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EXPANSION OF HUMAN AND MURINE ERYTHROID PROGENITORS

The role of steroids and thyroid hormone

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Verfasserin / Verfasser:	Ramona Seba
Matrikel-Nummer:	0164126
Studienrichtung /Studienzweig (lt. Studienblatt):	442
Betreuerin / Betreuer:	Ao. Univ.-Prof. Mag. Dr. Ernst Müllner

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Zusammenfassung

Erythrozyten sichern die Sauerstoffversorgung aller Körperzellen und sorgen gleichzeitig für den Abtransport von CO_2. Der intensive Kontakt mit Sauerstoff führt zu Schädigungen, die die ständige Neubildung von Erythrozyten unerlässlich machen. Die lebenslange Produktion roter Blutzellen setzt das Vorhandensein von hämatopoetischen Stammzellen voraus, die nicht nur die Fähigkeit besitzen müssen, sich während der gesamten Lebensspanne eines Individuums zu teilen (Proliferation), sondern auch jene, durch Reifung (Differenzierung) alle Typen von Blutzellen hervorzubringen. Die molekularbiologischen Prozesse die während der Entwicklung von der hämatopoetischen Stammzelle zum reifen Erythrozyten ablaufen sind bis heute nicht vollständig bekannt. Bei der Erforschung dieser Vorgänge kommt der Kultivierung erythroider Vorläuferzellen (Progenitoren) eine entscheidende Rolle zu. *In vitro* Kulturen ermöglichen die Untersuchung spezifischer Faktoren und deren Einfluss auf erythroide Progenitoren unter kontrollierten Bedingungen. So konnte beispielsweise gezeigt werden, dass Dihydrotestosteron die Zellteilung weiblicher Erythroblasten anregt, ein Effekt, der in männlichen Zellen nicht beobachtet werden konnte. Das in dieser Arbeit verwendete erythroide Zellkultursystem für humane Zellen ermöglicht die Langzeit-Kultivierung sowie Differenzierung von aus Nabelschnur- und peripherem Blut gewonnenen eryhtroiden Progenitoren. Dieses Zellkultursystem sieht die Verwendung von humanem Serum während der Differenzierung vor. Da Serum eine Vielzahl an Stoffen enthält, die unterschiedlich auf die Zellen wirken, ist sein Einsatz problematisch. Aus diesem Grund wurde in dieser Diplomarbeit die Wirkung von im Serum enthaltenen Faktoren auf differenzierende erythroide Zellen genauer untersucht, mit dem Ergebnis, dass ein für die Differenzierung wesentlicher Faktor eine molekulare Masse zwischen 3 und 6kD aufweist. Neben diesem Faktor, den es noch zu identifizieren gilt, stellt im Menschen, im Gegensatz zur Maus, das Schilddrüsenhormon T3 einen weiteren wesentlichen Differenzierungsfaktor dar. Das Hauptaugenmerk dieser Diplomarbeit lag in der Untersuchung zweier von T3 beeinflusster Gene, nämlich denen für das Oberflächenprotein *Spectrin ß* und *Caspase 7*, einem der Hauptregulatoren des programmierten Zelltodes. Es konnte eine Regulierung dieser Gene durch T3 auf Proteinebene nachgewiesen werden. Weiters wurden lentivirale Partikel produziert mit deren Hilfe eine verminderte Expression dieser Gene erreicht werden sollte.

Abstract

Erythrocytes guarantee the oxygensupply of all body cells and remove simultaneously CO_2 from tissues. These vital processes lead to damages of the red blood cells, due to permanent contact with high oxygen levels. This makes the constant replenishment of the erythrocyte pool an essential challenge. The life-long production of red blood cells requires the existence of haematopoietic stem cells not only capable to self-renew throughout the entire life-span of an individual (proliferation), but also to develop into the whole spectrum of mature blood cells (differentiation). Until now the molecular biological processes during development from the haematopoietic stem cell to the mature erythrocyte are still not fully elucidated. Cultivation of erythroid progenitor cells plays a critical role in the systematic study of these processes. *In vitro* cultures allow analysis of specific factors and their influence on erythroid progenitors under defined conditions. For example, we observed a proliferation-promoting effect of dihydro-testosterone on female erythroblasts, which could not be observed in erythroid cells derived from male donors. The erythroid cell culture system used for human cells, derived either from umbilical cord blood or peripheral blood, allowed both long-time proliferation and differentiation of erythroid progenitors. This cell culture system avoided the use of human serum during differentiation. Due to the fact that serum contains multiple components affecting erythroid cells in diverse ways, its use is suboptimal. In this diploma thesis the effect of serum constituents onto differentiating erythroid cells was analysed, with the result that one novel, so far undescribed essential factor for successful erythroid differentiation has an apparent molecular weight between 3 and 6kD. Beside this factor, which has to be identified, also free thyroid hormone (T3) represents an important maturation-promoting factor. The main focus of this diploma thesis laid on the analysis of two genes influenced by T3, the membrane-protein spectrin ß and caspase 7, one of the key regulators of apoptosis. Western blot analysis showed a regulation of these genes by T3 at the protein level. Additionally, lentiviral particles were produced to knock down the expression of these genes in human erythroid cells.

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1 Introduction

1.1 Haematopoiesis

Haematopoiesis is the process through which all mature blood cells arise. The cells giving blood its red colour are the haemoglobin-containing erythrocytes. They are responsible for oxygen transport through the body. This is a stressful process causing oxidative damage to the erythrocytes. Because of that their lifespan is limited to about 90-120 days. Thus it is essential for the organism to produce new red blood cells efficiently throughout the whole life of an individual (about 30g a day). For this challenge cells are needed, which are competent in both critical processes: proliferation and differentiation. This is achieved by haematopoietic stem cells (HSCs). They are able to self-renew and can undergo differentiation into all blood lineages (Weissman 2000). Both processes, proliferation and differentiation, have to be controlled very precisely to avoid anaemia or erythrocytosis. Haematopoiesis is managed by strictly coordinated patterns of gene expression. Growth factors and hormones play an important role in this context. They assist HSCs in choosing one of their diverse fates, which are not only self-renewal and commitment to differentiation but also commitment to senescence or cell death. The ability to self-renew guarantees a constant number of HSCs and simultaneously production of the corresponding mature cells.

During embryogenesis, the original HSC pool has to be established. In this process, multiple anatomic sites are involved (Figure 1.1). One of these sites, which is the first source of definitive HSCs, is the yolk sac (Li, Johnson et al. 2003; McGrath and Palis 2005). Cells from the yolk sac are able to reconstitute multi-lineage, long-term haematopoiesis in genetically HSC-deficient newborn mice (Yoder 2004) but fail in adult bone marrow repopulation (Lensch and Daley 2004). Another important site in the context of HSC generation is the aorta-gonad mesoneprhros region or AGM (Muller, Medvinsky et al. 1994; Medvinsky and Dzierzak 1996; Godin and Cumano 2002). The AGM is an anatomic region consisting of the dorsal aorta, its surrounding mesenchyme and the urogenital ridges. Beside the AGM, also the umbilical and vitelline arteries are additional sites for HSC development (de Bruijn, Speck et al. 2000), as well as the placenta (Alvarez-Silva, Belo-Diabangouaya et al. 2003; Mikkola, Gekas et al. 2005; Rhodes, Gekas et al. 2008). The main organ for HSC expansion and red blood cell generation during late embryonic development is the

foetal liver. During that period the foetal liver grows rapidly and has to be supplied with HSCs from other sites.

After the expansion of HSCs in the foetal liver, they finally become quiescent in their ultimate niche, the bone marrow. During postnatal life, it is important that a steady state is established, in which the balance between self-renewal and differentiation of HSCs is regulated according to the needs of the organism.

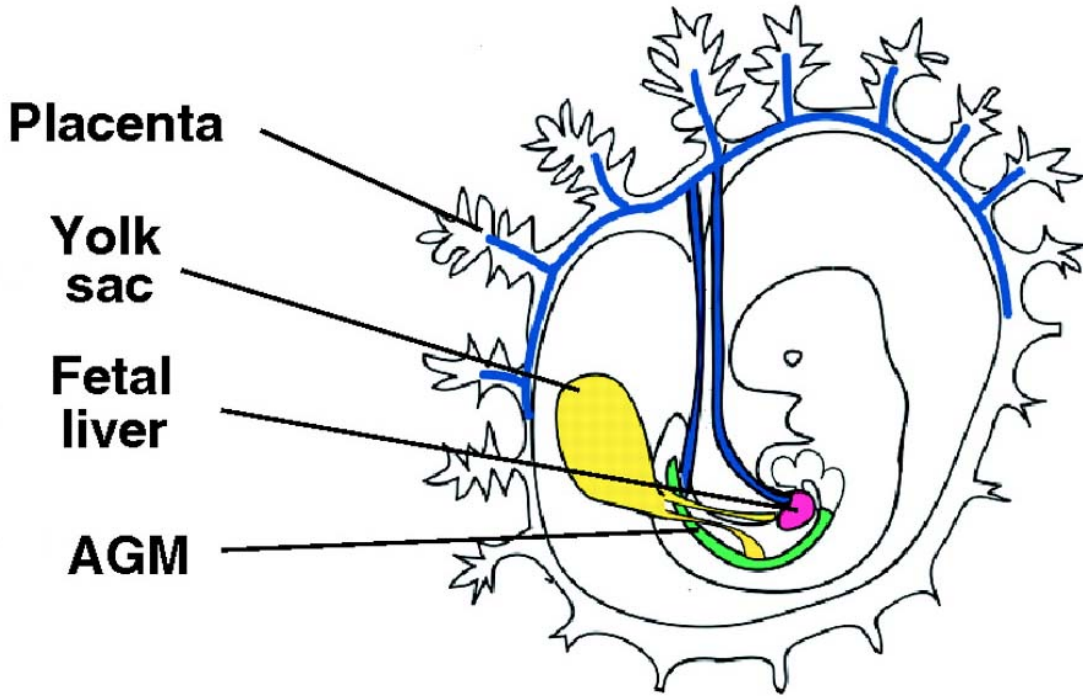


Figure taken from Mikkola and Orkin 2006

Figure 1.1: Sites of haematopoietic development during embryogenesis

Different anatomic sites are involved in the establishment of foetal blood. Depending on the stage of development, these sites alter their activity. The first HSCs could be observed in the yolk sac, during late embryogenesis the foetal liver is the most important haematopoietic organ.

1.1.1 HSCs & their niche

To maintain their self-renewal property, HSCs need a special subsidiary microenvironment, the so called stem cell niche. The niche of HSCs in adult mammals is the bone marrow.

Bone marrow is highly organized, forming a three-dimensional microenvironment with different cell types and an extra cellular matrix, consisting mainly of fibronectin and collagen. Many HSCs reside near the endosteum (Petit, Szyper-Kravitz et al. 2002). This is a fine stratum of vascular connective tissue at the interface between bone and bone marrow, where different cell types can be found: Bone-synthesizing osteoblasts and bone-resorbing osteoclasts are typical for this region as well as vascular cells. It is supposed that interaction of these cells regulates both, bone forming and haematopoiesis. As mentioned before, special factors are required for HSC maintenance. Some of them are already identified, like angiopoietin (Arai, Hirao et al. 2004), osteopontin (Nilsson, Johnston et al. 2005), stem cell factor (SCF) and thrombopoietin (Qian, Buza-Vidas et al. 2007).

Angiopoietin is a growth factor predominantly promoting the formation of blood vessels. It has been shown that a combined loss of the angiopoietin-receptors Tie1 and Tie2 causes defects in postnatal haematopoiesis (Puri and Bernstein 2003).

Osteopontin is synthesized by different cell types, like for example osteoblasts. This matrix glycoprotein negatively regulates the HSC pool size (Stier, Ko et al. 2005).

SCF is an important proliferation factor of HSCs as well as more mature haematopoietic progenitors. The receptor of SCF, c-Kit, is already expressed on the surface of early haematopoietic progenitors and also interacts indirectly with the cytoplasmic domain of the Epo-Receptor (Broudy, Lin et al. 1998).

Thrombopoietin is, among others, synthesized by osteoblasts and stromal cells of the bone marrow (Guerriero, Worford et al. 1997; Yoshihara, Arai et al. 2007). Mice deficient in thrombopoietin or its receptor c-Mpl have a severe reduction in the amount of HSCs (Kimura, Roberts et al. 1998; Kirito, Fox et al. 2003).

As osteoblasts produce both positive (angiopoietin) and negative (osteopontin) regulators for the maintenance of HSCs, they seem to be essential for haematopoiesis.

1.2 Erythropoiesis

1.2.1 From HSCs to erythrocytes

In adult individuals the immediate place where erythroblasts turn into erythrocytes is the erythroblastic island (Allen and Dexter 1982). These structures are placed throughout the bone-marrow and are made up of a central macrophage and varying numbers of erythroblasts in different developmental stages. During their maturation erythroblasts shrink in size and turn into normoblasts. At this stage, the nucleus becomes smaller and moves to the periphery of the cell. Finally, the nucleus is coated with a fine cell plasma layer and extruded. After the extrusion of the nucleus, the cell is released into the blood stream, where it develops into a reticulocyte and finally, assuming its typical biconcave shape, matures into an erythrocyte.

The erythroblastic island supports the maturation of erythroblasts. The central macrophage secretes several cytokines needed for differentiation, like for example insulin-like growth factor, IGF-1 (Kurtz, Hartl et al. 1985). Furthermore, erythropoietin mRNA was observed in macrophages (Rich, Vogt et al. 1988). Another role of the central macrophage is phagocytosis of the extruded erythroid nuclei (Seki and Shirasawa 1965). Adhesion of erythroblasts to the macrophage and vice versa is directed by adhesion molecules, like erythroblast macrophage protein (Emp) found on the surface of macrophages as well as on the surface of erythroblasts (Hanspal and Hanspal 1994). The attachment is accomplished by homophilic binding. It has been shown that this interaction is simultaneously a protection against apoptosis and a support for differentiation (Hanspal, Smockova et al. 1998).

Development of HSCs into mature erythrocytes is a complex process which proceeds via several intermediate stages (Figure 1.2). These intermediates cannot be discriminated properly by morphology alone but by flow cytometry (see section “Cell surface markers for haematopoietic progenitors”).

Long-term HSCs (LT-HSCs) have the ability to self-renew during the whole life-time of an organism. They give rise to short-term HSCs (ST-HSCs). Following this transition, the capacity for self-renewal can be observed only for 8 weeks. ST-HSCs differentiate to multi-potent progenitors (MPPs) of limited proliferation capacity but still constantly supplying the organism with cells from the whole spectrum of hematopoietic lineages (Morrison, Wandycz et al. 1997). After the identification of

common myeloid progenitors (CMPs) (Akashi, Traver et al. 2000) and common lymphoid progenitors (CLPs) (Kondo, Weissman et al. 1997) it was postulated that the first lineage restriction takes place when MPPs commit either to the lymphoid or the myeloid lineage. Why a single MPP commits to one of these two lineages still remains unclear. CMPs, however, develop into an erythroid and a myeloid branch, represented by the megakaryocyte/erythroid progenitor (MEP) and the granulocyte/macrophage progenitor (GMP). CLPs differentiate either into T- and B-lymphocytes or into Natural Killer Cells (NKC).

The earliest distinct progenitor within the erythroid lineage is the burst forming unit-erythroid, (BFU-E). BFU-Es are able to generate a burst of about 500 red cells in a time of 6-10 days (Heath, Axelrad et al. 1976; Iscove et al. 1974). BFU-Es differentiate into colony forming units-erythroid (CFU-Es). A CFU-E is able to form a colony with up to 32 cells within 2-3 days (Stephenson, Axelrad et al. 1971). After transition from CFU-Es to the stage of erythroblasts, development ends with mature red blood cells.

1.3 Erythroid cells in culture

To study the complex processes during red cell development, a tissue culture system is required which allows both biochemical and molecular biological analyses (Figure 3). This system has to meet several requirements. First, large numbers of homogenous progenitors have to be generated. Several kinds of primary cell model systems were already reported in the 1980s (Udupa, Crabtree et al. 1986). The problems of these systems were either insufficient cell production or heterogeneity of the cells obtained. Second, the cultured cells should behave similarly as those *in vivo*. Therefore, experiments using erythroleukaemic cell lines resulted in some insights but did not reflect the *in vivo* situation (Kabat, Sherton et al. 1975; Dean, Erard et al. 1981). Third, it should be possible to control the amount of growth factors in the medium precisely.

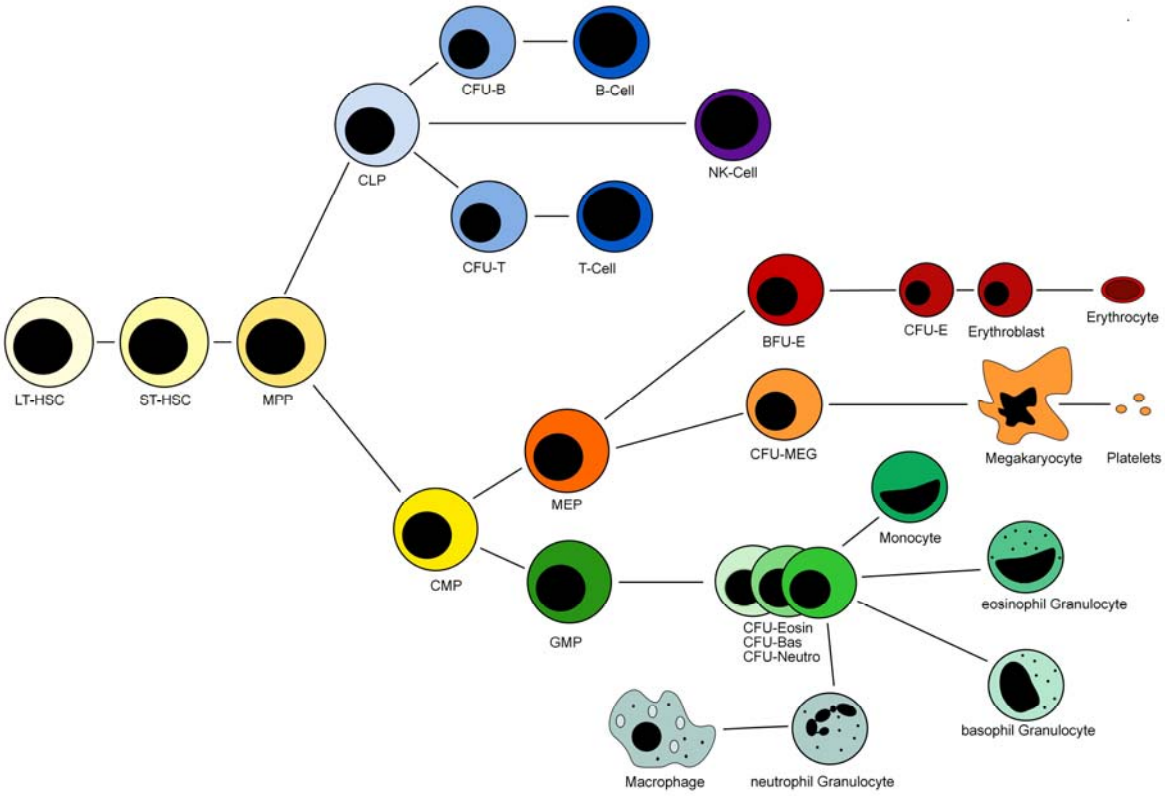


Figure 1.2: Simplified scheme of haematopoiesis

The pluripotent HSC is able to self renew throughout the whole lifespan of an individual. The proliferation rate is coordinated with the needs of the body and therefore tightly regulated. Beside proliferation, HSCs can also undergo differentiation into more and more committed progenitors, which ends with the creation of all kinds of mature blood cells.

Some earlier primary cell culture systems for erythroid progenitors originated from chicken (bone marrow), mouse (foetal liver) and human (umbilical cord blood) worked with serum plus a cocktail of factors (Epo, dexamethasone [Dex], SCF) involved in stress erythropoiesis (Bauer, Tronche et al. 1999; von Lindern, Zauner et al. 1999; Wessely, Bauer et al. 1999). In order to control the amount of the added proliferation factors precisely, the use of serum was suboptimal, because it contains a variety of different factors like hormones and proteins at unknown concentrations. For this reason, serum-free erythroid culture systems were established (Lebkowski, Schain et al. 1995; Neildez-Nguyen, Wajcman et al. 2002).

Sources for $CD34^+$ erythroid progenitors also used here are the murine foetal liver and, for human cells, peripheral (Fibach and Prus 2005) as well as umbilical cord blood (Sakatoku and Inoue 1997; Panzenbock, Bartunek et al. 1998; Leberbauer, Boulme et al. 2005). Although erythropoiesis takes place primarily in the bone marrow, a small number of HSCs is also present in the peripheral blood of adult humans (Goodman and Hodgson 1962). Umbilical cord blood, however, is more frequently used as source of human erythroid progenitors (Broxmeyer, Srour et al. 2006).

The pattern of cultured erythroid progenitors during expansion resembles the *in vivo* process of stress erythropoiesis (von Lindern, Deiner et al. 2001), which takes place after heavy blood loss and in case of anaemia or hypoxia. Cultured erythroid progenitors are conditioned to the factors active during stress erythropoiesis and undergo apoptosis if any single of these factors is omitted from the medium (Dozing, Kolbus et al. 2005).

1.3.1 Growth factors for *in vitro* proliferation

For *in vitro* proliferation of erythroid progenitor cells a specific factor mix, composed of Epo, Dex and Igf1 is needed.

The glycoprotein hormone Epo is one of the key regulators in erythropoiesis. In mice, knock out of Epo or its receptor leads to death *in utero* at embryonic day 13 (Wu, Liu et al. 1995). Although the amounts of BFU-Es and CFU-Es are comparable with the wild type situation, there is a loss of mature erythrocytes. Because of this Epo, respectively its receptor must be essential during or directly after the CFU-E stage. The Epo receptor (EpoR) belongs to the cytokine receptor superfamily and is

a homodimeric type I membrane protein. Its extra cellular domain is able to bind Epo and is therefore called Epo-binding domain. During normal conditions, only approximately 6% of EpoRs are occupied (Syed, Reid et al. 1998). This overspill of EpoRs is necessary for instance in case of severe blood loss, when Epo levels can increase up to 1000fold. $EpoR^{+/-}$ mice show normal haematocrit but have problems to deal with stress erythropoiesis (Jegalian, Acurio et al. 2002). Furthermore, it was observed, that Epo also protects early erythroid progenitors against apoptosis (Koury and Bondurant 1990). The binding of Epo causes activation of the transcription factor *signal transducer and activator of transcription 5* (STAT5) which results in the translation of the anti-apoptotic protein Bcl-$X_{L.}$ The protective effect of Epo seems to be needed not only during proliferation but also during differentiation.
It has been shown that Epo cooperates with SCF during proliferation which results in an increased number of cell divisions until terminal maturation (Wessely, Bauer et al. 1999). This is true in the case of primitive as well as more mature haematopoietic progenitor cells. During differentiation, SCF has a retarding effect (Muta, Krantz et al. 1995). Due to alternative splicing, SCF occurs in two isoforms. One consists of 165 amino acids and is water soluble, the other one consists of 220 amino acids and is membrane-bound. SCF is encoded in the Steel locus (Zsebo, Williams et al. 1990) localised on chromosome 12 in human and on chromosome 7 in mice (Anderson, Williams et al. 1991). Mutations in the locus for SCF or its receptor, c-Kit, result in similar phenotypes characterized by severe anaemia (Broxmeyer, Maze et al. 1991; Williams, de Vries et al. 1992). c-Kit is expressed on 1-5% of the cells in the bone marrow (Papayannopoulou, Brice et al. 1991). Besides haematopoietic progenitors, also platelets (Grabarek, Groopman et al. 1994), magakaryocytes (Briddell, Bruno et al. 1991) and some non-haematopoietic cells express c-Kit. Mutations in the gene encoding c-Kit are associated with some kinds of cancer and modifications in the c-kit expression pattern. For example, in case of acute myeloid leukaemia (AML) blast cells express c-kit (Ikeda, Kanakura et al. 1991). Epo has the ability to stimulate c-kit, which could be an indication for a relationship between these two proteins in haematopoietic target cells (Briddell, Bruno et al. 1991). Interactions between Epo/EpoR and SCF/c-Kit are required for long-term proliferation of erythroblasts *in vitro* (Wessely, Bauer et al. 1999). Furthermore, SCF also cooperates with the synthetic glucocorticoid hormone Dex. This supports the proliferation of chicken bone marrow cells and causes proliferation to last for about

27 days *in vitro*. In cooperation with Epo and SCF, Dex induces mouse erythroid progenitors to undergo 15-22 cell divisions, corresponding to a 10^5-10^6-fold amplification of erythroid cells (von Lindern, Zauner et al. 1999; Dolznig, Habermann et al. 2002). Furthermore von Lindern showed the delaying effect of Dex in terminal differentiation into erythrocytes.

Another frequently used growth factor for proliferation is Igf1. It is a protein hormone whose molecular structure is similar to insulin. At physiological concentrations, Igf1 stimulates erythroid colony formation of murine foetal liver and adult bone marrow cells (Kurtz, Jelkmann et al. 1982) and inhibits apoptosis of *in vitro* cultures (Rodriguez-Tarduchy, Collins et al. 1992; Muta, Krantz et al. 1994).

A rather unexpected support for proliferation of human erythroid progenitors comes from the androgen dihydro-testosterone. This effect is observed exclusively in cells derived from umbilical cord blood of female donors. Cells originating from male donors show no response to the hormone (Leberbauer, Boulme et al. 2005). That sex hormones might play a role in haematopoiesis was already supposed after the observation that dysfunctions in the level of sex steroids correlate with haematological abnormalities (Besa and Bullock 1981; Mooradian, Morley et al. 1987; Orwoll and Orwoll 1987; Claustres and Sultan 1988) but never rigorously tested.

For outgrowth of erythroid progenitors derived from peripheral blood addition of interleukin-6 (IL-6) and Flt3-ligand (Fibromyalgia syndrome like tyrosine kinase 3) is necessary for the first 6 days in culture.

Interleukin 6 is secreted by T cells, macrophages and osteoblasts and acts as both pro- and anti-inflammatory cytokine to trigger immune responses. It has been shown that the IL-6 receptor in synergy with Epo or Flt3 plays a role in HSC expansion (Ueda, Yoshida et al. 2001).

Flt3 has anti-apoptotic effects and promotes rapid cell divisions in erythrocyte progenitors (Poloni, Douay et al. 1997; Murray, Young et al. 1999).

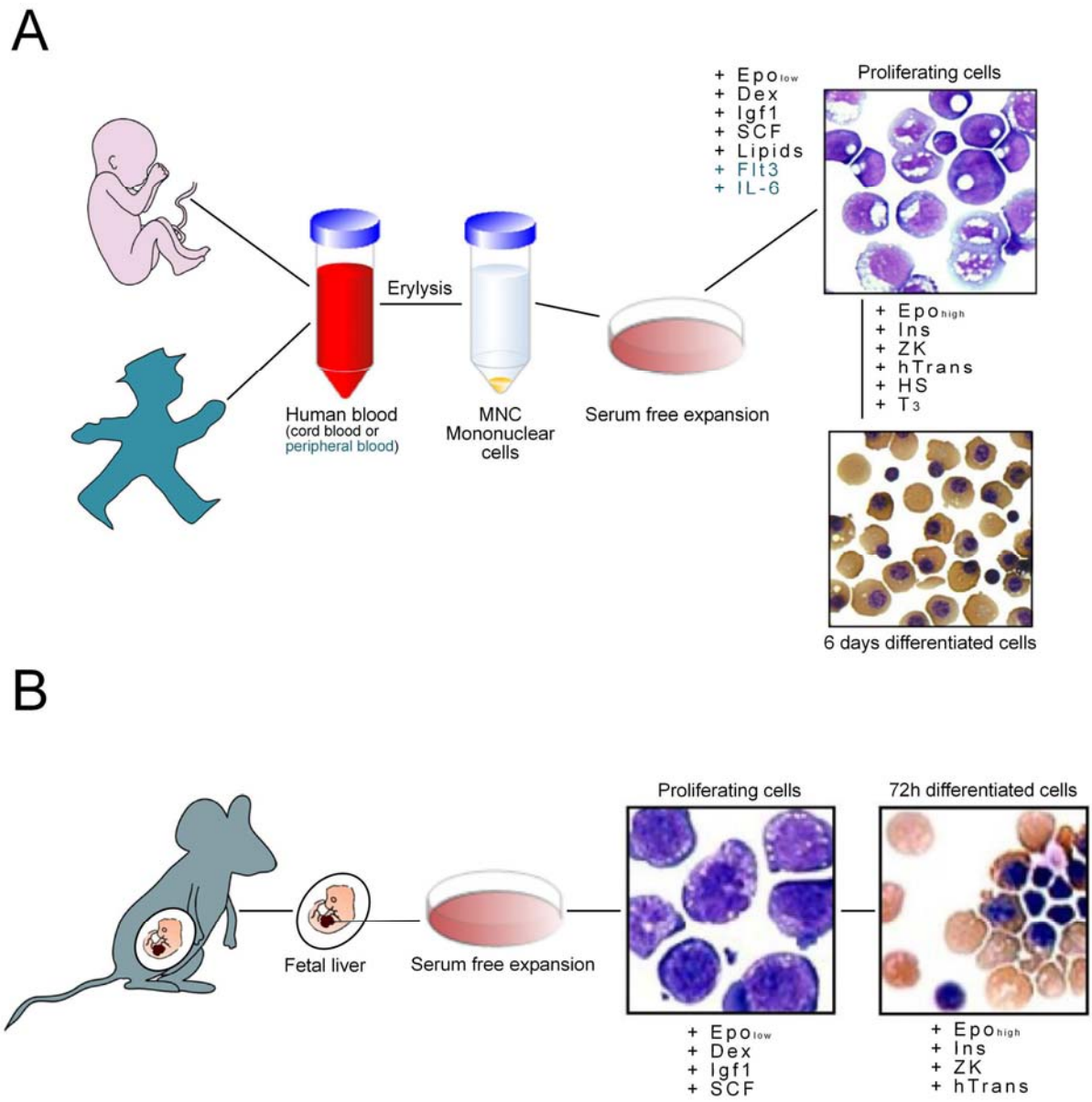


Figure 1.3: Human & murine erythroid cell culture systems

A) Human blood is collected and erythrocytes removed by lyses. The resulting cells represent a heterogeneous population of all mononuclear cells plus thrombocytes. After about 8 days (CB) and 10 days (PB) in culture with the appropriate medium enriched with proliferation growth factors, a homogenous erythroblast culture is established. Erythroblasts obtained from peripheral blood additionally need supplementation with Flt3 and IL-6 for the first 6 days in culture.

B) Murine foetal livers are harvested between E11.5 and E13.5. Comparable to the human system, the initial preparation contains different cell types. The outgrowth of homogenous erythroblasts takes about 4 days.

1.3.2 Growth factors for *in vitro* differentiation

Erythroblasts have a restricted ability to proliferate for a short time period. For mouse *in vitro* cultures, the factor mix for proliferation (Epo^{low}, Dex, Igf1, SCF) has to be changed to the one inducing differentiation (Epo^{high}, insulin, ZK, human transferrin) before erythroblasts lose their self-renewal capacity (Dolznig, Bartunek et al. 1995; Dolznig, Boulme et al. 2001). If this is not done, erythroblasts will undergo apoptosis. After addition of differentiation factors, erythroblasts initiate terminal maturation into erythrocytes in a synchronous and effective manner. During differentiation cell size decreases, haemoglobin is accumulated and the nucleus gets condensed and finally extruded.

In the presence of differentiation factors, murine erythroid progenitors complete differentiation within 3 days and become morphologically similar to erythrocytes *in vivo*. They are fully haemoglobinized and approximately 70% of the cells extrude their nucleus. Differentiation of human erythroid progenitors, however, takes about twice as long. The generation of enucleated cells takes six days and even then the majority of cells still contain their nucleus.

Epo is not only needed for proliferation, it plays also a key role in differentiation: Epo alone has the power to induce differentiation of erythroid progenitors (Muta, Krantz et al. 1994; Muta, Krantz et al. 1995). The most effective dose of erythropoietin during differentiation is about five times higher than during proliferation. The interaction partner of c-Kit during differentiation is no longer SCF, it is replaced by Insulin (Ins).

As mentioned before, glucocorticoids like Dex retain erythroid cells in an immature state. To avoid this effect the glucocorticoid-receptor antagonist ZK112993 (ZK) is added to the differentiation mix.

Another differentiation factor for human and avian erythroid cells but not for murine ones, is the thyroid hormone 3,5,3'-triiodothyronine (T3). Apparently, lack of thyroid hormone can be associated with anaemia in humans (Carmel and Spencer 1982), as already observed more than 25 years ago. In chicken, T3 is required by erythroblasts and sufficient to switch from sustained proliferation to terminal differentiation (Bauer, Mikulits et al. 1998). In case of human erythroblasts, T3 improves terminal maturation (Figure 1.4), resulting in an increased percentage of enucleated cells after six days of differentiation. Additionally, T3 accelerates the rate of haemoglobin accumulation and improves synchrony of maturation, which means

that the mean cell size variation is reduced (Leberbauer, Boulme et al. 2005). Nevertheless, mice lacking thyroid hormone receptor alpha (TRα), thyroid hormone receptor beta (TRβ) or both, show no recognisable variations in standard haematopoiesis (Forrest, Erway et al. 1996; Fraichard, Chassande et al. 1997; Wikstrom, Johansson et al. 1998).

In our culture system for human erythroid cells, also the addition of human serum is needed to promote successful differentiation. At present it remains unclear which serum factor is responsible for this effect. Thus, although the role of some factors essential for erythroid differentiation has become elucidated over time, apparently not all of them are identified yet.

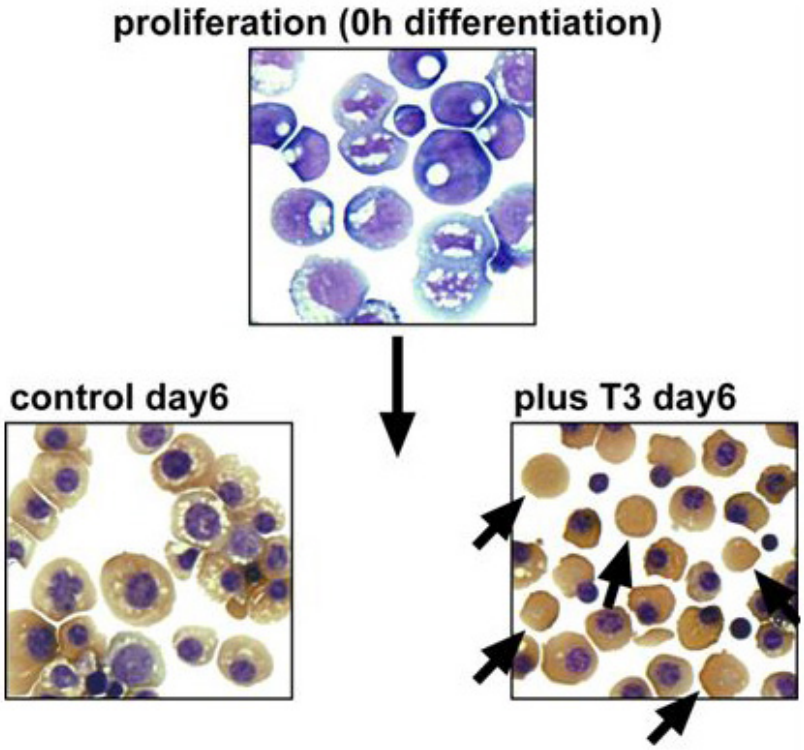


Figure 1.4: Differentiation of human erythroblasts with and without T3
In the presence of T3, the amount of enucleated cells increases significantly.

1.4 Cell-surface markers for haematopoietic progenitors

To distinguish different maturation steps during haematopoiesis, cell surface markers can be used. There is a long list of different markers, especially for haematopoietic cells. Their names are mostly composed of the letters CD for "Cluster of Differentiation" and a number.

Already in the 1980s, CD34 was described as a marker for HSCs (Ueda, Yoshida et al. 2001; Engelhardt, Lubbert et al. 2002). This glycoprotein is expressed on 1-5% of cells in the bone-marrow. It has been shown that $CD34^+$ cells are able to form colonies in short term assays (Civin, Strauss et al. 1984) and keep this potential even in long term assays in *in vitro* culture (Sutherland, Eaves et al. 1989). Furthermore it was demonstrated that $CD34^+$ cells are capable to repopulate immune-deficient mice (Bhatia, Wang et al. 1997). Despite these findings and the continuing wide-spread use of CD34 as an HSC marker, lately it has became clear that true HSCs are CD34-deficient (Bhatia, Bonnet et al. 1998). There is strong evidence that $CD34^-$ cells are more primitive than $CD34^+$ cells (Nakamura, Ando et al. 1999; Engelhardt, Lubbert et al. 2002).

Another frequently used marker for HSCs and early progenitors is c-Kit, the tyrosine kinase receptor for SCF, also called CD117. There is evidence that c-Kit is essential for HSC proliferation: SCF deficient mice have a 2.5-fold decrease in the absolute number of HSCs (Ikuta and Weissman 1992). Furthermore intraperitoneal injection of a c-Kit antagonist (anti-c-Kit) results in a dramatic reduction of HSCs and myeloid precursors in the bone marrow (Ogawa, Matsuzaki et al. 1991).

After the transition of HSCs to multi-potent progenitors they, start to express lineage-restricted markers like CD33 and CD38. The transmembrane-receptor CD33 is a marker for myeloid cells, whereas the glycoprotein CD38 is expressed on lymphoid cells.

Ter 119 (mouse) and glycophorin A (GPA; human), frequently together with CD71 (transferrin receptor) are well described erythroid markers, used to distinguish between immature, maturing and mature erythroid cells (Auffray, Marfatia et al. 2001).

1.5 Thyroid hormone and its targets

It remains unclear so far which molecular mechanisms cause the more mature phenotype of human erythroid cells differentiated in the presence of T3. mRNA expression profiling was performed to get a first idea of genes influenced by T3 (Leberbauer et al, unpublished data). The used microarray chip included 1.176 genes, 474 of which are expressed in erythroid cells. The profile was obtained from proliferating as well as differentiating cells at various time points (6, 30, 96 hours after induction of differentiation). Changes of gene expression, depending on time, were classified into four categories: “late up” (up-regulation after 30 hours), “early up” (up-regulation within the first 6 hours), “late down” (down-regulation after 30 hours) and “early down” (down-regulation within the first 6 hours). Significant changes in expression caused by T3 could be observed for several genes which may be essential for differentiation (Figure 1.5). The data suggest that T3 plays a role in enucleation. An indication for that is the up-regulation of spectrin-beta (SPTB) and cytokeratine 2. T3 could also be needed for accelerated down-regulation of genes that repress erythroid differentiation, like IMP-dehydrogenase. The finding that caspase 3 (C3) is required for erythroid differentiation (Zermati, Garrido et al. 2001) in connection to the observation that T3 represses caspase 7 makes the latter an interesting candidate for further investigation.

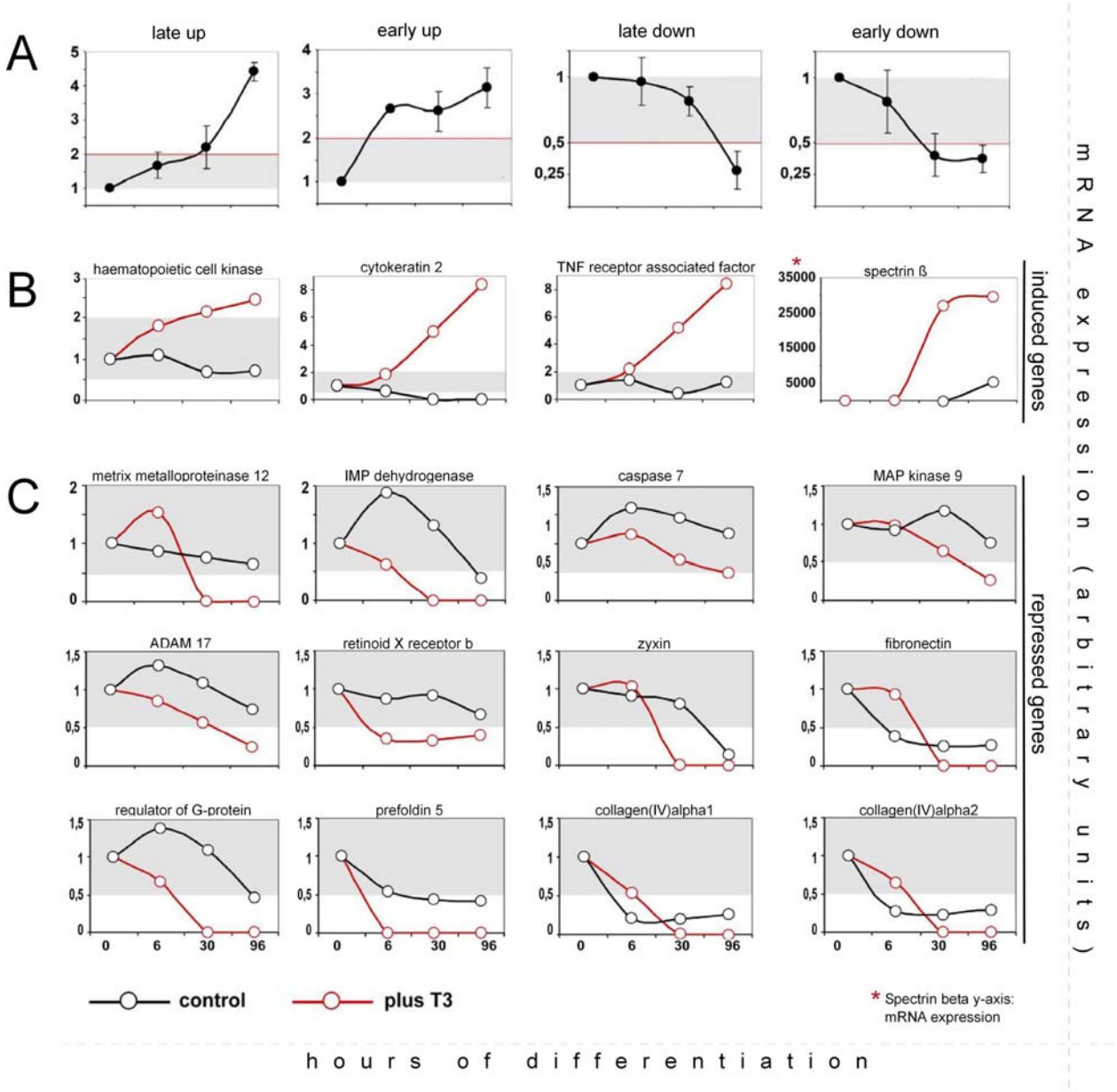


Figure 1.5: List of putative erythroid T3-targets

A) To classify T3 induced changes in mRNA-expression four categories ("late up", "early up", "late down", "early down") were defined.

B) Four of the putative erythroid target genes of T3 validated by induction of mRNA expression. One of them is the effector caspase C7.

C) 12 genes on the array known to play a role in haematopoiesis showed a T3-induced repression, including the erythrocyte membrane protein spectrin ß.

1.5.1 Spectrin

For their travel through the blood vessels red blood cells have to be elastic, flexible and equipped with good streaming properties. These features are almost due to one single protein: spectrin. Spectrin is the most abundant protein in the erythrocyte membrane skeleton, it makes up approximately 75% of its mass (Cohen 1983). This membrane protein in its simplest form is a heterodimer, consisting of two subunits: α (MW 240) and ß (MW 224) (Knowles, Marchesi et al. 1983) connected to each other at two positions. These heterodimers are linked by protein 4.1 and actin polymers to form a two-dimensional network (Figure 1.6). The chains of spectrin α and ß are antiparallel and both exhibit a repeating 106 amino acid long motif over most of their length that fold into a triple α-helical structure (Speicher and Marchesi 1984). The spectrin network lies on the cytoplasmic side of the erythrocyte membrane (Goodman, Krebs et al. 1988). The high affinity of the protein ankyrin with spectrin beta chains and the integral protein band 3 of the lipid bilayer result in the connection between the spectrin membrane and the cell membrane.

Abnormalities in spectrin structure, quantity or function are associated with several inherited haemolytic anaemias in humans, including hereditary spherocytosis and elliptocytosis (Palek 1984; Becker and Lux 1985). Despite these erythrocyte shape-changing variations, there are four known polymorphisms without any phenotype. Because of the ethnic origin of the used blood samples, we concentrated on spectrin type 1. Type 2, 3 and 4 are found exclusively in black people.

During erythropoiesis, the synthesis of spectrin occurs in an asynchronous manner. Differentiated mouse erythroleukemia (MEL) cells, for example, accumulate spectrin in an amount comparable to that in mature red blood cells (Lehnert and Lodish 1988). Human bone marrow cells from healthy donors, however, do not change their spectrin expression during erythropoiesis. It starts between the CFU-E and the pro-erythroblast stage (Nehls, Zeitler-Zapf et al. 1993).

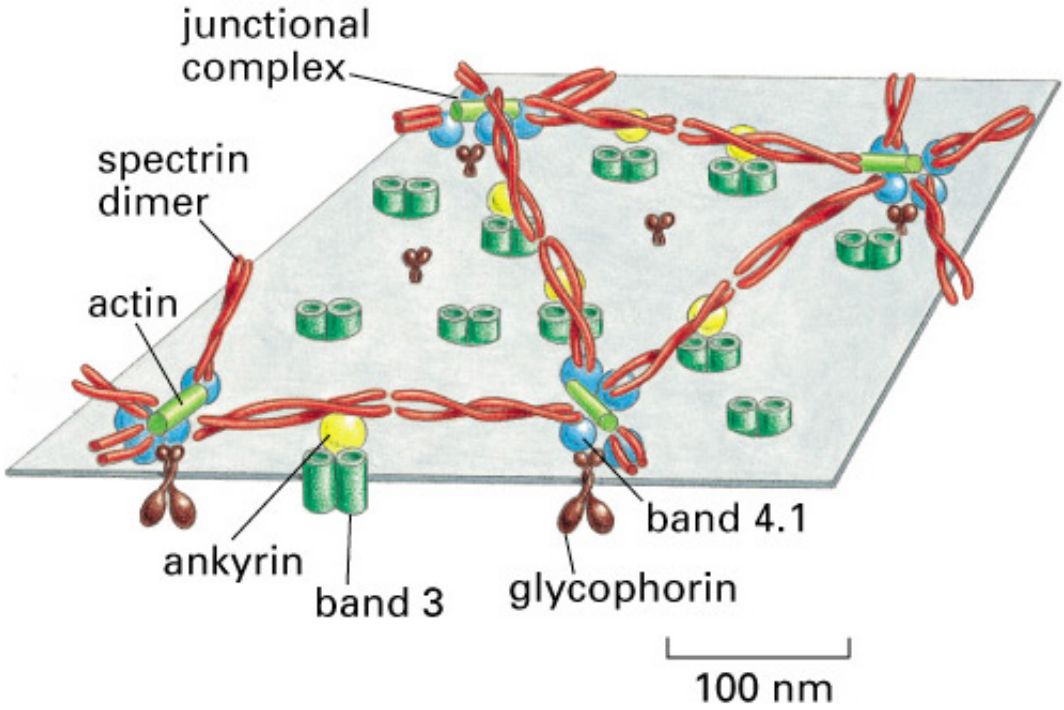


Figure taken from: “Molecular Biology of the Cell”, Alberts, Johnson, Walter, Lewis. Taylor & Francis. 2002

Figure 1.6: Spectrin-based cytoskeleton of red blood cells

Spectrin dimers together with ankyrin, actin and other components of the cell membrane cytoskeleton form a 2-dimensional meshwork, which lies beneath the plasma membrane.

1.5.2 Caspases

Caspases are a group of evolutionarily conserved proteases found in nearly all animals from Caenorhabditis elegans to humans. The term caspase reflects the two key characteristics of this protein-family. They use cysteine as the nucleophilic group for substrate cleavage, therefore they are cysteine proteases. This is the basis for the letter “C”. “Aspase” refers to their ability to cleave after aspartic acids (Alnemri, Livingston et al. 1996). Caspases are key regulators of apoptosis, one of the most important biological programs. Apoptosis is essential for development and viability of multi-cellular organisms. Apoptotic cells are characterized for example by chromatin and nucleus condensation and cell body shrinkage (Zamzami and Kroemer 1999). These features are also observed on differentiating erythroid cells (Morioka, Tone et al. 1998).

Caspases are synthesized as inactive precursors named procaspases. The formation of the two repeating heterodimeric catalytic domains of active caspases is carried out by proteolytic processing. On the basis of differences in procaspase-structure, substrate specificity and kinetic data, caspases can be classified into initiators and effectors. Initiator-caspases function as activators of effector-caspases. At least one of these effector-caspases is affected by T3, namely caspase 7 (C7). It has been shown that some effector-caspases (C3, C6, C7) are transiently activated through erythroid differentiation and that they cleave proteins involved in nuclear integrity (lamin B) and chromatin condensation (acinus) without inducing cell death and cleavage of GATA-1 (Zermati, Garrido et al. 2001). GATA-1, a haematopoietic transcription factor, is essential for survival of progenitors and terminal differentiated erythroid cells (Ohneda and Yamamoto 2002). Reduced expression of C3 leads to incapacity of CD34+ cells to complete the transition from pro-normoblasts to basophilic normoblasts (Carlile, Smith et al. 2004). Interestingly, caspase inhibition delays erythroid differentiation (Kolbus, Pilat et al. 2002).

1.6 Aim of this work

Although the constant supply of the body with mature erythrocytes is of essential importance, the molecular biological mechanisms during erythroid differentiation are not fully understood. Murine and human erythroid cell culture systems provided the basis for the study of these mechanisms. The human culture system, in contrast to the murine one, requires the use of serum during differentiation. One aim of this work was to remove specific constituents out of the serum and the performance of experiments in the presence of the obtained serum fractions. This should allow conclusions on the chemical character of essential differentiation factors within human serum.

An already identified differentiation factor for human erythroid cells is thyroid hormone T3 (Leberbauer, Boulme et al. 2005). The second aim of this work was to show the power of T3 in the regulation of the two genes SPTB and C7 to validate data from mRNA expression profiling. Furthermore the design of shRNA constructs for the knock down of SPTB and C7 and their integration in lentiviral particles were additional goals in this work. Furthermore, the production and use of lenitiviruses should be established as a tool for down-knock of specific genes in erythroid cells during this diploma thesis.

2 Methods

2.1 Cell Lines

2.1.1 K-562

Fresh cultures were started with $1x10^5$ viable cells/ml, later the density was increased to $1x10^6$ cells/ml. Medium (RPMI with 10% FCS; Sigma-Aldrich) was renewed every 2-3 days. Cells were propagated at 37°C with 5% CO_2.
Differentiation, characterized by haemoglobin accumulation and size-decrease, was induced with haemin (Lozzio and Lozzio 1975).

2.1.2 MCF-7, Hela, HEK 293T & 293FT

As basal medium Isocove's Modified Dulbecco's Medium (DMEM; Sigma-Aldrich) with 10% FCS was used. Cells were propagated at 37°C with 5% CO_2.
When the cells had reached a confluence of 80-90%, medium was removed. Cells were washed with PBS at room temperature. 1ml trypsin-EDTA was added and the mixture was incubated until the cell layer started to dispense. After addition of 9ml DMEM, cells were aspirated by gently pipetting. Aliquots of the suspension were put into new plates with the appropriate volume of medium. Usually the cells were split in a ration of 1:5 to 1:10. Medium was renewed every 2 to 3 days.

2.2 Human & murine erythroid cell culture

Umbilical cord and non-mobilized peripheral blood from healthy donors was collected in heparinised tubes (BD Vacutainer^{TM}) and stored at room temperature to prevent agglutination. Both peripheral and cord blood were processed within 24 hours.
Cord blood was originated from Caesarean sections, which took place in the period between May 2007 and June 2008 in the Division of Gynecological Endocrinology of the General Hospital, Vienna.

2.2.1 Isolation of mononuclear blood cells

To remove mature erythrocytes, aliquots of blood were mixed well with 1x erylysis-Buffer 1/25 (v/v) and incubated for 10-20 minutes at room temperature. During this time, lysis of red blood cells takes place. Because of that the suspension loses its viscosity and its colour turns from dark to light red. The mononuclear cells were collected by centrifugation at 250 g for 10min. The red supernatant was removed and the cell pellets washed 4-6 times with ice cold PBS (50ml 1xPBS, 250 g, 5min) to remove remaining non-lysed erythrocytes. After each washing step, 2-3 cell pellets were pooled. Finally, the pellet consists only of mononuclear blood cells and is almost white. Figure 2.1 shows a comparison between blood cells before and after this procedure. At the end the cell pellet was resuspended in an appropriate amount of 1xPBS. Cell concentration of this mixture was determined and the cells were seeded in proliferation medium at $5x10^6$ cells/ml.

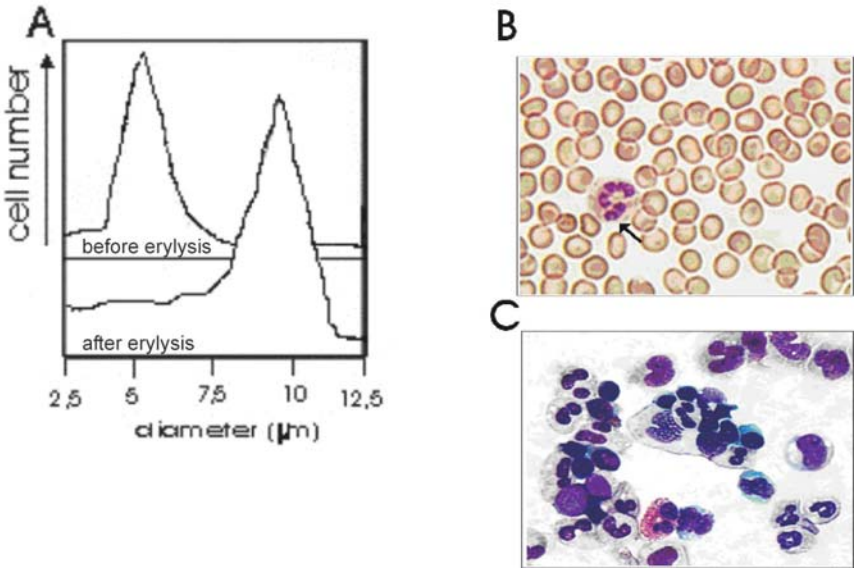


Figure 2.1: Establishment of erythroid mass culture originating from umbilical cord blood

A) Cell profiles with differences in mean cell size of blood cells before and after lysis and removal of erythrocytes.

B) Peripheral blood smear shows mainly erythrocytes and one granulocyte (indicated by an arrow).

C) Cytospin after erylysis shows mononuclear blood cells, i.e. granulocytes, monocytes and lymphocytes.

2.2.2 Determination of cell number & volume

To monitor size and number of cultured cells, they were characterized daily with a CASY^{TM} cell analyzer (Schärfe-System, Reutlingen, Germany). For this purpose, 50µl of cell suspension were diluted 1:100 with CASYton (Schärfe-System, Reutlingen, Germany). An aliquot of 200µl of the mixture was measured. The obtained data were used to control the proliferation of both, primary cells and immortalized cell lines.

2.2.3 Establishment of erythroblast mass cultures

After lysis of erythrocytes, the cell suspension contains different types of mononuclear blood cells, mainly leukocytes (granulocytes, lymphocytes, monocytes) and thrombocytes. Human erythroblasts were expanded from cord and peripheral blood after removing erythrocytes within 10 days in serum free medium (StemSpan, Cell Systems) with following proliferation factors:

- Epo 2 units (U)/ml Sigma-Aldrich
- Dex 1µM Sigma-Aldrich
- Igf1 40ng/ml Sigma-Aldrich
- SCF 100ng/ml R&D Systems
- Lipids 40µg/ml Sigma-Aldrich

For the outgrowth of erythroblasts from peripheral blood two additional factors were added for the first six days:

- human IL-6 10ng/ml Peprotech
- human Flt3-L 50ng/ml Peprotech

For the first 24 hours cells, were cultivated in medium with a double concentration of proliferation factors. During the following days, there is a mixed population of cells. From day 8 on the culture contains a homogenous population consisting predominantly of erythroblasts (Figure 2.1). Proliferation kinetics and size distribution were monitored daily using an electronic cell counter (see “Determination of cell number & volume”). Partial medium changes were performed every 24h to ensure optimal factor supply. This proliferation mix was not only used during initial

outgrowth of erythroid progenitors but also for long time culture. Once an erythroblast culture became visible, cells were cultivated at a density of $2x10^6$ cells/ml at 37°C, 5% CO_2 and 100% humidity.

2.2.4 Freezing erythroid progenitor cells

10-50x10^6 cells were centrifuged at 250 g for 5 minutes. The pellet was re-suspended in 900µl FCS. After that, 900µl of a mixture of FCS and 20% DMSO was added (final concentration of DMSO: 10%). DMSO protects the cell membranes during the freezing procedure. The cells were slowly cooled down by incubation at -80°C in a styrofoam box and subsequently stored in liquid nitrogen.

2.2.5 Thawing erythroid progenitor cells

Frozen cell batches were quickly thawed in a 37°C waterbath. 10ml of medium without any factors or PBS were added drop by drop. After that, another centrifugation step at 250 g for 5 minutes was performed to remove all DMSO, which could be toxic to thawed cells. Cells were cultivated in StemSpan medium containing 2x proliferation factors over night. Subsequently, the culture was handled as during establishment/expansion of erythroid mass cultures (see above).

2.2.6 Induction of terminal differentiation of human erythroid cells

To switch cells from proliferation to differentiation, the medium containing proliferation factors was removed by centrifugation (250 g, 5min). The pellet was washed with ice-cold PBS and resuspended in fresh medium containing differentiation factors:

- Epo 10U/ml Sigma-Aldrich
- Ins $4x10^{-4}$ IE/ml Actrapid HM
- hTransferrin 10mg/ml Sigma-Aldrich
- ZK $3x10^{-6}$M Schering Corp.
- T3 1µM Sigma-Aldrich

Because of size reduction of erythroid cells during differentiation, cells were maintained at a density of 3x10^6 cells per ml in differentiation medium.

2.2.7 Preparation of murine foetal livers

We used wild type foetal livers at E11.5-13.5 as a source for murine erythroid progenitors. Pregnant mice were killed by cervical dislocation. To avoid contamination, the fur was sprinkled with 70% ethanol before the body wall was opened. After exposure of the peritoneum, the uterus was isolated and placed in a tissue culture plate containing ice cold PBS. The embryos were isolated and killed immediately by decapitation. The clearly visible foetal liver was taken after the removal of surrounding tissue and placed in a Stem-Pro-34^{TM} (SFM, Invitrogen Gibco, 10639-011) containing tube. Foetal livers, collected from one pregnant mouse were homogenized by pipetting up and down. The medium was separated from the cells by centrifugation (250 g, 5min) and discarded. The pellet was resuspended with FCS and FCS/20% DMSO as described above ("Freezing of erythroid progenitor cells"). The suspension was filled into cryo-tubes so that every tube contained the equivalent of 1-2 foetal livers and stored like human erythroid cells.

2.2.8 Outgrowth of murine erythroid progenitors

Cells were quickly thawed in a 37° water bath, transferred to a 15ml-tube and 5ml medium or PBS was added. After centrifugation (250 g, 5min) and removal of the supernatant, collected cells were resuspended in medium enriched with growth factors:

- Epo 2U/ml
- SCF 100 µg/ml
- Igf 1 40µg/ml
- Dex 1µM

The cells were seeded into tissue culture dishes and cultured at 37°C with 5% CO_2. Partial medium change was performed every 24h, the cell density was adjusted to 2 million cells/ml.

2.2.9 Induction of terminal differentiation of murine erythroid cells

Before terminal differentiation was induced, cells were purified using density gradient centrifugation. Through this procedure, spontaneously differentiated cells can be removed and a homogenous population of proliferating cells is obtained. After

another washing step with PBS, cells were reseeded ($2x10^6$ cells/ml) in Stem-Pro-34^{TM}, enriched with the following differentiation factors:

- Epo 10U/ml
- Insulin 10µg/ml
- ZK $3x10^{-6}$ µM
- hTransferrin 1mg/ml

Murine erythroid cells were handled like human ones, which corresponds to daily partial media changes and maintenance of a cell-density of 3 million cells/ml.

2.2.10 Ficoll-Hypaque gradient centrifugation

Ficoll-Hypaque (lymphocytes separation medium, Eurobio) gradient centrifugation allows the removal of dead and spontaneously differentiated cells out of the culture. The purification should be done with 10-$20x10^6$ cells. The separation of the cell types takes place on different sedimentation density levels. Ficoll inserts into the cell membrane of dead and differentiated cells. Because of that, these cells sink to the bottom of the tube during centrifugation.
The cell-suspension was transferred into a tube. 2ml Ficoll were layered under the medium. The gradient was centrifuged at 600 g for 7 minutes. Immature erythroblasts accumulating at the inter-phase between Ficoll and medium were collected. Dead and differentiated cells formed a pellet at the bottom of the tube. Erythroblasts were transferred into another tube, counted and washed with 1x PBS to remove Ficoll, centrifuged and cultivated as described.

2.2.11 Photometric haem determination

A reaction of O-phenylene-diamine dihydrochloride (Sigma-Aldrich) with haem is used to determine haem levels in erythroid cells (Kowenz, Leutz et al. 1987).
3x50µl of cultured cells were transferred individually into 96well plates, centrifuged at 250 g for 5 minutes and washed once with 100µl ice-cold 1x PBS. The pellet was re-suspended in H_2O for cell lyses. The plates were stored at -20°C until further processing.
After thawing, lysates were incubated with 125µl haemoglobin-assay-dye-solution for three minutes. During incubation, the colour of the solution turns from clear to yellow. The reaction was stopped with 25µl 8N H_2SO_4. Haemoglobin concentration

was measured at a wavelength of 492nm using a 620nm filter as reference wavelength. For normalization of the haemoglobin levels, the obtained ODs were divided by cell-number and –volume. Relative haem levels were determined by using diluted chicken blood (1:3000) as standard.

2.2.12 Histological staining of erythroid cells with benzidine

To assess the condition of erythroid cells during different developmental stages, cells were centrifuged onto glass slides (150 g, 7min) and stained with histological dyes: DIFF red and blue (Fisher Scientific) for differentiation and O-diamisidine Meton (benzidine) for haemoglobin.

Working step	Time
Incubation in methanol	4min
Incubation in 1% benzidine solution	2min
Incubation in H_20_2 solution	1.5min
Washing in H_20	0.5min
Stain with Diff Quick Red (I)	4min
Stain with Diff Quick Blue (II)	40sec
Rinse in water	
Dry with hair dryer or by air	

The stain was covered with mounting media (Entellan®, Merck) and an appropriate cover slip.

2.3 Working with proteins

To analyse changes in gene expression on the protein level, Western blot analysis (Burnette 1981) was performed. For this purpose, cell extracts of proliferating and differentiating erythroid cells were used. 5-10x10^6 cells were pelleted, washed once in 1x PBS, shock frozen in liquid nitrogen and subsequently stored at -80°C.

2.3.1 Total protein extract

Depending on the size of the pellet, 20-50µl NP-40 lysis buffer was added. The solution was incubated for 3 minutes, vortexed and centrifuged for 2 minutes at 14.000 g. The supernatant was taken for Western blot analysis and Bradford protein assay.

2.3.2 Protein content determination with Bradford protein assay

To measure protein concentration, 2µl of protein extract were added to 1 ml of Bradford solution (BioRad) diluted 1:5 in H_2O. The mixture was set up in a plastic cuvette. The extinction at 595nm was measured against a blank only containing diluted Bradford solution. Protein concentration can be calculated using the formula:

OD595nm 8.65 = Xµg/µl protein

2.3.3 Protein content determination with SDS-Lowry

The advantage of this assay is the usage of SDS: SDS separates membrane proteins from contaminating membrane constituents and denatures the proteins, resulting in better reproducibility.

All glassware was washed with distilled water to remove detergents. A standard curve was prepared by adding 0, 5, 10, 15, 20 and 25µl of 0.1% BSA in water to the test tubes. 1ml of solution A was added to the cell extracts. The extracts were vortexed and incubated for 15 minutes at room temperature. After the addition of 100µl of solution B, the mixture was immediately vortexed and incubated for 30 minutes at room temperature. The extinction was measured at 650nm.

2.3.4 Western blot analysis

SDS gel electrophoresis

Generally, a SDS-polyacrylamide gel consists of two parts: A lower part (resolving gel) and an upper part (stacking gel). The concentration of acrylamide of a resolving gel depends on the respective protein size. For resolving proteins between 30 and 80kD, a 12% gel was used. For larger proteins, as for example SPTB, the percentage of acrylamide was decreased to 6%.

The cell extracts were mixed with 5x protein loading buffer and incubated at 95°C for five minutes to denature the proteins. The extracts were then separated on SDS-polyacrylamide gels with 100-150V at 4°C for 1-1.5h.

Western transfer

Subsequently, protein was transferred to nitrocellulose membranes (0.2µm Protran, Schleicher & Schuell) with 250mA for 2 hours in Harlow transfer buffer at 4°C. Subsequently, membranes were stained in Ponceau S (Serva) for about 10 minutes. The resulting stain was scanned to assess equal protein loading.

Antibody incubation

To block unspecific antibody binding to the nitrocellulose membrane, it was incubated with 5% milk powder in 1x TBS containing 0.1% Tween-20 (TBS-T) for 1h at room temperature. The first antibody (Table 2.1) was diluted to the appropriate concentration and added to the membrane and incubated overnight (o/n) at 4°C. After 3 washing steps with 10ml TBS-T for 10 minutes each, the membrane was incubated with the appropriate second antibody (either for immuno-detection or infrared imaging) for 1h at room temperature. This was followed by another three washing steps with 1x TBS-T.

Table 2.1: Concentrations of Western Blot Antibodies

1st antibody	2nd antibody	Block solution	Wash solution
Spectrin-ß 1:10.000	Anti mouse 1:10.000	TBS, 5% dry low fat milk	TBS 0.2% Tween
Caspase 7 1:5.000	Anti rabbit 1:10.000	TBS, 5% dry low fat milk	TBS 0.2% Tween
eIF4E 1:10.000	Anti rabbit 1:10.000	TBS, 5% dry low fat milk	TBS 0.2% Tween

Immuno-detection by chemoluminescence

The membrane was transferred into a transparent plastic foil and incubated for one minute in equal amounts of enhanced chemoluminescence (ECL) solution 1 and 2 (PerkinElmer). A light sensitive X-ray film (Fuji) was put in an exposure box together

with the membrane for appropriate time periods (20sec - 10min). The film was developed using a Curix 60 unit (Agfa).

Protein quantification with infrared imaging

The membrane was scanned on an Odyssey Infrared Imaging System. For quantification, Odyssey 2.1 software was used.

2.4 Working with Bacteria

2.4.1 Generation of chemically competent E. coli

E. coli (Stable 3; Invitrogen) were grown as overnight 3ml culture in LB medium at 37°C. 500ml SOB++ medium were added and the bacteria-culture was incubated at 30°C until the absorbance at 600nm was approximately 0.5 (between 0.4 and 0.6). When the proper optical density was reached, the culture was chilled on ice for 30 minutes. Bacteria were centrifuged in a pre-cooled Sorvall GSA rotor (4.500rpm, 10min, 4°C). The supernatant was decanted and the pellet gently resuspended in 100ml ice-cold TB buffer. The suspension was incubated for 30 minutes on ice, centrifuged in a pre-cooled Sorvall SS-34 rotor (3.000 rpm, 10min, 4°C) and resuspended in 18.6ml ice cold TB buffer. After that, 1.4ml DMSO were added. The cell suspension was incubated overnight on ice and subsequently aliquoted into sterile, pre-cooled Eppendorf tubes (400µl per tube) and shock frozen in liquid nitrogen. Chemically competent bacteria were stored at -80°C.

2.4.2 Transformation of competent bacteria (heat shock)

200µl chemically competent bacteria were thawed on ice and 10µl ligation solution added. After an incubation time of 30 minutes on ice, heat shock transformation was performed: The bacteria-ligation mixture was put into a 42°C warm water bath for 45 seconds followed by chilling on ice for 2 minutes. 1ml SOB medium was added and bacteria incubated for 45 minutes with shaking at 37°C. The transformed bacteria were plated onto LB plates with appropriate antibiotics and incubated overnight at 37°C.

2.5 Working with DNA & RNA

2.5.1 Preparation of plasmid DNA

A single bacterial colony was picked, inoculated into a starter-culture of 3ml LB containing the appropriate antibiotics and incubated over night at 37°C with shaking.
For midipreps, the starter-culture was used to inoculate a 50ml main culture (LB + antibiotics), again grown over night at 37°C. Midipreps were worked up according to the QIAfilter® Midiprep Handbook (Qiagen).
If a Miniprep was performed, 1.5ml of the overnight-culture were transferred into a 2ml tube, centrifuged and further processed as described in the QuickLyse Miniprep Kit-Handbook (Qiagen).

2.5.2 DNA Precipitation

For removal of protein contaminations, the DNA solution was mixed with an equal volume of phenol (= TRIS-saturated phenol-chloroform-iso-amylalcohol), vortexed and centrifuged for 2 minutes at 12.000 rpm at 4°C. The supernatant was transferred to a fresh tube. Aspiration of the interlayer or organic phase was avoided. To remove phenol, an equal volume of chloroform was added to the phenol/DNA mixture. The mixture was vortexed and centrifuged for 2 minutes at 12.000 rpm at 4°C. The supernatant was transferred to a fresh tube. 0.1 volume of 3M sodium acetate and 2.5 volumes of 100% ethanol were added. After vortexing, precipitation was performed either at -20°C overnight or -80°C for one hour. DNA was pelleted via centrifugation (20min, 12.000 rpm, 4°C) and washed with 1ml ice-cold 70% ethanol. The DNA pellet was air-dried for 10 minutes at room temperature, dissolved in H_20 and stored at -20°C.

2.5.3 Enzymatic reactions

2.5.3.1 DNA digestion with restriction endonucleases

DNA-digestions were performed with restriction enzymes and their recommended reaction buffers (New England Biolabs). Usually, 1µg of DNA was cut with 5U enzyme at 37°C for 1-2h in a total volume of 20-100µl. The resulting DNA was precipitated in 96% EtOH, washed with 70% EtOH and then resolved in H_2O (all reagents RNase free).

2.5.3.2 Ligation of DNA Fragments with T4 Ligase

For ligation of DNA fragments, T4 ligase (Invitrogen) and the supplied 5x buffer were used. In general, a molar "vector to insert" ratio between 1:3 and 1:6, estimated from agarose gel electrophoresis, was applied. The total DNA amount was kept between 10-50 ng for sticky end ligation. DNA was mixed with T4 5x buffer and 1µl (5U) of T4 ligase enzyme in a total volume of 20µl dH2O. The reaction was incubated overnight at 16°C.

2.5.4 Agarose Gel Eectrophoresis

Agarose gels with 0.8-2% agarose in 1x TAE were prepared depending on the size of DNA fragments of interest. Agarose was melted in a microwave oven, cooled down, mixed with 0.5 mg/l ethidium bromide and poured into a gel tray. Prior to loading, the DNA solution was mixed with 6x DNA loading buffer and separated at 2-8 V/cm.

2.5.5 Excision and purification of DNA from agarose gels

The DNA band of interest was excised with a clean scalpel under low-intensity UV light to avoid DNA damage and placed into a 1.5ml microcentrifuge tube. For DNA purification, Wizard® SV Gel and PCR Clean-Up System (Promega) was used. Excision and purification of DNA was performed according to the manufacturer's guidelines.

2.6 Working with lentiviral particles

The used system for lentivirus production belongs to the 2nd safety generation and was generated at the Ecole Polytechnique Fédérale de Lausanne (Wiznerowicz and Trono 2003) and distributed by Addgene. 2nd generation vectors have a deletion of the packaging sequence and furthermore also of the 3'LTR. Because of that, two recombination events would have to take place simultaneously to generate a competent, "wild-type-like" virus.

Therefore, three different vectors (Figures 2.1 and 2.2) are needed to produce viral particles: One, encoding Gag, for packaging (psPAX2), one envelope vector (pMD2.G) and of course a target vector (pLVTHM).

The mammalian vector pLVTHM allows direct cloning of shRNA (short hairpin RNA). For this purpose, the plasmid contains a single MluI site, which was used together with the ClaI site for inserting of shRNA-constructs (List 2.1). These constructs were designed with the Promega siRNA Target Designer (http://www.promega.com/siRNADesigner/program/), checked for target-specificity via blast search and cloned into pLVTHM using the recommended restriction sites (MluI and ClaI). Restriction enzymes were obtained from New England Biolabs.

2.6.1 Oligo annealing

The corresponding forward and reverse DNA-oligonucleotides (Eurofins MWG GmbH) were dissolved in water to a concentration of 0.5µg/µl. 1µg of each oligonucleotide was added to the annealing buffer to a final volume of 20µl. After incubation at 80°C for 1 hour and slow cooling, annealed oligonucleotides were stored at -20°C.

2.6.2 Production of lentiviral particles using calcium phosphate transfection

24 hours before transfection, $3x10^5$ 293T or 293FT cells were seeded on six-well plates to reach about 70% confluency the next day. Two hours before transfection, medium was replaced by 2ml fresh preheated medium.

For each well the following transfection mix was prepared:

6.7µg pLVTHM (target vector)
2µg pMD2G (envelope plasmid)
5µg psPAX2 (packaging plasmid)
20µl Ca_2PO_4
H_20 to a final volume of 167µl

167µl 2x HBS were transferred into an Eppendorf tube. Transfection mix was added drop-wise to 2x HBS, simultaneously HBS was bubbled using a pipette boy with an extracted Pasteur pipette. After 5 minutes of incubation at room temperature, the HBS-DNA-mixture was added drop-wise to the cells and the plate rotated gently.
16 hours after transfection, medium was changed. Transfection efficiency was determined by fluorescence microscopy (pLVTHM contains GFP). Supernatants were harvested every 12 hours, usually 2 or 3 times, and pooled. The lentivirus-

containing supernatant was centrifuged at 300 g for 5 minutes to remove cell debris. The cleared supernatants were either used directly or stored at -80°C.

2.6.3 Production of lentiviral particles with Lipofectamine™

24 hours before transfection, cells were seeded into six-well plates to reach 60-80% confluency the next day, using antibiotic-free medium. 10µl Lipofectamine™ (Invitrogen) were mixed with 250µl OptiMEM medium (Invitrogen) and incubated for 5min at room temperature. For each transfection, 4µg DNA (2µg pLVTHM, 0.5µg pMD2.G and 1.5µg psPAX2) were mixed gently with 250µl Optimem and added to the Lipofectamine™ mix. After 20 minutes of incubation at room temperature, the DNA-Lipofectamine™ mixture was added drop-wise to the cells, followed by gentle rocking of the plates. The transfection medium was removed after about 16 hours of incubation and exchanged by cell culture medium containing antibiotics. Determination of transfection efficiency, harvesting and storage of the lentivirus-supernatant was performed as described above.

2.6.4 Lentiviral infection of human cells using polybrene

Adherent cells were seeded at the day before the infection into six-well plates, erythroid cells were centrifuged (250 g, 5min) and the supernatant removed. Lentiviral supernatant was thawed in a 37°C water bath and added to the adherent cells. Erythroid cells were resuspended in 300µl virus-supernatant, additionally 100µl of stem span medium with 4-fold growth factor concentration was added.
Cells were incubated for 12 hours in the lentiviral supernatant. Polybrene (5µg/ml, Sigma-Aldrich) was added to the virus supernatant to allow the viral glycoproteins to bind more efficiently to their receptor on the cell surface. After incubation, the supernatant was removed and changed to the appropriate standard culture medium.

2.6.5 Lentiviral infection of human cells using RetroNectin®

The use of RetroNectin® has the advantage that contaminating substances from the culture, which could affect the infection negatively, are removed from the virus solution.

Preparing of RetroNectin® coated plates:

RetroNectin® lyophilized powder was dissolved in water by gentle swirling to a concentration of 1 mg protein/ml. The solution was filtered through a 0.22µl filter and stored at -20°C. Prior to coating, the solution was diluted with sterilized PBS to a concentration of 50µg Retronectin®/ml.

2ml of diluted sterile RetroNectin® solution were dispensed into each well of a six-well plate. The covered plate was allowed to stand for 2 hours. After this incubation, the RetroNectin® solution was removed and 2 ml of sterile PBS containing 2% bovine serum albumin (BSA) was added to each well for blocking. The BSA solution was removed after 30 minutes of incubation at room temperature and the wells were washed once with PBS.

Gene transduction with the RetroNectin®bound virus (RBV) infection method

Lentiviral supernatant was pre-loaded onto RetroNectin® coated plates and incubated for 5 hours at 37°C in a 5% CO_2 incubator to promote attachment of virus particles to RetroNectin®. Virus-depleted supernatant was discarded and the plate washed with PBS to further remove undesirable substances originating from the supernatant.

Viral infection

Target cells were collected, counted, and suspended in growth medium at a concentration of $1x10^5$ cells/ml. The suspension was added to the RetroNectin® coated plate immediately after removing of PBS. Cells were incubated for 2 days with 2x factor proliferation medium.

2.6.6 Fixation of lentiviral-infected cells for flow cytometry

$1\text{-}5x10^{5-}$cells were centrifuged at 250 g for 5 minutes, resuspended in 1ml of 4% para-formaldehyde (PFA) in 1x PBS and incubated for 30 minutes at room temperature. For FACS analysis, cells were centrifuged again (250 g, 5 min), washed once with PBS and resuspended in 500µl 1x PBS/FCS and transferred to FACS-tubes. Quantification of GFP-positive cells was performed as described.

2.7 Manipulation of human serum

2.7.1 Dialysis

Regenerated cellulose tubular membrane (ZelluTrans Roth) was cut into 15cm long pieces and boiled for 10 minutes in 1 liter 2% sodium bicarbonate with 1mM EDTA (pH 8). The tubing was rinsed thoroughly in distilled water and boiled again for 10 minutes in 1l 1mM EDTA (pH 8). The tubing pieces were washed with distilled water inside and outside and used immediately.
Human serum was dialysed for 72 hours against PBS. PBS was changed every 12 hours.

2.7.2 Fractionating of human serum using Vivaspin tubes

Serum was transferred to Vivaspin tubes with a molecular cut-off of 30kD and 50kD and centrifuged for 30 minutes at 4.000 g. For differentiation assays, only the upper fraction was used.

3 Material

3.1 Buffers & other solutions

Solution	Ingredients	Additional information
100x haemin	65mg haemin (Sigma-Aldrich) 10ml 0,2M KOH 1ml 0,2M Tris (pH 7,8) adjust pH to 7.8 with 1N HCl	adjust pH to 7.8 with 1N HCl long-time-storage: -20°C short-time-storage: 4°C
10x Erylysisbuffer	89.9g ammonium chloride 10g $KHCO_3$ 0.37g EDTA 1000ml H_20	adjust pH to 7.3 autoclave, dilute 1:10 with H_20 and filter sterilize store at room temperature
10x PBS	80g NaCl 2g KCl 11.5g $Na_2HPO_4.2H_2O$	H_2O ad 1000ml store at room temperature
1000x 3,3',5-triiodo-L-tyronine	6.7µg tri-iodo-thyronine (Sigma-Aldrich) 1ml 25mM NaOH	dilute 1:1 with H_20 and filter sterilize long time storage: -80°C short time storage: 4°C
haem assay dye solution	1 volume 0.1M citric acid 1 volume 0.1M Na_2HPO_4 0.5mg/ml o-phenylene-diamine-dihydrochloride (DAPI, Merck) 1µl/ml H_2O_2 8N H_2SO_4 1 volume concentrated H_2SO_4 3.6 volumes of H_20	

Benzidine staining solutions		
H_20_2 solution	75ml EtOH 75ml H_2O 4,4ml H_2O_2	
1% O-diamisidine Meton (benzidine)	1g O-diamisidine Meton 100ml dH_20	store light protected at -20°C

50x TAE (pH 8.5)	424g Tris Base 57.1ml glacial acetic acid 100ml 0.5M EDTA pH8	
Oligo Annealing buffer	15mM Tris (pH 8) 0.5M NaCl	dissolve in dH_20
NP-40 Lysis Buffer	25mM Hepes (pH 7.5) 25mM Tris-HCl (pH 7.5) 150mM NaCl 10mM EDTA 0.1% Tween20 0.5% NP-40 10mM Beta-Glycerophosphate	

SDS-Lowry solutions		
Solution A	20ml 2% Na_2CO_3 20ml 1% $CuSO_4$ 50ml 20% SDS	on the day of the assay: add 2% Na_2CO_3
Solution B	Folin diluted with H_20 at a 1:1 ratio	

Western blot solutions		
resolving gel	2.5ml 1.5M Tris (pH 8.8) 1.3-5ml 30% acryl amide 50µl 20% SDS 50µl 10% APS (fresh) 5µl TEMED	dH_2O ad 10ml (the amount of acryl amide depends on the respective protein size)
stacking gel	3ml H_2O 1.25ml 0.5M Tris (pH 6.8) 650µl 30% acryl amide 25µl 20% SDS 25µl 10% APS (fresh) 5µl TEMED	
30% acryl amide	150g acryl amide 4g N,N-methylen-bis-acryl amide	H_2O ad 500ml
5x SDS electrophoresis Buffer	30.2g Tris 188g glycin 1.800ml dH_2O 100ml 10% SDS	H_2O ad 2.000ml
1x Harlow transfer buffer	5.8g Tris 29g glycin 1.600ml H2O 400ml methanol	Harlow transfer buffer can be re used up to 3 times store at 4°C
10x Ponceau	2g Ponceau S 30g tri-chlor-acetic acid 30g sulfosalicylic acid	H_2O ad 100ml
10x TBS	100ml 1M Tris (pH 8) 300ml 5M NaCl	H_2O ad 600ml
TBS-T	500ml 1x TBS 2.5ml 20% Tween20	

LB medium	20g/l bacto-tryptone 5g/l yeast extract 5g/l NaCl	
TB buffer	10mM HEPES (pH 6.7) 15mM CaCl2 250mM KCl adjust pH to 6.7 with KOH 55mM MnCl2	filter sterilize the mixture using 0.22µl filter
SOB++ medium	20g/l bacto-tryptone 5g/l yeast extract 0.5g/l NaCl 0.186g/l KCl 10mM MgCl2 10mM MgSO4	

3.2 shRNA constructs

3.2.1 shRNA constructs for caspase 7

casp7_01-a (s)
CGCGT CCCC **GGGCAAATGCATCATAATA** TTCAAGAGA **TATTATGATGCATTTGCCC** TTTTT GGAA AT
casp7_01-b(as)
CGA TTTCCAAAAA **CCCGTTTACGTAGTATTA**T AAGTTCTCT **ATAATACTACGTAAACGGG** GGGGA

casp7_02-a (s)
CGCGT CCCC **GGGCAAATGCATCATAATA** TTCAAGAGA **TATTATGATGCATTTGCCC** TTTTT GGAA AT
casp7_02-6(as)
CGA TTTCCAAAAA **CCCGTTTACGTAGTATTA**T AAGTTCTCT **ATAATACTACGTAAACGGG** GGGGA

casp7_03-a (s)
CGCGT CCCC **GGGCAAATGCATCATAATA** TTCAAGAGA **TATTATGATGCATTTGCCC** TTTTT GGAA AT
casp7_03-b(as)
CGA TTTCCAAAAA **CCCGTTTACGTAGTATTAT** AAGTTCTCT **ATAATACTACGTAAACGGG** GGGGA

casp7_04-a (s)
CGCGT CCCC **GGGCAAATGCATCATAAT**A TTCAAGAGA **TATTATGATGCATTTGCCC** TTTTT GGAA AT
casp7_04-b(as)
CGA TTTCCAAAAA **CTCACTCGACTCTGTCTAT** AAGTTCTCT **ATAGACAGAGTCGAGTGAG** GGGGA
casp7_05-a (s)
CGCGT CCCC **GTCTGTTACCTTGTTAATA** TTCAAGAGA **TATTAACAAGGTAACAGAC** TTTTT GGAA AT
casp7_05-b(as)
CGA TTTCCAAAAA **CAGACAATGGAACAATTAT** AAGTTCTCT **ATAATTGTTCCATTGTCTG** GGGGA

c7_scramb-a
CGCGT CCCC **ACTGTCTAACGTTTTATT** TTCAAGAGA **AATAAAACGTTAGACAGTC** TTTTT GGAA AT
c7_scramb-b
CGA TTTCCAAAAA **TGACAGATTGCAAAATAA** AAGTTCTCT **TTATTTTGCAATCTGTCAG** GGGGA

3.2.2 shRNA constructs for spectrin ß

spec-ß_01-a (s)
CGCGT CCCC **GCCGCATCACCGATCTCTA** TTCAAGAGA **TAGAGATCGGTGATGCGGC** TTTTT GGAA AT
spec-ß_01-b(as)
CGA TTTCCAAAAA **CGGCGTAGTGGCTAGAGAT** AAGTTCTCT **ATCTCTAGCCACTACGCCG** GGGGA

spec-ß_02-a (s)
CGCGT CCCC **GACTGAGAAGATGATTGAA** TTCAAGAGA **TTCAATCATCTTCTCAGTC** TTTTT GGAA AT
spec-ß_02-b(as)
CGA TTTCCAAAAA **CTGACTCTTCTACTAACTT** AAGTTCTCT **AAGTTAGTAGAAGAGTCAG** GGGGA

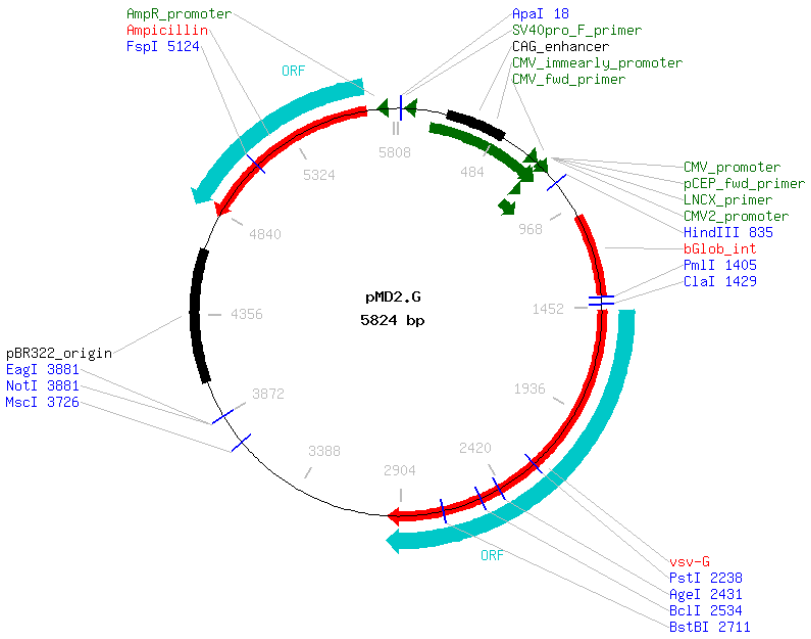
spec-ß_03-a (s)
CGCGT CCCC **GTGTACACCTGGAGAACAT** TTCAAGAGA **ATGTTCTCCAGGTGTACAC** TTTTT GGAA AT
spec-ß_03-b(as)
CGA TTTCCAAAAA **CACATGTGGACCTCTTGTA** AAGTTCTCT **TACAAGAGGTCCACATGTG** GGGGA

spec-ß_04-a (s)
CGCGT CCCC **GCAGGCTACCCTCATGTTA** TTCAAGAGA **TAACATGAGGGTAGCCTGC** TTTTT GGAA AT
spec-ß_04-b(as)
CGA TTTCCAAAAA **CGTCCGATGGGAGTACAAT** AAGTTCTCT **ATTGTACTCCCATCGGACG** GGGGA

spec-ß_05-a (s)
CGCGT CCCC **GCATCACCGATCTCTACAA** TTCAAGAGA **TTGTAGAGATCGGTGATGC** TTTTT GGAA AT
spec-ß_05-b(as)
CGA TTTCCAAAAA **CGTAGTGGCTAGAGATGTT** AAGTTCTCT **AACATCTCTAGCCACTACG** GGGGA

spec_scramb-a
CGCGT CCCC **GCATCACCGATCTCTACAA** TTCAAGAGA **TTGTAGAGATCGGTGATGC** TTTTT GGAA AT
spec_scramb-b
CGA TTTCCAAAAA **CGTAGTGGCTAGAGATGTT** AAGTTCTCT **AACATCTCTAGCCACTACG** GGGGA

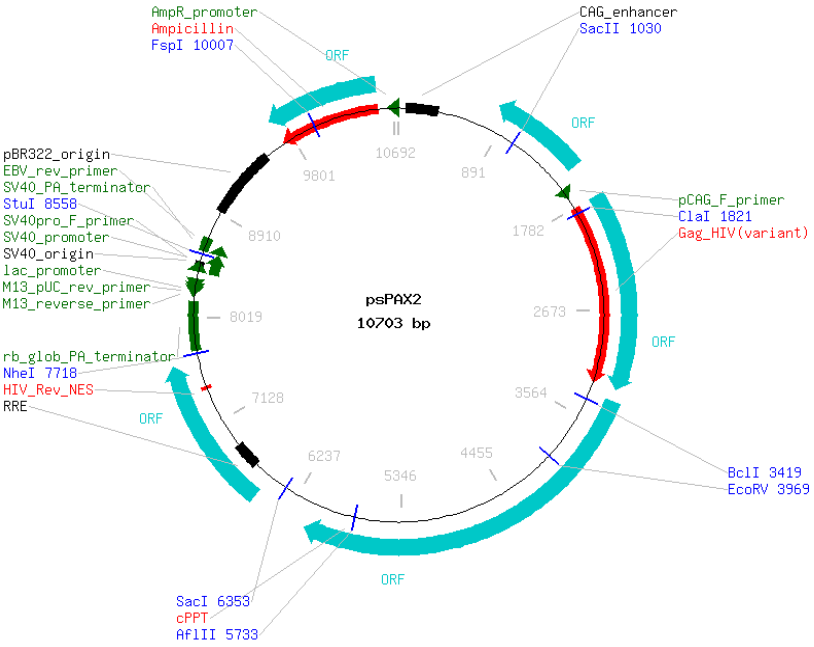
3.3 Maps of used plasmids



Maps taken from: www.addgene.org

Figure 3.1: Map of pMD2.G

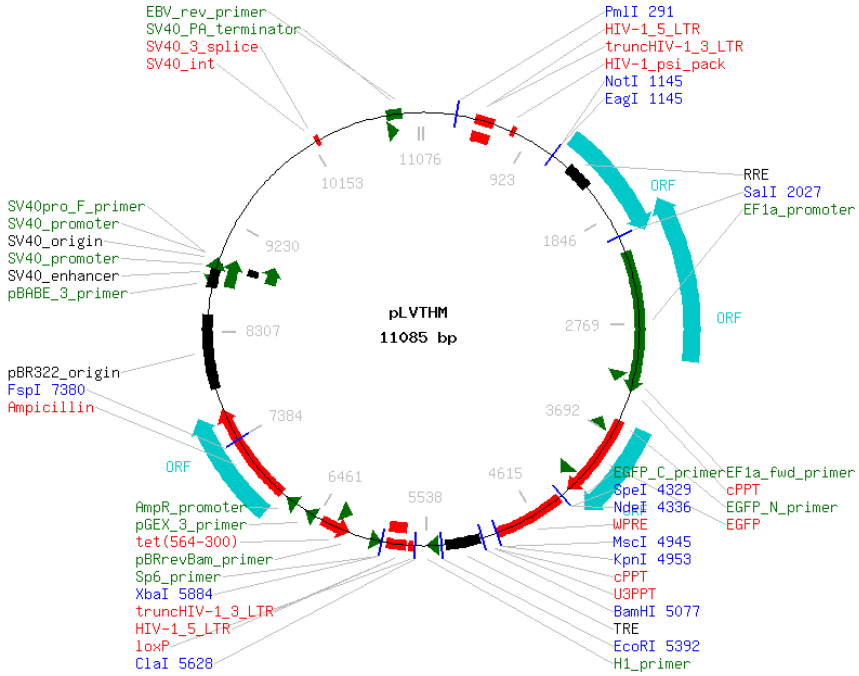
pMD2.G is needed to envelop the virus DNA in the producer cells (envelope plasmid)



Map taken from: www.addgene.org

Figure 3.2: Map of psPAX2

psPAX2 is a high-copy 2nd generation packaging vector recommended for lentivirus production.



Map taken from: www.addgene.org

Figure 3.3: Map of pLVTHM

The vector pLVTHM contains two ClaI sites: one is used for direct cloning of shRNA, the other one is blocked via Dam methylation. Because of that it is necessary to use Dam+ bacteria strains for plasmid propagation.

4 Results

4.1 Outgrowth & proliferation of erythroid progenitors

In this diploma thesis, outgrowth, proliferation and differentiation of erythroid progenitors were carried out in the specific serum-free media StemSpan (for human cells) and Stem-Pro-34 (for murine cells). Sources for erythroid progenitors were the murine foetal liver as well as human umbilical cord and unmobilized peripheral blood. The performance of the progenitor cells differ, depending on their origin (Figure 4.1.1 & 4.1.2). Whereas the culture system of human cells starts with the removal of erythrocytes to obtain mononuclear cells, murine foetal liver cells require no further preparation after their harvest and homogenization in medium. During the first days of cultivation, however, murine cultures have a tendency to differentiate spontaneously. To obtain a homogenous immature erythroblast cell population, a purification step is needed. In the human system such a purification step is not required and establishment of a homogenous erythroblast population occurs simply by outgrowth of progenitors triggered with the appropriate growth factors. As peripheral blood contains much fewer CD34+ cells than cord blood, the yield of erythroblasts proportional to the used amount of blood is smaller. To improve the outgrowth of erythroid progenitors from peripheral blood, cultures receive Flt3 and IL-6 for the first 6 days in addition to the other factors. Casy cell counter size profiles and cytospins illustrated the differences in outgrowth and composition of the different culture types (4.1.2.B). Due to the absence of haemoglobin in proliferating cells, the cytosol appears blue to violet after staining. Red-brownish staining is the result of a reaction of benzidine with haemoglobin. The cytospins of murine foetal liver cells after 4 days in culture show haemoglobinized cells, which were removed by gradient centrifugation. After this purification step, the cultures consisted almost exclusively of proliferating erythroblast (Figure 3.1).

During proliferation, DNA has to be replicated incessantly. Therefore the nucleus is relatively large. This could be observed in human as well as murine erythroid cells. Casy-profiles illustrate the differences in cell size of murine versus human immature erythroblasts: murine cells (approx. 9µm diameter) are smaller than human ones (approx. 11µm).

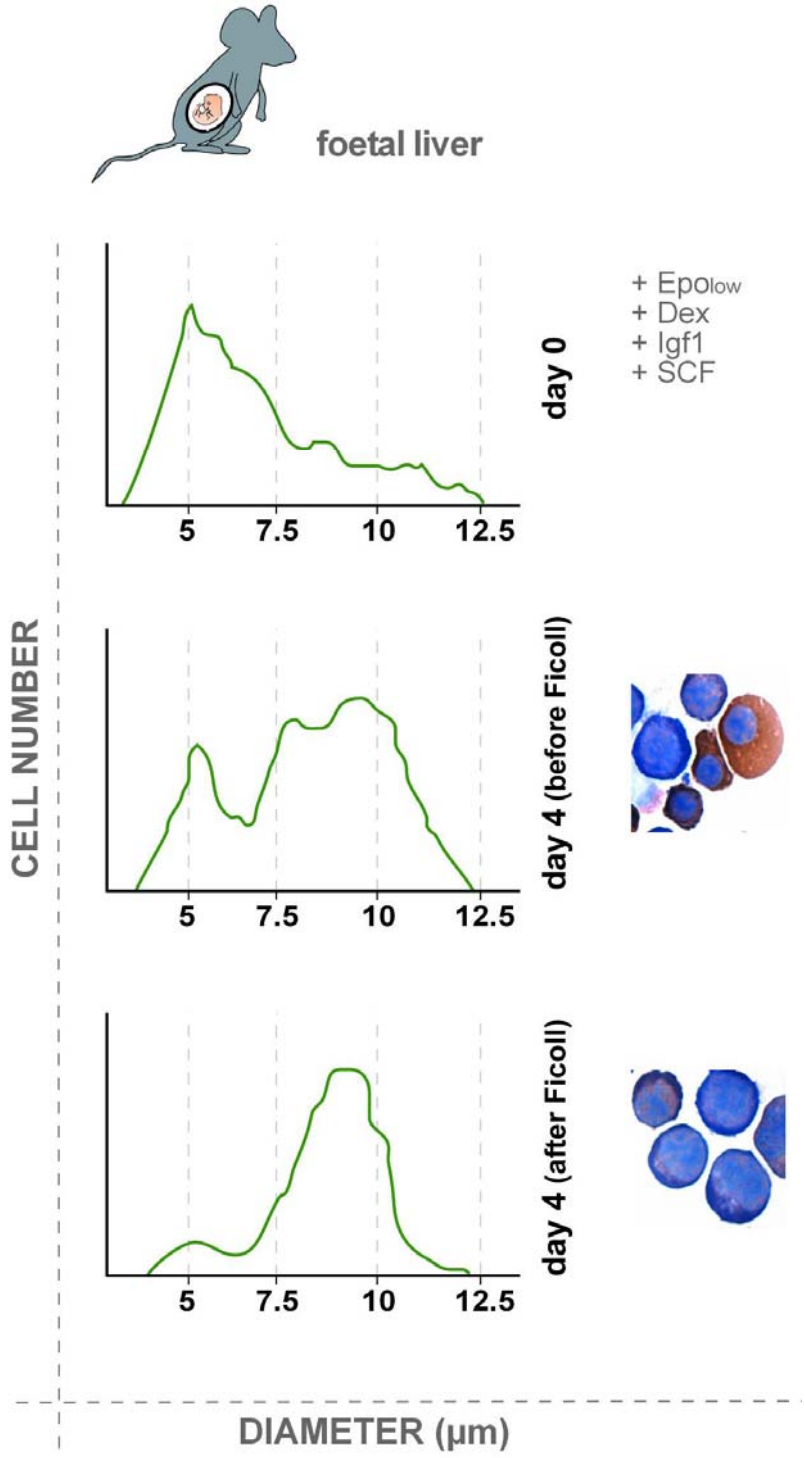


Figure 4.1: Establishment of murine erythroid mass cultures

Cells were cultivated as described in “Methods”. Cell numbers and volumes were determined daily with an electronic cell analyser. During the first days, the cultures are heterogenous. After 4 days, the amount of erythroblasts had increased significantly in the culture. Spontaneously differentiated cells were removed by density gradient centrifugation. Cytospins were stained with benzidine to reveal haemoglobin.

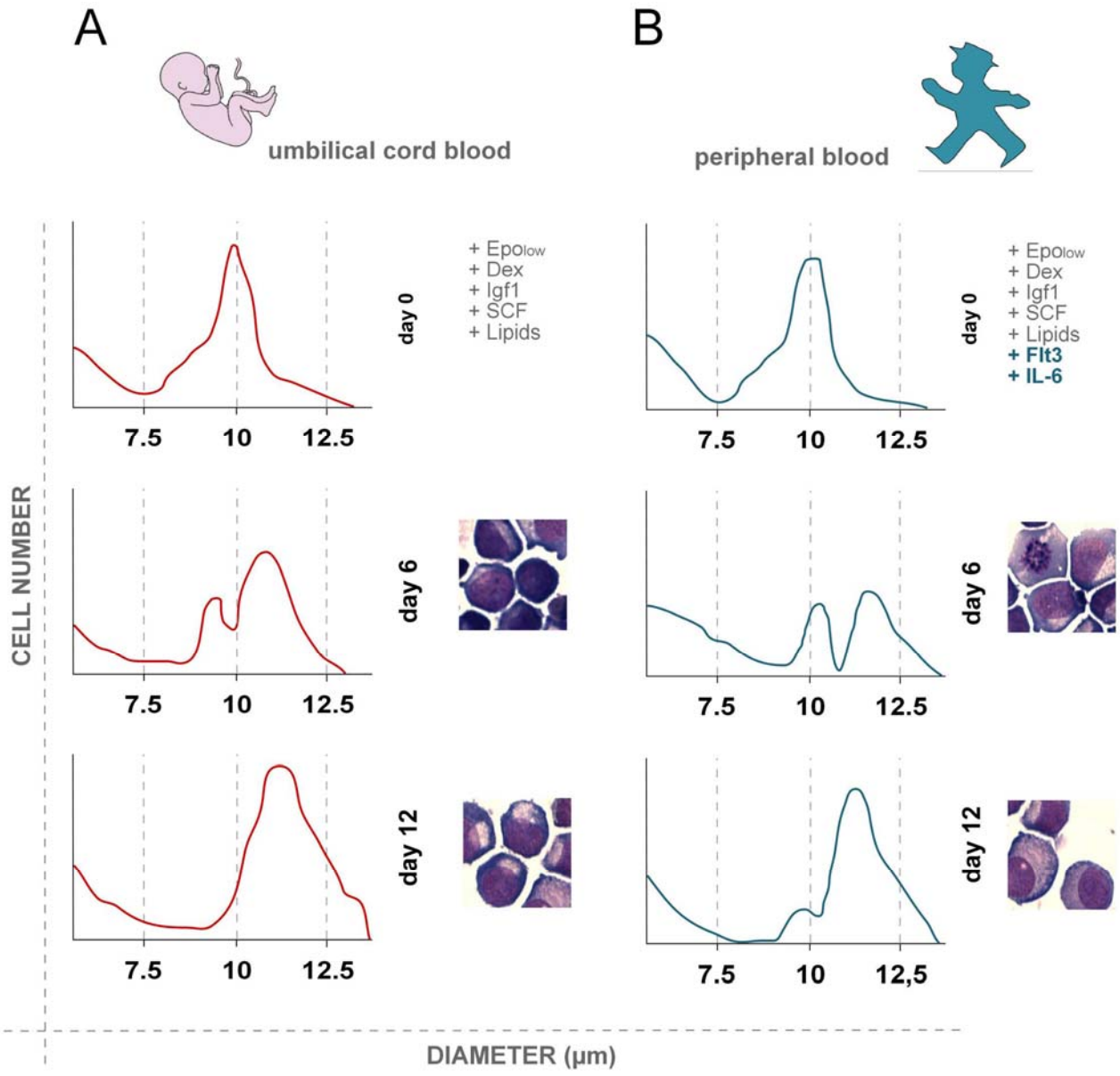


Figure 4.2: Establishment of human erythroid mass culture

Cell volume changes in cord blood derived (A) and peripheral blood (B) derived erythroid cell culture were monitored daily with an electronic cell analyser. Cells were cyto-centrifuged onto glass slides and stained with cytological dyes. During the first few days, multiple cell types like macrophages, leukocytes and only few erythroblasts are present. The proportion of the latter increases upon cultivation in specific erythroid factors (Epo, Dex, SCF, and IGF-1). At day 12, the majority of cells in the rapidly proliferating cultures shows an erythroblast phenotype.

4.2 The effect of testosterone on human erythroblasts

To check if there is any effect of steroid hormones on erythroid cells in our culture system, dihydro-testosterone (DHT) and progesterone were added to proliferating erythroblasts (Figure 3.1). Both hormones were added to the cells at a concentration of 1µM. The erythroid cells used had already been in culture for at least 10 days before starting the addition of hormones.

Erythroblasts expanded from male donors showed no difference in their proliferation rate after treatment with DHT or progesterone compared to the negative control. The calculation of cumulative cell numbers from female-derived erythroblasts showed no response to steroid treatment during the first five days of the experiment. From about day 6 (culture day 16) on, a proliferation-promoting effect of DHT was observed in female erythroblasts.

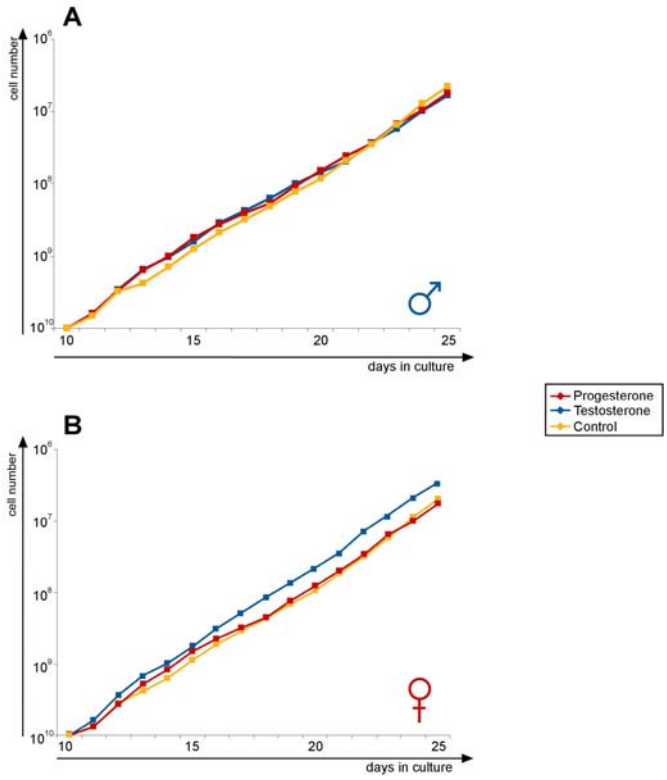


Figure 4.3: The influence of steroid hormones on human erythroid proliferation

Cell numbers from cultures of male (A) versus female (B) cord blood donors (pre-grown for 10 days in the absence of sex steroids) were counted daily in an electronic cell counter and cumulative cell numbers were determined. Cells were cultivated either under standard proliferation conditions (yellow), or in the presence of 1 µM testosterone (blue) or in the presence of 1µM progesterone (red).

4.3 Terminal differentiation of erythroid progenitors

Differentiation was induced in human cells on culture day 14 and lasted 6 days (Figure 3.2 – upper & middle panel). During this time, cells had shrunk in size from about 11 to 7.5µm (Figure 3.2.A). This size reduction was also observed on cytospins, which illustrated furthermore the condensation of the nuclei (Figure 3.2.B). After 72 hours of differentiation, human erythroid cells were already massively haemoglobinized. The increase of the haem synthesis rate was not linear and reached its maximum after a rapid progression between 69 and 120 hours of differentiation (Figure 3.2.C).

The same trends were observed in differentiating murine erythroblasts (Figure 3.2.A – bottom panel). Benzidine staining of cytospins (Figure 3.2.B) clearly illustrated the accumulation of haemoglobin during the process of maturation, indicated by the red-brownish staining of the cytosol. Besides haemoglobin production, morphological changes take place as well. The nuclei of proliferating cells are relatively large. This did not change during the first 24 hours after initiation of differentiation. First alterations in phenotype were observed after 48 hours in differentiation medium. Both murine and human cells showed strong reduction in cell size, massive condensation of nuclei and accumulation of haemoglobin. In addition, one of the final and most striking events during erythroid differentiation, the extrusion of nuclei could in some cells already be observed after 48 hours of differentiation.

At 72 hours, most changes accompanying terminal erythroid development were almost completed in murine cells. The nuclei were maximally condensed and approximately 30% of the cells were already enucleated. Under the culture conditions for murine erythroid cells, haem-synthesis reached its maximum after 48 hours of differentiation (Figure 3.2.C – bottom panel). Data from haemoglobin-assays and Casy cell size measurements underlined the visible morphological changes visible in cytospins. After enucleation, the mean cell size of murine erythroid cells reached values around 6µm.

As indicated by all used assays murine erythroblasts differentiated faster and with respect to enucleation also more efficiently than human erythroblasts *in vitro*.

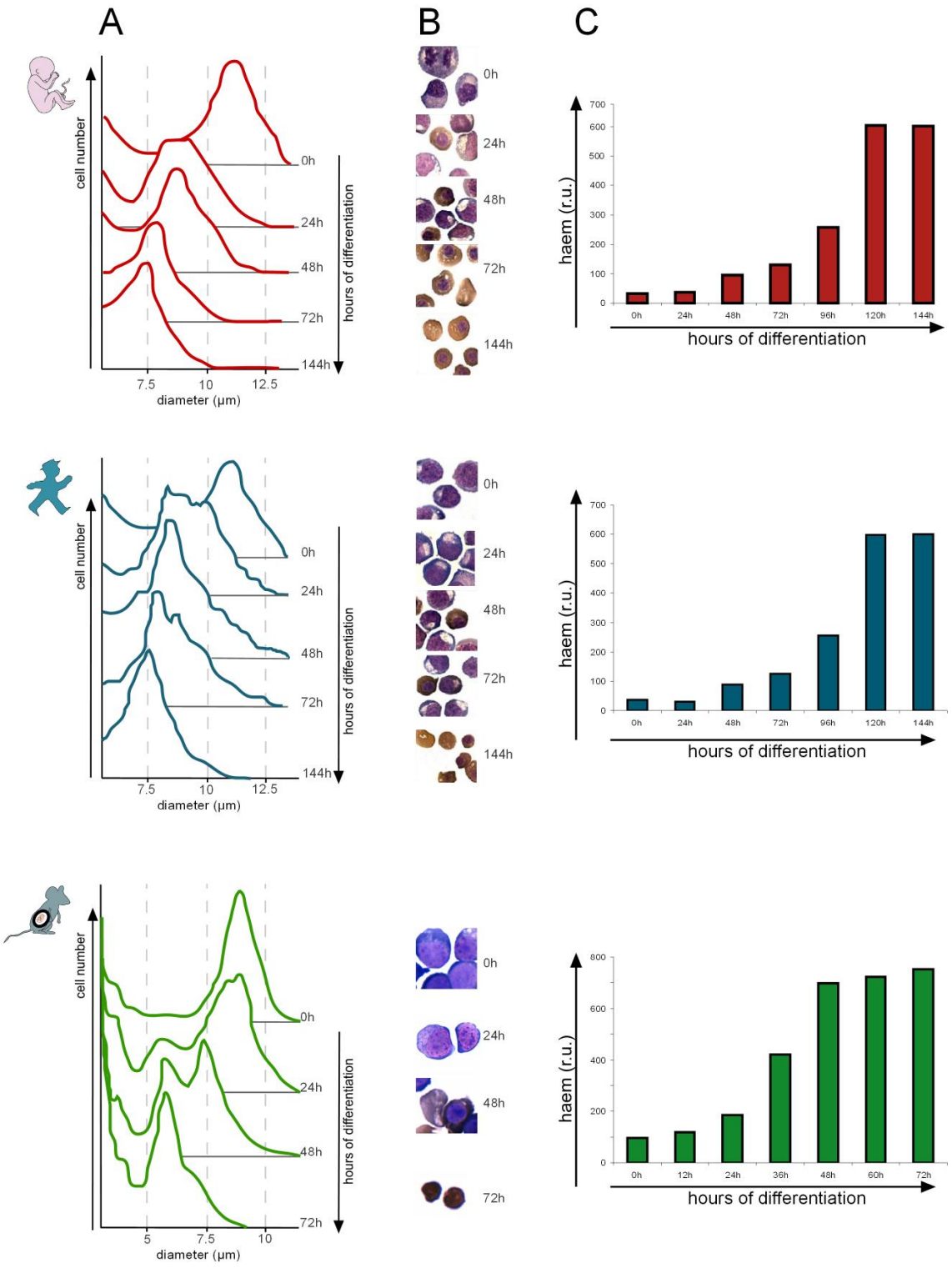


Figure 4.4: Terminal differentiation of murine and human erythroid cells

A) Cell numbers and cell volumes of differentiating human cord blood and peripheral blood derived cells as well as murine foetal liver derived cells were monitored daily with an electronic cell counter.

B) Differentiated cells were cyto-centrifuged onto glass slides and stained with cytological dyes.

C) Haemoglobin accumulation was determined by a photometric assay during differentiation every 24 hours in case of human cells and every 12 hours in case of murine cells.

4.4 Terminal differentiation of human erythroid cells with different fractions of serum

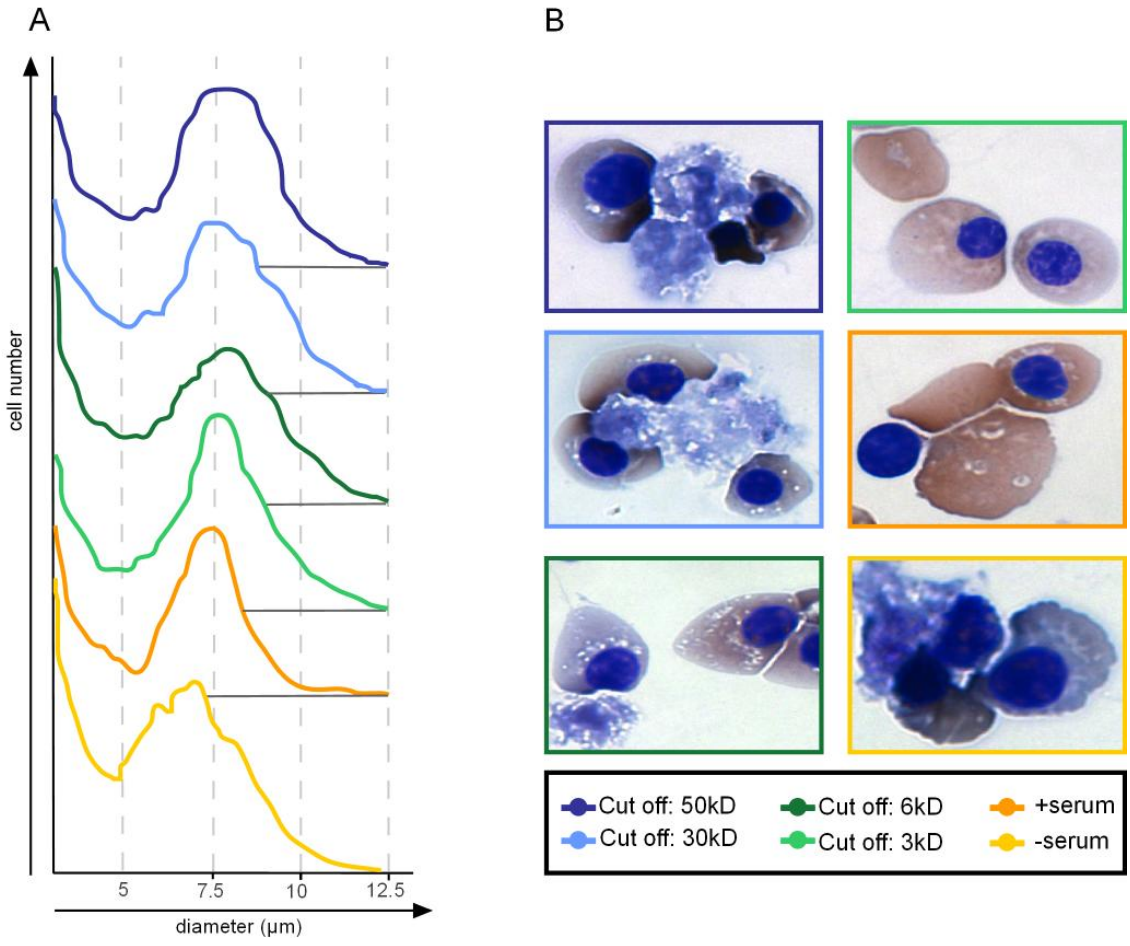
Until now it remained unclear which additional factor(s) in the serum promote human erythroid cell differentiation and inhibit proliferation. In our human cell culture system 3% human serum is needed to promote erythroid cell maturation in a synchronous and efficient manner. To get a first indication what kind of factor or factors are responsible for this observation, serum was dialysed to obtain samples of different average molecular weight.

Figure 3.1.A depicts Casy^{TM} profiles of human erythroid cells from umbilical cord blood differentiating for 6 days. The development of cells differentiated with whole serum (orange) and the one of erythroblasts treated with dialysed serum without components smaller than 3kD (light green) were quite similar. Both profiles showed homogenously differentiated cell populations with a mean cell size of 7.5µm. All other profiles showed a broader peak, indicating inefficient differentiation. Compared to the positive control, there were more cells bigger than 7.5µm, corresponding to more immature cells. In addition, there was also a population of particles smaller than 7.5µm, representing dead cells and cell components. As negative control, (yellow) another culture was kept in the absence of any serum. The mean size of these cells shifted to the left compared to differentiation in serum-containing medium.

The corresponding cytospins supported the trends indicated by the Casy profiles. Cells differentiated with full serum (orange framed) showed strongly haemoglobinized cells. The depicted section shows two enucleated cells with one extruded nucleus. The development of cells differentiated in the presence of the dialysed serum fraction with a cut off of 3kD (light green framed) was comparable to the positive control: it contained strongly haemoglobinized cells with properly condensed and sometimes even extruded nuclei. The cell shown in the middle has its nucleus already located at the cell periphery, apparently entering the process of enucleation. The other cytospins show exclusively nucleated cells and cell fragments.

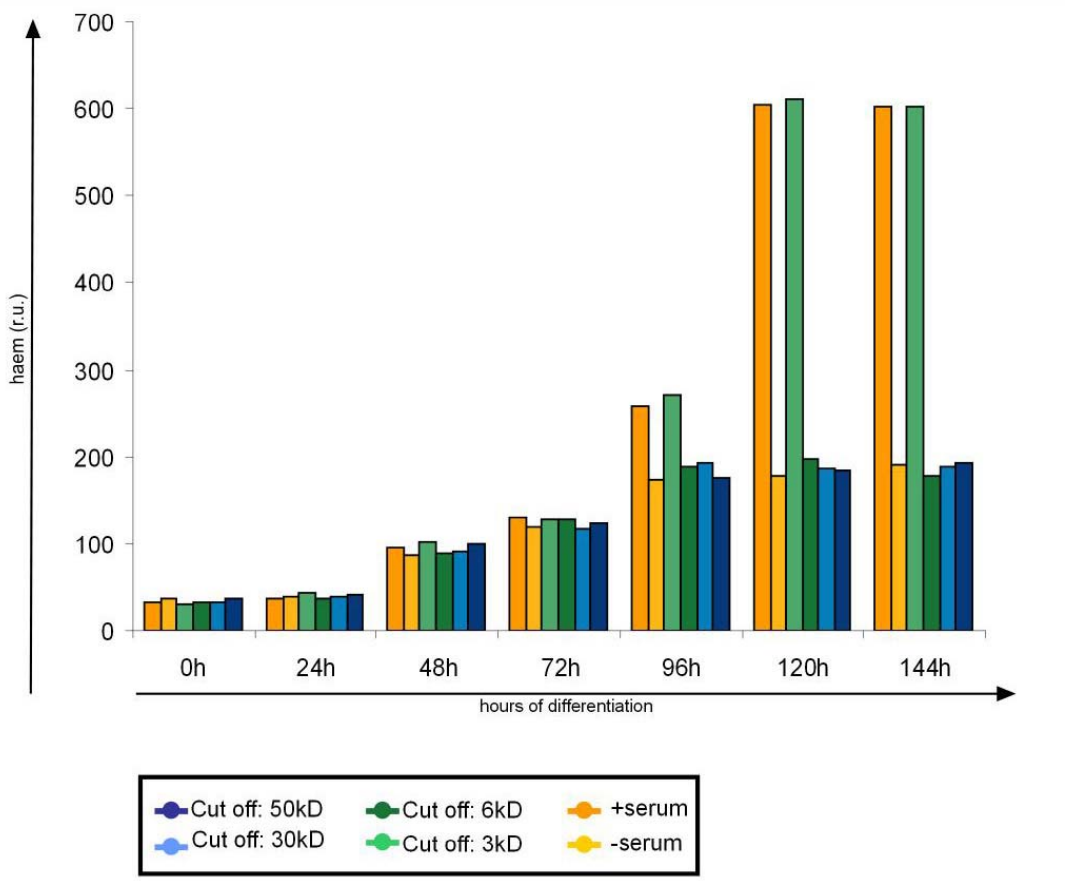
The most striking difference caused by the serum fractions used became apparent in the differences of the haem synthesis rates. During the first 72 hours of differentiation, erythroblasts exhibited comparable rates of synthesis. The first slight

aberration appeared after 96 hours in the presence of different serum fractions. Erythroid cells differentiated with sera dialysed with a bigger cut off than 3kD had no higher elevation of haem levels after 120 hours. A comparable performance was observed in cells maturing in the absence of serum. The haem synthesis rates of cells with complete serum and those receiving dialysed serum (cut-off 3kD), however, reached its maximum after 120 hours of differentiation. Furthermore, they showed the characteristic accelerated increase of haemoglobin levels between 96 and 120 hours.



4.5: Differentiation with diverse fractions of human serum

Human serum was depleted from constituents smaller than 50kD (dark blue) and 30kD (light blue) by centrifugation with Vivaspin tubes. For the removal of constituents smaller than 6kD (dark green) and 3kD (light green) dialyses tubes with appropriate molecular cut-off were used. Cord blood-derived erythroid progenitors were differentiated in the presence of these different serum fractions. As positive and negative controls, respectively, cells were differentiated either with full serum supply or in the absence of any serum. Erythroblasts differentiated for 6 days show differences in mean cell size distribution (A) and in the degree of differentiation (B).



4.6: Differences in haem-synthesis rates caused by the use of different serum fractions

Haem levels of differentiating cells were determined by colorimetry (see methods). During the first 72 hours of differentiation, there was no difference in haem synthesis rates of erythroblasts due to the diverse serum fractions. First effects became visible after 96 hours. By 120 hours, haem levels of erythroblasts differentiated with full serum and with serum dialysed with a cut off of 3kD was approximately 3 times higher than that of all other samples.

4.5 Haemoglobin negatively influences optical density measurement of protein concentration

The increasing amount of haemoglobin in differentiating cells causes problems with optical density (OD) measurements (Figure 4.3.1.B). Because of this observation we compared two different methods. First, the reliability of values obtained with Bradford and SDS-Lowry was tested. Figure 4.3.1.A depicts the comparison between the values obtained with these two protocols using specific amounts of BSA (0.1%). With SDS-Lowry an almost linear increase of OD-values was observed. Data obtained with the Bradford reagent had a stronger increase with BSA amounts lower than 15µl as compared to above this amount of BSA. In the determination of total protein amount in samples of proliferating and 6 days differentiated erythroid cells both measurements failed (Figure 4.3.1.B). Cell extracts separated by gel electrophoresis demonstrated that the amount of loaded protein was not comparable between the two growth conditions. The proliferation samples contained multiple protein bands in comparison to the differentiation sample, where haemoglobin dominates the mixture. After that a Bradford correction factor was determined for adjustment of aberrant raw data. We used increasing sample amounts referring to the increase of haemoglobin during differentiation. After that it was tested which amounts would result in comparable band intensities in Western blots using immuno-detection of eIF4E, which is known to stay at the same absolute abundance under the various conditions (Figure 4.3.1.C). For the sample with 2 days differentiated cells 20% (x 1.2) more extract volume was loaded than determined from Bradford OD measurements, 40% more (x1.4) for 4 days and 80% more (1.8) for 6 days.

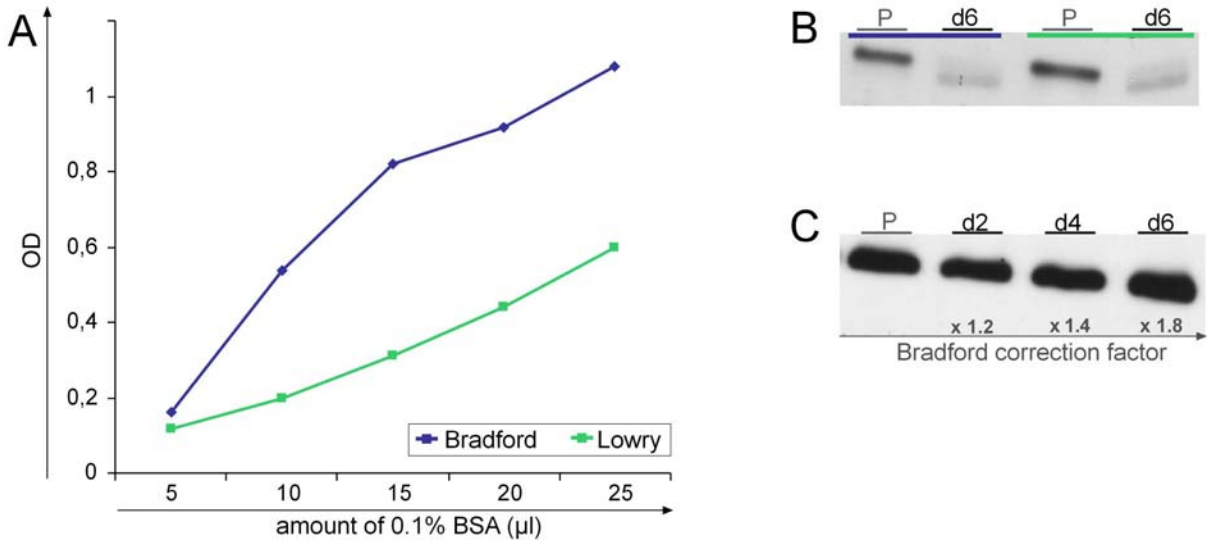


Figure 4.7 Correcting the amount of protein from erythroid cells for Western blotting

A) OD values of different amounts (5 to 25µl) of 0.1% BSA were measured with Bradford and Lowry assays and a standard curves was generated.

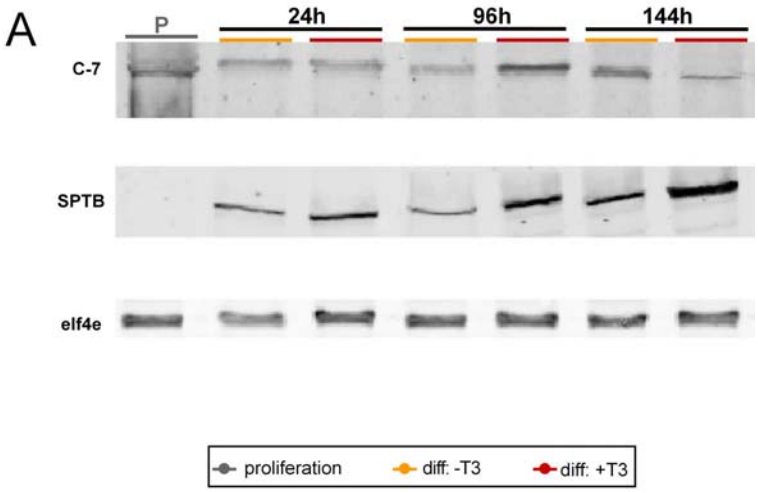
B) Western blotting was performed with proliferating (P) and terminally differentiating cord blood-derived erythroid cells (d6). The amount of protein was defined either with Bradford (left, blue) or SDS-Lowry (right, green).

C) For the adjustment of the inherent “erythroid” error in OD measurements caused by increasing amounts of haemoglobin, comparable amounts of protein were obtained by inclusion of “Bradford correction factors” based on experimental data.

4.6 Expression of Caspase 7 & Spectrin ß in erythroid cells is influenced by T3

Because of the promising results from the mRNA expression profiling and the known functions of C7 and SPTB in erythroid cells, the expression patterns of these two proteins were analyzed by Western blotting. First, differentiation was induced with two different media, one containing T3, the other without the hormone. For Western analyses, corresponding cell pellets were used, together with an additional one from proliferating starting culture as internal control. The C7 expression in erythroid cells differentiated without T3 decreased during the first 48 hours of differentiation, stayed rather constant thereafter and exhibited a strong increase at the last time point. Cells which were differentiated in the presence of T3 had another expression pattern: after the first 24 hours, during which no significant difference between the T3-treated and non-treated cells was observed, C7 expression went up about 3-fold after 96 hours of differentiation. At the end of differentiation, T3 treated cells showed a decrease of C7, reaching an amount below that of untreated cells (Figure 4.4.1).

SPTB is not present at detectable abundance in proliferating erythroblasts. Its amount increases constantly during differentiation, both in hormone-treated and non-treated erythroid cells. T3-treated cells, however, had a faster and more pronounced increase in SPTB expression than non-treated ones.



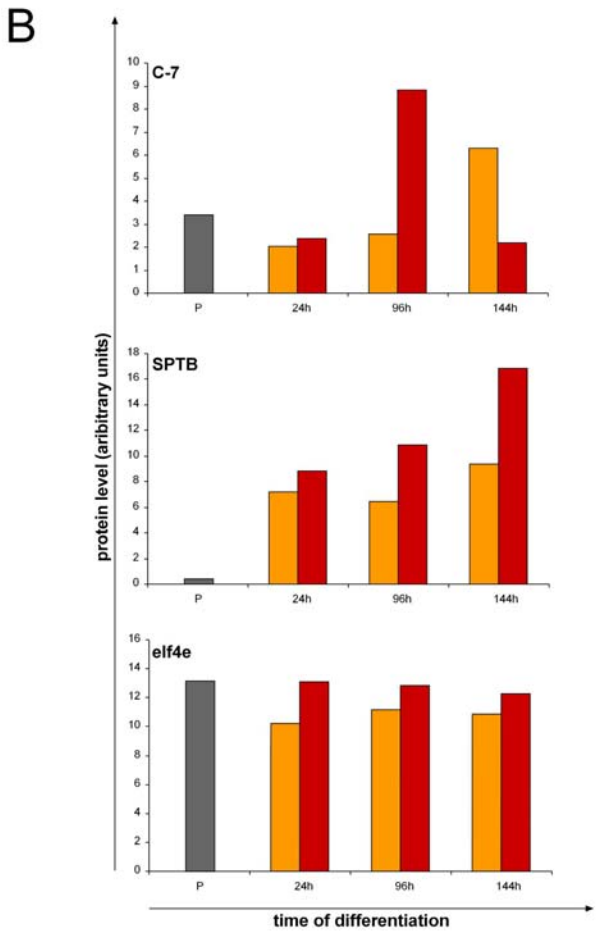


Figure 4.8: Expression of C7 & SPTB during erythroid differentiation

C-7 and SPTB protein expression levels during differentiation of T3 treated and non-treated cells were determined, eIF4e was used as loading control. For Western Blot (A) proliferating and differentiating cells of different time points (24h, 96h, 144h) were used. B shows the quantification of the signals shown in A. The graph illustrates the changes of the protein levels during differentiation of eryhthroblasts.

4.7 Caspase 7 & Spectrin ß expression in human cell lines

After validation of the mRNA expression profiling concerning C7 and SPTB in cord blood derived erythroid cells, we tried to find human cell lines which also express the genes of interest to validate the function of corresponding sh-RNA constructs. The use of cell lines was expected to be both, time and cost saving.
In a first step, the expression pattern of C7 and SPTB was assessed in HeLa, 293T and Mcf-7 cells (Figure 4.4.1.A). Especially the latter showed strong expression of C7. None of these cell lines, however, express any detectable SPTB.
Thus, in a second step differentiation was induced in the erythroid cell line K-562 using haemine. The cells started to accumulate haemoglobin (Figure 4.4.1.B) and showed a decrease in cell size (Figure 4.4.1.C). To proof whether this clear evidence for differentiation also had an effect on SPTB expression the Western blot analysis was repeated (Figure 4.4.1.D). As positive control 3 days differentiated primary erythroid cells were used. Also K-562 erythroleukaemic cells did not show any expression of SPTB during differentiation, possibly owing to their transformed phenotype precluding expression of late erythroid proteins.

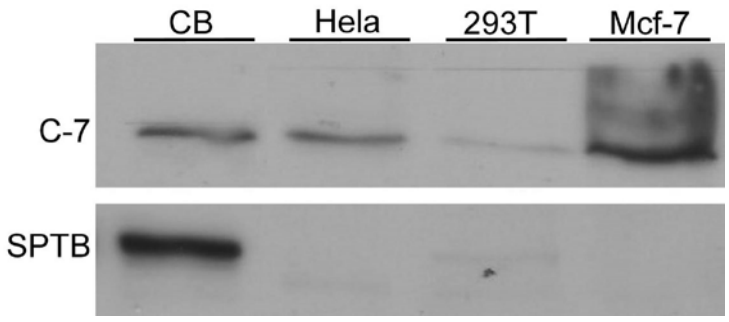


Figure 4.9: Expression of C7 & SPTB in human cell lines
Western blots presenting the expression of C7 and SPTB in proliferating cord blood-derived erythroblasts (CB) and different human cell lines (HeLa, 293T, MCF-7). The amount of loaded protein was estimated with Bradford assay.

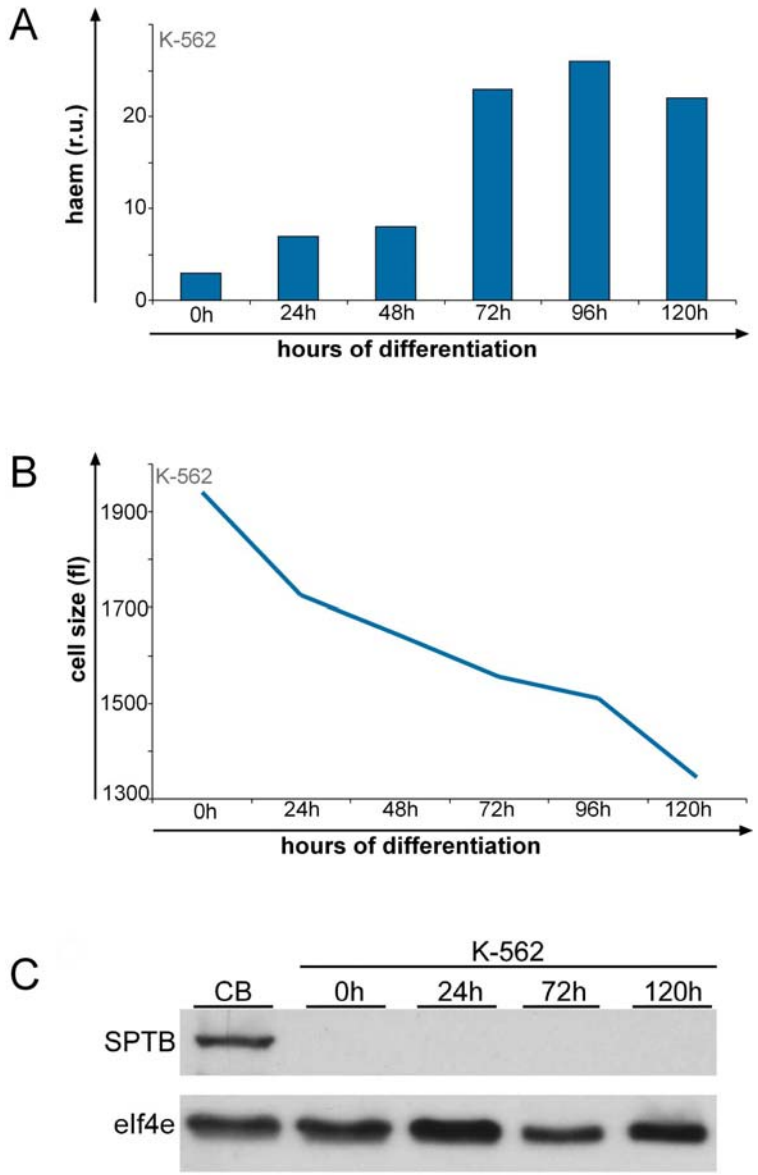


Figure 4.10: Characterization of K-562 differentiation

A) Differentiation of K-562 cells was induced with haemin. The accumulation of haemoglobin was determined by a photometric assay as described above.

B) The cell size of differentiating K-562 cells was monitored daily with a CASY^{TM} cell analyzer.

C) Western blot showing the expression of SPTB in cord blood derived erythroid cells differentiated for three days versus proliferating and differentiating K-562 cells at various time points (24h, 72h, 120h). Neither in proliferating nor in differentiating K-562 cells SPTB expression could be observed.

4.8 Lentiviral infection of human erythroid cells

For the production of lentiviral particles we used either Lipofectamine™ or Calcium Phosphate. The transfection efficiency was determined with luminescence microscopy and generally resulted in about 90% green-luminescent cells. However, there were big differences in the infectiousness of the obtained lentiviral supernatants, which ranged from about 5 to 20% infected cells. Differences caused by the different transfection methods were not observed. Figure 4.5.1 shows infection (transfection with Lipofectamine™), which results in the highest amount of infected erythroblasts.

Despite these low infection rates we performed western blot analyses (Figure 4.5.2.A) and induced differentiation (Figure 4.5.3.C). In western blot we could not detect any reduction of C7 expression. Cell profiles of proliferating infected erythroblasts (Figure 4.5.3.B) and the negative control (uninfected cells) show no significant differences. The peak of the uninfected cells of the negative control was slightly broader compared with the infected erythroblasts. Furthermore there was no difference in the performance of differentiation between infected erythroid cells and control cells detectable in the cell profiles.

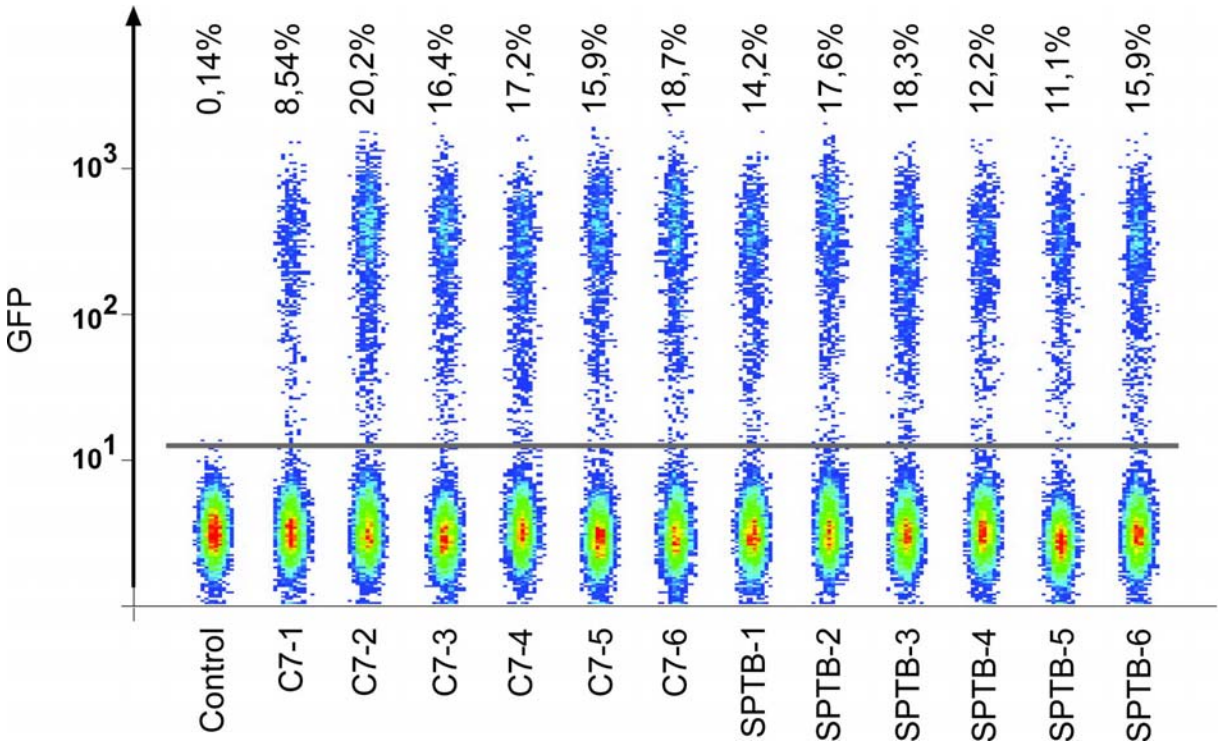


Figure 4.11: Efficiency of lentiviral infection of human erythroblasts

For lentiviral infection cord blood-derived erythroid cells were incubated for 12 hours with lentiviral supernatants containing 5µg/ml polybrene and medium with growth factors. The infection rate was determined by flow cytometry. All cells above the bold grey line are classified as GFP positive, respectively infected.

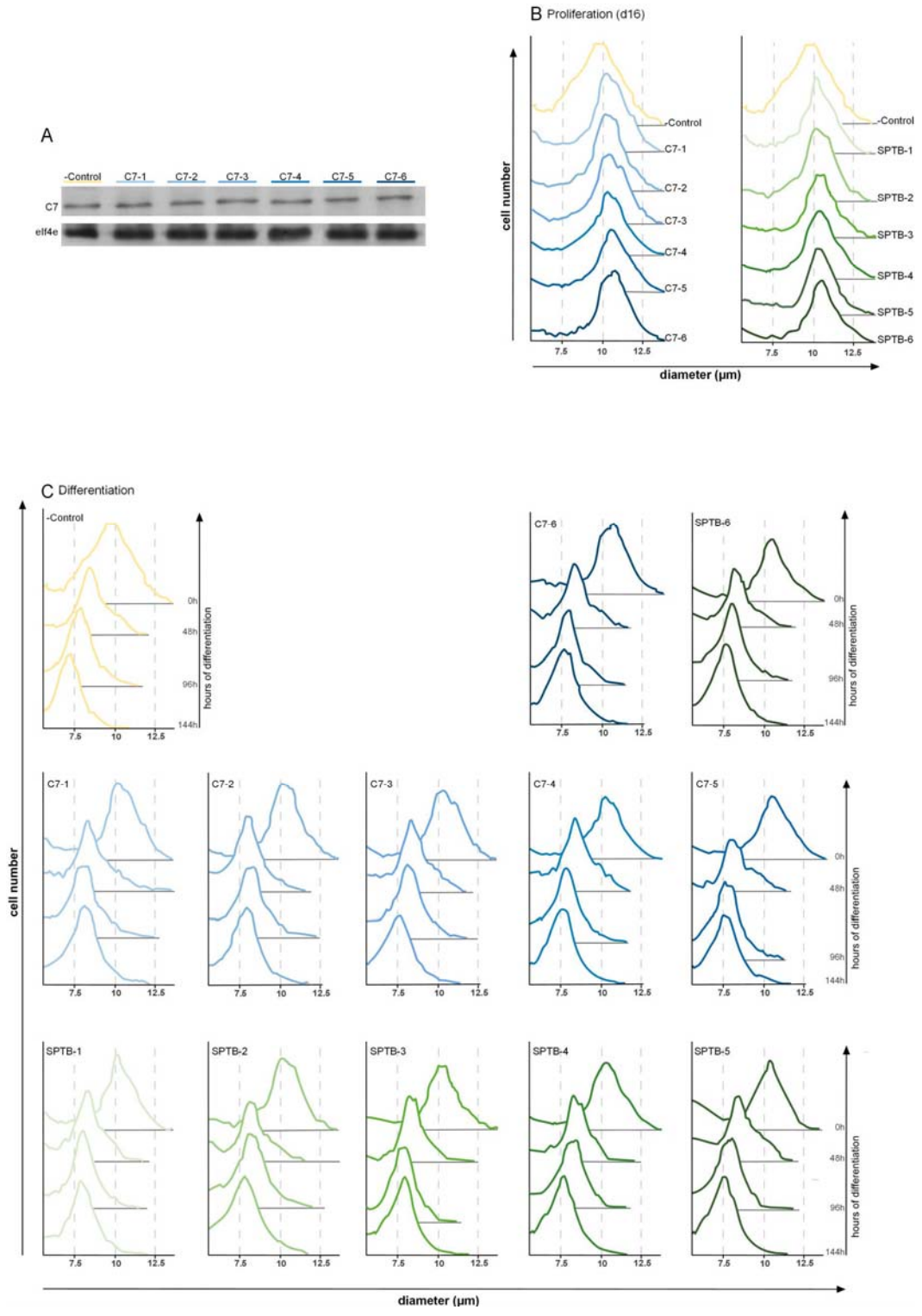


Figure 4.12: Cell profiles of lentiviral infected eryhroblasts

A) Western blots of lentivirally infected erythroblasts showed no knock down of C7 protein expression.

B) Cell number and size of lentivirally infected proliferating erythroblasts were monitored with an electronic cell counter.

C) Differentiation of infected and uninfected erythroblasts was comparable.

5 Discussion

5.1 Features of the human cell culture system: DHT & serum

During the last decades, culture systems for HSC contributed to a number of findings in diverse haemotopoietic conditions, like quiescence, commitment to special lineages, maturation and apoptosis. Key signalling pathways and essential factors for HSC maintenance were described improving the techniques for haematopoietic *in vitro* cultures. In this thesis we used different sources for HSCs of both murine and human origin. The human cell culture conditions were based on the mouse cell system (Dolznig, Boulme et al. 2001) and adapted to the special needs of human erythroid progenitors (Leberbauer, Boulme et al. 2005). First, we used different media for murine and human cells. Second, the factor-mix for proliferating human erythroid progenitors was enriched with lipids, which prolongs proliferation and survival of the cells from 30 to 60 days. The addition of lipid alone was not efficient to cultivate the desired number of erythroid cells from peripheral blood. The abundance of erythroid progenitors in peripheral blood is much lower than in umbilical cord blood. Outgrowth of erythroid progenitors from peripheral blood needed further support via IL-6 and Flt 3. Furthermore, in the cell culture system described here, human cells needed human serum during differentiation. Serum improved proliferation and also acted in protection against apoptosis.

One observation in human cord blood-derived erythroblasts was a sex-specific proliferation-promoting effect of DHT. This was only observed in female erythroid cells. It is obvious that sex-specific differences in all relevant blood parameters exist. One of the main indicators is the amount of cellular components in the blood volume, the haematocrit. It mainly results from erythrocytes, which are the most abundant cell type in blood. The haematocrit level of healthy women ranges from 4–5.5x10^6/µl, whereas the level in healthy men ranges from 4.5–6x10^6/µl. Corresponding to haematocrit levels, there is a difference in the amount of erythrocytes: 35–40 Vol.% (women) and 40–50 Vol.% (men). In addition to these general variations, anaemia caused by iron deficiency affects predominantly females. One reason for this is a loss of iron during menstruation and giving birth. Up to 20% of the female population in Austria is affected by sub-acute anaemia and the concurrent general fatigue. One of the reasons why men have higher amounts of erythrocytes and a lower risk to

develop anaemia could be explained by an erythropoietic potency of androgens, the male steroid hormones. It has been shown that females treated with DHT reach haematocrit levels similar to males (Fried and Gurney 1968). The increase of blood cells observed *in vivo* and *in vitro*, could be caused through accelerated erythroid differentiation or increased proliferation of HSCs or haematopoietic progenitors. The proliferation-promoting effects were observed exclusively in female erythroblasts. If DHT had a general erythropoietic potency, this effect would be expected to occur in cells of both sexes.

The fact that human erythroblasts in our cell culture system needed the presence of serum for differentiation pointed out the complexity of erythroid differentiation. Consuetudinary cell culture is performed in the presence of serum (Panzenbock, Bartunek et al. 1998). Under our culture conditions, the cells accumulated more haemoglobin and showed a higher and more synchronous size decrease in the presence of serum. Furthermore, serum seemed to prevent apoptosis. The use of serum is suboptimal, however, for various reasons: It contains still unidentified factors, which drive cells to differentiation. Furthermore, the concentration of the serum constituents may differ from batch to batch, because of that essential factors could not be controlled.

To get a first indication what kind of factors within serum might play the described crucial role during differentiation, serum-constituents were removed depending on size, for example by dialysis. The results suggested that one or more relevant factors for differentiation have a size between 3 and 6kD. Although efficiency of removal of components by dialysis is influenced not only by the size of constituents but also by chemical attributes, these possible side-effects were avoided by long dialysis times.

It is presumed that serum contains both differentiation promoting and inhibiting factors. It could be that the most important factors with these diverse functions are in the same size range. Another possibility is a different balance of promoting and inhibiting factors in the serum fractions. If there were factors essential for the maintenance of cells in the size range between 3 and 6kD, effects caused by the absence of other factors (for example inhibiting ones) would not be observed, because the cells should undergo apoptosis. To study these possibilities more precisely dialysis should be performed so that differentiation-inhibiting and -promoting factors are separated if they differ in size. Additionally, dialysis the other

way round could be performed (PBS in a dialysis tubing with a cut-off of 6kD dialyzed against serum already dialysed first with a cut-off with 3kD). Alternatively, also a combination between dialysis (cut-off 3kD) and centrifugation with Vivaspin tubes (cut-off 6kD) should be performed. If differentiation with the obtained fraction would be comparable to the positive control, this would support the finding of the first experiment. In a next step specific types of constituents within this fraction should be removed and tested again. One possibility would be the removal of lipophilic constituents with Freon, a chlorofluorocarbon (CFC) refrigerant. Other promising experiments would be the fractionation of serum with HPLC (high pressure liquid chromatography) and silver staining of the different fractions after gel electrophoresis. If these experiments resulted in further indications, mass spectrometry could be used for the final identification of the factor(s) of interest.

5.2 SPTB, C7 and the lentiviral infection of human erythroblasts

Western blot analysis supported the findings from the mRNA expression profiling: In human, T3 regulates the expression of SPTB and C7 during erythroid differentiation. Cells maturing in the presence of T3 showed faster and stronger increase of the major erythroid protein SPTB. In particular, it is responsible for the typical bi-concave shape of erythrocytes. As SPTB expression increased during differentiation it could be seen as a sign for the level of maturation of erythrocytes. Erythroid cells differentiated in the presence of T3 showed a more mature phenotype, especially in cytospins. Higher amounts of SPTB in these cells were therefore expected and observed.

The expression of C7 during differentiation was more complex in comparison to SPTB. Whereas cells differentiated in the absence of T3 exhibited an increase in C7 expression, C7 levels in T3-treated cells peaked after 96 hours followed by a rapid decrease. C7 could be essential for the condensation of the nucleus, a crucial action during differentiation and needed for the following process of enucleation. Caspases usually contribute to apoptosis, consequently C7 expression decreased at the end of differentiation. The T3-caused up- and late down-regulation of C7 could be the main reason for the presence of more enucleated erythroid cells in the corresponding erythroid cultures.

To elucidate the role of SPTB and C7 in erythroid differentiation more precisely, it would be interesting to know which effects are caused by a knock-down of these genes. Because of previous experiences in our group (transfection and retroviral infections of human erythroblasts failed) we looked for alternative methods. We decided to use lentiviral vectors to utilise the RNAi mechanism. After infection of human erythroblasts inefficient infection-rates were observed (best infection: approx. 20%). Because of these inefficient rates we were not able to detect any knock down, much less a detectable effect. Because of restrictions in the biosafety guidelines of the Division of Gynecological Endocrinology of the General Hospital Vienna (see Appendix), as well as the sensitivity and restricted life time of human erythroblasts, we were not able to sort the cells. Some improvement in the infection rate could be obtained with alternative infection protocols like for example spin infection (Tripp, Liu et al. 2003) or different ratios of the 3 vectors used for lentivirus-production. The increase in infection rates reached by these improvements, however, is not sufficient to see effects on gene expression. In addition to a general higher infection rate also multiple infections of the erythroblasts are desirable. That would cause multiple integrations into the genome of the erythroblasts and therefore an increased amount of shRNA in the cell, which should result in a more efficient knock down of the gene of interest. To achieve this, the lentivirus supernatant has to be concentrated, for example 100fold. The concentration can be done by ultracentrifugation, as described in the literature. During this diploma thesis it was not possible to concentrate virus supernatants because of absent approbations in the biosaftey level II approvals of the Max F. Perutz Laboratories and our collaboration partners in the IMP and AKH Vienna.

The consequential combination of the presented data creates a promising future perspective. The data suggest that until now unidentified factors for erythroid differentiation in human serum can be found in a well-defined small size-range. Furthermore, findings of the mRNA expression profiling were confirmed at the protein level. Future experiments are necessary to elucidate these unidentified differentiation factors and the specific role of SPTB and C7 in erythropoiesis.

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6 List of Abbreviations

ADAM	A disintegrin & metalloprotease
AGM	Aorta-gonad mesoneprhros region
AKH	Allgemeines Krankenhaus Wien
AML	Acute myeloid leukaemia
BFU	Burst forming unit
BFU-E	BFU-erythroid
BSA	Bovine serum albumin
C3	Caspase 3
C7	Caspase 7
Caspase	**C**ysteinyl **as**partate **p**rotein**ase**
CD	Cluster of differentiation
CFC	Chlorofluorocarbon
CFU	Colony forming unit
CFU-E	CFU-erythroid
CLP	Common lymphoid progenitor
CMP	Common myeloid progenito
DEMEM	Dulbecco's modified Eagle's medium
Dex	Dexamethasone
DHT	Dihydro-testosterone
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
EDTA	Ethylenediaminotetraacetic acid
eIF4E	Eukaryotic translation initiation factor 4E
EMEM	Eagle's minimal essential medium
Emp	Erythroblast macrophage protein
Epo	Erythropoietin
EpoR	Epo receptor
Flt3	Fibromyalgia syndrome like tyrosine kinase 3
FMS	Fibromyalgia syndrome
G-CSF	Granulocyte-colony-stimulating factor
GMP	Granulocyte/macrophage progenitor
GPA	Glycophorin A
G-protein	Guanine nucleotide-binding protein
HCK	Haematopoietic cell kinase
HSC	Haematopoietic stem cell
Igf 1	Insulin-like growth factor
IL-6	Interleukin-6
IMP	Inosine monophosphat
IMP	Institute of Molecular Pathology
kD	kilo Dalton
Lin	Lineage
LT-HSCs	Long term-HSC
MAP	Mitogen-activated protein
MEL	Mouse erythroleukaemia
MEP	Megakaryocyte/erythroid progenitor
MFPL	Max F. Perutz Laboratories
MMP	Matrix metalloproteinase
MPP	Multipotent progenitor

NKC	Matural killer cell
OD	Optical density
o/n	Over night
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
RBV	Retronectin bound virus
RNA	Ribonuleic acid
RNAi	RNA interference
SCF	Stem cell factor
SDF-1	Stromal-derived-factor-1
shRNA	Short hairpin RNA
Sl	Steel locus
SPTB	Spectrin beta
STAT5	Signal transducer and activator of transcription 5
ST-HSC	Short term-HSC
T3	3,5,3'-triiodothyronine
TNF	Tumour necrosis factor
TRα	Thyroid hormone receptor alpha
TRß	Thyroid hormone receptor beta
U	Units

7 References

Akashi, K., D. Traver, et al. (2000). "A clonogenic common myeloid progenitor that gives rise to all myeloid lineages." Nature **404**(6774): 193-7.

Allen, T. D. and T. M. Dexter (1982). "Ultrastructural aspects of erythropoietic differentiation in long-term bone marrow culture." Differentiation **21**(2): 86-94.

Alnemri, E. S., D. J. Livingston, et al. (1996). "Human ICE/CED-3 protease nomenclature." Cell **87**(2): 171.

Alvarez-Silva, M., P. Belo-Diabangouaya, et al. (2003). "Mouse placenta is a major hematopoietic organ." Development **130**(22): 5437-44.

Anderson, D. M., D. E. Williams, et al. (1991). "Alternate splicing of mRNAs encoding human mast cell growth factor and localization of the gene to chromosome 12q22-q24." Cell Growth Differ **2**(8): 373-8.

Arai, F., A. Hirao, et al. (2004). "Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche." Cell **118**(2): 149-61.

Auffray, I., S. Marfatia, et al. (2001). "Glycophorin A dimerization and band 3 interaction during erythroid membrane biogenesis: in vivo studies in human glycophorin A transgenic mice." Blood **97**(9): 2872-8.

Bauer, A., W. Mikulits, et al. (1998). "The thyroid hormone receptor functions as a ligand-operated developmental switch between proliferation and differentiation of erythroid progenitors." Embo J **17**(15): 4291-303.

Bauer, A., F. Tronche, et al. (1999). "The glucocorticoid receptor is required for stress erythropoiesis." Genes Dev **13**(22): 2996-3002.

Becker, P. S. and S. E. Lux (1985). "Hereditary spherocytosis and related disorders." Clin Haematol **14**(1): 15-43.

Besa, E. C. and L. P. Bullock (1981). "The role of the androgen receptor in erythropoiesis." Endocrinology **109**(6): 1983-9.

Bhatia, M., D. Bonnet, et al. (1998). "A newly discovered class of human hematopoietic cells with SCID-repopulating activity." Nat Med **4**(9): 1038-45.

Bhatia, M., J. C. Wang, et al. (1997). "Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice." Proc Natl Acad Sci U S A **94**(10): 5320-5.

Briddell, R. A., E. Bruno, et al. (1991). "Effect of c-kit ligand on in vitro human megakaryocytopoiesis." Blood **78**(11): 2854-9.

Broudy, V. C., N. L. Lin, et al. (1998). "Analysis of c-kit receptor dimerization by fluorescence resonance energy transfer." Blood **91**(3): 898-906.

Broxmeyer, H. E., R. Maze, et al. (1991). "The kit receptor and its ligand, steel factor, as regulators of hemopoiesis." Cancer Cells **3**(12): 480-7.

Broxmeyer, H. E., E. Srour, et al. (2006). "Cord blood stem and progenitor cells." Methods Enzymol **419**: 439-73.

Burnette, W. N. (1981). ""Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A." Anal Biochem **112**(2): 195-203.

Carlile, G. W., D. H. Smith, et al. (2004). "Caspase-3 has a nonapoptotic function in erythroid maturation." Blood **103**(11): 4310-6.

Carmel, R. and C. A. Spencer (1982). "Clinical and subclinical thyroid disorders associated with pernicious anemia. Observations on abnormal thyroid-stimulating hormone levels and on a possible association of blood group O with hyperthyroidism." Arch Intern Med **142**(8): 1465-9.

Civin, C. I., L. C. Strauss, et al. (1984). "Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells." J Immunol **133**(1): 157-65.

Claustres, M. and C. Sultan (1988). "Androgen and erythropoiesis: evidence for an androgen receptor in erythroblasts from human bone marrow cultures." Horm Res **29**(1): 17-22.

Cohen, C. M. (1983). "The molecular organization of the red cell membrane skeleton." Semin Hematol **20**(3): 141-58.

de Bruijn, M. F., N. A. Speck, et al. (2000). "Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo." Embo J **19**(11): 2465-74.

Dean, A., F. Erard, et al. (1981). "Induction of hemoglobin accumulation in human K562 cells by hemin is reversible." Science **212**(4493): 459-61.

Dolznig, H., P. Bartunek, et al. (1995). "Terminal differentiation of normal chicken erythroid progenitors: shortening of G1 correlates with loss of D-cyclin/cdk4 expression and altered cell size control." Cell Growth Differ **6**(11): 1341-52.

Dolznig, H., F. Boulme, et al. (2001). "Establishment of normal, terminally differentiating mouse erythroid progenitors: molecular characterization by cDNA arrays." Faseb J **15**(8): 1442-4.

Dolznig, H., B. Habermann, et al. (2002). "Apoptosis protection by the Epo target Bcl-X(L) allows factor-independent differentiation of primary erythroblasts." Curr Biol **12**(13): 1076-85.

Dolznig, H., A. Kolbus, et al. (2005). "Expansion and differentiation of immature mouse and human hematopoietic progenitors." Methods Mol Med **105**: 323-44.

Engelhardt, M., M. Lubbert, et al. (2002). "CD34(+) or CD34(-): which is the more primitive?" Leukemia **16**(9): 1603-8.

Fibach, E. and E. Prus (2005). "Differentiation of human erythroid cells in culture." Curr Protoc Immunol **Chapter 22**: Unit 22F 7.

Forrest, D., L. C. Erway, et al. (1996). "Thyroid hormone receptor beta is essential for development of auditory function." Nat Genet **13**(3): 354-7.

Fraichard, A., O. Chassande, et al. (1997). "The T3R alpha gene encoding a thyroid hormone receptor is essential for post-natal development and thyroid hormone production." Embo J **16**(14): 4412-20.

Fried, W. and C. W. Gurney (1968). "The erythropoietic-stimulating effects of androgens." Ann N Y Acad Sci **149**(1): 356-65.

Godin, I. and A. Cumano (2002). "The hare and the tortoise: an embryonic haematopoietic race." Nat Rev Immunol **2**(8): 593-604.

Goodman, J. W. and G. S. Hodgson (1962). "Evidence for stem cells in the peripheral blood of mice." Blood **19**: 702-14.

Goodman, S. R., K. E. Krebs, et al. (1988). "Spectrin and related molecules." CRC Crit Rev Biochem **23**(2): 171-234.

Grabarek, J., J. E. Groopman, et al. (1994). "Human kit ligand (stem cell factor) modulates platelet activation in vitro." J Biol Chem **269**(34): 21718-24.

Guerriero, A., L. Worford, et al. (1997). "Thrombopoietin is synthesized by bone marrow stromal cells." Blood **90**(9): 3444-55.

Hanspal, M. and J. S. Hanspal (1994). "The association of erythroblasts with macrophages promotes erythroid proliferation and maturation: a 30-kD heparin-binding protein is involved in this contact." Blood **84**(10): 3494-504.

Hanspal, M., Y. Smockova, et al. (1998). "Molecular identification and functional characterization of a novel protein that mediates the attachment of erythroblasts to macrophages." Blood **92**(8): 2940-50.

Heath, D. S., A. A. Axelrad, et al. (1976). "Separation of the erythropoietin-responsive progenitors BFU-E and CFU-E in mouse bone marrow by unit gravity sedimentation." Blood **47**(5): 777-92.

Ikeda, H., Y. Kanakura, et al. (1991). "Expression and functional role of the proto-oncogene c-kit in acute myeloblastic leukemia cells." Blood **78**(11): 2962-8.

Ikuta, K. and I. L. Weissman (1992). "Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation." Proc Natl Acad Sci U S A **89**(4): 1502-6.

Jegalian, A. G., A. Acurio, et al. (2002). "Erythropoietin receptor haploinsufficiency and in vivo interplay with granulocyte-macrophage colony-stimulating factor and interleukin 3." Blood **99**(7): 2603-5.

Kabat, D., C. C. Sherton, et al. (1975). "Synthesis of erythrocyte-specific proteins in cultured friend leukemia cells." Cell **5**(3): 331-8.

Kimura, S., A. W. Roberts, et al. (1998). "Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin." Proc Natl Acad Sci U S A **95**(3): 1195-200.

Kirito, K., N. Fox, et al. (2003). "Thrombopoietin stimulates Hoxb4 expression: an explanation for the favorable effects of TPO on hematopoietic stem cells." Blood **102**(9): 3172-8.

Knowles, W., S. L. Marchesi, et al. (1983). "Spectrin: structure, function, and abnormalities." Semin Hematol **20**(3): 159-74.

Kolbus, A., S. Pilat, et al. (2002). "Raf-1 antagonizes erythroid differentiation by restraining caspase activation." J Exp Med **196**(10): 1347-53.

Kondo, M., I. L. Weissman, et al. (1997). "Identification of clonogenic common lymphoid progenitors in mouse bone marrow." Cell **91**(5): 661-72.

Koury, M. J. and M. C. Bondurant (1990). "Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells." Science **248**(4953): 378-81.

Kowenz, E., A. Leutz, et al. (1987). "ts-oncogene-transformed erythroleukemic cells: a novel test system for purifying and characterizing avian erythroid growth factors." Haematol Blood Transfus **31**: 199-209.

Kurtz, A., W. Hartl, et al. (1985). "Activity in fetal bovine serum that stimulates erythroid colony formation in fetal mouse livers is insulinlike growth factor I." J Clin Invest **76**(4): 1643-8.

Kurtz, A., W. Jelkmann, et al. (1982). "A new candidate for the regulation of erythropoiesis. Insulin-like growth factor I." FEBS Lett **149**(1): 105-8.

Leberbauer, C., F. Boulme, et al. (2005). "Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors." Blood **105**(1): 85-94.

Lebkowski, J. S., L. R. Schain, et al. (1995). "Serum-free culture of hematopoietic stem cells: a review." Stem Cells **13**(6): 607-12.

Lehnert, M. E. and H. F. Lodish (1988). "Unequal synthesis and differential degradation of alpha and beta spectrin during murine erythroid differentiation." J Cell Biol **107**(2): 413-26.

Lensch, M. W. and G. Q. Daley (2004). "Origins of mammalian hematopoiesis: in vivo paradigms and in vitro models." Curr Top Dev Biol **60**: 127-96.

Li, W., S. A. Johnson, et al. (2003). "Primary endothelial cells isolated from the yolk sac and para-aortic splanchnopleura support the expansion of adult marrow stem cells in vitro." Blood **102**(13): 4345-53.

Lozzio, C. B. and B. B. Lozzio (1975). "Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome." Blood **45**(3): 321-34.

McGrath, K. E. and J. Palis (2005). "Hematopoiesis in the yolk sac: more than meets the eye." Exp Hematol **33**(9): 1021-8.

Medvinsky, A. and E. Dzierzak (1996). "Definitive hematopoiesis is autonomously initiated by the AGM region." Cell **86**(6): 897-906.

Mikkola, H. K., C. Gekas, et al. (2005). "Placenta as a site for hematopoietic stem cell development." Exp Hematol **33**(9): 1048-54.

Mikkola, H. K. and S. H. Orkin (2006). "The journey of developing hematopoietic stem cells." Development **133**(19): 3733-44.

Mooradian, A. D., J. E. Morley, et al. (1987). "Biological actions of androgens." Endocr Rev **8**(1): 1-28.

Morioka, K., S. Tone, et al. (1998). "The apoptotic and nonapoptotic nature of the terminal differentiation of erythroid cells." Exp Cell Res **240**(2): 206-17.

Morrison, S. J., A. M. Wandycz, et al. (1997). "Identification of a lineage of multipotent hematopoietic progenitors." Development **124**(10): 1929-39.

Muller, A. M., A. Medvinsky, et al. (1994). "Development of hematopoietic stem cell activity in the mouse embryo." Immunity **1**(4): 291-301.

Murray, L. J., J. C. Young, et al. (1999). "Thrombopoietin, flt3, and kit ligands together suppress apoptosis of human mobilized CD34+ cells and recruit primitive CD34+ Thy-1+ cells into rapid division." Exp Hematol **27**(6): 1019-28.

Muta, K., S. B. Krantz, et al. (1995). "Stem cell factor retards differentiation of normal human erythroid progenitor cells while stimulating proliferation." Blood **86**(2): 572-80.

Muta, K., S. B. Krantz, et al. (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**(1): 34-43.

Nakamura, Y., K. Ando, et al. (1999). "Ex vivo generation of CD34(+) cells from CD34(-) hematopoietic cells." Blood **94**(12): 4053-9.

Nehls, V., P. Zeitler-Zapf, et al. (1993). "Different sequences of expression of band 3, spectrin, and ankyrin during normal erythropoiesis and erythroleukemia." Am J Pathol **142**(5): 1565-73.

Neildez-Nguyen, T. M., H. Wajcman, et al. (2002). "Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo." Nat Biotechnol **20**(5): 467-72.

Nilsson, S. K., H. M. Johnston, et al. (2005). "Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells." Blood **106**(4): 1232-9.

Ogawa, M., Y. Matsuzaki, et al. (1991). "Expression and function of c-kit in hemopoietic progenitor cells." J Exp Med **174**(1): 63-71.

Ohneda, K. and M. Yamamoto (2002). "Roles of hematopoietic transcription factors GATA-1 and GATA-2 in the development of red blood cell lineage." Acta Haematol **108**(4): 237-45.

Orwoll, E. S. and R. L. Orwoll (1987). "Hematologic abnormalities in patients with endocrine and metabolic disorders." Hematol Oncol Clin North Am **1**(2): 261-79.

Palek, J. (1984). "Disorders of red cell membrane skeleton: an overview." Prog Clin Biol Res **159**: 177-89.

Panzenbock, B., P. Bartunek, et al. (1998). "Growth and differentiation of human stem cell factor/erythropoietin-dependent erythroid progenitor cells in vitro." Blood **92**(10): 3658-68.

Papayannopoulou, T., M. Brice, et al. (1991). "Isolation of c-kit receptor-expressing cells from bone marrow, peripheral blood, and fetal liver: functional properties and composite antigenic profile." Blood **78**(6): 1403-12.

Petit, I., M. Szyper-Kravitz, et al. (2002). "G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4." Nat Immunol **3**(7): 687-94.

Poloni, A., L. Douay, et al. (1997). "The potential role of FLT3 ligand in progenitor and primitive hematopoietic cell expansion." Boll Soc Ital Biol Sper **73**(3-4): 55-62.

Puri, M. C. and A. Bernstein (2003). "Requirement for the TIE family of receptor tyrosine kinases in adult but not fetal hematopoiesis." Proc Natl Acad Sci U S A **100**(22): 12753-8.

Qian, H., N. Buza-Vidas, et al. (2007). "Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells." Cell Stem Cell **1**(6): 671-84.

Rhodes, K. E., C. Gekas, et al. (2008). "The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation." Cell Stem Cell **2**(3): 252-63.

Rich, I. N., C. Vogt, et al. (1988). "Erythropoietin gene expression in vitro and in vivo detected by in situ hybridization." Blood Cells **14**(2-3): 505-20.

Rodriguez-Tarduchy, G., M. K. Collins, et al. (1992). "Insulin-like growth factor-I inhibits apoptosis in IL-3-dependent hemopoietic cells." J Immunol **149**(2): 535-40.

Sakatoku, H. and S. Inoue (1997). "In vitro proliferation and differentiation of erythroid progenitors of cord blood." Stem Cells **15**(4): 268-74.

Seki, M. and H. Shirasawa (1965). "Role of the reticular cells during maturation process of the erythroblast. 3. The fate of phagocytized nucleus." Acta Pathol Jpn **15**(4): 387-405.

Speicher, D. W. and V. T. Marchesi (1984). "Erythrocyte spectrin is comprised of many homologous triple helical segments." Nature **311**(5982): 177-80.

Stephenson, J. R., A. A. Axelrad, et al. (1971). "Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro." Proc Natl Acad Sci U S A **68**(7): 1542-6.

Stier, S., Y. Ko, et al. (2005). "Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size." J Exp Med **201**(11): 1781-91.

Sutherland, H. J., C. J. Eaves, et al. (1989). "Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro." Blood **74**(5): 1563-70.

Syed, R. S., S. W. Reid, et al. (1998). "Efficiency of signalling through cytokine receptors depends critically on receptor orientation." Nature **395**(6701): 511-6.

Tripp, A., Y. Liu, et al. (2003). "Human T-cell leukemia virus type 1 tax oncoprotein suppression of multilineage hematopoiesis of CD34+ cells in vitro." J Virol **77**(22): 12152-64.

Udupa, K. B., H. M. Crabtree, et al. (1986). "In vitro culture of proerythroblasts: characterization of proliferative response to erythropoietin and steroids." Br J Haematol **62**(4): 705-14.

Ueda, T., M. Yoshida, et al. (2001). "Hematopoietic capability of CD34+ cord blood cells: a comparison with CD34+ adult bone marrow cells." Int J Hematol **73**(4): 457-62.

von Lindern, M., E. M. Deiner, et al. (2001). "Leukemic transformation of normal murine erythroid progenitors: v- and c-ErbB act through signaling pathways activated by the EpoR and c-Kit in stress erythropoiesis." Oncogene **20**(28): 3651-64.

von Lindern, M., W. Zauner, et al. (1999). "The glucocorticoid receptor cooperates with the erythropoietin receptor and c-Kit to enhance and sustain proliferation of erythroid progenitors in vitro." Blood **94**(2): 550-9.

Weissman, I. L. (2000). "Stem cells: units of development, units of regeneration, and units in evolution." Cell **100**(1): 157-68.

Wessely, O., A. Bauer, et al. (1999). "A novel way to induce erythroid progenitor self renewal: cooperation of c-Kit with the erythropoietin receptor." Biol Chem **380**(2): 187-202.

Wikstrom, L., C. Johansson, et al. (1998). "Abnormal heart rate and body temperature in mice lacking thyroid hormone receptor alpha 1." Embo J **17**(2): 455-61.

Williams, D. E., P. de Vries, et al. (1992). "The Steel factor." Dev Biol **151**(2): 368-76.

Wiznerowicz, M. and D. Trono (2003). "Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference." J Virol **77**(16): 8957-61.

Wu, H., X. Liu, et al. (1995). "Generation of committed erythroid BFU-E and CFU-E progenitors does not require erythropoietin or the erythropoietin receptor." Cell **83**(1): 59-67.

Yoder, M. C. (2004). "Generation of HSCs in the embryo and assays to detect them." Oncogene **23**(43): 7161-3.

Yoshihara, H., F. Arai, et al. (2007). "Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche." Cell Stem Cell **1**(6): 685-97.

Zamzami, N. and G. Kroemer (1999). "Condensed matter in cell death." Nature **401**(6749): 127-8.

Zermati, Y., C. Garrido, et al. (2001). "Caspase activation is required for terminal erythroid differentiation." J Exp Med **193**(2): 247-54.

Zsebo, K. M., D. A. Williams, et al. (1990). "Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor." Cell **63**(1): 213-24.

8 Appendix

8.1 Bio-saftey guidlines for working with lentiviral vectors

MEDIZINISCHE UNIVERSITÄT WIEN

Working with Lentiviral vectors - safety regulations

Be sure that you read and signed the safety rules before starting your work with lentiviruses!!!

A: Working with lentiviruses and cells

1. work with lentiviral particles may only be done in the reserved laminar flow hood in room 5Q.9.06
2. start/duration of work with viral particles must be noted on the biohazard sign on the hood, together with the name of the responsible person
3. all relevant informations regarding virus production, target cell infection, nature of constructs must be noted in the log book immediately after completion of work.
4. all cell culture vessels must be labelled with date, name and room number (e.g. 5Q.012)
5. NO open vessels may be taken out of the hood
6. NO aerosol-producing steps (vortexing, resuspension of cells by pipetting) may be done outside the laminar flow hood
7. for transfer of culture vessels from the hood to the incubator or to the inverted microscope, leak-proof boxes have to be used to avoid spillage
8. all liquid waste has to be decontaminated with 10 % hypochloride solution and autoclaved
9. all cell culture plasticware has to be decontaminated with 10% hypochloride and autoclaved
10. full (liquid/solid) waste containers must be autoclaved immediately (use 2 waste bags for solid waste).
11. all working materials (unused tubes, pipetboys, tip boxes) which are removed from the hood must be cleaned with a viricidal disinfectant
12. after completion of work, the hood must be cleaned with a viricidal disinfectant

B: Personal protective equipment

13. lab coats and double gloves (the lower pair taped to the coat) are mandatory when working with virus

14. upper set of gloves must be removed and discarded when leaving the hood and discarded in the plastikwaste and autocleved.

15. safety goggles should be worn

16. double pair of gloves, laboratory coats and security glasses have to be used by every researcher

C: In case of spillage:

17. be prepared for spillage!!!

18. this means that paper towels and viricidal disinfectant must be available when working with virus

19. inform other people and close off the area

20. suck up spills with paper towels, apply disinfectant, leave for the recommended time, remove disinfectant, wipe dry

D: Further guidelines:

21. plan your experiments thoroughly

22. inform person responsible for lentiviral working area before starting work with lentivirus

23. check for the availability of hypochloride, viricidal disinfectant, waste bags etc. BEFORE starting your work

24. work slowly and concentrated

25. be prepared for spills

26. transfer all materials needed to the hood before starting work

E: Rules for flow cytometry:

27. virally infected cells must be fixed before flow cytometry and the cytometer must be flushed thoroughly with bleach after analysis

28. capped FACS tubes should be used

29. cell sorting: sorting of virally infected cells has to be arranged with sorting facility according to their safety regulations; some institutions allow cell sorting 7 passages after infection.

CURRICULUM VITAE

Ramona Seba

Kandlgasse 14/14
A-1070 Wien
www.mfpl.ac.at/mullner

e-mail: ramona.seba@univie.ac.at
phone: +43 1 4277-61761
mobile: +43 699 1100 7528

PERSONAL DATA

Date of Birth: 18.04.1982
Place of Birth: Steyr, Austria
Citizenship: Austria

EDUCATION

2007-2008	Diploma thesis in the lab of Ernst Müllner at the Max F. Perutz Laboratories (MFPL), Medical University of Vienna
2003 – 2008	Study of Biology at the University of Vienna
2001 – 2003	Study of “Quality Journalism & New Technologies“ at the Danube University Krems (Austria) Graduated in January 2003 with distinction
2001	University Entrance Diploma at the “Höhere Bundeslehranstalt für wirtschaftliche Berufe“ (HLW) in Stadt Haag (Austria)

FURTHER EXPERIENCES

December 2007	Tutor for the lab course “Forward and reverse genetics” at the University of Applied Sciences Vienna
September 2007	Congress of the „Society of Hematology and Stem Cells“ in Hamburg (Germany)
Summer 2006	Internship in the lab of Irute Meskiene (MFPL)