performed student's t-test showed that the differences are statistically significant (p-value was 0,004).

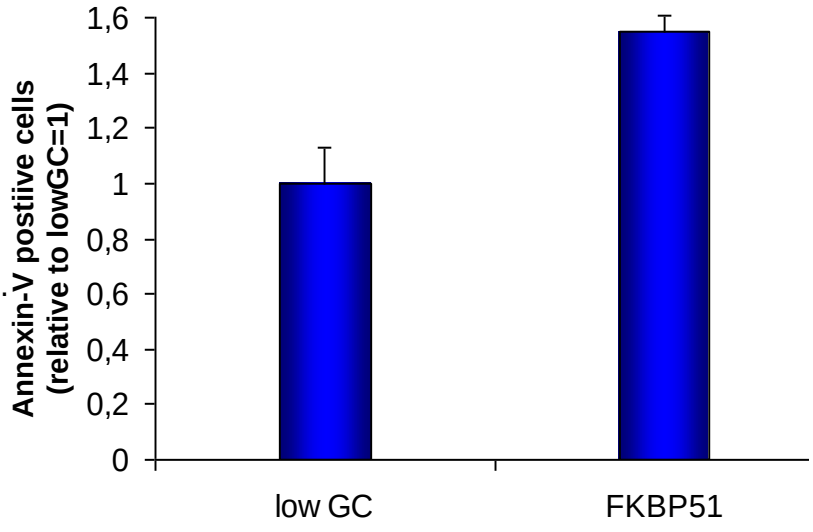


Figure 42: FKBP51 knock-down induces apoptosis

FKBP51 knock-down led to an 1,55 fold increase of Annexin-V-positive cells (relative to lowGC=1). Data of three independent experiments, performed with three different EPC-cultures, isolated from three different cord blood samples.

3.5.4 Effect of FKBP51 knock-down on NF-kB levels

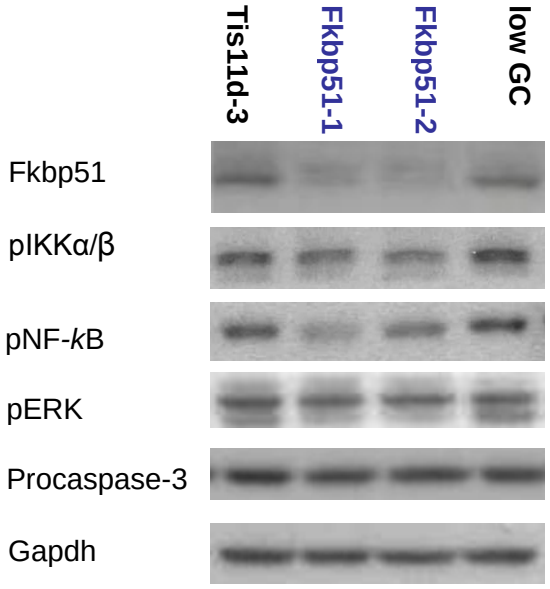
FKBP51 is a multifunctional protein and its expression is induced by glucocorticoids. It has been reported that FKBP51 might be implicated in the regulation of NF-kB activation and that FKBP51 links the glucocorticoid signaling to the NF-kB signaling pathway (Komura et al., 2005; Park et al., 2007; Romano et al.). When FKBP51 was overexpressed in megakaryocytes of human idiopathic myelofibrosis (IMF) patients, Komura et al. could demonstrate that this overexpression led to resistance to apoptosis. And at the same time overexpression of FKBP51 in this hematopoietic cell line, induced a sustained NF-kB activation. But this relationship between FKBP51 overexpression and a constitutive NF-kB activation in megakaryocytes needs further approval (Komura et al., 2005).

Therefore, also in our studies we analysed the effects of the FKBP51 downregulation on the NF-kB levels in primary human erythroid progenitor cells.
FKBP51 knock-down was performed in three independent experiments with different FKBP51 siRNAs (FKBP51-1,-2,-3) in 2 various EPC cultures (LT11 and CB14) isolated from 2 different cord blood samples. The non-coding lowGC-, med-GC-siRNAs and one approach not electroporated at all (no elpo) served as negative controls. The knock-down efficiency of FKBP51 varied depending on the EPC culture and on the analysed time-point. 72h and 96h after electroporation the downstream effects of the FKBP51 downregulation on the phosphorylated and unphosphorylated IKKα/β-, IKBα-, NF-kB- and the Procaspase-3- levels were analysed by Westernblotting.

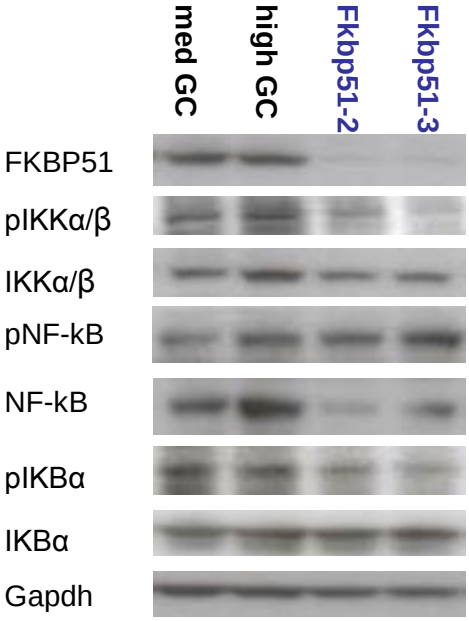
As shown in Figure 43, we could demonstrate that in hEPCs the phosphorylation of NF-kB and as well the unphosphorylated NF-kB-levels were reduced after FKBP51 downregulation. Additional to the NF-kB- levels, we found that the pIKKα- and pIKBα- levels were downregulated, whereas Caspase-3 levels seemed not be affected (see Figure 43). These effects could be reproduced several times (data not shown).

Downstream effects of FKBP51 knock-down

(A) FKBP51 knock-down in LT11 (72h)



(B) FKBP51 knock-down in CB14 (72h)



(C) FKBP51 knock-down in CB14 (96h)

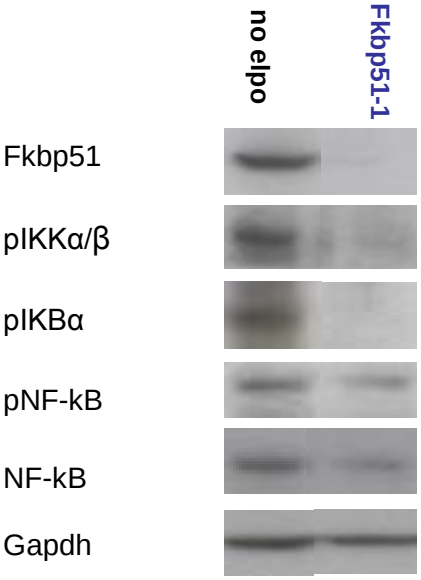


Figure 43: Fkbp51 knock-down led to downregulation of pNFkB levels
*FKBP51 knock-down was performed in three independent experiments with different FKBP51 siRNAs (FKBP51-1,-2, -3) in 2 EPC cultures (**(A)** LT11 and CB14 **(B)** and **(C)**) isolated from 2 different cord blood samples. The non-coding lowGC- and med-GC-siRNA and one approach not electroporated at all (no elpo) served as negative controls. The downstream effects of the FKBP51 downregulation on the phosphorylated and unphosphorylated IKKα/β-, NFkB-, the IKBα-, and the Procaspase-3- levels were analysed by Westernblotting 72h or 96h after electroporation.*

3.6 Tis11d knock-down leads to FKBP51 downregulation

After we performed a Tis11d knock-down and FKBP51 knock-down together in one experiment, we could show that reduction of the Tis11d levels, led also to a downregulation of FKBP51.

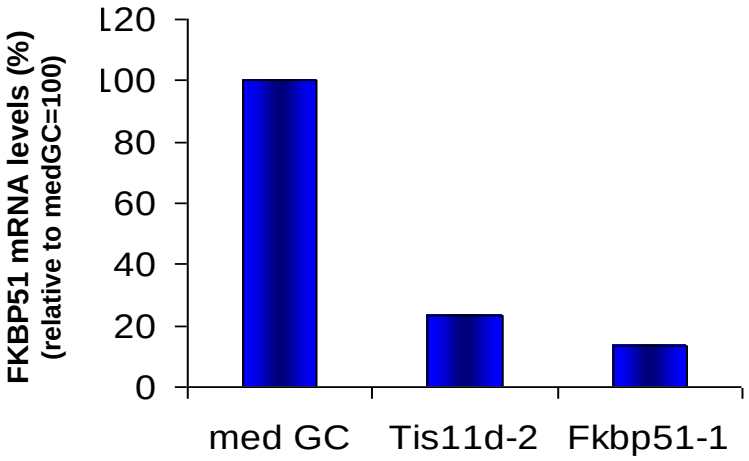


Figure 44: FKBP51 mRNA levels after Tis11d and FKBP51 electroporation *Electroporation was performed with Tis11d- and FKBP51-siRNAs, medGC-siRNA was used as a control. The FKBP51 mRNA levels were analysed by Q-PCR and shown in % relative o medGC-siRNA.*

In this experiment the non-coding medGC-siRNA was used as a control and the knock-down efficiency on mRNA level was analysed 96h after the electroporation by Q-PCR shown in figure 44. We could show that Tis11d knock-down led also to a dramatically decrease of FKBP51 on mRNA level and on protein level (data not shown). FKBP51 levels were reduced up to 85% in the approach, where Tis11d has been knocked down. A possible interaction between those two GR-target genes is estimated.

4. DISCUSSION

4.1 Raf-1

The family of Raf kinases consists of 3 different isoforms: A-Raf, B-Raf and Raf-1, which are expressed during the embryonic development. The 3 Raf isoformes are regulated differentially by upstream activators and show differences in their ability to activate MEK (Mikula et al., 2001). The best studied Raf isoform is Raf-1, which has been shown to be implicated in mechanisms regulating proliferation, differentiation and survival of mouse erythroblasts, by preventing apoptosis and and promoting proliferation.

Mikula et al. reported that Raf-1 deficient mouse embryos are anaemic, have hypocellular livers and die at E12.5 due to hepatoblast failure. Raf-1 deficient fetal livers contained fewer erythroid progenitors than WT livers (Mikula et al., 2001).
Additionally it has been shown that during differentiation of primary mouse erythroblasts Raf-1 protein is degraded and that overexpression of Raf-1 can delay terminal erythroyid differentiation by inhibiting activation of Caspases (Kolbus et al., 2002). Thus, the anaemic phenotype of the embryos lacking Raf-1 could be linked to the premature differentiation of erythroblasts.

Furthermore it has been reported that Raf-1-deficient mouse erythroblasts differentiated much faster than the wild-type erythroblasts. In the cytospins of Raf-1 deficient cells immature, mature and hemoglobinized cells could be found, whereas the control cells consisted mainly of large and undifferentiated cells. When differentiation was initiated (by high EPO and insulin) in erythroblasts, cells lacking Raf-1, showed a dramatic increase of nucleated and enucleated erythrocytes with accelerated differentiation compared to WT cells (Kolbus et al., 2002).

Those *in vivo* and *in vitro* studies revealed that in the mouse system Raf-1 seems to be involved in the regulation of differentiation and proliferation of erythroid progenitor cells.

4.1.1 Raf-1 knock-down in hEPCs led to more spontaneous differentiation

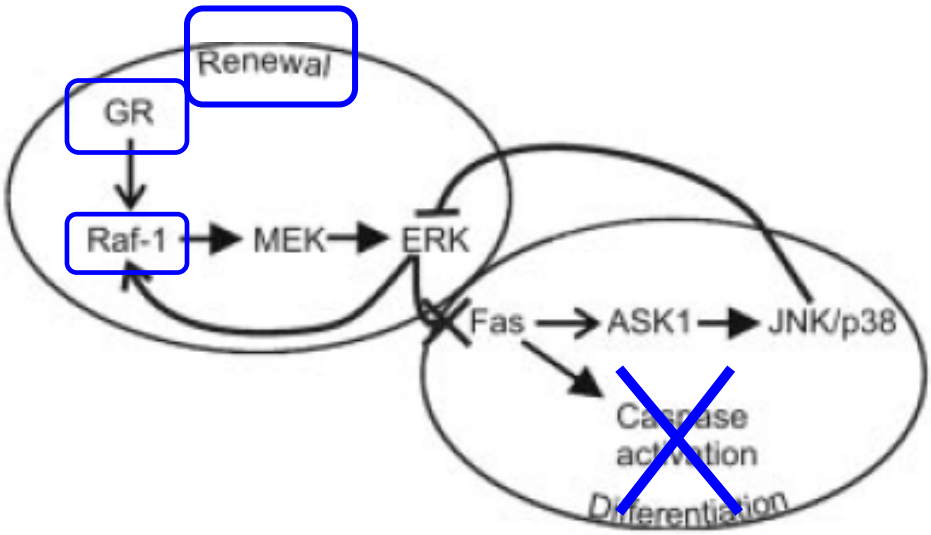
Little is known about the function of Raf-1 in human erythropoesis. Thus, in our study we analysed whether Raf-1 is also implicated in the differentiaton and proliferation process of human erythroid cells by using Raf-1 knock-down approaches in primary human erythroid progenitor cells.
We could show that cells with reduced protein levels of Raf-1 proliferated slower when compared to control cells. This effect was better detectable, when the cells were cultivated at higher cell densities. When the cell morphology was analysed via cytospins, Raf-1 knock down cells showed more spontaneously differentiated cells. The CASY profiles of Raf-1 downregulated cell-cultures were shifted to the left, indicating a decrease in cell size which is characteristic for terminal erythroid differentiation. Unfortunately, in the experiments performed in the presented study, the difference between the control cells and the cells with low Raf-1 levels were not as prominent as in the mouse system. This could be due to the fact that the used control cells showed an increased spontaneous differentiation, which may superimpose the effect of Raf-1 downregulation.

In erythroid cells Caspases are not only involved in apoptotic mechanisms, but they have been shown to play an important non-apototitc role in the differentiation mechanisms. Carlile et al. for example demonstrated that reduction of Caspase activity during early human erythropoiesis resulted in a block of erythroid maturation. Without Caspase activation human erythroblasts did not develop beyond the pronormoblast stage (Carlile et al., 2009). Caspases are activated by Fas, which is a member of the tumor necrosis factor family of receptors. As Raf-1 downregulation has been shown to correlate with up-regulation of Fas in the erythroid progenitors, it has been concluded that Raf-1 might therefore be involved indirectly in Fas activation (Rubiolo et al., 2006). Furthermore Raf-1 has also been shown to act as a MEK/ERK activator.

Figure 45 is showing a scheme of the current hypothesis of the function of Raf-1 in mouse erythroblasts reported by Rubiolo et al. According to this model in the mouse system ERK and Raf-1 are involved in a positive feedback loop.

Working model in the mouse system:

When Raf-1 is present: ⟶ caspase dependend differentiation is inhibited



When Raf-1 is absent: ⟶ caspase dependend differentiation is activated

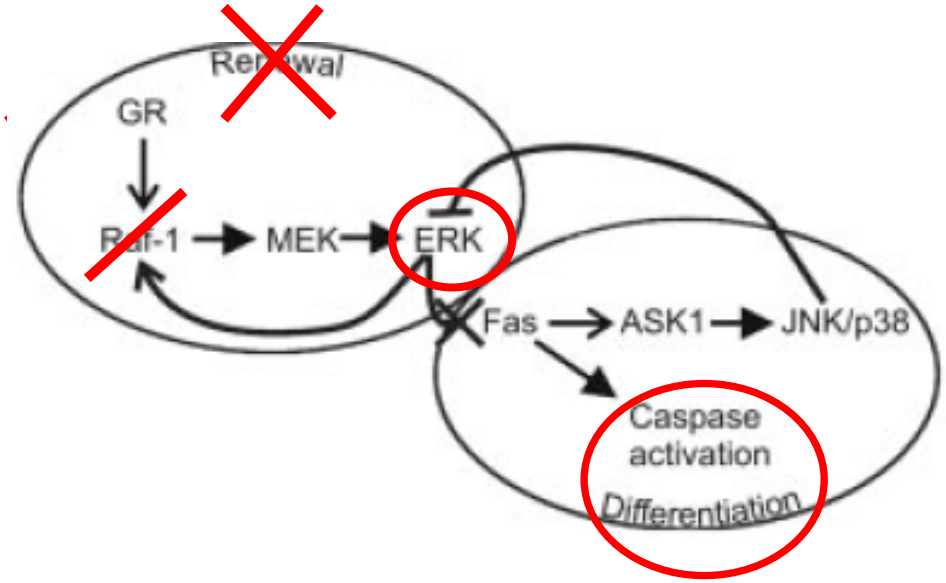


Figure 45: Raf-1 stimulates proliferation and inhibits differentiation in mouse erythroid cells
Glucocorticoids maintain the Raf-1 expression, which in order stimulates ERK acivation and thereby inhibits FAS. In this way Caspases activation is inhibited and EPCs undergo renewal. When the renewal stimulus is absent, Raf-1 is degraded, and ERK can not be activated. Thus Fas is present and induces Caspase activation and thereby induces differentiation (Rubiolo et al., 2006).

Raf-1 is required for ERK activation and activated ERK on the other hand maintains Raf-1 expression. Activated ERK inhibits FAS and thereby also the Caspase activation, whereby Caspase-mediated differentiation is repressed. This suggests, that in prescence of Raf-1, erythroblasts are maintained in an immature state (renewal). When Raf-1 is knocked down, the renewal stimulus is absent, as ERK can't be activated and therefore Fas is present and induces Caspase activation and thereby differentiation.

Also in our studies the downstream effects of the Raf-1 knock-down in hEPCs were analysed in the human system. According to the findings in the mouse system, in absence of Raf-1 we expected a downregulation of the pERK levels and an activation of the Caspase-3 levels. Caspase activation would explain the slight increase of spontaneous differentiation in our experiments.
But again the effects were not as prominent as in the mouse system, the Caspase-3 activation was very low and we didn't notice remarkable effects on the phosphorylatian of ERK (pERK). Then there was the discrepancy that Caspase activation and pERK downregulation could not be detected in the same approach, either the Caspase-3 levels were affected or the pERK levels were downregulated. And even more remarkable was that in some experiments we couldn't detect any effects on both, the Caspase-3 and the pERK-levels, although Raf-1 has been knocked down efficiently. Furthermore the effects of the knock-down on the spontaneous differentiation were not reproducible, as the effects couldn't be reported in other experiments, where Raf-1 has been knocked down.
Those findings therefore argue against the hypothesis that in human erythroid system Raf-1 has the same role in differentiation processes as reported in the mouse system.

4.2 TIS11d

As mentioned above Tis11d is a zinc-finger-like protein and member of the Tis11-family. The Tis11-family consists of four mammalian members:

- TTP (Tristetraprolin)
- Tis11b (ZFP36l1 in the mouse system)
- Tis11d (ZFP36l2 in the mouse system)
- Zfp36L3

The fourth family member Zfp36L3 has been shown to be expressed only in rodents and in the mouse placenta, but has not yet been detected in human tissues so far. Characteristics of Tis11- like proteins are two tandem repeat zinc finger regions. The Tis11-like proteins have been shown to induce degradation of specific mRNAs (see figure 46). Through their zinc finger regions they are able to bind to the adenine-uridine (AU) rich elements (ARE) in the 3'-untranslated region of mRNAs and thereby they first promote deadenylation, then decapping and finally degradation of a variety of different mRNAs containing AU-rich elements (Baou et al., 2009; Wilusz et al., 2001).

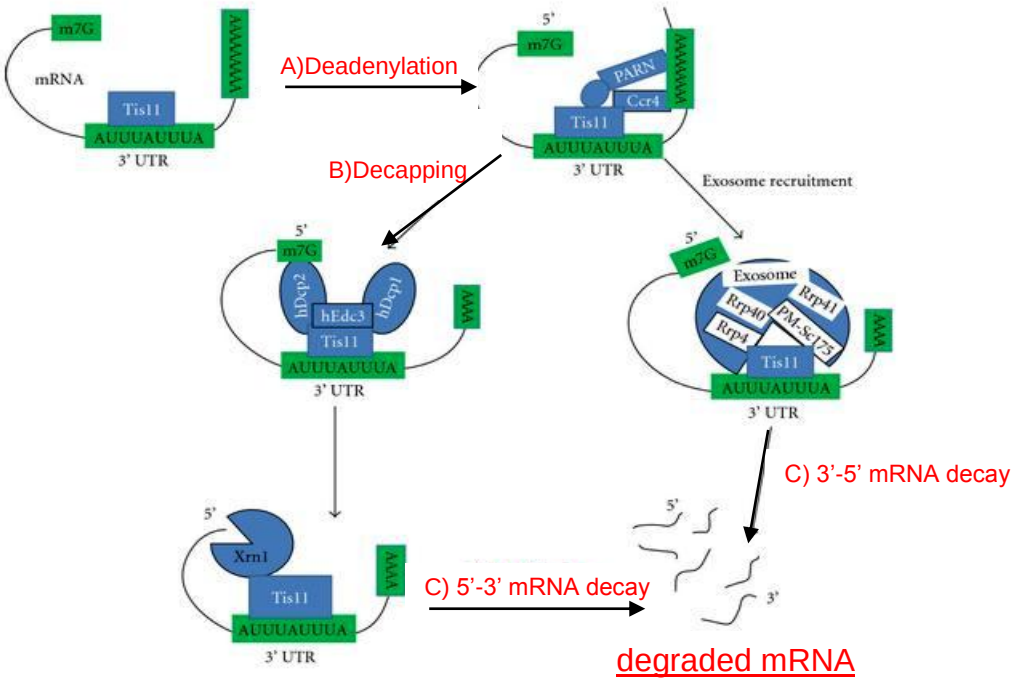


Figure 46: Members of the Tis11- family induce mRNA degradation
Through their zinc finger region Tis11-like proteins bind to adenine-uridine (AU) rich elements of mRNAs and thereby promote deadenylation, decapping and finally degradation of mRNA (picture taken from: Baou et.al., 2009).

Interestingly, such AREs are also found at the 3' end of each of the mRNAs of the Tis11- proteins, which suggests that they might regulate themselves by a negative feedback loop. The Tis11 family of proteins have an important role in posttranscriptional gene regulation and in the control of a variety of developmental and functional processes (Baou et al., 2009; Johnson et al., 2000).

Little is known about the function of Tis11d in humans in general or specifically in erythropoiesis. In adult mouse tissues Tis11d is expressed at high levels in thymus, spleen, lung, uterus, ovary, small and large intestine, fat and bone marrow and lower levels in heart, kidney, eye, fetal liver, brain and skeletal muscle (Ino et al., 1995).

After we analysed the expression levels of all of the three Tis11-like proteins in human EPCs, we found that Tis11d was the highest expressed zinc-finger-like protein in erythroid cells. The high expression level of Tis11d in erythroid cells indicates that Tis11d is the most prominent member of the three Tis11 like family genes, and plays the most important role in these cells.

Tis11b knock-out mice are embryonically lethal; they die in utero between day E8 and E13, probably due to defective extraembryonic vasculogenesis (Bell et al., 2006).

Stumpo et al. demonstrated that in the mouse system Tis11d is involved in erythropoiesis and hematopoiesis. Zfp36l2 knock-out mice appeared normal until birth, but two weeks after birth they died of severe anemia. These knock-out mice showed significantly reduced levels of red and white blood cells, hemoglobin, hematocrit and platlets in peripheral blood analysis. Bone marrow analysis showed decreased myeloid and megakaryocyte cell populations. Loss of Zfp36l2 also led to a decreased level of hematopoietic progenitor cells (HPCs) and hematopoietic stem cells. Zfp36l2$^{-/-}$ fetal liver cells were unable to reconstitute the hematopoietic systems in lethally irradiated mice, which suggests that Zfp36l2 might be required for self-renewal and maintenance of adult HSCs. By performing colony forming assays out of fetal liver cells from Zfp36l2 deficient cells, a significant lower number of erythroid BFU-E (burst formung units) and CFU-GM (granulocyte macrophage units) was observed (Stumpo et al., 2009). These studies indicate that Tis11d plays an important role in erythropoiesis and in definitive hematopoiesis during mouse development.

4.2.1 Downregulation of Tis11d in primary hEPCs induces apoptosis

In our studies the downregulation of Tis11d in primary hEPCs resulted in an apoptotic phenotype. We could demonstrate that compared to the control cells, Tis11d-deficient cells showed an increase of approximatley 12% of dead /apoptotic cells in the cytospins 72h to 96h after the electroporation. Data of three independent experiments with three different hEPCs-cultures (isolated from three different cord blood samples) revealed that the downregulation of Tis11d led to an up to 1,7 fold increase of Annexin-V-positive cells, when compared to the cells transduced with lowGC-siRNA (relative to lowGC=1).

Apoptosis can be mediated either by the instrinsic or extrinsic pathway (see chapter 1.6). As already mentioned the extrinsic pathway is initiated through the stimulation of the transmembrane death receptors, such as TNF-α-Receptor, wheareas the intrinsic pathway is initiated through the release of signal factors by mitochondria within the cell. In the extrinsic pathway Caspase-8 is directly inducing the cleavage of Procaspase-3 leading to the active form Caspase-3. And in the intrinsic pathway the activated Caspase-8 is first activating BID, which in turn activates BAK and BAX. Both pathways have at the end of the signalling cascade, an activated Caspase-3, which then induces apoptosis by cleaving several proteins in various cellular compartments.
We could show that in every approach where Tis11d has been downregulated, the Procaspase-3 levels were decreased most probably by cleavage, leading to activation of Caspase-3. This clearly demonstrated that the cells undergo apoptosis. We also could show that the BAX-levels were down-regulated in the cells with reduced Tis11d levels, which was quite surprisingly, as we expected either no effect on the BAX levels, if cells die through the extrinsic pathway, or an upregulation of BAX if apoptosis is induced through the intrinsic pathway. Thus it remains still unclear whether the erythroid cells undergo apoptosis through the intrinsic or the extrinsic pathway.

4.2.2 Targets of Tis11-like proteins

Studies from TTP-, Tis11b- and Tis11d- knock-out mice revealed a lot of different mRNA- targets of Tristetraprolin in humans (see figure 47), but little is known about specific Tis11d- mRNA targets. The reported Tis11d- targets in humans are (Baou et al., 2009):

→ **TNF-α** (Tumor Necrosis Factor alpha)
→ **GM-CSF** (granulocyte-monocyte colony-stimulating factor)
→ **IL-3** (interleukin-3)

The most studied member of the Tis11-family is **Tristetraprolin (TTP)**. As shown in figure 47 the most prominent mRNA- target of TTP in humans is the tumor necrosis factor alpha (TNF-α).

In 1998 Carballo et al. reported a possible interaction of TTP and TNF-α in studies of TTP-deficient mice. TTP-deficient mice appeared normal at birth, but after two weeks they were suffering of syndroms of infammatory arthritis, autoimmunity, and extramedullary hematopoiesis, which were typical syndroms of TNF-α- excess (Taylor et al., 1996). These findings suggested that TNF-α seemed to be target of Tristetraprolin and that TTP might induce degradation of TNF-α-mRNA. Abscence of TTP led to abnormal accumulation of TNF-α- mRNA and protein. It has also been demonstrated that macrophages isolated from $TTP^{-/-}$ fetal livers secreted five-times more TNF-α compared to the control cells (Carballo et al., 1998). Furthermore, the cellular levels of TNF-α-mRNA were two times higher compared to the wild-type macrophages. The half-life of the TNF-α mRNA in macrophages increased from 12 minutes (WT) up to 39 minutes in macrophages lacking TTP. Therefore they could show that TTP regulates the amount of TNF-α mRNA postranscriptionally and not by inhibiting gene expression. This TNF-α accumulation was responsible for the development of a systemic inflammatory syndrome characterized by arthritis, skin lesions, autoimmunity and myeloid hyperplasia (Carballo et al., 1998; Lai et al., 1999; Taylor et al., 1996).

TABLE 1: Human TIS11 family members and reported mRNA targets of the TIS11 family.

Gene	Alternative names	Chromosomal location	Reported mRNA targets	reference	mechanism
TIS11	*ZFP36, TTP, Nup475, GOS24*	19q13.1	**TNF***	[8]	mRNA stability
			GM-CSF	[15]	mRNA stability
			IL-3	[16]	mRNA stability
			IL-6	[17]	mRNA stability
			cyclooxygenase	[18]	mRNA stability
			PAI type 2	[19]	mRNA stability
			Pitx2	[20]	mRNA stability
			TIS11	[14]	mRNA stability
			IL-2	[21]	mRNA stability
			1,4galactosyltransferase	[22]	mRNA stability
			IL-12	[23]	?
			Ccl2	[24]	mRNA stability
			Ccl3	[24]	mRNA stability
			c-myc	[25]	mRNA stability
			cyclin D1	[25]	mRNA stability
			Fos	[26]	mRNA stability
			Ier3	[27]	mRNA stability
			Genome analysis 250 mRNAs	[27]	mRNA stability
			MIP-2	[23]	?
			p21	[26]	mRNA stability
			E47	[28]	mRNA stability
			VEGF	[29]	mRNA stability
			IL-10	[30]	mRNA stability
			Genome analysis 137 mRNAs	[30]	mRNA stability
			polo-like kinase 3	[31]	mRNA stability
TIS11b	*ZFP36L1, Berg36, ERF-1, BRF-1*	14q22-24	TNF	[32]	mRNA stability
			GMCSF	[33]	mRNA stability
			IL-3	[34]	mRNA stability
			VEGF	[35]	mRNA stability
			c-IAP2	[36]	mRNA stability
			VEGF	[37]	translation
			STAR	[38]	mRNA stability
TIS11d	*ZFP36L2, ERF-2, BRF-2*	2p22.3-p21	TNF	[32]	mRNA stability
			GM-CSF	[33]	mRNA stability
			IL-3	[33]	mRNA stability

* Targets in bold confirmed in cells derived from knockout animals are so-called "physiological" targets.

Figure 47: Human Tis11 family members and their reported mRNA targets
Tristetraprolin is the most studied member of this family, with the most known mRNA-targets in humans. For Tis11d there are just 3 known mRNA-targets reported in humans: TNF-α, GM-CSF and IL-3. (*Baou et. al., 2009)*

TNF-α affects a number of different cell types and it is mainly produced by activated macrophages and lymphocytes. TNF-α can act either directly or indirectly by inducing other cells to produce cytokines, like hematopoietic growth factors. In hematopoiesis TNF-α acts as a positive and as a negative regulator of myeloid cell proliferation and differentiation (Rusten and Jacobsen, 1995).
It has been demonstrated that TNF-α suppresses erythropoiesis by inhibiting the generation of Glycophorin-A+ cells derived from CD34+ cells (Xiao et al., 2002). TNF-α therefore inhibits the differentation of CD34+ cells into mature eryhthroid cells. It has been reported that TNF-α maintains erythropoiesis *in vivo* and *in vitro* by constraining the proliferative capacity of mature erythroid progenitor cells (Li et al., 1989). When CD34+ cells were treated with TNF-α, this resulted in inhibition of growth of BFU-E and CFU-E-cultures, which suggests that TNF-α not only inhibits the differentiation but also the proliferation of hEPCs (Xiao et al., 2002).

As it has been reported that TNF-α is also one of the Tis11d-targets as shown in figure 47, and that TTP-knock-down in the mouse system led to an accumulation of TNF-α, we also expected that the absence of Tis11d in erythroid cells would lead to an accumulation of TNF-α since the TNF-α-mRNA can't be degraded anymore. TNF-α accumulation would lead to an increased Caspase-8 activation and in turn also to an increased Caspase-3 activation, exactly what we found after the downregulation of Tis11d by siRNA.
Unfortunatley we couldn't detect any significant effects of Tis11d on the TNF-α levels, neither on mRNA-level, nor in the supernatant of different appraoches, since the TNF-α levels were below the detection limit.
According the protocols described in Materials and Methods, a partial medium change and an adjustment of the cell number to a concentration of $1x10^6$ cells/mL was performed daily. Therefore every day part of the supernatant was discarded. This could be a possible explanation why the TNF-α content was below the detection limit of the ELISA assay and couldn't be measured. Thus, in further experiments the single proliferation-factors should be added in the appropriate concentration directly to the cell suspenstion, without performing a partial medium change. Thereby a possible TNF- α accumulation in the supernatant could be achieved, which would be high enough for measuring by an ELISA assay.

On the other side, another possibility could be that in human erythroid cells no soluble TNF-α is produced. It could also be that Tis11d is even involved in other than the expected signaling pathways and thus Tis11d has no effect on the TNF-α levels at all. Figure 48 shows a possible model on how Tis11d could be involved in the apoptosis protection in primary human erythroid cells.

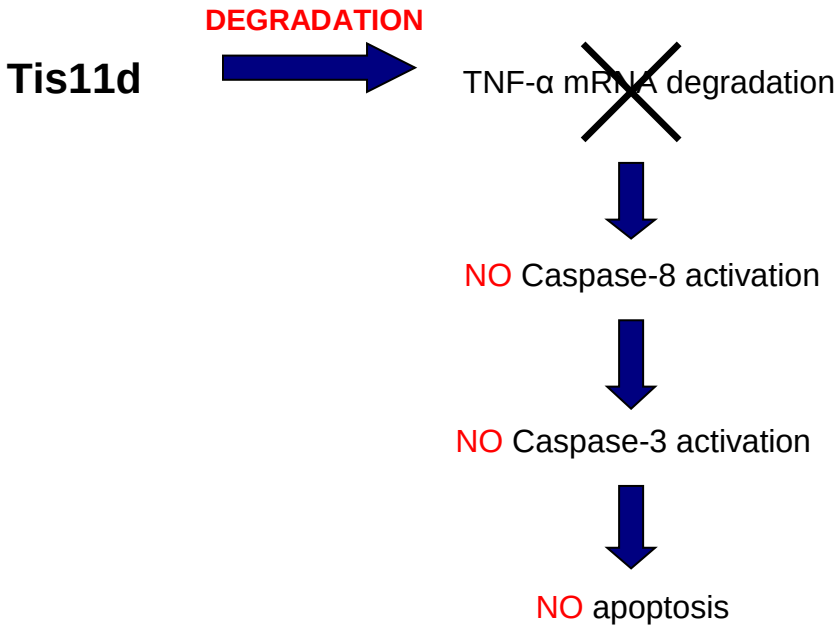


Figure 48: Possible ivolvement of Tis11d in apoptosis-protection
When Tis11d is present TNF-α is degraded and Caspases are not activated. Without activated caspases, apoptosis is not induced.

4.2.3 Tis11d might have anti-apoptotic effects in hEPCs

In summary we could show that the reduction of Tis11d siRNA induces apoptosis in human EPCs. As it has been reported that Tis11 proteins are involved in the deadenylation and destabilization of a variety of different mRNAs, we suggest that the reduction of Tis11d may lead to an abnormal stabilization of one or more mRNAs, whose protein products may have a direct or indirect affect on apoptosis induction in erythroid cells.

But as we couldn't demonstrate any effect on the TNF-α levels, maybe Tis11d acts on the stability of other ARE-containing mRNAs. A lot of mRNAs encoding cytokins, transcription factors, apoptosis regulators contain AU- rich elements (ARE) at their 3' untranslated region. It is estimated that approximatley 8% of mRNAs may contain AREs (Bakheet et al., 2003). As the Tis11d knock-down induces apoptosis, the unknown mRNA-target is probably directly or indirectly involved in the induction of apoptosis. On the other hand, when Tis11d is inducing the degradation of this unknown target, it implies that Tis11d might be responsible for apoptosis protection in human erythroid progenitor cells.

As already mentioned, studies with Tis11d knockout mice revealed an involvment of Tis11d in erythropoiesis and in definitive haematopoiesis (Stumpo et al., 2009).
During my diploma thesis, I could also show that Tis11d plays an essential role in human erythroid progenitor cells. The exact function of Tis11d in humans still needs to be further investigated.
Analysis using microarrays showed that in Tis11d deficient mice, Cxc11-mRNA was upregulated (Stumpo et al., 2009). In mice Cxc11 is a homolog of human IL-8, which is a chemokine that has been shown to play a critical role in stem cell survival, development and homeostasis (Romagnani et al., 2004). Thus, IL-8 could be a possible target of Tis11d in human cells, and IL-8 could be also involved in the apoptotic process.
Further studies are required, to find out the mechansism behind the observed induction of apoptotis. Microarray analyses of Tis11d deficient cells could reveal other upregulated mRNAs, which may be involved in apoptosis or other regulatory mechansisms.

4.3 FKBP51

In a recent genome-wide profiling of human lung biopsies, FKBP51 has been identified as the **most highly glucocorticoid-induced gene** (Woodruff et al., 2007). Glucocorticoids bind to the GR, a transcription factor, which is situated in the cytoplasm in absence of glucocorticoids. In the cytoplasm the GR is associated with the heat shock protein 90 chaperone (HSP90), which keeps the receptor in an inactive form (Paakinaho et al., 2010).

FK506-binding protein 51 is a multifunctional co-chaperone protein and a component of the GR complex, as it can bind to the HSP90 chaperone protein. FKBP51 is also a member of the HSP90 steroid receptor complex, which is a cellular chaperone protein that is preventing protein aggregation. The HSP90 steroid receptor complex maintains the GR in a conformation able to bind to steroids (Rees-Unwin et al., 2007).

As depicted in figure 49, during the formation of the GR complexes, FKBP51 competes with its homolog FKBP52 for binding to HSP90 (Davies et al., 2002). When FKBP51 and FKBP52 are bound to the GR-complex, they differentially modulate the interaction of glucocorticoids with the GR. It has been reported that FKBP51 decreases the affinity of the glucocorticoids for binding to their receptor (Park et al., 2007).

Davies et al. demonstrated that replacement of FKBP51 by FKBP52 in the GR complex induces the translocation of the activated GR complex into the nucleus, where it leads to the activation or the inactivation of specific target genes. Thereby glucocorticoids can influcence metabolic and developmental processes (Davies et al., 2002).

The FKBP51 expression in response to glucocorticoids has been studied in several cell types and tissues *in vivo* and *in vitro*. In mononuclear cells isolated from peripheral blood it has been demonstrated that dexamethasone-treatment of individuals led to a 30-fold increase of FKBP51 mRNA (Vermeer et al., 2004).

J. Park et al. infected chickens with the Newcastle disease virus (NDV), which is a highly immunogenic virus that induces glucocorticoid secretion, to study the glucocorticoid-dependent- FKBP51 expression. He observed a 130-fold induction of FKBP51-mRNA levels in the liver of NDV-infected chickens. Thereby he

demonstrated that glucocorticoids are the main cause of systemic induction of FKBP51 (Park et al., 2007).

It is suggested that FKBP51 is involved in the negative feedback regulation of the GR signaling, which means that at high levels of glucocorticoids, the expression of FKBP51 is stimulated, whereas high levels of FKBP51 limit the GR activation.

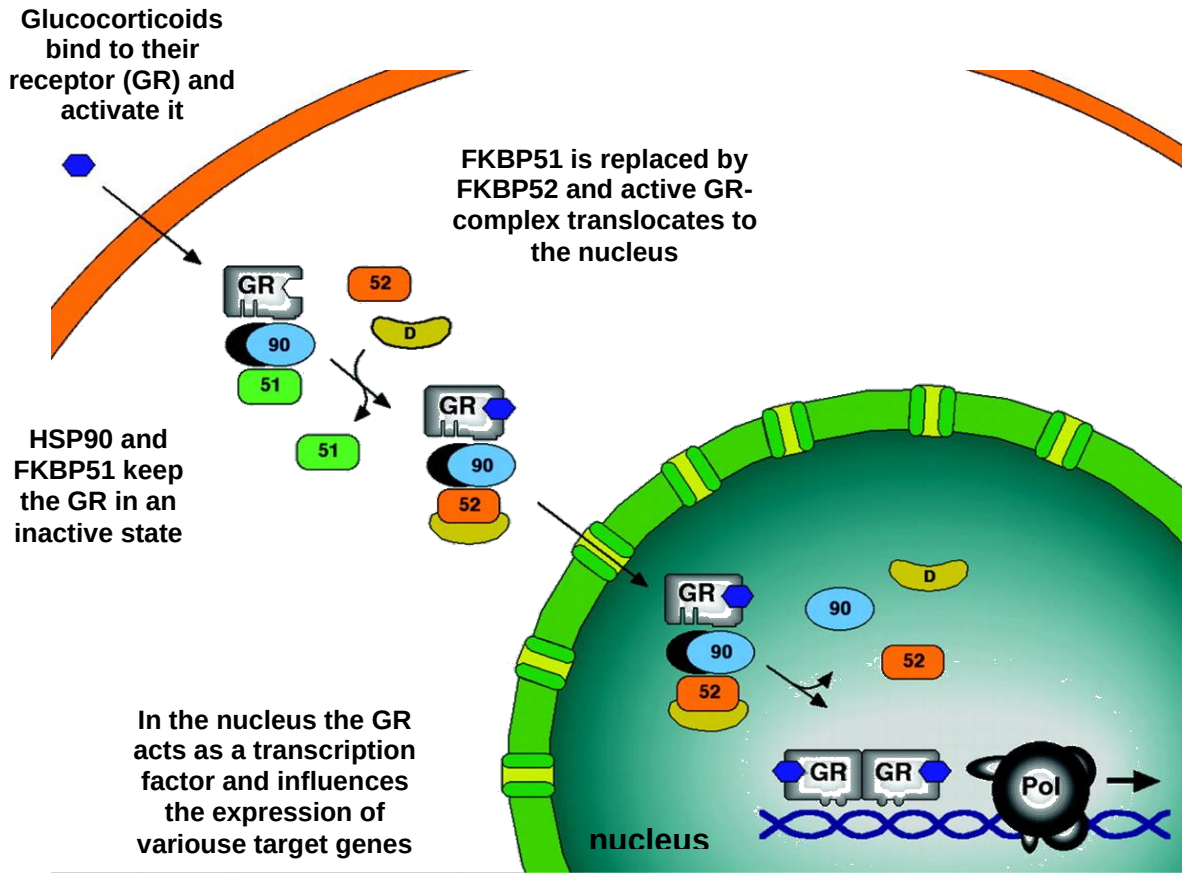


Figure 49: FKBP51 is involved in the GR-complex-activation *(picture taken and modified from: Davies et al., 2002).*

It has been reported in several publications that the multifunctional FKBP51 might be implicated in the control of apoptosis (Bouwmeester et al., 2004; Giraudier et al., 2002; Komura et al., 2005).

Giraudier et al. demonstrated that overexpression of FKBP51 induced a resistance to apoptosis with no effect on proliferation. In this study, FKBP51 was overexpressed in human megakaryocytes derived from CD34+ cells and after the overexpression apoptosis was induced by cytokine deprivation. This resulted in a

significant increase in survival of CD34+ cells. Cells remained alive for more than five days, whereas 50% of the control cells died after two days of cytokine deprivation. Relative to the control cells, the number of Annexin-V-positive cells was remarkley increased in cells overexpressing FKBP51. This resistance to apoptosis was further confirmed by analysing the Procaspase-3 levels: cells overexpressing FKBP51 showed less Caspase-3 activation relative to the control cells (Giraudier et al., 2002).

Down-regulation of the FKBP51 levels in malignant melanoma cells by siRNAs led to induction of apoptosis. Romano et al. could demonstrate that after irradiation of FKBP51-downregulated malignant melanoma cells this resulted in massive apoptosis *in vivo* and *in vitro*. Cell death was measured by Annexin-V-analysis. They could clearly show that irradiation of cells induced a remarkable increase of Annexin-V-positive cells compared to the control cells. Typical biochemical markers for appoptosis, such as cleaved Caspase-9 and -3 were observed in irradiated cells, where FKBP51 has been knocked down. The same results could be reported *in vivo*: When nude mice (tumor-xenograft mice) were treated with a single dose of FKBP51- siRNA before the irradiation, this resulted in an extensive apoptosis. All those data demonstrated that FKBP51 has a relevant role in counteracting apoptotis (Romano et al., 2010).

4.3.1 Downregulation of FKBP51 in primary hEPCs induces apoptosis

In accordance with the published data, also in our studies we could show that reduction of the FKBP51 levels in primary hEPCs resulted in an apoptotic phenotype. In the cytospins of cells with reduced FKBP51 levels a two fold higher amount of dead and apoptotic cells was observed. This induction of apoptosis could be reproduced in a second independent experiment. Measuring the Annexin-V-positive cells, we obtained similar results in even three independent experiments using erythroid cultures of three different cord blood samples. The reduction of FKBP51 led to a 1,55 fold increase of Annexin-V- positive cells when compared to the lowGC- control (lowGC=1).

4.3.2 FKBP51 is involved in the NF-kB pathway

Glucocorticoid-mediated FKBP51 expression has been demonstrated to play a central role in the activaton of the **NF-kB-pathway** (Bouwmeester et al., 2004; Komura et al., 2005; Romano et al., 2010).

Park et al. demonstrated that expression of FKBP51 in NDV (Newcastle disease virus) infected chickens results in induction of an NF-kB luciferase reporter gene even in abscence of glucocorticoids. This supported the existance of an FKBP51-mediated NF-kB activation pathway (Park et al., 2007).

As already mentioned in chapter 1.6.3, NF-kB is located in the cytoplasm as an inactive form, where it is associated with its inhibitor IkBα. IkBα can be phosphorylated by an active IKKα, which leads to the degradation of IkBα by a proteasome. Thereby NF-kB is released and translocates into the nucelus, where it stimulates the expression of multiple antiapoptotic genes, like Bcl-2, Bcl-XL and BFL1.

Romano et al. reported that FKBP51 knockdown in malignant melanoma cells prevented the translocation of NF-kB into the nucleus. In FKBP51-silenced cells the lack of NF-kB induction permitted the activation of the apoptotic machinery, leading to the cellular suicide. The downregulation of FKBP51 induced by siRNA, inhibited the activation of NF-kB by tumor necrosis factor alpha, demonstrating that FKBP51 is a key regulator of the NF-kB pathway (Romano et al., 2010).

In 2005 Komura et al. compared the NF-kB activation in the factor dependendent UT7/Mpl cell line overexpressing FKBP51 (UT7/FKBP51) with the WT-cell-line (UT7/Mpl). They could show that overexpression of FKBP51 led to constitutive NF-kB activation after cytokine deprivation and a strong reduction of the cytoplasmic IKBα in comparison with the wild-type cell line (Komura et al., 2005).

Our analysis on the levels of the components of the NF-kB pathway in hEPCs after reduction of FKBP51 showed a decrease of the phosphorylation of NF-kB- and the phosphorylation of IKK-α/β.

Recently it has been reported that FKBP51 is also an essential co-factor for IKKα, the inhibitor of IkBα (Bouwmeester et al., 2004).

Therefore in our case, in absence of FKBP51, IKKα could not any longer be phosphorylated, whereas IkBα would not be degraded and thus NF-kB would

remain in its inactive form in the cytoplasm and could therefore no longer be released into the nucleus to inhibit apoptosis.
This scheme would fit exactly with our results (reduced pNF-kB and pIKK α/β-levels). The data of our study demonstrate that also in erythroid cells FKBP51 is involved in the NF-kB activation and that it might act as well as a cofactor of IKKαβ, since also these levels were down-regulated in the abscence of FKBP51.

4.3.3 FKBPP51 might be involved in the JAK/STAT pathway

FKBP51 can not only phosphorylate IKKαβ and thereby induce the activation of NF-kB, it has also been shown to be able to inhibit **Calcineurin (CaN)** (Baughman et al., 1995; Nair et al., 1997).
Calcineurin is a calcium-calmodulin-regulated serine/threonine phosphatase, which has been reported to dephosphorylate IkBs (Frantz et al., 1994). Thus, Calcineurin regulates the NF-kB pathway by dephosphorylating IkB and preventing its degradation by the proteasome and thereby promoting apoptosis. It has been shown that overexpression of FKBP51 in a UT-7/Mpl cell line induced the resistance to apoptosis through inhibition of the calcineurin signal transduction pathway (Giraudier et al., 2002). FKBP51 regulates Calcineruin activity either alone or by binding to an unknown cellular protein.

On the other side Calcineurin is also involved in the control of the JAK/STAT pathway, as it has been shown to dephosphorylate STAT3 (Liang et al., 1999).
It has been demonstrated that overexpression of FKBP51 in pathologic megacaryoctes from IMF patients (idiopathic myelofibrosis) induced a sustained activation of the JAK2/STAT5 pathway and a sustained activation of STAT5 (Komura et al., 2003). This STAT5 activation was associated with a persistent Bcl-XL expression and an absence of Caspase-3 cleavage after cytokine deprivation. It was also reported that in this FKBP51-overexpressing cell-line, STAT5 was dephosphorylated much slower, which was related to a persistent JAK2 phosphorylation. Calcineurin may act directly or indirectly on JAK2 phosphorylation to modify the STAT5 phosphorylation state.

Figure 50 shows a possible model for downstream signaling of FKBP51, it hypothesizes how FKBP51 might inhibit apoptosis: either through inhibition of Calcineurin or through IKK-αβ activation.

When FKBP51 is present:

In presence of FKBP51 Calcineurin is inhibited and can't lead to dephosphorylation of JAK2. Active JAK2 in turn phosphorylates STAT5 or STAT3, which act as transcription factors and induce the expression of antiapoptotic genes like Bcl-2, which protect cells from apoptosis.

When FKBP51 is absent:

On the other hand, in the absence of FKBP51, Calcineurin is no longer inhibited and leads to dephosphorylation of JAK2 and IkB, which blocks NF-kB. Inactive JAK2 in turn can't phosphorylate STAT5/STAT3, which thus can no longer stimulate the expression of antiapoptotic genes, whereby cells undergo apoptosis.

The role of the Calcineurin pathway in the myeloid lineages is poorly understood. In our studies in primary human erythroid progenitor cells we didn't yet analyse the relation of FKBP51 to Calcineurin and the JAK/STAT pathway. We would expect that the absence of FKBP51 would lead to an increase of Calcineurin, -if it is even expressed in hEPCs-, and therefore decreased levels of phosphorylated STAT3, JAK2 and STAT5 and as well as decreased expression of the antiapoptotic genes Bcl-2, Bcl-XL and BFL1 are expected. The decrease of the NF-kB-levels could already be demonstrated, but in further studies the expression of Bcl-2, Bcl-XL and BFL1 should be analysed.

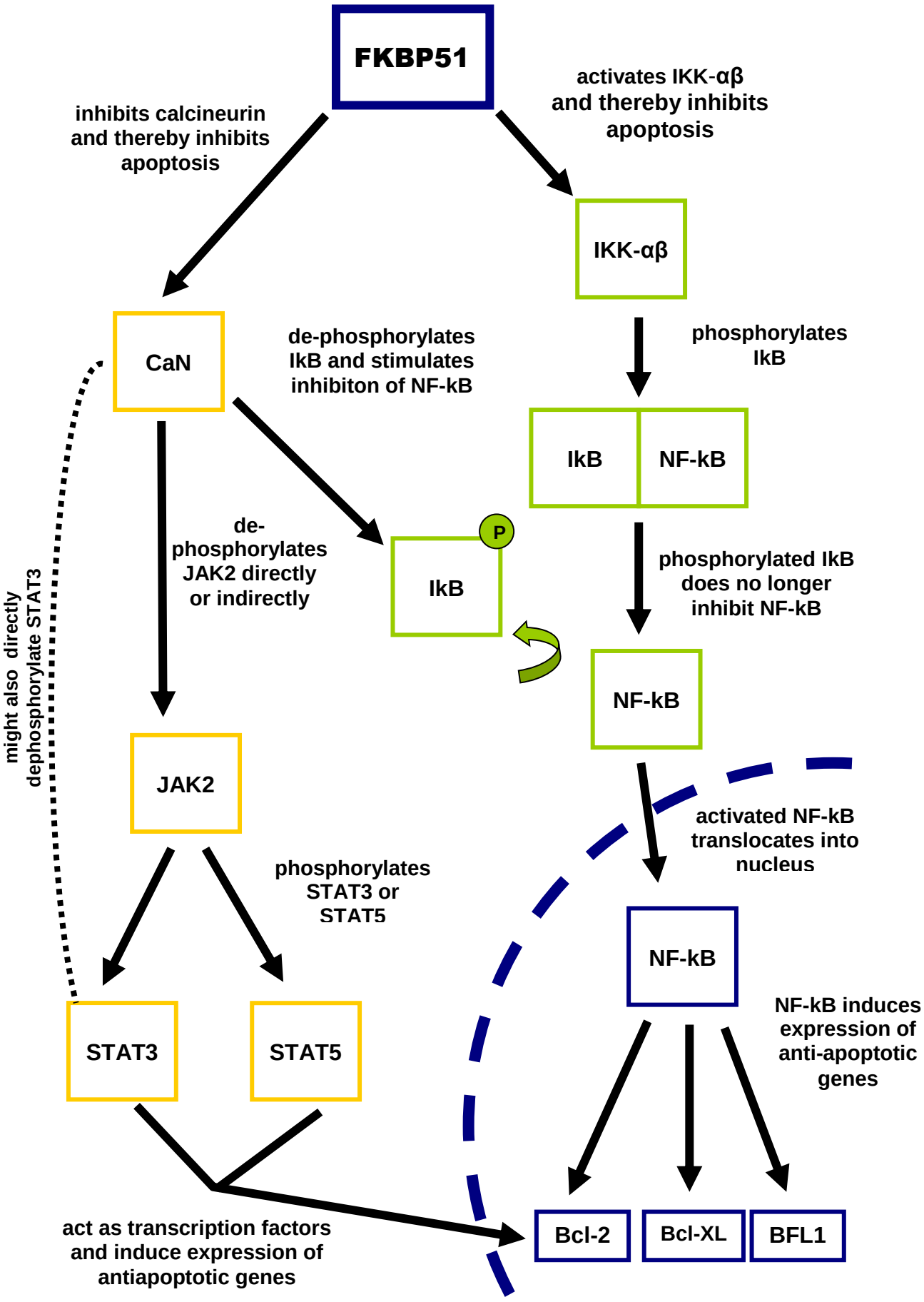


Figure 50: Possible model for downstream signaling of FKBP51
FKBP51 might inhibit apoptosis either through Calcineurin inhibition or IKK-αβ activation.

Another issue in our experiments was that the seen apoptotic phenotype (1,5- fold increase of apoptotic cells relative to the control) was not as prominent as in other reported FKBP51-silenced cells (Romano et al., 2010). In further experiments apoptosis could therefore be induced by factor deprivation -as reported in experiments with other factor dependent cell lines (Giraudier et al., 2002) - to further excite the effects of the FKBP51 knock-down in erythroid cells.

Taken together, we could show that the reduction of FKBP51 in primary human erythroid progenitor cells leads to an increase of apoptosis. FKBP51 seems to be involved in apoptosis protection, which might be mediated through IKKαβ-interaction and subsequent NF-kB activation. Whether FKBP51 in hEPCs is also involved in the JAK/STAT-pathway and is interacting with Calcineurin remains still unclear. Further studies will be required in which also the expression of the antiapoptotic genes (Bcl-2, Bcl-XL and BFL1) are analysed.

4.4 Glucocorticoids prevent apoptosis in hEPCs

Glucocorticoids are steroid hormones involved in several developmental and biological processes. Binding of glucocorticoids to their Receptor leads to activation and translocation of the GR into the nucleus, where it regulates different target genes.

Nuclear steroid receptors, such as the GR, were reported to modulate apoptosis in different cell types (Amaral et al., 2009). It has been demonstrated that GR supress spontaneous apoptosis of neutrophils and TNF-α mediated apoptosis of mouse fibroblasts (Pagliacci et al., 1993). Also in fibrosarcoma development the GR has been shown to inhibit apoptosis by transcriptionally activating the antiapoptotic gene Bcl-XL (Gascoyne et al., 2003). Even in human mammary epithelial cell lines, GR can activate several survival pathways (Moran et al., 2000). Furthermore, GR are involved in both, the induction and prevention of apoptosis depending on the cell type. Several studies were performed to identify GC-induced genes by microarray analyses, but the menchanism how exactly many of the target genes modulate apoptosis remains still unclear.

In my diploma thesis I analysed the function of three different GR-targets genes: Raf-1, Tis11d and FKBP51 by siRNA mediated knock-down (downregulation). While there were no remarkable effects after the Raf-1 downregulation, the reduction of Tis11d and FKBP51 led to an increase of apoptotis. Thus, the latter GR-targets seem to be involved in anti-apoptotic mechanisms of erythroid progenitor cells.

How both regulate the survival pathway reamains still unclear. We propose that Tis11d leads to the destabilisation of one or more mRNAs, whose protein products may have a direct or indirect affect on apoptosis induction in erythroid cells. Whether the mRNA target involved in the apoptotic process, is TNF-alpha, as we first assumed, needs further investigation. There is strong evidence that the antiapoptotic effect of FKBP51 is mediated either through inhibition of the calcineurin activity, or over a glucocorticoid-dependent-FKBP51-induced-NF-kB signaling pathway.

As we could show that Tis11d knock-down at the same time led also to a dramatically decrease of FKBP51 on mRNA level, a possible interaction of those two GR target genes, both involved in antiapoptotic mechanisms, could be feasible. We found out that the 3'-untranslated region of the FKBP51 mRNA contains four adenine-uridine (AU) rich elements (ARE), which have been reported to be the binding side of Tis11d. But if Tis11d is capable to bind to FBKP51 and thereby to induce its degradation needs to be analysed. Thus, the absence of Tis11d should lead to an accumulation and not to a down-regulation of FKBP51. This possible interaction is still entirely speculative and requires further experiments and analysis.

In summary we could show that in human erythroid progenitor cells glucocorticoids are involved in the antiapoptotic mechanism.

5. REFERENCES

Adli, M., Merkhofer, E., Cogswell, P., and Baldwin, A.S. IKKalpha and IKKbeta each function to regulate NF-kappaB activation in the TNF-induced/canonical pathway. PLoS One *5*, e9428.

Allen, T.D., and Dexter, T.M. (1982). Ultrastructural aspects of erythropoietic differentiation in long-term bone marrow culture. Differentiation *21*, 86-94.

Amaral, J.D., Sola, S., Steer, C.J., and Rodrigues, C.M. (2009). Role of nuclear steroid receptors in apoptosis. Curr Med Chem *16*, 3886-3902.

Arai, F., Hirao, A., and Suda, T. (2005). Regulation of hematopoietic stem cells by the niche. Trends Cardiovasc Med *15*, 75-79.

Arai, F., Yoshihara, H., Hosokawa, K., Nakamura, Y., Gomei, Y., Iwasaki, H., and Suda, T. (2009). Niche regulation of hematopoietic stem cells in the endosteum. Ann N Y Acad Sci *1176*, 36-46.

Auffray, I., Marfatia, S., de Jong, K., Lee, G., Huang, C.H., Paszty, C., Tanner, M.J., Mohandas, N., and Chasis, J.A. (2001). Glycophorin A dimerization and band 3 interaction during erythroid membrane biogenesis: in vivo studies in human glycophorin A transgenic mice. Blood *97*, 2872-2878.

Bakheet, T., Williams, B.R., and Khabar, K.S. (2003). ARED 2.0: an update of AU-rich element mRNA database. Nucleic Acids Res *31*, 421-423.

Baou, M., Jewell, A., and Murphy, J.J. (2009). TIS11 family proteins and their roles in posttranscriptional gene regulation. J Biomed Biotechnol *2009*, 634520.

Bauer, A., Tronche, F., Wessely, O., Kellendonk, C., Reichardt, H.M., Steinlein, P., Schutz, G., and Beug, H. (1999). The glucocorticoid receptor is required for stress erythropoiesis. Genes Dev *13*, 2996-3002.

Baughman, G., Wiederrecht, G.J., Campbell, N.F., Martin, M.M., and Bourgeois, S. (1995). FKBP51, a novel T-cell-specific immunophilin capable of calcineurin inhibition. Mol Cell Biol *15*, 4395-4402.

Bell, S.E., Sanchez, M.J., Spasic-Boskovic, O., Santalucia, T., Gambardella, L., Burton, G.J., Murphy, J.J., Norton, J.D., Clark, A.R., and Turner, M. (2006). The RNA binding protein Zfp36l1 is required for normal vascularisation and post-transcriptionally regulates VEGF expression. Dev Dyn *235*, 3144-3155.

Bernstein, A., Forrester, L., Reith, A.D., Dubreuil, P., and Rottapel, R. (1991). The murine W/c-kit and Steel loci and the control of hematopoiesis. Semin Hematol *28*, 138-142.

Bessis, M. (1958). [Erythroblastic island, functional unity of bone marrow]. Rev Hematol *13*, 8-11.

Bessis, M.C., and Breton-Gorius, J. (1962). Iron metabolism in the bone marrow as seen by electron microscopy: a critical review. Blood *19*, 635-663.

Bonifer, C., Faust, N., Geiger, H., and Muller, A.M. (1998). Developmental changes in the differentiation capacity of haematopoietic stem cells. Immunol Today *19*, 236-241.

Bouwmeester, T., Bauch, A., Ruffner, H., Angrand, P.O., Bergamini, G., Croughton, K., Cruciat, C., Eberhard, D., Gagneur, J., Ghidelli, S.*, et al.* (2004). A physical and functional map of the human TNF-alpha/NF-kappa B signal transduction pathway. Nat Cell Biol *6*, 97-105.

Broxmeyer, H.E., Maze, R., Miyazawa, K., Carow, C., Hendrie, P.C., Cooper, S., Hangoc, G., Vadhan-Raj, S., and Lu, L. (1991). The kit receptor and its ligand, steel factor, as regulators of hemopoiesis. Cancer Cells *3*, 480-487.

Carballo, E., Lai, W.S., and Blackshear, P.J. (1998). Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science *281*, 1001-1005.

Carlesso, N., and Cardoso, A.A. Stem cell regulatory niches and their role in normal and malignant hematopoiesis. Curr Opin Hematol *17*, 281-286.

Carlile, G., Smith, D.H., and Wiedmann, M. (2009). A non-apoptotic role for Fas/FasL in erythropoiesis. FEBS Lett.

Chasis, J.A. (2006). Erythroblastic islands: specialized microenvironmental niches for erythropoiesis. Curr Opin Hematol *13*, 137-141.

Chasis, J.A., and Mohandas, N. (2008). Erythroblastic islands: niches for erythropoiesis. Blood *112*, 470-478.

Cole, T.J., Blendy, J.A., Monaghan, A.P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K., and Schutz, G. (1995). Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev *9*, 1608-1621.

Congdon, C.C. (1957). Bone marrow transplantation in animals exposed to whole-body radiation. J Cell Physiol Suppl *50*, 103-108.

Davies, T.H., Ning, Y.M., and Sanchez, E.R. (2002). A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. J Biol Chem *277*, 4597-4600.

Dolznig, H., Grebien, F., Deiner, E.M., Stangl, K., Kolbus, A., Habermann, B., Kerenyi, M.A., Kieslinger, M., Moriggl, R., Beug, H.*, et al.* (2006). Erythroid progenitor renewal versus differentiation: genetic evidence for cell autonomous, essential functions of EpoR, Stat5 and the GR. Oncogene *25*, 2890-2900.

Dolznig, H., Habermann, B., Stangl, K., Deiner, E.M., Moriggl, R., Beug, H., and Mullner, E.W. (2002). Apoptosis protection by the Epo target Bcl-X(L) allows factor-independent differentiation of primary erythroblasts. Curr Biol *12*, 1076-1085.

Dolznig, H., Kolbus, A., Leberbauer, C., Schmidt, U., Deiner, E.M., Mullner, E.W., and Beug, H. (2005). Expansion and differentiation of immature mouse and human hematopoietic progenitors. Methods Mol Med *105*, 323-344.

Dorn, I., Lazar-Karsten, P., Boie, S., Ribbat, J., Hartwig, D., Driller, B., Kirchner, H., and Schlenke, P. (2008). In vitro proliferation and differentiation of human CD34+ cells from peripheral blood into mature red blood cells with two different cell culture systems. Transfusion *48*, 1122-1132.

Droin, N., Cathelin, S., Jacquel, A., Guery, L., Garrido, C., Fontenay, M., Hermine, O., and Solary, E. (2008). A role for caspases in the differentiation of erythroid cells and macrophages. Biochimie *90*, 416-422.

Frantz, B., Nordby, E.C., Bren, G., Steffan, N., Paya, C.V., Kincaid, R.L., Tocci, M.J., O'Keefe, S.J., and O'Neill, E.A. (1994). Calcineurin acts in synergy with PMA to inactivate I kappa B/MAD3, an inhibitor of NF-kappa B. EMBO J *13*, 861-870.

Gangenahalli, G.U., Singh, V.K., Verma, Y.K., Gupta, P., Sharma, R.K., Chandra, R., and Luthra, P.M. (2006). Hematopoietic stem cell antigen CD34: role in adhesion or homing. Stem Cells Dev *15*, 305-313.

Gascoyne, D.M., Kypta, R.M., and Vivanco, M.M. (2003). Glucocorticoids inhibit apoptosis during fibrosarcoma development by transcriptionally activating Bcl-xL. J Biol Chem *278*, 18022-18029.

Giraudier, S., Chagraoui, H., Komura, E., Barnache, S., Blanchet, B., LeCouedic, J.P., Smith, D.F., Larbret, F., Taksin, A.L., Moreau-Gachelin, F.*, et al.* (2002). Overexpression of FKBP51 in idiopathic myelofibrosis regulates the growth factor independence of megakaryocyte progenitors. Blood *100*, 2932-2940.

Gunsilius, E., Gastl, G., and Petzer, A.L. (2001). Hematopoietic stem cells. Biomed Pharmacother *55*, 186-194.

Heissig, B., Hattori, K., Dias, S., Friedrich, M., Ferris, B., Hackett, N.R., Crystal, R.G., Besmer, P., Lyden, D., Moore, M.A.*, et al.* (2002). Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. Cell *109*, 625-637.

Ino, T., Yasui, H., Hirano, M., and Kurosawa, Y. (1995). Identification of a member of the TIS11 early response gene family at the insertion point of a DNA fragment containing a gene for the T-cell receptor beta chain in an acute T-cell leukemia. Oncogene *11*, 2705-2710.

Johnson, B.A., Geha, M., and Blackwell, T.K. (2000). Similar but distinct effects of the tristetraprolin/TIS11 immediate-early proteins on cell survival. Oncogene *19*, 1657-1664.

Jordan, E.T., Collins, M., Terefe, J., Ugozzoli, L., and Rubio, T. (2008). Optimizing electroporation conditions in primary and other difficult-to-transfect cells. J Biomol Tech *19*, 328-334.

Kolbus, A., Blazquez-Domingo, M., Carotta, S., Bakker, W., Luedemann, S., von Lindern, M., Steinlein, P., and Beug, H. (2003). Cooperative signaling between cytokine receptors and the glucocorticoid receptor in the expansion of erythroid progenitors: molecular analysis by expression profiling. Blood *102*, 3136-3146.

Kolbus, A., Pilat, S., Husak, Z., Deiner, E.M., Stengl, G., Beug, H., and Baccarini, M. (2002). Raf-1 antagonizes erythroid differentiation by restraining caspase activation. J Exp Med *196*, 1347-1353.

Komura, E., Chagraoui, H., Mansat de Mas, V., Blanchet, B., de Sepulveda, P., Larbret, F., Larghero, J., Tulliez, M., Debili, N., Vainchenker, W.*, et al.* (2003). Spontaneous STAT5 activation induces growth factor independence in idiopathic myelofibrosis: possible relationship with FKBP51 overexpression. Exp Hematol *31*, 622-630.

Komura, E., Tonetti, C., Penard-Lacronique, V., Chagraoui, H., Lacout, C., Lecouedic, J.P., Rameau, P., Debili, N., Vainchenker, W., and Giraudier, S. (2005). Role for the nuclear factor kappaB pathway in transforming growth factor-beta1 production in idiopathic myelofibrosis: possible relationship with FK506 binding protein 51 overexpression. Cancer Res *65*, 3281-3289.

Kondo, M., Wagers, A.J., Manz, M.G., Prohaska, S.S., Scherer, D.C., Beilhack, G.F., Shizuru, J.A., and Weissman, I.L. (2003). Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annu Rev Immunol *21*, 759-806.

Krakstad, C., and Chekenya, M. Survival signalling and apoptosis resistance in glioblastomas: opportunities for targeted therapeutics. Mol Cancer *9*, 135.

Kurtz, A., Jelkmann, W., and Bauer, C. (1982). A new candidate for the regulation of erythropoiesis. Insulin-like growth factor I. FEBS Lett *149*, 105-108.

Lai, W.S., Carballo, E., Strum, J.R., Kennington, E.A., Phillips, R.S., and Blackshear, P.J. (1999). Evidence that tristetraprolin binds to AU-rich elements and promotes the deadenylation and destabilization of tumor necrosis factor alpha mRNA. Mol Cell Biol *19*, 4311-4323.

Lai, W.S., Carballo, E., Thorn, J.M., Kennington, E.A., and Blackshear, P.J. (2000). Interactions of CCCH zinc finger proteins with mRNA. Binding of tristetraprolin-related zinc finger proteins to Au-rich elements and destabilization of mRNA. J Biol Chem *275*, 17827-17837.

Leberbauer, C., Boulme, F., Unfried, G., Huber, J., Beug, H., and Mullner, E.W. (2005). Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. Blood *105*, 85-94.

Li, X.F., Anderson, J., Hutzler, D., and Roodman, G.D. (1989). Hemin-induced erythroid differentiation changes the sensitivity of K562 cells to tumor necrosis factor-alpha. Exp Hematol *17*, 1059-1062.

Liang, H., Venema, V.J., Wang, X., Ju, H., Venema, R.C., and Marrero, M.B. (1999). Regulation of angiotensin II-induced phosphorylation of STAT3 in vascular smooth muscle cells. J Biol Chem *274*, 19846-19851.

Lin, C.S., Lim, S.K., D'Agati, V., and Costantini, F. (1996). Differential effects of an erythropoietin receptor gene disruption on primitive and definitive erythropoiesis. Genes Dev *10*, 154-164.

Lin, H. (2002). The stem-cell niche theory: lessons from flies. Nat Rev Genet *3*, 931-940.

Merkhofer, E.C., Cogswell, P., and Baldwin, A.S. Her2 activates NF-kappaB and induces invasion through the canonical pathway involving IKKalpha. Oncogene *29*, 1238-1248.

Mikula, M., Schreiber, M., Husak, Z., Kucerova, L., Ruth, J., Wieser, R., Zatloukal, K., Beug, H., Wagner, E.F., and Baccarini, M. (2001). Embryonic lethality and fetal liver apoptosis in mice lacking the c-raf-1 gene. EMBO J *20*, 1952-1962.

Miura, O., Nakamura, N., Quelle, F.W., Witthuhn, B.A., Ihle, J.N., and Aoki, N. (1994). Erythropoietin induces association of the JAK2 protein tyrosine kinase with the erythropoietin receptor in vivo. Blood *84*, 1501-1507.

Moran, T.J., Gray, S., Mikosz, C.A., and Conzen, S.D. (2000). The glucocorticoid receptor mediates a survival signal in human mammary epithelial cells. Cancer Res *60*, 867-872.

Muszynski, K.W., Ruscetti, F.W., Gooya, J.M., Linnekin, D.M., and Keller, J.R. (1997). Raf-1 protein is required for growth factor-induced proliferation of primitive hematopoietic progenitors stimulated with synergistic combinations of cytokines. Stem Cells *15*, 63-72.

Muta, K., Krantz, S.B., Bondurant, M.C., and Dai, C.H. (1995). Stem cell factor retards differentiation of normal human erythroid progenitor cells while stimulating proliferation. Blood *86*, 572-580.

Muta, K., Krantz, S.B., Bondurant, M.C., and Wickrema, A. (1994). Distinct roles of erythropoietin, insulin-like growth factor I, and stem cell factor in the development of erythroid progenitor cells. J Clin Invest *94*, 34-43.

Nair, S.C., Rimerman, R.A., Toran, E.J., Chen, S., Prapapanich, V., Butts, R.N., and Smith, D.F. (1997). Molecular cloning of human FKBP51 and comparisons of immunophilin interactions with Hsp90 and progesterone receptor. Mol Cell Biol *17*, 594-603.

Nakamura, Y., Tajima, F., Ishiga, K., Yamazaki, H., Oshimura, M., Shiota, G., and Murawaki, Y. (2004). Soluble c-kit receptor mobilizes hematopoietic stem cells to peripheral blood in mice. Exp Hematol *32*, 390-396.

Nocka, K., Buck, J., Levi, E., and Besmer, P. (1990). Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. EMBO J *9*, 3287-3294.

Orkin, S.H., and Zon, L.I. (2008). Hematopoiesis: an evolving paradigm for stem cell biology. Cell *132*, 631-644.

Paakinaho, V., Makkonen, H., Jaaskelainen, T., and Palvimo, J.J. Glucocorticoid receptor activates poised FKBP51 locus through long-distance interactions (2010). Mol Endocrinol *24*, 511-525.

Pagliacci, M.C., Migliorati, G., Smacchia, M., Grignani, F., Riccardi, C., and Nicoletti, I. (1993). Cellular stress and glucocorticoid hormones protect L929 mouse fibroblasts from tumor necrosis factor alpha cytotoxicity. J Endocrinol Invest *16*, 591-599.

Palis, J. (2008). Ontogeny of erythropoiesis. Curr Opin Hematol *15*, 155-161.

Palis, J., and Yoder, M.C. (2001). Yolk-sac hematopoiesis: the first blood cells of mouse and man. Exp Hematol *29*, 927-936.

Panzenbock, B., Bartunek, P., Mapara, M.Y., and Zenke, M. (1998). Growth and differentiation of human stem cell factor/erythropoietin-dependent erythroid progenitor cells in vitro. Blood *92*, 3658-3668.

Park, J., Kim, M., Na, G., Jeon, I., Kwon, Y.K., Kim, J.H., Youn, H., and Koo, Y. (2007). Glucocorticoids modulate NF-kappaB-dependent gene expression by up-regulating FKBP51 expression in Newcastle disease virus-infected chickens. Mol Cell Endocrinol *278*, 7-17.

Rahman, M.M., and McFadden, G. (2006). Modulation of tumor necrosis factor by microbial pathogens. PLoS Pathog *2*, e4.

Rajasagi, M., Vitacolonna, M., Benjak, B., Marhaba, R., and Zoller, M. (2009). CD44 promotes progenitor homing into the thymus and T cell maturation. J Leukoc Biol *85*, 251-261.

Rees-Unwin, K.S., Craven, R.A., Davenport, E., Hanrahan, S., Totty, N.F., Dring, A.M., Banks, R.E., G, J.M., and Davies, F.E. (2007). Proteomic evaluation of pathways associated with dexamethasone-mediated apoptosis and resistance in multiple myeloma. Br J Haematol *139*, 559-567.

Renstrom, J., Kroger, M., Peschel, C., and Oostendorp, R.A (2009). How the niche regulates hematopoietic stem cells. Chem Biol Interact *184*, 7-15.

Revollo, J.R., and Cidlowski, J.A. (2009). Mechanisms generating diversity in glucocorticoid receptor signaling. Ann N Y Acad Sci *1179*, 167-178.

Rich, I.N., Vogt, C., and Pentz, S. (1988). Erythropoietin gene expression in vitro and in vivo detected by in situ hybridization. Blood Cells *14*, 505-520.

Romagnani, P., Lasagni, L., Annunziato, F., Serio, M., and Romagnani, S. (2004). CXC chemokines: the regulatory link between inflammation and angiogenesis. Trends Immunol *25*, 201-209.

Romano, S., D'Angelillo, A., Pacelli, R., Staibano, S., De Luna, E., Bisogni, R., Eskelinen, E.L., Mascolo, M., Cali, G., Arra, C*., et al (2010).* Role of FK506-binding protein 51 in the control of apoptosis of irradiated melanoma cells. Cell Death Differ *17*, 145-157.

Rubiolo, C., Piazzolla, D., Meissl, K., Beug, H., Huber, J.C., Kolbus, A., and Baccarini, M. (2006). A balance between Raf-1 and Fas expression sets the pace of erythroid differentiation. Blood *108*, 152-159.

Rusten, L.S., and Jacobsen, S.E. (1995). Tumor necrosis factor (TNF)-alpha directly inhibits human erythropoiesis in vitro: role of p55 and p75 TNF receptors. Blood *85*, 989-996.

Shiozawa, Y., Havens, A.M., Pienta, K.J., and Taichman, R.S. (2008). The bone marrow niche: habitat to hematopoietic and mesenchymal stem cells, and unwitting host to molecular parasites. Leukemia *22*, 941-950.

Skutelsky, E., and Danon, D. (1972). On the expulsion of the erythroid nucleus and its phagocytosis. Anat Rec *173*, 123-126.

Smoak, K., and Cidlowski, J.A. (2006). Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. Mol Cell Biol *26*, 9126-9135.

Srivastava, A.S., Kaushal, S., Mishra, R., Lane, T.A., and Carrier, E. (2006). Dexamethasone facilitates erythropoiesis in murine embryonic stem cells differentiating into hematopoietic cells in vitro. Biochem Biophys Res Commun *346*, 508-516.

Stellacci, E., Di Noia, A., Di Baldassarre, A., Migliaccio, G., Battistini, A., and Migliaccio, A.R. (2009). Interaction between the glucocorticoid and erythropoietin receptors in human erythroid cells. Exp Hematol *37*, 559-572.

Stumpo, D.J., Broxmeyer, H.E., Ward, T., Cooper, S., Hangoc, G., Chung, Y.J., Shelley, W.C., Richfield, E.K., Ray, M.K., Yoder, M.C*., et al.* (2009). Targeted disruption of Zfp36l2,

encoding a CCCH tandem zinc finger RNA-binding protein, results in defective hematopoiesis. Blood *114*, 2401-2410.

Taylor, G.A., Carballo, E., Lee, D.M., Lai, W.S., Thompson, M.J., Patel, D.D., Schenkman, D.I., Gilkeson, G.S., Broxmeyer, H.E., Haynes, B.F.*, et al.* (1996). A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. Immunity *4*, 445-454.

Testa, U. (2004). Apoptotic mechanisms in the control of erythropoiesis. Leukemia *18*, 1176-1199.

Tsiftsoglou, A.S., Vizirianakis, I.S., and Strouboulis, J. (2009). Erythropoiesis: model systems, molecular regulators, and developmental programs. IUBMB Life *61*, 800-830.

Tulac, S., Dosiou, C., Suchanek, E., and Giudice, L.C. (2004). Silencing lamin A/C in human endometrial stromal cells: a model to investigate endometrial gene function and regulation. Mol Hum Reprod *10*, 705-711.

Udupa, K.B., Crabtree, H.M., and Lipschitz, D.A. (1986). In vitro culture of proerythroblasts: characterization of proliferative response to erythropoietin and steroids. Br J Haematol *62*, 705-714.

Van Herreweghe, F., Festjens, N., Declercq, W., and Vandenabeele, P. Tumor necrosis factor-mediated cell death: to break or to burst, that's the question (2010). Cell Mol Life Sci *67*, 1567-1579.

Vermeer, H., Hendriks-Stegeman, B.I., van der Burg, B., van Buul-Offers, S.C., and Jansen, M. (2003). Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: a potential marker for glucocorticoid sensitivity, potency, and bioavailability. J Clin Endocrinol Metab *88*, 277-284.

Vermeer, H., Hendriks-Stegeman, B.I., van Suylekom, D., Rijkers, G.T., van Buul-Offers, S.C., and Jansen, M. (2004). An in vitro bioassay to determine individual sensitivity to glucocorticoids: induction of FKBP51 mRNA in peripheral blood mononuclear cells. Mol Cell Endocrinol *218*, 49-55.

von Lindern, M., Zauner, W., Mellitzer, G., Steinlein, P., Fritsch, G., Huber, K., Lowenberg, B., and Beug, H. (1999). The glucocorticoid receptor cooperates with the erythropoietin receptor and c-Kit to enhance and sustain proliferation of erythroid progenitors in vitro. Blood *94*, 550-559.

Weissman, I.L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. Cell *100*, 157-168.

Wessely, O., Deiner, E.M., Beug, H., and von Lindern, M. (1997). The glucocorticoid receptor is a key regulator of the decision between self-renewal and differentiation in erythroid progenitors. EMBO J *16*, 267-280.

Wilson, A., and Trumpp, A. (2006). Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol *6*, 93-106.

Wilusz, C.J., Wormington, M., and Peltz, S.W. (2001). The cap-to-tail guide to mRNA turnover. Nat Rev Mol Cell Biol *2*, 237-246.

Witthuhn, B.A., Quelle, F.W., Silvennoinen, O., Yi, T., Tang, B., Miura, O., and Ihle, J.N. (1993). JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell *74*, 227-236.

Woodruff, P.G., Boushey, H.A., Dolganov, G.M., Barker, C.S., Yang, Y.H., Donnelly, S., Ellwanger, A., Sidhu, S.S., Dao-Pick, T.P., Pantoja, C.*, et al.* (2007). Genome-wide profiling identifies epithelial cell genes associated with asthma and with treatment response to corticosteroids. Proc Natl Acad Sci U S A *104*, 15858-15863.

Xiao, W., Koizumi, K., Nishio, M., Endo, T., Osawa, M., Fujimoto, K., Sato, I., Sakai, T., Koike, T., and Sawada, K. (2002). Tumor necrosis factor-alpha inhibits generation of glycophorin A+ cells by CD34+ cells. Exp Hematol *30*, 1238-1247.

Yin, T., and Li, L. (2006). The stem cell niches in bone. J Clin Invest *116*, 1195-1201.

Zamore, P.D., and Aronin, N. (2003). siRNAs knock down hepatitis. Nat Med *9*, 266-267.

DANKSAGUNG:

Mein Dank gilt vor allem Dr. Mario Mairhofer, welcher mich als hervorragender und liebenswerter Betreuer durch meine ganze Arbeit hindurch begleitet hat und mir immer mit Rat und Tat zur Seite stand, wenn ich ihn gebraucht hab. Besonderer Dank auch an Univ.Doz.Dr. Andrea Kolbus für die Betreuung und stetige Korrektur meiner Diplomarbeit, sowie für die Möglichkeit, an diesem interessanten Thema in ihrer Arbeitsgruppe forschen zu können. In diesem Zusammenhang möchte ich mich auch bei der gesamten Arbeitsgruppe Endokrinologie für ihre wertvollen Tipps und das produktive Arbeitsklima bedanken.

Nicht zuletzt möchte ich mich bei meinen Eltern für die finanzielle sowie auch für die mentale Unterstützung in all meinen Lebensbereichen bedanken. Großer Dank gilt auch meinem Freund, sowie all meinen Freunden, die ich oft mit meinen faszinierenden Forschungsergebnissen gelangweilt habe. Vielen Dank für Eure Unterstützung!

Curriculum Vitae:

Persönliche Daten:

Vorname:	Michela
Nachname:	Parth
Geschlecht:	weiblich
Geburtsdatum:	22.09.1983
Geburtsort:	Schlanders

Kontaktdaten:

Straße:	Staudgasse 56/4
Wohnort:	1180 Wien
Email:	michela_parth@yahoo.de

Universitäre Ausbildung:

Studium: 2002 – 2011:	Studium der Biologie mit Fachrichtung Genetik in Wien
10. 2008 – 03. 2009:	Auslandsaufenthalt an der Universität in Leicester, England, im Rahmen des Erasmusprogrammes

Schulische Ausbildung:

1997 – 2002:	Realgymnasium Schlanders - wissenschaftliche Fachrichtung
1994 – 1997:	Mittelschule in Laas
1989 – 1994:	Volksschule in Laas

Praktika:

Oktober 2009 – Dezember 2009:	Praktikum im Gynäkologisch - Endokrinologischen Forschungslabor der Universitätsklinik für Frauenheilkunde, Medizinische Universität Wien
Jänner 2009 - März 2009:	Praktikum im Krebsforschungslabor der University of Leicester (UK) bei Prof. Nick Barlev
Sommer 2008:	Praktikum im Labor für Molekularbiologie am Land- und Forstwirtschafltichen Versuchszentrum Laimburg, Provinz Bozen
2002- 2006:	Während des Studiums diverse Praktika in biochemischen und molekularbiologischen Labors

Sprachliche Kenntnisse:

Deutsch:	Muttersprache
Italienisch:	gut, Diplom: Zweisprachigkeitsprüfung A
Englisch:	gut, Diplom: First Certificate, Level C