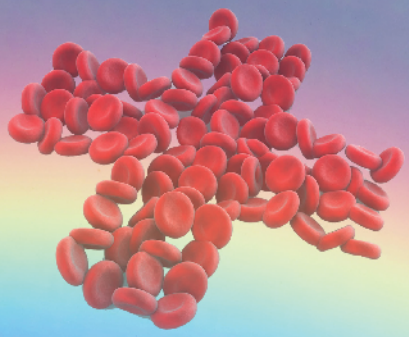

Erythropoiesis and Hemoglobin Regulation: A journey from laboratory to disorders

Sahar Esteghamat

Erythropoiesis and Hemoglobin Regulation: A journey from laboratory to disorders

Fatemehsadat (Sahar) Esteghamat Hanzaei

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Hemoglobin Regulation and Erythropoiesis: A journey from laboratory to disorders

Hemoglobine regulering en erytropoiese: Een reis van het laboratorium naar ziektes

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The intellect combed the fields high and low
But splitting hairs was all it could show
In my heart a thousand suns shone
Yet the mystery of the atom was not undone

(Avicenna, a Persian polymath, 980– 1037)

-- Rendered from Persian to English by A.M.A. Imam

To my ultimate sources of motivation,
my younger sister, Sepideh
and all those who like her merit welfare

AND

to my deeply respected parents,
greatly appreciating their unflinching support in all my endeavors

Contents

List of Abbreviations

AGM	Aorta-Gonad-Mesonephros
Bcl11a	B-cell CLL/lymphoma 11A
BFU-E	Burst-Forming Unit-Erythroid
BMCP	Basophil Mast cell Common Progenitor
BMT	Bone Marrow Transplantation
CBC	Complete Blood Count
CD	Cluster of Differentiation
CDA	Congenital Dyserythropoietic Anemia
CFU-E	Colony-Forming Unit-Erythroid
CLP	Common Lymphoid Progenitor
CMP	Common Myeloid Progenitor
CoREST	Rest Repressor Complex
DMSO	Dimethyl Sulfoxide
DNMT	DNA Methyl-Transferase
DRED	DR-binding protein
EPO-R	Eryhtropoietin receptor
ES-EP	Embryonic Stem cell derived Erythroid Progenitor
FL	Fetal Liver
GFP	Green Fluorescent Protein
GMP	Granulocyte Myeloid Progenitor
GPA	Glycophorin A
GWAS	Genome Wide Association Studies
Hb	Hemoglobin
HbA	major Adult Hemoglobin
HbA_2	minor Adult Hemoglobin
HBB	Human beta globin
HBD	Human delta globin
HBE	Human epsilon globin
HbF	Fetal Hemoglobin
HBG	Human Gamma Globin
HCT	Hematocrit
HDAC	Histone Deacetylase
HEP	Human Erythroid Progenitor
HFL	Human Fetal Liver
HGB	Hemoglobin
HMG20A	High Mobility Group 20A
HMG20B	high Mobility Group 20B
HPLC	High Performance Liquid Chromatography
HS	Hypersensitivity Site
HTN-Cre	His-TAT-NLS Cre recombinase
HU	Hydroxyurea
KI	Knock In
Klf1	Kruppel-like factor
KO	Knock Out
LCR	Locus Control Region
LDB	LIM Domain Binding protein
Lmo2	LIM domain Only 2
LSD1	Lysine (K)-Specific Demethylase 1A
LT-HSC	Long term-Hematoopoietic Stem Cell
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Cell Volume
MEP	Megakaryocyte Erythroid Progenitor
MEP	Mouse Erythroid Progenitor
MFL	Mouse Fetal Liver
MPP	Multipotent Progenitor
NK	Natural Killer cell
NLS	Nuclear Localization Signal
PLT	Platelets
RBC	Red Blood Cell
RCOR1	REST corepressor 1
SCA	Sickle Cell Anemia
SCD	Sickle Cell Disease
shRNA	short hairpin RNA
ST-HSC	Short term-Hematopoietic Stem Cell
WBC	White Blood Cell
YS	Yolk Sac

Scope of this thesis

The hematopoietic system provides one of the most attractive systems for studies on development at both levels of molecular characterization and systems biology. Among the single-gene related disorders, hemoglobinopathies ranked on top with the prevalence of 4.83% in the world population (Urbinati et al., 2006). These reasons underlined the need of discovering the mechanisms underlying hemoglobin regulation and erythropoiesis. This thesis describes a journey beginning with an attempt to establish an *in vitro* hematopoietic progenitor cell system, passing through the transcriptional complexes regulating general aspects of erythropoiesis including the regulation of globin gene expression. It ends with a proposal to select patients with β-thalassemia for hydroxyurea treatment.

Chapter 1 provides a general introduction to research questions addressed in the experimental Chapters:

2: Are HoxB4 cells a useful model for multipotent hematopoietic progenitors?

3: Hmg20b is a repressor of erythroid differentiation.

4: The role of the Klf1/Bcl11a axis in erythropoiesis and hemoglobin regulation.

5: Proposal for selection of patients with β-thalassemia for hydroxyurea treatment.

Chapter 6 is a general discussion of the achievements described in this thesis, providing possible directions for continuation of this journey.

Introduction

General aspects of hematopoiesis

Hematopoiesis is the process through which all mature blood cells are produced. Hematopoietic stem cells (HSCs) reside at the apex of the hematopoietic hierarchy. HSCs are capable of self-renewing and generating all lineages of blood cells throughout life. To support growth of the embryo, hematopoietic cells are generated early during development (Morrison and Spradling, 2008; Steinberg, 2009).

HSCs are maintained as a quiescent population and their numbers in the bone marrow and circulation are tightly regulated (Jude et al., 2008). The HSC pool is heterogeneous and is generally divided into two populations: clonogenic long-term self-renewing HSC (LT-HSC) and transiently self-renewing HSC (short-term HSC) (Morrison and Weissman, 1994). The immediate progeny of HSCs are multipotent progenitor cells (MPPs) which maintain full lineage potential but have limited self-renewal capacity. MPPs produce pluripotent progenitors which are more restricted in lineage development (Warr et al., 2011).

The final lymphoid lineage products include natural killer (NK) cells and two major types of lymphocytes: firstly, B lymphocytes or B cells, in which antigen confrontation results into their activation and further differentiation into antibody-producing plasma cells; and secondly, T lymphocytes or T cells, which are involved in cell-mediated immunity (Janeway, 2001). Lymphocytes appear in human embryos in the fetal liver, gut-associated lymphoid tissue, thymus and lymphatic plexuses around week 7 after gestation (Steinberg, 2009; Wojchowski et al., 1999). In mice, the first T-lymphocytes, with no known function, appear in the yolk sac at E8.5 (Eren et al., 1987a; Eren et al., 1987b; Liu and Auerbach, 1991) and the first B lymphoid potential cells are found at E9.5 (Ogawa et al., 1988; Steinberg, 2009).

The first monocyte-macrophage committed cells are observed in human embryos at week 4-5 (Steinberg, 2009; Wojchowski et al., 1999). Two separate populations of macrophages have been reported in mouse embryos: primitive macrophages which appear first at E9 in the yolk sac, supposedly arising from

local precursors, and the monocytic lineage of macrophages arising from fetal liver and yolk sac at E10 (Naito et al., 1996). It has been proposed that the origin of adult macrophages is from the aorta-gonad-mesonephros (AGM) monocytic precursors (Bonifer et al., 1998; Steinberg, 2009). In adults, the myeloid progenitor is the precursor of the granulocytes, macrophages, dendritic cells, and possibly mast cells of the immune system (Janeway, 2001).

Dendritic cells may originate from either myeloid- or lymphoid- committed progenitors. The origin of mast cells and basophils is still a mystery in hematology. Mast cells and basophils are supposed to be closely related since they both are enriched with histamine and heparin granules. It is suggested that they come from the same ancestor called basophil/mast cell progenitors (BMCP). Alternative models propose a distinct mast cell committed progenitor giving rise to mature mast cells (Arinobu et al., 2009).

Figure 1 summarizes the hematopoietic hierarchy. Different roadmaps have been proposed for the hematopoietic hierarchy. The conventional hypothesis defines the origin of common myeloid and lymphoid progenitors (CMP/CLP) from the multipotent progenitor cells. More recent findings demonstrate the derivation of megakaryocyte-erythroid progenitors and lymphoid-primed multipotent progenitors from multipotent progenitors (Adolfsson et al., 2005; Arinobu et al., 2009).

Erythrocytes are the first differentiating hematopoietic cells appearing in mouse embryos at E7-7.5 (Houssaint, 1981; Johnson and Moore, 1975; Suzuki et al., 2007) and in human embryos at E16-20 (Perutz et al., 1960; Steinberg, 2009; Wojchowski et al., 1999). Red blood cells and megakaryocytes originate from the same ancestor megakaryocyte erythroid progenitor (MEP) cells. In adults, red blood cells are produced in a specialized niche in the bone marrow, which are called erythroblast islands, adjacent to macrophages and other cell types constituting the hematopoietic system. Per second, every healthy adult human generates two million red blood cells. In mammals, erythrocytes expel their nucleus and remove their organelles before entering the circulation (Palis, 2004).

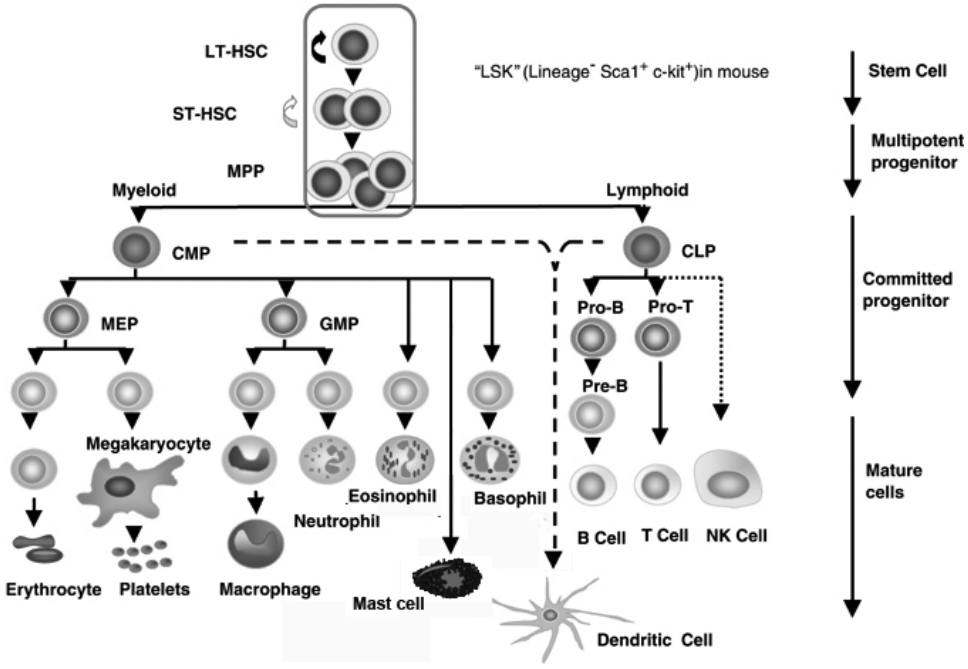

Figure 1: The hematopoietic system hierarchy; depicting the various hematopoietic cells derive from the same HSC ancestors. The HSCs generate multipotent progenitors which are committed to a specific lineage. HSC, hematopoietic stem cell; LT, Long term; ST, short term; HSCs, Hematopoietic stem cells; MPP, multipotent progenitors; MEP, megakaryocytic/erythroid progenitor; CLP, common lymphoid progenitor ; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; NK, natural killer; Lin, lineage markers. [Drawing has been adapted and modified from (Larsson and Karlsson, 2005)].

Hematological disorders include various autoimmune disorders, malignancies such as leukemias and lymphomas, erythroid-related disorders and dozens of other types of disorders.

This thesis focuses on general aspects of erythropoiesis, transcription factors regulating this process, globin switching and finally hemoglobin-related disorders.

Animal models to study the ontogeny of hematopoiesis

In non-mammalian vertebrates, the onset of erythropoiesis has been widely studied in chicks and frogs. Concerning the studies on early chick embryos, It has been

known for almost a century that the first blood cells originate from the yolk sac (Sabin, 1920). In 1975, using quail-chick chimeric embryos, Francoise Dieterlen-Lievre showed that adult hematopoiesis has its origin inside the chick embryo (Dieterlen-Lievre, 1975). In addition, *Xenopus* frogs have been widely employed as a model system to investigate the embryonic origins of hematopoietic stem cells. The early developmental stages of frogs are outside the mother and easy to approach thus providing an attractive model for experimentation. Accordingly, it facilitates the manipulation and grafting of cells to investigate the early commitment and fates of hematopoietic lineages (Miale, 1982). Hematopoietic emergence sites in frog, chick and mouse have been depicted in Figure 2.

Studies on mice and humans marked the yolk sac as the first resource of hematopoietic cells. Detailed analysis of mice depicted the first intra-embryonic derived hematopoietic cells originating from mesodermal germ layer cells and throughout development they migrate to at least four hematopoietic colonization sites (Steinberg, 2009). Remarkably, there are two main waves of erythropoiesis known as primitive and definitive erythropoiesis. These display distinct phenotypes which will be discussed below.

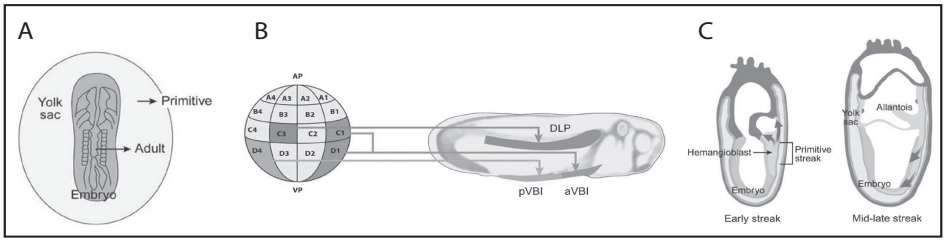

Figure 2: the onset of hematopoiesis in chick (A), frog (B) and mouse (C). A) Grafting a quail-chick embryo body points at the yolk sac generating primitive erythrocytes and later, adult erythrocytes generate from the conceptus. B) Tracing the cell fates of Xenopus using the genetic labeling approach at an embryonic 32-cell stage (left) shows that C3 blastomere gives rise to the dorsal aorta and circulating cells at stage 41-cell. C) Early in gestation, the first hemangioblasts derives from the primitive streak giving rise the primary HSCs (left) and later, these cells migrate from the primitive streak to the mid- to late-streak stage (right). The arrows indicate the emigration path of the first HSCs**.** [Drawing has been adapted and modified from (Dzierzak and Speck, 2008)].

Primitive erythropoiesis

It has been proposed almost a century ago that primitive erythrocytes and endothelial cells are derived from the same ancestors, the hemangioblasts (Sabin, 1920). The first primitive wave of erythropoiesis happens in yolk sac at E7.5. It is unlikely that these cells share a common ancestor with definitive hematopoiesis appearing from the AGM at E10.5 (Dzierzak and Speck, 2008). Further attempts to delineate the origin of the hemangioblasts using mesodermal markers such as Brachyury and fetal liver kinase1 ($Bra^+/Flk1^+$) suggested that these cells first appear in the mid-streak of gastrulating mouse embryo (Huber et al., 2004). It may imply that the first stage of hematopoiesis development takes place before the cells migrate to the yolk sac mesoderm (Orkin and Zon, 2008). In the yolk sac, the hemangioblasts form blood islands (Haar and Ackerman, 1971) and transform to primitive erythrocytes and endothelial cells (Palis and Yoder, 2001). Morphologically, these cells are enucleated and larger than definitive erythrocytes. They express embryonic globins, which will be discussed later in the hemoglobin switching part of this thesis.

Definitive erythropoiesis

The primitive erythrocytes are only generated transiently and this is followed by the production of definitive hematopoietic progenitors (Palis and Koniski, 2005; Palis et al., 1999; Palis and Yoder, 2001). The onset of definitive erythropoiesis is at E10 which is linked to the appearance of small enucleated bi-concaved red cells in the circulation (Houssaint, 1981; Johnson and Moore, 1975).

At embryonic day E8.5, the first signs of long term-hematopoietic stem cells (HSCs) appear in the AGM region, which are originating from hemogenic endothelial cells (Medvinsky and Dzierzak, 1996). All HSCs in the AGM are positively characterized by $CD45^+/CD41^+/CD34^+/Sca\text{-}1^+/c\text{-}Kit^+$ surface markers (de Bruijn et al., 2002; Ma et al., 2002; Mikkola et al., 2003; North et al., 2002). However, genetic labeling of Runx1 and further using it to trace HSCs confirmed the

presence of definitive erythrocytes in the yolk sac. This observation considered the yolk as the primary source of HSCs rather than the AGM (DeWitt, 2007). However, it is debatable whether tracingg the Runx1 expression provides correct information on the ontogeny of HSCs.

Further residences of HSCs are the placenta and fetal liver (Ottersbach and Dzierzak, 2005). The placenta mediates fetal-maternal exchange during pregnancy and produces cytokines and growth factors which may play a role in hematopoiesis (Cross et al., 2003; Dancis et al., 1977; Gekas et al., 2010). Early multi-lineage progenitors appear in the placenta at E9 and mature HSCs are present at E10.5-11 (Alvarez-Silva et al., 2003; Gekas et al., 2005; Mikkola et al., 2005). Placental HSCs either generate *de novo*, or originate from the AGM (Gekas et al., 2010; Mikkola and Orkin, 2006; Sadler and Langman, 2010). After E13.5, the HSC pool in the placenta decreases while it is expanding in the fetal liver (Gekas et al., 2005; Ottersbach and Dzierzak, 2005).

The fetal liver does not generate HSCs *de novo* and circulating hematopoietic stem cells emerging from the yolk sac, the AGM, the placenta comprise the first HSCs pool in mouse fetal liver (Houssaint, 1981; Johnson and Moore, 1975; Kumaravelu et al., 2002; Ottersbach and Dzierzak, 2005).

Shortly before birth, HSCs move to bone marrow and spleen, and thymus where they remain quiescent and further stimulating signals propel them to generate lineage-restricted hematopoietic progenitors (Dzierzak and Medvinsky, 1995; Medvinsky et al., 2011). The ontogeny of hematopoiesis has been summarized in Figure 3.

Hematopoietic stem cell niches

The cells existing adjacent to the hematopoietic stem cells form the hematopoietic stem cell niche (Schofield, 1978). Studies on genetically modified mice demonstrated the influence of osteoblasts and endothelial cells on HSCs function through Notch, BMP, Tie2, Ang1, NF-kappaB signaling (Arai et al., 2004; Arai et al., 2009; Calvi et

al., 2003; Ehninger and Trumpp, 2011; Jeong et al., 2009; Levesque and Winkler, 2011; Nilsson et al., 1997; Xiao et al., 2009; Yoshihara et al., 2007; Zhang et al., 2003a).

Erythropoiesis and mammalian experimental models

Erythropoiesis

Human blood contains approximately $5x10^6$ erythrocytes per microliter and their average lifespan is 120 days in a healthy adult individual (Steinberg, 2009). Proerythroblasts are the earliest morphologically recognizable cells committed to the erythroid lineage. During their development towards enucleated red cells, proerythroblasts differentiate to basophilic, polychromatic and orthochromatic erythroblasts (Figure 4). Eventually, orthochromatic erythroblasts undergo a series of remarkable changes including enhanced chromatin condensation, expelling their nucleus and degradation of all other organelles. Coordinated regulation of the genes involved in hemoglobin synthesis and membrane assembly propel the cells to transform to the highly hemoglobinized bi-concave cells with a large surface area for gas exchange. This ordered differentiation process is commonly referred to as terminal erythroid differentiation (An and Mohandas, 2011).

Erythroid differentiation incudes changes in the expression of cell surface markers such as decreased in cKit expression, while transferrin receptor (CD71) expression is high from proerythroblasts to orthochromatic erythroblasts. The final maturation step of reticulocytes includes suppression of CD71 and overexpression of Ter119 in mouse and GPA in human erythrocytes. These markers are widely used to study mouse and human erythropoiesis (Chen et al., 2009a) (Figure 4).

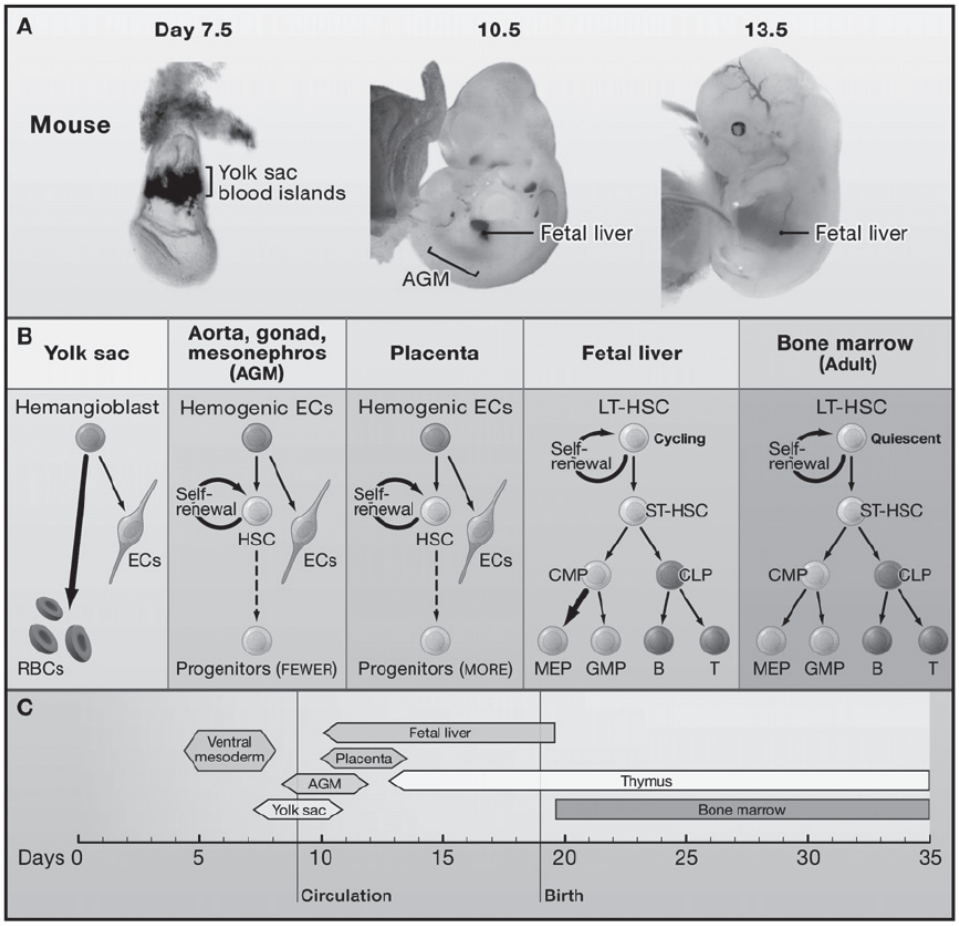

Figure 3: Hematopoietic development in mouse. A) Hematopoiesis firstly is visualized in the yolk sac (YS) at E7.5, afterward hematopoietic stem cells (HSCs) migrate to aorta-gonad mesonephros region (AGM), placenta and fetal liver (FL). B-C) It shows the different blood lineage (B) and the duration of hematopoiesis in each related sites (C). RBC, red blood cells; LT-HSC, long-term hematopoietic stem cell; ST-HCS, short-term hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megacaryocyte, erythroid progenitor; GMP, granulocyte/macrophage progenitor (Orkin and Zon, 2008).

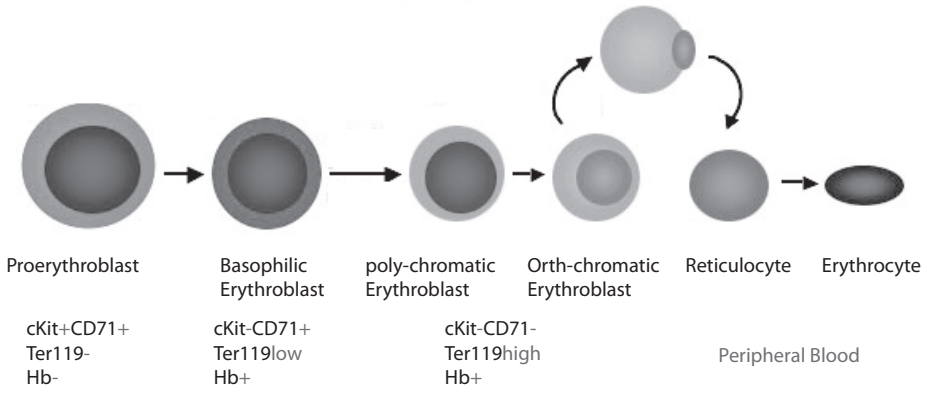

Figure 4: Definitive erythroid differentiation in the mouse. Definitive erythropoiesis involves the progressive differentiation steps from a basophilic, polychromatic, to an orthochromatic erythroblasts. The orthochromatic erythroblasts expel their nucleus to become reticulocytes that structurally change to bi-concave mature erythrocytes. Peripheral blood consists of reticulocytes and erythrocytes.

Hemoglobin

Hemoglobin (Hb), the major component of red cells, is a metalloprotein composed of four globin peptides and mediates oxygen delivery to all tissues and CO_2 to the lungs (Beaven and Gratzer, 1959a, b). The globin protein includes 8 stretches of α-helices (Antonini and Brunori, 1971). These helices are folded into a compact globule that heterodimerizes and then forms the tetrameric structure (Figure 5). In highly specialized mammalian enucleated erythrocytes, these molecules are expressed at a very high level (Schechter, 2008); each human erythrocyte contains ~$250x10^6$ hemoglobin molecules.

Mammalian erythropoiesis models

Animal model

The mouse is widely used as the model organism most closely related to humans. Its small size, short duration of pregnancy and high number of pups makes it suitable as an experimental model. Genetically engineered knockout (KO)

or knockin (KI) mice have facilitated the tracing of erythroid tissues at different developmental stages. If absence of a certain gene results in lethality (Brakebusch and Pihlajaniemi, 2011).

Human/Mouse primary cell culture

Bone marrow and spleen cells can be resuspended in a properly defined medium and expanded in a culture. They are also employed for direct colony assay on mouse/human bone marrow and spleen for burst forming unit-erythroid (BFU-E) and colony forming unit-erythroid (CFU-E) assays in methyl cellulose cultures. BFU-Es are the earliest and CFU-Es are late progenitors with exclusive erythroid potential (Steinberg, 2001, 2009).

Human or mouse erythroid progenitors can be obtained from different sources and expanded for a limited time in culture. Some examples are listed below:

- MFL: Proerythroblasts obtained from mouse fetal liver at E12.5-13.5 which can be expanded up to 10^7 fold (Dolznig et al., 2001; von Lindern et al., 2001)

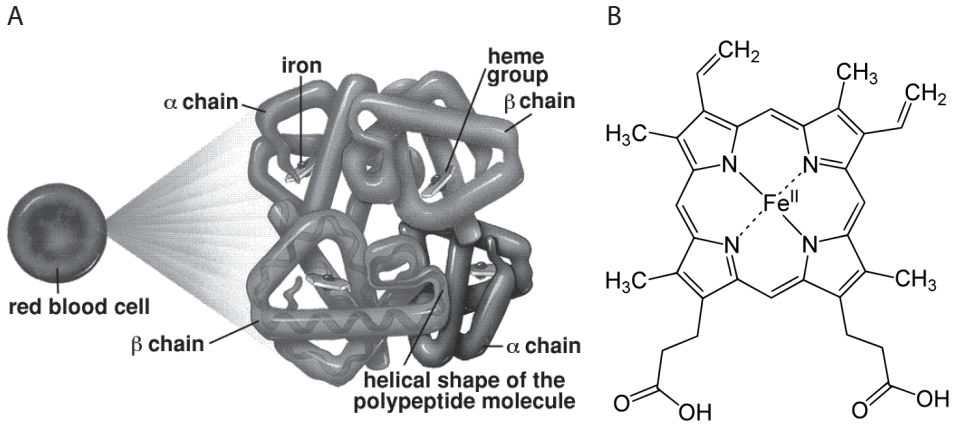

Figure 5; Hemoglobin molecule structure. A) Hemoglobin molecule contains four globin subunits, with an embded heme group in each globin polypeptide chain (Sylvia S.Mader, inquiry info life, 8th edition). Heme binds to one oxygen molecule. B) Molecular structure of heme group (http://rmgh.net/wiki/index.php?title=Hemoglobin)

- HFL: Proerythroblasts obtained from human fetal liver cells in week 16-19 of gestation (http://encodeproject.org/ENCODE/protocols/cell/human/FetalPBDE_Farnham_protocol.pdf)
- MEP/HEP: erythroid progenitors derived from human/mouse adult bone marrow or mononuclear cell fraction from peripheral blood. (Giarratana et al., 2005; Leberbauer et al., 2005; van den Akker et al., 2010)

Cell lines

MEL (murine erythroleukemia) cells, firstly introduced by Friend et al in the 1970s, reproduce some aspects of normal erythroid differentiation after induction by di-methyl sulfoxide (DMSO) (Friend et al., 1971). MEL cells are widely used as an *in vitro* model for analysis of erythropoiesis (Boyer et al., 1972; Scher et al., 1971; Scher et al., 1973). Other hematopoietic cell lines cells for the study of erythropoiesis were introduced later, as listed below:

- K562: the first human immortalized myelogenous leukemia cell line derived from a 53 year old female patient with chronic myeloid leukemia (CML) (Lozzio and Lozzio, 1975)
- HEL: human erythroid leukemia cell line (Papayannopoulou et al., 1983)
- MB-02: human megakaryocytic leukemia cell line (Munshi et al., 1993)
- UT-7: A murine bone marrow-derived stromal cell line (Komatsu and Fujita, 1993)
- G1E cells: erythroblasts derived from Gata1-null embryonic stem cells, exogenously expressing human Bcl-2 and, in the case of G1E-ER4 cells, estrogen-inducible Gata1 (Weiss et al., 1997)
- ES-EP: embryonic stem cell-derived erythroid progenitors (Dolznig et al., 2001)
- I/11 cells: Immortalized proerythroblasts derived from p53 *null* mouse fetal liver (Dolznig et al., 2001).
- ES-HoxB4 cells: Hematopoietic cells derived from HOXB4-expressing

mouse embryonic stem cells; it has been shown that they recapitulate some hematopoietic stem cell characteristics in culture (Pilat et al., 2005).

The terminal differentiation process includes 3-4 cell divisions with loss of cell size control, after which the erythroblasts arrest in the G1 stage of the cell cycle and eventually enucleate (Dolznig et al., 2002). Stimulating MEL cells with DMSO results in modest hemoglobinization and only a small percentage of the cells may enucleate. In contrast, induction of I/11 cells using Epo results in strong hemoglobinization and enucleation of the majority of the cells (Dolznig et al., 2001). I/11 cells therefore represent a more physiological model of erythropoiesis than MEL cells.

Chapter 2 provides the data of an attempt to establish a hematopoietic system in vitro using ES-HOXB4 cells. The original embryonic stem cells were genetically modified using knock in technology to express HA-BirA. BirA is an abbreviation for the bacterial biotin ligase which biotinylates any protein tagged with a bio-peptide. This system could serve as a model to trace a transcription factor and its interacting partners during hematopoietic cell fate decisions and in committed lineages.

Regulation of erythropoiesis

Erythropoiesis is mediated by several signaling pathways including phosphinositide-3 (PI-3) kinase, JAK-STAT, Src-kinases and phospholipase Cγ (Larsen and Ropke, 2002; Rane and Reddy, 2002; Richmond et al., 2005). In addition, several hormones are known to modulate erythropoiesis, as exemplified by the high co-incidence of thyroid disorders with anemia due to tri-iodothyronine, the effect of glucocorticoids on proliferation of proerythroblasts, the response of bone marrow to parathyroid hormone (PTH) release, and the role of T_3 receptor signaling in transient spleen erythropoiesis in mice (Angelin-Duclos et al., 2005; Dainiak et al., 1978; Dolznig et

al., 2001; Golde et al., 1977; Perris et al., 1971; Steinberg, 2009; Tang et al., 2011; von Lindern et al., 2001).

Hypoxia activates a cascade of transcription factors including the HIF1 complex which results in Epo secretion from the kidneys (Grimm et al., 2002; Lee and Percy, 2011; Mazure et al., 2004). Epo is a major physiological cytokine binding to the erythropoietin receptor (EpoR) on erythroid progenitors (Elliott et al., 2010). This leads to phosphorylation of Jak2 resulting in JAK/STAT activation; it also might be involved in RAS signaling (Fried, 2009; Marzo et al., 2008; Steinberg, 2009). This promotes survival and proliferation of the erythroid progenitors, thus enhancing erythroid output.

Hemoglobin regulation

The human β-globin locus is comprised of five developmentally regulated genes in the order 5'-ε-^{G}γ-^{A}γ-δ-β-3'. This is similar to the mouse β-globin locus which consists of 4 genes in the order 5'-εy-βh1-β1-β2-3'. The α–globin locus in human and mouse are composed of an embryonic ζ- and two α-globin genes (Liebhaber and Russell, 1998). In mice εy-, βh1-, ζ- and α-globins are expressed in primitive erythrocytes. Their expression is silenced in the definitive erythrocytes with dominant expression of α-, β1- and β2 globins, in both of the fetal and adult stages of development (Brotherton et al., 1979; Whitelaw et al., 1990). Human primitive erythrocytes express Hb Gower1 ($\zeta_2\varepsilon_2$), Hb Gower2 ($\alpha_2\varepsilon_2$), and Hb Portland ($\zeta_2\gamma_2$).

During development, mice complete one switch from embryonic globins immediately to fetal/adult globins. In contrast, humans firstly switch from ε andζ, the major primitive red cell globins, to α and γ at the fetal stage. Shortly after birth, a second switch occurs when γ-globin is replaced by β- and δ-globin. Thus, in humans HbF ($\alpha_2\gamma_2$) is the dominant hemoglobin when the fetal liver is the site of erythropoiesis. After birth, HbA ($\alpha_2\beta_2$) becomes the dominant hemoglobin when the major site of erythropoiesis shifts to the bone marrow. In a healthy adult, HbA ($\alpha_2\beta_2$), HbA_2 ($\alpha_2\delta_2$), HbF ($\alpha_2\gamma_2$) account for 97%, ~2% and <1% of all globins, respectively (Schechter, 2008) (Figure 6).

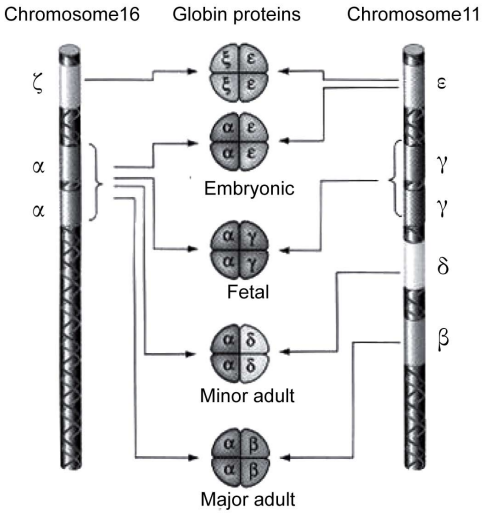

Figure 6: Human globin expression upon development. Embryonic globins: Hb Gower1 ($\zeta_2\varepsilon_2$), Hb Gower2 ($\alpha_2\varepsilon_2$); fetal globin: HbF ($\alpha_2\gamma_2$); Adult globins: HbA ($\alpha_2\beta_2$), HbA_2 ($\alpha_2\delta_2$) (Gilbert, 2010).

Hemoglobin switching

Considering that re-activation of γ-globin expression is a very attractive therapeutic approach for patients with β-thalassemia and sickle cell disease, discovering the mechanisms underlying hemoglobin switching is of outstanding demand. Accordingly, human β-globin locus transgenic mice have been established as models for developmental regulation of globin gene expression. These mice complete hemoglobin switching before birth around E13.5 in the fetal liver. While this is different from the developmental timing of switching in humans, such mice still provide very valuable models to obtain more knowledge of the underlying molecular regulatory mechanisms (Behringer et al., 1990; Chan et al., 2008; Dillon and Grosveld, 1991; Patrinos et al., 2004) (Figure 7). It has been proposed that this different pattern of globin switching in human and mice is due to the divergent stage specific regulation by Bcl11a (Sankaran et al., 2009). It will be discussed more in detail later in this chapter.

LCR and globin regulation

Developmental and cell lineage-specific regulation of gene expression relies not only on gene-proximal elements such as promoters, enhancers, and silencers, but also on long-range interactions of various cis-regulatory elements and dynamic chromatin alterations. Locus control regions (LCRs) are a class of long-distance cis-regulatory elements that enhance the expression of linked genes at any ectopic chromatin sites to tissue-specific levels and do so in a copy number-dependent manner (Li et al., 2002).

The human β-globin LCR, first described in 1987, is located at 5-25kb upstream of the ε-globin gene promoter and is found associated with the active globin genes (Fraser and Grosveld, 1998; Grosveld et al., 1987). It comprises five DNase I hypersensitive sites (HS1-HS5) that each encompass a ~300bp region containing binding sites for erythroid-specific transcription factors and other ubiquitous DNA-binding proteins (Grosveld et al., 1990; Philipsen et al., 1990; Talbot et al., 1990) (Figure 8). The human α-globin LCR, termed HS-40, has been identified by DNase I hypersensitive site mapping (Higgs et al., 1990). Deletions of HS-40, resulting in severely reduced α-globin expression, have been reported in patients with α-thalassemia (Hatton et al., 1990; Liebhaber et al., 1990). Deletion of the β-LCR alters chromosomal replication timing (Forrester et al., 1990) and globin expression. For example deletion of 5'HS1 (5'ΔHS1) resulted in 16-fold upregulation of ε-globin expression but it did not alter γ-globin expression in primitive erythrocytes. In contrast, γ-globin expression was reduced 4-fold in definitive erythrocytes (Fedosyuk and Peterson, 2007). 5'ΔHS2 does not affect timing of globin expression, but it results in decreased ε-, γ-, and β-globin expression at all developmental stages (Fiering et al., 1995; Peterson et al., 1996). Remarkably, it is the only HS showing very strong enhancer activity due to the tandem binding of NF-E2-AP1 (Ney et al., 1990). 5'ΔHS3 results into a significant decrease in ε-globin and dramatic increase of γ-globin expression (Peterson et al., 1996).

Mice carrying 5'ΔHS4 demonstrated a two days delay in globin switching and

slightly decreased in β-globin expression (Fedosyuk and Peterson, 2007). Finally, studies on mice carrying a human β-locus with a conditional HS5 deletion (5'ΔHS5) demonstrated that HS5 has no discernable activating activity in adult erythroid cells. HS5 functions as a developmental stage-specific border in erythroid cells (Wai et al., 2003).

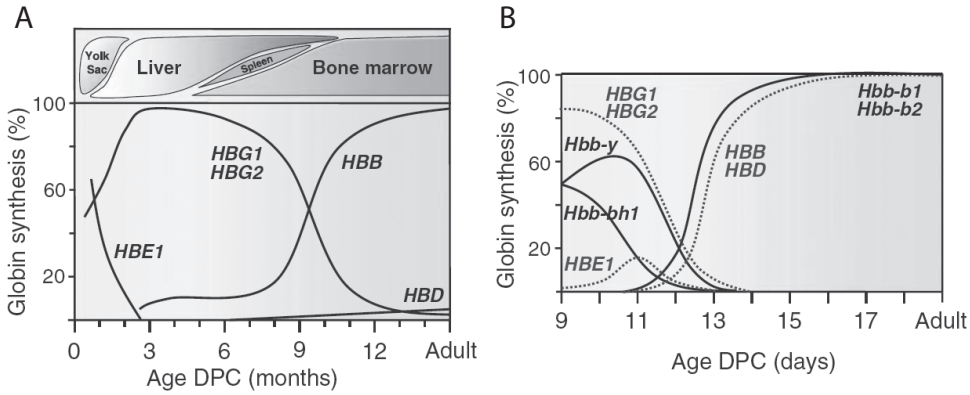

Figure 7: Hemoglobin switching in human and human β-globin locus carrying mice. A) Hemoglobin switching takes place two times in human, 1- Around 6 weeks when expression of embryonic globin silences and fetal globin actively expresses upon migration of hematopoietic site to the fetal liver; 2- After birth, when fetal globin expression declines to <1% of total hemoglobin. B) The dynamics of mouse and human globins expression in mice carrying a β-globin locus transgene. Drawings adapted from (Sankaran et al., 2010).

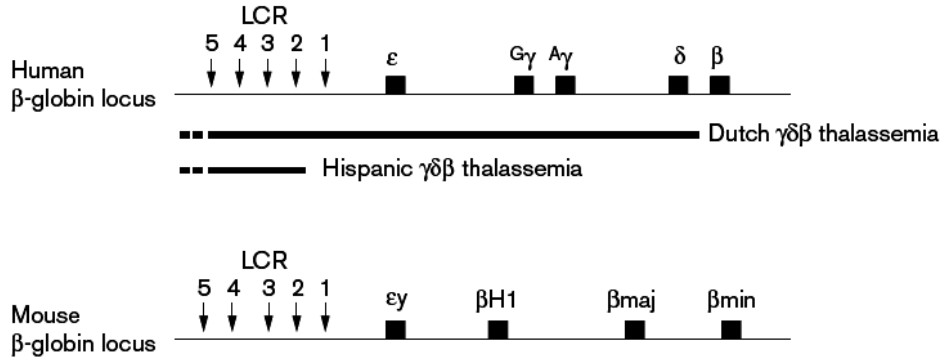

Figure 8: The position of the locus control region in the human and mouse β-globin locus. DNase I hypersensitive assay showed the position of locus control regions at upstream of globin genes, in both human and mouse 5 hypersensitive sites are recognizable at upstream of ε-globin region (Grosveld, 1999).

Transcriptional regulation of erythropoiesis

The establishment of cell identity and its maintenance is dependent on the activity of specific transcription factors during development and the action of epigenetic mechanisms that globally regulate gene expression. This network is comprised of different transcription factors which recognize blocks of *cis*-regulatory elements. Hematopoietic lineage commitment is achieved by fine-tuned expression of certain transcription factors. For example, short-time expression of PU.1 leads to the formation of immature eosinophils, whereas low level expression generates the lymphoid lineage and long-term expression results into myeloid commitment of hematopoietic stem cells (Engel and Murre, 1999; Nerlov and Graf, 1998; Scott et al., 1994). Tal1 (a basic helix loop helix factor), Gata2 and Lmo2 (LIM-only domain family) are examples of essential transcription factors involved in both primitive and definitive hematopoiesis. Tal1$^{-/-}$ mice die at E9.5 and similarly Lmo2$^{-/-}$ embryos at E10.5, both displaying aberrant hematopoiesis (Bockamp et al., 2009; Patterson et al., 2007; Tijssen et al., 2011; Warren et al., 1994). Gata2 is expressed early in stem cells and is down-regulated as erythroid differentiation occurs (Tsai et al., 1994; Tsai and Orkin, 1997). In contrast, Runx1 is not necessary for primitive erythropoiesis, but it plays an essential role in generation of definitive hematopoietic progenitors and long-term repopulating hematopoietic stem cells (North et al., 2002). Runx1$^{-/-}$ mice die at E12.5 due to lack of fetal liver erythropoiesis and they show dramatically reduced Gata1 expression (Chen et al., 2009b; Yokomizo et al., 2008). Gata1 is expressed in erythroid-megakaryocytic committed cells, eosinophils, mast cells, dendritic cells and in the Sertoli cells of the testis (Ferreira et al., 2005). Mouse embryos with a Gata1 *null* mutation die at E10.5-11.5 due to severe anemia (Fujiwara et al., 1996). Remarkably, GATA1 suppresses the expression of GATA2 (Bostick et al., 1999) which is highly expressed in the hematopoietic stem cells and plays a major role in their maintenance (Tsai and Orkin, 1997). Interestingly, it has been shown that Gata2 expression can rescue the lethal phenotype of Gata1-null

mice. This highlights the importance of Gata transcription factor expression levels, rather than their identity, in hematopoietic development (Ferreira et al., 2007).

Many multi-protein chromatin complexes are involved in erythroid differentiation e.g. NuRD, SWI/SNF, LDB1 and CoREST (Bultman et al., 2005; Kim and Bresnick, 2007; Laurent et al., 2009). For the purpose of this thesis, the CoREST complex is described in more detail below.

CoREST complex

The co-repressor for repressor element 1-silencing transcription factor (CoREST) complex was discovered in neural cells in association with the REST transcription factor, which is mediating recruitment of the complex to the RE1 sequences of its target genes. In general, the CoREST complex negatively regulates the differentiation process. It is comprised of different subunits, such as HDACs 1/2, lysine specific histone demethylase I (LSD1), REST corepressors 1, 2 and 3 (RCOR1/2/3), high mobility group 20A (HMG20A), HMG20B, and BRAF-HDAC complex-80 (BHC80) (Lakowski et al., 2006). This complex is recruited by GFI1B to its target sites in erythroid cells, thus repressing erythroid differentiation (Saleque et al., 2002; Saleque et al., 2007). Table 1 describes the CoREST complex subunits in human and mouse.

Briefly, the function of each CoREST subunit in erythropoiesis can be summarized as follows:

- *GFI1B*: Although not a direct subunit of CoREST complex, the zinc finger protein GFI1B plays a critical role in erythroid differentiation via recruitment of CoREST to its binding site TAAATCAC(A/T)GCA (Saleque et al., 2007; Tong et al., 1998)
- *RCOR1/2/3:* It is an integral protein which recruits the other interacting partners to the CoREST complex. RCOR1 knockdown does not affect erythroid differentiation, which may be explained due to redundancy with other RCOR family members (Saleque et al., 2007).

- *HDAC1/2*: They deacetylate the histone proteins, which results in chromatin compression and further gene repression. HDAC inhibitors result in embryonic globin reactivation, therefore they are prescribed frequently for the patients with β-thalassemia and sickle cell disease.
- *LSD1*: It is a demethylase which specifically demethylase the lysine residues in histones.LSD1 knockdown impairs erythroid differentiation (Saleque et al., 2007).
- *BHC80*: BHC80 knockout mice show neonatal lethality due to inability to suckle (Iwase et al., 2006). The role of BHC80 in erythropoiesis is unknown.
- *HMG20A* and *HMG20B*: Structural proteins associated with CoREST complex (Hakimi et al., 2002), with unknown roles in erythropoiesis. The inhibitory role of HMG20B in neural cell differentiation has been shown. Moreover, it is proposed that HMG20A and HMG20B compete with each other to bind to the other CoREST subunits which they further change the DNA conformation through their HMG domain and define the other cofactors assemble to the CoREST complex (Lakowski et al., 2006).

Chapter 3 of this thesis describes the role of Hmg20b in proerythroblast differentiation using the knockdown approach. In addition, it describes deregulated genes in Hmg20b-depleted cells using microarray analysis which is followed by functional analysis of one of the Hmg20b target genes, Hrasls3.

Table 1: CoREST complex subunits synonyms, locations and Entrez Gene name and IDs in human and mouse (https://analysis.ingenuity.com/pa/launch.jsp)

Symbol	Synonym(s)	Entrez Gene Name	Location	Human Gene ID	Mouse Gene ID
KDM1A	1810043O07RIK, AA408884, AOF2, BHC110, D4Ertd478e, KDM1, KIAA0601, LSD1, mKIAA0601, p110B, RGD1562975	lysine (K)-specific demethylase 1A	Nucleus	23028	99982
RCOR1	5730409O11, 6720480E22Rik, AU042633, COREST, D12WSU95E, KIAA0071, mKIAA0071, RCOR	REST corepressor 1	Nucleus	23186	217864
RCOR2	1A13, AW122124, COREST, LOC283248, MGC105529, Rcor, Rcor1, Rcor2l1, RGD1565031	REST corepressor 2	Nucleus	283248	104383
RCOR3	4921514E24Rik, C730034D20RIK, E130101E15Rik, FLJ10876, FLJ16298, KIAA1343, MGC28186, RP11-318L16.1	REST corepressor 3	Nucleus	55758	214742
HDAC1	DKFZp686H12203, EG15181, ENSMUSG00000061062, Gm1824, GON-10, HD1, HDAC, Hdac1-ps, HISTONE DEACETYLASE-1, MGC102534, MGC118085, MommeD5, RPD3, RPD3L1	histone deacetylase 1	Nucleus	3065	433759
HDAC2	D10Wsu179e, HD2, HISTONE DEACETYLASE-2, mRPD3, RGD: 619976, RPD3, YAF1, Yy1bp	histone deacetylase 2	Nucleus	3066	15182
HMG20A	1200004E06Rik, 5730490E10Rik, FLJ10739, HMGX1, HMGXB1, Ibraf, RGD1564760	high-mobility group 20A	Nucleus	10363	66867
HMG20B	AW610687, BRAF25, BRAF35, FLJ26127, HMGX2, HMGXB2, PP7706, pp8857, SMARCE1r, SOXL	high-mobility group 20B	Nucleus	10362	15353
PHF21A	Bhc, BHC80, BM-006, D030065N23Rik, KIAA1696, MGC123676, MGC29190, PFTF1, RGD1560612	PHD finger protein 21A	Nucleus	51317	192285
KIAA0182	2210013I18Rik, GSE1, MGC58013, mKIAA0182, RGD1562686	KIAA0182	unknown	23199	382034

Transcriptional regulation of hemoglobin switching

Hemoglobin switching is regulated by the transcription factors that either directly bind to globin promoters or modulate the interaction of the LCR with embryonic, fetal or adult globin promoters (Figure 9). Some of these main regulators are listed below:

- FOP: it is not clear whether it interacts directly with the β-globin locus or acts indirectly by regulation of other genes involved in γ–globin regulation (van Dijk et al., 2010a; van Dijk et al., 2010b).
- Sox6: Through long-range interaction with BCL11A, it is involved in γ-globin silencing (Xu et al., 2010).
- TR2/TR4 or DR-binding protein (DRED): they bind to the DR sequences upstream of ε-globin gene and lead its repression in definitive erythrocytes (Tanabe et al., 2002; Tanimoto et al., 2000). Interacting with CoREST/NuRD, they directly bind to embryonic globin genes and promote embryonic to fetal globin switching (Cui et al., 2011).
- DR binding site have been recognized upstream of the γ-globin gene, in which mutation influences the binding of TR2/TR4 and results in HPFH (hereditary persistence of hemoglobin) condition (Berry et al., 1992).
- Coup-TFII: like TR2/TR4, it binds to γ–globin DR sequence and results into its repression (Filipe et al., 1999)
- HBS1L-MYB: Genome wide association analysis (GWAS) confirmed the association of certain SNPs in the intergenic HBS1L-MYB region with altered regulation of γ-globin (Farrell et al., 2011; Nuinoon et al., 2010).
- P22 NF-E4: It has been known as an activator of γ-globin which interacts with CP2 and directly binds to the γ-globin promoter (Zhou et al., 2004).
- CP2: It is the major enhancer of α-, ε-, and γ-globin expression (Chae and Kim, 2003).

Two other key regulators, BCL11a and KLF1, will be discussed in detail below.

BCL11A

B-Cell/Lymphoma 11A (*BCL11A*) is a C2H2 zinc finger protein. It was initially found as a myeloid or B cell proto-oncogene in mice and humans (Fell et al., 1986; Li et al., 1999). Genome wide association studies (GWAS) revealed a strong correlation between HbF levels with several SNPs located in the *BCL11A* gene (Lettre et al., 2008; Menzel et al., 2007; Uda et al., 2008).

BCL11A has been reported as a critical mediator of fetal γ-globin silencing in theadult (Sankaran et al., 2009). It functions as a repressor through binding to *cis*-regulatory elements in the β-globin locus, and interacts with the NuRD repressor complex and the GATA1 and the FOG1 transcription factors. It also binds to the third hypersensitivity site (5'HS3) in the β-globin locus control region (LCR) and a region downstream of the ^{A}γ gene. BCL11A downregulation in sorted and expanded CD34^{+} human hematopoietic progenitor cells elevates γ-globin expression. This depletion does not affect the expression of the well-known transcription factors regulating erythropoiesis such as KLF1, NF-E2 and GATA-1 (Sankaran et al., 2008). Bcl11a knockout mice fail to repress mouse embryonic Hbb-y and Hbb-bh1 globin genes after the primitive stage. Breeding of Bcl11a knock out mice with the human β-globin locus transgene (as a yeast artificial chromosome clone, β-YAC) demonstrated robust expression of human γ-globin in the definitive erythroid lineage (Sankaran et al., 2009).

KLF1

KLF (Krüppel-like factor) family members share three conserved zinc finger DNA binding domains recognizing similar DNA motifs (Kaczynski et al., 2003). Erythroid-specific KLF1 (a.k.a. EKLF) was discovered as an activator through a highly conserved CACCC motif in the promoter of the adult human β-globin gene (Miller and Bieker, 1993). it plays a critical and specific role in erythroid development, and its expression increases three-fold during definitive erythropoiesis in mice (Donze et al., 1995). KLF1 plays a major role in regulating erythropoiesis through

three distinct controlling steps. 1-it is critical in erythroid lineage commitment of the megakaryocyte-erythroid progenitor; 2- helping erythroblasts to exit the cell cycle and propel them towards terminal differentiation; 3-promoting definitive erythropoiesis and hemoglobin switching (Siatecka and Bieker, 2011). The first two functions will be briefly discussed and because of the purpose of this thesis the third role will be discussed in more detail.

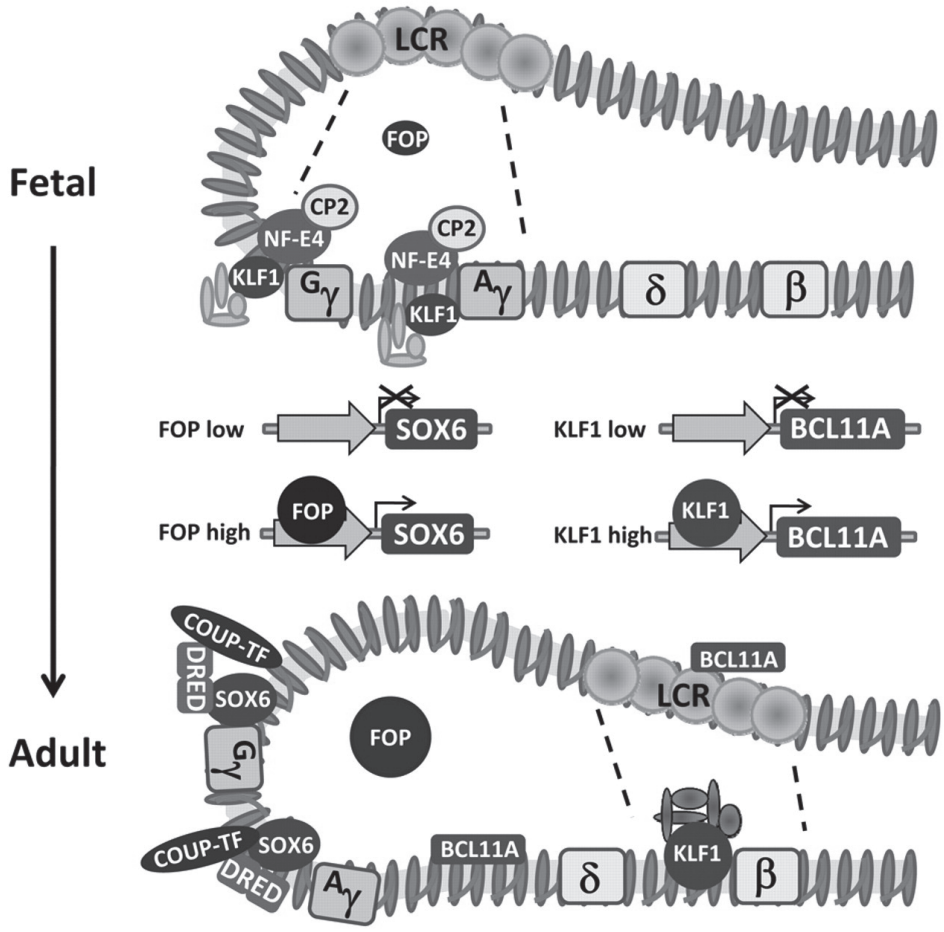

Figure 9: LCR interaction with fetal/adult globin during development is being mediated by stage-specific transcription factors. All transcription factors have been described in the text (Wilber et al., 2011).

Erythroid lineage commitment is regulated by KLF1 through repressing FLI1 which is a critical megakaryocytic activator (Frontelo et al., 2007). E2F2 as a direct target of KLF1 plays a critical role in erythroid cell cycle and terminal differentiation. Insufficient expression of E2F2 due to the absence of KLF1 blocks the transition of erythroid precursors towards terminal differentiation (Pilon et al., 2008; Siatecka and Bieker, 2011; Tallack et al., 2009; Zhang et al., 2003b)

Studies on *Klf1* knockout mice revealed lethality at day 14.5 of embryonic development (E14.5) due to anemia caused by disrupted fetal liver erythropoiesis. *Klf1 null* mice appeared to be embryonic-lethal due to a marked reduction of *Hbb* expression, while embryonic globin genes were not affected (Nuez et al., 1995; Perkins et al., 1995).

Subsequent studies of *Klf1 null* mice indicated that it is also active during the primitive stage of erythropoiesis. An LCR-β-globin promoter reporter transgene, which is normally expressed in primitive erythroid cells, is not expressed in the absence of Klf1, indicating that Klf1 is also a transcriptional activator in primitive erythropoiesis (Guy et al., 1998; Tewari et al., 1998).

Mutations in human *KLF1* are associated with different phenotypes, such as HPFH (hereditary persistence fetal hemoglobin), inhibition of Lutheran blood group expression (In(Lu)), congenital dyserythropoietic anemia (CDA) (Arnaud et al., 2010; Borg et al., 2010; Singleton et al., 2008) and red cell zinc protoporphyria (Satta et al., 2011) (Figure 10).

Remarkably, KLF1 acts as a key activator of BCL11A and this KLF1-BCL11A axis has been proposed to have an important role in γ-globin suppression (Borg et al., 2010; Zhou et al., 2010).

Chapter 4 will address the role of Bcl11a and Klf1 in erythropoiesis and mouse/human hemoglobin regulation in vivo.

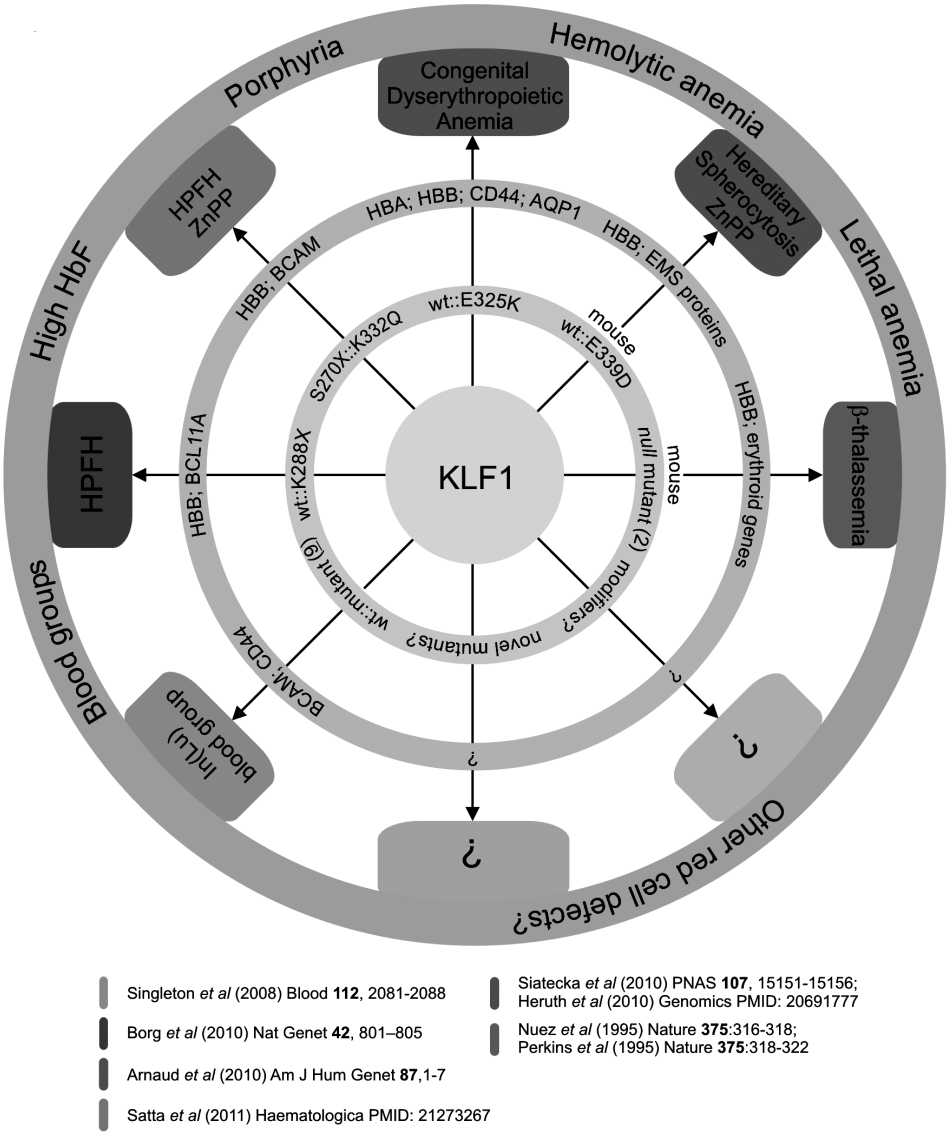

Figure 10: Phenotypes caused by KLF1 mutations. The inner ring displays KLF1 mutations and potential modifiers. The number of different mutants reported is shown. The middle ring displays critical KLF1 target genes/loci whose expression is affected by the KLF1 mutation(s). The outer ring displays phenotypes. Clinical conditions are in the boxes; the colors refer to the publications shown below. HBA = α-globin locus; HBB = β-globin locus; EMS=erythrocyte membrane skeleton, ZnPP = zinc protoporphyrin (Borg et al., 2011).

Hemoglobinopathies

Hemoglobinopathies refer to any inherited mutation of the globin genes leading to a qualitative or quantitative abnormality of globin synthesis. The major etiology of the disorders is the imbalance between α- and β-globin polypeptides leading to precipitation of excess unbound globins in the cells and further damage and destruction of the erythrocytes. The carrier prevalence of hemoglobinopathies worldwide is 7% and the most common types are β-globin related disorders β-thalassemia and sickle cell disease (SCD) (Urbinati et al., 2006; Weatherall et al., 2001). As carriers are somewhat resistant to malaria, in particular the form caused by *Plasmodium falciparum*, the diseases are prevalent in malaria-endemic areas such as the Mediterranean, Middle East, Africa, India and Southeast Asia (Angastiniotis and Modell, 1998; Clegg and Weatherall, 1999). Some hemoglobin related disorders are:

SCD is caused by a single nucleotide substitution (A to T) in the codon for amino acid 6, leading to the replacement of a glutamic acid residue by a valine residue in the β-globin gene (HbS). This abnormal globin results in abnormality in the red cell and they sickle and morphologically they show a crescent shape.

Locating polar amino acid moieties on the surface avoids interactions between individual hemoglobin molecules within a red blood cell. Non-polar residues on the inside of hemoglobin create a non-oxidative environment for binding the oxygen to the heme molecule (Beutler and Williams, 2001). HbS is problematic under hypoxic conditions when the non-polar valine residue, exposed on the surface of the hemoglobin molecule instead of polar glutamic acid residue in HbA causes HbS polymerisation through hydrophobic interactions. The presence of polymerized HbS results in deformation and rigidity of the RBCs. Blocking of microarteries by sickled cells causes painful crises and resulting in permanent organ damage (Wintrobe and Greer, 2009).

SCD patients are subjected to moderate to severe anemia, strokes, vaso-occlusive

episodes, acute chest syndrome, priapism, hepatibilliary and liver diseases, splenic infarction, life-threatening infection, renal disease, retinopathy, and shortened lifespan (Madigan and Malik, 2006).

Hemoglobin C disease is a situation when the 6th glutamic acid residue in β-globin gene is replaced by a lysine residue. People with this mutation carry more erythroid target cells which are erythrocytes with increased surface area to volume ratio. Carrying hemoglobin C mutation does not affect hemoglobin expression and those people will not have any symptoms of anemia (Vella, 1966). It mainly causes a severe phenotype in accompany with other β-globin gene mutations (Bender and Hobbs, 1993).

Hemoglobin E disease is caused by a mutation in the 26th residue of the β-globin chain which is glutamic acid changing to a lysine residue. The patients carrying this mutation are distinguished by mild haemolytic anemia. Conjunction of hemoglobin E mutation with the other mutations of β-globin gene results in a sever phenotype (Colah et al., 2010).

β-thalassemia is the second most common monogenic disorder worldwide after SCD (Angastiniotis and Modell, 1998). There are three major phenotypes which are summarized in Table 1. The most severe type is β–thalassemia major which is characterized by severe anemia, decreased hemoglobin synthesis and shortened RBC life span, splenomegaly, skeletal abnormalities and mental retardation (Urbinati et al., 2006).

α-thalassemia is caused by mutations in the α-globin genes. There are three major sub-groups: 1- α-thalassemia minor and silent carriers (asymptomatic and not anemic) (Urbinati et al., 2006); 2- HbH disease (deletion or mutation of three α-globin genes leading to moderate anemia) (Chan et al., 1997), 3- the most deleterious form of α-thalassemia, called Hb Bart's, is caused by mutation of all four α-globin genes. The majority of cases will die before birth and those who are born will be transfusion-dependent for life (Chan et al., 1985; Chan et al., 1997).

δβ-thalassemia is caused by either large gene deletions in various extent

encompassing the δ- and β-genes or by non-deletional mechanisms. Carriage of nondeletion δβ-thalassemia is characterized by high levels of hemoglobin F (Hb F; range, 10%-20%) containing mainly A2 chains and normal levels of Hb A2. (Atweh et al., 1987; Edington and Lehmann, 1955; Esteghamat et al., 2007; Gerald and Diamond, 1958).

Hb Lepore patients carry a 7.2 Kb deletion which results in the fusion of δ- and β-globins. The patients express normal HbA2, reduced levels of HbA level and increased level of HbF (Steinberg, 2009). In the heterozygous patients, fusion globin comprises 6-15% of the total haemoglobin (Bunn and Forget, 1986; Ropero et al., 1999).

Hereditary persistence fetal hemoglobin (HPFH) is an inherited condition in which the underlying mutation is likely to be the direct cause of increased HbF in adult life. The first person with HPFH was recognized in South Africa with 100% HbF and normal phenotype (Steinberg, 2009). Mutations resulting in this condition include deletions within the β-globin cluster, mutations in the promoters of the γ-genes (Wood, 1993). Association of heterocellular HPFH, beta (+)-thalassaemia, and delta beta(0)-thalassaemia results to a less severe/normal transfusion-independent condition (Cianetti et al., 1984).

There is a heterogeneous group of conditions characterized by small increases in HbF which appear to be inherited. They are unlinked to the β-globin genes and their effect may not be directly on γ-gene expression but could be secondary to subtle changes in erythroid differentiation. In those conditions in which only small increases in HbF (< 5%) are observed and in some of the γ-gene promoter mutants, only a proportion of the red cells contain detectable HbF. This is referred to as heterocellular HPFH (Wood, 1993).

Characterizing the red cell

Complete Blood Count (CBC) This blood test utilizes an automated cell counter to measure the number of red blood cells (RBC), white blood cells (WBC) and

platelets in the blood.

Blood samples can be collected in a tube containing anticoagulants, usually EDTA or heparin, and employed for complete blood count (CBC) analysis within 24 hours. CBC analysis can be performed manually or automatically. The results of the CBC usually include several values regarding the basic components of blood:

- RBC (red blood cell count): RBC displays the number of red blood cells in 1 liter of blood. A high RBC may indicate the lack of oxygen in the blood environment or a bone marrow problem. Generally, low level RBC indicates that the patient is suffering from anemia. High RBC represents excess of red blood cells which is called erythrocytosis. Normal value for human is 3.7-5.6x10^6/μL.
- WBC (white blood cell count): a high WBC count indicates that there is an infection or it may indicate the presence of a malignancy. A low WBC count may signal a problem in the bone marrow or it could be a side effect of chemotherapy. Normal value for human is 6-10x10^3/μL.
- HGB (hemoglobin): responsible for binding and releasing oxygen. High and low HGB indicate dehydration and anemia, respectively. Normal value for human varies between 10.5 and18 g/dL.
- HCT (hematocrit)/packed cell volume (PCV)/erythrocyte volume fraction (EVF): represents the percentage of blood volume that is occupied by red blood cells. Low value of hematocrit points at anemia, and high HCT increases the risk of thrombosis. Normal value for human varies between 33 and 50%.
- MCV (mean corpuscular volume): average size of red blood cells. Normal value for human is 70-98 fL.
- MCH (mean corpuscular hemoglobin): average weight of hemoglobin in each red blood cell. This value should always be considered together with MCV. Increased or decreased of MCV and MCH levels point at vitamin B12 deficiency or anemia. Normal value for human is 23-33.6 pg.
- MCHC (mean corpuscular hemoglobin concentration): the average percentage of hemoglobin concentration in each red blood cell. Low MCHC indicates

anemia, while high level are indicative of excess iron in the body which might be deleterious to the liver. Normal value for human is 30-35.7 g/dL.

- PLT (platelets): A high PLT count may indicate severe inflammation or a bone marrow disease while a low platelet count may the reason for prolonged hemorrhage. Normal value for human and mouse are 250-400x10^3/μL.

Reticulocyte counts provide important information on hematopoiesis and hemolysis. Reticulocytes released from bone marrow to blood circulation, they continue hemoglobin synthesis for one or two consequent days. A healthy person carries less than 2% reticulocytes in the circulation; this is increased in stress hematopoiesis (Urrechaga et al., 2011).

Globin chain synthesis provides information on the balance of α- versus non-α-globin chains which is the most important characteristics of thalassemia. This test is usually performed on reticulocytes or bone marrow through incorporation of labeled amino acid residues. However, the results are not always unequivocal (Kan et al., 1969).

HPLC (high performance liquid chromatography) is a precise test to measure the percentage of different types of hemoglobin in blood. In recent years, it has become a reference method for the study of hemoglobin (Hb) abnormalities (Wajcman, 2003). The HPLC reference values for a healthy person are ~96.5% HbA, ~2.8% HbA2 and ~0.4% HbF (Ondei et al., 2007).

Therapeutic approaches for hemoglobinopathies

Different therapeutic methods are in use to treat β-thalassemic and SCD patients. The only permanent curative treatment at the moment is bone marrow transplantation with the limitation of finding an HLA-matched donor for the bone marrow cells. Regular blood transfusion is the other approach to treat the patients. Because the hemoglobin in red blood cells is an iron-rich protein, regular blood transfusions lead to iron overload in blood which damages the liver, heart, and other parts of the body. To prevent this damage, iron chelation therapy is needed

to remove excess iron from the body.

HbF induction in adults would also be of a great value to these patients as this would compensate for the reduced HbA levels and improve the anemia (Azarkeivan et al., 2010; Sankaran and Nathan, 2010). Recently, gene therapy has been proposed as another useful approach to treat β-thalassemia. All aforementioned methods will be discussed below.

Bone marrow transplantation: The first successful bone marrow transplantation (BMT) for β-thalassemia major was performed in 1981 in Seattle, Washington (Thomas et al., 1982). Currently, high-resolution HLA typing has enabled physicians to perform transplants from both related and unrelated volunteer donors. The probability of obtaining thalassemia-free survival after transplant is around 85% (Gaziev et al., 2008; Pakakasama et al., 2008; Sumboonnanonda et al., 2009).

Chemical compounds known to increase the HbF level currently include Hydroxycarbamide (hydroxyurea or HU), 5-azacytidine (5-Aza), and short chain fatty acid derivatives.

- HU is a ribonucleotide reductase inhibitor which inhibits DNA replication. HU is The first approved drug by the US food and drug administration (FDA) for treatment of SCD and β-thalassemia (Charache et al., 1995); however the mechanisms underlying γ-globin reactivation in patients using this chemical is unknown. It has been proposed that HU selectively kills proerythroblasts in the bone marrow, except those expressing HbF (Bhagavan, 2002). It efficiently ameliorates disease manifestation in ~50% of β-thalassemic cases by increasing HbF levels, thus reducing the need for blood transfusion and hospitalization (Gambari, 2010). Revealing the molecular mechanisms underlying the response to HU would give a better guide to select patients for HU treatment.
- 5-Aza-2'-deoxycitidine (decitibine) is a DNA methyltransferase (DNMT) inhibitor that can increase HbF levels by unknown mechanisms. Based on recent reports it can modulate γ-globin expression through post-

translational modifications (Mabaera et al., 2008).

- Sodium butyrate, a short-chain fatty acid derivative, is an HDAC inhibitor (Constantoulakis et al., 1989a, b; Fathallah and Atweh, 2006; Perrine et al., 1989a; Perrine et al., 1989b) which induces HbF by increasing γ-globin mRNA transcription (Fathallah et al., 2008; Fathallah et al., 2007; Trompeter and Roberts, 2009).

Gene therapy: expressing a normal β-like globin gene in erythroid cells following permanent gene transfer into hematopoietic stem cells can result in a permanent cure (May et al., 2000; Persons, 2003; Persons and Nienhuis, 2003). Notably, a major drawback of this approach is clonal expansion of progenitors, similar to a pre-leukemic condition (Cavazzana-Calvo et al., 2010). The first β-thalassemic patient that received a correct version of the β-globin gene by gene therapy was cured of anemia. However clonal expansion of a hematopoietic progenitor with a vector insertion site at the *HMGA2* gene (Cavazzana-Calvo et al., 2010) raises questions about the safety of this method.

Hydroxyurea (HU) is the most frequently used drug to treat SCD and β-thalassemic patients, with a positive response in ~50% of cases. Distinguishing non-responders before treatment would be very helpful to select patients for HU treatment. Chapter 5 of this thesis provides data on prognostic factors to distinguish between these two groups of patients.

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Are HOXB4 cells a useful model for multipotent hematopoietic progenitors?

Are HoxB4 cells a useful model for multipotent hematopoietic progenitors?

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Abstract

Background

The hematopoietic system is comprised of a hierarchy of different cell types in the bone marrow cavity of all adult mammals. Mimicking the hematopoietic system in vitro provides a good opportunity to trace and manipulate the transcription factors involved in early hematopoietic lineage commitment or in final differentiation. It has been shown that differentiated embryonic stem cells ectopically expressing HOXB4 recapitulate adult hematopoietic stem cell growth and differentiation characteristics in vitro and in vivo.

Design and Methods

In this study we expressed HOXB4 ectopically in an embryonic stem cell line expressing HA-BirA (ES-HA-BirA) from the Rosa26 locus.

Results

We found that ES-HA-BirA-HOXB4 cells fully differentiate to myeloid lineages including macrophages and granulocytes. The majority of differentiated granulocytes in culture were polymorphonuclear neutrophils. In line with other reports, we found that high expression of HOXB4 inhibited erythroid and megakaryocyte differentiation. To approach definitive erythropoiesis, we produced erythroid progenitors derived directly from ES-HA-BirA cells.

Conclusions

We established blast cultures with myeloid or erythroid differentiation potential, capable of metabolically biotinylating an ectopically introduced Avi-tagged protein.

Keywords: HOXB4, ES-HA-BirA, HSCs, Myelopoiesis, Erythropoiesis, Megakaryopoiesis

Introduction

All of the mature blood cells in the body are generated from a relatively small number of hematopoietic stem cells (HSCs) and progenitors that are located in specialized niches in the bone marrow (Lemischka, 2001; Weissman, 2000). Hematopoiesis is the highly orchestrated process of blood cell development. Understanding the biology and molecular mechanisms regulating hematopoietic stem cell generation, expansion and maintenance holds promise to improve treatment of malignancies and congenital disorders (Smith, 2003). Mouse models, particularly those involving short- and long-term transplant studies, have given remarkable insights into the biology and detailed molecular characterization of HSCs and progenitors (Eaves et al., 1997; Jones et al., 1996).

Transcription factors play a key role in keeping the balance between HSCs proliferation and differentiation (Tantos et al., 2011) and their further lineage commitment (Starnes and Sorrentino, 2011). Among the transcription factors supporting stem cell maintenance (Thorsteinsdottir et al., 2002; Varnum-Finney et al., 2000), HOXB4 has been widely studied in hematopoiesis. It has been confirmed through different studies that ectopic expression of HOXB4 maintains self-renewal capacity of hematopoietic cells in vitro and in vivo (Amsellem et al., 2003; Antonchuk et al., 2001; Pilat et al., 2005; Sauvageau et al., 1995; Schiedlmeier et al., 2007; Sharma et al., 2006). HOXB4 belongs to the clustered family of homeobox genes (class I homeobox genes) which are the DNA-binding transcription factors. This gene family comprises an evolutionarily highly conserved set of genes initially identified as key regulators of positional identity along the anterior-posterior body axis of animal embryos (Ahn and Ho, 2008; Krumlauf, 1994). Hoxb4 is expressed in primitive mouse and human hematopoietic cells (Pineault et al., 2002; Sauvageau et al., 1994) and its enforced expression is sufficient to stimulate marked HSC expansion in vivo and ex vivo without promoting leukemia (Amsellem et al., 2003; Antonchuk et al., 2001, 2002; Sauvageau et al., 1995). Furthermore, HOXB4 overexpression

in monkey CD34+ cells promotes the expansion and engraftment of short-term repopulating cells but has a less pronounced effect on long-term repopulating cells (Zhang et al., 2006). In contrast, it has been proposed that HOXB4 maintains long-term repopulating of hematopoietic stem cells due to regulation of several signalling pathways including Wnt, Notch, FGF and Hedgehog (Sharma et al., 2006).
Both bone marrow and embryonic stem cells ectopically expressing HOXB4 expand as a bulk of different clones representing some of the bone marrow characteristics in vitro (Pilat et al., 2005). Combined with newly developed proteomics techniques this system may provide a useful tool to discover hematopoietic regulatory mechanisms and lineage commitment. In this study, we applied HOXB4 overexpression in an embryonic stem cell line expressing HA-BirA from the Rosa26 locus, which allows biotinylation of Avi-tagged proteins ubiquitously in all cell types (Driegen et al., 2005).

Materials and Methods

His-Tat-Cre recombinase purification

HTN-Cre recombinase was purified according to a described protocol (Peitz et al., 2007). Purified HTN-Cre recombinase was diluted in medium and thoroughly mixed before adding to the cultures.

Western blotting and antibodies

Whole cell extracts were prepared following a published protocol (Esteghamat et al., 2011). The extracts of 3x105 cells were loaded on 10% SDS-PAGE, and after blotting on nitrocellulose membrane incubated with primary antibodies at a dilution of 1:1000 in blocking buffer (PBS containing 1% (w/v) BSA). The applied primary antibodies included Gata-1 (sc-265), Gata-2 (sc-9008), Pu-1 (sc-352), Prmt-1 (sc-13393), Runx1 (sc-28679) and Cebp-β (sc-150). Secondary staining was performed using goat-anti-mouse/rabbit IR-Dye 680 or 800 antibodies in PBS with

5% (w/v) blotting grade non-fat dry milk powder (Bio-Rad Laboratories, Hercules, USA) and 0.05% (v/v) tween 20 (Roche Diagnostics).

Southern blot analysis

Southern blot analysis of EcoRI-digested DNA was performed according to a published protocol (Driegen et al., 2005).

Retroviral transduction and cell culture

The generation of the embryonic stem (ES) cells expressing HA-BirA (ES-HA-BirA) from the Rosa26 locus has been described (Driegen et al., 2005). The ES-HA-BirA cells were transduced with retroviruses carrying SF91-eGFP2AHOXB4-wPRE as described (Pilat et al., 2005). ES-HA-BirA cells ectopically expressing HOXB4 were sorted with a FACS sorter and they were further expanded on a gelatinized (0.1%) dish without feeder cells in DMEM (GIBCO/BRL), supplemented with BRL medium, 2mM L-glutamine (GIBCO/BRL), 100 units/ml penicillin and 100 mg/ml streptomycin (GIBCO/BRL), 15% (vol/vol) FCS, 1% (vol/vol) leukemia inhibitory factor (LIF)-supernatant, 0.02% β-mercaptoethanol, and non-essential amino acids (NEAA) (GIBCO) (1%) (Wiles and Keller, 1991). Two days before differentiation in vitro, ES cells were transferred to IMDM (GIBCO/BRL) supplemented with the same components as DMEM.

Biotinylation and tagging and proteomics analysis

Avi-HA-HMG20B was cloned into a modified 5pRRLsin.sPPT.CMV.GFP.Wpre lentiviral vector (Esteghamat et al., 2011). The ES-HA-BirA-HOXB4 cells were transduced with Avi-HA-HMG20B and Avi-HA-empty lentiviruses. The transduced cells were selected using puromycin (1μg/ml) and expanded in culture.

ES-HA-BirA-HOXB4 cell differentiation

To differentiate the ES-HA-BirA-HOXB4 cells to embryonic bodies (EBs), a “hang-

ing drop" protocol was followed using 200-300 cells seeded per 20 μl drops of differentiation medium consisting of IMDM (Gibco/BRL), L-glutamine (2mM; Gibco/BRL), 100 units/ml penicillin and 100 mg/ml streptomycin (GIBCO/BRL), FCS (15%), L-ascorbic acid (0.5 μg/ml; 50-81-7, Sigma) , Holotransferrin (0.5 μg/ml; T101-5, SCIPAC Ltd, Sittingbourne, UK), β-mercaptoethanol (0.02%). At day four all EBs were collected and seeded in 20ml fresh differentiation medium for two more day and then they were trypsinized to obtain the single cells. The single cells were washed two times with PBS and then the ES-HA-BirA-HOXB4 cells were seeded in Stempro34plus (Invitrogen, Carlsbad, USA) supplemented with stem cell factor (SCF) (100ng/ml; R&D, 455-MC), mouse Flt-3 Ligand (10ng/ml; R&D, 427-FL) mouse IL-6 (5ng/ml; R&D, 406-ML), mouse IL-3 (4ng/ml), mouse IGF-1 (40ng/ml; Promega, G5111) and dexamethasone (1 μM; Sigma, D4902).

ES-HA-BirA-HOXB4 cells were differentiated to the myeloid and erythroid lineages as described below:

Granulocyte differentiation, ES-HA-BirA-HOXB4 cells were washed two times with PBS and then seeded in Stempro34plus supplemented with G-CSF (5ng/ml).

Macrophage differentiation, ES-HA-BirA-HOXB4 cells were washed two times with PBS and seeded in Stempro34plus supplemented with M-CSF (R&D, 416-ML) (50ng/ml).

Erythroid differentiation, ES-HA-BirA-HOXB4 cells were washed two times with PBS and seeded in Stempro34plus supplemented with Erythropoietin (EPO) (10u/ml), and transferrine (50 ng/ml).

Megakaryocyte differentiation, HOXB4 cells were washed two times with PBS and they were seeded in Stempro34plus supplemented with thrombopoietin (Tpo) (R&D, 488-TO) (10 ng/ml) and SCF (50 ng/ml).

ES-EP differentiation, differentiated ES-HA-BirA ES cells were seeded in Stem-Pro34plus (Invitrogen, Carlsbad, USA) supplemented with EPO (1U/ml, Janssen-Cilag BV, Tilburg, NL), SCF (100ng/ml; R&D, 455-MC) and dexamethasone (1 μM; Sigma, D4902).

Results and Discussion

Deletion of the puromycin resistance gene using HTN-Cre recombinase

ES-HA-BirA cells were a kind gift of Dies Meijer (Driegen et al., 2005). Providing more flexibility to choose a selection marker, the puromycin resistance gene was removed from the ES-HA-BirA cells using HTN-Cre recombinase (His-tag-TAT-Nuclear localization signal). HTN-Cre recombinase can be efficiently produced and purified from E. coli and it provides a rapid and efficient approach to recombine between two adjacent LoxP sites (Xu et al., 2008). The amino-terminal His-tag facilitates purification from E. coli lysates. The TAT peptide, comprising mainly basic residues (GRKKRRQRRRPQ), is derived from the transactivator of transcription (TAT) of human immunodeficiency virus (HIV). The 9 highly charged residues underlined in the TAT sequence plays the major role in cell penetration and enabling the delivery of cargo to the cells (Brooks et al., 2005). Upon intraperitoneal injection of TAT-β-galactosidase (β-gal) into mice, β-gal activity was observed in different organs such as brain, kidney, liver, spleen and lung (Schwarze et al., 1999).

The ES-HA-BirA cells were incubated 16 hours with purified and concentrated HTN-Cre recombinase in normal culture conditions using two different concentrations, 10- and 5 μM. Using 10 μM HTN-Cre recombinase, we observed a better recombination efficiency (7 out of 8 clones) comparing to that of 5 μM. The major disadvantage of using the high concentration of HTN-Cre recombinase was toxicity leading to increased cell death. Addition of 5 μM HTN-Cre recombinase had less lethal effect on the cultured cells with a decreased efficiency of recombination. Previously, Peitz et al reported >95% recombination efficiency using 2 μM HTN-Cre recombinase on fibroblast cells within 16 hours incubation. Up to 50% efficiency can be obtained within 30 min incubation time and it increased up to 95% within 8 hours (Peitz et al., 2002). When Using 5 μM HTN-Cre recombinase for 16 hours with the HOXB4-ES cells, recombination was 33% efficient (3 recombined clones

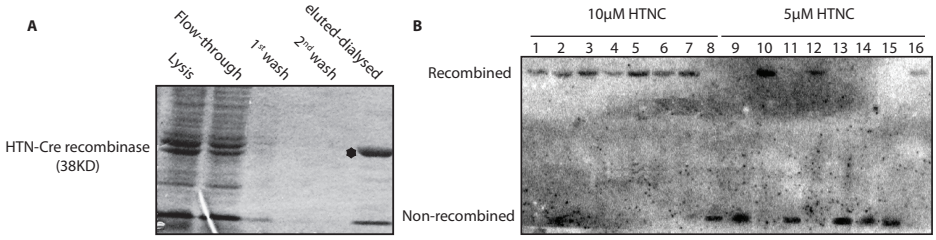

Figure 1. HTN-Cre recombinase purification and recombination efficiency. A) 10 µl of the purified and concentrated enzyme was loaded on 10% SDS-PAGE to test for purification efficacy. B) Two different concentrations, 10 and 5 µM, of HTN-Cre recombinase were introduced to the ES-HA-BirA cells. To assess recombination efficiency, a southern blot was used. Size of the recombined allele was 11.5Kb and that of the un-recombined allele was 2Kb.

out of 9). This indicates that the concentration of HTN-Cre recombinase and the incubation time should be optimized for each cell line (Figure 1A-B).

Several studies observed cytotoxicity of Cre recombinase transduction on different established cell lines including NIH 3T3, COS-7 and HeLa. This detrimental effect is probably due to pseudo- or cryptic LoxP sites naturally present throughout the genome, which could be recombined after Cre recombinase transduction (de Alboran et al., 2001; Huh et al., 2010; Loonstra et al., 2001; Pfeifer et al., 2001; Schmidt et al., 2000; Silver and Livingston, 2001). Therefore, to select an ES-HA-BirA clone lacking obvious rearrangements, karyotyping was carried out on several recombined ES-HA-BirA clones. In line with previous reports (Peitz et al., 2007), No abnormalities in the chromosome counts were found. Finally, the recombined ES-HA-BirA clones became sensitive to puromycin selection, providing further evidence that the puromycin cassette had been removed.

Transduction of ES-HA-BirA cells with HOXB4 retroviruses

The recombined ES-HA-BirA cells were transduced with the SF91-eGFP2AHOXB4+wPRE retroviral vector. GFP positive cells were further sorted and subsequently differentiated into embryoid bodies for 6 days (Pilat et al., 2005).
The embryoid bodies were then dissociated and expanded in medium supple-

mented with essential factors for hematopoietic cell growth (Pilat et al., 2005). To assess their morphology, ES-HA-BirA-HOXB4 cells were stained with Giemsa. We found that the cultures were a mixture of different cell types, as indicated by their size. However, the majority of cells contained a relatively large nucleus adjacent to the cell membrane and appeared morphologically as blast-like cells (Figure 2A).

To determine the percentage of HOXB4-expressing cells, FACS analysis was performed. HOXB4 is flanked by 2A estrerase of foot-and-mouth disease virus (2A) followed by eGFP. The 2A esterase peptide ensures coordinate expression of multiple introduced proteins. On translation such polyproteins would self-process or self-dissociate at the carboxy terminus of 2A to yield discrete protein products that then can be independently targeted to a variety of sub-cellular locations (Halpin et al., 2001). This allowed the use of GFP expression as an indirect marker to follow HOXB4 expression in the ES-HA-BirA-HOXB4 cells. We found that almost all cells were highly positive for GFP four weeks after EB dissociation (Figure 2B). These observations show a selective growth advantage for cells expressing high levels of HOXB4 (Pilat et al., 2005).

In conclusion, we find that ES-HA-BirA-HOXB4 cells are a heterogenous population displaying a relatively high ectopic expression of HOXB4 after several weeks in culture. Most cells carry a large nucleus close to the membrane and show a blast-like phenotype. These ES-HA-BirA-HOXB4 cell cultures include some morphologically recognizable myeloid-lineage committed cells, as well.

Characterization of ES-HA-BirA-HOXB4 cells

Previous characterization of ES-HA-BirA-HOXB4 cells by immunostaining assay for some cell surface markers has demonstrated that these cells are maintained as a bulk or clonal cultures. At day 75 after dissociation, around 80% of these cells were positive for CD31 (an endothelial marker), and 17% were positive for CD34 (a hematopoietic stem cell marker). From the bulk of the cells, 7% were

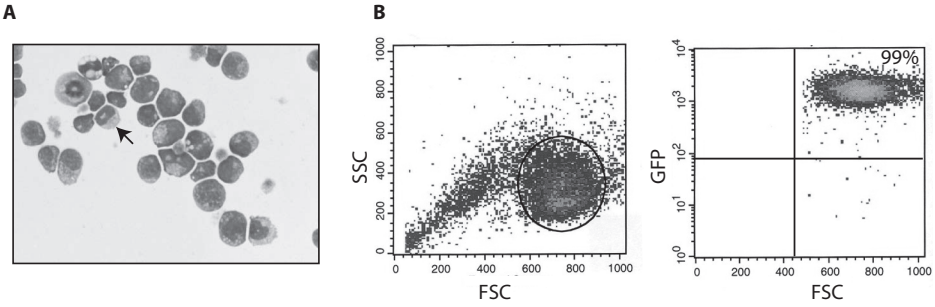

Figure 2. ES-HA-BirA-HOXB4 morphology and HOXB4 expression. A) cytospin followed by Giemsa staining showed ES-HA-BirA-HOXB4 cells are a heterogenous population with different cell sizes. However the majority of the cells are blast-like containing a relatively large nucleus attached to the membrane. The culture displayed some morphologically obvious myeloid cells differentiated from the progenitors (shown by arrow). B) FACS analysis for GFP expression (representing HOXB4 expression) showing high expression of GFP in four weeks after embryoid body dissociation.

positive for Flt3, a myelo-proliferative marker, and 13% were double positive for Gr1 and CD11b which are expressed in myeloid lineages. Other hardly detectable markers with expression around 1% or less were Ter119, cKIT, B220 and CD19 (Pilat et al., 2005).

To delineate whether the ES-HA-BirA-HOXB4 cells expressed some erythroid specific proteins, we carried out western blot analysis. As a control, mouse erythroid leukemia (MEL) cells were employed. Runx1 was hardly detectable in ES-HA-BirA-HOXB4 cells and these cells lack Gata-1 and Gata-2, which are crucial erythroid transcription factors. Ubiquitously expressed proteins including Prmt1 and Cebp-β served as loading controls. Expression profile analysis revealed the highest expression of Cebp-β in whole blood, placenta, monocytes and lung cells (http://biogps.org/#goto=genereport&id=1051). ES-HA-BirA-HOXB4 cells were also highly positive for the myeloid marker Pu.1, a transcription factor with an important role in granulocyte-macrophage proliferation and differentiation (DeKoter et al., 1998). Altogether, the ES-HA-BirA-HOXB4 cells lack erythroid progenitor markers and appear to be strongly skewed towards the myeloid lineage (Figure 3A).

Efficient in vivo biotinylation tagging in ES-HA-BirA-HOXB4 cells

To test whether ES-HA-BirA-HOXB4 cells are able to biotinylate Avi-tagged proteins, they were transduced with lentiviruses carrying Avi-Hmg20b. Avi-Hmg20b was cloned into a modified 5pRRLsin.sPPT.CMV.GFP.Wpre lentiviral vector (Esteghamat et al., 2011; Follenzi et al., 2002). Avi-Hmg20b expressing cells were selected in medium supplemented with puromycin. Whole cell lysates of the Avi-Hmg20b transduced and control cells were loaded on a 10% SDS-PAGE gel and stained with streptavidin. This revealed the presence of biotinylated Hmg20b exclusively in ES-HA-BirA-HOXB4 cells transduced with lentiviruses carrying Avi-Hmg20b (Figure 3B).

We conclude that the ES-HA-BirA-HOXB4 cells can be efficiently transduced with lentiviruses and puromycin can be used effectively for selection of transduced cells. Importantly, the ES-HA-BirA-HOXB4 cells are able to biotinylate proteins endowed with an Avi-tag.

Erythroid/Megakaryocyte/Myeloid differentiation of ES-HA-BirA-HOXB4 cells

To investigate the multipotent capacity of the ES-HA-BirA-HOXB4 cells, they were differentiated towards different lineages. Using a two-step differentiation protocol, we observed a homogenous population of differentiating ES-HA-BirA-HOXB4 cells to granulocytes. To obtain the granulocytes at the same stage of differentiation we established a two-step differentiation protocol. The ES-HA-BirA-HOXB4 cells were first maintained for two days in medium containing IL3, IGF-1 and G-CSF, and then ransferred and maintained for four more days in medium containing G-CSF only. At day 6, the majority of the cells were positive for the Gr-1 surface marker (Data not shown). Analysis of cell morphology using Wright-Giemsa staining revealed granulocytic differentiation predominantly towards polymorphonuclear neutrophils

(PMNs). We only rarely detected basophils in these cultures (Figure 4A).

To test the ability of the ES-HA-BirA-HOXB4 cells towards macrophage differentiation the cultures were supplemented with M-CSF and the cells were analysed after 10 days of culture. FACS analysis using Mac1 revealed a homogenous population of macrophages in the culture (Data not shown). Further Giemsa staining confirmed the presence of cells with the typical morphological characteristics macrophages, e.g. large vacuolated cytoplasm and relatively small nucleus (Figure 4A).

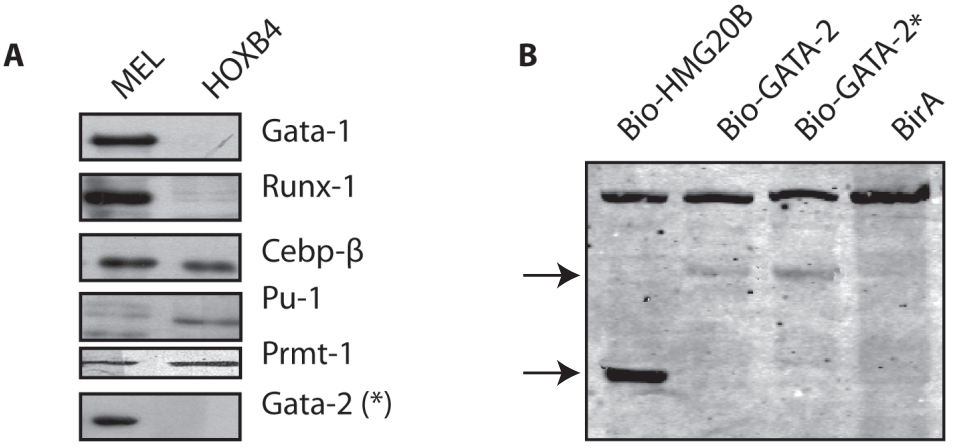

Figure 3. Transcription factor and biotinylation ability of the ES-HA-BirA-HOXB4 cells. A) Characterization of ES-HA-BirA-HOXB4 cells for some transcription factors regulating different aspects of hematopoiesis. GATA-1, GATA-2 and Runx-1 regulate erythropoiesis. However, PU.1 and Cebp-β are important in myelopoiesis. Prmt1 served as a loading control. * indicates the MEL cells overexpresing Avi-GATA-2 (made by Harald Braun) B) Whole cell lysates from Avi-Hmg20b transduced and control cells were tested loaded on 10% SDS-PAGE and the blot was stained with a streptavidin. * indicates the HOXB4 cells overepresseing a Gata-2 with a point mutation (L359V).

Our attempts towards differentiating the ES-HA-BirA-HOXB4 cells to erythroid and megakaryocyte lineages consistently failed. Maintaining the cells in the defined cultural conditions for erythro- and megakaryopoiesis resulted in cell death two days after induction. The negative influence of high levels of HOXB4 expression on erythropoiesis has been shown previously in erythroid progenitors derived from embryonic stem cells. It specifically attenuates the last stages of erythroid

differentiation and perturbs generation of Ter119+ cells (Pilat et al., 2005).

Ectopic HoxB4 expression has been suggested for an efficient expansion of megakaryocytes for the production of platelets for use in medical transfusion (Zhong et al., 2010). Because megakaryocytes originate from the same ancestor cells as erythrocyte, like erythropoiesis, high levels of HOXB4 probably cause the lack of megakaryopoietic ability of the ES-HA-BirA-HOXB4 cells.

Next, we cultured the bulk of the ES-HA-BirA-HOXB4 cells in ES-EP differentiation medium (Dolznig et al., 2005) to enrich the population of erythroid progenitors out of the other clones for further homogenous erythroid differentiation; that eventually failed. Apparently, HOXB4 accumulation in the cells maintain them as common myeloid progenitors (CMPs) which in hematopoietic hierarchy implies monomyeloid differentiation from multipotent progenitors, away from erythroid progenitors.

Other studies also found that HOXB4 expression attenuates the capacity of hematopoietic progenitors to erythroid lineage commitment (Milsom et al., 2005). Based on previous reports describing the suppression of T-lymphoid differentiation by ectopic expression of HOXB4 (Pilat et al., 2005), we did not attempt to differentiate the ES-HA-BirA-HOXB4 cells to B or T lymphocytes.

Tracing HOXB4-YFP in mice confirmed the expression of HOXB4 in CD45+ CD144+ aorta-gonad-mesonephrous-derived cells enriched for HSCs, and also yolk sac and placental HSC populations. Bone marrow LSK (Lin- Sca1+ c-Kit+) cells express Hoxb4-YFP and long-term repopulation assays demonstrated that definitive HSCs express Hoxb4 (Hills et al., 2011). In contrast, HOXB4 overexpression in monkey CD34+ cells promoted the expansion and engraftment of short-term hematopoietic stem cells only (Zhang et al., 2006). Interestingly, Hoxb4 expression in the fetal liver HSCs is lower than in the bone marrow, reaching negligible levels in some HSCs, suggesting an insignificant role of Hoxb4 in the expansion of fetal liver HSCs. Hoxb4 expression therefore would not appear to correlate with the cycling status of fetal liver HSCs, although highly proliferative HSCs from young

bone marrow show strong Hoxb4 expression (Hills et al., 2011). Fetal liver HSCs are mainly required for the supply of erythroid progenitors and lack of Hoxb4 in

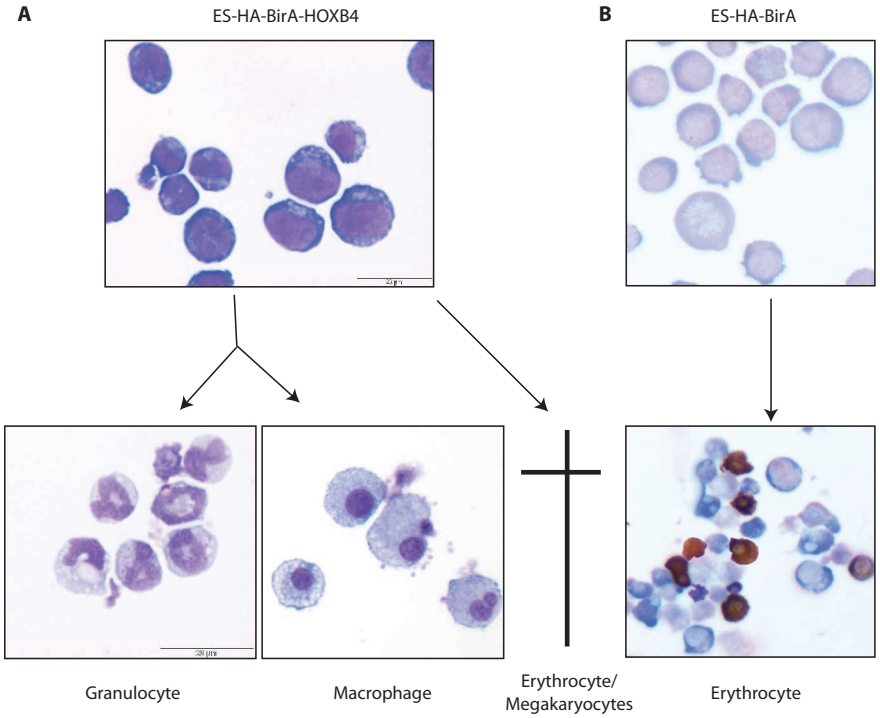

Figure 4. ES-HA-BirA differentiation. A)ES-HA-BirA-HOXB4 cells were differentiated to granulocytes and macrophages. Differentiating cell morphology confers to segmented polymorphonuclear neutropils and vacuolated macrophages. Our attempts to differentiate the HOXB4 expressing cells to erythroid and megakaryocyte lineages failed. B) Therefore, we established erythroid progenitors directly from ES-HA- BirA cells.

these cells might aid commitment towards erythroid lineage potential. We conclude that HOXB4 accumulation in the ES-HA-BirA-HOXB4 cells strongly skews the cells to a myeloid fate. ES cell-derived erythroid progenitors resemble primary, definitive erythroid progenitors in cell morphology, molecular markers, growth, and differentiation kinetics (Carotta et al., 2004). To establish the erythropoiesis aspect of hematopoiesis, we used the same clone of ES-HA-BirA cells, not transduced

with HOXB4, to produce erythroid progenitors. We followed an established protocol to derive erythroid progenitors from embryonic stem cells (Carotta et al., 2004). Twenty days after dissociation the embryoid bodies, the established erythroid progenitors differentiated to definitive erythrocytes (Figure 4B).

Conclusion

To obtain the repopulating hematopoietic progenitors with the ability to differentiate concordantly into erythroid and myeloid lineages, an inducible HOXB4 expression system could be tried. This allows tight regulation of HOXB4 levels in the progenitors which should help to maintain erythroid potential. HOXB4 overexpression accompanied by other HOXB family members, HOXB5 and HOXB2, converted fibroblasts to hematopoietic cells (Harris et al., 2011). This suggests that concerted overexpression of HOXB2, 4 and 5 may provide an opportunity to obtain hematopoietic progenitors. Finally, there are also reports on overexpression of other transcription factors including Mxd4, HOXA10, IKAROS and NF-Ya which result in in vitro generation of hematopoietic progenitors (Boros et al., 2011; Magnusson et al., 2007; Zhu et al., 2005).

In conclusion, HOXB4 overexpressing ES cells could fully recapitulate the bone marrow characteristics if the HOXB4 expression could be tightly regulated. In addition high level of HOXB4 expression provides a helpful tool to expand the myeloblast cells in culture which are efficiently differentiating towards the granulocytes and macrophages.

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The DNA binding factor Hmg20b is a repressor of erythroid differentiation

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The online version of this article contains a supplementary appendix.

Abstract

Background

In erythroblasts, the CoREST repressor complex is recruited to target promoters by the transcription factor Gfi1b, leading to repression of genes mainly involved in erythroid differentiation. Hmg20b is a subunit of CoREST, but its role in erythropoiesis has not been established yet.

Design and Methods

To study the role of Hmg20b in erythropoiesis, we performed knockdown experiments in a differentiation-competent mouse fetal liver cell line, and in primary mouse fetal liver cells. The effects on globin gene expression were determined. We used microarrays to investigate global gene expression changes induced by Hmg20b knockdown. Functional analysis was carried out on Hrasls3, an Hmg20b target gene.

Results

We show that Hmg20b depletion induces spontaneous differentiation. To identify the target genes of Hmg20b, microarray analysis was performed on Hmg20b knockdown cells and controls. In line with its association to the CoREST complex, we found that 85% (527 out of 620) of the deregulated genes are upregulated when Hmg20b levels are reduced. Among the few downregulated genes was Gfi1b, a known repressor of erythroid differentiation. One of the consistently upregulated targets were embryonic β-like globins and the phospholipase HRAS-like suppressor 3 (Hrasls3). We show that Hrasls3 expression is induced during erythroid differentiation and that knockdown of Hrasls3 inhibits terminal differentiation of proerythroblasts.

Conclusions

We conclude that Hmg20b acts as an inhibitor of erythroid differentiation, through the downregulation of genes involved in differentiation such as Hrasls3, and activation of repressors of differentiation such as Gfi1b. In addition, Hmg20b suppresses embryonic β-like globins.

Keywords: Hmg20b, CoREST, erythropoiesis, Gfi1b, Hrasls3, embryonic β-like globins

Introduction

Erythropoiesis is a multi-step developmental process that commences with pluripotent hematopoietic stem cells in the bone marrow and culminates in the release of enucleated and hemoglobinized reticulocytes in the circulation (Magnon C, 2008). Erythroid progenitors undergo massive expansion and pass through several differentiation steps including the morphologically recognizable proerythroblast, basophilic and orthochromatic erythroblast stages (Tsiftsoglou et al., 2009). These final steps involve a series of three to five differentiation-specific cell divisions before the cells arrest in G1, enucleate and undergo terminal maturation to erythrocytes (Dolznig et al., 2002).

Studies on erythropoiesis have revealed the basics of tissue-specific gene regulation, in which transcription factors and chromatin modifying complexes play a major role (Ney, 2006). CoREST (corepressor of repressor element-1-silencing transcription factor) is a chromatin-modifying complex that was first described as a regulator of neuronal gene expression, playing a crucial role in neuronal differentiation (Andres et al., 1999). In these cells, the CoREST complex is recruited by REST (RE1-silencing transcription factor) that recognizes the 21-nucleotide RE-1 sequence in the promoter of more than 1700 different target genes in human and mouse (Bruce et al., 2004). The CoREST complex is composed of multiple subunits, including RCOR1, lysine-specific demethylase I (Lsd1), histone deacetylases 1/2 (HDACs 1/2) and two very similar proteins from the High Mobility Group (HMG) family, Hmg20a and Hmg20b (Hakimi et al., 2002). In erythroblasts the transcriptional regulator Gfi1b recruits the CoREST complex to the majority of its target gene promoters in vivo. Similar to neuronal cells, inhibition of CoREST disturbs differentiation of erythroid progenitors, as well as megakaryocytic and granulocytic precursors (Saleque et al., 2007).

Several studies have shown the function of various CoREST complex subunits in erythropoiesis (Hu X, 2009; Laurent et al., 2009) , but the role of Hmg20a

and Hmg20b in erythropoiesis has so far not been established. It is believed that Hmg20b-containing CoREST complexes mediate chromatin remodelling and repression of specific genes involved in neuronal differentiation through modulation of chromatin structure (Abrajano et al., 2009; Lakowski et al., 2006). In erythroblasts the transcriptional regulator Gfi1b recognizes TAAATCAC(A/T)GCA (Tong et al., 1998; Zweidler-Mckay et al., 1996) and recruits the CoREST complex to the majority of its target gene promoters in vivo. Inhibition of Gfi1b and the CoREST subunit Lsd1 disturbs differentiation of erythroid progenitors, as well as megakaryocytic and granulocytic precursors (Randrianarison-Huetz et al., 2010; Saleque et al., 2007).

Hmg20b is an HMG domain protein with the ability of non-specific DNA binding. It is expressed in various tissues with relative high levels in the brain (Sumoy et al., 2000), and is classified in a new group of HMG proteins due to the presence of a kinesin-like coiled-coil domain with no known function (Wattler et al., 1999). The only known protein with relatively high homology to Hmg20b is BAF-57 (BRG1-associated factor 57) (Sumoy et al., 2000). BAF-57 is part of the SWI/SNF remodelling complex in association with the RCOR1 protein and plays a crucial role in cell proliferation and cell cycle progression (Hah et al., 2010).

To investigate the role of Hmg20b during erythroid differentiation, we employed primary proerythoblasts from mouse fetal liver, as well as I/11 cells, a cell line derived from p53 knockout mouse fetal liver (von Lindern et al., 2001). These cells can be expanded in the presence of erythropoietin (EPO), stem cell factor (SCF) and dexamethasone (Arcasoy and Jiang). In the absence of SCF and dexamethasone, the majority of the cells hemoglobinize and enucleate within three days. This differentiation process requires the presence of EPO and transferrin (Dolznig et al., 2001).

Here, we studied the role of Hmg20b protein in the balance of erythroid proliferation and differentiation by performing knockdown experiments. We used whole genome expression profiling to further investigate the role of Hmg20b in maintain-

ing this balance, and we assessed the impact of Hmg20b knockdown on globin gene expression.

Design and Methods

Cell culture

I/11 erythroid progenitors and primary mouse fetal liver cells were cultured as described (von Lindern et al., 2001). Primary mouse fetal liver cells were collected on embryonic day 12.5 and expanded for three days before lentiviral transduction according to an established protocol (Dolznig et al., 2001). To induce differentiation, proliferating erythroblasts were washed in ice-cold PBS and reseeded at 1-1.5x106 cells/ml in StemPro (Invitrogen, Carlsbad, USA) supplemented with EPO (10U/ml, Janssen-Cilag BV, Tilburg, NL) and holo-transferrin (1mg/ml; SCIPAC Ltd, Sittingbourne, UK). Differentiating I/11 cells were maintained at 2-3x106 cells/ml. To determine the morphological changes, cells were cytospun and stained with histological dyes (Diff-Quick staining set; Medion Diagnostics International Inc, Miami, USA) and neutral benzidine (Beug et al., 1982). To measure cell size distribution, a CASY instrument (Roche Innovatis AG, Bielefeld, D) was used. Hemoglobin assays were performed according to a published protocol (Bakker et al., 2004). All experiments involving mice were approved by the Erasmus MC Animal Ethics Committee.

Biotinylation and tagging and protemics analysis

Bio-HA-HMG20B was cloned into a modified 5pRRLsin.sPPT.CMV.GFP.Wpre lentiviral vector (Follenzi et al., 2002). BirA-expressing I/11 cells were transduced with Bio-HA-HMG20B and Bio-HA lentiviruses. The transduced cells were selected using puromycin (1µg/ml) and were expanded in culture. Sample preparation and mass spectrometry analyses were carried out as described previously (Soler et al., 2011).

Virus transduction

Short hairpin constructs were from the TRC library (Moffat et al., 2006); Sigma Aldrich, St. Louis, USA). For knockdown of Hmg20b, TRCN0000081665 (Hmg20b shRNA#1), and TRCN0000081664 (Hmg20b shRNA#2) were used. For Hrasls3 knockdown TRCN0000077660 (Hrasls3 shRNA#1), and TRCN0000077662 (Hrasls3 shRNA#2) were used. SHC002 was employed as a control. Lentiviruses were produced by transiently transfecting 293T cells with helper plasmids, the virus-containing supernatants were collected for three days and concentrated by ultracentrifugation (Zufferey et al., 1997). Five days after transduction, the cells were collected and whole cell lysates prepared for western blotting. RNA extraction was carried out for Quantitative Real-Time PCR (QRT-PCR) analysis. For differentiation, cells at day five after transduction were washed twice with PBS and cultured in differentiation medium for four days.

Western blotting and antibodies

Whole cell extract was prepared by incubating the cells with RIPA buffer (20mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 5mM EDTA) supplemented with complete protease inhibitors cocktail (Roche Diagnostics BV, Almere, NL). After two minutes centrifugation at 13 krpm, the supernatant was transferred to an equal volume of 2x sample buffer, with a final concentration of 107 cells per ml. To assess Hmg20b knockdown, whole cell protein extracts of 3x105 cells were loaded on 10% SDS-PAGE, and after blotting on nitrocellulose membrane incubated with Hmg20b antibody (WH0010362M1, Sigma-Aldrich) at a dilution of 1:1000 in blocking buffer (PBS containing 1% (w/v) BSA). Secondary staining was performed using goat-anti-mouse/rabbit IR-Dye 680 or 800 antibodies in PBS with 5% (w/v) blotting grade non-fat dry milk powder (Bio-Rad Laboratories, Hercules, USA) and 0.05% (v/v) tween 20 (Roche Diagnostics).

QRT-PCR analysis and statistical analysis

RNA was extracted using TRI reagent (Sigma-Aldrich) in accordance with the

protocol provided by the company. 2 µg of RNA was used to synthesize cDNA, using oligo dT, RNase OUT, SuperScript reverse transciptase (Invitrogen) in a total volume of 20 µl, and 0.3 µl of cDNA was used for further amplification by QRT-PCR. Amplification was performed with the primers listed in Supplementary Table 1 using Platinum Taq DNA polymerase (Invitrogen) and 40 cycles consisting of 94°C for 30 sec, 60°C 30sec and 72°C 30 sec. All statistical analyses including chi-square tests, ANOVA and Bonferroni correction for globin expression analysis and Mann Whitney U p-value for ChIP experiments, were performed using Stata 11.1software (StataCorp, College Station, TX, USA).

Microarray analysis

RNA samples were extracted from biological triplicates using shRNA#1, control SHC002 and non-transduced I/11 cells three days after transduction. RNA samples were qualitatively controlled and microarray analysis was performed as described (Hou et al., 2010; Soler et al., 2010). The differentially regulated genes between control SHC002 and non-transduced I/11 cell cells were excluded from the final comparison with Hmg20b knockdown I/11 cell cells. Microarray data have been deposited in the NCBI GEO database (GSE29169).

ChIP analysis

To perform chromatin immunoprecipitation (ChIP) 2x107 of either 1/11 or MEL cells were crosslinked with 1% formaldehyde for 10 min. The reaction was stopped with 0.125 M glycine at room temperature and the cells were washed two times with PBS. The cells were resuspended in sonication buffer (10 mM Tris pH 8, 1 mM EDTA and 0.5 mM EGTA) and sonicated with amplitude 7 for 30 cycles (10 sec on, 45 sec off, Soniprep150, MSE, UK).

After centrifugation (10 min at 13 krpm) the supernatants were incubated overnight with Hmg20b (14582-1-AP, Proteintech Group Inc, Chicago, USA) or Lsd1 (ab17721, Abcam, Cambridge, UK) antibodies and Protein A Agarose/Salmon

Sperm DNA beads (Millipore, Billerica, USA). Washing and elution was performed according to the protocol provided by the manufacturer (Millipore).

Results

Hmg20b is part of the CoREST complex in I/11 cells.

Hmg20b has been reported as a subunit of two different complexes in HeLa cells, CoREST and BRCA2 (Hakimi et al., 2002). In mouse erythroleukemia (MEL) cell line Hmg20b acts as part of the CoREST complex (Saleque et al., 2007). To identify Hmg20b interaction partners in growth factor-dependent erythroid progenitors we used I/11 cells, a cell line derived from p53 knockout mouse fetal liver (von Lindern et al., 2001). In contrast to MEL cells, I/1 cells are dependent on EPO, SCF and dexamethasone for expansion, and can be induced to undergo terminal differentiation including enucleation upon withdrawl of SCF and dexamethasone (Dolznig et al., 2001). Thus, these cells recapitulate normal erythropoiesis more closely than MEL cells. We used I/11 cells expressing the E. coli BirA biotin ligase to metabolically label a tagged version of Hmg20b (Bio-HA-Hmg20b). We selected a cell population expressing Bio-HA-Hmg20b at a level similar to that of the endogenous protein. This did not have major effects on the proliferation of the cells (Online Supplementary Figure 1A-B). Protein extracts, streptavidin pull-down of Bio-HA-Hmg20b and associated proteins, and mass spectrometry analysis were performed as described (de Boer et al., 2003; Rodriguez et al., 2006; Soler et al., 2011). Streptavidin pull-down of Bio-HA-Hmg20b followed by mass spectrometry analysis revealed that Hmg20b is part of the CoREST complex in proliferating and differentiating I/11 cells. Consistent with previous data (Saleque et al., 2007), we also identified the transcriptional repressor protein Gfi1b in the mass spectrometry data. Very few other interacting proteins were identified, and we therefore conclude that CoREST is the major complex in which Hmg20b participates in I/11 cells (Figure 1A-B). We also studied the expression changes of Hmg20b and Gfi1b in

differentiating I/11 cells. Western blot and QRT-PCR analyses showed a modest decrease in Hmg20b and Gfi1b expression during I-11 cell differentiation (Figure 1C-E).

Knockdown of Hmg20b induces spontaneous erythroid differentiation.

To assess the role of Hmg20b in erythroid differentiation, we applied lentiviral-mediated knockdown of Hmg20b expression in I/11 cells. Western blot and QRT-PCR analysis confirmed that two independent shRNA constructs reduced Hmg20b expression to less than 20% of wild-type levels at day three after transduction (Fig

A

Proliferating I/11-BirA cells		
Hmg20b-associated proteins		
Score	Protein ID	Protein
673	IPI00323315	**Hmg20b**
640	IPI00453837	Lsd1
617	IPI00396700	Gse1
415	IPI00265217	Rcor1
276	IPI00753751	Bhc80
192	IPI00120110	Hmg20a
150	IPI00114232	Hdac1
78	IPI00116423	Gfi1b

B

Induced I/11-BirA cells		
Hmg20b-associated proteins		
Score	Protein ID	Protein
833	IPI00396700	Gse1
817	IPI00453837	Lsd1
719	IPI00323315	**Hmg20b**
470	IPI00265217	Rcor1
270	IPI00114232	Hdac1
207	IPI00120110	Hmg20a
122	IPI00116423	Gfi1b
55	IPI00124469	Bhc80

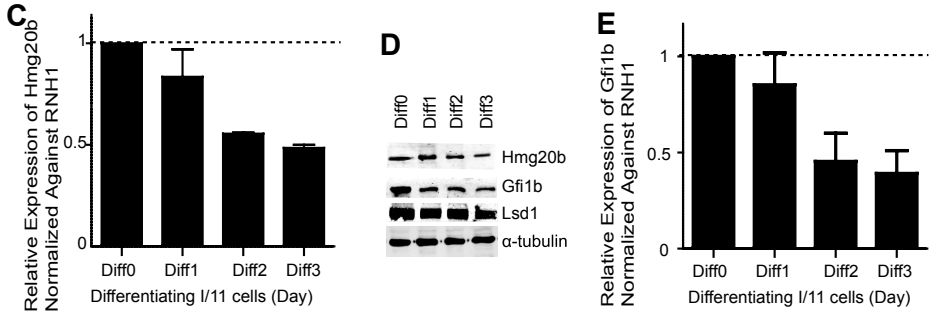

Figure 1. Bio-HA-Hmg20b pull-down and mass spectrometry analysis. (A-B) Hmg20b interacting proteins in proliferating (A) and differentiating (B) I-11 cells. CoREST complex subunits are shown in grey. (C) Hmg20b mRNA expression during I/11 cell differentiation. (D) Western blot of differentiating I/11 cells depicted that Lsd1 is expressed at a constant level, while a modest decrease in Gfi1b and Hmg20b expression occurs; β-tubulin serves as a loading control. (E) Gfi1b mRNA expression decreases during I/11 cell differentiation. Error bars indicate standard deviations. Ribonuclease inhibitor 1 (RNH1) was used for normalization of gene expression.

ure 2A-B). Cell counting revealed a slower expansion of Hmg20b knockdown cell (Figure 2C). Differentiating erythroblasts go through three to five cell divisions before entering G1 arrest and terminal differentiation (Dolznig et al., 2002). Hmg20b depletion resulted in G1 accumulation of I/11 cells grown in expansion medium, with concomitant decrease in proliferation rate (Online Supplementary Figure 2). In line with reduced proliferation, differentiating cells were clearly detected by CASY cell counting profiles at day five to seven after transduction (cells <10 μm; Figure 2D). Knockdown by shRNA #1 was more efficient than that obtained with shRNA #2 with the latter resulting in the most efficient I/11 cell differentiation. This suggests that a low level of Hmg20b protein augments completion of the differentiation and enucleation process, possibly because this allows completion of the 3-5 cell divisions required for normal differentiation.

To study the effects of Hmg20b knockdown in more depth, cytospins were prepared at different time points and stained with histological dyes and neutral benzidine (Beug et al., 1982). The results show that by day seven after transduction, Hmg20b knockdown cells displayed increased differentiation with more than 50% hemoglobinized and enucleated cells, while only 10% of control cells were observed at these late stages of differentiation (Figure 2D). Direct measurement of hemoglobin content showed elevated hemoglobin levels in Hmg20b-depleted cells, further supporting the requirement of Hmg20b for renewal divisions of proerythroblasts.

To confirm that the spontaneous differentiation upon Hmg20b knockdown was not specific to the immortalized p53 null I/11 cell line, Hmg20b expression was knocked down in primary mouse fetal liver proerythroblasts (pMFL). The results obtained were comparable to those observed in the I/11 cells. Hmg20b depletion resulted in a marked proliferation arrest and an increased percentage of hemoglobinized cells compared to control cells (Figure 2F-G). Thus, shRNA-mediated knockdown of Hmg20b in pMFL cells induced differentiation under self-renewal conditions. Collectively, these results indicate that Hmg20b maintains proliferation and suppresses differentiation of mouse proerythroblasts.

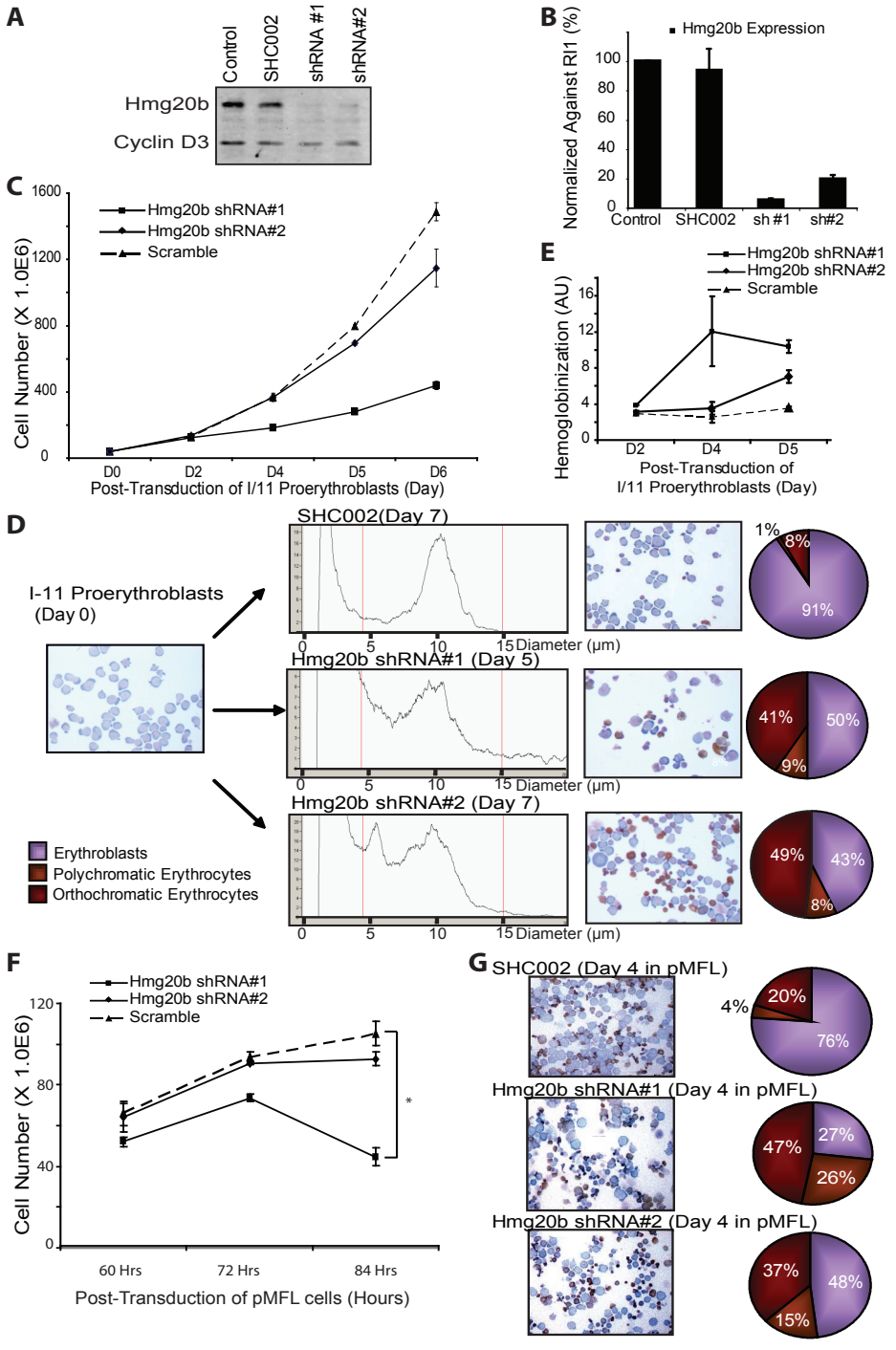
A
Control
SHC002
shRNA #1
shRNA#2
Hmg20b
Cyclin D3
B
Hmg20b Expression
Normalized Against RI1 (%)
100
80
60
40
20
0
Control
SHC002
sh #1
sh#2
C
Hmg20b shRNA#1
Hmg20b shRNA#2
Scramble
Cell Number (X 1.0E6)
1600
1200
800
400
0
D0
D2
D4
D5
D6
Post-Transduction of I/11 Proerythroblasts (Day)
E
Hmg20b shRNA#1
Hmg20b shRNA#2
Scramble
Hemoglobinization (AU)
16
12
8
4
0
D2
D4
D5
Post-Transduction of
I/11 Proerythroblasts (Day)
D
I-11 Proerythroblasts
(Day 0)
SHC002(Day 7)
Hmg20b shRNA#1 (Day 5)
Hmg20b shRNA#2 (Day 7)
0
5
10
15 Diameter (μm)
1%
8%
91%
41%
50%
9%
49%
43%
8%
Erythroblasts
Polychromatic Erythrocytes
Orthochromatic Erythrocytes
F
Hmg20b shRNA#1
Hmg20b shRNA#2
Scramble
Cell Number (X 1.0E6)
120
80
40
0
*
60 Hrs
72 Hrs
84 Hrs
Post-Transduction of pMFL cells (Hours)
G
SHC002 (Day 4 in pMFL)
4%
20%
76%
Hmg20b shRNA#1 (Day 4 in pMFL)
47%
27%
26%
Hmg20b shRNA#2 (Day 4 in pMFL)
37%
48%
15%

Figure 2. Hmg20b knockdown induces spontaneous differentiation of proerythroblasts. (A) Hmg20b knockdown in I-11 cells was mediated with two different shRNA constructs. Whole cell lysates were prepared and used for western blot analysis of Hmg20b protein, cyclin D3 served as a loading control. (B) Hmg20b knockdown was confirmed with QRT-PCR. (C) The proliferation rate of Hmg20b knockdown cells decreased compared to that of cells transduced with control shRNA virus. (D) five to seven days after virus transduction spontaneous differentiation was observed, as shown by cell size profiles. Cytospins were used to determine the percentage of differentiating cells, tabulated in the pie charts (>300 cells counted). (E) Increased hemoglobin content of Hmg20b knockdown cells. (F,G) Hmg20b knockdown induces spontaneous differentiation in primary mouse fetal liver cells, slightly decreases primary proerythroblast proliferation (F) and increases in hemoglobinization (G). The cytospin pictures were taken with 40x magnification using an Olympus BX40F4 microscope (Olympus Optical Co. Ltd). * indicates $p<0.05$. Error bars indicate standard deviations.

Hmg20b regulates Gfi1b expression during erythropoiesis.

Hmg20b knockdown resulted in Gfi1b downregulation in I/11 cells (Figure 3A). Since it is known that the CoREST complex regulates Gfi1b expression in erythroblasts (Saleque et al., 2007) and Hmg20b is part of the CoREST complex in I/11 cells (Figure 1A-B), we performed chromatin immunoprecipitation (ChIP) analysis to clarify whether Hmg20b is part of the CoREST complex that regulates Gfi1b expression in proerythroblasts. To be able to compare previously reported results in MEL cells (Saleque et al., 2007) with those obtained in I/11 cells, we first performed ChIP of the CoREST subunit Lsd1 in I/11 cells. Similar to the observations reported for MEL cells (Saleque et al., 2007), Lsd1 enrichment on the Gfi1b promoter was stronger in proliferating than in differentiating I/11 cells (Figure 3B). In MEL and I/11 cells, Hmg20b followed a pattern of enrichment on the Gfi1b promoter similar to that of Lsd1 (Figure 3C). These results suggest that Hmg20b regulates Gfi1b expression in erythroid progenitors as part of the CoREST complex.

The majority of genes deregulated upon Hmg20b depletion are upregulated.

To explore the changes in whole genome expression profiles of Hmg20b-depleted cells in comparison with control cells, microarray experiments were performed in biological triplicates. RNA for expression profiling was harvested from I/11 cells

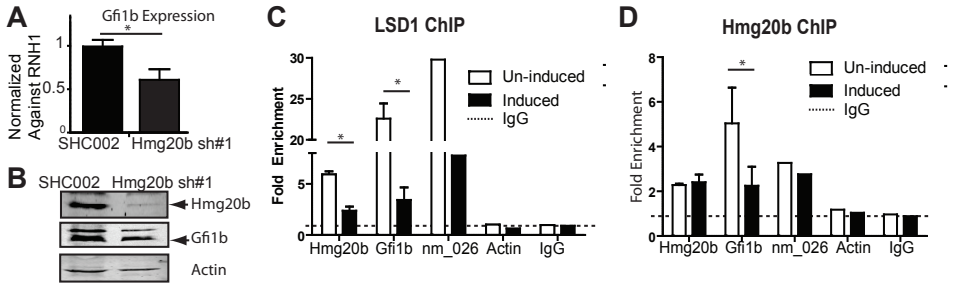

Figure 3. Hmg20b regulates Gfi1b expression during I/11 cell differentiation. (A) Gfi1b mRNA expression is downregulated three days after Hmg20b knockdown. (B) Gfi1b expression at the protein level is decreased after Hmg20b knockdown after five days of virus transduction. (C) Lsd1 enrichment on the Gfi1b promoter in I/11 cells. (D) Hmg20b enrichment on the Gfi1b promoter. The Nm_026543 (nm_026) gene was used as a positive control (Saleque et al., 2007). White bars: proliferating cells; black bars: differentiating cells. * indicates $p<0.05$. Error bars indicate standard deviations.

three days after transduction with lentiviral shRNA vectors. Following array hybridization, we first ensured that the triplicate samples were closely correlated (Figure 4A). To validate the microarray data, QRT-PCR was performed for several selected target genes including upregulated genes such as Hrasls3, Trp53inp1, Cited2, Ccng2, and downregulated genes such as Rcor2 and Kit. This analysis showed a generally good correlation between the microarray expression data and QRT-PCR results on the same genes (Online Supplementary Figure 3 and Online Supplementary Table 2). Taking a 1.5-fold change in gene expression with p-value <0.01 as a cut off, the analysis of the microarray data showed deregulation of 620 genes, the majority of which (85%; 527 out of 620) had been upregulated (Online Supplementary Table 3). This strongly points at a generally repressive role of Hmg20b in erythroid gene expression. However, some of the deregulated genes (15%; 93 out of 620) were downregulated indicating that Hmg20b may have a dual role as either an activator or a repressor in erythroid cells (Figure 4 B-C).

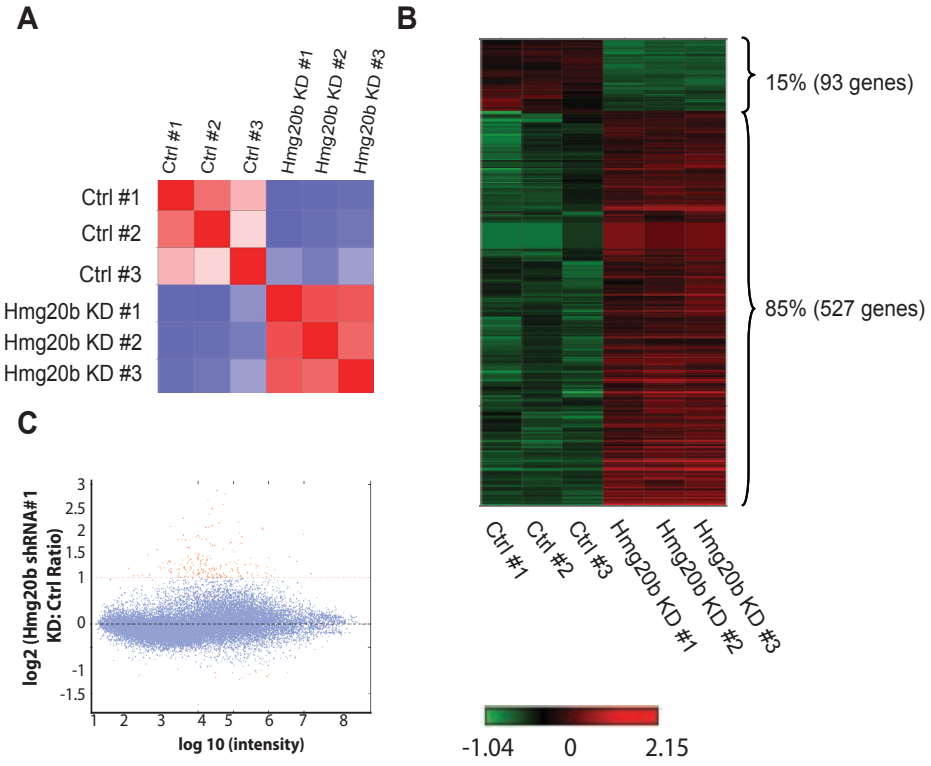

Figure 4. Expression profiling of Hmg20b knockdown proerythroblasts. (A) Correlation analysis of biological triplicates of control- and Hmg20b knockdown cells. Red: positive correlation; blue: negative correlation. (B) Heatmap of differentially regulated genes with known function (GO terms) in Hmg20b knockdown cells compared with control I/11 cells. Downregulated genes: green, upregulated genes: red. (C) MA plot of Hmg20b knockdown proerythroblasts. Blue: constantly expressed genes; red: differentially expressed genes (>2-fold).

Knockdown of Hrasls3 inhibits erythroid differentiation

We noted that the Hrasls3 tumor suppressor gene was among the upregulated genes in the Hmg20b-depleted proerythroblasts. Microarray analysis showed ~1.6-fold upregulation of Hrasls3 in Hmg20b depleted I/11 cells, which was confirmed by QRT-PCR (Online Supplementary Figure 3 and Online Supplementary Table 2). Hrasls3 is involved in cell cycle arrest (Mason et al., 2010; Sers et al., 1997) and its role in adipocyte differentiation has been established (Hummasti et al., 2008). It

might therefore perform similar roles during erythroid differentiation.

To investigate this, we first established the expression pattern of Hrasls3 during erythroid differentiation. QRT-PCR showed a sharp increase (>6-fold) in Hrasls3 expression at day two of differentiation (Figure 5A). This is consistent with a positive contribution of Hrasls3 to erythroid differentiation. To test this putative role, we performed shRNA-mediated knockdown experiments of Hrasls3 expression. Using two different shRNA constructs, we found that Hrasls3 depletion did not interfere with cell proliferation, as Hrasls3 knockdown I/11 cells grew without any significant difference in proliferation rate compared to cells transduced with the control SHC002 shRNA virus (Figure 5B-C). Interestingly, Hrasls3 depletion severely affected differentiation of pMFL cells. The percentage of undifferentiated proerythroblasts was ~2-fold higher in Hrasls3 knockdown cells than that observed in controls transduced with the SHC002 shRNA construct (Figure 5D).

Next, we depleted I/11 cells for both Hmg20b and Hrasls3 to address the question if depletion of Hrasls3 rescues the spontaneous differentiation of I/11 cells induced by Hmg20b knockdown. Double knockdown of Hmg20b and Hrasls3 resulted in a marked decrease of differentiating proerythroblasts and the cells grew at rates similar to those observed in SHC002-transduced control cells (Figure 5E-F). As expected, expression of globin mRNAswas significantly increased in spontaneously differentiating Hmg20b-depleted cells. Notably, the fold-change in embryonic εy-globin mRNA expression was the highest (Figure 5G). This considerable increase in expression was not observed in double knockdown Hmg20b / Hrasls3 cells. In these cells, the expression of εy–globin mRNA still increased to some extent, but significantly less than in Hmg20b-only depleted cells (Figure 5G).

Collectively, we conclude that Hrasls3 contributes positively to erythroid differentiation, and that the observed spontaneous differentiation upon Hmg20b knockdown can at least partially be attributed to increased expression levels of Hrasls3.

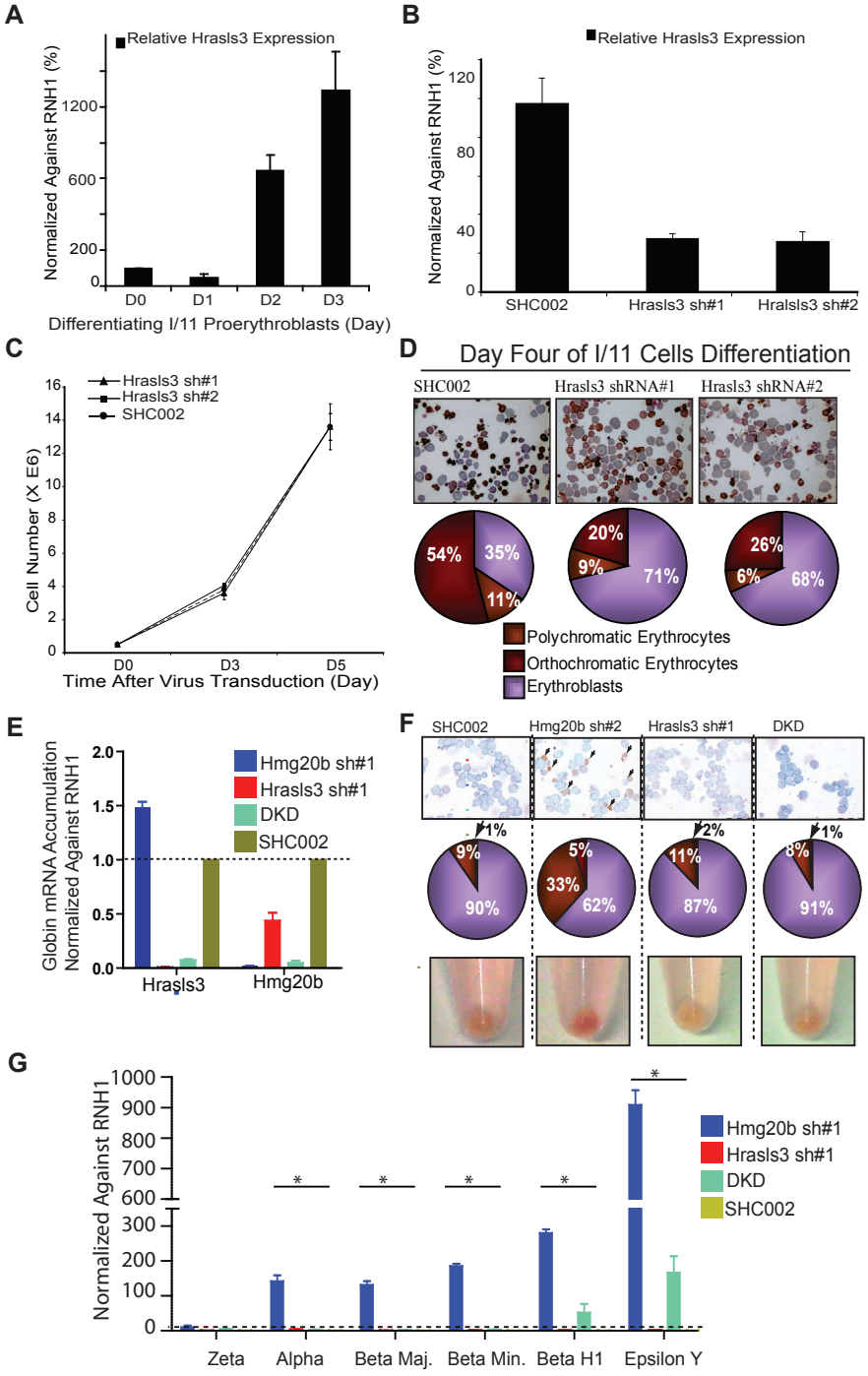
A
Relative Hrasls3 Expression
Normalized Against RNH1 (%)
1200
600
200
0
D0
D1
D2
D3
Differentiating I/11 Proerythroblasts (Day)
B
Relative Hrasls3 Expression
Normalized Against RNH1 (%)
120
80
40
0
SHC002
Hrasls3 sh#1
Hralsls3 sh#2
C
Hrasls3 sh#1
Hrasls3 sh#2
SHC002
Cell Number (X E6)
16
14
12
10
8
6
4
2
0
D0
D3
D5
Time After Virus Transduction (Day)
D
Day Four of I/11 Cells Differentiation
SHC002
Hrasls3 shRNA#1
Hrasls3 shRNA#2
54%
35%
11%
20%
9%
71%
26%
6%
68%
Polychromatic Erythrocytes
Orthochromatic Erythrocytes
Erythroblasts
E
Globin mRNA Accumulation Normalized Against RNH1
2.0
1.5
1.0
0.5
0.0
Hmg20b sh#1
Hrasls3 sh#1
DKD
SHC002
Hrasls3
Hmg20b
F
SHC002
Hmg20b sh#2
Hrasls3 sh#1
DKD
1%
9%
90%
5%
33%
62%
2%
11%
87%
1%
8%
91%
G
Normalized Against RNH1
1000
900
800
700
600
300
200
100
0
*
Hmg20b sh#1
Hrasls3 sh#1
DKD
SHC002
Zeta
Alpha
Beta Maj.
Beta Min.
Beta H1
Epsilon Y

Figure 5. Hrasls3 and erythroid differentiation. (A) QRT-PCR showing upregulation of Hrasls3 in differentiating I-11 cells. (B) Two shRNA constructs induced efficient Hrasls3 knockdown. (C) Normal proliferation rates of I/11 cells knocked down for Hrasls3. (D) Hrasls3 knocked down I/11 cells on day four of differentiation. The pie charts represent the percentage of differentiating cells (>300 cells counted). (E) mRNA expression levels of Hmg20b and Hrasls3 in single- and double knockdown cells (Hmg20b, Hrasls3). (F) cytospins, pie charts and cell pellets from single- and double knockdown cells (Hmg20b, Hrasls3) compared with SHC002-transduced cells. (G) Changes in globin mRNA expression levels were determined upon Hmg20b, Hrals3 and double knockdown compared with SHC002-transduced cells. DKD: double knockdown. The cytospin pictures were taken with 40x magnification using an Olympus BX40F4 microscope (Olympus Optical Co. Ltd). * indicates $p<0.05$. Error bars indicate standard deviations.

Discussion

The current study has elucidated Hmg20b as a repressor of terminal erythroid differentiation. To address the function of this protein in erythroid differentiation we depleted Hmg20b in I/11 cells using shRNA-mediated knockdown. Interestingly, Hmg20b downregulation induced spontaneous differentiation of I/11 cells which was manifested by hemoglobinization and enucleation of the cells. Notably, previous studies showed that other subunits of the CoREST complex, including Rcor1 and Lsd1, are involved in erythro- and megakaryopoiesis (Saleque et al., 2007). Knockdown of Lsd1 but not of Rcor1 impaired erythroid differentiation in MEL cells and reduced expression of erythroid differentiation markers (Saleque et al., 2007). Redundant expression of Rcor family members may explain the apparent absence of a phenotype after Rcor1 depletion in erythroblasts (Saleque et al., 2007). From our data on spontaneous differentiation induced by Hmg20b knockdown in I/11 cells and pMFL cells we conclude that Hmg20b plays a crucial role in maintaining the proliferative status of erythroid pregenitors. Presumably, it does so in concert with the other subunits of the CoREST complex; this notion is supported by our proteomics data indicating that CoREST is the major complex in which Hmg20b participates in these cells. Differentiating erythroblasts complete a series of differentiation-specific cell divisions before undergoing G1 arrest (Dolznig et al., 2002). Thus, our data suggest that Hmg20b is involved in the switch from renewal to dif-

ferentiation divisions.

We found that Hmg20b depletion results in accumulation of different globin mRNAs, including embryonic β-like globins. It has been shown that CoREST complex subunits like Hdac1, Lsd1 and Rcor1 bind to the promoters of the embryonic εy and βH1 genes, but not to the promoter of the adult β-major gene (S. Cui et al, unpublished data, 2011). In addition, Lsd1 and Rcor1 do not bind to the promoters of the β-like globin genes, providing a rationale for our observation that the embryonic β-globin gene is not derepressed upon Hmg20b knockdown. Our data on Hmg20b suggest that it plays a role in the recruitment of the CoREST repressor complex on the embryonic β-like globin genes in adult erythroid cells. Accordingly, Hmg20b depletion removes the repressor complex giving rise to relieve of suppression of the embryonic β-like globin genes. This suggests that Hmg20b maintains a repressive function on some of its targets during terminal differentiation of definitive erythroid cells. We note that the expression levels of the embryonic β-like globin genes remain low compared to those of the adult β-like globin genes, indicating that even in upon knockdown of Hm20b the transcription factor milieu of adult erythroid cells is not permissive for full expression of the embryonic genes. This could be due for example to the presence of the Bcl11a and Sox6 repressor proteins (Xu et al., 2010). Collectively, the data on the role of CoREST subunits indicate that modulation of CoREST activity is essential for normal erythropoiesis ((Saleque et al., 2007) and this paper).

To identify more potential targets of Hmg20b in proerythroblasts, we carried out microarray analysis on Hmg20b-depleted I/11 cells. Whole genome expression profile analysis of Hmg20-depleted cells strongly supports the inhibitory role of Hmg20b in erythroid cells, since we found that 85% of differentially expressed genes are upregulated upon Hmg20b knockdown. Apparently, in the presence of Hmg20b the majority of its target genes are repressed which enables proerythroblasts to retain their undifferentiated proliferative status. In the absence of Hmg20b those targets are derepressed, leading to spontaneous terminal differentiation of

the cells. Genes deregulated by Hmg20b knockdown include those involved in cell cycle arrest, globin synthesis and the erythroid differentiation process, indicating a global regulatory role of Hmg20b in erythropoiesis.

To extend our understanding on the regulatory function of Hmg20b protein, we searched for the factors that can be directly regulated by Hmg20b. Hmg20b has been purified as a subunit of the CoREST complex, mediating repression of neurogenesis through recruitment by REST protein to the RE1 sequence of the target genes (Andres et al., 1999). In erythroid cells, Gfi1b represses gene expression

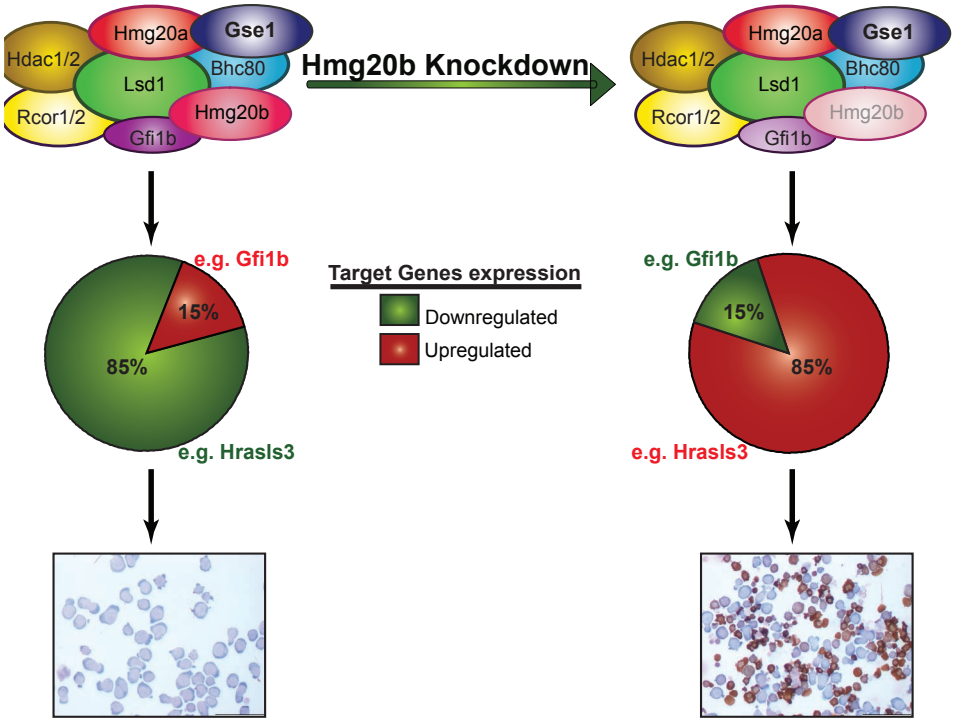

Figure 6. Proposed regulatory mechanism for Hmg20b in erythroid differentiation. Hmg20b, as part of the CoREST complex, plays a dual regulatory role in proliferating proerythroblasts. It regulates the repressor Gfi1b, which guides the CoREST complex to genes involved in differentiation, resulting in their repression. Decreased Hmg20b expression leads to reduced Gfi1b expression and release of repression of Gfi1b/CoREST target genes, including Hrasls3, allowing erythroid differentiation to ensue.

through recruitment of the CoREST complex to its target genes (Saleque et al., 2007). Studies on GFP-Gfi1b-knockin mice demonstrated that Gfi1b is highly expressed in mouse fetal liver cells, the anatomical site where hematopoiesis takes place at midgestation (Vassen et al., 2007). In the absence of Gfi1b, only a small fraction of erythroblasts is able to differentiate and the accumulation of immature erythrocytes is observed (Saleque et al., 2002; Vassen et al., 2007). In vitro studies demonstrated that Gfi1b knockdown impairs erythroid differentiation as well (Randrianarison-Huetz et al., 2010; Saleque et al., 2007). Gfi1b expression is autoregulated, and it recruits the CoREST complex to its promoter (Anguita et al., 2010; Saleque et al., 2007). Proteomics analysis demonstrated association of the Hmg20b protein with the CoREST complex in erythroid cells, and we showed direct binding of Hmg20b to the Gfi1b promoter. We therefore conclude that Gfi1b-mediated CoREST recruitment to the Gfi1b promoter regulates Gfi1b expression ((Anguita et al., 2010; Saleque et al., 2007), Figure 6).

One of the consistently upregulated genes upon Hmg20b depletion was the Hrasls3 tumor suppressor gene. Hrasls3-overexpressing cells can not be expanded in culture, demonstrating the role of Hrasls3 in suppressing cellular proliferation (Nazarenko et al., 2007; Uyama et al., 2009). In fibroblasts, overexpression of Hrasls3 augments adipogenesis and accordingly Hrasls3 downregulation inhibits adipocyte differentiation (Han et al., 2010; Hummasti et al., 2008; Yanatatsaneejit et al., 2008). We find that, similar to the observations in adipogenesis, Hrasls3 expression increases during erythroid differentiation. We demonstrated that shRNA-mediated downregulation of Hrasls3 inhibits erythroid differentiation. It therefore appears that Hrasls3 plays a key role in distinct differentiation processes, as exemplified by adipogenesis and erythropoiesis (Figure 6). Additionally, we found that the spontaneous differentiation phenotype observed upon Hmg20b depletion is dependent on the upregulation of Hrasls3.

In conclusion, we found that Hmg20b is a repressor of erythroid differentiation. It acts in the autoregulatory loop of Gfi1b expression, helping to maintain the Gfi1b/

CoREST activity that serves to repress erythroid differentiation. Upon Hmg20b knockdown the majority of differentially expressed genes are upregulated and the terminal differentiation program ensues. Furthermore, Hmg20b knockdown leads to derepression of the embryonic β-like globin genes. We discovered that Hrasls3 is an important gene repressed by Hmg20b, and that upregulation of Hrasls3 plays an important role during terminal erythroid differentiation.

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Online Supplementary Design and Methods

Cell cycle analysis

To analyze cell cycle profiles, at different time points after virus transduction I/11 cells were washed once with cold PBS, and fixed in 70% ethanol. Fixed cells were pelleted and stained with a propidium iodide (PI) solution (50 µg/ml, Invitrogen) containing 1U/µl RNase A and 0.1% Triton X-100 (Sigma-Aldrich) for 30 min at room temperature. Flow cytometric quantification of DNA was performed using a FACScan (Becton Dickinson) (Vindelov LL,et al,. 1983).

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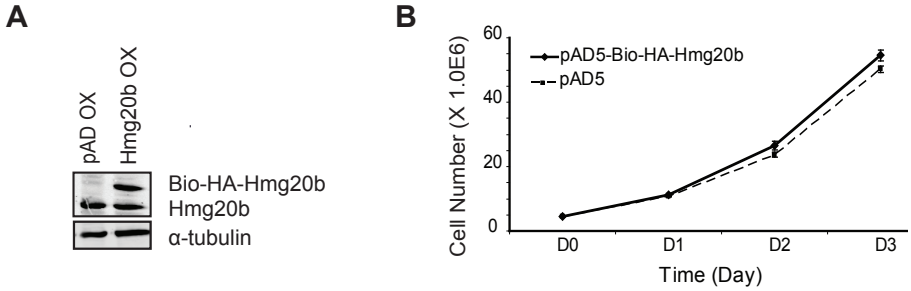
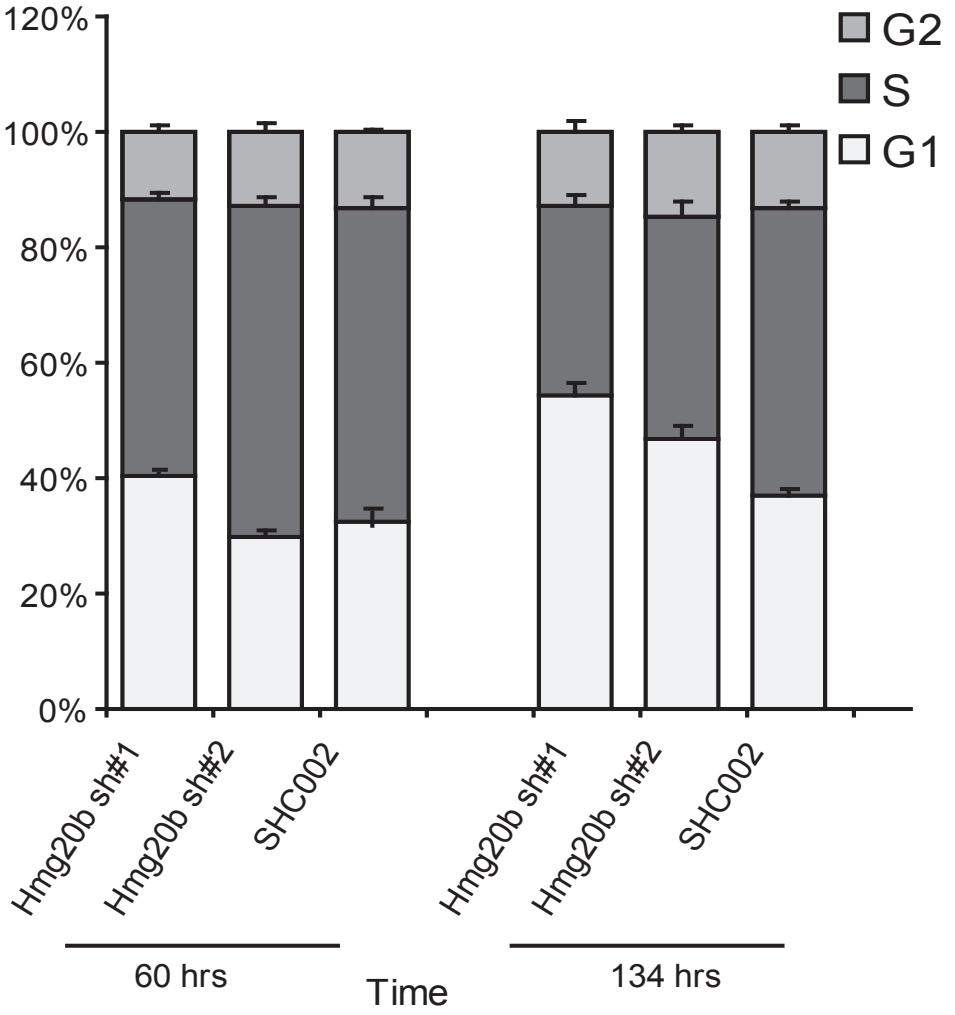
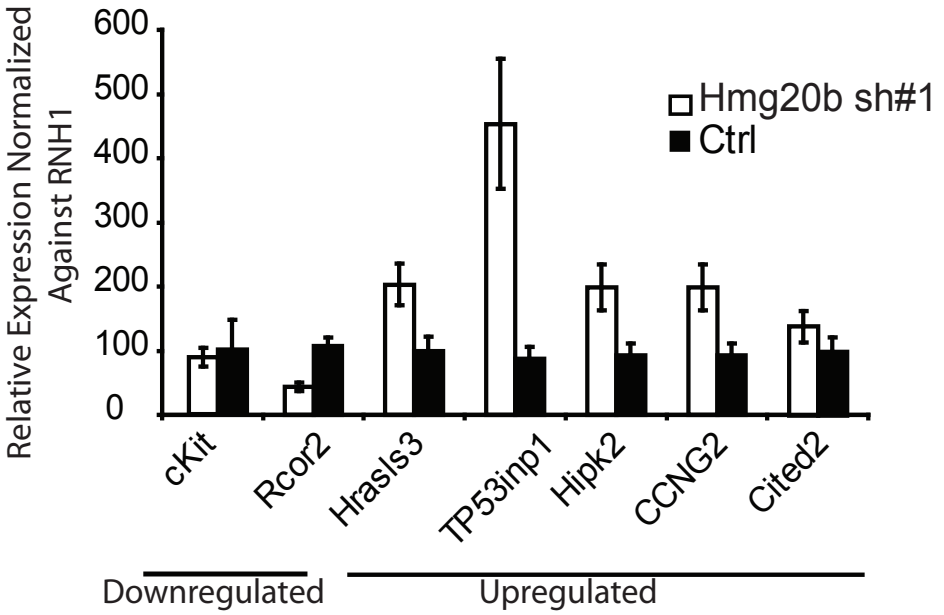


Supplementary Figure 1. Bio-HA-Hmg20b overexpression. A) Hmg20b cDNA N-terminally tagged with Bio-HA was cloned in 5pRRLsin.sPPT.CMV.GFP.Wpre (pAD5) and exogenously expressed in BirA expressing I/11 cells (Hmg20b-Bio-HA-Hmg20b). B) Bio-HA-Hmg20b overexpression does not affect I/11 cell proliferation rate compared with control cells.



Supplementary Figure 2. Cell cycle analysis using PI in Hmg20b knockdown I/11 cells at day 3 and 5 after transduction showed higher percentage of Hmg20b-depleted cells arrested in G1 compared with control SHC002 transduced cells.



Supplementary Figure 3. Validation of microarray data by QRT-PCR. QRT-PCR analysis of genes deregulated according to the microarray analysis. Gene expression has been normalized against mouse Ribonuclease Inhibitor1.

Supplementary Table 1: Oligonucleotides used in this study

Name	Sequence	Purpose
Ccng2-S	AAGCAAGACCATCTGTATTAGCTC	qRT-PCR
Ccng2-A	GTGTCGCTGAGCTTCAAATGT	qRT-PCR
Cited2-S	AAGCTCAACAACCAGTATTTCAAC	qRT-PCR
Cited2-A	ATCTCGGAAGTGCTGGTTTGT	qRT-PCR
Hipk2-S	GATTGAGAACACAAGCAGCGT	qRT-PCR
Hipk2-A	TTCACTGTTGGAGCCACTGTT	qRT-PCR
Hmg20b-S	CACGGGGCCTTTGTAGTG	qRT-PCR
Hmg20b-A	CAGCCTCGCTTCTTCACTG	qRT-PCR
Hrasls3-S	TCCAAGTGAAATCGCAGGAG	qRT-PCR
Hrasls3-A	TACTCCTCGTCATGTTTGTTATTG	qRT-PCR
Rcor2-S	GCTATAACATTGAGCAGGCACT	qRT-PCR
Rcor2-A	CAGCACCTTGTCCTCCACC	qRT-PCR
Trp53inp-S	ACTTCATAGATACCTGCCCTGG	qRT-PCR
TRP53inp-A	TTCCAAAGATGCAGGTAAACAG	qRT-PCR
Hmg20b-S	AAGTGGAATGTGAGAATGGTTT	ChIP
Hmg20b-A	AAACAAAAATCAGAAAGAAAAGAGA	ChIP
Amylase-S	CTCCTTGTACGGGTTGGT	ChIP
Amylase-A	AATGATGTGCACAGCTGAA	ChIP
Gfi1b-S	CGCCAGATTTTGACACAAATAA	ChIP
Gf1b-A	CTGCACAGACAGACACTTCTCC	ChIP
Nm_026-S	AAACACGTAGGAACACCAGCTC	ChIP
Nm_026-A	CATCCCCTGACAAGCATAAAA	ChIP

Supplementary Table 2: online published in haematologica journal website

Supplementary Table 3: Correlation of QRT-PCR and microarray data

Name	Probe set	Fold change in microarray	Correlation
Trp53inp1	10503259	3,88	0,69
Hrasls3	10461093	1,60	0,80
Ccng2	10523297	2,31	0,89
Cited2	10361828	1,89	0,55
Hipk2	10544114	1,86	0,85
Rcor2	10461057	0,66	0,98
cKit	10522530	0,49	0,30

Erythropoiesis and globin switching in compound Klf1-Bcl11a mutant mice

(Manuscript in preparation)

Erythropoiesis and globin switching in compound Klf1-Bcl11a mutant mice

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Abstract

B-Cell/Lymphoma 11A (BCL11A) down-regulation in human primary adult erythroid progenitors results in elevated expression of fetal γ-globin. Recent reports showed that BCL11A expression is activated by KLF1, leading to γ-globin repression. Using PAC8.1 mouse line with combinations of Bcl11a conditional knockout, EpoR-Cre knockin, and Klf1 knockout alleles we studied regulation of erythropoiesis and globin expression by Bcl11a and Klf1 in an in vivo model. We found a mild anemia in Bcl11a mutant mice and higher percentage of reticulocytes in adult Klf1+/- mice, which was more pronounced in combination with the Bcl11a-/- genotype. Analysis of Klf1+/-, Bcl11a-/- and Klf1+/-::Bcl11a-/- mutant embryos demonstrated increased expression of mouse embryonic globins during fetal development. The combination of Klf1+/- with Bcl11a-/- further augmented γ-globin expression before birth, but γ-globin expression decreased after birth and was eventually expressed at a low level in all genotypes studied. Collectively, we showed that Bcl11a mutation result in mild anemia and together with Klf1 haploinsufficiency it results in enhanced γ-globin expression.

Introduction

Sickle cell anemia (SCA) and β-thalassemia are the most common monogenic disorders in the human population, with an estimated 300,000 seriously affected children born annually (Angastiniotis and Modell, 1998). SCA is caused by expression of a pathological β-globin missense mutant (Glu6Val), while β-globin insufficiency underlies β-thalassemia. The symptoms of these diseases are alleviated by high levels of expression of γ-globin, a β-like globin that is expressed at the fetal stages of human development. HbF ($\alpha 2\gamma 2$) is the dominant hemoglobin when the fetal liver is the site of erythropoiesis. After birth, when the site of erythropoiesis shifts to the bone marrow, γ-globin is gradually replaced by β-globin and HbA (α2β2) becomes the major hemoglobin. In adults, HbF levels normally decrease to less than 1% and HbA2 (α2δ2) accounts for less than 2% of total hemoglobin (Stamatoyannopoulos and Grosveld, 2001). For this reason, newborn patients with β-thalassemia or SCA will start manifesting disease symptoms during the first year of life. Since HbF can substitute for HbA in adults, HbF induction would alleviate the symptoms of β-thalassemia and SCA. Safe pharmacological reactivation of γ-globin expression therefore remains a very attractive approach (Stamatoyannopoulos, 2005). Currently, hydroxyurea is used in the clinic with considerable success, elevating HbF levels in ~50% of patients with β-thalassemia (Gambari, 2010). However, long-term treatment of thalassemic and SCA patients with hydroxyurea may give rise to a series of abnormalities such as leukemia, rash and nail changes (Strouse et al., 2008). Thus, revealing novel targets to develop new therapeutic intervention emerges understanding the molecular details of γ- to β-globin switching as an important goal.

To study the mechanisms underlying switching from γ- to β-globin, human β-globin locus transgenic mice have been established as models for developmental regulation of globin gene expression (Stamatoyannopoulos and Grosveld, 2001). The human β-globin locus contains five developmentally regulated β-like globin

genes in the order 5'-ε (embryonic) -^{G}γ-^{A}γ (fetal) -δ-β (adult) -3', while the mouse β-globin locus harbors four genes in the order 5'-εy-βh1 (embryonic) -βmaj-βmin (fetal/adult) -3' (Stamatoyannopoulos and Grosveld, 2001). The α-globin loci in human and mouse contain an embryonic ζ- and two fetal/adult α-globin genes (Higgs and Gibbons, 2010). In mice, εy, βh1 and ζ are embryonic globins expressed in primitive erythrocytes. Their expression is silenced in definitive erythrocytes which express α- and β-globins at the fetal and adult stages of development (Brotherton et al., 1979; Palis, 2008). In PAC8.1 human β-globin locus transgenic mice, the γ-globin genes behave like embryonic/early fetal genes. Switching to β-globin expression takes place between embryonic day (E) 12 and E14 (de Krom et al., 2002), when the fetal liver is the major site of erythropoiesis. It has recently been demonstrated that this difference in developmental timing of globin switching is linked to alterations in the expression of the B-Cell/Lymphoma 11A (BCL11A) repressor protein, creating a *trans*-acting environment in the mouse fetal liver that is non-permissive for γ-globin expression (Sankaran et al., 2009).

In humans, genome-wide association studies (GWAS) revealed a strong correlation of HbF levels with several SNPs located in the *BCL11A* gene (Menzel et al., 2007; Uda et al., 2008). Subsequently, BCL11A was reported as a critical mediator of fetal γ-globin silencing in the adult (Sankaran et al., 2008; Sankaran et al., 2009). It functions as a repressor though binding to *cis*-regulatory elements in the β-globin locus, and interacts with the NuRD repressor complex and the GATA1 and FOG transcription factors. It binds to the third hypersensitivity site (5'HS3) in the β-globin locus control region (LCR) and a region downstream of the ^{A}γ gene. BCL11A downregulation in sorted and expanded CD34^{+} human hematopoietic progenitor cells elevates γ–globin expression. BCL11A depletion does not affect the expression of well-known transcription factors regulating erythropoiesis such as KLF1, NF-E2, GATA1 and FOG (Sankaran et al., 2008). In contrast, it was recently shown that KLF1 activates BCL11A expression (Borg et al., 2010; Zhou et al., 2010). KLF1 (previously known as EKLF (Borg et al., 2011; Siatecka and Bieker, 2011)

has a critical role in erythroid development, and in mice its expression increases three-fold upon the transition from primitive to definitive erythropoiesis (Donze et al., 1995). Analysis of *Klf1* knockout embryos revealed lethality at E14.5 due to anemia caused by disrupted fetal liver erythropoiesis (Nuez et al., 1995; Perkins et al., 1995). Remarkably, Klf1 is absolutely required for activation of β-globin expression, while the α-like and embryonic β-like genes are still highly expressed in *Klf1* knockout embryos (Nuez et al., 1995; Perkins et al., 1995). Furthermore, *Klf1* knockout embryos carrying a human β-globin locus transgene fail to activate the human β-globin gene, while the γ-globin genes are fully expressed (Perkins et al., 1996; Wijgerde et al., 1996).

Mutations in human *KLF1* are associated with a spectrum of phenotypes, such as the In(Lu) blood group (Singleton et al., 2008), zinc protoporphyria (Satta et al., 2011), congenital dyserythropoietic anemia (CDA) (Arnaud et al., 2010) and hereditary persistence fetal hemoglobin (HPFH) (Borg et al., 2010). Analysis of the HPFH phenotype has led to the proposal that KLF1 has a dual role in γ-globin suppression, through its preferential activation of the β-globin gene and ,as a key activator of expression of the BCL11A repressor protein (Borg et al., 2010; Zhou et al., 2010). Here, we used mice carrying a human *β-globin* locus transgene (PAC8.1) (de Krom et al., 2002) and crossed these with mice carrying *Klf1* knockout (Nuez et al., 1995), *Bcl11a* conditional knockout, and *EpoR-Cre* knockin (Heinrich et al., 2004) alleles, to interrogate the impact of these two key molecules, Klf1 and Bcl11a, on erythropoiesis and globin gene regulation.

Materials and Methods

Mice

All animal studies were approved by the Erasmus MC Animal Ethics Committee. Transgenic mouse strains used were: human β-globin locus transgenic line PAC8.1 (de Krom et al., 2002); *Klf1* knockout allele (Nuez et al., 1995); knockin of Cre

recombinase in the Epo receptor locus (*EpoR-Cre*) (Heinrich et al., 2004) and *Bcl11a* conditional knockout allele (Bilic and Busslinger, manuscript in preparation). Genotyping was performed by PCR using DNA isolated from tail snips using primers listed in Supplementary Table 1. Embryos were collected at different time points after gestation; head DNA was used for genotyping by PCR. Inactivation of the *Bcl11a* gene was analyzed using PCR to detect Cre-mediated recombination at the *Bcl11a* locus.

Blood analysis

Peripheral blood was collected from the mandibular vein of >6-week old mice and standard blood parameters were measured using an automated hematologic analyzer (Scil Vet ABC, Viernheim, Germany). Blood smears were stained with Wright-Giemsa and scored double-blinded by KvL. Cytospins were made using single-cell suspensions prepared from fetal liver, spleen and bone marrow and stained with Wright-Giemsa.

Statistical tests

Statistical analyses used ANOVA with Bonferroni correction using Stata 11.1 software (StataCorp, College Station, TX, USA). GraphPadPrism software (GraphPad Software, Inc. version 5.02, CA, USA) was employed to draw the graphs.

RNA isolation, S1 nuclease protection assays and QRT-PCR analyses

RNA was isolated from mouse erythroid tissues including fetal liver, blood, and bone marrow using TRI reagent (Sigma-Aldrich). 2.5 µg of RNA was used for each S1 nuclease protection assay as described previously (Antoniou, 1991; Borg et al., 2010). Synthesis of cDNA and quantitative real-time PCR (QRT-PCR) were performed as described (Esteghamat et al., 2011). Expression of different globin genes was measured using the Ct values; Ct values obtained for α-globin

expression was used for normalization. The oligonucleotides used for QRT-PCR are listed in Supplementary Table 1.

Protein extraction and western blotting

Whole cell lysates were prepared using RIPA buffer as described previously (Esteghamat et al., 2011). To visualise γ-globin expression at the protein level, whole cell lysates of ~$3x10^5$ red cells were loaded on 12.5% SDS-PAGE , the gels were transferred to nitrocellulose membranes and probed with γ-globin antibody (51-7, sc-21756, Santa Cruz Biotechnology). Staining for actin served as loading control (I-19, sc-1616, Santa Cruz Biotechnology).

Flowcytometry analysis

Whole blood and single cell suspensions collected from bone marrow and spleen were washed twice with phosphate buffered saline (PBS) and then resuspended in PBS containing 1% (w/v) bovine serum albumin and 1mM EDTA. ~10^6 cells were incubated for 30 min with CD71-FITC (553266, BD Pharmingen) and Ter119-PE (553673, BD Pharmingen) antibodies (diluted 1:200) in a final volume of 100 µl. The cells were washed and live cells distinguished negatively by 7-aminoactinomycin D (A1310, Invitrogen) staining. Cells were analyzed on a FACScan instrument (Becton Dickinson).

Erythropoietin assays

Whole blood was centrifuged at 4 krpm for 10 min, sera were collected and stored at -20°C. Erythropoietin concentrations were measured using an ELISA-based assay (mouse/rat Epo immunoassay, Quantikine, R&D systems, USA).

Immunohistochemistry

Immunohistochemistry for γ-globin expression was performed as described (van Dijk et al., 2010).

Results

Generation of compound transgenic animals

The role of Klf1 in erythropoiesis has been widely studied (Borg et al., 2011; Siatecka and Bieker, 2011), while less is known about the role of Bcl11a. The interplay of Klf1 and Bcl11a in the switching of fetal to adult *β-like* globin gene expression is of particular interest (Borg et al., 2010; Zhou et al., 2010). Since mice do not have a fetal *β-like* globin gene, we used animals carrying a human *β-globin* locus transgene (line PAC8.1 (de Krom et al., 2002)). In these animals, the human γ-globin genes are expressed at the embryonic and early fetal stages; the switch from γ-globin to β-globin expression takes place between E12.5 and E14.5 when the fetal liver is the major site of erythropoiesis (Palis, 2008). Such mice have been widely used as a model to study developmental expression of the human *β-like* globin genes (Stamatoyannopoulos, 2005). We crossed the PAC8.1 mice with mice carrying a knockout allele of *Klf1* (Nuez et al., 1995) and a conditional knockout allele of *Bcl11a* (Bilic and Busslinger, manuscript in preparation). To obtain erythroid-specific ablation of Bcl11a expression we introduced a knockin allele of Cre recombinase in the *EpoR* locus (Heinrich et al., 2004). We studied mice with four genotypes: (1) *PAC8.1::Bcl11a*$^{cko/cko}$; (2) *PAC8.1::Klf1*$^{ko/wt}$*::Bcl11a*$^{cko/cko}$; (3) *PAC8.1::Bcl11a*$^{cko/cko}$*::EpoR*$^{Cre/wt}$; and (4) *PAC8.1::Klf1*$^{ko/wt}$*::Bcl11a*$^{cko/cko}$*::EpoR*$^{Cre/wt}$. For reasons of clarity, we will refer to these mice as (1) control; (2) *Klf1*$^{+/-}$; (3) *Bcl11a*$^{-/-}$ and (4) *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$. To ascertain recombination efficiency, CD71^{+} bone marrow cells from control and *Bcl11a*$^{-/-}$ mice were sorted. PCR amplification demonstrated efficient Cre-mediated recombination at the *Bcl11a* locus in CD71+ bone marrow cells (Supplementary Figure 1). Mice with ubiquitous *Bcl11a* deficiency die prenatally from unknown causes (Liu et al., 2003). In contrast, erythroid-specific ablation of *Bcl11a* did not affect viability since we obtained *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ animals at the expected Mendelian ratios (Supplementary Table 2). These animals appeared healthy, were fertile and displayed no gross morphological abnormalities. We therefore first analyzed standard hematological parameters of adult mice.

Hematological parameters of mutant mice

To investigate the impact of Bcl11a mutation either with or without Klf1 mutation on adult erythropoiesis, we determined standard hematological parameters of the mutant mice (Figure 1 and Supplementary Table 3). White blood cell and platelet counts were only significantly altered in compound *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice. Furthermore, the presence of *Bcl11a* mutation in *Klf1*$^{+/-}$ background significantly affected all hematological parameters with the exception of MCHC. *Bcl11a*$^{-/-}$ animals displayed significantly lower RBC, HCT and Hgb counts and higher values for MCH. These differences were more pronounced in the *Klf1*$^{+/-}$ background (p-values< 0.05). We also observed a tendency towards increased MCV in *Bcl11a*$^{-/-}$ mice; MCV was significantly higher in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice (Figure 1) . We note that the observed differences were unrelated to the gender of the mice (p> 0.05, data not shown).

Collectively, these results demonstrate a role of Bcl11a in adult erythropoiesis; and in combination with adverse effect that can be caused by mutations in both Bcl11a and Klf1.

Blood morphology

Morphological alterations of the erythrocytes were investigated using blood smears stained with Wright-Giemsa. This revealed slightly increased numbers of spherocytes, elliptocytes, polychromatic- and target cells in peripheral blood of *Bcl11a*$^{-/-}$ mice. Blood smears of *Klf1*$^{+/-}$ mice displayed abnormalities in cell shape and in combination with the *Bcl11a*$^{-/-}$ mutation more dramatic effects on red cell morphology were found. We observed increased numbers of Howell-Jolly bodies, elliptocytes and target cells in the blood of *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice, compared with blood from *Bcl11a*$^{-/-}$ or *Klf1*$^{+/-}$ mutant mice (Figure 2A-B). Collectively, these results show a mild anemia in Bcl11a mutant mice which is enhanced in Klf1 haploinsuffcient background.

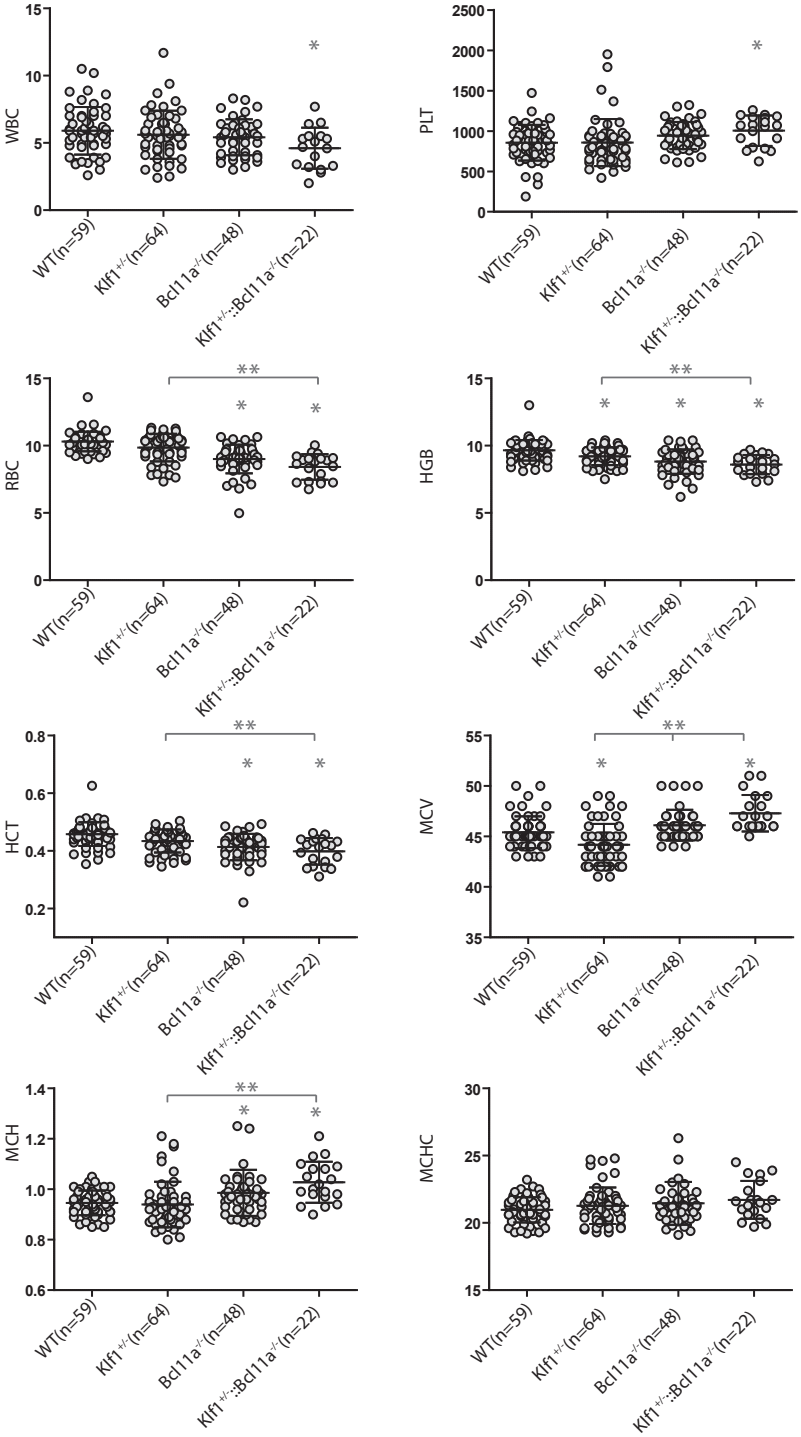

WBC
PLT
RBC
HGB
HCT
MCV
MCH
MCHC
WT(n=59)
Klf1+/-(n=64)
Bcl11a-/-(n=48)
Klf1+/-::Bcl11a-/-(n=22)
*
**

Figure 1: Blood parameter analysis of control, Klf1$^{+/-}$, Bcl11a$^{-/-}$, and Klf1$^{+/-}$::Bcl11a$^{-/-}$ mice. Hematological parameter analysis revealed a mild anemia in *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$mice.* indicates p<0.05 between a specific genotype compared with the wt. ** p<0.05 between the observed values in knockout mice.

A

	Sferocytes	Elliptoycytes	Howell-Jolly bodies	Polychromasia	Target cells
wt	<+	<+	-	-	
Klf1ko/wt	<+	<+	-	<+	<+
Bcl11acko/cko	+	++	+	+++	+
Bcl11acko/cko-klf1ko/wt	<+	++	+	+	++

B

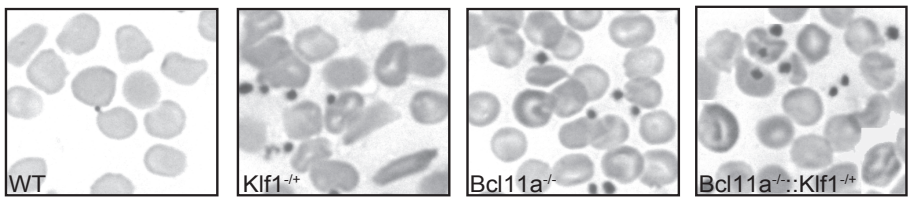


Figure 2: Blood morphological analysis of control, Klf1$^{+/-}$, Bcl11a$^{-/-}$, and Klf1$^{+/-}$::Bcl11a$^{-/-}$ mice. A) Red cells with abnormal morphology are more frequently observed in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice. B) Blood smears stained with Wright-Giemsa; pictures were taken with 40x magnification using an Olympus BX40F4 microscope (Olympus Optical Co. Ltd).

Klf1$^{+/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice display reticulocytosis

Reticulocytes are normally measured by flow cytometry analysis of the RNA content of blood cells (Piva et al., 2010). The transferrin receptor (CD71) is expressed by erythroid precursor cells committed to differentiation. Its expression peaks at the orthochromatic normoblast stage and gradually decreases during terminal differentiation and maturation. Although the CD71 molecule is absent on mature erythrocytes, it is still present on the surface of circulating reticulocytes (Ponka and Lok, 1999). Therefore, it can be used as a marker for reticulocytes. Reticulocyte counts are higher in young mice and stabilize after 6 weeks of age (Antonchuk et al., 2004). Therefore, we only included >6-week old mice for reticulocyte analysis using CD71 expression as the marker (Figure 3A).

We found increased percentages of reticulocyte counts in all mutant animals. *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$ mice displayed significantly increased numbers of reticulocytes

in their peripheral blood (2.1% and 4.2% respectively comparing with 1.8% in controls). We observed a further increase in the percentage of reticulocytes in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice (11.63%) compared with *Bcl11a*$^{-/-}$ or *Klf1*$^{+/-}$ animals, and this difference reached statistical significance compared with the other groups (Figure 3B).

Reticulocytosis is usually observed in response to anemic stress; this stimulates erythropoiesis thereby increasing the number of reticulocytes released in the circulation. We therefore determined the levels of erythropoietin (Epo), the major growth factor driving erythroid expansion, in the serum. Despite the higher

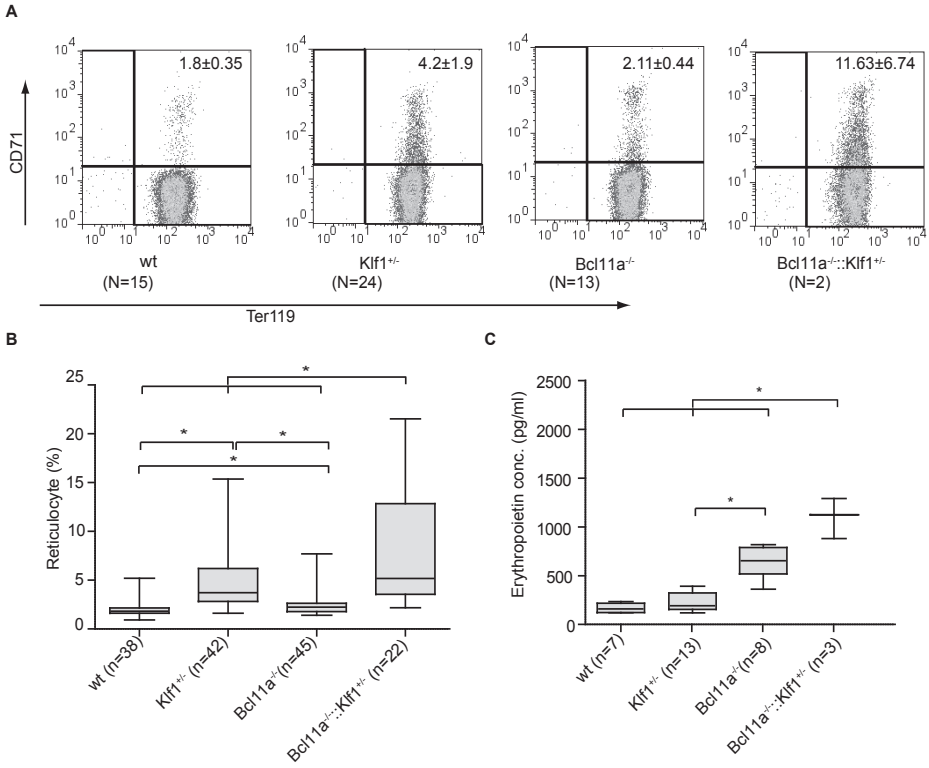


Figure 3: Reticulocyte counts and serum erythropoietin assays. A) CD71 marker representing the percentage of reticulocytes in peripheral blood. B) Statistical analysis confirms higher percentage of reticulocytes in all knockout mice with the most pronounced effect in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice. C) Erythropoietin concentration in serum samples was significantly higher in *Bcl11a*$^{-/-}$, *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ genotypes comparing with the controls.

percentage of reticulocytes in peripheral blood, Epo levels in *Klf1*$^{+/-}$ mice were similar to those observed in control mice. This suggests that the observed reticulocytosis is not due to compensated anemia, but most likely reflects prolonged erythroid maturation. Surprisingly, *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice displayed increased Epo levels (Figure 3C), suggesting that they were under anemic stress. In these animals spleen size and cellularity were not significantly different from those observed in the control animals, indicating that stress erythropoiesis had not been fully activated in order to compensate for the apparently minor anemia.

FACS analysis of adult bone marrow

Next, we investigated the erythroid compartment in the bone marrow by FACS analysis using the transferrin receptor (CD71) and Ter119 as markers (Socolovsky, 2007). This did not reveal any remarkable differences in the ProE (Ter119^{med} CD71^{high} FCS^{high}) and Ery.A (Ter119^{high} CD71^{high} FCS^{high}) populations, based on the described criteria by Socolovsky et al (Socolovsky, 2007). However, there was a tendency towards an increased percentage of cells in the Ery.C (Ter119^{high} CD71^{low} FCS^{low}) population in *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice. We observed a clear difference in the Ery.B (Ter119^{high} CD71^{high} FCS^{low}) populations; in the *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ and *Bcl11a*$^{-/-}$ mice these numbers were lower than those in the controls (Figure 4). Collectively, these results suggest that Bcl11a, alone or in conjunction with Klf1 haploinsufficinecy, plays a role in erythroid maturation at the bone marrow.

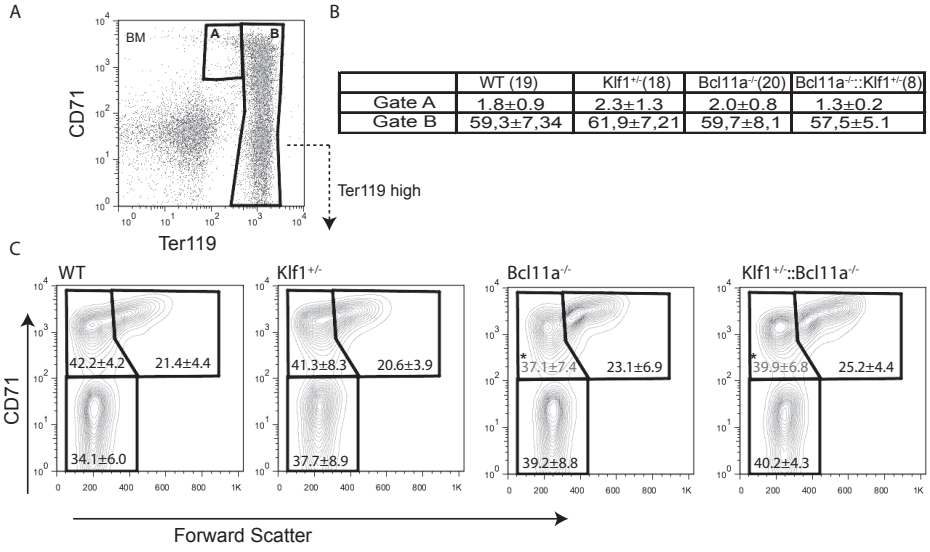
Erythropoiesis in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice during development

To extend these observations on adult mice, we analyzed embryonic blood and fetal livers at E18.5, just prior to birth. At this stage, erythropoiesis is highly active to supply the demand of the rapidly growing embryo for more oxygen-carrying capacity. We prepared cytospins of E18.5 embryonic blood and stained thesewith Wright-Giemsa in order to determine the percentage of reticulocytes. We observed that 42-45% of the erythrocytes from *Bcl11a*$^{-/-}$, *Klf1*$^{+/-}$ and control E18.5 blood

were basophilic cells. This percentage increased to 60% in blood from *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ E18.5 embryos (Fig. 5A). We did not find any remarkable differences in erythropoiesis of mutant embryos at E14.5 (data not shown).

FACS analysis of E18.5 blood revealed a small but consistently increased percentage of CD71^{+} cells in peripheral blood of *Bcl11a*$^{-/-}$ embryos (72% vs. 70%). This percentage was further increased in *Klf1*$^{+/-}$ blood samples (81% vs. 70%) and was highest in blood from *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ embryos (86% vs. 70%). Similar results were obtained following FACS analysis of fetal liver cells (Fig. 4B-D).

Consequently, the percentage of mature CD71^{-}/Ter119^{+} cells in fetal liver and peripheral blood of E18.5 *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ embryos was significantly lower than that observed in *Bcl11a*$^{-/-}$, *Klf1*$^{+/-}$ and control embryos (Figure 5B-D). Collectively, these results indicate that haploinsufficiency for Klf1 prolongs erythroid maturation, and that this phenotype is further exacerbated in combination with Bcl11a deficiency.



	WT (19)	Klf1$^{+/-}$(18)	Bcl11a$^{-/-}$(20)	Bcl11a$^{-/-}$::Klf1$^{+/-}$(8)
Gate A	1.8±0.9	2.3±1.3	2.0±0.8	1.3±0.2
Gate B	59,3±7,34	61,9±7,21	59,7±8,1	57,5±5.1

Figure 4: FACS analysis of bone marrow erythroblasts. A) Proerythroblast (CD71-high, Ter119 med) population is shown in Gate A and Ter119-high cells in Gate B. B) statistical analysis of A- and B-gate population do not show remarkable difference between the four groups. C) Erythrocyte distribution in Ter119-high population shows higher percentage of immature erythrocytes in bone marrow site. The numbers in red represent p<0.05.

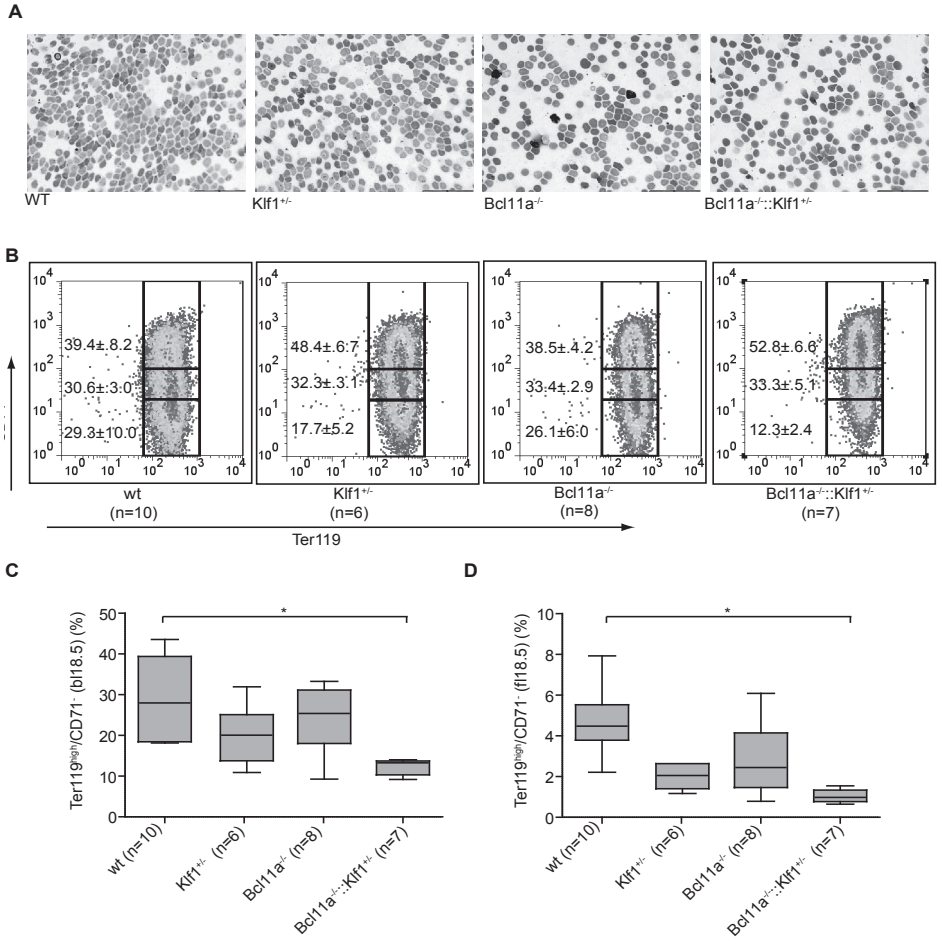


Figure 5: Blood and fetal liver analysis at E18.5. A) Giemsa staining of blood samples reveals more basophilic red cells in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice. B,C) Percentage of mature red cells reached a significant decrease in *Klf1*$^{+/-}$ mice and it decreased further in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice. D) Between the different genotypes, analysis of mature erythrocytes in fetal liver at E18.5 shows a similar trend as observed for the blood samples.

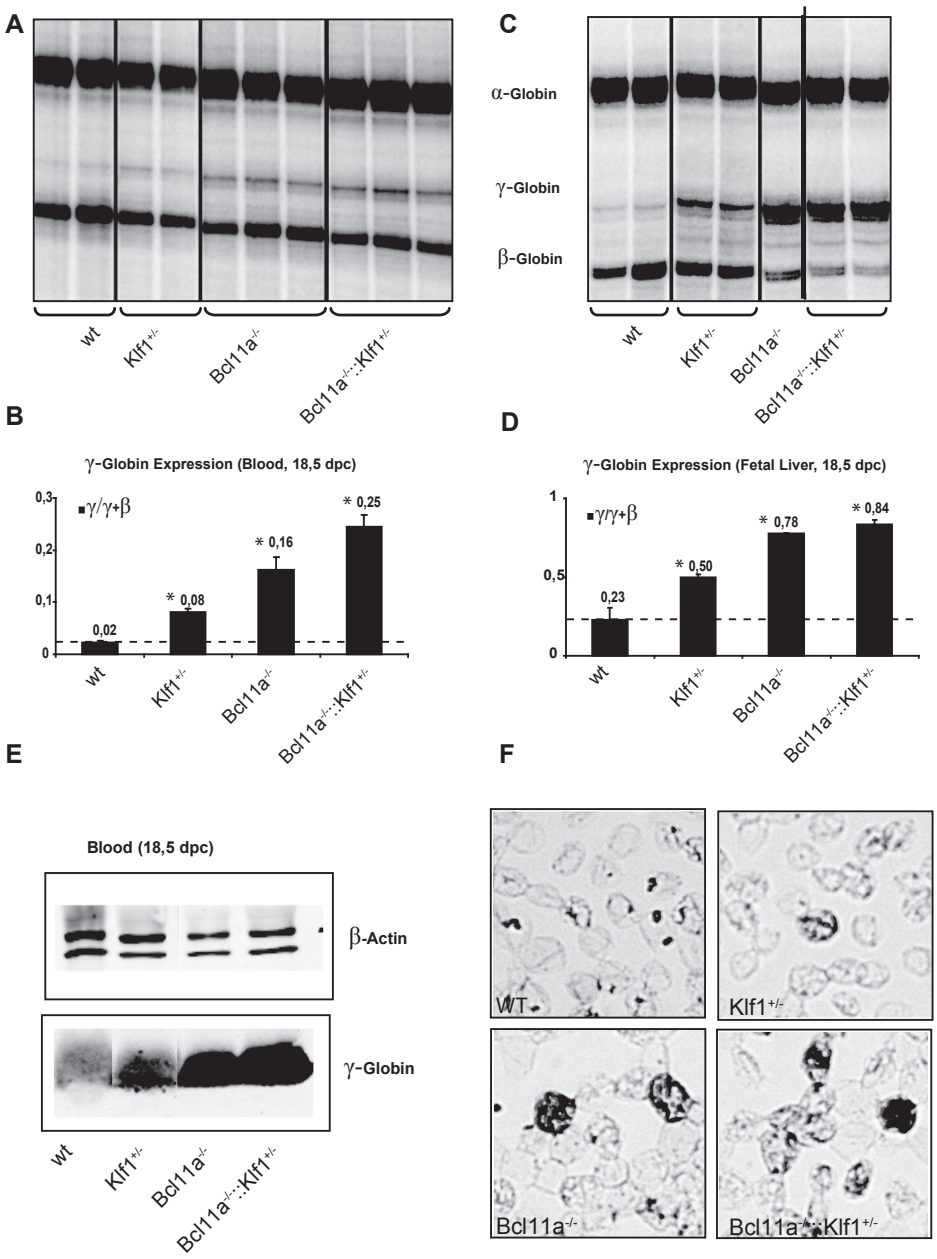


Figure 6: γ-globin expression analysis. A) S1 nuclease protection assay on E18.5 blood RNA. B) γ/ γ+β ratio is significantly higher in all knockout mice comparing with the controls. C) S1 nuclease protection assay on E18.5 fetal liver RNA. D) γ/ γ+β ratio follows the similar trend as blood samples at

Western blot E18.5. E) on blood at E18.5 confirms higher expression of γ-globin at the protein level in different genotypes; *Klf1+/-::Bcl11a*$^{-/-}$ mice display the highest level. F) γ-globin immunohistochemistry analysis showing that γ-globin expression levels correlate with the number of positive cells.

Despite this, the impact on erythropoiesis is modest and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice do not suffer from overt anemia even at prenatal stages when the demand for erythroid expansion is high.

Regulation of γ-globin expression during fetal development

Previously, it has been demonstrated that Klf1 preferentially activates the adult β-globin gene (Donze et al., 1995; Nuez et al., 1995; Perkins et al., 1995). Using mice carrying a human β-globin locus transgene, it was shown that switching from γ- to β-globin expression was delayed in a *Klf1*$^{+/-}$ background (Wijgerde et al., 1996). In addition, it was recently found that Klf1 is a direct activator of Bcl11a expression (Borg et al., 2010; Zhou et al., 2010). To assess the impact of the Klf1-Bcl11a axis on expression of the β-like globin genes, we determined globin expression at different developmental stages in erythroid cells derived from mice of all four genotypes. Since γ-globin expression is silenced around E13.5 in PAC8.1 mice (de Krom et al., 2002; Patrinos et al., 2004), we assessed globin expression after E13.5 at the latest possible time before birth. We used total RNA extracted from fetal liver samples collected at E18.5. The γ/(γ+ β) ratios were calculated from data obtained with S1 nuclease protection assays (Antoniou, 1991), and these results were validated with QRT-PCR assays (Borg et al., 2010). Compared to *Bcl11a*$^{-/-}$ embryos, which express high levels of γ-globin (Sankaran et al., 2009), *Klf1*$^{+/-}$ embryos displayed lower levels of γ-globin expression but these levels were still significantly higher than those observed in the control samples. The increase in γ-globin expression was less pronounced in E18.5 *Klf1*$^{+/-}$ embryos (Figure 6A-D), consistent with previous results (Wijgerde et al., 1996). Notably, the highest γ-globin mRNA expression was observed in *Klf1*$^{+/-}$*:: Bcl11a*$^{-/-}$ embryos. In addition, western blot analysis revealed higher levels of γ-globin protein in E18.5 blood

samples from *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ embryos compared to those observed in *Bcl11a*$^{-/-}$ embryos (Figure 6E), while for both genotypes these levels were much higher than in the control samples. Consistent with previously reported data (Wijgerde et al., 1996), γ-globin expression was low in *Klf1*$^{+/-}$ E18.5 erythroid cells, although still significantly higher than in the control samples. Finally, to determine whether the increased γ-globin expression had a pancellular or heterocellular distribution, we performed immunohistochemistry on cytospins of E18.5 blood. The percentage of cells staining positive for γ-globin expression correlated well with the γ-globin levels determined by the S1 nuclease protection- and QRT-PCR assays (Fig. 6F). This is consistent with a pancellular distribution of γ-globin. Collectively these data emphasize the dominant role of Bcl11a in γ-globin silencing during prenatal development in mice (Sankaran et al., 2009; Xu et al., 2010). Furthermore, the observation that γ-globin levels are highest in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ embryos lends support to the proposed role of the Klf1-Bcl11a axis in this process (Borg et al., 2010; Liu et al., 2003).

Dynamics of γ-globin expression from embryonic to adult stage

Next, we investigated γ-globin expression dynamics pre- and post-natally, using S1 nuclease protection assays on fetal liver RNA samples at E14.5 and E18.5. The obtained results were further compared with γ-globin expression of adult bone marrow using QRT-PCR on total mRNA. γ-globin expression was significantly higher in all mutant mice compared with the controls at E14.5, which remained statistically significant at E18.5. Although γ-globin expression decreased after birth in all mutant mice, it remained significantly higher in *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ young adult mice compared to *Klf1*$^{+/-}$ and control mice. Remarkably, in aging mice γ-globin expression was eventually silenced in all genotypes studied (Fig. 7). Collectively, these results support the proposed role of the Klf1-Bcl11a axis of γ-globin regulation, but the marked decline in γ-globin expression upon

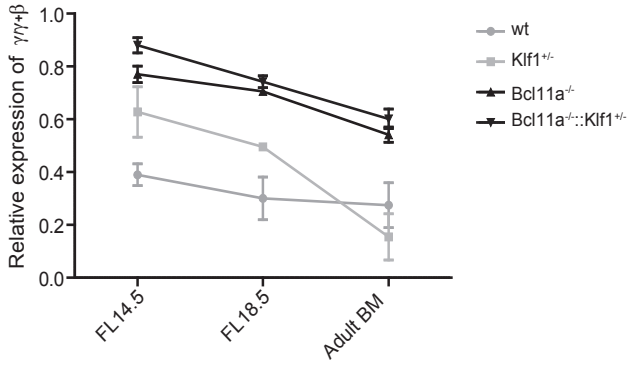


Figure 7: γ-globin expression analysis in the mutant mice. *Bcl11a*$^{-/-}$ mice display high γ/ γ+β ratio during embryonic stages. This ratio is highest in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice.

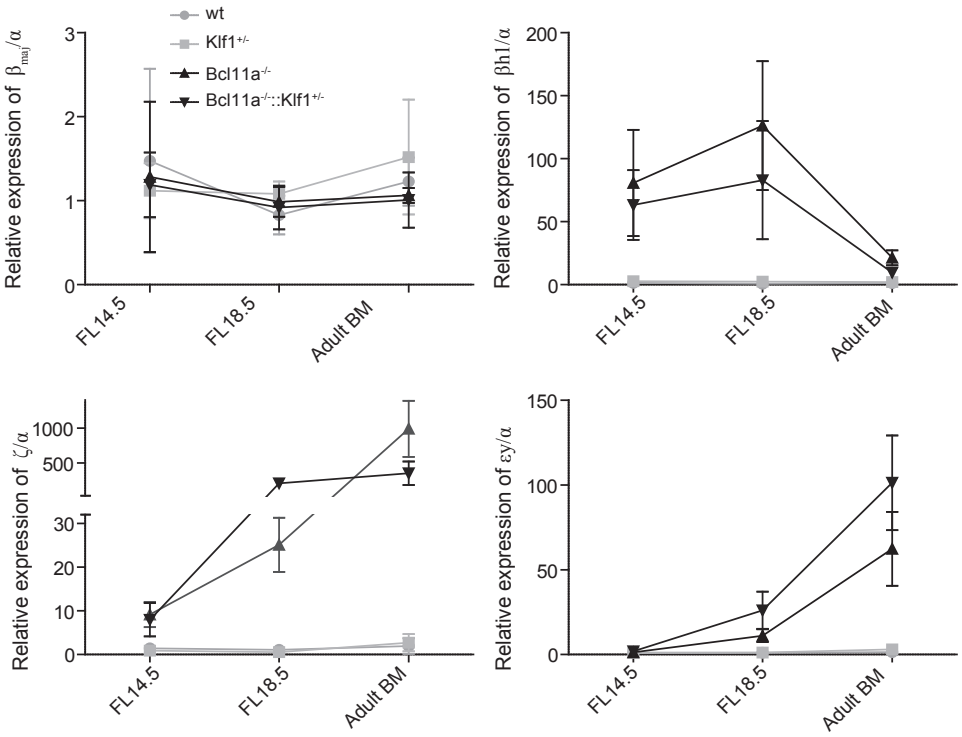


Figure 8: **Mouse globin expression analysis in the mutant mice.** *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice fail to repress embryonic globin expression.

aging suggests that additional silencing mechanisms exist that prevent high level γ-globin expression in adult mice.

Dynamics of mouse globin expression

The decline in γ-globin expression in adult *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice raised the question whether the endogenous mouse embryonic globin genes would display a similar expression pattern. We therefore investigated the expression of mouse globins at different developmental stages by QRT-PCR. Firstly, we observed that, relative to α-globin, the expression of β_{maj}-globin did not change significantly between all genotypes and developmental stages studied. This is consistent with the notion that β_{maj} is the major fetal/adult β-like globin in the mouse. In agreement with previous reports, the expression of embryonic εy- and βh1-globin was significantly higher in E14.5 and E18.5 fetal liver of *Bcl11a*$^{-/-}$ mice (Sankaran et al., 2008). In addition, we found that expression of these genes was even higher in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ fetal livers (Fig. 8). Similar to the observations on γ-globin expression, we found that expression of the endogenous mouse embryonic β-like globin, βh1, declined after birth in both *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice. However the expression level of βh1-globin remained 10-fold higher than that observed in the controls.

Compared to the controls, expression of εy-globin remained relatively high in *Bcl11a*$^{-/-}$ mice and the highest levels were observed in *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice (Fig. 8). In adult mice this level was similar to that observed in E18.5 fetal liver. Interestingly, we also observed persistent expression of embryonic ζ-globin in *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ mice (500-fold and 150-fold increased relative to the controls).

Collectively, the data on expression of the endogenous mouse globin genes are congruent with the observations on expression of the γ- and β-globin genes residing in the human β-globin locus PAC8.1 transgene. Bcl11a has a dominant role in silencing of the embryonic/fetal globin genes during mouse ontogeny, which is further augmented by the preferential activation of β-globin expression by Klf1.

Expression of embryonic/fetal globin genes nevertheless declines to low levels in adult *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ animals, indicating that additional silencing mechanisms are operational in adult mouse erythropoiesis.

Discussion

In this report we focused on the role of two transcription factors, Klf1 and Bcl11a, in erythropoiesis and developmental regulation of globin expression. Klf1, an erythroid-specific protein, directly activates β-globin expression through binding to CACCC box sequences in the LCR and the β-globin promoter (Chen and Bieker, 1996; Feng et al., 1994; Hodge et al., 2006). While high level expression of β-globin requires the presence of Klf1, the other globins, including γ-globins contained on human β-globin locus transgenes, are still highly expressed in the absence of Klf1 (Nuez et al., 1995; Perkins et al., 1996; Perkins et al., 1995; Wijgerde et al., 1996). In addition to a critical activator of β-globin expression, genome-wide gene expression analyses have revealed that Klf1 is a key regulator of genes activated during terminal erythroid differentiation (Drissen et al., 2005; Hodge et al., 2006; Pilon et al., 2008). These target genes include heme synthesis enzymes, cell cycle regulators and erythroid membrane and cytoskeleton proteins. It is therefore not surprising that *Klf1 null* embryos succumb to severe anemia. Embryonic lethality is not rescued by expression of a γ-globin transgene (Nilson et al., 2006; Perkins et al., 2000); this corrects globin chain imbalance but not hemolysis caused by deregulated expression of essential erythroid membrane and cytoskeleton proteins.

In humans, *KLF1* mutations result in a spectrum of erythroid phenotypes (Borg et al., 2011). Haploinsufficiency for KLF1 is the underlying cause of the In(Lu) blood group (Singleton et al., 2008), is associated with red cell zinc protoporphyria (Satta et al., 2011) and with HPFH (Borg et al., 2010). Very high HbF levels (22.1% and 30.9%) were observed in compound heterozygote individuals who carried a *null* mutation on one *KLF1* allele and a missense mutation p.K332Q, affecting

the DNA binding properties of KLF1, on the other (Satta et al., 2011). A dominant KLF1 missense mutation p.E325K also affects DNA binding and was reported to cause CDA (Arnaud et al., 2010). The two CDA patients with the p.E325K mutation displayed HbF levels of 31.6% and 44%. Remarkably, embryonic ζ- and ε-globin were also increased to very high levels (Arnaud et al., 2010; Tang et al., 1993). The ethylnitrosurea-induced mouse mutant *Nan* (neonatal anemia) carries a missense mutation, p.E339D, in the homologous position in mouse Klf1 (Heruth et al., 2010; Siatecka et al., 2010). This mutation causes a dominant hemolytic anemia, with markedly increased expression of the embryonic βh1-globin gene in adult *Nan* animals (Siatecka et al., 2010). Collectively, these data support a model in which KLF1 activates β-globin expression and suppresses the embryonic/fetal β-like globin genes. Recently, it has been shown that expression of BCL11A is regulated by KLF1, suggesting an intricate mechanism for the developmental regulation of the β-like globin genes co-ordinately mediated by KLF1 and BCL11A (Borg et al., 2010; Zhou et al., 2010). Ablation of Bcl11a in mice demonstrated that it is the major repressor of embryonic/fetal β-like globin genes during ontogeny (Sankaran et al., 2009; Xu et al., 2010). Interestingly, the timing of expression of full-length BCL11A differs between mouse and human (Sankaran et al., 2009), providing an explanation for the observation that in mice carrying a human β-globin locus transgene γ-globin silencing is already completed at the fetal liver stage (Stamatoyannopoulos, 2005; Stamatoyannopoulos and Grosveld, 2001).

In this paper, we have investigated the interplay of Klf1 and Bcl11a in erythropoiesis and developmental regulation of globin gene expression. We find that Bcl11a is the dominant repressor of embryonic/fetal globin genes in the mouse embryo from midgestation to term. Whilst the endogenous embryonic globin genes are normally silenced at the onset of definitive erythropoiesis in the fetal liver, their expression is markedly increased in the absence of Bcl11a. Their expression is still significant at E18.5, just prior to birth. These data are in agreement with those reported on mouse embryos with a systemic Bcl11a *null* mutation (Sankaran et

al., 2009), demonstrating that this phenotype is intrinsic to the erythroid lineage. In combination with Klf1 haploinsufficiency, we observed a further increase in the expression of the embryonic globin genes. This shows that the preferential activation of β-globin expression by Klf1 still occurs in the absence of Bcl11a. To achieve efficient silencing of the embryonic globin genes in fetal liver erythropoiesis, an intact Klf1-Bcl11a axis is required: Klf1 activates Bcl11a expression (Borg et al., 2010; Zhou et al., 2010); Bcl11a represses the embryonic globin genes (Sankaran et al., 2009) thereby unleashing the full potential of Klf1 to activate β-globin expression. It is interesting to note that Bcl11a also represses the embryonic ζ-globin gene, whilst Klf1 is not essential for high levels of α-globin expression. This provides another example of the contrasting mechanisms regulating the α-like and β-like globin loci (Craddock et al., 1995; Garrick et al., 2008).

In mice carrying a human β-globin locus transgene, the switch from γ- to β-globin expression occurs at the early fetal liver stage (Stamatoyannopoulos, 2005; Stamatoyannopoulos and Grosveld, 2001). This is dependent on the presence of Bcl11a (Sankaran et al., 2009); and in our experiments with erythroid-specific ablation of Bcl11a we obtained similar results. In agreement with previous reports using a different human β-globin locus transgene (Wijgerde et al., 1996), we find that haploinsufficiency for Klf1 delays γ- to β-globin switching leading to a 1.5-fold increase in the γ/(γ+β) ratio at E14.5 and E18.5, respectively. This can be attributed to diminished Bcl11a expression in Klf1 haploinsufficient embryos (Zhou et al., 2010). Remarkably, in compound *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ embryos there is a further increase in the γ/(γ+β) ratio, in particular at E18.5. Collectively, these data support the proposed role of the KLF1-BCL11A axis in γ-globin regulation (Borg et al., 2010; Borg et al., 2011; Zhou et al., 2010).

Unlike constitutive deficiency, which results in perinatal lethality (Liu et al., 2003), erythroid-specific ablation of Bcl11a does not affect post-natal survival even in combination with haploinsufficiency for Klf1. GWAS analysis has shown an association between BCL11A SNPs and MCV (Ganesh et al., 2009), suggesting

a role for BCL11A in adult eythropoiesis beyond globin rgulation. We therefore determined the effects on steady-state erythropoiesis and globin expression in adult animals. We found only minor deviations in the hematological parameters of *Klf1*$^{+/-}$, *Bc11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ animals. *Klf1*$^{+/-}$ animals display mild reticulocytosis but do not have increased Epo levels, indicating that the production of erythroid cells is adequate but that the maturation of reticulocytes takes more time. Mild reticulocytosis is also one of the hallmarks of individuals from a Maltese pedigree with KLF1 haploinsufficiency (Borg et al., 2010). *Bcl11a*$^{-/-}$ animals were also displaying reticulocytosis and a trend towards lower RBC counts. In the *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ animals these two traits are exacerbated and the lower RBC counts reach statistical significance. The most straightforward explanation for these observations is that Bcl11a deficiency provokes a mild anemia, which is compensated by increased Epo levels. Consistent with this notion, Epo levels are increased in *Bc11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ animals. In addition, blood smears of *Klf1*$^{+/-}$, *Bc11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ animals reveal increased numbers of erythrocytes with morphological abnormalities, suggesting a role for these factors in erythroid maturation. Klf1 has a well established role in the expression of erythroid membrane and cytoskeleton proteins (Drissen et al., 2005; Heruth et al., 2010; Hodge et al., 2006; Nilson et al., 2006; Perkins et al., 2000; Siatecka and Bieker, 2011; Siatecka et al., 2010; Singleton et al., 2008) while a putative role for Bcl11a in erythroid maturation remains to be further investigated. Importantly, *Bc11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ animals were obtained at the expected Mendelian ratios and thus far none of them developed leukemia (n = 28). This is in contrast to animals reconstituted with Bcl11a *null* hematopoietic cells which succumb to T cell leukemia at a high frequency (Liu et al., 2003). We conclude that erythroid-specific ablation of Bcl11a, even in combination with Klf1 haploinsufficiency, only mildly affects steady-state erythropoiesis in adult mice. This supports proposals for the modulation of the KLF1-BCL11A axis in β-thalassemia and SCA patients for reactivation of γ-globin expression (Borg et al., 2010; Sankaran et al., 2010; Zhou

et al., 2010).

At first glance, the observation that expression of γ-globin declines in aging *Bcl11a*$^{-/-}$ and *Klf1*$^{+/-}$*::Bcl11a*$^{-/-}$ animals is counterintuitive to this notion. However, there is evidence for species-specific differences in developmental regulation of globin expression. In mice, full-length Bcl11a is already expressed in the fetal liver, while human fetal liver cells only express short isoforms of BCL11A (Sankaran et al., 2009). The full-length isoform is first observed in human adult erythroid progenitors. This difference in developmental timing of BCL11A expression provides a molecular explanation for the observation that γ- to β-globin switching occurs at the fetal liver stage in mouse embryos carrying a human β-globin locus transgene (Perkins et al., 1996; Sankaran et al., 2009; Wijgerde et al., 1996). Furthermore, treatment with drugs that raise HbF cells in human subjects fail to do so in human β-globin locus transgenic mice (Buller et al., 1999; Pace et al., 1994; Sloane-Stanley et al., 2006), suggesting that silencing of the γ-globin genes is much tighter in mice than it is in humans. We therefore strongly feel that this intrinsic property of the mouse model does not provide a convincing argument against proposals to target the KLF1-BCL11A axis as a therapeutic approach to increase HbF levels in β-thalassemia and SCA patients (Borg et al., 2011; Sankaran et al., 2010). Rather, we suggest that this provides an opportunity to identify additional factors involved in the silencing mechanism at the adult stage. Enforcement of repression of the embryonic/fetal programme in adult erythropoiesis may be executed by, for instance, the transcription factors MYB (Thein et al., 2007) and SOX6 (Xu et al., 2010), the chromatin-bound FOP/CHTOP protein (van Dijk et al., 2010) and NuRD complex (Gnanapragasam et al., 2011), the orphan nuclear receptors TR2/TR4 (Cui et al., 2011) and the protein arginine methyl transferase PRMT5 (Rank et al., 2010), and is likely to include epigenetic mechanisms such as PcG complex recruitment and DNA methylation. Future work will be aimed at further elucidating the multi-layered repressive network of the embryonic/fetal program in the adult erythroid environment.

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Online Supplementary Design and Methods

Supplementary Table 1: oligonulceotide sequences

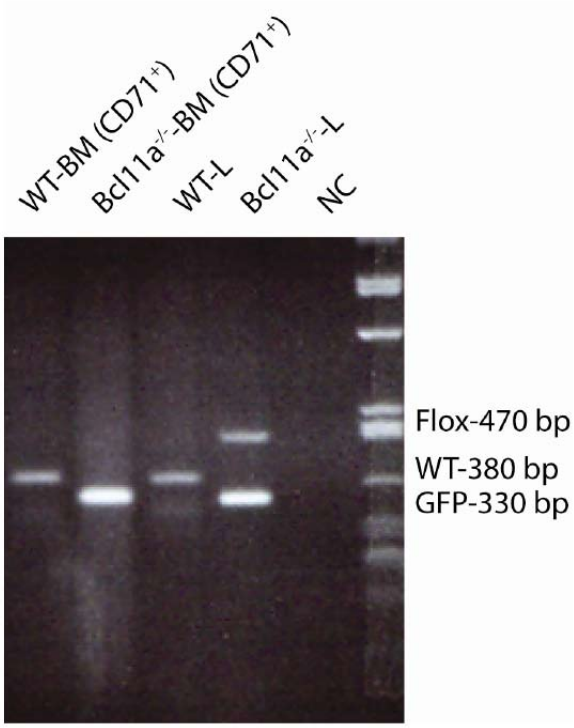
Name	Sequence	Purpose
Mouse α-globin S	TTGGCTAGCCACCACCCT	QRT-PCR
Mouse α-globin A	CCAAGAGGTACAGGTGCA	QRT-PCR
Mouse β_{maj}-globin S	TTSAGGCTCCTGGGCAATAT	QRT-PCR
Mouse β_{maj}-globin A	TGCCAACAACTGACAGATGC	QRT-PCR
Mouse ε-globin S	GTTTTGGCTAGTCACTTCGG	QRT-PCR
Mouse ε-globin A	CAAGGAACAGCTCAGTATTC	QRT-PCR
Mouse ζ-globin S	GAAGCCTGGGACAAGTTCAT	QRT-PCR
Mouse ζ-globin A	GGGTTCAATAAAGGGGAGGA	QRT-PCR
Mouse βh1-globin S	TTGCCAAGGAATTCACCCCA	QRT-PCR
Mouse βh1-globin A	CTCAATGCAGTCCCCATGGA	QRT-PCR
Hum β-globin S	CTGCCTATCAGAAAGTGGTG	QRT-PCR
Hum β-globin A	ATTGGACAGCAAGAAAGCGA	QRT-PCR
Hum γ-globin S	AGGTGCTGACTTCCTTGGG	QRT-PCR
Hum γ-globin A	GGGTGAATTCTTGCCGAA	QRT-PCR
Actin S	GATTACTGCTCTGGCTCCT	QRT-PCR
Actin A	TGGAAGGTGGACAGTGAG	QRT-PCR
Bcl11a Common	AGTGGCACAGAGCTGAAATGAC	Genotyping
Bcl11a Flox	AAGTTGTACATGTGTAGCTG	Genotyping
Bcl11a GFP	AAGCAAGGGGAAGGGTGGTTAGAA	Genotyping

Supplementary Table2: genotyping of 74 litters confirmed that genotype distributions follow the Mendelian ratio in all knockout mice.

Genotype	Expected frequency (%)	Observation (%)	P-value
WT	18.5 (25%)	21 (28.4%)	0.67
$Klf1^{+/-}$	18.5 (25%)	20 (27%)	0.78
$Bcl11a^{-/-}$	18.5 (25%)	18 (24.3%)	1.00
$Klf1^{+/-}$::$Bcl11a^{-/-}$	18.5 (25%)	15 (20.3%)	0.64

Supplementary Table 3: Mean ± SD of hematological parameters of >6-week old mice

Genotype	No	WBC ($x10^9$/L)	RBC ($x10^{12}$/L)	HGB (mmol/L)	HCT (L/L)	PLT ($x10^9$/L)	MCV (fL)	MCH (fmol)	MCHC (mmol/L)
WT	51	5.7 ± 1.6	10.2 ± 0.6	9.7 ± 0.6	0.46 ± 0.03	865.2 ± 175.3	45.2 ± 1.3	0.94 ± 0.05	20.9 ± 0.9
$Klf1^{+/-}$	51	5.4 ± 1.5	10.0 ± 0.8	9.2 ± 0.6*	0.43 ± 0.04*	801.1 ± 184.5	44.0 ± 1.9*	0.92 ± 0.06	21.0 ± 0.1
$Bcl11a^{+/-}$	6	4.9 ± 0.7	9.3 ± 0.6	9.2 ± 0.7	0.43 ± 0.04	967.3 ± 311.5	46.4 ±1.7	0.99 ± 0.03	21.3 ± 1.0
$Bcl11a^{-/-}$	45	5.3 ± 1.2	9.1 ± 0.8*	8.9 ± 0.8*	0.42 ± 0.04*	927.0 ± 151.4	45.7 ± 0.9	0.99 ± 0.09*	21.1 ± 0.9
$KLF1^{+/-}$::$Bcl11a^{+/-}$	6	6.3 ± 1.7	8.4 ± 0.6*	8.6 ± 0.9*	0.40 ± 0.03*	821.2 ± 187.1	47.8 ± 1.1*	1.03 ± 0.06	21.4 ± 1.0
$KLF1^{+/-}$::$Bcl11a^{-/-}$	17	4.4 ± 1.3*	8.4 ± 0.9*	8.6 ± 0.7*	0.40 ± 0.05*	1052.6 ± 168.8*	46.9 ± 1.4*	1.02 ± 0.07*	21.6 ± 1.3



Supplementary Figure 1: Genotyping of the CD71+ sorted cells from bone marrow of wt and Bcl11a-/- mice. the genotyping reveals an efficient Cre-mediated recombination of the Bcl11a conditional knockout allele in CD71+ bone marrow cells. DNA amplification of liver served as the control.

Gene expression analysis of the hydroxyurea response in primary erythroid progenitors derived from β-thalassemia patients

(Manuscript in preparation)

Gene expression analysis of the hydroxyurea response in primary erythroid progenitors derived from β-thalassemia patients

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Abstract

The β-thalassemia is caused by mutations in the β-globin gene locus that cause loss or reduction of β-globin gene expression. Hydroxyurea (HU) increases expression of fetal β-globin in postnatal life, and is therefore an appealing therapeutic approach to the β-thalassemias. Patients treated with HU fall into three categories: (i) 'Good responders' increase hemoglobin to therapeutic levels (ii) 'Moderate responders' increase hemoglobin levels but still need transfusions at longer intervals, and (iii) 'non-responders', who remain transfusion-dependent. The mechanisms underlying these differential responses remain unclear.

We generated RNA expression profiles of erythroblasts grown in the presence or absence of HU that were expanded from blood of 16 β-thalassemia patients that responded either very poor (non-responders) or very good (responders) to HU treatment. RNA expression profiles showed that the cells derived from responders, follow the pattern of genes involved in terminal erythroid differentiation stronger than non-responders. Many genes were upregulated in response to HU in erythroblasts from non-responders, including γ-globin. However, the γ-globin starting level was low in these cases and HU treatment induced significant cell death. In contrast, HU treatment of erythroblasts from responders had little impact on their gene expression profiles. Their γ-globin was upregulated, but the baseline level of γ-globin was already high. Interestingly, part of the gene program that was upregulated by HU in non-responder erythroblasts was already highly expressed in the erythroblasts of responders before HU treatment. We conclude from the gene expression profiles of responders that the cells of these patients have adapted well to constitutive stress conditions and have more potential to follow the erythroid differentiation program.

Introduction

Hemoglobin disorders, particularly β-thalassemia and sickle cell disease, are the most common single gene disorders worldwide (Weatherall, 2001). They are caused by mutations in the β-globin locus resulting in abnormal or reduced rates of hemoglobin production (Stamatoyannopoulos and Grosveld, 2001). Clinical symptoms include anemia, α/β globin chain imbalance, infarction, bone marrow expansion and splenomegaly. Currently the patients have a life expectancy of about four decades.

During human development, fetal γ-globin and adult β-globin are the main β-like globins. They associate with β-globin chains to produce HbF ($\alpha 2\gamma 2$) during the fetal period and HbA ($\alpha 2\beta 2$) in adult life. The mechanism responsible for this developmentally regulated gene expression pattern, known as globin switching, has been the subject of intense research efforts during the last 30 years, mainly because reactivation of γ-globin expression would be beneficial to β-hemoglobinopathy patients. In the case of β-thalassemia, γ-globin protein can reduce α-globin chain precipitation and compensate for the lack of β-globin chains. In sickle cell patients, high γ-globin expression reduces hemoglobin polymerization in erythrocytes. This prevents sickling and improves the life span of the cells, thereby ameliorating the symptoms of the disease (de Paula et al., 2003; Hajjar and Pearson, 1994; Hoppe et al., 2000; Loukopoulos and Pavlides, 1998; Poillon, 1993; Zeng, 1995).

Several drugs can induce γ-globin gene expression and ameliorate the disease phenotype. Three well-known HbF inducing agents are sodium butyrate (a histone deacetylase inhibitor) (Constantoulakis et al., 1989; Perrine et al., 1989; Perrine et al., 1987), 5-azacytidine (a DNA demethylating agent) (DeSimone, 1982; Dover, 1983; Ley 1983; Ley, 1982) and hydroxyurea (a ribonucleotide reductase inhibitor) (Veith et al., 1985). Of these, only hydroxyurea (HU) is currently approved by FDA for treatment of patients with β-hemoglobinopathies. How HU induces γ-globin gene expression is poorly understood. Mechanisms proposed for the induction of

HbF by HU include rapid erythroid regeneration, increased erythropoietin (EPO) production, apoptosis, nitric oxide (NO) production (Cokic et al., 2007), increased guanylate cyclase activity (Cokic et al., 2008), and activation of the p38 MAPK pathway (Park et al., 2001). Induction of HbF by HU in β-thalassemic patients was reported to be of similar magnitude as found in the cells of normal individuals (1.3- to 3.5-fold) and sickle cell patients (2- to 5-fold). HbF induction by HU was reported using erythroid cell cultures (Moi and Kan, 1990). In erythroid progenitor cells treated with HU in vitro, HbF induction was comparable to the increase of HbF in peripheral blood *in vivo* of sickle cell disease patients following HU therapy (Yang et al., 1997). HU increases RBC survival from 18.6 +/- 11 days to 70 +/- 21 days, as a result of decreased hemolysis (Ballas et al., 1999). This is presumably due to the reduced intracellular HbS polymerization as a result of increased γ -globin expression, and better hydration of the cells.

The majority of patients increase HbF production upon HU treatment (Steinberg et al., 1997), but baseline HbF levels and the magnitude of the response vary widely among patients. The absolute response to HU and the HbF baseline likely depend on genetic factors, which modulate different regulatory pathways, including the trans-acting factors.

In order to prevent ineffective HU treatment, the ability to predict the HbF response to HU would be useful for the selection of patients. A correlation of single nucleotide polymorphisms (SNPs) with high HbF induction has been reported in several studies. The XmnI (G)γ SNP at -158 (C>T) is associated with high HbF response to HU in sickle cell disease and β-thalassemia (Alebouyeh, 2004; Bradai et al., 2003; Dixit et al., 2005; Karimi et al., 2005; Neishabury et al., 2008; Panigrahi et al., 2005; Verma et al., 2007; Yavarian et al., 2004). Furthermore, a SNP association study reported 17 and 20 SNPs significantly associated with the percentage of HbF and the response to HU, respectively (Ma et al., 2007). A recent study in a large group of sickle cell disease children under HU tretment revealed no significant association between maximum HU tolerated dose and HbF response

(Ware et al., 2011).

Regulation of γ-globin gene expression is complex and can be influenced by different regulatory pathways, genetic and environmental factors. The net outcome of these determines the response to HU (reviewed in (Bank, 2006; Stamatoyannopoulos and Grosveld, 2001)). We hypothesized that the activity of the regulatory mechanisms may be deduced from the comparison of the expression profiles of erythroid progenitor cells from HU 'responder' and 'non-responder' groups. In addition, data from such an approach may help to understand the mechanism by which HU induces γ-globin expression. It may also explain the difference between 'responders' and 'non-responders' regarding base line levels of HbF and factors that are involved in high γ-globin induction. One study reported expression profiling of SCD patients with mild and severe phenotype. This study used peripheral blood for expression profiling that mainly contains erythrocytes. These are terminally differentiated cells without a nucleus and the program necessary for globin production has finalized. The expression profile of these cells may therefore not be very informative with respect to this process (Jison et al., 2004).

Here we studied two patient groups of β-thalassemic patients that did not respond to HU treatment and remained fully dependent on regular blood transfusions and those that responded well and became transfusion-independent upon HU treatment. Erythroblasts were expanded from peripheral blood, and proliferation, hemoglobin production and gene expression were compared in the presence and absence of HU.

Materials and Methods

Patients

16 β-thalassemic patients were selected from a large Iranian collection of more than 3000 β-thalassemic patients based on their clinical manifestation and response to hydroxyurea (HU). After clinical consultation, globin levels and other hematological

parameters were measured, and mutations in the α- and β-globin loci were determined. Patients were further selected based on the following criteria: absence of β-globin gene deletions; the patients were divided in two categories, namely group I, characterized based on complete absence of a HU response, 'non-responder', and group II 'responders' characterized by a good response to HU resulting in transfusion independence (Table 1 and Suppelmentary Table 1). Patients in the responder group have a mild thalassemic phenotype; they are transfusion independent and respond to HU. Patients in the non-responder group have more severe phenotype, they are transfusion dependent and do not respond to HU. Age and sex were distributed similarly between the groups (group I: 4/8 male, 15-30 yrs median age 22.5; group II, 5/8male, 8-41 yrs median age 25) (Table 1 and Supplementary Table 1).

Cell culture

HEPs were cultured essentially as published (Leberbauer et al., 2005). 40 ml of blood was collected per patient, and buffy coats isolated by centrifugation. White cell were isolated from the inter phase after Ficoll gradient purification, and washed with PBS. For initial expansion, $5x10^6$ cells/ml were cultivated at a density of $1\text{-}2x10^6$ cells/ml in serum-free medium (StemSpan; Stem Cell Technologies, Vancouver, BC, Canada) supplemented with lipids (40 ng/ml cholesterol-rich lipid mix; Sigma, St Louis, MO) and erythropoietin (2 U/ml, a kind gift of Orthobiotech, Tilburg, The Netherlands), dexamethasone (1 μM; Sigma) and SCF (50 ng/ml, supernatant of CHO producer cells)(Leberbauer et al., 2005). The erythroblast cultures were expanded until day 10 by daily partial medium changes, addition of fresh factors and keeping cell densities between 1.5–2 × 10^6 cells/ml. Proliferation kinetics and cell size distributions were monitored daily using an electronic cell counter (CASY-1, Schärfe-System, Reutlingen, Germany). To induce terminal differentiation erythroblasts were washed with PBS and reseeded at 1.5-2 x 10^6 cells/ml in lipid-enriched StemSpan supplemented with Epo (5U/ml) and iron-loaded transferrin (1 mg/ml;

SCIPAC Ltd, UK) (Leberbauer et al., 2005). Differentiating erythroblasts were maintained at 2-3 x 10^6 cells/ml and harvested 48 hours after induction. After day 10, the cells were divided into 2 groups: proliferation and proliferation + 100 μg/ml HU. The medium was refreshed every other day. RNA was isolated 48 hours after HU treatment, the remainder of the cells were kept for another 3 days in culture to determine total hemoglobin, HbF and growth rates.

Cell morphology

Cell morphology was analyzed using cytospins stained with histological dyes and neutral benzidine (Beug et al., 1982) Pictures were taken with an OlympusBx40 microscope (40x objective, NA 0.65) equipped with an Olympus Dp50 CCD camera and Viewfinder Lite 1.0 acquisition software.

Hemoglobin content

Aliquots of approximately 2x10^6 cells of the original cultures were removed and analyzed for hemoglobin content by photometry as described (Bakker et al., 2004). The relative ratios of HbA, HbA_2 and HbF were determined by HPLC (BioRad, Hercules, CA).

Globin locus mutation analysis

α- and β-globin locus mutations in thalassemic patients were determined as described (Najmabadi et al., 2001). The XmnI SNP (C>T substitution at position -158 upstream of the Gγ-globin gene) was detected by PCR and enzymatic digestion with Asp700, an isoschizomer of XmnI, followed by gel electrophoresis (Sutton et al., 1989).

RNA purification and real-time RT-PCR analysis

Total RNA was extracted from cells using the TRI reagent (Sigma). For quantitative

RT-PCR, cDNA was synthesized from 1 μg of total RNA using random hexamers and SuperscriptTM II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA). RNase-free DNaseI (Invitrogen) was used to degrade contaminating DNA and primers were designed spanning an intron. PCR was performed as described below.

Microarray analysis

EPs were lysed using the TRIzol Reagent (Invitrogen), and then incubated at room temperature for 5 minutes before adding 0.2 μl of chloroform to each 1ml sample. After centrifugation at full speed (12000 rpm) for 20 minutes, the RNA was precipitated with iso-propanol collected by centriguation. The RNA pellets were washed with 75% ethanol and dissolved in RNase-free water. RNA samples were qualitatively controlled and microarray analysis was performed as described (Hou et al., 2010). Functional annotation was processed using Ingenuity Pathway Analysis with Fisher's Exact Test p-value and threshold value of 0.05. (Ingenuity, Mountain View, CA, USA, http://ingenuity.com/).

Quantitative PCR conditions and primers

Quantitative real-time PCR (MyIQ, Bio Rad) was performed using 0.75 μl of SYBR Green I (Sigma S9430; 1/2500 dilution in DMSO). 0.5U Platinum Taq (Invitrogen), 10 pmol of each primer and 4 μl cDNA sample, in a final volume of 25 μl, were used under the following cycling conditions: 3 min. at 95°C followed by 40 cycles of 30 s at 95°C, 20 s at 56°C, 40 s at 60°C, 15 s at 75°C. Human GAPDH and USP14 were used as endogenous references for normalization. Enrichment of specific sequences was calculated using the comparative CT method (Livak and Schmittgen, 2001). The oligonucleotide sequences is listed below:

BCL11A/F	5′-GTCTCGCCGCAAGCAAGG	BCLxl/F	5′-ACCTGAATGACCACCTAGAGC
BCL11A/R	5′-GCCGTGGTCTGGTTCATCATC	BCLxl/R	5′-CAGCGGTTGAAGCGTTCC
ARG1/F	5′-CAAGAAGAACGGAAGAATCAGC	STAT5B/F	5′-CATCCAGTACCAGGAGAGC
ARG1/R	5′-CCAGATGACTCCAAGATCAGG	STAT5B/R	5′-AGAGACACCTGCTTCTGC
IKZF1/F	5′-GGACCTCTCCACCACCTC	PRMT5/F	5′-CCATCAAAGCAGCCATTCTCC
IKZF1/R	5′-AATCCTCCGCACATTCTTCC	PRMT5/R	5′-TGGTGGTTGGTGCCTGTG
HIPK2/F	5′-GCCAGCCACGTCTCCAAGG	MYB/F	5′-CAGTGACGAGGATGATGAGG
HIPK2/R	5′-CACAGCCCAGGGACCACATG	MYB/R	5′-TGTTCCACCAGCTTCTTCAG
BCL6/F	5′-CTGAGGAGATGGGAGAGACC	Gamm/F	5′-AGGTGCTGACTTCCTTGGG
BCL6/R	5′-CAGCGTGTGCCTCTTGAG	Gamma/R	5′-GGGTGAATTCTTTGCCGAA
ARG2/F	5′-TGAGGTGGTTAGCAGAGC	P15 INK4b/F	5′-ATCACATGAGGTCAGGAGTTCG
ARG2/R	5′-AACCCAGACAACACAAAGG	P15 INK4b/R	5′-CCAGGTTCAAGCGAGTCTCC
FOXO3/F	5′-CGTTGCGTGCCCTACTTC	P14 ARF/F	5′-GGTTTTCGTGGTTCACATCC
FOXO3/F	5′-CTCTTGCCAGTTCCCTCATTC	P14 ARF/R	5′-CCTAGACGCTGGCTCCTC
SOX6/F	5′-CGAGACAACAGCAGCAACTTC	P16 INK4a/F	5′-CCCCTTGCCTGGAAAGATAC
SOX6/R	5′-GAGTCCGCTGGTCATGTGG	P16 INK4a/R	5′-AGCCCCTCCTCTTTCTTCCT
SOX4/F	5′-GTCCCACTCCTCCTCTTCC	GAPDH/F	5′-GCCAAAAGGGTCATCATCTC
SOX4/R	5′-CCGACGACGAACTGAAGC	GAPDH/R	5′-GGTGCTAAGCAGTTGGTGGT
KLF10/F	5′-ACCCAGGATGTGGCAAGAC	USP14/F	5′-AACGCTAAAGGATGATGATTGGG
KLF10/R	5′-TTCATCAGAACGGGCAAACC	USP14/R	5′-TTTGGCTGAGGGTTCTTCTGG

Results

HU induces hematopoietic differentiation and reduces cell proliferation

Human erythroid progenitor cells (HEPs) were expanded from peripheral blood mononuclear cells as described (Leberbauer et al., 2005). We first titrated HU treatment to determine the concentration-dependent effects on cell survival and proliferation, and on accumulation of HbA and HbF. We used cells from two unrelated healthy donors for these experiments. HEPs were expanded from peripheral blood mononuclear cells for 10 days before HU was added in concentrations ranging from 0 to 400 μM and cell proliferation was monitored daily. Hemoglobin production, the percentage of HbA and HbF, and cell morphology were analyzed after 8 days when the experiment was terminated (Figure1).

Cell density was maintained between 1-2 million/ml by daily dilution and cumulative cell numbers were calculated. Increasing HU concentrations progres-

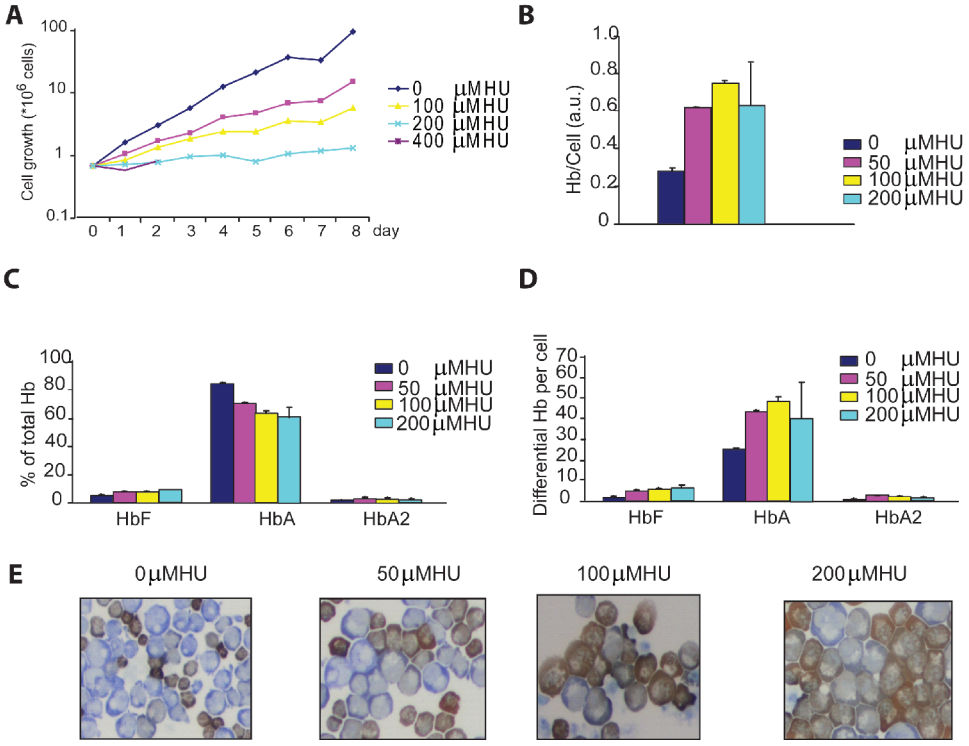

Figure 1. Hydroxyurea (HU) dose-response curve on healthy donor cells. A) Human Erythroid progenitor (Conley et al.) cells growth curves in different Hydroxyurea dose from 0 to 400 µM. B) The effect of different HU concentrations on total hemoglobin production after 8 days of treatment. Experiments were performed using cells from two healthy donors in triplicate. Hb levels are represented in arbitrary units (a.u.) C) HPLC Hb analysis of samples in 1B. D) Representation of Hb subtypes by distributing total hemoglobin (1B) in to Hb F, HB A and HbA2 (1C); expressed in arbitrary units (a.u.). E) Representative cytospins of HEPs treated with different HU concentrations, stained with histological dyes and neutral benzidine. Hemoglobinized cells are stained brown.

sively decreased cell proliferation, but only 400 μM was immediately toxic to the cells (Figure 1A). HU induced a concentration-dependent increase in total hemoglobin that reached a maximum at 100 μM HU (Figure 1B). Analysis of hemoglobin subtypes by HPLC indicated that HU increased the HbF percentage from 1.7% in non-HU treated to 6% in 100 μM HU-treated samples (Figure 1C). Using total Hb levels, the accumulation of hemoglobin can be calculated (Figure1D). This shows that both HbA and HbF were induced by HU. Finally, we analyzed hemoglobinisation at the cellular level. Under proliferation conditions, addition of HU increased the percentage of hemoglobinized cells from 30% in 0 μM HU to 40% at 50 μM HU and up to 50% at 200 μM HU concentration(Figure 1E). Based on these observations and in agreement with previous publications (Budzowska et al., 2004; Fibach et al., 1993; Nagai et al., 2003; Rodrigue et al., 2001) we conclude that HU induces erythroid differentiation and hemoglobinization of these cells. Since the best response with least cell toxicity was observed at 100 μM HU, we used 100 μM HU for all other experiments reported here.

Selection and characterization of patients

The selection of two groups of β-thalassemia patients was based on either the complete absence of response to HU treatment (Group I, Non-Responders; NR), or a good response to HU treatment resulting in transfusion-independence (Group II, Responders; R). The XmnI (G)γ SNP (-158C>T) has been associated with increased γ-globin expression and was present in 19% of the non-responders and in 81% of the responders chromosomes. All of the patients were screened for α- and β-globin gene mutations. β-globin mutations were detected in all of the samples and no mutation of α-globin locus were observed (Table 1 and Supplementary Table 1).

Erythroblasts derived from non-responders are more sensitive to HU treatment and express lower HbF baseline. First, we examined how HU sensitivity at the cellular level corresponds to the response of β-thalassemic patients. HEPs were

Table 1: Thalassemia patients included in this study

Number	Date Of Birth	Sex	Thalassemia type	Mutation	Xmn1
Non-Responders					
NR1	1983	Female	Major	IVSII-I/IVSI-110	-/-
NR2	1982	Female	Major	IVSII-I/IVSII-I	-/-
NR3	1987	male	Major	C44/Cd 27	-/-
NR4	1977	Female	Major	IVSII-I/IVSII-I	+/+
NR5	1992	Female	Major	IVSI-I/IVSI-I	-/-
NR6	1984	male	Major	IVSI-25/IVSI-5	+/-
NR7	1986	male	Major	C39/C39	-/-
NR8	1985	male	Major	C22/C30	-/-
Number	**DOB**	**Sex**	**Thalassemia type**	**Mutation**	**Xmn1**
Responders					
R1	1966	male	Major	IVSII-I/IVSII-I	+/+
R2	1977	Female	Major	C8/C8	+/+
R3	1999	Female	Intermediate	IVS II-I/IVS II-I	+/+
R4	1985	male	Major	IVSII-I/IVSI-5	+/-
R5	1982	male	Intermediate	cd25-26/cd25-26	+/+
R6	1986	male	Intermediate	IVSII-I/IVSII-I	+/+
R7	1977	Female	Intermediate	IVSII-I/IVSII-I	+/+
R8	1982	male	Intermediate	IVSII-I/29bp deletion	-/-

expanded from blood mononuclear cells of the 16 selected β-thalassemic patients (Table 1). After 10 days of culture, the HEPs were further expanded in the presence or absence of 100 μM HU. Cell numbers were monitored daily. Total hemoglobin production, ratio of hemoglobin types, and cell morphology were analyzed at day 15 when the cultures were terminated (Figure 2A).

The first difference observed between cultures derived from group I (NR) and group II (R) was the relatively poor growth rate of NR HEPs (Figure 2B-C and supple-

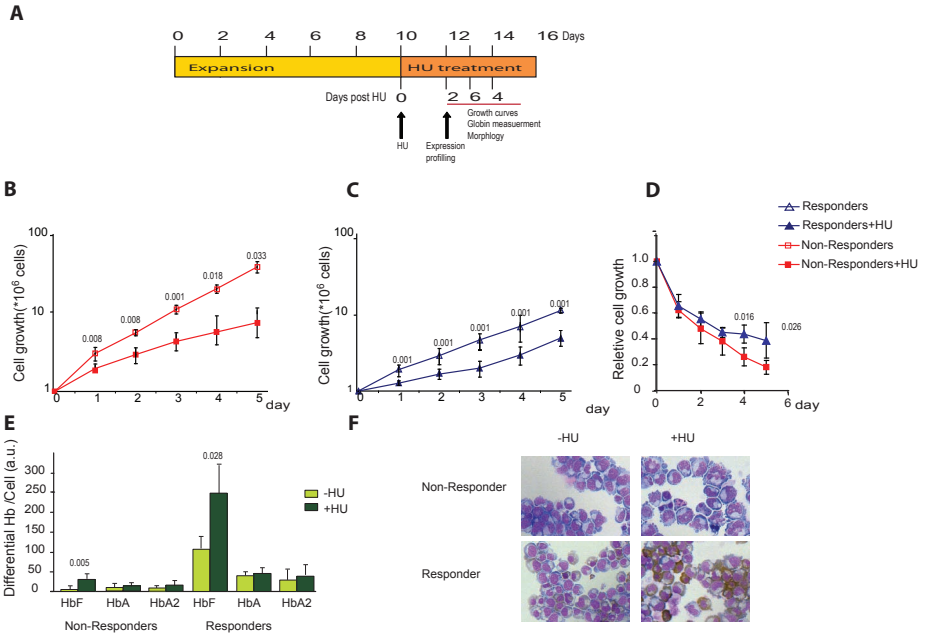

Figure 2. Characteristics of cultured HEPs from β-thalassemia patients. A) Schematic diagram of culture system time course during the experiments. The days of culture are indicated on top. Days of HU treatment, expression profiling and the performed studies time points are indicated at the bottom. B and C) HEPs from non-responders in B, grow faster than those from responders in C. After HU treatment, growth curves of non-responder HEPs (B) decline more than those of responders (C). D) HU sensitivity curve for HEPs from responders and non-responders, normalized by taking the ratio of cell proliferation of HU treated cells over non-treated cells. (E)Hemoglobin induction by HU (5 days) in responders and non-responders. (B-E) p-values <0.05 are indicated. (F) Representative cytospins of responder and non-responder HEPs treated with HU for 3 days. Cytospins were stained with histological dyes and neutral benzidine. Hemoglobinized cells are stained brown.

mentary Figure 2). Strikingly, addition of HU barely affected the slow growth rate of NR HEP cultures, while NR HEP cultures were very sensitive to HU treatment (Figure 2D).

Total hemoglobin (in arbitrary units, a.u.) and the ratios of fetal (HbF) versus adult (HbA) hemoglobin expression were measured 5 days after the start of HU treatment (day 15 of culture), and the absolute distribution of different hemoglobins was calculated (Figure 2E).Interestingly, HU treatment did not alter the expression of adult HbA but resulted in increased expression of HbF (NR from 8 to 33 a.u., R from 106 to 248 a.u.). Since the basal level of total hemoglobin is much higher in R HEPS, these cells express the highest Hb F levels upon HU treatment (Figure 2E).

Erythroblasts derived from responders hemoglobinize more than non-responders upon HU treatment.

Morphological analysis of HEPs treated with HU for 3 days, and stained for hemoglobin in combination with histological dyes showed that R HEP cultures accumulated more hemoglobinized cells after HU treatment (25% in R HEPs versus 10% in NR HEPs; Figure 2F). Cultures of NR HEPs accumulated more pyknotic cells (20% in NR HEPs versus 10% in R HEPs; Figure 2F).

In conclusion, compared to R HEPs, NR HEPs have a higher propensity to succumb to cell death in response to HU. Furthermore, although the fold-change in HbF levels upon HU treatment is higher in NR HEPs than that observed in R HEPs, the low baseline levels of HbF preclude induction of HbF to significant amounts in NR HEPs.

Responder erythroblasts constitutively express a stress-program that is induced by HU in non-responders HEPs.

To carry out gene expression profiling analysis, HEPs were expanded for 10 days and subsequently treated with 100μM HU or solvent for 2 days. RNA expression

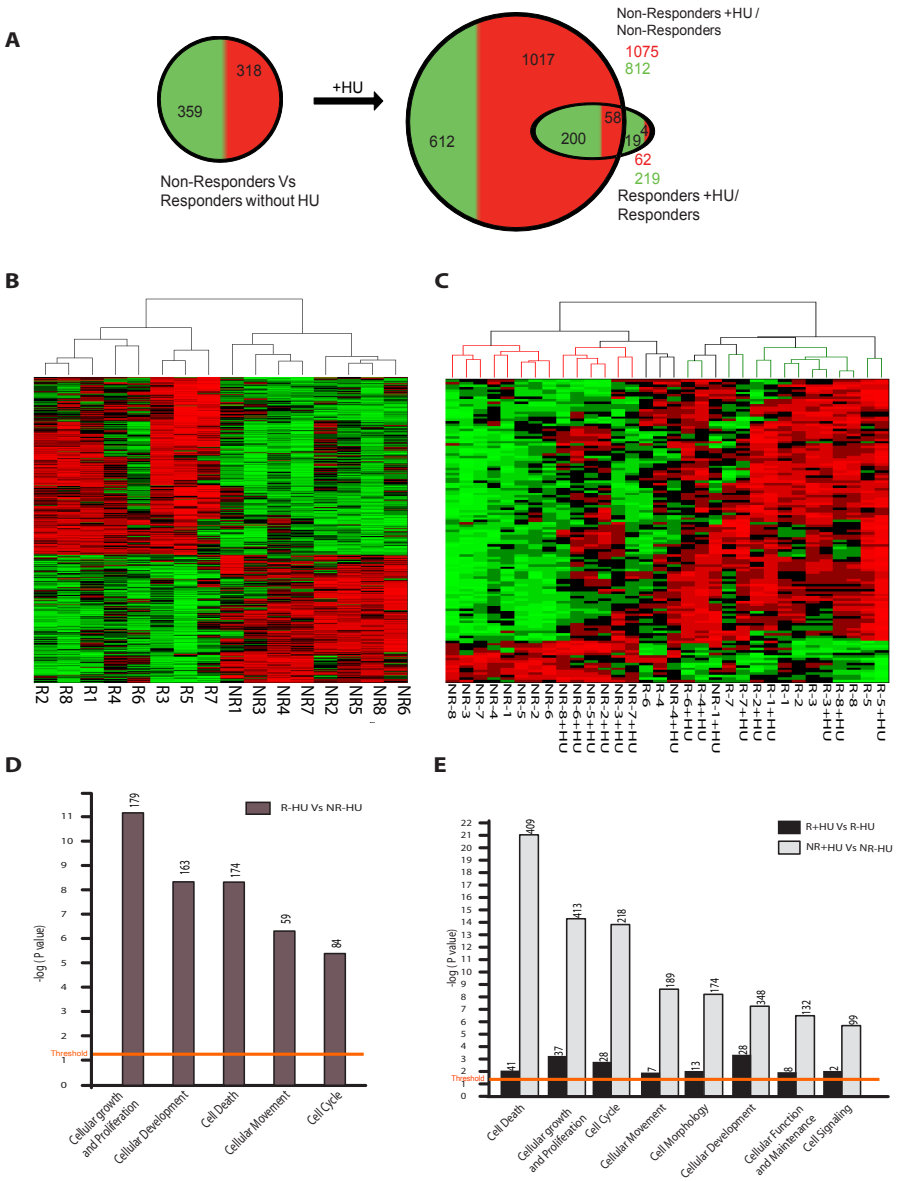

Figure 3. Gene expression profiling of thalassemic patients. A) The number of differentially expressed genes between responders and non-responders before and after HU treatment. B and C) Supervised clustering of differentially expressed genes without (B) and with (C) HU treatment. Responders: R, non-responders: NR, HU treated: +HU. (D) Functional annotation of differentially expressed genes in responders versus non-responders before HU treatment. E) Functional annotation of differentially expressed genes in responders versus non-responders after HU treatment. Functional annotation for biological processes were processed by ingenuity pathway analysis ($P < 0.05$). See Supplementary excel file for extensive lists).

profiles were compared between 8 R and 8 NR HEP cultures. The expression profiles of the samples were compared using the SAM algorithm to identify HU response-associated genes. A striking difference between the NR and R profiles is their response to HU at the gene expression level. HU induced many changes in gene expression in NR HEPs (1887 genes, 2664 differentially expressed probe sets) whereas far fewer changes were observed in R HEPs (281 genes, 320 differentially expressed probe sets) (Figure 3A).

Comparison of the expression profiles between NR and R HEPs showed a moderate number of differentially expressed genes in the absence of HU (677 genes,1105 differentially expressed probe sets) (Figure 3A-B) and the differences became smaller when the cells were treated with HU (661 genes, 899 differentially expressed probe sets) (Figure 3A). In total, 1105 probe sets were differentially expressed between the two groups of HEPs in absence of HU treatment (see the supplementary excel file for extensive list of genes). The expression data were used for cluster analysis, clusters R HEPs and NR HEPs separately (Figure 3B). Gene enrichment analysis of R and NR HEPs before HU treatment indicates that the genes involved in regulation of cellular proliferation and apoptosis are already expressed in much higher level compare to NR HEPs (Figure 3D). In another word R HEPs are adopted to stress pathways.

Upon HU treatment the NR cluster separately from samples treated with HU (Figure 3C). In contrast, the R samples plus or minus HU cluster independent of HU treatment. HU treatment barely affected expression of these genes in samples R5, R8, R3, R1 and R7 and only moderately in samples R2, R4 and R6 (Figure 3C). HU treatment doesn't seem to change R HEPs expression, while it has a dramatic effect on NR HEPs transcriptome. Gene enrichment analysis of HU treatment indicates that the majorities of differentially expressed genes in NR HEPs are involved in apoptosis and cell cycle regulation (Figure 3E). This suggests that cells from R patients constitutively express a stress program that is activated in NR HEPs upon exposure to HU, and that may be involved in induction of γ-globin.

Validation of gene expression data by RT-QPCR

Regulation of genes involved in stress response, γ-globin expression and erythroid maturation

The INK4b-ARF-INK4a locus is differentially regulated between responders and non-responders. It was striking that the proliferation rate of erythroid cultures expanded from R patients was much slower compared to those from NR patients. The INK4b-ARF-INK4a locus is known to be involved in stress responses, but probe sets on the microarrays cannot discriminate between the overlapping ORFs of p14ARF and p16INK4a. Therefore, we analyzed expression of p15INK4b, p14ARF and p16INK4a encoded by the INK4b-ARF-INK4a locus in more detail (Fig. 4A). Expression of p16INK4a was increased upon HU treatment for both NR and R HEPs. In contrast, expression of p14ARF was increased in NR compared to R HEPs, and treatment with HU further increased expression in NR, but not in R HEPs. Expression of p14ARF in NR HEPs treated with HU is on average 10-fold higher when compared to R HEPs treated with HU. Expression of p15INK4b is lower in NR HEPs compared to R HEPs before HU treatment. However, HU significantly enhanced p15INK4b expression in NR HEPs, but not in R HEPs. In conclusion, the failure of R HEPs to upregulate p14ARF and p15INK4b may contribute to their relative resistance to HU treatment, however the differential expression of the INK4b-ARF-INK4a locus does not explain the slow proliferation of R HEPs.

Stress response genes

From the array data we selected a number of genes with a role in the adaptation to stress responses that were consistently differentially expressed between cultures derived from NR and R HEPs (see the supplementary excel file for extensive list of genes). Expression of these genes was analyzed using qRT-PCR on cDNA samples. Forkhead box O3 (FOXO3) is a transcription factor inducing genes that

enforce the oxidative firewall. Arginase 1 and 2 (ARG1, ARG2) compete with NO synthase (NOS) for the substrate L-arginine (Durante et al., 2007), and thereby protect against oxidative stress from NO. Homeodomain interacting protein kinase 2 (HIPK2) is involved in apoptosis, differentiation and also activation of CBP/p300 (Steinmann et al., 2009; Yoshida and Kitabayashi, 2008). Arg2 and Hipk2 are known Foxo3a target genes (Bakker et al., 2007). These genes were all consistently upregulated in R compared to NR HEPs, and further upregulated upon treatment with HU in both groups (Figure 4B). Notably, expression levels of ARG1 and ARG2 were higher in the untreated R HEP cultures than in HU-treated NR HEP cultures and may play an important role in protection from HU-induced cell death in R HEPs (see Discussion). Kruppel like factor 10 (KLF10) (Døsen-Dahl et al., 2008) was identified as a protein that protects ALL blasts and stromal cells against chemotherapy. KLF10 was expressed at elevated levels in R HEPs compared to NR HEPs and reached similar levels in both groups upon HU treatment.

Apoptosis genes

BCLXL protects erythroblasts from apoptosis. Both in the absence and presence of HU, BCLXL levels are higher in R HEPs. BCLXL is under control of STAT5B (Moucadel and Constantinescu, 2005)which decreased in NR HEPs upon HU treatment, although the difference is not as marked as BCLXL (Fig. 4C and not shown). Whether BCL6 is involved in apoptosis or in a different process is not clear. During VDJ rearrangement in B-cells it is responsible for methylation of the ATR gene and thus prevents the activation of the DNA damage response during the rearrangement process (Ranuncolo et al., 2007). Expression of BCL6 is not increased by HU in both groups, but it is expressed almost 10-fold higher in R HEPs than in NR HEPs (Figure 4C).

γ-globin expression and erythroid maturation

Whereas erythropoietic stress induces γ-globin expression, several transcription

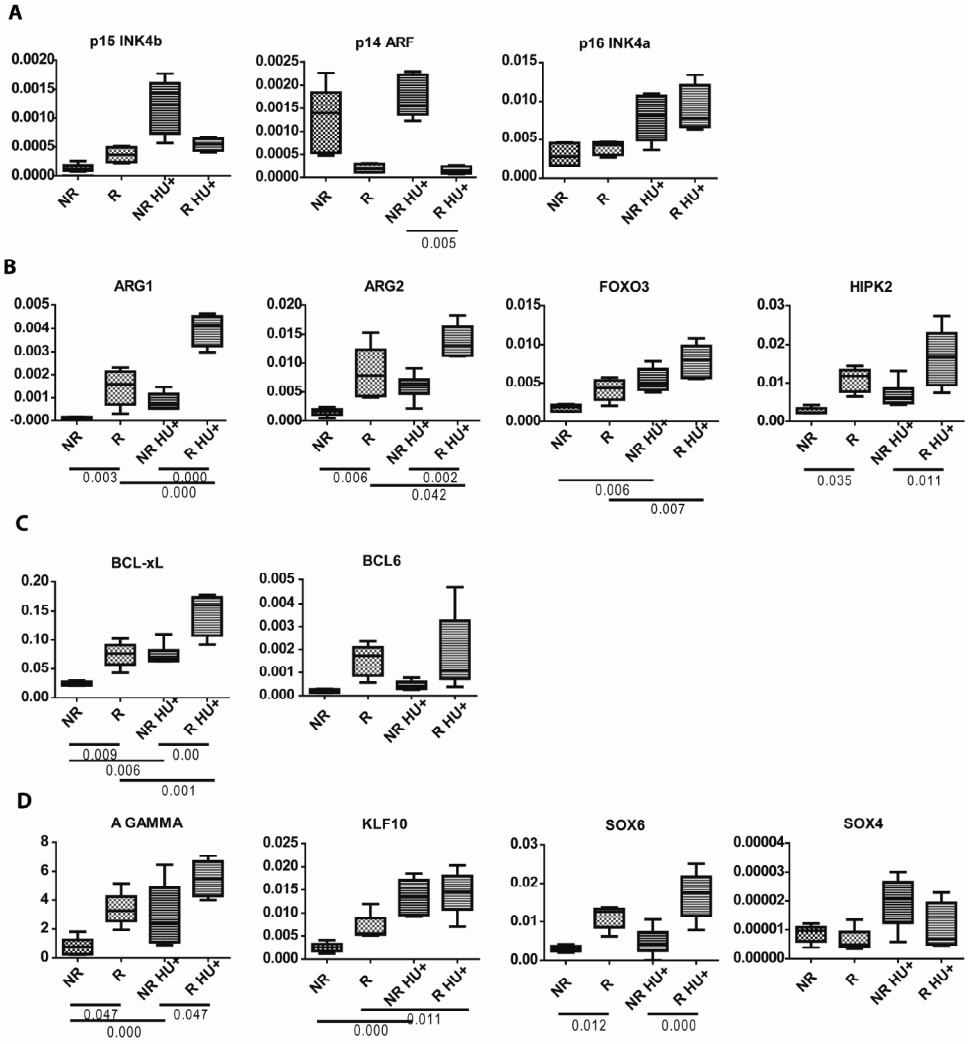

Figure 4. RT-QPCR validation of selected genes. A) INK4b-ARF-INK4a locus B) stress response genes C) apoptosis response genes D) globin regulation related genes. Responders: R, non-responders: NR, HU treated: HU+. p-values < 0.05 are indicated. At least 5 patients were analyzed in each group (n≥5).

A

Up regulated

ALAS2	2.19394322	OPTN	1.539197
ANXA5	2.554261323	PAQR9	2.080337
ARG1	10.17353714	PDZK1IP1	3.31088
ARG2	2.433526283	PRO2852	1.636954
ARHGEF12	1.725042656	RAP1GAP	1.720793
BCL6	3.617914509	RASD1	2.425198
BEX1	2.884296864	RHCE	1.792361
BSG	1.632699043	RHD	2.020121
BTG1	1.662100185	RIOK3	1.717625
BTG2	1.960629193	RNF11	1.606764
C17orf103	1.668589911	RSAD2	3.53973
C17orf91	3.645792185	RUNDC3A	2.531284
C19orf22	1.602514204	SEC14L1	1.749137
C5orf41	2.034547926	SESN3	1.669384
CPEB4	2.610951537	SGK1	1.686945
CR1	1.578687965	SLC14A1	2.320063
CTSB	1.715553361	SLC25A37	1.903562
DNAJB9	1.543289851	SLC2A1	2.252239
FHDC1	1.5607789	SLC30A1	2.321541
FOXO3	1.965608804	SLC4A1	2.28137
FOXO3B	1.740705455	SMOX	2.444033
GABARAPL1	2.43782498	SRGAP2P1	1.648432
GADD45A	1.705556074	TBCEL	1.551772
GDF15	2.202646277	TMCC2	2.412047
GSTM3	1.535454104	TNS1	3.226844
GYPA	1.839129639	TRAK2	1.979956
GYPB	1.660170365	TRIB3	2.237891
HEMGN	1.51065715	TRIM10	1.805231
HIST1H2AC	1.713853539	TRIM58	1.738083
HMOX1	1.667268189	TSC22D3	1.585707
IL6ST	3.444774902	TSPAN5	1.919801
KAT2B	1.571593609	UBE2H	2.728712
KLF9	1.625917092	WDR26	1.854971
KLHL24	1.662304668	WDR45	1.695834
LPIN2	1.530152036	XPO7	3.320614
MBNL2	1.863300835	YPEL3	2.09024
NEAT1	1.899383213	YPEL4	2.113776
NUDT4	1.556181393	ZBTB16	1.747955

Down regulated

CDCA7	0.571967
CDCA7L	0.630156
CDK6	0.504975
EPCAM	0.440324
FABP5	0.541622
FAM45A	0.559767
LAPTM4B	0.656124
ME2	0.654102
MGST1	0.651254
NSMAF	0.650295
PRKACB	0.619356
SCARB2	0.516909
SKP2	0.586068
SLC39A8	0.615998
SYNGR1	0.649989

B

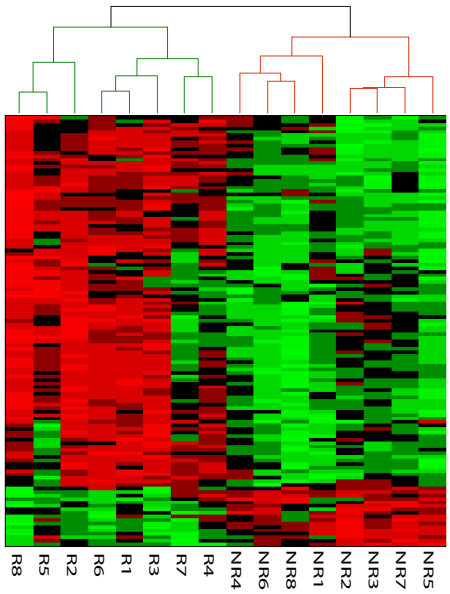

Figure 5. Clustering analysis of differentially expressed erythroid differentiation-related genes. A) Overlap of differentially expressed genes in responders: R, versus non-responders: NR, with genes involved in erythroid differentiation program (91 out of 327). The expression dynamics of these genes are characteristic of the terminal erythroid differentiation program (Merryweather-Clarke et al., 2011a). B) Clustering analysis of genes involved in the erythroid differentiation program that are also differentially expressed between R and NR.

factors have been reported to modify γ-globin expression, including BCL11A (Sankaran et al., 2008) and SOX6 (Xu et al., 2010). Expression levels of SOX6 are approximately 3.5-fold higher in R HEPs compared to NR HEPs, whereas expression of SOX4 was slightly less. BCL11A expression was not different between the two groups. Expression of the γ-globin gene itself at the transcript level is 4-fold higherin R HEPs compared to NR HEPs before HU treatment (Figure 4D). Finally genes important to maintain expansion of erythroblasts such as MYB (Emambokus et al., 2003), PRMT5 (Rank et al., 2010), IKAROS (Bank et al., 2005; Bottardi et al., 2009) and STAT5B (Boosalis et al., 2001) remained constant (data not shown).

Merryweather-Clarke et al. recently reported Global gene expression analysis of human erythroid progenitors.(Merryweather-Clarke et al., 2011b) Comparison of highly differentially expressed genes towards terminal erythroid differentiation from Merryweather-Clarke et al(Merryweather-Clarke et al., 2011b). with the list of differentially expressed genes between R and NR HEPs without HU treatment in this study reveals that 28% overlap (91 out of 327 genes) (see the supplementary excel file for extensive list of genes). The dynamic of all these overlapping genes are toward terminal erythroid differentiation (Figure 5A). Clustering analysis of these 91 genes, clusters R and NR HEPs in two distinct groups (Figure 5B). This would indicate the HEP cells derived from responders are more prompted towards differentiation.

Discussion

Our data shows that cells derived from responders express relatively high baseline levels of HbF compared to cells derived from non-responders. Cells from non-responders change the expression pattern of a large number of genes upon HU treatment, while cells from responders displayed only minor changes after treatment. Differential gene expression profiles of these two groups indicated that high HbF was associated with a continuously activated stress response, and with genes that protect from stress-induced apoptosis. This suggests that HU is effective in 'responder' patients because their baseline HbF levels are relatively high and their erythroblasts already activated a stress response program which protects them from the cytotoxic effects of HU. Moreover, cells derived from responders have more potential to follow terminal erythroid differentiation.

Reactivation of γ-globin expression in patients suffering from β-thalassemia or sickle disease would ameliorate the severity of the disorders, and sufficiently high expression could even cure the patients. However repression of γ-globin after birth is well controlled and it has proven difficult to alleviate repression by pharmacological means. Proliferative stress, such as induced by HU, can increase γ-globin

expression, but not all patients respond with an increase in HbF that renders them less dependent on regular transfusions. We characterized erythroblasts expanded from β-thalassemia patients that do or do not respond to HU, with respect to proliferation and differentiation kinetics, and gene expression profiles. The subjects included in this study were selected from a large cohort of patients either because they remained fully transfusion-dependent upon HU treatment (non-responders) or because they became essentially transfusion-independent (responders). All patients stopped using the drug two weeks before blood sampling and the cells were cultured from freshly isolated peripheral mononuclear cells for more than 10 days. Erythroblasts expanded from patients that did not respond to HU were very sensitive to HU treatment, and the initial γ-globin levels were low. HU treatment up-regulated γ-globin expression and that of many other genes, but this eventually resulted in cell death rather than in maturation to hemoglobinized cells. Erythroblasts from patients responding well to HU treatment were characterized by relatively high initial γ-globin levels, and HU treatment had a relatively minor impact on their gene expression profiles. HU treatment provoked cell death to a lesser extent, allowing maturation to hemoglobinized cells. The gene expression profiles indicated that high HbF was associated with signatures of terminal erythroid differentiation, activated stress response and protection from stress-induced apoptosis.

Factors regulating γ-globin expression

The -158 XmnI SNP in the promoter of the γ-globin gene has been linked to HbF expression (Alebouyeh, 2004; Bradai et al., 2003; Dixit et al., 2005; Karimi et al., 2005; Neishabury et al., 2008; Panigrahi et al., 2005; Verma et al., 2007; Yavarian et al., 2004). In our patient cohort, 13/16 (81% of chromosomes) of the responders carried the -158 XmnI C>T SNP. In the non-responder only 3/16 (19% of chromosomes) carried this SNP. Although there is a correlation between the presence of the mutation and HbF expression, this SNP does not solely determine HbF and HU response in this patient group.

Several factors were found to control γ-globin expression. In vitro cultured erythroid cells increase γ-globin expression in response to SCF and glucocorticoids (Gabbianelli et al., 2008) (Sripichai et al., 2009b), but the cultures of both groups were exposed to these conditions.

Recently, expression of BCL11A was negatively associated with HbF expression. BCL11A is differentially expressed in pre- and postnatal erythroid cells and reduced expression of BCL11A is associated with increased γ-globin expression (Sankaran et al., 2008). We found that NR- and R-derived HEP cultures express similar levels of BCL11A. However, some other transcription factors involved in γ-globin expression are increased in R HEP cultures. QRT-PCR confirmed regulation of SOX6 (3.8-fold up in R versus NR) which is in contradiction with other studies (Cohen-Barak et al., 2007; Yi et al., 2006). KLF10 (2,5-fold up in R versus NR, 5-fold up in response to HU) is a TGFβ immediate early response gene (Subramaniam et al., 1995), possibly linking KLF10 to the observed induction of γ-globin by TGFβ (Sripichai et al., 2009a).

The array data reveal more genes differentially expressed in R versus NR that may have an effect on the regulation of HbF such as ID1 and HHEX (Supplementary Table 2). We note that genes involved in erythroid differentiation are expressed at higher levels in R HEPs (Fig. 5, (Merryweather-Clarke et al., 2011a)). This suggests that R HEPS have a propensity to enter the terminal differerentiation pathway while NR HEPs maintain a proliferative state. This difference may explain decreased survival of NR HEPs upon HU treatment, whilst R HEPs survive by undergoing terminal differentiation.

The role of stress factors in γ-globin expression

The low proliferation rate of the R HEPs and their expression profiles suggest that these cells have adapted to permanent stress conditions. The observation that HU treatment did not have a major impact on gene expression in the R samples, while 2664 probe sets were differentially expressed in response to HU in the NR

samples, supports this notion.

The most striking observation from the expression profiles is the expression of stress proteins in R HEPs. An example validated by Q-PCR is expression of FOXO3. FOXO3 is upregulated during erythroid differentiation (Bakker et al., 2004) and in response to various types of stress such as ROS and DNA damage (Hattangadi and Lodish, 2007). The increased FOXO3 expression in R HEP compared to NR HEP cultures and the further upregulation in response to HU indicates increased levels of cellular stress. Numerous FOXO3 target genes are also upregulated among which HIPK2 and BTG1(Bakker et al., 2007). Interestingly, the function of stress-induced FOXO3 protein is not to induce cell death, but to increase the potential of cells to prevent and repair oxidative damage and to slow down the cell cycle to allow for DNA repair before replication.

HU is a ribonucleotide reductase inhibitor that stalls cells in S phase (Szekeres et al., 1997). Stalled replication forks are potent inducers of senescence or apoptosis, but apparently also of HbF. Defects in the Fanconi anemia pathway result in increased replication fork stalling. It is therefore interesting to note that Fanconi anemia is associated with high HbF levels (Gumruk et al., 2008; Miniero, 1981). Importantly, a stalled replication fork activates ATR kinase, which is crucial to all downstream events. During B-cell development, BCL6 is required to methylate and silence the ATR locus to prevent ATR activation during VDJ recombination. It is interesting that increased tolerance to HU and high HbF levels are associated with high BCL6 levels. It would be worthwhile to test the role of BCL6 in cell survival upon HU treatment. Increased expression of BCLXL could also contribute to enhanced survival in presence of HU in R HEP cultures. In normal erythroid progenitors BCLXL is induced by EPO to maintain viability of erythroid cells during terminal maturation (Motoyama et al., 1999; Silva et al., 1996).

ARG1 and ARG2

HU also increases NO production through phosphorylation and activation of NOS

(Cokic et al., 2006). Although NO has been implicated in upregulation of HbF through activation of γ-globin expression (Cokic et al., 2007; Lou et al., 2009), NO also inhibits growth of erythroid primary cells and colony cultures (Maciejewski et al., 1995). Arginase hydrolyzes L-arginine to urea and L-ornithine in the urea cycle and inhibits nitric oxide (NO) production via competition with NOS for the substrate L-arginine (Durante et al., 2007). During erythroid differentiation NO levels decreased significantly (Kucukkaya et al., 2006). NO donors inhibit hemoglobinization (Chénais et al., 1999). NO is inhibited more by fetal RBCs when compared to adult RBCs suggesting that fetal RBCs have a higher level of NO scavengers (Calatayud et al., 1998). On the other hand high NO concentrations promote apoptosis, while low NO concentrations result in resistance to apoptosis.

The Arginase 1 (ARG1), Arginase 2 (ARG2) and Argininosuccinate synthetase 1(ASS1) genes are differentially expressed between NR HEPs and R HEPs. Their expression is 10.2-, 2.4- and 1.9-fold increased in R HEPs compared to NR HEPs. In a SNP association study ARG1 and ARG2, ASS1, NOS1 and NOS2A were reported to be significantly associated with response to HU treatment (Ma et al., 2007). This suggests that high expression of ARG1, ARG2 and ASS1 protects hematopoietic progenitor cells against the excessive amounts of NO after HU treatment to prevent apoptosis, and scavenge the extra NO at the later stages of differentiation.

In conclusion, the biological and molecular analysis of erythroblast cultures of β-thalassemia patients suggests that several mechanisms are involved in high HbF expression and HU responsiveness. Although the basal HbF level and XmnI (Gγ) SNP (-158C>T) polymorphism are correlated with HbF response, cell survival upon HU tretment, stress response and propensity toward trminal erythroid differentiation are also playing important roles. Collectively our data shows HU induces HbF induction and Hemoglobinization of human erythroid progenitors. Responder HEPs, compare to non-responder HEPs, proliferate with slower rate, have more potential to undergo terminal erythroid differentiation and are adapted to constitu-

tive stress response. Once HEPs are treated with HU, NR HEPs suffer from their high proliferation rate, especially because HU stalls replication and since they are less adapted to stress response, they go to apoptotic pathways. Responders on the other hand proliferating slowly and are adapted to stress, therefore they do not suffer from HU cytotoxic effects and moreover, they are prone to follow their terminal erythroid differentiation. Our data further suggest that drugs activating the stress response may increase HbF, provided that cell survival is not simultaneously compromised and cells have more erythroid differentiation potentials.

The differential expression profile of responders and non-responders can be very useful to predict HU response in sickle cell and thalassemia patients. A list of consistently differentially expressed genes can be deduced from their expression profiles to identify an expression fingerprint (predictor). This HU response predictor can be developed from the selected gene list presented in supplementary Table 2 and should be validated by analysis of independent cohorts of patients.

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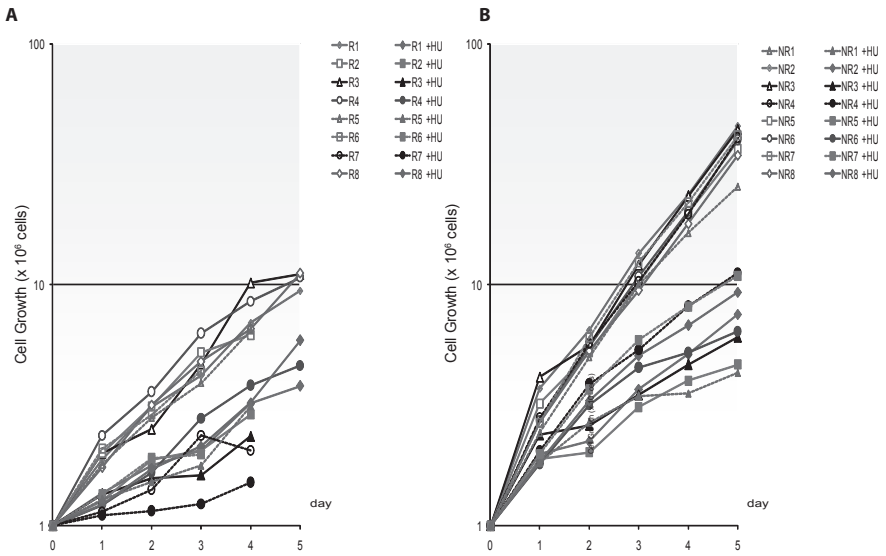

Supplementary Figure 1. Growth curves of HEPs from β-thalassemic patients. A) Responder HEPs grown under proliferation conditions. B) Non-responder HEPs grown under proliferation condi-

Patient number	Birth date	Blood group	Sex	Hight cm	Weight kg	Talassemia type	Transfusion after HU treatment	Phenotype	Mutation	Xmn1 polymorphism	Alpha chain genotype
NR1	1983	A+	F	162	50	Major	YES	Good	IVSII-I/IVSI-110	-/-	αα/αα
NR2	1982	A+	F	162	51	Major	YES	Good	IVSII-I/IVSII-I	-/-	αα/αα
NR3	1987	O+	M	120	48	Major	YES	Good	C44/Cd 27	-/-	αα/αα
NR4	1977	O+	F	168	48	Major	YES	Medium	IVSII-I/IVSII-I	+/+	αα/αα
NR5	1992	O+	F	135	35	Major	YES	Good	IVSI-I/IVSI-I	-/-	αα/αα
NR6	1984	O+	M	168	55	Major	YES	Good	IVSI-25/IVSI-5	+/-	αα/αα
NR7	1986	O-	M	164	50	Major	YES	Good	C39/C39	-/-	αα/αα
NR8	1985	AB+	M	163	57	Major	YES	Medium	C22/C30	-/-	αα/αα
R1	1966	AB+	M	175	48	Major	NO	Very Good	IVSII-I/IVSII-I	+/+	αα/αα
R2	1977	O+	F	149	50	Major	NO	Very Good	C8/C8	+/+	αα/αα
R3	1999	A+	F	137	27	Intermedia	NO	Very Good	IVS II-I/IVS II-I	+/+	αα/αα
R4	1985	O+	M	164	49	Major	NO	Good	IVSII-I/IVSI-5	+/-	αα/αα
R5	1982	B+	F	156	57	Intermedia	NO	Very Good	cd25-26/cd25-26	+/+	αα/αα
R6	1986	O+	M	178	62	Intermedia	NO	Very Good	IVSII-I/IVSII-I	+/+	αα/αα
R7	1977	A+	F	162	52	Intermedia	NO	Very Good	IVSII-I/IVSII-I	+/+	αα/αα
R8	1982	AB+	M	155	61	Intermedia	NO	Good	IVSII-I/29bp deletion	-/-	αα/αα

Patient number	Splenectomy	Sex Maturity Rate 1: unmture 5: fully mature	Skeletal deformity	Desferal week/startage	WBC	Hgb	MCV	MCH	Platelet	Hb Elect Hb A	Hb Elect Hb F	Hb Elect HbA2	Ferritin
NR1	Not done	4	Negetive	20/2	47000	7.9	86.0	28.3	368				1976
NR2	Done	3	Negetive	20/2	40200	8.4	90.0	32.0	520				405.6
NR3	Not done	2	Negative	24/3	32000	7.3	82.0	28.0	880				1781
NR4	Done	2	Posetive	14/10	10300	8.2	87.0	30.0	506	84	11.5	4.5	2391
NR5	Not done	2	Negetive	12/5	13000	8.5	86	28	450				2600
NR6	Not done	2	Negative	24/8	5000	6.5	76.0	25.0	324				1128
NR7	Done	2	Negetive	16	5500	9.4	83	28	227	12		2	1186
NR8	Not done	2	Negetive	24	5000	10	86	29	244	18	450	1	1524
R1	Done	4	Posetive	3/28	28000	11.9	90.1	31.0	699	21.6	75	3.4	140
R2	Done	4	Negetive	24/2	113000	7.8	94.2	29.7	433	28.9	68	3.1	405
R3	Not done	3	Negative	NO	10500	8.9	65.6	21.8	400	43.1	54	2.9	212
R4	Done	2	Negetive	24	31000	9.2	75	24	677	15.3	83	2.7	2924
R5	Not done	5	Negetive	NO	6600	9.3	68.4	23.0	235			3.3	616
R6	Not done	4	Negetive	NO	9100	12.8	73.4	23.4	450	10.9	87.9	1.9	698
R7	Done	2	Posetive	12	8800	8.9	73	25	755	12	84.3	3.7	399
R8	Done	4	Negetive	NO	42800	12.4	84	26.4	581	39.1	56	4.9	430

tions. +HU: 100 μM hydroxyurea added to the cultures. Note that the y-axis has a logarithmic scale.

Supplementary Table 1. Clinical data of Thalassemia patients included in this study (NR: non-re

Supplementary Table 2. Selected differentially expressed genes between non-responders and responders. The

	Gene Title	Gene Symbol	R vs. NR	qRT Confirmed	Function
A	**Group A genes**				
A	Hemoglobin, Epsilon	C	6,3		
A	Hemoglobin, gamma A	HBG1	3,0	3,5	
A	Hematopoietically expressed homeobox	HHEX	1,5		Scicchitano et al., 2003
A	Inhibitor of DNA binding 1, dominant negative helix-loop-helix	ID1	1,5		Scicchitano et al., 2003
A	SRY (sex determining region Y)-box 6	SOX6	2	3.8	Scicchitano et al., 2003
B	**Differentiation related genes**				
B	Arginase, liver	ARG1	10,1	12.7	Ma et al., 2007
B	Arginase, type II	ARG2	2,4	5.1	Ma et al., 2007
B	Argininosuccinate synthetase 1	ASS1	1,9		Ma et al., 2007
B	cAMP responsive element binding protein 3-like 1	CREB3L1	2,3		sequence-specific DNA binding transcription factor
B	Glycophorin A	GYPA	1,839		Glycophorin
B	Glycophorin B	GYPB	1,66		Glycophorin
B	Interleukin 6 signal transducer (gp130, oncostatin M receptor)	IL6ST	2,5		proliferation, growth, differentiation
B	Interleukin 7	IL7	-1,9		proliferation, survival,
B	Interleukin 7 receptor	IL7R	-1,6		proliferation, differentiation, growth
B	Interleukin 8	IL8	2,9		chemotaxis, activation, migration
B	Kruppel-like factor 10	KLF10	2,0	2.7	proliferation, differentiation
B	Kruppel-like factor 9	KLF9	1,6		transcription factor that binds to GC box elements
B	Mitogen-activated protein kinase kinase 6	MAP2K6	-2,1		apoptosis, proliferation
B	Nuclear factor (erythroid-derived 2), 45kDa	NFE2	1,5		sequence-specific DNA binding transcription factor
B	RAR-related orphan receptor A	RORA	1,8		differentiation
D	Sorbitol dehydrogenase	SORD	-3.4		enzyme in sorbitol pathway
B	SRY (sex determining region Y)-box 4	SOX4	-1,7	-1.4	sequence-specific DNA binding transcription factor
B	Tripartite motif-containing 10	TRIM10	1,8		differentiation, Blaybel et al., 2008
B	WD repeat domain 26	WDR26	1,855		facilitate multiprotein complexe formation
B	WD repeat domain 45	WDR45	1,696		facilitate multiprotein complexe formation
B	Exportin 7	XPO7	3,321		nuclear export signal receptor
B	Zinc finger and BTB domain containing 16	ZBTB16	1,748		differentiation, binds SP1, BCL6 etc.
C	**Stress and prolifration related genes**				
C	BCL2-like 1	BCL2L1	2,0	3,0	pro-apoptotic
C	B-cell CLL/lymphoma 6 (zinc finger protein 51)	BCL6	3,6	7.3	apoptosis, proliferation
C	BTG family, member 1	BTG1	1,7		apoptosis, proliferation
C	BTG family, member 2	BTG2	2,0		apoptosis, proliferation
C	Caspase 4, apoptosis-related cysteine peptidase	CASP4	-1,62		apoptosis, proliferation
C	Caspase 6, apoptosis-related cysteine peptidase	CASP6	-1,8		apoptosis, proliferation
C	Cyclin-dependent kinase 6	CDK6	-2.1		apoptosis, proliferation, growth, differentiation
C	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	CDKN2B	2,3	3,0	apoptosis, proliferation, growth, differentiation
C	DnaJ (Hsp40) homolog, subfamily C, member 12	DNAJC12	-2,0		complex assembly, protein folding, and export
C	Forkhead box O3	FOXO3	1,9	2.3	apoptosis, expression in, cell cycle progression
C	Homeodomain interacting protein kinase 2	HIPK2	2,5	3.9	apoptosis, proliferation, growth

genes included in this table belong to one of the following categories: A) involved in γ-globin regulation B) differentiation related genes or possibly involved in γ-globin regulation based on literature or similarities with genes in the same gene family C) stress response or apoptosis genes. Differential expression is calculated as fold-changed comparing responders over non-responders. Expression profiling data were confirmed in selected cases by QRT-PCR.

General Discussion

Hematopoiesis

Hematopoiesis is the process by which mature blood cells of distinct lineages are produced from pluripotent hematopoietic stem cells. Elucidation of the mechanisms that regulate hematopoietic stem/progenitor cell self-renewal and various hematopoietic-lineage commitments would be facilitated by ectopic expression of specific genes and/or identification of defined culture conditions. To study the hematopoietic system in vitro, we used an established system based on overexpression of HOXB4 in embryonic stem cells.

ES-HA-BirA-HOXB4 cells as an in vitro model to study the hematopoiesis

Both bone marrow and embryonic stem cells ectopically expressing HOXB4 recapitulate some characteristics of hematopoietic stem/progenitor cells in vitro through regulation of different signaling pathways including Wnt, Notch, FGF and Hedgehog (Pilat et al., 2005; Schiedlmeier et al., 2007; Sharma et al., 2006).

In Chapter 2 we proposed that these cells in combination with Avi-tag biotinylation system would be a suitable in vitro model to study the regulatory role of transcriptional complexes in hematopoietic lineage commitment. The ES-HA-BirA-HOXB4 cells are able to metabolically biotinylate a protein of interest which in this case was Hmg20b. In contrast to previous reports, we found that ES-HA-BirA-HOXB4 cells are primarily committed to the myeloid lineage. A possible explanation for this observation would be a high level of HOXB4 expression in these cells.

It has been shown that a high expression level of ectopic HOXB4 enforces myeloid development whereas the lymphoid and erythroid differentiation are suppressed (Pilat et al., 2005). To overcome this limitation one possible approach would be to introduce an inducible HOXB4 expression vector. This method would

enable control of the HOXB4 expression level.

Considering that Hox genes are tightly regulated during development (He et al., 2011; Ikuta, 2011; Kuraku, 2011), combining ectopic expression of HOXB4 with other HOX family members may result in obtaining the cells phenotypically more close to the apex of the hematopoietic hierarchy. Very recent studies showed that overexpression of HOXB-2, -4 and -5 in human mesenchymal and skin fibroblast cells changes their phenotype towards hematopoietic cells with multipotent progenitor characteristics (Harris et al., 2011). HOXB2 is most likely involved in leukemogenesis (Ivins et al., 2003) and HOXB5, which is hypermethylated in adult mice, is expressed in mouse embryonic hematopoietic tissues (Hershko et al., 2003; Sachan and Raman, 2006; Wu et al., 2003). Notably, HOXA10 is a critical regulator of erythroid/megakaryocyte development. Its expression is tightly regulated during erythro-/megakaryopoiesis and sustained expression blocks differentiation (Magnusson et al., 2007). The ectopic expression of HOXA10 in addition of HOXB4 in a dose dependent manner could be helpful in obtaining hematopoietic progenitors in vitro. Apparently, ectopic expression of HOX family members provides a promising road to maintain hematopoietic progenitor cells however their expression must be tightly regulated.

Another possibility to control HOXB4 expression would be to use the trimeric regulatory complex nuclear factor Y (NF-Y) that recognizes the Hox response element 1 (HxRE-1) and HxRE-2/E-box located in the HOXB4 promoter (Giannola et al., 2000; Zhu et al., 2003). Studies on the HSCs overexpressing NF-Ya showed a bias towards primitive hematopoiesis in vitro. Moreover, these cells displayed a strikingly increased in vivo repopulating capacity after single and sequential bone marrow transplantations, suggesting that NF-Ya is a cellular regulator of HSC self-renewal (Zhu et al., 2005). Recent reports describe that overexpression of other transcription factors e.g. Mxd4 and IKAROS results in the expansion of hematopoietic progenitor cells in vitro (Boros et al., 2011; Papathanasiou et al., 2009).

In conclusion, ectopic expression of HOXB4 has been repeatedly reported as

a tool to generate and expand hematopoietic stem/progenitor cells in vitro. However its expression should be tightly regulated. To achieve this goal, we propose to use an inducible HOXB4 system, or overexpression of (combinations of) other transcription factors.

Studies on the erythropoiesis at the transcriptional regulation level and hemoglobin switching

Thalassemia and sickle cell disorders (SCD) are the most prevalent single-gene disorders worldwide, occurring particularly frequently in the Middle East, Southeast Asia, the Mediterranean region and Africa (Angastiniotis and Modell, 1998; Weatherall, 2010). Mutations in the β-globin genes are the major etiology of β-thalassemia and SCD (Madigan and Malik, 2006; Urbinati et al., 2006; Weatherall, 1976).

β-globin is one of the major components of the hemoglobin molecule in the red blood cells. Hemoglobin is a tetramer comprised of two α-globins and two β-globins and it is involved in oxygen-transport to the whole body (Poyart et al., 1992). These disorders point to the importance of studies on erythropoiesis at least at three different levels; erythropoiesis regulation, hemoglobin regulation and therapeutic approaches at the molecular level.

Transcriptional regulation of erythropoiesis by the CoREST Complex

Like any other cell, the identity of erythroid cells is determined by specific transcription factors regulating gene activity (Cantor and Orkin, 2002; Perry and Soreq, 2002). They perform their regulatory task globally with the aid of covalent modifications of DNA and associated histones (Goll and Bestor, 2005). Histone modifications include phoshphorylation, ubiquitination, ADP ribosylation, acetylation and

lysine and arginine methylation (Lakowski et al., 2006). Among different chromatin modifying complexes, the CoREST complex is a well-known repressor that is comprised of different structural subunits such as Hmg20b, Hmg20a and BHC80, and other subunits with enzymatic activity including Lysine-specific histone demethylase 1 (LSD1) and histone deacetylases (HDACs) (Hakimi et al., 2002). Both histone modifications including deacetylation by HDACs or/and demethylation by LSD1 result in gene repression (Marmorstein and Trievel, 2009; Wang et al., 2009). In erythroid cells the transcription factor Gfi1b recognizes specific sequence and represses gene activity through recruitment of the CoREST complex (Saleque et al., 2007).

To investigate which subunits of the CoREST complex are involved in repression, we applied RNAi against CoREST complex subunits using mouse p53-/- fetal liver I/11 cells. In line with studies performed in MEL cells, LSD1 knockdown disturbs erythroid differentiation in I/11 cells.

Chapter 3 delineates the repressory role of one of the CoREST complex subunits, Hmg20b. Hmg20b depletion drives proerythroblast towards spontaneous differentiation. Microarray analysis showed that the absence of the Hmg20b protein is accompanied by upregulation of genes which are involved in the differentiation process. Increased expression of the majority of deregulated genes (85%) supports the hypothesis that the Hmg20b protein acts as a repressor in the context of the CoREST complex.

Hmg20b has been previously described mediating the Rest-dependent repression of neural differentiation (Hakimi et al., 2002). A related component of the CoREST complex is HMG20A which is proposed to compete with Hmg20b for biding to RE-1 element in neural cells. It has also been shown that Hmg20a mediates the recruitment of the methyltransferase mixed-lineage leukemia (MLL), resulting in gene activation in the brain (Wynder et al., 2005). In contrast, in erythroid cells we found both Hmg20a and Hmg20b in association with the CoRest complex. The role of Hmg20a in erythropoiesis should be elucidated in the further studies. We

found Hrasls3 as a secondary target of Hmg20b. similar to adipocytes (Hummasti et al., 2008), its expression dramatically increases upon erythroid differentiation and its depletion inhibits this process.

In conclusion, this study brought some interesting insights into how Hmg20b directs the negative regulatory role of the CoREST complex on its target genes. In addition, a number of questions were raised about the role of the Hmg20b in the CoREST complex;

• Changes in the global histone methylation and acetylation patterns induced by the other subunits of the CoREST complex in the absence of Hmg20b remain to be determined.

• It is unknown whether the remaining CoREST subunits are still bound to Gfi1b and its target genes.

• Does any protein compete with Hmg20b to be a part of this complex; does reduced availability of Hmg20b open up space for other proteins such as Hmg20a?

After knocking-down Hmg20b, we found an increase in the percentage of the cells arrested in the G1 stage of cell cycle. Hmg20b has been reported as a subunit of BRCA-2 complex in HeLa cells, leading transition from G2 into mitosis (Lee et al., 2011; Marmorstein et al., 2001). In association with KIF4, HMG20B also localizes to mitotic chromosomes (Lee and Kim, 2003). The proteomics data on proerythroblast did not confirm the association of Hmg20b either with BRCA-2 or KIF4. Further fluorescent immunohistochemisrtry analysis of I/11 proerythroblasts did not show the association of Hmg20b with mitotic chromosomes, either.

Hmg20b plays a major role in the completion of cell division (Lee et al., 2011). We did similar observations in erythroblasts: depletion of Hmg20b inhibited cell division but activated genes involved in hemoglobinization.

All these finding suggest a distinct role for Hmg20b in erythroblast proliferation and depletion of Hmg20b results in cell cycle stop and further activation of differentiation. It should still be determined whether Hmg20b carries out this role in the context of the CoREST complex or in association with other molecules.

Transcriptional regulation of hemoglobin switching by the Bcl11a and Klf1 transcription factors

A unique property of erythropoiesis is developmental stage-specific hemoglobin expression. A major distinguishing feature of primitive and definitive erythropoiesis is the type of hemoglobin expressed by the cells. More interesting is the globin switching that happens during definitive erythropoiesis in both human and mouse. Discovering the molecular mechanisms underlying globin switching is clinically important due to the potential therapeutic advantage of fetal globin reactivation in adults with β-globin disorders such as β-thalassemia and sickle cells disease (Miller, 2002; Peterson, 2003; Sankaran et al., 2010; Wilber et al., 2011).

The discovery of BCL11A as the main stage-specific regulator of hemoglobin switching in human and mouse was a major breakthrough in the field (Sankaran et al., 2008; Sankaran et al., 2009). Shortly after this discovery, KLF1 was reported as a regulator of BCL11A (Borg et al., 2010; Zhou et al., 2010). KLF1, an erythroid tissue-specific zinc finger protein, is the major regulator of genes controlling various aspects of erythroid differentiation (Drissen et al., 2005; Hodge et al., 2006; Pilon et al., 2008). Mouse Klf1 null embryos die due to failure of β-globin activation, but this can not be rescued by expression of a γ-globin transgene (Nilson et al., 2006; Perkins et al., 2000); In humans, KLF1 mutations result in a spectrum of erythroid phenotypes (Borg et al., 2011).

Chapter 4 describes a study on the interplay of KLF1 and BCL11A in erythropoiesis and hemoglobin regulation.

KLF1-BCL11A interplay in erythropoiesis

Blood morphology: KLF1 has a well established role in the expression of erythroid membrane and cytoskeleton proteins (Drissen et al., 2005; Heruth et al., 2010; Hodge et al., 2006; Nilson et al., 2006; Perkins et al., 2000; Siatecka and Biek-

er, 2011; Siatecka et al., 2010; Singleton et al., 2008). Therefore, it was not that surprising to find increased numbers of erythrocytes with morphological abnormalities in blood smears of Klf1 haploinsufficient mice. In addition, Bc11a-/- and Klf1$^{+/-}$::Bcl11a$^{-/-}$ animals revealed abnormal red cells pointing to a possible role for Bcl11a in regulating genes involved in erythropoiesis.

Hematological parameters: We found only minor deviations in the hematological parameters of Klf1$^{+/-}$, Bc11a$^{-/-}$ and Klf1$^{+/-}$::Bcl11a$^{-/-}$ animals. In Klf1+/- animals production of erythroid cells is adequate but the maturation of reticulocytes takes more time. Mild reticulocytosis has been observed in the subjects with KLF1 haploinsufficiency (Borg et al., 2010). Bcl11a$^{-/-}$ animals do not display reticulocytosis but have a trend towards lower RBC counts. In the Klf1$^{+/-}$::Bcl11a$^{-/-}$ animals these two traits are exacerbated and the lower RBC counts reach statistical significance. BCL11a deficiency provokes a mild anemia, which is compensated by increased Epo levels. Consistent with this notion, Epo levels are increased in Bc11a$^{-/-}$ and Klf1$^{+/-}$::Bcl11a$^{-/-}$ animals.

In conclusion, these data provide a first analysis of the role played by the KLF1-BCL11A axis in erythropoiesis. The genes that are regulated by the KLF1-BCL11A axis remain to be discovered; this will provide a deeper insight in the molecular regulation of erythropoiesis.

KLF1-BCL11A and hemoglobin regulation

In line with published observations we found that Bcl11a$^{-/-}$ mice fail to repress the endogenous embryonic and human fetal globin genes that are normally silenced at the onset of definitive erythropoiesis in the fetal liver (Sankaran et al., 2009). In combination with Klf1 haploinsufficiency, we observed a further increase in the expression of those genes. This shows that the preferential activation of β-globin expression by KLF1 still occurs in the absence of BCL11A. Interestingly, BCL11A also represses the embryonic γ-globin gene, whilst KLF1 is not essential for high

levels of γ-globin expression. This provides another example of the contrasting mechanisms regulating the α-like and β-like globin loci (Craddock et al., 1995; Garrick et al., 2008).

In conclusion, to achieve efficient silencing of the embryonic globin genes in fetal liver erythropoiesis, an intact KLF1-BCL11A axis is required: KLF1 activates BCL11A expression (Borg et al., 2010; Zhou et al., 2010); BCL11A represses the embryonic globin genes (Sankaran et al., 2009) thereby unleashing the full potential of KLF1 to activate β-globin expression.

Breeding to BCL11A$^{-/-}$ mice rescued the anemia of SCD mice (S.H. Orkin, Gordon research conferences, Red Cell, 2011), suggesting a promising road towards therapeutic intervention in SCD and β-thalassemia patients. However the ubiquitous expression of BCL11A is a major limitation to targeting this molecule in patients. Finding additional genes that are regulated by the KLF1-BCL11A axis may guide towards more specific targets for development of future therapeutic intervention.

Another potentially interesting topic is the identification of the interplay of KLF1-BCL11A and additional factors involved in the silencing mechanism at the adult stage. Such factors may include transcription factors such as MYB (Thein et al., 2007) and SOX6 (Xu et al., 2010), the chromatin-bound FOP/CHTOP protein (van Dijk et al., 2010) and NuRD complex (Gnanapragasam et al., 2011), the orphan nuclear receptors TR2/TR4 (Cui et al., 2011), and the protein arginine methyl transferase PRMT5 (Rank et al., 2010). Furthermore, it is likely that epigenetic mechanisms such as PcG complex recruitment and DNA methylation are also involved. Future work will be aimed at further elucidating the multi-layered repressive network of the embryonic/fetal programme in the adult erythroid environment.

Hydroxyurea therapy of β- thalassemic patients

Hydroxyurea is the first approved drug by the US food and drug administration

(FDA) for treatment of SCD and β-thalassemia (Charache et al., 1995); It ameliorates disease manifestation in ~50% of β-thalassemic cases by increasing HbF levels, thus reducing the need for blood transfusion and hospitalization (Gambari, 2010).

HU is a ribonucleotide reductase inhibitor which inhibits DNA replication (Baliga et al., 2000). It has been proposed that HU activates fetal hemoglobin through activation of the nitric oxide pathway (Cokic et al., 2003) or it might selectively kill proerythroblasts in the bone marrow, except those expressing HbF (Baliga et al., 2000; Bhagavan, 2002). However the details are still unknown. Revealing the molecular mechanisms underlying the response to HU would give a better guide to select patients for HU treatment and avoiding the side effects introduced to the non-responders.

In Chapter 5, we compared the transcriptome of proerythoblasts from patients responding to HU with those from non-responders, before and after administering HU to the cultured cells. HU-responders and non-responders demonstrate strikingly different gene expression patterns. Applying stringent selection criteria yielded a set of differentially regulated genes which could be used to stratify potential responders and non-responders. Comparing the transcriptomes of responders and non-responders before and after HU treatment guides us towards finding the fetal hemoglobin regulatory elements. The results obtained and future perspectives are categorized below.

1-The proliferation rate of proerythroblasts from responders is lower than that of proerythroblasts from non-responders. One of the differentially regulated genes is located in the INK4b-ARF-INK4a locus which is required for the maintenance of stem/progenitor cells populations. Inappropriately reduced expression is often observed in oncogenesis through the aberrant saturation of the INK4b-ARF-INK4a locus with PcG complexes (Aguilo et al., 2011). P15INK4b is a cyclin-dependent kinase inhibitor (CDKi) of cyclin/cdk4 (or cdk6) complexes thus preventing pRB phosphorylation and inhibiting cell cycle progression (Simboeck et al., 2011). Upon addition of HU to the cultures, the expression of p15INK4b is higher in non-re-

sponders than in responders, which might explain the reduced survival of the cells from non-responders after drug treatment. Such molecular details should be studied in more detail in the future.

2-Deregulation of the genes involved in apoptosis and stress response (nitric oxide pathway) including BCL-xL, BCL-6 and ARG-1/-2 and those which are involved in both stress response and normal erythroid differentiation such as FOXO-3 and some of its target genes (HIPK2, BTG1, and p27KIP) (Marinkovic et al., 2007), suggested a possible role of HU in fetal globin activation through different pathways. BCL-6 is important to retain the proliferative capacity of erythroblasts in the spleen (Asari et al., 2005), while BCL-xL, another member of the BCL family, is an anti-apoptotic molecule which is involved in cell survival by downrergulation of acetyl co-enzyme A (Yi et al., 2011). Increased expression of Foxo3a, p27, and Btg1 during normal erythropoiesis induces cell cycle arrest followed by terminal differentiation including overexpression of globin genes (Bakker et al., 2007). Finding the differentially regulated genes involved in these pathways not only can be used a tool to predict HU responders and to avoid the prescription of HU to non-responders, but also for finding a new targets for therapeutic purposes.

3-Other groups of deregulated genes including transcription factors that may regulate the fetal globin genes directly, such as SOX4 and SOX6. Notably, most of the well-known fetal globin regulators, MYB, BCL11A, CHTOP, PRMT5 and IKAROS were not differentially expressed between the responders and non-responders. Since the transcription factors mostly regulate the expression of a various genes, finding the specific targets of those transcription factors is important. It might provide new leads towards specific therapy for the β-thalassemic and SCD patients.

In conclusion, the data obtained from this investigation can be used to expand studies on hemoglobin regulation at the molecular level, and the set of differentially regulated genes between responders and non-responders may be developed into a prognostic test to select patients for HU treatment.

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Summary
(Samenvatting)

Summary

Thalassemia and sickle cell disorders, as the most prevalent monogenic disorders worldwide, necessitate detailed studies on hematopoiesis with special focus on erythropoiesis. In addition, the hematopoietic system provides one of the most attractive systems for investigation of development and differentiation, both at "systems biology" level and at the level of individual molecules. These are the two major reasons to investigate the mechanisms underlying hemoglobin regulation and erythropoiesis. In Chapter 1 (introduction) this is introduced in detail.

Chapter 2 (hematopoietic system); delineates attempts to mimic the hematopoietic system in vitro by combining two well-established biological systems HOXB4 overexpression and Avi-tag biotinylation. HOXB4 was ectopically expressed in an embryonic stem cell line expressing HA-BirA (ES-HA-BirA) from the Rosa26 locus. We found that ES-HA-BirA-HOXB4 cells could be fully differentiated into myeloid lineages including macrophages and granulocytes. Of the granulocytes, the majority were polymorphonuclear neutrophils. Due to the erythroid differentiation blockade caused by expression of HOXB4, we utilized ES-HA-BirA ES cells for direct differentiation into definitive erythrocytes.

Chapter 3 (transcriptional regulation of erythropoiesis); describes the role of the Hmg20b in erythropoiesis as a subunit of the CoREST complex. To study the role of Hmg20b in erythroid differentiation, we performed knockdown experiments followed by microarray analysis in a differentiation-competent mouse cell line. Hmg20b knockdown results in spontaneous differentiation of the $p53^{-/-}$ fetal liver cell line which was further confirmed in primary mouse fetal liver cells. Analyzing the whole genome expression profile, we found that 85% (527 out of 620) of the deregulated genes are upregulated when Hmg20b levels are reduced. We concluded that Hmg20b acts as an inhibitor of erythroid differentiation,through the downregulation of genes involved in differentiation, and activation of repressors of differentiation. In addition, Hmg20b suppresses embryonic beta-like globins.

Chapter 4 (transcriptional regulation of globin expression and erythropoiesis); genome-wide association studies (GWAS) revealed a strong correlation of HbF levels with several SNPs located in the BCL11A gene. BCL11A is regulated by KLF1

and it act as a repressor of γ-globin expression. To study erythropoiesis and globin expression by Bcl11a and its regulator, KLF1, in an in vivo model, we generated the mice carrying a human β-globin locus transgene with combinations of Bcl11a conditional knockout, EpoR-Cre knockin, and Klf1 knockout alleles. Analysis of $Bcl11a^{-/-}$ mutant embryos demonstrated higher expression of mouse embryonic globins. We found a higher percentage of reticulocytes in adult $Klf1^{+/-}$ and $Bcl11a^{-/-}$ mice, which was more pronounced in the compound genotype, $Klf1^{+/-}$::$Bcl11a^{-/-}$. We found that the combination of $Klf1^{+/-}$ with $Bcl11a^{-/-}$ augment γ-globin expression, significantly. Klf1, as a transcription factor, plays a global role in regulating the genes involved in definitive and primitive erythropoiesis. Blood smear analysis of the Klf1 haploinsufficient mice showed abnormalities in cell morphology. This phenotype was stronger in $Klf1^{+/-}$::$Bcl11a^{-/-}$ mice. $Bcl11a^{-/-}$ and $Klf1^{+/-}$::$Bcl11a^{-/-}$ mice had mild anemia which points to a slight defect in erythroid maturation. Collectively, our data support an important role of the Klf1-Bcl11a axis in erythroid maturation and developmental regulation of globin expression.

Chapter 5 (a step forward to the therapeutic application); abnormal β-globin expression in patients with β-thalassemia and sickle cell disease can be compensated by fetal globin expression. Hydoxyurea (HU) is the only FDA approved drug to treat adult patients with the aim to increase γ-globin expression. HU treatment is beneficial to many of the adult patients, but ~50% of the β-thalassemic patients do not respond to this drug. In order to begin to understand this difference, we performed whole genome expression profiling. We defined criteria that could be used to stratify responders and non-responders before they receive HU treatment. In addition, the expression profiles may help to uncover the mechanisms underlying HU treatment, providing scope for the development of novel therapies.

Chapter 6 (discussion); is a general discussion and a passage through all experimental chapters pointing to future perspectives and the questions remaining to be answered.

Samenvatting

Wereldwijd gezien zijn thalassemie en sikkelcelziekte de meest voorkomende ziektes waaraan een enkel defect gen ten grondslag ligt. Daarom zijn gedetailleerde studies naar bloedcelvorming nodig, in het bijzonder naar erythropoiese. Verder vormt het hematopoietisch systeem één van de meest aantrekkelijke systemen om ontwikkeling en differentiatie te bestuderen, zowel op het niveau van "systems biology", als van individuele moleculen. Dit zijn de twee belangrijkste redenen om de mechanismen van hemoglobine regulatie en erythropoiese te bestuderen. In Hoofdstuk 1 (Introductie) worden deze onderwerpen nauwgezet geïntroduceerd.

Hoofdstuk 2 (het hematopoietisch systeem); beschrijft de poging het hematopoietisch systeem na te bootsen door twee gevestigde biologische systemen te combineren: de overexpressie van HOXB4 en de biotinylering van een Avi-tag. HOXB4 werd ectopisch tot expressie gebracht in embryonale stamcellen die ook HA-BirA tot expressie brengen vanuit het Rosa26 locus (ES-HA-BirA). We konden de ES-HA-BirA cellen laten differentiëren tot myeloïde cellen, vooral macrofagen en granulocyten. Van de laatste groep waren de meeste cellen polymorphonucleaire neutrofielen. Omdat hogere HOXB4 expressie de erythroïde differentiatie bleek te remmen, hebben we ES-HA-BirA cellen met een versneld protocol laten differentiëren tot rode bloedcellen.

Hoofdstuk 3 (de transcriptionele regulatie van erythropoiese); beschrijft de rol van Hmg20b, een component van het CoREST complex, in erythropoiese. Om de rol van dit eiwit op de differentiatie te bestuderen, hebben we knockdown - en microarray experimenten gedaan in een erythroïde muizencellijn die terminale differentiatie kan ondergaan. De knockdown van Hmg20b leidde tot spontane differentiatie van de cellen, een resultaat dat bevestigd kon worden in primaire erythroblasten gegroeid uit de foetale lever. De analyse van de microarray data liet zien dat de expressie van 85% (527 van de 620) van de gedereguleerde genen omhoog gaat bij verlaagde Hmg20b expressie. We concludeerden dat Hmg20b de spontane differentiatie van erythroïde voorlopers remt door de genen te remmen die belangrijk zijn voor differentiatie.

Hoofdstuk 4 (de transcriptionele regulatie van globine regulatie en erythropoiese);

genoom-wijde associatie studies (GWAS) laten een sterke correlatie zien tussen HbF levels met een aantal SNP's in het BCL11A gen. BCL11A blijkt inderdaad een remmer van γ-globine expressie te zijn, terwijl het zelf wordt gereguleerd door KLF1. Om de rol van BCL11A en KLF1 op erythropoiese en globine expressie in vivo te bestuderen, hebben we muizen met een humaan β-globine locus gecombineerd met de conditionele Bcl11a knockout, de EpoR-Cre knockin en de Klf1 knockout. In de Bcl11a$^{-/-}$ embryo's bleek de expressie van de embryonale globines (muis) verhoogd. We vonden een hoger percentage reticulocyten in volwassen Klf1$^{+/-}$ en Bcl11a$^{-/-}$ muizen, en een nog sterker effect in de compound heterozygoten. De combinatie van Klf1$^{+/-}$ met Bcl11a$^{-/-}$ verhoogde ook de expressie van γ-globine. De transcriptie factor Klf1 speelt een belangrijke rol in de regulatie van genen die essentieel zijn voor primitieve en definitieve erythropoiese. Dit bleek onder meer uit de abnormale morfologie van rode bloedcellen in Klf1 haploinsufficiënte muizen. Dit fenotype was sterker in Klf1$^{+/-}$::Bcl11a$_{-/-}$ muizen. Bcl11a$^{-/-}$ en Klf1$^{+/-}$::Bcl11a$^{-/-}$ muizen hadden een milde anemie, iets dat wijst op een defect in de uitrijping van erythroïde voorlopers.

Hoofdstuk 5 (een stap voorwaarts naar een therapeutische toepassing); abnormale β-globine expressie in patiënten met β-thalassemie en sikkelcelziekte kan worden gecompenseerd door verhoogde γ-globine expressie. Hydroxyureum (HU) is momenteel het enige door de FDA goedgekeurde medicijn om patiënten te behandelen met het doel de expressie van γ-globine te verhogen. Dit werkt voor een deel van de patiënten, maar ~50% reageert niet op de behandeling. Om een verklaring voor dit verschil te vinden, hebben we de genexpressie profielen van 'responders' en 'non-responders' bepaald en vergeleken. De uitkomsten hiervan kunnen ook gebruikt worden om 'responders' en 'non-responders' te herkennen voordat met de HU-behandeling wordt gestart.

Hoofdstuk 6 (discussie); is een algemene discussie waarin alle experimentele hoofdstukken worden behandeld. Hierbij wordt stil gestaan bij de vooruitzichten van de relevante onderzoeksvelden en worden niet-beantwoorde – en nieuwe vragen benoemd.

Curriculum Vitae

NAME
Fatemehsadat (Sahar) Esteghamat Hanzaei

DATE AND PLACE OF BIRTH
July 03, 1981– Tehran, Iran

EDUCATION

- PhD, Department of Cell Biology, Erasmus MC, (25th Oct 2007–20th Oct 2011)
- MSc in Cellular and Molecular biology (Oct 2002 – Mar 2005), Khatam Institute for Higher Education, Tehran-Iran, Average=17.6/20
- BSc in Botany (Oct 1998 – Jul 2002), Shahid Beheshti University, Tehran – Iran, Average=17.06/20

PROFESSIONAL EXPERIENCE

- Research Assistant in Research Centre for Gastroenterology and Liver Disease (RCGLD), Shahid Beheshti University of Medical Sciences (Prof. Dr. Mohammad Reza Zali)
  - Research Assistant in the group of Liver (Oct 2002 – May2005)
    - The role of SNPs in CTLA4 and VDR SNPs in Autoimmune hepatitis
    - Screening the HCV patients for occult hepatitis B
  - Research Assistant in the group of Cancer (Oct 2006 – Oct 2007)
    - The association between COX2 SNPs and colorectal carcinom
- Research Assistant in Clinical Genetics (May 2005– Oct 2007) (Prof. Hossein Najmabadi)
    - The association between mutation in different loci and Hearing loss.
    - Molecular analysis of globin regulation

HONORS

- MSc
  - Best oral presentation award; C77G mutation in protein tyrosine phosphatase CD45 gene and autoimmune hepatitis. Esteghamat F, et al. 2nd international congress From Bed to Bench in Gastroenterology and Liver Diseases, Tehran, Iran, 2004.
  - Student award for: first Author in C77G mutation in protein tyrosine phosphatase CD45 gene and autoimmune hepatitis.

HOBBY

- Playing Santoor (a Persian traditional musical instrument)

Publication

- Esteghamat F, Gillemans N, van Gent T, van Lom K, Bilic I, Klingmüller U, Busslinger M, van Dijk TB, Grosveld F, Philipsen S. Erythropoiesis and globin switching in compound Klf1-Bcl11a mutant mice. (Manuscript in preparation).
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PhD Portfolio

Summary of PhD training and teaching

Name PhD student: Fatemehsadat (Sahar) Esteghamat Hanzaei Erasmus MC Department: Cell Biology Research School: MGC graduate school	PhD period: 25th Oct 2007-20th Oct 2011 Promotor(s): Prof.dr. Sjaak Philipsen Prof.dr. Frank Grosveld Supervisor: Dr. Thamar van Dijk

1. PhD training

	Year	Workload (Hours)
General courses		
- Course on laboratory animal science (Art9)	2007	108
- Experimental approach to molecular and cell Biology	2008	168
- Introduction to Data-analysis (ESP03)	2009	70
- Safety working in the laboratory	2009	8
- Epigenetic regulation	2010	16
- EuTRACC proteomics Course	2010	18
- Biomedical writing in English	2010	84
- Development, Stem Cell Disease	2011	32
Specific courses (e.g. Research school, Medical Training)		
- 7th Winter School of the International Graduiertenkolleg GRK767 "Transcriptional Control in Developmental Processes" Kleinwalsertal, Germany (Oral presentation)	2009	72
- 1st Winter School of the Collaborative Research Centre TRR81, "Chromatin Changes in Differentiation and Malignancies" Kleinwalsertal, Germany (Oral presentation)	2011	72
Seminars and workshops		
- MGC PhD Workshop (Oral presentation)	2009	32
- The 19th MGC-Symposium, Rotterdam, the Netherlands	2009	12
- Workshop on basic data analysis on gene expression Array IV	2010	32
- Browsing genes and genomes with Ensemble workshop	2010	16
- MGC PhD Workshop	2010	32
- Photoshop and Illustrator CS5 Workshop	2010	8
- Workshop Writing Successful Grant Proposals	2010	8
- The 20th MGC-Symposium, Leiden, the Netherlands	2010	12
- Workshop on InDesign CS5	2011	4
- The 21st MGC-Symposium, Leiden, the Netherlands	2011	12
- BIOBASE hands-on training course on: Functional Annotation of Experimental Data (including human NGS data) using TRANSFAC® Professional, HGMD® Professional, and Genome TraxTM	2011	12
- Erasmus lectures in cell biology and development	2007-2011	60
- Erasmus lectures on stem cell and regenerative medicine	2010-2011	45
- Monday Morning Meetings	2007-2011	176
Presentations		
- Monday Morning Meetings	2007-2011	320
- Work discussion	2007-2011	240
International conferences		
- EUrythron annual meeting, Groningen, the Netherlands	2008	24

- ISSCR and NIRM meeting, Amsterdam, the Netherlnads	2011	8
Oral presentation		
- EUrythron annual meeting, Lisbon, Portugal	2009	64
- 5th Dutch hematology meeting, Arnhem, the Netherlands	2011	48
Poster presentation		
- Hemoglobin Switching meeting, Asilomar, CA, USA	2008	80
- Hemoglobin switching meeting, Oxford, UK	2010	80
- Gordon research conferences, Red cells, Andover, NH, USA	2011	88
- Chromatin changes in differentiation and malignancies, Giessen, Germany	2011	56
2. Teaching	**Year**	**Workload (Hours)**
Supervising practicals and excursions, Tutoring		
- High school students	2010	40
Other		
- PhD students	2011	100

I could not have enjoyed being in the Netherlands without accompany of my fellow-travellers throughout this journey of 4-year.

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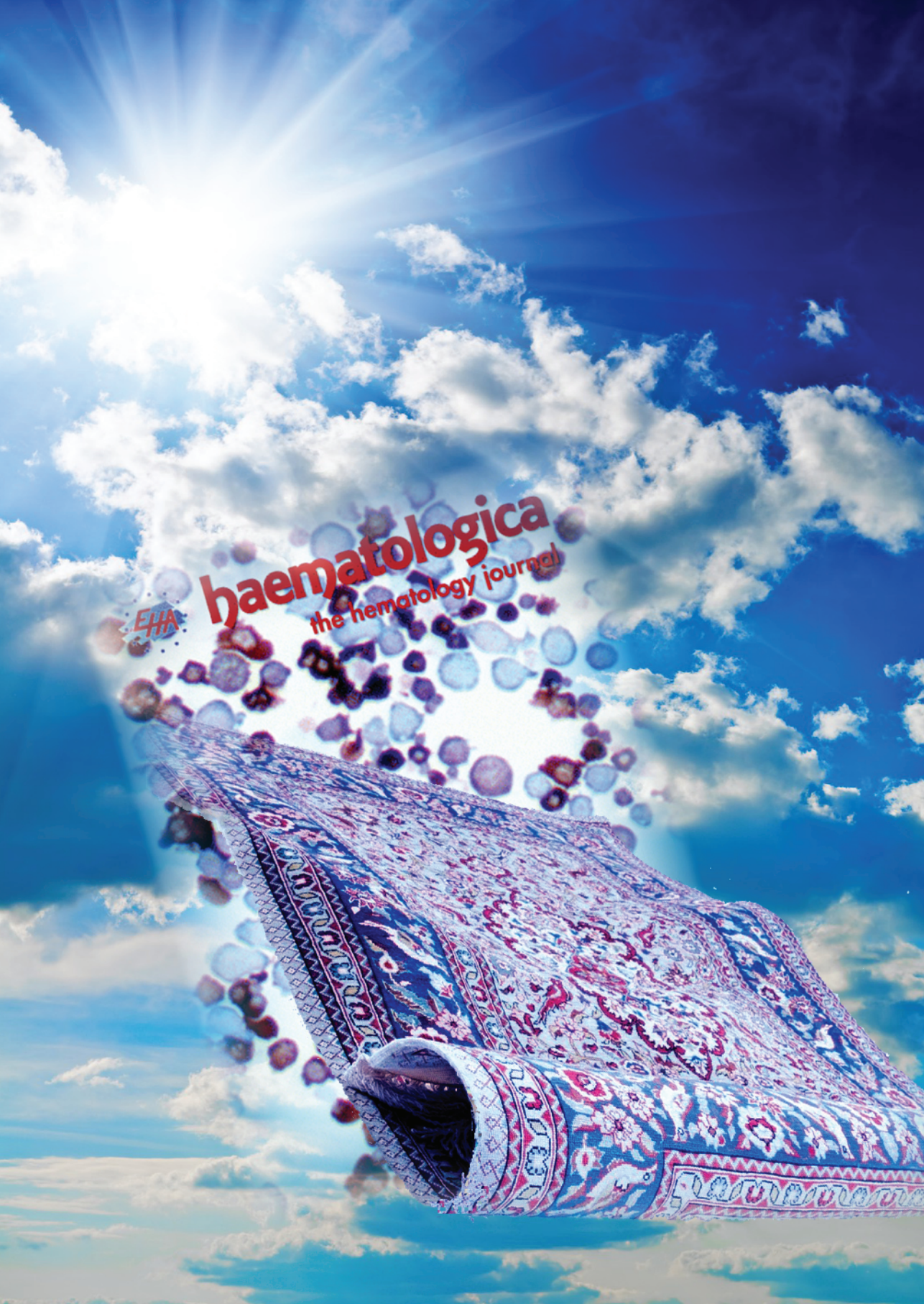
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