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Over-expression of AHSP in bone marrow-derived $CD34^+$ cells is not effective in ameliorating the thalassemic phenotype

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Chapter 1

GENERAL INTRODUCTION

1. HEMATOPOIESIS

Hematopoiesis is a continuous process in which stem/progenitor cells develop into mature blood cells. Hematopoietic stem cells (HSCs) are self-renewing, multipotent progenitors that give rise to all types of mature blood cells. HSCs are thought to reside in a quiescent state in specialized stromal cells containing niches (Wilson et al., 2007). Through mechanisms that are only partially understood, the integrity of stem cells is maintained throughout life. Hematopoietic cells can be broadly classified as transiting through three compartments: (1) stem cell compartment, (2) committed progenitor cells, and (3) mature functional-end cells (Fig. 1.1). The cells in each succeeding compartment are the progeny of, and more numerous than, the cells of the preceding compartment.

The stem cell compartment is composed of very rare cells with specific properties mainly they maintain competence to self-renew and to generate progenitors capable of making billions of blood cells each day (Wilson and Trumpp, 2006). Several physical characteristics have been ascribed to stem cells, and these have aided both their purification and the assay used to define them in different transplantation systems (Morrison et al., 1995). Lineage markers are absent from these cells, and they normally are found in a quiescent state or are turning over very slowly. Stem cells are also equipped with a regimen of critical transcription factors that are important in the execution of their fundamental cellular functions of cell renewal and multilineage differentiation.

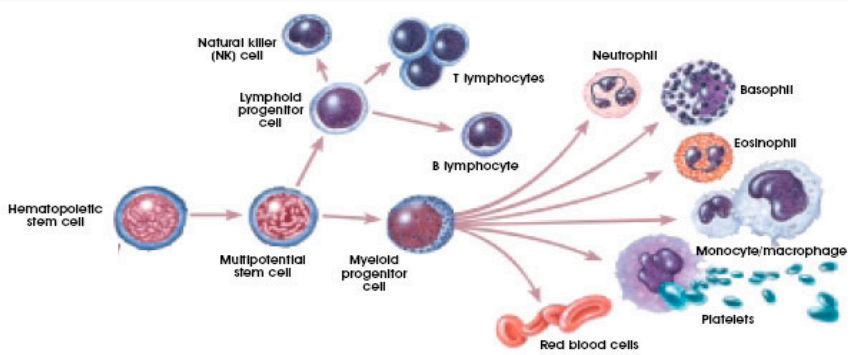

Figure 1.1. Development of mature blood cells through the sequential restriction of the cell fate potential of oligopotent progenitor cells derived from multipotent HSC.

The progenitor cell compartment contains cells that are found at a higher frequency than the stem cell pool and, like the stem cell, are not morphologically distinguishable. Their existence is revealed by their ability to give rise to differentiated progeny *in vitro* in well-defined functional assays. The progenitor cell compartment is derived from stem cells trough a process of commitment to different lineage pathways. Transition of stem cells to cells of the committed compartment is achieved not by acquisition of new characteristics or new proteins but by enhancement of certain molecular pathways, already primed in these cells, and abrogation of others (Hu et al., 1997). Within each lineage, a spectrum of progenitor cells exists, and these are hierarchically categorized on the basis of their proliferative potential, maturation time,

response to a set of cytokines and type of differentiated progeny as revealed through *in vitro* clonogenic cultures. For example, multilineage progenitors giving rise to multiple lineages in vitro (e. g., colony-forming unit- granulocytic, erythroid, eosinophil, macrophage, megakariocyte [CFU-GEMM]) are presumed to be more primitive than unilineage progenitors committed only to a single lineage. As progenitor cells differentiate, they acquire more distinctive features characteristic of each lineage and move away from shared primitive progenitor characteristics: they show the enhancement of lineage-specific features, with a diminished or absence of expression of multilineage properties. Progenitor cells of each lineage are internally programmed to ensure their own survival, but external cues are frequently impinged on these internal program that control differentiation, maturation, survival and migration (Wagers et al., 2002).

The precursor cell compartment, in contrast to stem and progenitor compartments, is defined by morphological criteria and contains cells at different maturation stages; the cells may be capable of undergoing a few rounds of cell division, or they may be end-stage, non-mitotic cells with a finite life span. The morphological characteristics of these cells reflect the accumulation of lineage-specific proteins, and organelles and the decline of nuclear activity, which gives them a unique appearance. Furthermore, precursor cells for each lineage follow a unique maturation sequence; for example, in erythroid cells, the end product is an enucleated red cell, as the nuclei are expelled before terminal maturation. By contrast, terminally mature white cells remain nucleated. Also, cells of megakaryocytic

lineage undergo unique endoreduplication cycles, forming large cells with multilobulated nuclei. The maturation sequence for each lineage requires a specific time frame, but there is enough plasticity to allow for faster than normal production of end-stage effector cells. Such deviations from the normal sequence are dictated by stress, which demands quick delivery of specialized (mainly white) cells into the periphery.

1.1 The Hematopoietic Stem Cell

Hematopoietic Stem Cells (HSCs) are probably the best characterized stem cell population. The properties that define these cells are the capacity to generate differentiated progeny of multiple blood cell lineages and the potential to produce more stem cells through a process known as self-renewal. It is this capacity to self-renew that enables the stem cell population to sustain hematopoiesis over extended periods of time (perhaps indefinitely) *in vivo* and, in fact, distinguishes them from virtually all other cells within the hematopoietic system (Jordan and Lemischka, 1990; Keller and Snodgrass, 1990; Morrison et al., 1996). The theory of HSCs was first put forward in 1964 (Till and McCulloch, 1964), but to date it has not been comprehensively identified and isolated. Indeed, the only means of assaying stem cells is by their ability to reconstitute the bone marrow of an irradiated host and give rise to the hematopoietic system (radioprotection assay). This has been demonstrated primarily in humans undergoing bone marrow transplantation and also in experimental animal models, where the endogenous hematopoietic system was ablated by a lethal dose of

radiation (Aguila et al., 1997). The frequency of HSCs in the bone marrow is argued to be $1x10^{-4}$ to $1x10^{-5}$ (Murray et al., 1994). Due to its rarity, a robust effort has been dedicated to enrich HSCs to allow further characterization studies. HSCs have been enriched using a variety of techniques, including density centrifugation, activation and/or cell-cycle status and surface antigen expression, but no unique characteristics have been found to identify these elusive cells specifically.

Although $CD34^+$ cells represent a large section of the hematopoietic microenvironment, they have been demonstrated to possess reconstitution potential (Peault et al., 1991). $CD34^+$ cells injected into a SCID-hu (thymus model) mouse gives rise to engraftment and generation of CD3, CD4, CD8 and T cells and they are routinely used for humans, thus confirming their potential.

$CD34^+$ $Thy\text{-}1^+$ Lin^-, $CD34^+$ $Thy\text{-}1^+$, $CD34^+$ HLA-DR- and $CD34^+$ $CD38^+$ $CD33^+$ cells have all been demonstrated to reconstitute mice and sheep. The $CD34^+$/HLA-DR^- cell population was demonstrated to reconstitute sheep, giving rise to virtually all the hematopoietic lineages over at least 7 months. The $CD34^+$/$Thy\text{-}1^+$ cell population is routinely used for SCID and SCID-hu experiments, giving rise to both myeloid and B-lymphoid cells. The $CD34^+$ $CD38^+$ $CD33^+$ cell population has obtained popularity for reliably reconstituting NOD/SCID mice (Larochelle et al., 1996). However, all of these phenotypes are rare, and it is difficult to plan extensive experiments employing these cell types, due to the difficulty and low efficiency in obtaining them.

The most widely used phenotype for animal experiments, believed to represent the repopulating stem cell, is the $CD34^{+}/CD38^{-}$ cell, which has been used extensively for the reconstitution of mice (Verfaillie et al., 2002).

1. 2 Molecular control of Hematopoiesis

The complex orchestration of hematopoiesis to produce the elaborate array of blood cells requires three physiological components, each of which is essential. These are: (1) the stem cell pool itself; (2) hematopoietic cytokines, that regulate hematopoiesis through both endocrine and paracrine mechanisms; and (3) the hematopoietic inductive microenvironment, which is made up of the bone marrow stroma and vasculature. In addition to these extracellular cues, it is evident that critical steps in the control of HSC fate, from the very earliest swiching of mesenchymal cells to the hematopoietic lineage to differentiation into the various lineages, are transcriptionally regulated. Most studies of HSC fate determination focus on the choice between stem cell self-renewal or differentiation. Over the years, several models have been advanced proposing that hematopoietic lineage determination is driven extrinsically (through growth factors, stroma or other external influences), intrinsically (as described in stochastic models), or both. While several studies support an instructive role for cytokines in the maturation of terminally differentiated cells from progenitor and precursor cells (Kaushansky et al., 1995; Kondo et al., 2000; Metcalf and Burgess, 1982) the initial divisions of HSC are likely to involve stochastic fate choices (Ogawa,

1993; Ogawa et al., 1999). In fact, it has been postulated that stochastic determination of HSC cell fate may be important for HSC function, in that this scheme allows for the maintenance of production of all blood cell lineages even in the face of substantial demand for one particular lineage (Enver and Greaves, 1998). Moreover, it appears that the regulation of hematopoiesis is the result of multiple processes involving cell-cell and cell-extracellular matrix interactions, the action of specific growth factors and other cytokines, as well as intrinsic modulators of hematopoietic development.

1.2.1 Role of the microenvironment in HSC fate determination

In nature HSC are located mainly in the bone marrow where they interact within a specific microenvironment called the stem cell niche, which regulates their fate in terms of quiescence, self-renewal and differentiation (Calvi et al., 2003; Scadden, 2006). Recent data suggest that quiescent HSC are located in the trabecular endosteum (the osteoblastic niche) whereas dividing ones reside in sinusoidal perivascular areas (the vascular niche) of the bone marrow (Kiel and Morrison, 2006) (Fig. 1.2). An orchestra of signals mediated by soluble factors and/or cell-to-cell contact regulates the balance and homeostasis of self-renewal, proliferation, and differentiation in vivo (Blank et al., 2008) by determinig cell cycle status and gene expression profile. This complexity is due in large part to the fact that HSCs do not grow as independent autonomous units. Rather, these cells are surrounded in all dimensions by the marrow microenvironment, the so-called “stem cell

niche" (Fig. 1.2). The hypothetical function of the HSC niche is to provide HSC survival and self-renewal factors, either through direct contact with HSC or through secreted factors. HSC niches are formed from a subset of mesenchymal cells within the bone marrow. These so-called "stromal cells", which include specialized fibroblasts, endothelial cells, osteoblasts, and perhaps adipocytes, may play a further role in "translating" external signals to influence HSC developmental decisions (Deryugina et al., 1995) (Fig. 1.2).

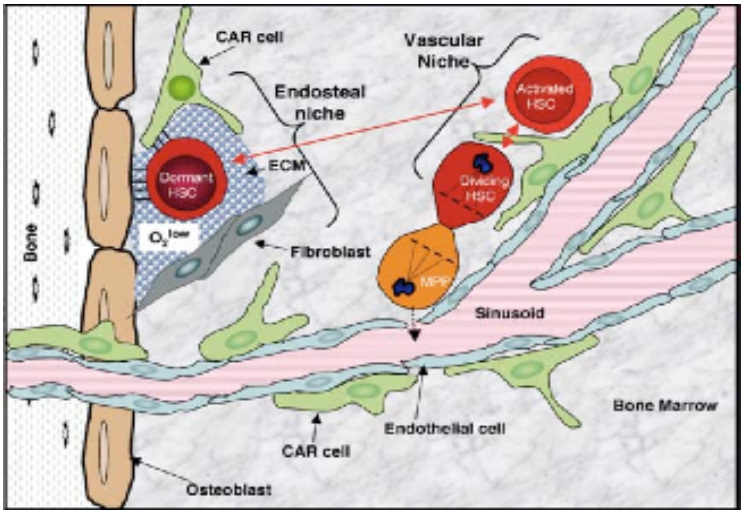

Figure 1.2. Dormant and self-renewal HSCs in their specif microenvironments called "stem-cell niche".

HSC are exposed *in situ* to many different growth factors, some soluble, some bound to extracellular matrix (ECM) and others bound to adjacent

cells. Due to the inherent difficulty in studying these cells in their microenvironment, most of what we know about how different soluble growth factors control HSC fate has been deduced from *in vitro* studies (Ema et al., 2000; Goff et al., 1998). The primary question that has been addressed is whether we can simulate *in vitro* the conditions that tell the HSC to self-renew. Unfortunately, the expansion of HSC in vitro is difficult to achieve because as the cells proliferate they tend to differentiate (Sorrentino, 2004). This is presumably caused by a lack of appropriate cues that are provided in vivo by the microenvironment.

Within the context of a supportive microenvironment, cell adhesion and extracellular matrix molecules also play an important role in HSC development. Within the adult bone marrow, these molecules mediate interactions between stromal and hematopoietic cell components that in turn regulate cell proliferation, differentiation and migration. Extracellular matrix (ECM) is composed of three major classes of molecules: structural proteins such as collagen and elastin, specialized proteins such as fibronectin and laminin, and proteoglycans which consist of a protein core to which is attached long chains of repeating disaccharide units, termed glycosaminoglycans (GAGs). The ECM acts in concert with cell-cell interactions and soluble factors to regulate the HSC. In general, adhesion of hematopoietic stem and progenitor cells to the marrow ECM inhibits cellular proliferation and prevents apoptosis, both of which lead to long-term survival of quiescent hematopoietic stem cells. Clues are beginning to emerge regarding how these effects are mediated. For example, binding of integrins on hematopoietic stem or progenitor cells

has been shown to lead to increased p27 expression, and p27 halts progression of the cell cycle by inhibiting a cyclin dependent kinase (Cheng et al., 2000).

1.2.2 Transcriptional regulation of HSC fate

Transcription factors represent a nodal point of hematopoietic control through the integration of the various signaling pathways and subsequent modulation of the transcriptional machinery. Transcription factors can act both positively and negatively to regulate the expression of a wide range of hematopoiesis-relevant genes including growth factors and their receptors, other transcription factors, as well as various molecules important for the function of developing cells. It is the alternative expression of specific combinations of transcription factors that determines the survival, proliferation, commitment, and differentiation responses of hematopoietic progenitors to such signals, whether they arise from extrinsic or intrinsic regulatory factors. Increasing evidence suggests that different families of transcription factors regulate the developmental program of stem cells (Shivdasani and Orkin, 1996) and when their expression is disrupted, leukaemic proliferation is initiated (Rabbitts, 1994).

Since purifying and biochemically investigating very rare HSC populations is so difficult, knowledge of the role of specific transcription factors in HSC fate decisions has been derived largely from genetic strategies, primarily gene-targeting (knockout) and retroviral infection/overexpression experiments. From this growing body of

literature, several transcription factors have been found to play critical roles in HSC physiology, and some of them are described in the sections below depending on the time and the influence of their expression during different steps of HSC development.

2. THE ERYTHROPOIESIS

Erythropoiesis is the process by wich mature red blood cells (erythrocytes) differentiate from hematopoietic cells occurring in the bone marrow and provides aboutb 2 x 10^{11} new erythrocytes daily to replace the 1% of old cells removed from the circulation.

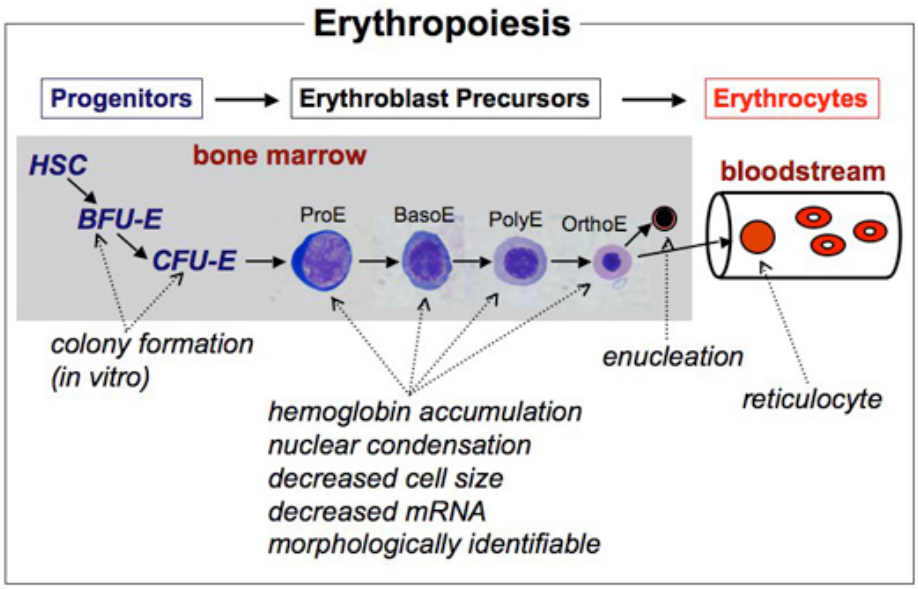

Figure 2.1 : ERYTHROPOIESIS. Erythroid cells begin as pluripotential stem cells. Cell that is recognizable as specifically leading down the red cell pathway is the proerythroblast. As development progresses, the nucleus becomes somewhat smaller and the cytoplasm becomes more basophilic, due to the presence of ribosomes. In this stage the cell is called a basophilic erythroblast. The cell will continue to become smaller throughout development. As the cell begins to produce hemoglobin, the cytoplasm attracts both basic and eosin stains, and is called apolychromatophilic erythroblast. The cytoplasm eventually becomes more eosinophilic, and the cell is called an orthochromatic erythroblast. This orthochromatic erythroblast will then extrude its nucleus and enter the circulation as a reticulocyte. Reticulocytes are so named because these cells contain reticular networks of polyribosomes. As reticulocytes loose their polyribosomes they become mature red blood cells.

Like all blood cells, erythroid cells begin as pluripotential stem cells. The earliest erythroid progenitor, the BFU-E (burst forming unit-erythroid), is small and without distinguishing histologic characteristics. BFU-Es express the cell surface antigen, CD34, as do all other early hematopoietic progenitors allowing for its isolation using anti-CD34 antibodies. The stage after the BFU-E, right before hemoglobin production begins is the CFU-E (colony forming unit-erythroid), which is larger than the BFU-E.

The first cell that is well recognizable as specifically leading down the red cell pathway is the proerythroblast characterized by the presence of a euchromatic nucleus and visible nucleoli. As development progresses, the nucleus becomes somewhat smaller, due to the progressive chromatin condensation and the cytoplasm becomes more basophilic, due to the presence of ribosomes. In this stage the cell is called a basophilic erythroblast. The cell will continue to become smaller throughout development. As the cell begins to produce hemoglobin, the cytoplasm attracts both basic and eosin stains, and is called a polychromatophilic erythroblast. The cytoplasm eventually becomes more eosinophilic, and the cell is called an orthochromatic erythroblast. This orthochromatic erythroblast will then extrude its nucleus and enter the circulation as a reticulocyte. Reticulocytes are so named because these cells contain reticular networks of polyribosomes. As reticulocytes lose their polyribosomes they become mature red blood cells (Fig. 2.1).

2.1 THE ERYTHROBLASTIC ISLAND

Erythroblastic islands are specialized niches composed of erythroblasts surrounding a central macrophage niches in which erythroid precursors proliferate, differentiate, and enucleate (Chasis and Mohandas, 2008) (Fig. 2.2). For several decades the importance of erythroblastic islands remained unrecognized as erythroid progenitors were shown to possess an autonomous differentiation program with a capacity to complete terminal differentiation in vitro in the presence of erythropoietin but without macrophages. However, as the extent of proliferation, differentiation, and enucleation efficiency documented in vivo could not be recapitulated in vitro, a resurgence of interest in erythroid niches has emerged. We now have an increased molecular understanding of processes operating within erythroid niches, including cell-cell and cell-extracellular matrix adhesion, positive and negative regulatory feedback, and central macrophage function.

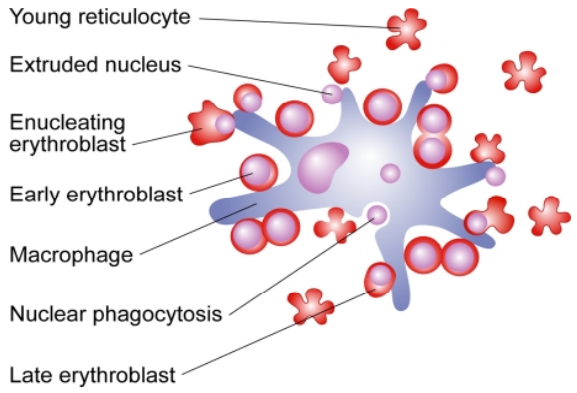

Figure 2.2. An erythroblastic island with a centered macrophage. There is evidence of large areas of closely opposed erythroblat and macrophage membranes. At the conclusion of terminal differentiation, expelled nuclei were phagocytosed by the central macrophage.

Bone marrow contains a heterogeneous population of monocytes/macrophages at various differentiation stages with diverse phenotypes. Island macrophage, derived from monocyte precursors, are likely to be a subset of resident macrophages in hematopoietic tissue. Central macrophages harvested from human bone marrow islands express CD4, CD11a, CD11c, CD18, CD31, HLA-DR and FcRI, FcRII, FcRIII (Lee et al., 1988).

During definitive erythropoiesis, erythroblasts are known to express a diverse array of adhesion molecules that undergo dynamic variation during differentiation. These proteins mediate both erythroblast/erythroblast and erythroblast/macrophage interactions, as well as attachments to extracellular matrix components such as fibronectin and laminin. Historically, Emp (erythroblast macrophage protein) was the first molecule identified in both erythroblast and macrophage membranes that appears capable of forming macrophage/erythroblast attachments via homophilic binding (Hanspal and Hanspal, 1994).

The second receptor/counterreceptor identified as mediating cell-cell interactions within erythroid islands were $\alpha 4 \beta 1$ integrin in erythroblasts and VCAM-1 in central macrophages (Sadahira et al., 1995). More recently are discovered another set of adhesion molecules that contribute to island integrity; erythroid intercellular adhesion molecule-4 (ICAM-4) and macrophage αV integrin. ICAM-4S, a novel secreted isoform of

mouse ICAM-4, is up-regulated late in terminal differentiation (Lee and Juliano, 2004). A currently postulated function for ICAM-4S is to enable young reticulocytes to detach from central macrophages, thereby allowing them to enter the circulation.

A number of additional macrophage surface proteins have been identified as receptors for erythroblasts, although their counterreceptors remain uncharacterized and future studies will identify additional attachments between cells within erythroid islands.

A protective macrophage function intimately tied to phagocytosis of extruded nuclei has only recently been delineated; in mice DNase II in macrophages degrades the ingested nuclear DNA (Kawane et al., 2001). Another important role originally proposed for central macrophages is transfer of iron directly to attached erythroid progenitor (Bessis and Breton-Gorius, 1962). A very recent report suggests ferritin is synthesized by macrophages, secreted via exocytosis, and subsequently taken up by erythroblasts. After endocytosis, iron, released from ferritin by acidification and proteolysis, is used by erythroblasts for heme synthesis (Leimberg et al., 2008). Although the attractive hypothesis of transfer of iron from central macrophages to erythroblasts has not yet been conclusively proven, with the exciting advances in our knowledge of iron metabolism and iron transporters answers should soon be forthcoming.

Abnormalities in macrophage differentiation can lead to perturbations in the function of the erythroid niche. One important factor regulating macrophage differentiation is retinoblastoma tumor suppressor (Rb) protein (Iavarone et al., 2004) a nuclear protein that functions in

regulating the G1-to-S-phase transition of the cell cycle. A second protein affecting central macrophage function is cytoskeletal-associated protein paladin (Liu et al., 2007). This protein, localizing in focal adhesions of stress fibers along with alpha-actin, regulates actin cytoskeleton dynamics and cell attachment to extracellular matrix.

2.1.1 Regulatory factors in erythroid niche

Erythropoiesis is driven by the balance between positive and negative feedback mechanisms operating within the island niche involving both cell-cell interactions and soluble factors. The concentration of Epo is a predominant regulator of erythroid progenitor numbers by preventing apoptosis of cells in the colony-forming unit erythroid (CFU-E)/proerythroblast stages of differentiation (Koury and Bondurant, 1990). However, cell-cell interactions within islands also play crucial roles. Indeed, erythroblasts interacting with one another function to regulate erythroid lineage output. In a study of human erythropoiesis, Fas/Fas ligand binding appears to contribute to regulation of apoptosis of immature erythroblasts (De Maria et al., 1999a). Although Fas is expressed on human erythroblasts at all stages of terminal differentiation, only in immature erythroblasts does Fas crosslinking transduce a death signal. Fas ligand is not expressed until late in differentiation and orthochromatic erythroblasts demonstrate a Fas-based cytotoxicity against immature erythroblasts. However, in vitro, increased Epo consistent with levels measured in anemia, provides protection to early erythroblasts from Fas-induced cytotoxicity by late erythroblasts. By enabling more

immature erythroblasts to terminally differentiate, this mechanism effectively up-regulates erythropoiesis in the presence of elevated Epo. Moreover, there is a role for Fas/Fas ligand binding as an active regulator of apoptosis within erythroblastic islands (Niho and Asano, 1998).

Erythroid progenitor can be regulated not only by protection from apoptosis but also by a direct contact that established with macrophages (cell-cell interactions) that is important to enhance erythroblast proliferation (Rhodes et al., 2008).

Two potential candidate interactions have been identified as enhancing erythroblast proliferation at the CFU-E stage. Macrophage membrane protein Ephrin-2 (HTK ligand) interacting with its erythroid receptor EphB4 (HTK)54,55 and the c-kit ligand transmembrane protein binding c-kit on erythroid progenitor (Muta and Krantz, 1995).

Erythroblast-erythroblast attachments also regulate cell proliferation. It has long been recognized that the level of transcription factor GATA-1 is crucial to completion of normal differentiation (Gutierrez et al., 2004) and its activity and hence, gene expression, are regulated by intercellular signaling between erythroblasts.

Soluble factors secreted by macrophages add another layer of positive regulation within erythroblastic islands. Macrophage cytokines, including burst-promoting activity and insulin-like growth factor-1, induce growth of both burst forming unit-erythroid (BFU-E) and CFU-E (Kurtz et al., 1985; Sawada et al., 1989).

Soluble factors secreted by erythroblasts also have relevant functions within the erythroid niche. A very recent discovery is that Gas6, a

secreted protein that enhances proliferation and survival in nonerythroid cells, is released by erythroblasts in response to Epo. Secreted Gas6 binds its erythroblast receptor, and by activating PI3K and its effector Akt, enhances Epo receptor signaling (Angelillo-Scherrer et al., 2008). Differentiating erythroblasts also secrete angiogenic factors, vascular endothelial growth factor A (VEGF-A) and placenta growth factor (PIGF) (Tordjman et al., 2001). Erythroblasts have no receptors for VEGF-A or PIGF, hence these secreted angiogenic factors may serve as paracrine effectors, mediating crosstalk between receptor-expressing macrophages and developing erythroblasts that regulates island structure and/or localization. In addition, by affecting endothelial cell junctional integrity these secreted proteins may facilitate reticulocyte movement into bone marrow sinusoids.

Within erythroid niche there are elevated levels of circulating cytokines, chemokines, and interleukins, including interleukin 6 (IL6), transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ) because of direct secretion by central macrophages (Fig. 2.3). TNF-α inhibits erythropoiesis either by caspase-mediated cleavage of the major erythroid transcription factor GATA-1 resulting in apoptosis (De Maria et al., 1999b) and/or by retarding proliferation (Dai et al., 2003); the precise mechanism(s) remains somewhat controversial. TGF-β also perturbs erythropoiesis in various ways. It inhibits erythroblast proliferation by reducing the number of progenitors in cycle via a mechanism other than apoptosis and accelerates

differentiation of noncycling erythroid progenitors (Zermati et al., 2000). TGF-β triggers activation of Rho and Rac GTPases and could have effects on various cytoskeletal functions including membrane stability. Elevated levels of INF-γ induce both macrophages and erythroblasts to secrete soluble TRAIL (TNF-related apoptosis inducing ligand), which inhibits erythroblast differentiation (Zamai et al., 2000) mediated by activation of the intracellular ERK/MAPK pathway (Secchiero et al., 2004). Finally, IL6 up-regulates hepcidin expression, which inhibits iron export from macrophages by binding to the iron exporter ferroportin and inducing its internalization and degradation, thereby blocking availability of iron for erythropoiesis (Nemeth and Ganz, 2006).

Another negative feedback loop regulating erythropoiesis within islands involves a lesser known soluble factor secreted by bone marrow macrophages termed RCAS1, receptor binding cancer antigen expressed in SiSo cells (Matsushima et al., 2001). The binding of RASC1 to its receptor expressed on immature erythroblasts activates proapoptotic caspases 8 and 3. Hence, RASC1 represents another regulator of apoptosis of erythroid progenitors.

In sum, there are myriad means by which elevated levels of circulating cytokines, chemokines, and interleukins can disorder erythropoiesis and there is a pressing need to dissect out the various molecular mechanisms and their potential interplay to design novel therapies to stimulate effective erythropoiesis.

2.1.2 Extracellular matrix role in erythroid niche

Currently 2 extracellular matrix proteins, fibronectin and laminin, are strong candidates for regulating processes in terminally differentiating erythroblasts and reticulocytes. Fibronectin influences growth, differentiation, adhesion, and migration of multiple cell types, including hematopoietic cells. Erythroblasts express 2 integrins that bind fibronectin, α4β1 and α5β1 (Hanspal, 1997) (Fig. 2.3). However α5β1 expression is down-regulated during terminal differentiation and late-stage erythroblasts express almost exclusively α4β1 on their surface (Rosemblatt et al., 1991). α4β1 mediates adhesion to several sites on the fibronectin glycoprotein (Fig. 2.3). A recent exciting finding is that proliferation appears to be regulated by an early Epo-dependent phase followed by an α4β1 integrin/fibronectin-dependent phase (Eshghi et al., 2007). Both Epo and fibronectin promote proliferation by protecting cells from apoptosis, partly through antiapoptotic bcl-xL. Late in terminal differentiation Lutheran (Lu) glycoproteins that bind extracellular matrix laminin appear on the erythroblast surface (El Nemer et al., 1998; Zen et al., 1999). Lu is a specific, high-affinity receptor for laminins and various laminins localize to different regions of extracellular matrix (Chasis and Mohandas, 2008).

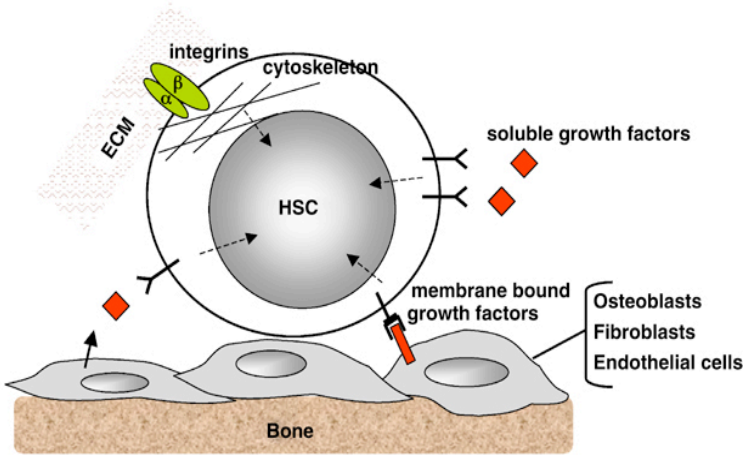

Figure 2.3. The principal regulatory entities within the HSC niche in the bone marrow microenvironment. Stromal fibroblasts, osteoblasts and endothelial cells as well as cell–extracellular matrix (ECM) components present growth regulatory signals to hematopoietic stem cells (HSCs). External signals from the BM microenvironment can be mediated by cell–cell interactions, cell–extracellular matrix (ECM) interactions (integrins) and by soluble growth factors

2.2 REGULATION OF ERYTHROPOIESIS

The appropriate regulation of erythropoiesis is essential for both embryonic development and adult red cell production

The regulation of red blood cell production involves three basic processes in erythroid progenitor and precursor cell population: (1) proliferation, (2) differentiation and (3) survival. Although they act in concert from the earliest cell committed to erythropoiesis, the burst-forming unit-erythroid (BFU-E), through the last cell division of erythroblasts, each of these three processes can be regulated independently of another. After blood loss or hemolysis, stem cell factor (SCF/Kit-ligand) and glucocorticoids

increase proliferation of cells in the BFU-E to colony-forming unit-erythroid (CFU-E) stages. However, SCF and glucocorticoids have little effect on survival or differentiation of these erythroid progenitor cells. Erythopoietin (EPO), the major physiologic regulator of erythropoiesis, is regulated by hypoxia at the level of its transcription (Koury, 2005). EPO promotes the survival of erythroid cells in the CFU-E through basophilic erythroblast stages without affecting their proliferation or differentiation. Contact with macrophages in erythroblastic islands regulates proliferation of these EPO-dependent cells without affecting their survival or differentiation (Rhodes et al., 2005). Differentiation of late-stage erythroblasts is characterized by the synthesis and accumulation of hemoglobin, the most abundant and major functional protein of mature erythrocytes. Accumulation of heme and globin chains, which is finely regulated to assure erythroblast survival (Chen et al., 2006; Weiss and Goodnough, 2005), has no effect on cellular proliferation, which has largely ceased by this stage of differentiation. In the last decade, utilizing genetic and biochemical approaches, several key intracellular as well as extracellular factors have been identified that regulate erythropoiesis. Cytokines and transcription factors have been extensively characterized as key players in regulating this process.

2.2.1 THE ROLE OF CYTOKINES DURING ERYTHROPOIESIS

It is clear that c-Kit/SCF and Epo-R/Epo signaling events are necessary for erythropoiesis, the signaling mechanisms by which these two receptor/ligand pairs regulate cellular responses in erythroid cells remain

poorly defined. However, there are recent advances made towards understanding the role of signaling pathways that regulate red cell production via c-Kit and Epo-R. The signaling events initiated by the binding of erythropoietin (Epo) to the Epo-receptor (Epo-R) induce proliferation, survival, as well as, differentiation of erythroid progenitors. Likewise, c-Kit and stem cell factor (SCF) signaling pathway also plays an essential role in erythroid cell development (Nocka et al., 1989; Russell and Cosimi, 1979).

c-kit/SCF signaling

In order to understand the importance of c-Kit/SCF interaction in erythroid linear two mutant mice are generated: White spotting (W) and Steel (Sl). These mice demonstrate erythroid and other lineage-specific defects due to inherited mutations within the c-Kit and SCF genes, respectively. The phenotypic abnormalities in mice with mutations affecting the Dominant White Spotting (W) and Steel (Sl) loci (reduction in pigment cells, sterility, and macrocytic anemia with mast cell deficiencies) demonstrate the critical nature of the proteins encoded by these loci in the normal development of hematopoietic stem and progenitor cells, melanocytes, and germ cells (Broudy, 1997; Nocka et al., 1989; Russell and Cosimi, 1979). The W locus encodes the receptor tyrosine kinase c-Kit (Chabot et al., 1988; Geissler et al., 1988), and the Sl locus encodes the soluble (S) and the membrane-associated forms of its ligand, known as stem cell factor (also called mast cell growth factor

[MGF], Kit ligand [KL], and Steel factor [SF]) (Copeland et al., 1990; Huang et al., 1990; Zsebo et al., 1990).

Similar to the mutants of W, the severity of the phenotype in Sl mutant varies considerably. Like the W mutant, a complete deletion of the Sl gene coding sequences results in death in utero due to severe anemia. On the other hand, Sl mutants encoding some form of SCF protein are compatible with life, but manifest varying degrees of abnormalities in the affected lineages, including red cell deficiency, mast cell deficiency, coat color abnormalities, and sterility (Galli et al., 1994; Russell and Cosimi, 1979).

Mice and humans encode two distinct isoforms of SCF as a result of mRNA splicing (Anderson et al., 1990; Brannan et al., 1991; Flanagan et al., 1991; Huang et al., 1990). A glycoprotein of 248 (SCF248) amino acids is rapidly cleaved from the cell to release a biologically active soluble protein of 164 aa. In contrast, a glycoprotein of 220 aa (SCF220), which lacks the proteolytic cleavage site encoded by differentially spliced exon 6 sequences, remains predominantly membrane associated. This isoform can also be slowly released from the cell surface via the use of an alternate proteolytic cleavage site in exon 7. The physiologic role of these isoforms in blood cell development is not completely understood. However, studies performed on naturally occurring mutants of Sl, as well as using transgenic and knock-in mice have revealed distinct functions of soluble versus membrane associated (MA) SCF isoforms in regulating blood cell development. Moreover, membrane form of SCF would appear

to be critical for normal function in the affected lineages (Russell and Cosimi, 1979).

Studies performed on transgenic mice that express an obligate membrane-restricted form of SCF in Sld or Sl17 mutant background have demonstrated a significant correction in both hematocrits and total red cell numbers compared with mutants expressing the soluble form of SCF as a transgene (Kapur et al., 1998). Further, studies performed in mice expressing only the slowly secreted form of SCF (SCF220) have also suggested an essential role for membrane presentation of SCF in normal erythroid cell development (Tajima et al., 1998). Moreover in vivo evidence, confirmed by in vitro studies, suggest that MA SCF may be an important regulator of c-Kit activation in vivo.

c-Kit plays a role in multiple hematopoietic lineages, the role of individual signaling pathways in regulating cellular responses in primary hematopoietic cells thus far has been characterized predominantly in the mast cell lineage (Serve et al., 1995; Tajima et al., 1998). The signaling pathways utilized by c-Kit in mast cells may not play a significant role in regulating c-Kit-induced functions in other hematopoietic lineages, including erythroid progenitor (Huddleston et al., 1995). Thus, it is likely that other signaling pathways play a more prominent role in regulating c-Kit functions in erythroid cells or additional signaling pathways might compensate for deficiency of these molecules in erythroid progenitors. To this end it is important to know that the binding of SCF to c-Kit results in dimerization and autophosphorylation of the receptor on several distinct cytoplasmic tyrosine residues which become binding sites for a variety of

Src homology 2 domain-containing enzymes and adaptor proteins such as phospholipase Cγ (PLC-γ), phosphatidylinositol 3-kinase p85 subunit (PI-3Kinase), Ras GTPase activating protein, SHP2 phosphatase, Src kinases, Grb2, Grb7, and Shc (Boissan et al., 2000; Gommerman et al., 2000; Lennartsson et al., 1999; Linnekin et al., 1997). In this manner, the phosphorylated tyrosine residues initiate signal transduction via several distinct early signaling pathways.

Studies in multiple cell types have shown that c-Kit carrying tyrosine to phenylalanine mutations at the critical residues fail to bind the associated signaling molecules and consequently fail to activate these pathways (Boissan et al., 2000; Linnekin et al., 1997). It is really difficult to perform functional and biochemical studies. Further, very few erythroid progenitor cell lines have been described that faithfully mimic c-Kit functions in primary erythroid progenitor cells. To this end, Weiss et al. and Gregory et al. have described an erythroid cell line G1E-ER2 that mimics primary pro-erythroblasts and is dependent on SCF and Epo for cell survival, proliferation and differentiation. These cells have been utilized extensively to study the role of c-Kit and various mutant isoforms of SCF in erythroid cell proliferation and survival (Kapur et al., 2002; Kapur et al., 1998; Kapur and Zhang, 2001).

In addition to a role for c-Kit in normal hematopoiesis, nearly 30 gain-of-function mutations of c-Kit have been identified which are associated with highly malignant tumors in humans. Most of the activating mutations in the juxtamembrane region of c-Kit (V560G) are associated with gastrointestinal tumors (GIST) and sinonasal NK/T cell lymphomas.

The activating mutations (D816V, D816Y, and D816F) in the second half of the kinase domain of c-Kit are associated with human mastocytosis and acute myeloid leukemia (AML) (Heinrich et al., 2002).

Epo-R/ Epo signaling

In addition to c-kit, erythropoiesis is controlled to a large extent by signals derived from erythropoietin and erythropoietin receptors. Epo-R belongs to the class I cytokine receptor family, and initiates signaling by activating Janus kinase (JAK) 2, which binds to Epo receptor dimers at a conserved Box 1 motif (Witthuhn et al., 1993). Activated JAK2 phosphorylates the Epo-R at multiple cytoplasmic tyrosine residues, which results in the recruitment of Src homology (SH)-2 domain-containing proteins to the receptor. Studies utilizing truncated and tyrosine to phenylalanine mutated murine Epo receptors have mapped the binding sites for several SH2 containing adaptor proteins as well as enzymes to phosphotyrosine sites within the Epo-R. These include, Stat5a/b, SHP1, SHP2, SHIP, p85α regulatory subunit of PI-3Kinase, Grb2, Lyn tyrosine kinase, and suppressor of cytokine signaling (SOCS) 3. In addition to these signaling molecules, other signaling molecules that bind Epo-R have also been identified, including tyrosine kinases Syk, Tec, PLC-γ, and adaptor proteins Shc, Cbl, Crkl, IRS-2, and Gab1/2, and the nucleotide exchange factors Sos and Vav (Klingmuller, 1997). In spite of the identification of binding sites for various signaling molecules, the physiologic role(s) of the signaling cascade initiated via the phosphorylation of these tyrosines in Epo-R remain poorly understood.

Activation of PI-3Kinase, Akt, and FKHRL1 forkhead transcription factor have been implicated as critical downstream effectors induced via tyrosine 479 in the Epo-R (Uddin et al., 2000). On the other hand, tyrosine 343 has been shown to primarily activate Stat5 and induce the expression of Bcl-xL (Pircher et al., 2001; Socolovsky et al., 1999). Studies utilizing mice deficient in Bcl-xL expression have demonstrated a critical role for this protein in the survival and terminal differentiation of committed erythroid progenitors (Motoyama et al., 1996). In some studies, induction of Bcl-xL expression by Epo has been shown to be dependent on Stat5 activation (Socolovsky et al., 1999). However, in other studies impaired binding of Stat5 in vivo did not result in an erythroid defect (Quelle et al., 1998), while a marked reduction in the number of CFU-E progenitors as well as embryonic anemia due to Stat5 deficiency has been reported (Damen et al., 1997; Pircher et al., 2001).
Moreover, the role of various tyrosine residues in the distal region of the Epo-R in proliferation, survival, and/or differentiation of erythroid progenitors is also poorly understood. In vivo expression of a truncated Epo-R (lacking the distal half) showed little defects in steady-state levels of erythropoiesis, suggesting that tyrosine residues in the distal half of the Epo-R may have only a minimal role in regulating Epo-R functions in vivo (Zang et al., 2001). Mutations of the human Epo-R that lack approximately 70–90 amino acids of the distal region are associated with erythrocytosis (Arcasoy et al., 1997; de la Chapelle et al., 1993; Li et al., 2003; Sokol et al., 1995). A truncation mutant of Epo-R that only retains one tyrosine residue at position 343 (Y343) in the distal region of the

receptor also demonstrates a significant increase in Stat5 activation, suggesting that perhaps the distal region of the Epo-R may negatively influence Stat5 activation.

Several potential mechanisms of action of JAK2 have been proposed for the cells expressing the truncated Epo-R that consist in the activation of PI-3Kinase or in the induction of c-Myc. In addition to these possibilities, it is conceivable that the truncated Epo-R mutant causes hyper-activation of JAK2 in these cells leading to the activation of downstream pathways in the absence of phosphotyrosine binding sites. Examples of activation of downstream signaling pathways in the absence of tyrosine binding sites in the receptor have been described previously in other cells systems.

To further elucidate the intracellular mechanisms by which Epo-induces the differentiation of human erythroid progenitors, Kubota et al. have analyzed the roles of Src and PI-3Kinase (Kubota et al., 2001). They demonstrated that Epo-induced differentiation is dependent on the cooperation between the Src and the PI-3Kinase pathways and argued that Src kinase likely provides the binding site(s) for the p85α subunit of PI-3Kinase in Epo-R by tyrosine phosphorylating the receptor.

Indeed, recent studies by Boudot et al. showed that Lyn Src family kinase binds to the unphosphorylated mouse Epo-R, and that its SH2 domain binds directly to the mouse Epo-R through its interaction with phosphorylated tyrosine 464 or 479 after Epo stimulation. In humans, Src may bind directly to these phosphorylated tyrosine residues in Epo-R through its SH2 domain, after Epo stimulation.

Studies have shown that Lyn pre-associates with Epo-R and is rapidly activated after Epo binding to its receptor. Therefore, Lyn may function as a critical protein tyrosine kinase in regulating erythroid development like JAK2. Taken together, cooperation between Src/Lyn kinase and PI-3Kinase may play a prominent role in regulating murine and human erythroid cell differentiation.

Huddleston et al. have recently demonstrated that PI-3Kinase have an essential role in erythroid cell differentiation and the deficiency of p85α subunit of PI-3Kinase results in defective fetal liver erythropoiesis, including reduced CFU-E and BFU-E production in response to Epo (Huddleston et al., 2003). Klingmuller et al. showed that tyrosine 479 is the binding site for p85α subunit of class IA PI-3Kinase, these results suggest that PI-3Kinase is involved in the erythroid differentiation of hematopoietic cells (Klingmuller, 1997).

The mechanisms by which Epo-R balances positive and negative signals in response to Epo depend in part on its ability to tyrosine phosphorylate and dephosphorylate the receptor. Tyrosine phosphorylation in response to Epo is transient and returns to basal levels within 30min of stimulation, suggesting that negative regulation dephosphorylates Epo-R rather quickly (Klingmuller et al., 1995; Linnekin et al., 1992; Miura et al., 1991; Quelle and Wojchowski, 1991). To this end, recruitment of a negative regulator of Epo-R signaling; SHP-1 phosphatase appears to play an essential role in terminating proliferative signals through dephosphorylation and inactivation of Jak2. Consistent with these observations, CFU-Es derived from mice lacking the expression of SHP-1

(motheaten) or those that are impaired in SHP-1 phosphatase activity (motheaten viable) are also sensitive to lower concentrations of Epo, and demonstrate increased numbers of splenic CFU-E in vivo (Van Zane et al.,1989). In humans, expression of a truncated Epo-R that lacks the C-terminal 70 amino acids cause erythrocytosis, elevated hematocrit and increase in the concentration of hemoglobin in the blood. Interestingly the receptor lacking the binding site for SHP-1 and erythroid progenitors from these patients hyper-proliferate at lower concentrations of Epo. Other mutations in the Epo-R gene that eliminates the binding of SHP-1 resulting in increased sensitivity of erythroid progenitors to Epo (Sokol et al., 1995). Collectively, these studies suggest an essential role for SHP-1 in the negative regulation of Epo-R functions. In addition to SHP-1, the SOCS family of negative regulators of cytokine signaling have also been described (Starr et al., 1997; Yoshimura et al., 1995). Expression of SOCS genes is rapidly modulated in response to a variety of cytokines and the SOCS family act via a negative feedback loop to suppress cytokine-induced signal transduction. CIS, the initial SOCS family member, was identified as an Epo-inducible molecule (Yoshimura et al., 1995). Expression of CIS is elevated upon activation of JAK2 and Stat5 (Matsumoto et al., 1995; Verdier et al., 1998). SOCS-1 has been shown to inhibit the activation of Jak2 (Endo et al., 1997; Naka et al., 1997).

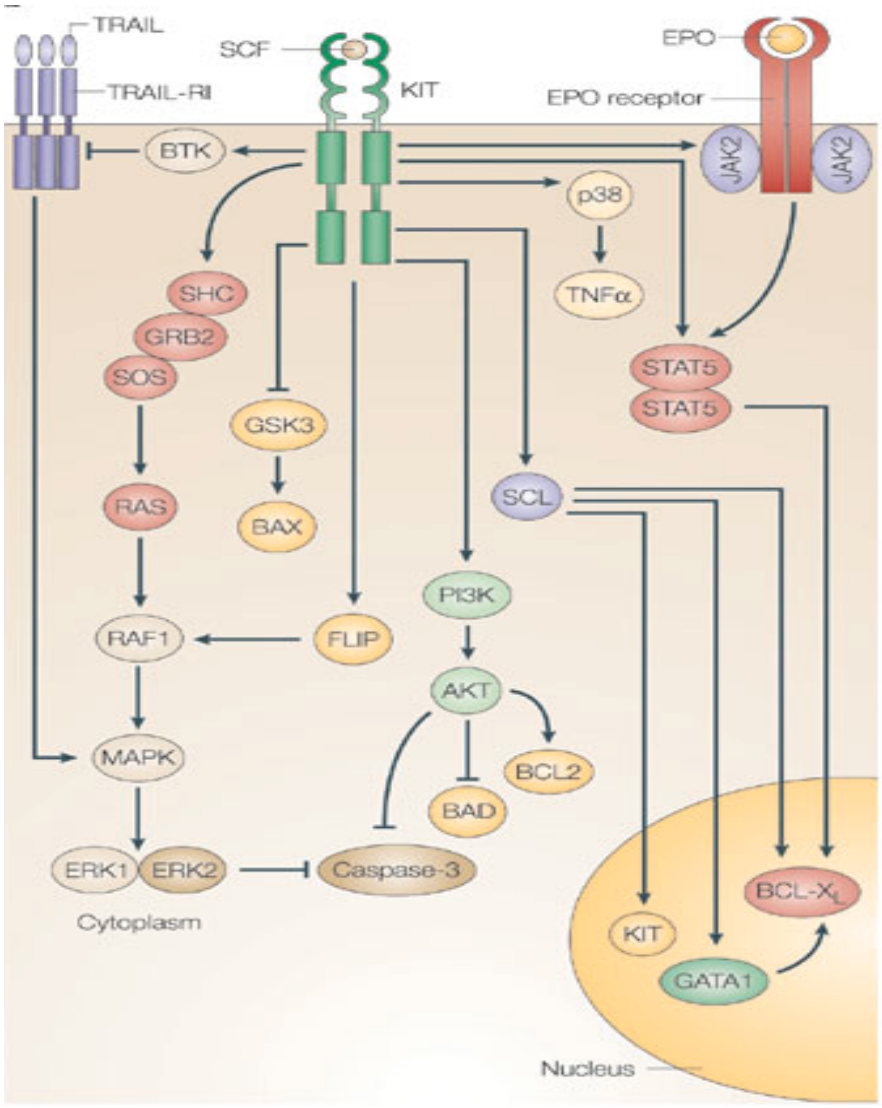

Figure 2.4. Signal transduction mediated by erythropoietin and stem-cell factor in erythroid progenitor cells.

SOCS-3 is tyrosine phosphorylated in response to Epo stimulation and has been shown to suppress Epo signaling by associating with the Epo

receptor and JAK2 (Cacalano et al., 2001; Cohney et al., 1999; Sasaki et al., 2000).

Three SOCS genes have been associated with erythropoiesis, including SOCS-1, CIS, and SOCS-3. Recruitment of CIS to the Epo-R via the SH2 domain represses proliferation. In addition, the SOCS box may be responsible for mediating apoptotic effects of CIS in fetal liver erythroid progenitors. Interestingly, however, CIS-/- mice show no detectable erythroid phenotype (Marine et al., 1999). In contrast, SOCS-1-/- mice demonstrate mild anemia and elevated numbers of nucleated erythroid cells in the spleen (Metcalf, 1999). This has been attributed to enhance sensitivity of SOCS-1-/- progenitors to Epo. In contrast, deletion of SOCS-3 results in embryonic lethality because of erythrocytosis and accumulation of nucleated erythroid cells in the fetal liver. Furthermore, transgene-mediated over-expression of SOCS-3 suppresses fetal liver erythrocytosis (Marine et al., 1999). Although it is not entirely clear why the deletion of one SOCS family member results in only mild anemia where as another leads to embryonic lethality; this may in part be due to the differential regulation of these genes during different stages of erythroid cell maturation.

Cooperation between c-kit and Epo-R

Although SCF has been shown to induce some growth and survival of erythroid progenitors, its response is profoundly amplified in combination with Epo. This type of synergy between c-Kit and Epo-R has been described in experiments attempting to enumerate BFU-E and CFU-E

formation in vitro as well as in vivo upon co-administering SCF and Epo. However, the basis for this synergism is poorly understood. To this end, biochemical studies have provided evidence for physical association between c-Kit and Epo-R via the box 2 region cytoplasmic domain of the Epo-R. SCF supports proliferation only in cells that co-express c-Kit and the Epo-R (Wu et al., 1997; Wu et al., 1995) and Wu et al. utilizing various truncation mutants of Epo-R have demonstrated that c-Kit stimulation by SCF does not activate the Epo-R by inducing its dimerization, but by phosphorylating tyrosine residues in the cytoplasmic domain of the Epo-R (Fig. 2.4). Recent studies by Tan et al. have demonstrated that the tyrosine residues 567 and 569 in the c-Kit receptor play an important role the synergy between c-Kit and Epo-R and the phosphorylation of Epo-R in erythroid cells. Other studies suggest that tyrosine residues within the Epo-R cytoplasmic tail may not be involved in contributing to the observed synergy between c-Kit and Epo-R. To this end, a truncated Epo-R (Y343F) lacking all the tyrosine residues in the Epo-R was able to synergize with c-Kit. Thus, although these results suggest that an Epo-R tyrosine-independent mechanism could contribute to Epo-R/c-Kit cooperation, one cannot rule out the possibility that compensation by other signaling pathways in a truncated Epo-R may contribute to the synergy between a truncated Epo-R and c-Kit. Additional studies will address this issue.

Sui et al. utilizing human erythroid colony-forming cells showed that although SCF induces the phosphorylation of Epo-R in purified human erythroid cells, it is not sufficient to transduce signals via Epo-R and

support cell growth (Sui et al., 1998). These authors demonstrated that SCF and Epo synergistically activate MAP kinase (Erk1/2) (Fig 2.4), which correlates with cell growth and thus may be responsible for the synergistic effects observed in response to SCF and Epo co-stimulation in erythroid cells. They further demonstrated that inhibiting MAP Kinase and the PI-3Kinase pathways inhibits the synergistic activation of MAP Kinase as well as erythroid cell growth. Kapur and Zhang utilizing an erythroid progenitor cell line G1E-ER2 cells demonstrated that c-Kit stimulation by SCF may play an essential role in the maintenance of Epo-R and Stat5 protein expression, which leads to increased expression of Bcl-xL and survival of erythroid progenitors in response to Epo stimulation (Fig. 2.4). Sato et al. have shown that SCF can activate the human Epo-R promoter containing the GATA and Sp1 binding sites, and mutations in the Sp1 binding site results in the abrogation of Epo-R mRNA in response to SCF stimulation. More recently, Boer et al. have shown that SCF can enhance Epo-mediated transactivation of Stat5 via the PKA/CREB pathway. They showed that Epo induces transactivation of Stat5, which is enhanced by SCF treatment. SCF pre-treatment prior to Epo stimulation leads to a significant increase in Stat5 transactivation, however SCF stimulation alone did not affect Stat5 transactivation. Recent studies also provide evidence for a role of cytoplasmic tyrosine kinase Bruton's tyrosine kinase (Btk) in c-Kit/Epo-R co-signaling. Btk gets phosphorylated by Jak2 and activated Btk phosphorylates Epo-R as well as downstream signaling molecules such as PLC γ and Stat5 (Von et al., 2004). In sum, there are several distinct mechanisms of synergy

between c-Kit and Epo-R in regulating normal erythroid cell development and remains to be determined whether these mechanisms are cell line specific or operational in primary erythroid progenitors.

2.2.2 THE TRANSCRIPTION FACTOR CONTROL OF ERYTHROPOISIS

The differentiation of hematopoietic stem cells (HSCs) into specific progenitor cells, and ultimately into diverse blood cell types, is intricately controlled by intercellular and intracellular signaling mechanisms (Kaushansky, 2006; Mikkola and Orkin, 2006). These mechanisms commonly target transcriptional regulators, which in turn establish complex transcriptional networks. Since a host of transcriptional regulators and signaling pathways that control erythropoiesis have already been identified, major efforts are focused on elucidating the underlying molecular mechanisms. Canonical transcriptional mechanisms involve sequence-specific binding of transcription factors to DNA motifs termed cis-elements in chromatin, followed by recruitment of additional regulatory proteins (coregulators) via direct protein–protein interactions (Kadonaga, 2004). Coregulators typically exist as large multiprotein complexes and either mediate activation (coactivators) or repression (corepressors) (Bresnick et al., 2006; Lee and Workman, 2007). Certain coregulator complexes mediate both activation and repression in a context-dependent manner (Crispino et al., 1999; Rogatsky et al., 2002). It is instructive to classify coregulators as chromatin remodeling or chromatin modifying enzymes, based on whether they lack or have the

capacity, respectively, to post-translationally modify histones that form the octameric core of the nucleosome. Since chromatin can be a formidable impediment to transcription factor access to nucleosomal DNA (Hager et al., 1993), remodeling enzymes regulate transcription factor access to chromatin. In addition, as nucleosomal filaments condense into higher-order structures (Felsenfeld and Groudine, 2003), remodeling enzyme-dependent chromatin structure transitions almost certainly regulate higher-order chromatin folding.

In contrast to remodeling enzymes, chromatin modifying enzymes catalyse a plethora of histone post-translational modifications, including acetylation, methylation, phosphorylation, ubiquitination, sumoylation and ADP ribosylation, which are termed epigenetic marks (Allfrey et al., 1964; Fischle et al., 2003). In addition to the canonical mechanisms noted above, certain transcription factors retain functionality upon disabling their sequence-specific DNA binding activity, indicating the importance of DNA binding-independent mechanisms in certain contexts (Porcher et al., 1999; Reichardt et al., 1998; Tuckermann et al., 1999). Given that transcriptional complexes assembled at promoters and distal regulatory elements, such as enhancers and locus control regions (LCRs), often contain a large cohort of factors that engage in a multitude of protein–protein interactions (Bresnick et al., 2006), it seems reasonable that certain transcription factors can integrate into such complexes without a critical DNA binding activity requirement. Important principles underlying transcriptional control in higher eukaryotes have regularly emerged from mechanistic studies on the regulation of the beta-like

globin gene cluster, a commonly used model system to elucidate cell type-specific and developmental-stage specific transcriptional mechanisms (Bresnick et al., 2006). Many works with the beta-globin system has provided important insights into perhaps the most fundamental aspect of transcriptional control, how trans-acting factors recognize and occupy functional sites in chromatin (Bank, 2006; Bresnick et al., 2006) (see Section 2.5.2). Extensive analyses of chromatin occupancy by the hematopoietic zinc-finger protein GATA-1, which is discussed below in detail (see Section 2.5.2), revealed that only a small fraction of high-affinity GATA motifs are occupied in chromatin (Grass et al., 2003; Grass et al., 2006; Im et al., 2005; Johnson et al., 2007; Johnson et al., 2002; Martowicz et al., 2005; Pal et al., 2004). Intriguingly, the cell type-specific coregulator Friend of GATA-1 (FOG-1), which mediates certain biological functions of GATA-1 (Tsang et al., 1998; Tsang et al., 1997), facilitates GATA-1 occupancy at certain, but not all, chromatin target sites (Letting et al., 2004; Pal et al., 2004). We refer to this FOG-1 activity as chromatin occupancy facilitator (COF) activity. Thus, mechanisms responsible for the selective recognition of a small subset of motifs represent a crucial primary mode of transcriptional control, and specific protein–protein interactions influence this decision-making process. Subsequent to transcription factor occupancy of chromatin, a multitude of regulatory events, including coregulator recruitment, coregulator-dependent chromatin structure transitions, dynamics of transcription factor and coregulator interactions with the template, and interactions between these components and RNA

Polymerase II (Pol II) dictate the magnitude and kinetics of the transcriptional response (see Section 2.5.2).

2.3 ERYTHROPOIESIS AND GLOBIN GENE EXPRESSION

During differentiation, mammalian red blood cells synthesize enormous quantities of hemoglobin, which consist of tetramer of 2 α-globin and 2 ß- globin. Both the α- and ß-globins are encoded by multiple genes, which are organized into distinct α- and ß-globin clusters; expression from within these clusters is developmentally controlled.

As reported more than 20 years ago by Nienhuis and Stamatoyannopoulos (Nienhuis and Stamatoyannopoulos, 1978), the phrase hemoglobin switching represents "the gradual replacement, in the blood stream, of red cells containing predominantly one hemoglobin with red cells which contain predominantly another". Their definition articulates the now classic correlation among changes in red cells, their site of production and their globin content during human ontogeny. This switching paradigm is illustrated in Figure 3.1 (Wood et al., 1976).

In humans, two gene clusters direct the synthesis of hemoglobins: the α locus, which contains the embryonic ζ gene and the two adult α genes encoded on chromosome 16; and the β locus, which consists of the ε, $^{G}\gamma$, $^{A}\gamma$, δ, and β genes that are arranged in a linear array on chromosome 11 and are expressed in a developmental stage-specific manner in erythroid cells (Fig. 2.5). While for the human alpha globin genes only one switch occurs, from the embrional zeta-globin to the fetal/adult α_1 (25%) e α_2 (75%) chains, two globin gene switches occur for the human beta globin

genes during development: the embryonic to fetal globin switch, which coincides with the transition from embryonic (yolk sac) to definitive (fetal liver) hematopoiesis and the fetal to adult switch, which occurs at the perinatal period (Fig. 2.5).

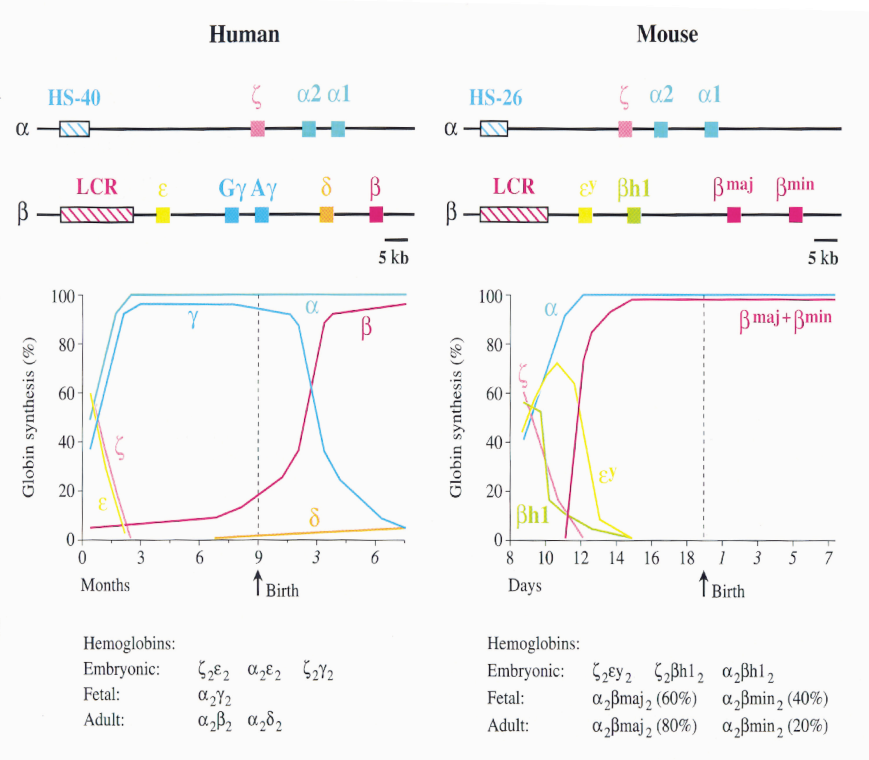

Figure 2.5 Genomic organization of human and murine globin genes (upper part). Hemoglobin synthesis and sites of hematopoiesis during human and murine fetal development (lower part).

The switches from ε to γ and from γ to β globin gene expression are controlled exclusively at the transcriptional level (Stamatoyannopoulos,

2005). The ζ to α switch is controlled predominantly at the transcriptional level, although posttranscriptional mechanisms also play a role. Differently, during the murine erythroid development, the switching from the embryonic (epsilon-y and beta-h1) to the beta chains (beta major and beta minor) occurs early before birth (Fig. 2.5). For this reason, mice lacking both copies of b1 and b2 globin genes are not viable (Yang et al., 1995). Genetic information governing the stage-specificity for all ß-like globin genes is located in gene proximal regions. These elements represent transcription factor-binding sites that recruit proteins or protein complexes in a stage-specific manner. Examples exist for the presence of both positive and negative acting factors that turn genes on or off at a specific developmental stage.

The complex program of transcriptional regulation leading to the differentiation and developmental stage-specific expression in the globin locus is mediated by DNA-regulatory sequences located both proximal and distal to the gene-coding regions. The most prominent distal regulatory element in the human ß-globin locus is the locus control region (LCR), located from about 6 to 22 kb upstream of the epsilon-globin gene (Forrester et al., 1987; Grosveld et al., 1987a). The LCR is composed of several domains that exhibit extremely high sensitivity to DNase I digestion in erythroid cells (called hypersensitive, or HS, sites), and is required for high-level globin gene expression at all developmental stages (Higgs, 1998).

The LCR confers lineage-specific expression on the globin genes; it acts as the major enhancer of the ß-locus and insulates the locus from

surrounding inactive chromatin. Moreover, on the basis of finding in transgenic mice, it was proposed that the LCR is responsible for opening the ß-locus chromatin domain.

2.4 HEMOGLOBINOPATHIES

Hemoglobinopathies are a group of inherited disorders characterized by the absence of functional α-like or β-like globin chains. Sickle cell disease (SCD) and ß-thalassemias are two of the most common categories of hematopoietic diseases. SCD include sickle cell anemia, sickle cell-hemoglobin C disease and sickle cell-ß-thalassemia. Millions worldwide are affected; one of 400 African Americans, over 70,000 victims, is afflicted. These diseases are major health problems, associated with severe morbidity, lower-than-average life expectancy and serious, long-term disability. Clearly, it is of interest to combat these deadly diseases.

In the circulatory system, erythrocytes (red blood cells) transport oxygen to bodily tissues and carbon dioxide to the lungs for exhalation. Within erythrocytes, this process is mediated by hemoglobin, a molecule that consists of two α-like and two β-like globin chains and four iron-coordinated heme moieties. As mentionated above, the human α-like and β-like globin loci, located on chromosomes 16 and 11 respectively, encode these protein chains. During development, different α- and β-globin genes are expressed to produce a developmental stage-specific hemoglobin molecule that meets the oxygen demand of the developing fetus (Fig. 2.5). Naturally occurring mutations within these loci cause the

production of abnormal hemoglobins and anemia. Abnormal hemoglobins appear in one of three basic circumstances:

1. Structural defects in the hemoglobin molecule. Often, mutations in the gene for one of the two hemoglobin subunit chains, α- or β, change a single amino acid building block in the subunit. Most commonly the change is innocuous, perturbing neither the structure nor function of the hemoglobin molecule. Occasionally, alteration of a single amino acid dramatically perturbs the behavior of the hemoglobin molecule and produces a disease state. Sickle hemoglobin exemplifies this phenomenon.

2. Diminished production of one of the two subunits of the hemoglobin molecule. Mutations that produce this condition are termed "thalassemias". Equal numbers of hemoglobin α- and β-chains are necessary for normal function. Hemoglobin chain imbalance damages and destroys red cells thereby producing anemia. Although there is a dearth of the affected hemoglobin subunit, with most thalassemias the few subunits synthesized are structurally normal.

3. Abnormal associations of otherwise normal subunits. A single subunit of the α chain (from the α-globin locus) and a single subunit from the β-globin locus combine to produce a normal hemoglobin dimer. With severe α-thalassemia, the ß-globin subunits begin to associate into groups of four (tetramers) due to the paucity of α-chain partners. These tetramers of ß-globin subunits are functionally inactive and do not transport oxygen. No comparable tetramers of α globin subunits form with severe ß-thalassemia. α subunits generate aggregates (Heinz bodies) and

increase ROS production that damage erythroid cell precursors in the absence of a partner from the ß-globin gene cluster (gamma, delta, beta globin subunits) (see section 4.2).

The Thalassemias

The thalassemias are defined as a heterogenous group of inherited disorders of hemoglobin synthesis, all characterized by the absence or reduced output of one or more of the globin chains of hemoglobin. They are most prevalent in the Mediterranean region, the Middle East, the Indian subcontinent and South-East Asia, representing a serious health problem in certain areas where gene frequencies reach 3-10% of the population (Weatherall and Clegg, 1996). They can be classified at several levels. First, there is a clinical classification, which simply describes the degree of severity. Second, the thalassemias can be defined by the particular globin chain that is synthesized at a reduced rate (genetic classification). Finally, it is now often possible to subclassify them according to the particular mutation that is responsible for defective globin chain synthesis (molecular classification).

Based on clinical features, the thalassemias are divided into the major forms of the illness, which are severe and transfusion-dependent and the symptomless minor forms, which usually represent the carrier state, or trait. Thalassemia major results either from the homozygous inheritance of a particular mutation or from the compound heterozygous state of two different mutations. Thalassemia intermedia describes conditions that are associated with a more severe degree of anemia than the trait, although

they are not as severe as the major forms. Finally, there are some forms of thalassemia trait that are clinically and hematologically completely silent; they are designated as a silent carrier state.

According to their genetic basis the thalassemias are classified into α, ß, γ, ∂ and εγ∂ß varieties, depending on which chain or chains are synthesized at a reduced rate.

As their molecular pathology has been ascertained, it is now feasible to develop a more accurate approach to the designation of different types of thalassemias. For example, in many cases, it is possible to describe the genotype of a patient with the clinical picture of ß-thalassemia major according to the particular mutations at the homologous pairs of ß-globin chain loci.

In particular, the ß-thalassemias are the most intensively studied monogenic disorders in man. Over 200 different mutations that giving rise to the clinical phenotype of ß-thalassemia have been identified (Olivieri, 1999). These mutations can be classified in two distinct categories: $ß^0$ mutations, in which no ß-globin chains are produced and $ß^+$ mutations, in which some ß chains are produced but at a reduced rate. The deficiency or absence of ß-globin chains reflects the action of mutations that affect every level of ß-globin gene function; that is, transcription, mRNA processing, translation and post-translational stability of the ß-globin chain product. These mutations are classified according to the mechanism by which they affect ß-globin gene expression.

In most cases, beta thalassemia is caused by point mutations affecting the beta globin gene or the flanking regions. Large deletions are extremely

rare. Mutations in the 5'UTR often determine a reduction of beta globin synthesis (beta+ phenotype), while only large deletions in 5' region can cause a complete transcriptional inactivation (beta0 phenotype). The most frequent mutations affect the splicing sites or activating cryptic splicing sites. Single base substitutions or small deletions in the 3'UTR, in the polyadenilation signal, have usually only a moderate effect on the beta globin expression level. Around 50% of all the mutations affecting the beta globin gene interfere with the translation process. These are frameshift mutations or single base substitutions that introduce stop codons and cause an early termination of the protein synthesis and a beta^0 phenotype.

The pathophysiology of ß-thalassemia is directly linked to the degree of imbalance in the production of alpha and beta-globin chains (Weatherall, 2001). In fact, the alpha chains in excess form aggregates and precipitate causing membrane damage of the erythroid bone marrow precursors that undergo apoptosis (ineffective erythropoiesis) (see 4.2). Red blood cells, which escape from apoptosis are sequestered and destroyed in the spleen. In the most severe form of ß-thalassemia, the anemia induces erytropoietin secretion leading to bone marrow erythroid hyperplasia. This marrow espansion can cause bone deformations and increased iron absorption.

In the most severe form of ß-thalassemia found in homozygotes and compound heterozygotes, ß-thalassemia major, the profound anemia requires regular lifelong blood transfusions; without treatment, the condition is lethal within the first year of life. In ß-thalassemia

intermedia, the anemia is less profound and may only require occasional transfusions. Chronic transfusions aimed to correct the anemia, suppress massive erythropoiesis and inhibit increased gastrointestinal absorption of iron. However, transfusion therapy leads to iron overload, which primarily affects the liver, heart and endocrine tissues. The iron overload is lethal if untreated and its prevention is the major goal of current patient management. At present, the only means to cure the disease definitively is through allogeneic bone marrow transplantation (Lucarelli et al., 2001).

2.5 THE ß-GLOBIN LOCUS CONTROL REGION

The ß-globin LCR (locus control region) is composed of five HS sites (HS1, HS2, HS3, HS4 and HS5) located upstream of the epsilon gene within a 20 kb region (Fig. 3.1). HS5 was found in different cell types, whereas the first four HS site (1-4) are erythroid-specific. The presence of HS indicates that *trans*-acting factors are binding to these regions and displacing or destabilizing nucleosomes. Expressed genes are located in an "open" chromatin context more accessible to trans-acting factors and in general containing nucleosomes with highly acetylated histones. In contrast, "closed" chromatin generally has deacetylated histones and is bound by the linker histone H1, is less accessible to DNA binding factors and genes in these regions are not expressed.

In transgenic mice, human ß-globin transgenes are silent at most integration sites or transcribed to about 1% of the endogenous mouse ß-major level. In contrast, addition of the LCR including all four erythroid

specific HSs sites (HS1, HS2, HS3 and HS4) to the ß-globin transgene results in expression to about 100% levels at all integration sites, and expression is copy number-dependent (Ellis et al., 1996a; Grosveld et al., 1987b; Talbot et al., 1989) (Ellis et al., 1996b; Grosveld et al., 1987a; Talbot et al., 1989). This copy number-dependent, position-independent transgene expression is the defining feature of LCR activity.

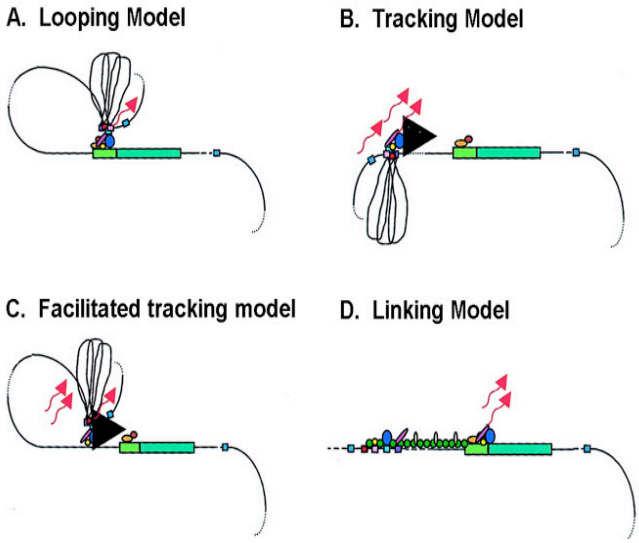

Figure 2.6 Proposed models for LCR-globin interaction. Green rectangular box represented the globin gene and the box lighter green indicated the promoter region. Transcription factors are shown as colored ovals and circe. The four erythroid hypersensitive site cores (HSs) are indicated by small colorated boxes. The flanking DNA sequences of the HSs are depicted as loops between the HS cores. Transcripts are denotated by wavy arrows.

Further investigation demonstrated that individual HS2, HS3 and HS4 elements and their smaller "cores" of approximately 200-300 bp, containing binding sites for ubiquitous and erythroid-specific

transcription factors such as GATA-1 and NF-E2 (Talbot and Grosveld, 1991), also direct copy number-dependent transgene expression but to lower levels (10-25%) (Philipsen et al., 1993; Talbot and Grosveld, 1991). The LCR is often referred to as an enhancer, but does not have classic enhancer activity because it does not function equally well in either orientation (Tanimoto et al., 1999). Rather, it appears that complete LCR activity requires all HS, and these have some distinct roles (Fraser et al., 1993) but at the end the HS function together as a unit, making the LCR sufficient to open chromatin and enhance full expression of ß-globin transgenes. The mechanism by which the LCR controls ß-globin gene expression has been extensively studied and four models of LCR function have been proposed: looping, linking tracking, and facilitated tracking (Li et al., 2006). Available data neither strongly support nor preclude any of them.

The looping model was first proposed to explain the interaction between the LCR and individual genes in the ß-globin locus. In this model, the ß-globin LCR acts as an integral unit (a "holocomplex") to stimulate the transcription of individual globin genes by looping through the nucleoplasm (Bungert et al., 1995; Choi and Engel, 1988; Wijgerde et al., 1995). Once open chromatin has been established, it is proposed that each of the HS then interacts with each other by DNA looping mediated via the bound factors to form an LCR holocomplex (Fig. 2.6 A). The holocomplex would then interact with a single globin gene in the cluster, and switching during development would be accomplished by stage-specific silencer elements associated with the ε- and γ-globin gene

promoters. In the linking model (Fig. 2.6 D) the function of the LCR is not required for chromatin opening at the endogenous ß-globin locus but is important to enhance ß-globin expression by ensuring that factors are bound at intervals across the cluster and that the gene is localized to the right nuclear compartment. Sequential binding of transcription factors along the DNA directs changes in chromatin conformation and defines the transcriptional domain. The transcription factors are linked to one another from the LCR to the gene promoter by non-DNA-binding proteins and chromatin modifiers. In the tracking, or scanning, model, erythroid-specific and ubiquitous transcription factors and cofactors bind recognition sequences in the LCR, forming an activation complex that migrates, or tracks, linearly along the DNA helix of the locus (Fig. 2.6 B) (Blackwood and Kadonaga, 1998; Tuan et al., 1992).

When this transcription complex encounters the basal transcription machinery located at the correct (according to the developmental stage) promoter, the complete transcriptional apparatus is assembled and transcription of that gene is initiated. The facilitated-tracking model incorporates aspects of both the looping and tracking models (Blackwood and Kadonaga, 1998; Tuan et al., 1992). A LCR bound transcription factor and coactivator complex loops to contact downstream DNA in promoter-distal regions, where the transcription factor complex is released. This complex then tracks in small steps along the chromatin until it encounters the appropriate promoter with its associated bound proteins. A stable loop structure is established and gene expression proceeds.

2.6 Regulation of the ß-globin genes

2.6.1 The beta globin gene: proximal elements

The ß-globin gene is a relatively small gene comprising three coding exons and two introns. The exons code for 146 amino acids and correspond to functional domains in the protein. Each globin gene has a number of regulatory elements (promoter, enhancer, or silencers) that are important for its precise developmental regulation. These elements are thought to interact with the more distant ß globin LCR to achieve high levels of gene expression. Each regulatory element is composed of binding motifs for multiple erythroid restricted and ubiquitously expressed transcriptional activators or suppressor. These factors interact and synergize with each other and other cofactors resulting in the formation of multimeric complexes that change chromatin structure to allow complex interactions giving rise to the action of the basic transcription machinery and the formation of an initiation complex.

The proximal part of the ß-globin gene contains an initiator sequence, a TATA box at -30, a G-rich sequence at -50, a CAAT box at -75, and two CACCC boxes at -90 to -110. The initiator element was discovered through *in vitro* analysis (Antoniou et al., 1995; Lewis and Orkin, 1995), the G-rich sequence called "ß DRE repeats" at -50 is highly conserved during evolution (Stuve and Myers, 1990). The CAAT box region is important for promoter function in erythroid cells (Antoniou and Grosveld, 1990), and has been shown to bind several different factors: the erythroid-specific protein GATA-1, the

ubiquitous CAAT-binding protein CP1 (also called NF-Y) and a DNA binding activity that was denoted DSFr (Delvoye et al., 1993). The CACCC box binds several factors *in vitro*, but the functional protein *in vivo* is the transcription factor EKLF. The ß-globin CACCC box has a higher binding affinity for EKLF than the ε or γ-globin CACCC boxes and mutations that substantially decrease the binding affinity of the CACCC box for EKLF produce a thalassemic phenotype (Donze et al., 1995).

The ß-globin gene has been reported to contain two enhancer elements, one located near the junction of the second intron and the third exon and another a 6 hundred base pairs downstream from the poly-A site of the gene (Antoniou et al., 1995; Behringer et al., 1987; deBoer et al., 1988; Wall et al., 1996).

2.6.2 Genetic regulation of ß-globin genes during erythropoiesis: transcription factors

The tissue- and developmental-specific expression pattern of the individual globin genes is achieved through the action of transcription factors on regulatory sequences that immediately flank the individual genes and on more distant sequences that are important for the regulation of all the genes of the locus. The factors that regulate globin genes are either tissue restricted or ubiquitous with respect to their expression pattern. Only a few factors have been studied in detail for their direct role in the transcription of the globin genes, these factors

bind to elements within the LCR and to β-globin gene promoters, although their functions are not necessarily the same in both contexts.

2.6.2.1 Transcriptional activator

A great deal of attention has been given to the genetic basis of the tissue- and developmental-specific expression of b-globin genes. Several transcriptional activators, limited in their own expression to erythroid cells and closely related myeloid cell types, have been identified and shown to be important for high-level β-globin expression; these factors bind to elements within the LCR and to β-globin gene promoters, although their functions are not necessarily the same in both contexts.

GATA-1

GATA-1 is a zinc finger transcription factor that is involved in the development of the megakaryocyte and mast cell lineage and plays a central role in erythroid development. It was first identified by its ability to bind functionally important DNA regulatory sequences found in globin genes (deBoer et al., 1988; Evans and Felsenfeld, 1989). Following the recognition that a common sequence motif [(A/T)GATA(A/G)] (GATA motif) exists within transcriptional regulatory regions at most, if not all, erythroid cell-specific genes (Evans and Felsenfeld, 1989), a dual zinc-finger transcription factor, GATA-1, that binds this motif (Ko and Engel, 1993; Merika and Orkin, 1993) was purified and cloned (Evans and Felsenfeld, 1989; Tsai et al., 1989) (Evans and Felsenfeld, 1989; Tsai et

al., 1989). This founding member of the GATA transcription factor family is expressed in erythroid, megakaryocytic, eosinophil, and mast cell lineages (Cantor and Orkin, 2005).

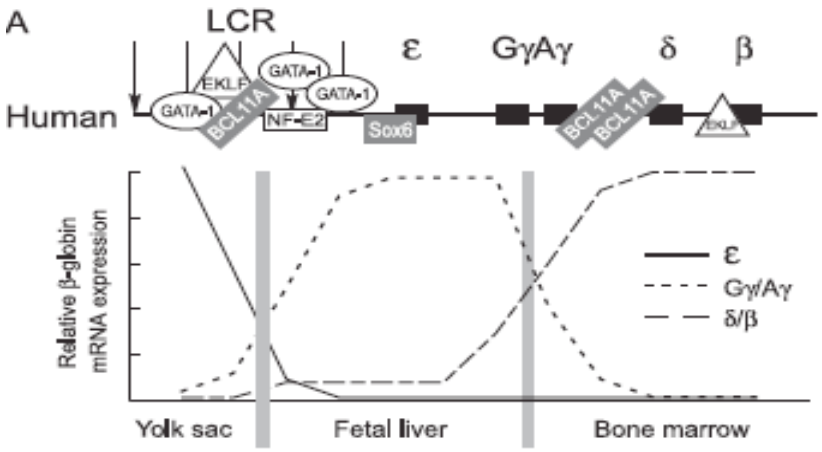

Figure 2.7 Developmental swiching and cofactors involvement at the human locus. The β-globin gene in human is depicted as black box, and arrows at the LCR (locus control region) represent DNase I hypersensitive sites. Binding sites that are occupied by transcriptional activator (white ovals, triangles, and rectangles) are represented at some of their know location. Gene loci and relative expression levels are not to scale.

GATA sites were also identified in the GATA-1 locus where DNase I hypersensitivity mapping of erythroid cell chromatin identified at least three regions (HS1-2-3). In particular, HS2 contains two high affinity GATA site, suggesting that GATA-1 protein plays an autoregulatory role in its own expression. Targeted disruption of *Gata1* in mice provided evidence for its essential function in stimulating erythropoiesis (Pevny et al., 1991,1995; Simon et al., 1992; Weiss et al., 1994). GATA-1

hemizygous male knock-out mice (GATA-1 is located on the X-chromosome) die at mid-embryonic gestation (E10.5) from severe anemia with arrest in erythroid maturation at a proerythroblast-like stage (Fujiwara et al., 1996. GATA-1 arrests cellular proliferation {Rylski, 2003 #193; Munugalavadla et al., 2005), and GATA-1-mediated survival of erythroid precursors involves induction of Bcl-$_{xL}$ expression (Gregory et al., 1999; Weiss and Orkin, 1995a). Differentiated *in vitro* GATA-1^{-} ES cells likewise fail to mature past the proerythroblast stage and undergo rapid apoptosis, indicating a role for GATA-1 in cell survival as well as maturation (Weiss and Orkin, 1995b). Loss of GATA-1 expression in megakaryocytes also leads to defects in maturation characterized by impaired endoreduplication and granule formation, disorganized platelet demarcation membrane synthesis and hyperproliferative growth (Shivdasani et al., 1997). In primitive erythroid cells, GATA-1 expression is regulated by a 5' enhancer, whereas its expression in definitive erythroid cells requires an additional element located in the first intron. Together, these two elements form the GATA-1 locus hematopoietic regulatory domain (HRD) (Shimizu et al., 2001).

GATA-1 contains two zinc fingers, both of the Cys-X2-Cys-X17-Cys-X2-Cys configurations. The C-terminal GATA-1 zinc finger (CF) recognizes GATA motifs (Martin and Orkin, 1990), whereas the N-terminal zinc finger (NF) enhances the specificity and stability of binding of the two-finger DNA binding domains to palindromic GATA recognition sequences (Trainor et al., 1996). Therefore, CF is necessary for GATA-1 function, while NF is required for definitive but not for

primitive erythropoiesis. This suggests that different GATA-1 functional domains are requires for target gene activation in primitive and definitive erythropoiesis (Shimizu et al., 2001). Thus, both transcriptional regulatory elements and protein functional domains may ensure proper lineage specification in primitive and definitive erythropoiesis.

Binding of erythroid-specific transcription factors, such as GATA-1, to enhancers of erythroid-specific genes early in development or differentiation could be a key factor in initiation and maintenance of active chromatin structures (Martin et al., 1996). GATA-1 orchestrates multiple programs of erythroid development, including control of the cell cycle and apoptosis, as well as differentiation. At the biochemical level, GATA-1 is thought to recruit critical proteins to target sites. The N-terminal zinc finger also directly binds FOG-1 (Crispino et al., 1999) and TRAP220 (Stumpf et al., 2006). GATA-1 also binds CBP/p300 (Blobel et al., 1998) (Blobel et al., 1998) and several transcription factors including PU.1 (Nerlov et al., 2000; Rekhtman et al., 1999), Sp1 (Merika and Orkin, 1995) and erythroid Kruppel-like factor (EKLF) (Merika and Orkin, 1995) (Fig 2.7). Furthermore, proteomics analysis of GATA-1 interactors identified diverse GATA-1-containing multiprotein complexes (Rodriguez et al., 2005). The interaction of GATA-1 with CBP/p300 suggests at least one mechanism by which histone acetylases might be brought to specific site (Blobel, 2000; Blobel et al., 1998). By modifying chromatin-bound histones or perhaps by modification of GATA-1 itself, acetylases could enhance transcriptional activity of erythroid-specific loci (Boyes et al., 1998).

Friend of GATA (FOG)

The FOG (Friend of GATA) family of proteins comprises a novel class of multi-type zinc finger nuclear polypeptides that interact physically with GATA factors and likewise serve essential functions in development. FOG-1, the founding member of this family, was identified through a yeast two-hybrid screening in which the amino zinc finger of GATA-1 was employed as bait (Tsang et al., 1997). The gene for FOG-1 encodes a 998-amino-acid polypeptide with nine predicted zinc fingers. Four of these zinc fingers (fingers 1, 5, 6, and 9) individually are able to mediate an interaction with GATA-1 (Fox et al., 1999). FOG-1 is expressed abundantly in erythroid and megakaryocytic cells and co-expressed with GATA-1 during embryonic development (Tsang et al., 1997). FOG-$1^{-/-}$ mice die in mid-embryonic gestation (10.5 to 11.5 days postcoitum) from severe anemia with an arrest in erythroid maturation at a stage similar to that observed with the GATA-1-null mice (Tsang et al., 1998). This has provided genetic evidence that FOG-1 and GATA-1 function through a common pathway in erythroid development. However, unlike the GATA-1^{-} mice, FOG-1 null mice failed to produce any megakaryocytes, suggesting that FOG-1 also has a GATA-1-independent role in early megakaryopoiesis. Point mutations of GATA-1 resulting in markedly reduced affinity for FOG-1 (but normal DNA binding activity), fails to rescue erythropoiesis in a GATA-1-deficient cell line (Crispino et al., 1999). A similar point mutation in humans leads to severe congenital dyserythropoietic anemia and thrombocytopenia (Nichols et al., 2000). Such patients have an overabundance of abnormal megakaryocytes

resembling GATA-1- megakaryocytes. Taken together, these findings demonstrate that a direct physical interaction between GATA-1 and FOG-1 is critical for normal erythropoiesis as well as late stages of megakaryopoiesis.

A second mammalian member of the FOG family (FOG-2) is expressed predominantly in heart, brain, lung, and gonadal tissues (Tevosian et al., 1999). FOG-$2^{-/-}$ mice die during embryogenesis from cardiac defects characterized by thin ventricular myocardium, Tetralogy of Fallot malformation, common atrioventricular valve and defective coronary vasculature (Tevosian et al., 1999). All GATA family members are capable of interacting with either FOG-1 or FOG-2 through conserved residues in their amino zinc fingers. Knock-in of a FOG non-interacting point mutation into the GATA-4 gene, one of the cardiac expressed GATA factors, results in embryonic death and a constellation of heart defects similar to that observed in the FOG-$2^{-/-}$ mice (Crispino et al., 2001). This indicates that interactions between GATA and FOG proteins are critical for multiple developmental processes.

Efforts have now been focused on understanding the mechanism by which FOG proteins influence GATA-mediated processes. Virtually all erythrocyte- and megakaryocyte-expressed genes contain GATA DNA-binding sites in both their promoters and enhancer elements (Orkin, 1992). Since a single FOG-1 molecule can potentially bind more than one GATA-1 molecule, FOG-1 might function as a molecular bridge between distant GATA-1-DNA complexes (Fox et al., 1999). This could bring distal enhancer elements into proximity of the promoter through DNA

looping, a mechanism invoked in models of enhancer-promoter interactions and also in the activity of the ß-globin LCR (Bulger and Groudine, 1999). However, mutants of FOG-1 that are capable of binding only a single molecule of GATA-1 rescue erythroid maturation from a FOG-1 deficient cell line as well as the wild type molecule, providing evidence against such a model in its simplest form (Cantor et al., 2002). Another possibility is that FOG provides a transcriptional activation domain either by itself or via interaction with another transcriptional coactivator. This predicts that protein domains outside of the GATA-binding zinc fingers would be required for its activity. However, FOG-1 molecules containing extensive and overlapping deletions spanning the entire molecule (but retaining at least one GATA-binding zinc finger) are also able to rescue erythroid maturation of a FOG-$1^{-/-}$ cell line (Cantor et al., 2002). This suggests that a simple interaction between FOG-1 and GATA-1 is by itself sufficient to activate GATA-1. This could occur through an allosteric change in GATA-1 or perhaps by the displacement of a repressor protein bound to GATA-1.

Nuclear Factor-erythroid 2 (NF-E2)

Nuclear Factor-erythroid 2 (NF-E2) was the second erythroid specific protein that was identified through DNA-binding studies *in vitro*. It was discovered through its binding to an AP-1 motif in the promoter of the porphobilinogen deaminase gene (Mignotte et al., 1989), and was somewhat ignored until it was shown that this factor could bind to the HS2 region of the ß-globin LCR (Ney et al., 1990). NF-E2 is expressed

in several hematopoietic lineages, including erythroid, mast, and megakaryocytic cells. It is also expressed in the intestine of anemic mice and may be involved in iron metabolism (Andrews et al., 1993). NF-E2 is a 45 kDa protein with a basic DNA-binding domain, an adjacent leucin zipper domain, an N-terminal proline-rich domain, and a cap'n collar (CNC) domain necessary for transcriptional activation (Bean and Ney, 1997). NF-E2 heterodimerizes with members of the family of small, ubiquitously expressed 18 kDa Maf proteins, which themselves have no transcriptional activation domain but which are essential for binding site recognition (Motohashi et al., 1997).

NF-E2 has been shown to play a vital role in ß-globin expression by facilitating the assembly of the preinitiation complex (Sawado et al., 2003; Sawado et al., 2001). In addition, NF-E2 is required to obtain the normal pattern of histone hyperacetylation and methylation of histone H3 lysine 4 (H3K4) at the active ß-globin promoter; the establishment of these modifications within the LCR, however, is independent of NF-E2 (Johnson et al., 2001). The overall stimulatory activity at the HS2 of the LCR in the chromatin environment of transgenic mouse or in stably integrated constructs in cells appears to depens on NF-E2 motifs (Caterina et al., 1994a; Caterina et al., 1994b; Ellis et al., 1993; Talbot and Grosveld, 1991). In particular, at HS2 of the LCR, NF-E2 participates in dynamic pattern of factor binding during erythroid differentiation. Prior to ß-globin gene activation and terminal erythroid differentiation, the MAREs (Maf responsive element or NF-E2 binding site) within HS2 are associated with a distinct factor composed of a small

Maf protein associated with the transcription factor Bach1. Bach1 may be able to maintain the locus in an open chromatin environment by serving as a ‘‘place-holder’’ for subsequent NF-E2 binding, but alone, it is not sufficient for ß-globin gene activation. As erythroid differentiation proceeds, the cell begins to synthesize large quantities of heme, which, in turn, associate with Bach1, leading to its ubiquitination and subsequent degradation. This would appear to be the basis by which NF-E2 is swapped for Bach1 at HS2, transforming a ‘‘potentiated’’ state into an active one (Fig. 3.3) (Igarashi et al., 1998; Zenke-Kawasaki et al., 2007). Furthermore, NF-E2 is critically involved in remodeling the nucleosome structure over the HS2 region, where it interacts with the tandem MARE sites (Armstrong and Emerson, 1996; Gong et al., 1996). In contrast with the study supporting a role for NF-E2 in ß-globin gene expression, p45 NF-E2-null mice had nearly normal levels of ß-globin protein, and deletion of HS2 had no significant effect on the timing or extent of expression of the gene (Fiering et al., 1995). Disruption of one of the genes coding for one of the Maf subunits shows no phenotype, most likely because of compensation by one of the other Maf proteins (Kotkow and Orkin, 1996).

Erythroid Kruppel-like factor (EKLF)

Erythroid Kru¨ppel-like factor (EKLF) is a transcription factor that was identified in a screen for erythroid-specific messenger RNA (Miller and Bieker, 1993) and is the most extensively studied stage-specific activator which is crucial for human ß-globin gene expression. EKLF interacts with

the ß-globin LCR and adult ß-globin gene promoter by binding to CACCC elements (Fig. 2.7), which are mutated in certain thalassemias. EKLF is required for the regulation of adult erythropoiesis, and is uniquely required for expression of adult ß-globin. It is, nevertheless, also expressed in primitive erythroid cells, where it has been shown to function in primitive erythropoiesis, activation of additional gene targets, and repression of megakaryopoiesis (Hodge et al., 2006; Lohmann and Bieker, 2008; Sengupta et al., 2008). EKLF is also required for nuclear colocalization of the LCR and the adult ß-globin genes; such colocalization is commonly thought to underlie the ability of the LCR to activate ß-globin transcription, despite its physical location far from the ß-globin gene promoters (Drissen et al., 2004). Targeted disruption of the EKLF gene in mice also results in lethal ß-thalassemia. EKLF knock-out mice die from severe anemia at the fetal stage due to failure of adult ß-globin gene activation. $EKLF^{-/-}$ mice containing a complete human ß-globin locus transgene have reduced levels of ß-globin, but elevated levels of γ-globin expression, compared to wildtype mice containing the same transgene (Perkins et al., 1996; Wijgerde et al., 1996). Taken together, these experiments suggest that EKLF participates in the switch from embryonic/fetal globin to adult ß-globin expression in humans. Moreover, loss of EKLF leads to the lost of the nuclease HSs that mark HS3 of the LCR and the adult ß-globin promoter. An erythroid-specific SWI/SNF-like chromatin remodeling complex, termed E-RC1, can be recruited by EKLF via direct interaction between EKLF and the SWI2/SNF2-like ATPase BRG1, which may be the basis for HS

formation (Armstrong et al., 1998; Kadam et al., 2000). The molecular basis for the stage-specific activation of the adult ß-globin gene by EKLF is unclear, especially given that the factor is present and functional in other contexts in primitive erythrocytes, but it has been proposed that these post-translational modifications are a possible mechanism by which such specificity might be achieved (Fig. 2.7). Reintroducing EKLF into an EKLF-null erythroid cell line, which harbors a copy of the human ß-globin locus, resulted in enhanced differentiation and hemoglobinization, as well as reduced proliferation. This may point to a role for EKLF in cell cycle regulation and hemoglobinization, in addition to regulation of ß-globin gene expression (Coghill et al., 2001).

The Fli-1 oncogene

A member of the Ets family of transcription factors, Fli-1, was identified in Friend virus-induced erythroleukemia and affects the self-renewal of erythroid progenitor cells (Howard et al., 1993). In pluripotent human hematopoietic cells, differentiation is followed by reduced Fli-1 expression and over expressing Fli-1 inhibits erythroid differentiation, impairs the cells' ability to respond to specific erythroid inducers such as hemin, and reduces the levels of GATA-1 (Athanasiou et al., 2000). In the erythroblastic cell line HB60, Fli-1 expression is downregulated by erythropoietin (Epo), which induces terminal erythroid differentiation. Constitutive expression of Fli-1 blocks Epo-induced differentiation and enhances cell proliferation in HB60 cells, suggesting that Fli-1 targets erythroid cells to either proliferation or differentiation, in response to Epo

(Tamir et al., 1999). Fli-1 binds a cryptic Ets consensus site within the retinoblastoma (Rb) gene promoter, repressing Rb expression, which results in impaired terminal erythroid maturation and continuous presence of nucleated erythrocytes in peripheral blood (Lee et al., 1992). Negative regulation of Rb by Fli-1 could destine erythroid progenitors to self-renewal, while Epo-induced repression of Fli-1 expression will enable differentiation (Tamir et al., 1999).

PU.1

The putative oncogene Spi-1 (PU.1) protein product is a hematopoietic-specific Ets factor, promoting differentiation of lymphoid and myeloid lineages (Scott et al., 1994a) (Scott et al., 1994b). PU.1 expression in erythroid progenitors can induce erythroleukemia in mice. Like Fli-1, PU.1 blocks erythroid differentiation and restoration of terminal erythroid differentiation in murine erythroleukemia (MEL) cells requires PU.1 suppression (Rekhtman et al., 1999).

PU.1 can interact directly with GATA-1 and repress GATA-1 mediated transcriptional activation. Both the PU.1 DNA binding domain and transactivation domain are required for GATA-1 suppression and for blocking terminal differentiation in MEL cells. PU.1 does not seem to affect binding of other factors, such as FOG, to GATA-1, nor does it prevent GATA-1 DNA binding (Rekhtman et al., 1999). It is likely that PU.1 binds to assembled, DNA-bound GATA-1 complexes and represses their activity. Ectopic expression of PU.1 in *Xenopus* embryos blocks erythropoiesis. Exogenous GATA-1 is able to relieve this blockage of

erythroid differentiation in MEL cells as well as in *Xenopus* embryos and explants, suggesting that lineage commitment decisions are regulated by their relative levels.

2.6.2.2 Transcriptional repressor

In transgenes in mice, consisting of human embryonic ε-globin or fetal γ-globin genes linked individually to the human ß-globin LCR, developmental silencing occurs normally (Dillon and Grosveld, 1991; Raich et al., 1990). This demonstrates that promoter-proximal sequences alone are sufficient for silencing, and suggests that silencing is an active process. The identities of trans-acting factors involved in the developmental silencing of ε- and γ-globin genes have been elusive, however, until recently.

BCL11A

BCL11A is a multizinc finger transcription factor with known roles in lymphocyte development, which functions at the human ß-globin locus as a transcriptional repressor. This factor was originally linked to γ-globin levels in humans by a genome-wide association strategy (Thein et al., 2009); subsequently, it was found that sequence variants in the BCL11A gene directly influence the levels of fetal hemoglobin in adult humans (Lettre et al., 2008)). In adult erythroid cells, BCL11A is expressed in 2 major isoforms, and it has been shown to associate with the erythroid transcription factors GATA-1 and FOG-1, as well as the chromatin remodeling NuRD complex (Sankaran et al., 2008). The induction of fetal

hemoglobin in the human embryonic/fetal cell line K562 correlates with reduced levels of BCL11A, and the overexpression of BCL11A in this cell line results in an approximately 50% reduction in fetal hemoglobin transcription (Chen et al., 2009). Small interfering RNA knockdown of BCL11A results in an increase in γ-globin RNA, without affecting the expression of other erythroid-specific proteins, such as GATA-1, FOG-1, NF-E2, and EKLF. BCL11A was not detected at the γ- or ß-globin promoters by ChIP, but was significantly enriched at HS3 of the LCR and at 2 sites between the Aγ- and δ-globin genes that were previously implicated in γ-globin gene silencing (Sankaran et al., 2008). A chromosome conformation capture (3C) assay shows that BCL11A reconfigures the ß-globin cluster by modulating chromosomal loop formation. BCL11A and the HMG-box-containing transcription factor SOX6 interact physically and functionally during erythroid maturation (Xu et al.).

Sox6

Sox6 is a member of a family of sry-related transcription factors, and was recently found to function during erythroid development. Sox6 is expressed at high-levels in skeletal and cardiac muscle, cartilage, testis, and neuronal tissue, and Sox6-null mice die perinatally due to heart defects (Hagiwara et al., 2000). More recently, however, it has been shown that Sox6 is expressed specifically in definitive erythroid cells, not in yolk-sac-derived primitive erythrocytes, and that Sox6-null mice exhibit elevated levels of the embryonic ε- and γ-globin (Yi et al., 2006).

Sox6 has been shown to bind to the εγ-globin gene promoter and to repress transcription from this promoter in reporter assays. Mice that were irradiated and then engrafted with embryonic liver stem cells from Sox6-deficient mice had high-levels of εγ-globin in their bone marrow, spleen, and circulating blood (Cohen-Barak et al., 2007). Moreover, Sox6-null mice exhibit an erythroid phenotype characterized by anemia and altered red blood cell morphology and maturation (Dumitriu et al., 2006a). Taken together, these results imply that Sox6 is a crucial factor involved in definitive erythropoiesis, and is required for the silencing of the embryonic εγ-globin gene in the adult. Recently Orkin et al., demonstrated that BCL11A and SOX6 co-occupy the human ß-globin cluster along with GATA-1, and cooperate in silencing γ-globin transcription in adult human erythroid progenitor (Xu et al.). (See 2.7.4)

2.6.2.3 Putting the puzzle together: a model for ß-globin gene regulation

A four-step model for human ß-globin gene regulation has been suggested (Levings and Bungert, 2002). The first step involves partial unfolding of globin chromatin structure and generation of a highly accessible LCR. It is mediated by erythroid-specific proteins, which bind to sequences throughout the globin locus. GATA-1, which is known to associate with histone acetyl-transferases, may be involved in this step. The disruption of the LCR chromatin structure allows binding of transcription factors such as EKLF, GATA family members and the HLH proteins to the LCR HS sites, and the recruitment of chromatin-remodeling complexes and

coactivators. In the third step, chromatin domains permissive for transcription are being established. Intergenic transcription was suggested to modify chromatin structure of an active gene domain, distinguishing it from an accessible but inactive one, that way separating the globin genes into developmental stage-specific chomatin domains. Finally, transcription complexes are being transferred from the LCR to individual globin gene promoters within transcriptionally permissive domains, allowing the developmental stage-specific pattern of globin gene expression.

2.7 THE SOXD subfamily: SOX5, SOX6 and SOX13

2.7.1 Structure and molecular function

The Sox family is comprised of 20 genes. These genes encode transcription factors with a high-mobility-group (HMG) box DNAbinding domain highly similar to that of the sex-determining region (Sry) protein. According to sequence identity inside and outside this domain, the Sox genes are classified into 8 groups, A to H. The SoxD group is composed of 3 genes – Sox5, Sox6, and Sox13 – in most vertebrates, and 1 gene in the D. melanogaster fly (Sox102F) and other invertebrates. They are highly conserved in the family-specific high-mobility-group (HMG) box DNA-binding domain and in a group-specific coiled-coil domain. The SoxD genes have overlapping expression and cell-autonomously control discrete lineages. Their gene and protein structures are highly identical to each other, but they are related to other Sox genes and proteins only in the HMG box. The human SOX5 and SOX6 genes are located in paralogous

chromosomal regions on 12p12.1 and 11p15.3–15.2, respectively, and are more closely related to each other than to SOX13, located on 1q32. The 3 genes have 12–16 coding exons. They are spread across 300–400 kb of genomic DNA in the case of SOX5 and SOX6, but across 12 kb only in the case of SOX13. With molecular weights of 48–89 kDa, the SoxD proteins are among the largest Sox proteins. They harbor two highly conserved functional domains. The family-specific HMG box DNA binding domain is located in the C-terminal half of the proteins. It is 87% identical among all human and mouse SoxD proteins, and less than 60% identical to that of other Sox proteins. The other domain is a group-specific coiled-coil; it is located in the N-terminal half of the proteins, and is 76% identical among all human and mouse SoxD proteins. The proteins display only short stretches of identity outside these two domains. Both Sox5 and Sox6 are expressed as short transcripts (2 and 3 kb, respectively) in adult testis and as long transcripts (6 and 8 kb, respectively) in other tissues. Both Sox6 transcripts appear to encode the full-length protein, but the short Sox5 transcript encodes a protein isoform that lacks the Nterminal half of the full-length protein. This protein was the first to be discovered, and was thus named Sox5. The full-length protein was originally named L-Sox5 or Sox5-L (Hiraoka et al., 1998; Lefebvre et al., 1998), but for simplicity most authors have referred to it as Sox5 because this long Sox5 isoform is structurally and functionally equivalent to Sox6 and Sox13.

The SoxD HMG box domain preferentially binds DNA sequences featuring an AACAAT motif in electrophoretic mobility shift assay

(EMSA) in vitro (Connor et al., 1994). The coiled-coil domain mediates homodimerization as well as heterodimerization of the SoxD proteins with each other (Lefebvre et al., 1998). It blocks binding of the full-length proteins to single recognition sites (Roose et al., 1998; Takamatsu et al., 1995), but strongly enhances binding to pairs of recognition sites (Lefebvre et al., 1998). The proteins efficiently bind in vitro and in vivo to sites harboring one or two mismatches in the preferred site. Moreover, they have little predilection for the relative orientation of the paired sites and for the length of the intervening sequence, from 0 to at least 19 bp (Han and Lefebvre, 2008).

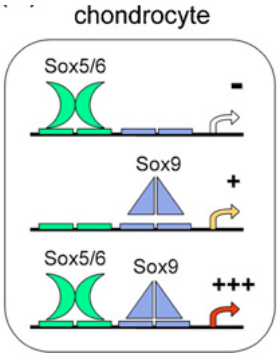

Figure 2.8 Sox5 and Sox6 cooperate with Sox9 in transactivating chondrocyte-specific genes. They bind to recognition sites nearby, but distinct from those of Sox9.

They are thereby more flexible than Sox9 and other Sox proteins in choosing DNA sequences. Taking such flexibility into account, putative SoxD-binding sites can be found in virtually any promoter or DNA regulatory region. It is therefore mandatory that solid experimentation be

performed to ascertain such sites as actual targets of SoxD proteins in vivo. The SoxD proteins have no known transactivation or transrepression domain, but they do participate in transcriptional activation and repression (Fig. 2.8). For instance, Sox5 and Sox6 synergize with Sox9 in activating many chondrocyte-specific extracellular matrix genes (Han and Lefebvre, 2008; Lefebvre et al., 1998). They bind to sites distinct from those of Sox9 on enhancers in these genes, and facilitate Sox9 DNA-binding through an as yet unknown mechanism. In contrast, Sox5 and Sox6 interfere with the activation of myelin genes by Sox9 and Sox10 in oligodendrocytes and with the activation of the Mitf and Dct marker genes by Sox10 in melanocytes (Stolt et al., 2008; Stolt et al., 2006) (Fig. 2.9). They do so by competing with these proteins for binding to Sox recognition sites in the promoter of differentiation markers, and they thereby block transactivation. Sox5 and Sox6 have been shown in vitro to be able to induce transrepression by directly binding to the CtBP2 corepressor (Iguchi et al., 2007; Stolt et al., 2008).

Sox6 and Sox13 have also been shown in vitro to be able to block canonical Wnt signaling in T cells and pancreatic beta-cells, respectively, but through different mechanisms (Iguchi et al., 2007) (Fig. 2.9). While Sox6 can physically interact with beta-catenin, Sox13 can physically interact with the Tcf1 transcription factor. These data thus show that the SoxD proteins can act as either positive or negative modulators of transcription and might do so through various mechanisms. In vivo confirmation of some of these mechanisms and additional biochemical studies are needed to fully uncover the mechanisms underlying these

activities. Additional studies are also needed to elucidate whether the differential activities of the SoxD proteins are dictated primarily by the nature of each specific target gene or by the molecular context characterizing each specific cell lineage.

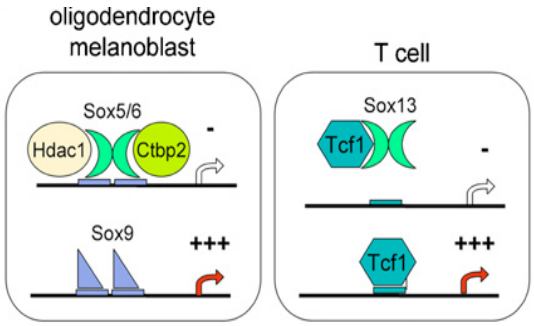

Figure 2.9 SoxD proteins repress transcription through various mechanisms. They compete with SoxE proteins in binding to recognition sites on oligodendrocyte- and melanocyte-specific genes, and recruit co-repressors. Sox13 interacts with Tcf1 in T cells, thereby preventing it from binding to target genes.

2.7.2 Expression and regulation

Like most Sox genes, each SoxD gene is expressed in a limited subset of cell types. Both Sox5 and Sox6 are highly expressed in spermatids, neurons, oligodendrocytes, and chondrocytes (Connor et al., 1995; Denny et al., 1992; Lefebvre et al., 1998; Stolt et al., 2005). Sox5 and Sox13 are co-expressed in pancreatic epithelial cells (Lioubinski et al., 2003). Sox5 alone is expressed in melanoblasts (Stolt et al., 2008) (Stolt et al., 2008), Sox6 in erythroid cells (Dumitriu et al., 2006a; Yi et al., 2006) and skeletal myoblasts (Shirai et al., 2005), and Sox13 in arterial

walls, kidney, and liver (Roose et al., 1998). The mechanisms underlying these specific expression patterns of the SoxD genes are virtually unknown. Sox5 and Sox6 are co-expressed with SoxE genes in chondrocytes, oligodendrocytes, and melanocytes, and require these genes for expression, but it is not known whether the SoxE proteins directly activate the SoxD genes. Similarly, data regarding the possibility that the SoxD proteins are controlled at the translational and post-translational levels are still missing. The proteins are nuclear in all cells that expressed their RNA, ruling out all but little regulation at the translational and nuclear translocation levels. Important directions of future research are thus to uncover themechanisms whereby the SoxD genes and their protein products are regulated.

2.7.3 Biological function

Key biological functions for the SoxD genes have been revealed through gene inactivation in the mouse. The first function to be discovered was that of Sox5 and Sox6 in chondrogenesis (Smits et al., 2001). Inactivation of Sox5 causes respiratory distress leading to death upon birth due to a cleft secondary palate and small thoracic cage, whereas inactivation of Sox6 is only occasionally lethal at birth, and skeletal defects are limited to a short sternum. Double inactivation of the genes, in contrast, is lethal 3 days before birth, apparently due to circulatory failure. The embryos have chondrocytes, but the cells fail to overtly differentiate and proliferate. Skeletal growth and ossification are thus severely impaired. As mentioned earlier, a main function for Sox5

and Sox6 in chondrocytes is to boost the ability of Sox9 to activate major chondrocyte markers. Sox5 and Sox6 also have redundant roles in oligodendrocytes, but their roles are very different from those in chondrocytes, as they repress specification and terminal differentiation, and influence migration patterns (Stolt et al., 2006). Their mechanism of action is also different, as they directly interfere with, rather than enhance, SoxE-mediated gene activation.

As expected from their expression pattern, each SoxD gene also has non-redundant functions. Sox5 is dispensable for melanogenesis, but its loss partially rescues the strongly reduced melanoblast generation and marker gene expression occurring in Sox10 heterozygous mice. Sox5 recruits CtBP2 and HDAC1 and binds to the regulatory regions of Sox10 target genes, thereby directly inhibiting Sox10 activity. Sox5 also ensures proper development of specific neuronal cell types by controlling the timing of critical cell fate and differentiation decisions (Kwan et al., 2008; Lai et al., 2008). It acts at least in part by directly binding and downregulating such genes as Fezf2 and Bcl11b. Finally, Sox5 overexpression studies in the chick embryo have suggested that Sox5 may also promote generation of the neural crest (Perez-Alcala et al., 2004).

Sox13-null mice are born alive and show no overt defects, but they rapidly develop severe growth abnormalities (Melichar et al., 2007). The reason is yet unknown. The analysis of fetuses with Sox13 gain-of-function and loss-of-function mutations has revealed that Sox13 plays a critical role in the emergence of gammadelta T cells in the thymus,

while opposing alphabeta T cell differentiation. It acts at least in part by inhibiting canonical Wnt signaling.

2.7.4 Sox6

Sox6 is known to be highly expressed in neuronal cells, chondrocytes, notochord and spermatid cells, and weakly in muscle cells (Connor et al., 1995; Hagiwara et al., 2000; Lefebvre et al., 1998; Takamatsu et al., 1995). $Sox5^{-/-}$ $Sox6^{-/-}$ fetuses develop severe skeletal dysplasia (Lefebvre et al., 1998; Smits et al., 2004; Smits and Lefebvre, 2003; Smits et al., 2001) as Sox5 and Sox6 redundantly promote chondrocyte proliferation, differentiation, and maturation and also promote notochord cell survival and maturation. $Sox6^{-/-}$ mice have mild skeletal defects. About half die at birth and the others fail to thrive after postnatal day 7 (P7) and die at approximately P14. The cause of death remains unclear. Mice with a chromosomal inversion (p100H) disrupting Sox6 have the same gross phenotype as $Sox6^{-/-}$ mice. They were shown to develop cardiac and skeletal myopathy, suggesting that Sox6 might promote myocyte maturation (Hagiwara et al., 2000; Hagiwara et al., 2005).

It has been also demonstrates that Sox6 is an important in erythropoiesis. $Sox6^{-/-}$ fetuses had many nucleated RBCs and liver hyperplasia and that Sox6 likely controlled erythropoiesis directly. Nucleated RBCs indeed are often a sign of anemia or hypoxia, but $Sox6^{-/-}$ fetuses were not pale or cyanotic. $Sox6^{-/-}$ pups had high RDW (Red blood cell Distribution Width) even though they compensated for

anemia, suggesting an intrinsic RBC problem. Finding high expression of Sox6 in proerythroblasts and erythroblasts and weak or no expression in erythroid precursors and other erythropoietic tissue cells strengthened this notion. The erythroid-specific inactivation of Sox6 provided definitive demonstration that Sox6 has cell-autonomous roles in erythroid cells. Sox6 acts in erythroid cells after activation of EpoR, thus after commitment to the erythroid lineage. Thus, in contrast to such factors as Gata-1 and Fog-1, Sox6 does not control lineage commitment. Sox6 was found similarly expressed in fetal liver, neonatal spleen, and bone marrow, which is consistent with roles in definitive erythropoiesis throughout life.

Inactivation of Sox6 did not block erythropoiesis because Sox6 works in erythroid cells with other proteins (Dumitriu et al., 2006a). Sox5, the closest relative of Sox6, is coexpressed with Sox6 in many cells but was not found expressed in erythropoietic tissues.

Accordingly, $Sox5^{-/-}$ mice had normal RBCs and $Sox5^{-/-}Sox6^{-/-}$ mice had the same erythroid phenotype as $Sox6^{-/-}$ mice.

2.7.4.1 Sox6 and EPO

The functions of Sox6 in erythroid cells thus resemble those of Sox5/Sox6 in chondrocytes and notochord cells (Roose et al., 1998; Smits and Lefebvre, 2003) in that it is not required for lineage commitment but boosts development along the cell differentiation pathway. So Dumitriu et al. demonstrated that Sox6 acts along the erythroid pathway by promoting proerythroblast survival, which is one

of Epo's primary functions, and found that Sox6 enhances this Epo function (Dumitriu et al., 2006a). Sox6$^{-/-}$ proerythroblasts were dying at the same rate as controls without Epo, not at a higher rate as would be expected if Sox6 and Epo were acting independently (Dumitriu et al., 2006a). Moreover, Sox6$^{-/-}$ proerythroblasts responded less efficiently than control cells to Epo, thus demonstrating that Sox6 enhances Epo's function by modulating expression of genes involved in Epo signaling and acting as targets (gene and/or protein) of Epo signaling. Therefore, Sox6 stimulates proerythroblast and erythroblast proliferation, acting here in parallel with Epo. Sox6$^{-/-}$ cells were indeed proliferating more slowly than controls without Epo and increased their proliferation rate in parallel with controls in response to Epo.

2.7.4.2 The role in erythroid cells

Sox6 has also a critical role for Sox6 in erythroid cell maturation but molecular mechanisms involved in this process remain largely unknown (Dumitriu et al., 2006a).

This role was suggested in vivo by the abundance of nucleated RBCs in Sox6 $^{-/-}$ fetuses, abnormal maturation and reduced survival of Sox6$^{-/-}$ RBCs, and high proportion of erythroblasts in Sox6$^{-/-}$ erythropoietic tissues. It was proven in vitro by showing that Sox6$^{-/-}$ erythroblasts started to differentiate as early as control cells but, in contrast to control cells, maintained a high proliferation rate and RNAlevel of erythroblast markers (Dumitriu et al., 2006a). No evidence of increased cell death was found in mutant erythroblasts, confirming that the increased ratio

of early/late erythroid cells in mutant cultures was due to a maturation delay or block.

Moreover, Sox6 controls erythroid maturation at least in part through F-actin. actin is important in cellular processes, including proliferation, transcription, and chromatin remodeling (Rando et al., 2000); erythroid cell enucleation (Koury and Bondurant, 1990); and RBC cytoskeleton integrity. $Sox6^{-/-}$ cells did not accumulate as much F-actin at the proerythroblast stage as controls and maintained less F-actin during erythroblast maturation. F- Similar levels of F-actin RNA were found in control and $Sox6^{-/-}$ cells, suggesting that Sox6 may control genes involved in F-actin assembly or stability (Dumitriu et al., 2006a).

In conclusion, Sox6 has key roles in definitive erythropoiesis. Sox6 amplifies the function of erythropoietin in promoting erythroid cell survival and stimulating cell proliferation. It also facilitates terminal maturation. It thereby enhances the rate, quantity, and quality of red blood cell production. Therefore, Sox6 could be involved in some forms of erythroleukemias and inherited anemia diseases and that Sox6 could be used as a new tool to stimulate erythropoiesis in the treatment of various diseases with anemia. Despite Sox6 promotes cell survival and proliferation in synergy with erythropoietin signaling and acts beyond erythropoietin signaling to facilitate erythroid maturation, the $Sox6^{-/-}$ cultures were hemoglobinizing slowly. Erythroid cells continue to accumulate hemoglobin actively after condensation and enucleation, it is likely that the slow hemoglobinization of $Sox6^{-/-}$ erythroid cells primarily reflects their maturation defect (Dumitriu et al., 2006a). There

is higher ratio of embryonic/adult globin chains in $Sox6^{-/-}$ RBCs than in controls. It seems that Sox6 represses expression of embryonic globin genes in erythroid cells at transcriptional level by binding to consensus sites in their proximal promoter (Yi et al., 2006) (Yi et al., 2006). The observation that Sox6-deficient mice ectopically express εγ genes in liver, where definitive erythroid cells mature, suggests that Sox6 has key roles in definitive erythropoiesis, directly contributes to repress embryonic globin genes (Yi et al., 2006) (Yi et al., 2006). A recent work in human erythroid progenitors suggests that variation in the level of Sox6 correlates with γ-globin gene expression (Sripichai et al., 2009).

While a precise role for Sox6 in hemoglobin switching remains to be determined, Sox6 appears to act as either an activator or a repressor, depending on its interacting proteins and promoter context (Lefebvre et al., 1998; Murakami et al., 2001). In addition, Sox transcription factors bind to the minor groove of DNA and cause a drastic bend of the DNA that leads to local conformational changes (Ferrari et al. 1992; Connor et al. 1994). Therefore, it has been suggested that Sox6 may perform part of its function as an architectural factor by organizing local chromatin structure and assembling other DNA-bound transcriptional factors into biologically active, sterically defined multiprotein transcriptional complexes (Yi et al., 2006).

Sox6 is coexpressed with BCL11A during erythroid development, interacts physically with BCL11A and GATA1. The coexpression of BCL11A and SOX6 during erythroid differentiation suggests that these

factors, both of which have been described previously as mediators of hemoglobin switching and silencing of mouse embryonic globin gene expression (Sankaran et al., 2009; Yi et al., 2006), may function together in regulating globin switching in a protein complex. Moreover, Sox6 interacts directly with BCL11A and Gata-1 under physiological conditions and cooccupies the human β-globin cluster in vivo in human erythroid progenitors. In particular, Sox6 interacts with BCL11A through multiple domains, likely through multiple zinc fingers, similar to the interactions between Gata-1 and Fog1 (Cantor and Orkin, 2005). Importantly, Sox6 strongly binds the Aγ and Gγ proximal promoters, reminiscent of the role of Sox6 in silencing mouse εγ-globin expression by association with its proximal promoter (Yi et al., 2006). In contrast, BCL11A does not detectably bind to γ-promoters. Furthermore, these two proteins physically interact and likely associate within a protein complex (or complexes) and this suggest that Sox6 may act as a partner of BCL11A in silencing γ-globin expression. These data also suggest that Sox6, by interacting with γ-proximal promoters, may help recruit BCL11A to the proximal regions of the γ-genes during hemoglobin switching and they may function collaboratively in their silencing (Sankaran et al., 2009; Yi et al., 2006). Moreover, depleting the expression of both SOX6 and BCL11A leads to substantial derepression of mouse embryonic β-like globins in MEL cells and reactivation of g-globins in adult human erythroid precursors. These results suggest that involvement of both BCL11A and Sox6 is preserved during mammalian

evolution; however, their exact roles in cellular function, such as silencing of the embryonic or HbF expression, can be divergent.

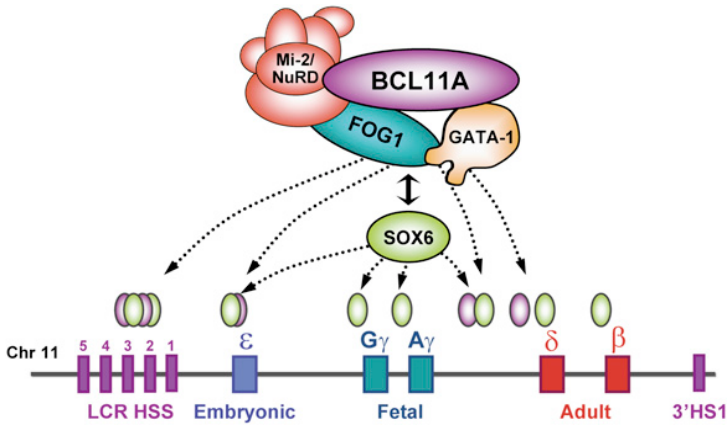

Figure 2.10 Model of BCL11A-mediated silencing of γ-globin genes. The diagram illustrates the physical interaction between BCL11A and the Mi-2/NuRD complexes, erythroid transcription factors GATA1 and FOG1, and the HMG-box protein SOX6. Rather than binding to the promoters of the g-globin or β-globin genes as these latter factors do, BCL11A protein occupies the upstream LCR and γδ-intergenic regions of the b-globin cluster in adult human erythroid progenitors. Our study suggests that transcriptional silencing of γ-globin expression by BCL11A involves long-range interaction within the β-globin cluster and local interactions with the chromatin associated SOX6 proteins at the proximal promoters of the γ-globin genes. (Xu et al., 2010).

Moreover it seems that some evolutionarily conserved transacting factors may perform divergent regulatory functions among different species. While the precise role of Sox6 in repressing mouse embryonic β-like globins and human γ-globins merits further study, Sox6 can act as either an activator or a repressor, depending on its interacting proteins and

promoter context (Lefebvre et al., 1998; Murakami et al., 2001). The physical interaction between BCL11A and Sox6 may help recruit BCL11A and NuRD repressor complexes to the proximity of γ-globin genes. Thus, Sox6 may act as an important cofactor of BCL11A in mediating globin switching and silencing of γ-globin transcription. BCL11A mediates silencing of γ-globin genes through both long-range interaction within the human β-globin cluster and local interactions with the chromatin-associated Sox6 proteins at the proximal promoters of the γ-globin genes (Fig. 2.10).

3. α-GLOBIN GENE EXPRESSION DURING ERYTHROPOIESIS

3.1 The α-globin gene locus

The human α-globin locus locates very close to the telomere of the short arm of chromosome 16 and lies immediately adjacent to several ubiquitously expressed genes. This cluster spans 26 kb and includes 3 functional α-like protein coding genes (ζ,α2 and α1), 2 expressed genes with unknown function (μ and θ1) and 3 pseudogenes (ψζ1, ψα1 and ψρ) (Figure 3.1) (Higgs, 1998). The three pseudogenes all carry inactivating mutations and are, therefore, not expressed.

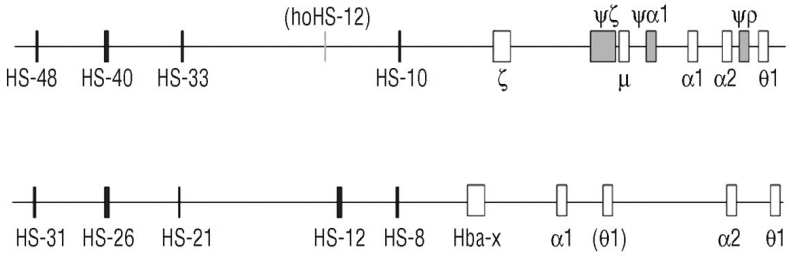

Figure 3.1 Structure of the human (upper part) and murine (lower part) α-globin locus. The genes are depicted as boxes and the DNase I hypersensitive sites (HSs) are shown as vertical arrows. In the the human α-globin locus four conserved DnaseI hypersensitive site known to bind erythroid transcription factors are located between 10-50 kb upstream of the start of the ζ-globin gene. The genes (and pseudogenes) of the α-globin cluster are arranged 5' to 3' in the order of developmental expression. The murine a-globin locus contains an additional DnaseI hypersensitive site (HS-12) which contributes to regulation of murine α-globin expression.

Expression of μ-globin was higher than θ or ζ but much lower than α-globin Like θ-globin, μ-globin demonstrates a highly regulated pattern of expression with no detectable transcription in non-erythroid tissues. Both

transcripts also yield no detectable protein product and deletions of θ or μ have no reported effects on clinical phenotypes so their function remains undefined (Goh et al., 2005). Of the highly expressed functional α-genes, ζ-globin constitutes the embryonic α-gene while α2 and α1 are the dominant α-genes throughout all other stages of development. Expression of ζ-globin is limited to the yolk-sac of primitive erythroblasts then silenced in the foetal liver concordant with the selective induction of α-globin expression (Liebhaber and Russell, 1998). This globin switch is dependent on two major factors; the transcriptional inactivation of ζ-globin (Liebhaber, 1997) and the selective destabilization of ζ-globin mRNA in fetal and adult erythroblasts (Liebhaber and Russell, 1998; Russell et al., 1998). Though α2 and α1 encode an identical protein, α2 is expressed at levels 2-3 times higher than α1 at both the transcriptional and translational level (Albitar et al., 1992; Liebhaber et al., 1986). The embryonic and adult α-genes share 58% homology at the amino acid level (Hughes et al., 2005) and it appears that ζ-globin can be exchanged for α-globin in an adult organism without significant deleterious effects. Interestingly, exchanging ζ-globin for α-globin in mice expressing an abnormal sickle β-globin gene, prevents erythrocyte sickling and improves the phenotype (He and Russell, 2004).

3.2 The α-globin regulatory elements

The α-globin regulatory elements include four conserved DnaseI HS sites which lie between 10 and 50 kb upstream from the start of the ζ-globin

gene (Hughes et al., 2005; Tufarelli et al., 2004). A recent study by De Gobbi et al. utilized tiled microarrays to analyze a 220 kb region surrounding the α-like genes (Higgs et al., 1989). The study confirmed that the four known DnaseI HS sites bind erythroid-specific transcription factors and found no additional erythroid-specific regulatory elements in this region. Of these four sites (HS-10, HS-33, HS-40 and HS-48) (De Gobbi et al., 2007; Flint et al., 2001), HS-40 forms the major regulatory element15-17 and is the only element capable of directing high level expression of the α-globin gene (Sharpe et al., 1992). Deletion of HS-40 from the human α-globin cluster leads to severe reductions in a-globin expression to <5% of normal (Higgs, 1998; Viprakasit et al., 2006). Analysis of the other elements in transgenic mice indicate that combinations of HS-10, HS-33 and HS-48 in small constructs are able to direct tissue and developmental stage specific expression but are unable to drive substantial levels of α-globin expression (Higgs et al., 2005). This has been further confirmed in a newly identified mutation in the enhancer which resulted in an α-thalassemia phenotype. The mutation involved the deletion of a 16 kb region including the HS-40 and HS-48 while leaving HS- 33 and HS-10 intact. Analysis of the affected chromosome indicated that α-globin expression was reduced to <1% of normal thus demonstrating that HS-33 alone or in combination with HS-10 has little or no positive effect on α-globin expression (Viprakasit et al., 2006). Furthermore, transgenic mice created with a human BAC containing only HS-48 but lacking all other conserved HS sites did not

express any detectable levels of human α-globin at any stage during development, strongly indicating that HS-48 alone is also unable to drive substantial expression of a-globin (Tang et al., 2008). It is interesting that though these four HS sites are conserved between mouse (HS-31, HS-26, HS-21 and HS-8) and human, the deletion of the mouse HS-26 (homologous to human HS-40) only results in a 50% reduction in mouse a-globin expression (Anguita et al., 2002). Analysis of the mouse α-globin locus revealed the presence of an additional HS-12 element capable of binding the pentameric erythroid complex essential for activating α-globin transcription while the human homologous region lacks these critical binding sites. In addition, the mouse α-globin promoter also binds GATA1, a crucial erythroid regulator, while the human promoter which lacks the consensus-binding site, does not (De Gobbi et al., 2007).

3.3 Transcriptional activation of α-globin

The binding of transcription factors in the pentameric erythroid complex to HS sites in the α-globin locus is essential for high level expression of α-globin (De Gobbi et al., 2007; Vernimmen et al., 2007). This complex (GATA-1, SCL, E2A, LMO-2 and Ldb-1) and other factors such as NF-E2, is thought to recruit Pol II to the α-globin locus then facilitate the transfer of Pol II to the promoters (Vernimmen et al., 2007). This is the final step in the activation of α-globin transcription which begins with a range of chromatin modifications. Priming of the α-globin cluster begins

in multipotent hematopoietic progenitor cells (Anguita et al., 2004). In mouse cells, the four main erythroid specific HS sites appear at this stage of differentiation concurrent with initiation of histone H3 and H4 acetylation. As cells differentiate further down the erythroid lineage, a number of other sites also become hypersensitive to DNaseI, including the α-globin promoter, while the domain of acetylation extends to become fully developed in mature erythroblasts (Anguita et al., 2004). In primary human erythroblasts, GATA-1, SCL and the entire pentameric erythroid complex were found bound to all four HS sites. In addition to this, both subunits of the NF-E2 transcription factor were also enriched at HS-40 and to a lesser extent at HS-48. Maximal binding of all transcription factors were detected in basophilic and polychromatophilic erythroblasts, coinciding with maximal levels of histone H3 and H4 acetylation (De Gobbi et al., 2007). At the onset of transcription, the promoter then becomes bound by SP/X-Kruppellike transcription factors (Sp/X-KLF) (Vernimmen et al., 2007). These factors behave as activators in erythroid cells and are known to interact with GATA-1. At this stage, the pre-initiation complex (PIC), which includes general transcription factors and Pol II, is also detectable at the remote HS sites and is highly enriched at the promoter. In the absence of upstream HS elements, PIC binding to α-globin promoter is greatly reduced suggesting that recruitment of the full PIC to the promoter is heavily dependent on upstream elements (Vernimmen et al., 2007). Studies on the β-globin locus indicate that Pol II is transferred to the promoter in an NF-E2 dependent manner, though the mechanism of transfer is unknown (De

Gobbi et al., 2007). However, in cells expressing α-globin, there is a detectable increase in interactions between upstream regulatory elements and the α-globin genes (Vernimmen et al., 2007). These results seem to suggest that there is a change in chromosome conformation which results in physical interaction between the HS elements and the α-globin promoter, and this interaction facilitates the delivery of activated Pol II from the enhancer elements to the promoter. Anguita et al. analyzed the pattern of histone acetylation across α-globin locus in cell lines and identified an erythroid-specific domain of acetylation within the locus (Anguita et al., 2001). Both murine and human α globin loci displayed a significantly increasing of histone modification level during globin gene switching, indicating that a conserved, extended chromatin opening occurs within the α-globin locus during development. It is well known that chromatin opening is a necessary step for initiating transcription. DNase I sensitivity and histone modification level are widely used to assess the status of chromatin (Tollefsbol, 2004). The pattern of histone modification along the murine and human α globin loci was measured during globin gene switching in vivo (Fu et al., 2005). In the murine α-globin locus, the level of histone modification, especially H3 acetylation and H3 K4 methylation, within the locus, with the exception of ζ-gene increased obviously during globin gene switching. Similarly, in the human locus, H3 acetylation, H4 acetylation, and H3 K79 methylation displayed a significant increasing during the switching. It indicates that a conserved, extended chromatin domain opening occurs in the α-globin

locus as globin gene switching proceeds. It is thought that the chromatin opening of the α-globin domain embodies at least three steps: potential, primitive, and definitive state, which coordinate with hematopoiesis. Firstly, the α-globin domain is characterized by a loose chromatin structure in hematopoietic progenitor cells (HPC), which is important for transcriptional potentiation. Then in primitive erythroid cells, the α-globin domain becomes looser and displays a ‘‘primitive’’ state. Some stage-specific transcriptional activators, which bind to promoter-proximal regulatory elements, as well as enhancers, may have a role in this step (Fu et al., 2002; Ho et al., 2002). It is well known that high-level gene expression is closely related to phosphorylation of the C-terminal domain (CTD) of PolII (Komarnitsky et al., 2000). Recent evidence indicates that one major role of β-globin locus control region (LCR) is CTD phosphorylation at Ser5, which is required for the transition from transcription initiation to elongation (Sawado et al., 2003). The αMRE (HS-40) may possess similar role with β-globin LCR. Therefore, it is feasible that PolII complex is recruited to the promoters of fetal/adult α-genes in primary cells, which display high-level active histone modification. However, because of gene competition, the aMRE cannot interact with α-genes, which makes PolII elongation inefficient and eventually leads to low-level expression of α-genes at this stage. Finally, during globin gene switching, the α-globin domain becomes much looser and displays a ‘‘definitive’’ state in definitive erythroid cells, which may be critical for high level expression of α-genes. So, the specific sequential

chromatin opening of α-globin locus serve as a more general model for the developmental activation of gene, especially for gene clusters in which the regulatory elements locate far from the targeted genes.

3.4. α-globin mRNA

Once the enhancer regions have co-ordinated the positioning of Pol II on the promoter, transcription of α-globin begins and transcripts rapidly accumulate. Active transcription occurs mainly in the basophilic erythroblast, the first committed erythroid precursor, and in the polychromatophilic erythroblast. By the next stage of differentiation, in orthochromatophilic erythroblast, the cellular chromatin has completely condensed and transcription has ceased as the cell prepares to extrude its nucleus prior to full maturation. It is interesting that although transcription of α-globin occurs only during the early stages of erythroid cell maturation, α-globin protein is synthesized for an additional 4-6 days after transcriptional silencing of erythropoietic progenitor cells (Waggoner and Liebhaber, 2003). This phenomenon is dependent on the high level stability of α-globin mRNA transcripts, with half-life of α-globin mRNA estimated to be greater than 24 hours in erythroid cells (Liebhaber and Russell, 1998; Waggoner and Liebhaber, 2003).

It appears that there are two independent mechanisms, both erythroid specific, which are important for the stability of α-globin mRNA. The first involves the ribosomal displacement of an RNA-protein (RNP) complex, bound to the a-globin 3'UTR, which stabilizes α-globin

transcripts (Ji et al., 2003; Kiledjian et al., 1995; Wang et al., 1995; Weiss and Liebhaber, 1995). In the second, it appears that ribosome extension far into the 3'UTR triggers deadenylation of the α-globin transcript. This new mechanism, termed ribosome-extension mediated decay (REMD) is thought to be related to more general mRNA surveillance machinery such as nonsense mediated decay or the non-stop decay pathway (Kong and Liebhaber, 2007).

A crucial RNP complexes at 3' terminus of mRNA important in maintenance of poly(A) tails and also for the deadenylation. The α-complex binds to three C-rich regions located at position 29-70 in the 3'UTR and deletions or mutations in this region which prevent α-complex formation significantly reduce the half-life of α-globin transcripts. The selective binding of the α-complex is mediated by two distinct but similar poly(C) binding proteins, αCP1 and αCP2 (Wang et al., 1995). The αCP proteins are ubiquitously expressed in human and mouse cells but appear to have different roles in other cell types (Waggoner and Liebhaber, 2003). In erythroid cells, αCP nucleates the formation of an α-complex which protects the α-globin transcript from premature degradation by an erythroid enriched endoribonuclease and helps stabilize the poly (A) tail thus prolonging the halflife of the transcript (Anguita et al., 2004). In addition to stabilizing mRNA, αCP also appears to have a nuclear role in the splicing of pre-mRNA α-globin transcripts. It was recently discovered that αCPs are loaded onto α-globin transcripts in the nucleus and in addition to the 3'UTR, also bind a Crich

region in intron 1.37 In HeLa cells selectively depleted of αCP, splicing efficiency of a truncated α-globin construct (comprising exon 1, intron 1 and exon 2) was increased approximately four-fold from 15% to 58%. Thus, it appears that nuclear αCP binding to intron 1 represses splicing of the α-globin transcript. Conversely, nuclear binding of αCP to the 3'UTR appears to enhance splicing of the α-globin pre-mRNA (Ji et al., 2007). Experiments were conducted in HeLa and MEL cells comparing WT α-globin and a mutated transcript with a modified 3'UTR which does not bind αCP (αDPR). In both cell types, it was found that WT transcripts were spliced with higher efficiency than mutated aDPR transcripts (Ji et al., 2007). Given the apparently contradictory roles of αCP binding in different regions of α-globin transcripts, it may be interesting to determine if the affinity of αCP for each site varies with stages of cell differentiation. It is tempting to speculate that late in differentiation, αCP bound to the 3'UTR enhances splicing and stabilizes a-globin transcripts in preparation for high levels of hemoglobin synthesis. However, in early stages of a-globin expression, αCP may bind to intron 1 and repress splicing and expression until the appropriate time. This could then potentially represent another level of control at the transcriptional stage, preventing inappropriate expression of α-globin which would be deleterious to the developing erythrocyte.

4. α-HEMOGLOBIN STABILIZING PROTEIN (AHSP) IN ERYTHROPOIESIS

4.1. Characteristics of AHSP

Free α-globin is an unstable, reactive molecule capable of destroying erythroid progenitor cells. A small pool of excess α-globin chains must be stabilizes to limit its reactivity. This is achieved by the binding of an abundant erythroid-expressed protein known as α-hemoglobin stabilizing protein (AHSP). This protein was identified in a screen for proteins regulated by GATA-1 and was found to bind to α-globin specifically (Kihm et al., 2002).

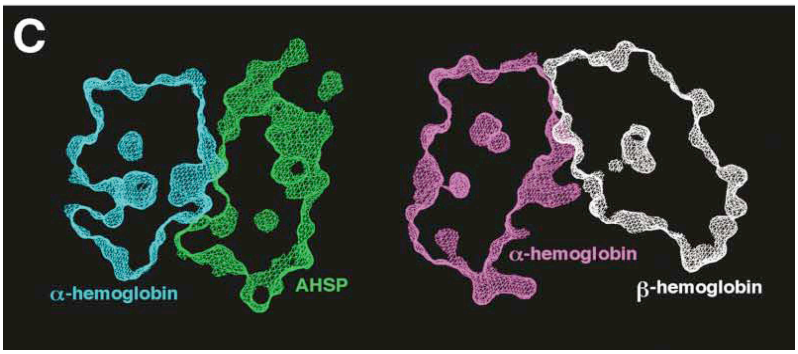

Figure 4.1 AHSP can efficiently and specifically capture free αHb. These nonoptimal αHb-AHSP interactions can be distrupted by incoming βHb for the formation of functional HbA.

AHSP is able to interact with multiple forms of α-globin including apo-ferrous and ferric states bound to a variety of ligands (Feng et al., 2004) and reduces oxidant-induced precipitation of α-globin in solution. AHSP

binds α-globin at the α1β1 dimer interface, opposite the haem binding pocket, with lower affinity than β-globin and is easily displaced by β-globin binding (Feng et al., 2004).

The initial binding of AHSP to free oxy-α-globin results in a number of structural changes including displacement of the proximal F8 histidine and co-ordination of the ferrous iron by the distal E7 histidine (Feng et al., 2004; Feng et al., 2005; Zhou et al., 2006). The oxygen molecule also binds to the proximal side of the haem group resulting in an overall structure which exposes the oxygen binding site (Feng et al., 2004; Zhou et al., 2006). This initial interaction is fully reversible and β-globin can readily displace AHSP to generate functional HbA under reducing conditions (Zhou et al., 2006).

However, if oxy-α-globin remains bound to AHSP, the exposure of the oxygen molecule predisposes it to the spontaneous loss of HO_2 and rapid auto-oxidation of the haem iron occurs. This reaction was shown to be oxygen dependent (Zhou et al., 2006) and results in a formation of a second, discrete, ferric bis-histidyl complex (Feng et al., 2005). In this state, the haem iron is oxidized to Fe(III) and becomes coordinated by both the proximal and the distal histidines (Feng et al., 2004; Weiss et al., 2005). This hemichrome structure is more resistant to denaturation and haemin loss compared to oxidized free α-globin and inhibits the reaction of Fe(III) α-globin with oxidants such as hydrogen peroxide. Both the ferrous and the ferric form of AHSP bound α-globin are capable of binding to oxy-β-globin to form adult hemoglobin (HbA) tetramers, but

the ferric form results in HbA products with reduced electrophoretic mobility. In addition, HbA containing Fe(III) a-globin was rapidly converted to cyanomet Hb upon exposure to cyanide while HbA generated from AHSP modified α-globin was resistant (Zhou et al., 2006). Together, these findings suggest that the AHSP-mediated rearrangement of α-globin to the bis-histidyl structure is retained upon binding to β-globin and the α-subunits retained a hemichrome formation which inhibits cyanide binding and alters the electrophoretic mobility. However, this alteration appears to be reversible as the AHSP-induced hemichrome formation can be reduced back to ferrous iron under appropriate conditions, generating functional hemoglobin capable of binding oxygen (Zhou et al., 2006). Therefore, AHSP can sequester α-globin in a reversible inert state if β-globin is unavailable or, alternatively, it can bind α-globin transiently during normal HbA formation. In fact, it appears that AHSP binding to α-globin actually facilitates the formation of HbA tetramers in vitro. Using a wheat germ-derived transcription and translation to express α-globin, it was found that addition of β-globin with AHSP augmented the formation of HbA in a dose-dependent fashion (Yu et al., 2007). This is consistent with previous observations that soluble haem-containing α-globin chains associate with apo-β-globin to promote formation of HbA (Adachi et al., 2003), and AHSP maintains the newly synthesized α-globin in a soluble state to facilitate this interaction (Weiss et al., 2005). In addition, similar to haem, AHSP also acts as a molecular chaperone to promote folding of nascent

apo-α-globin. AHSP was shown to stabilize α-globin in vitro, rendering it resistant to protease digest in a haem-independent manner. Consistent with the role of AHSP as a molecular chaperone, AHSP is also able to promote refolding of denatured α-globin (Yu et al., 2007). As AHSP and haem bind α-globin at different sites, the two molecules most likely synergize to stabilize the native structure to facilitate interaction with β-globin.

4.2 *In vitro* role of AHSP

The roles for AHSP are supported by the in vivo effects of AHSP loss. AHSP (–/–) homozygous knockout mice exhibit mild hemolytic anemia, shortened erythrocyte lifespan and high levels of reactive oxygen species (ROS) consistent with the presence of unstable α-globin (Kihm et al., 2002). These erythrocytes also display prominent eosinophilic inclusions (Heinz bodies) which contain both α-globin and β-globin precipitates (Feng et al., 2004; Kihm et al., 2002; Kong et al., 2004; Weiss et al., 2005). It was originally hypothesized that reduction of α-globin synthesis may ameliorate the AHSP phenotype since there would be lower levels of excessive toxic α-globin. However, surprisingly, the loss of a single α-globin gene (-+/++) in a AHSP–/– null background resulted in significantly more anemic phenotype. The compound AHSP –/– and α-KO (-+/++) mice were found to have significantly higher levels of both α-globin and apo-α-globin lodged in erythrocyte membranes compared to heterozygous α-KO mice (Yu et al., 2007). This finding supports the role

of AHSP in stabilizing the free α-globin pool and therefore facilitating integration with α-globin prior to formation of HbA tetramers. The loss of AHSP reduces the pool of stable α-globin chains available for HbA formation. Under these conditions, further reduction of α-globin expression would result in even fewer α-globin chains, which would be unstable, and therefore increased free α-globin. These results imply that mutant α-globin chains unable to bind AHSP may exhibit a more severe phenotype than simple loss of α-globin alone. Predictably, the loss of AHSP in thalassemic heterozygous α-KO mice also results in a poorer phenotype and increased α-globin precipitation (Kong et al., 2004). AHSP-KO, β-thalassemic mice had significantly impaired intrauterine survival and were approximately 20% more anemic than heterozygous α-KO mice with normal AHSP expression (Kong et al., 2004; Weiss et al., 2005). These studies in mice indicate that AHSP may be a possible modifier gene in human β-thalassemias. However, mutations which ablate AHSP in humans are rare (Viprakasit et al., 2006; Yu et al., 2007) and investigations into possible effects of reduced AHSP expression in β-thalassemia have so far been largely inconclusive. Preliminary reports indicate that AHSP may be a modifier in the human population (dos Santos et al., 2008; Galanello, 2003; Lai et al., 2006; Weiss et al., 2005) but as yet, there has been little definitive evidence that this is the case.

4.3 Excess of α-globin in β-thalassemia:the role of AHSP

In contrast to the situation with AHSP, the interaction of α-globin

mutations with β-thalassemia are exceedingly well characterized (Al Qaddoumi, 2006; Kulozik et al., 1993; Rund and Rachmilewitz, 2001; Steinberg, 1991; Thein, 2005; Voon et al., 2007; Weatherall, 2001).

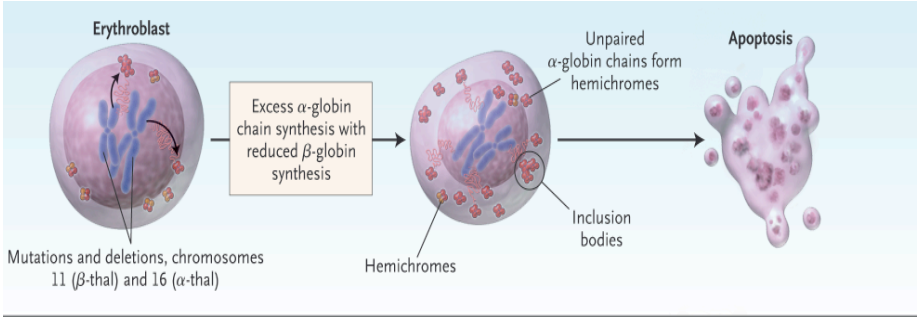

Figure 4.2 Cellular damages caused by free α-globin chains in β-thalassemic cells. α-globin is an unstable monomer that is prone to oxidation of cellular costituents and to precipitate and generate the inclusion bodies (Heinz bodies) and at the end apoptosis of the cell (ineffective erythropoisis).

Though β-thalassaemia arises from reduced expression of β-globin leading to decreased formation of functional hemoglobin tetramers, this plays a relatively minor role in contributing to the severely anemic phenotype (Steinberg, 1991; Weatherall, 2001). Instead, it is the damage caused at the cellular level by excess, improperly paired α-globin chains which leads to premature cell death and accounts for the majority of the pathology (Sorensen et al., 1990). In the absence of normal β-globin production, it is likely that α-globin is synthesized at levels which exceed the binding capacity of available AHSP. Under these conditions, free α-globin is able to accumulate in red blood cells and precursors leading

invariably to a variety of deleterious alterations. As previously noted, free α-globin is a highly unstable molecule and when devoid of stabilizing binding partners, forms large insoluble aggregates which can be visualized by light microscopy in an estimated one third of erythroid progenitor cells (Schrier, 1997).Much of the excess a-globin undergoes auto-oxidation to produce equal molar ratios of metHb and superoxide which can lead to an autocatalytic oxidative process in the cell. The oxidized ferric iron is bound more loosely to α-globin compared to the ferrous form which results in degradation of haem (Nagababu et al., 2008) and ultimately the release of iron which lodges in the cell membrane (Nagababu et al., 2008; Shinar and Rachmilewitz, 1990). In addition, oxidized haem-containing α-globin is also found bound to the cytoskeleton (Advani et al., 1992; Sorensen et al., 1990; Yuan et al., 1994). In this unstable conformation, both the haem group and the iron are able to participate in redox reactions leading to generation of ROS which can then oxidize adjacent membrane proteins (Schrier, 1997). Several critical membrane proteins including Band 4.1, spectrin and transmembrane Band 3 have been found to be oxidised in b-thalassemic erythroid precursors, leading to severe membrane.

SCOPE OF THESIS

The aims of this thesis are the study of the erythroid differentiation and the factors that could have relevance during this process in physiological and pathological conditions. Basic research about erythropoiesis, globin chains transcriptional regulation, hemoglobin formation and factors that cooperate in this process may have a strong impact on the medical treatment of a great variety of blood disease, such as β-thalassemia or sickle cell anemia. In a part of the work we investigated how the Sox6 transcription factor is involved in the regulation of erythroid differentiation, and hemoglobin switching process in a contest of normal erythropoiesis. In another part we focused to alpha hemoglobin stabilizing protein (AHSP) and their role in β-thalassemia, a genetic disorders caused by impaired production of the hemoglobin, in order to investigate the importance of this molecular chaperone in thalassemic cells. In both cases we utilized an over-expression approach to understand the function of the factors matter of interest, and, possibly, their molecular mechanism of action.

- **The transcription factor Sox6.** Sox6 is a transcription factor of the Sry-related HMG-box family and control cell fate specification of many cell types. This gene was over-expressed in a human model context (a cell line and a primary cells, $CD34^+$ then differentiated towards an erythroid lineage) and then was measured and described the phenotypic changes

consequent to its enforced expression. To better clarify the results obtained and so explain what is the molecular mechanism of action of Sox6 in erythroid cells it seems interesting to look for its early target genes on the DNA. Bioinformatic tools help us to find possible target binding sites within the human genome and then molecular tools validate in vitro what was found in-silico.

- **The molecular chaperone Alpha Haemoglobin Stabilizing protein (AHSP).** Hemoglobin synthesis is coordinated by homeostatic mechanism to produce a balanced level of α and β subunits. The α-globin is stable only when make a complex with the β-globin. β-thalassemia is a genetic anemia characterized by a reduced or absent synthesis of β-globin chains with consequent α-globin accumulation. The free α-globin precipitate and partecipate in chimical reactions that generate reactive oxigen species (ROS) responsible for cellular damages. Particolar attention is devoted to molecular interactions between α-globin and AHSP in erythroid cells. AHSP is a molecular chaperone that binds free α-globin chains probabily limiting their cytotoxic effect. Gene ablation studies in mice demonstrate that AHSP is required for normal erythropoiesis. AHSP-null erythrocytes are short-lived, contain Hb precipitates, and exhibit signs of oxidative damage and loss of AHSP exacerbates β-thalassemia in mice. These observation suggest that altered AHSP expression or function could modify thalassemic phenotype in human, a topic that is interesting to explore for possible clinical strategies

as therapeutic approach. So, our interest was to determine the effect of AHSP gene over-expression in experimental model of β-thalassemia in order to clarify if could ameliorate the pathogenic state of thalassemic cells.

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CHAPTER 2

SOX6 ENHANCES ERYTHROID DIFFERENTIATION IN HUMAN K562 CELLS AND CORD BLOOD-DERIVED ERYTHROID PROGENITORS

(manuscript submitted to Blood, under second revision)

SOX6 ENHANCES ERYTHROID DIFFERENTIATION IN HUMAN K562 CELLS AND CORD BLOOD-DERIVED ERYTHROID PROGENITORS

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Running title: Sox6 enhances erythroid differentiation

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ABSTRACT

Sox6 belongs to the Sry-related HMG-box family of transcription factors, which control cell fate specification of many cell types. Here we explore the role of Sox6 in human erythropoiesis by its over-expression in both the erytholeukemic K562 cell line and in primary erythroid cultures from human cord blood $CD34^+$ cells. Sox6 induces significant erythroid differentiation in both models: K562 cells undergo hemoglobinization and, despite their leukemic origin, die within 9 days after transduction; primary erythroid cultures accelerate their kinetic of erythroid maturation and increase the number of cells reaching the final enucleation step. Searching for direct Sox6 targets, we found SOCS3 (Suppressor of Cytokine signalling-3), a known mediator of cytokine response. Sox6 binds *in vitro* and *in vivo* to an evolutionary conserved regulatory SOCS3 element, inducing transcriptional activation. SOCS3 overexpression in K562 cells and in primary erythroid cells recapitulates the growth inhibition induced by Sox6, demonstrating that SOCS3 is a relevant Sox6 effector. The Sox6-mediated SOCS3 induction is, in K562, accompanied by a decreased expression of IGF-1 gene, a known inhibitor of apoptosis and survival factor for hematopoietic cells.

INTRODUCTION

Sox proteins are important transcriptional regulators of different developmental processes where they control the specification and differentiation of many cell types (Lefebvre; Schepers et al., 2002; Wegner, 1999).

In particular, Sox6, originally isolated form adult mouse testis (Connor et al., 1995), is required for the development of the central nervous system (Hamada-Kanazawa et al., 2004a; Hamada-Kanazawa et al., 2004b; Stolt et al., 2006), for chondrogenesis (Ikeda et al., 2004), and for cardiac and skeletal muscle formation (Hagiwara et al., 2000; Hagiwara et al., 2005). Recently, Sox6 has been demonstrated to be crucial for definitive erythropoiesis (Cohen-Barak et al., 2007; Dumitriu et al., 2006b; Xu et al.; Yi et al., 2006), a process in which committed progenitors progressively differentiate into BFU-E and CFU-E, that in turn, give rise to proerythroblasts, erythroblasts and finally to mature, enucleated red blood cells (RBCs). These differentiation stages are accompanied by profound maturational changes: within few cell divisions, in parallel with the accumulation of erythroid-specific markers (membrane proteins, enzymes required for the heme biosynthesis pathway, globins), cells undergo chromatin condensation and enucleate (Palis, 2008; Tsiftsoglou et al., 2009).

This complex spectrum of maturational steps is controlled, at the molecular level, by the integration of extrinsic (growth factors, oxigen

and iron availability) and intrinsic (growth factor receptors, signalling mediators, transcription factors) signals.

Several transcription factors are essential for erythroid commitment and for differential globins genes expression during development: their absence is associated with a wide spectrum of phenotypes ranging from mild perturbation to death due to a complete failure of erythropoiesis (Cantor and Orkin, 2002; Perry and Soreq, 2002).

Among them, Sox6 has been recently shown to stimulate erythroid cell survival, proliferation and terminal maturation during definitive murine erythropoiesis (Dumitriu et al., 2006b). Sox6-null fetuses and pups mice are anemic and present defective RBCs. Sox6 has recently been implicated in the silencing of embryonic globins genes: it directly silences the embryonic εy-globin gene in definitive erythroid cells by binding to the εy promoter (Cohen-Barak et al., 2007; Yi et al., 2006) and it cooperates with BCL11A in silencing γ-globin, possibly via direct physical interaction (Xu et al.).

In this paper we show that Sox6 over-expression in K562 and in primary erythroid cultures from coral blood derived-$CD34^+$ cells induces enhanced erythroid differentiation in both models. In K562 cells, differentiation is associated with reduced proliferation, leading the culture to exhaustion within 9 days after transduction.

Accordingly to the phenotypic changes observed in K562 cells, Sox6 over-expression in human primary erythroid cultures is accompanied by an accelerated kinetic of maturation and an increased number of cells that achieve enucleation.

Searching for direct Sox6 targets, we found an evolutionarily conserved potential double Sox6 binding site lying 2.7kb 5' to the SOCS3 (Suppressor of Cytokine Signalling-3) transcription start site. This element is bound by Sox6 in vitro and in vivo and is activated by Sox6 in cotransfection experiments in K562 cells. SOCS3 is a negative regulator of the cellular response to several cytokines and plays a crucial role in regulating the balance between proliferation and differentiation in different cell types (Krebs and Hilton, 2001). When SOCS3 is over-expressed in K562 cells and in primary cultures, cells stop growing with a kinetics similar to that observed upon Sox6 over-expression, suggesting that SOCS3 is indeed a relevant Sox6 target controlling cell proliferation. In K 562 cells, the Sox6-mediated SOCS3 induction is accompanied by decrease of IGF-1 mRNA level, suggesting that the IGF-1/IGF1R axis is modulated by Sox6 in erythroid cells.

EXPERIMENTAL PROCEDURES

Plasmids preparation

The Sox6 murine cDNA was kindly provided by Prof. Michiko Hamada-Kanazawa, Kobe-Gakuin University, Japan. The Sox6 cDNA was transferred into the pCMV-Tag 4B plasmid (Stratagene, La Jolla, CA), in frame with a 3' FLAG epitope, (EcoRI-EcoRV sites), to produce the Sox6FLAG expression vector used in transfection assays. The Sox6 recombinant protein lacks the 49 C-ter aminoacids: this shorter molecule fully retains Sox6 biological properties.[6] The Sox6-FLAG cassette (EcoRI – KpnI blunted sites) was then cloned immediately upstream to the IRES-Emerald GFP cassette (blunted BamHI site) of the pHR SIN BX IR/EMW (derived from pHR SIN CSGW lentiviral vector, (Demaison et al., 2002)). The TWEEN plasmid vector containing human SOCS3 cDNA was kindly provided by Dr. M.G. Francipane (Francipane et al., 2009). For transfection experiments, the human SOCS3 promoter region from nt. -393 to nt. +12 was obtained by direct amplification from genomic DNA with Phusion High-fidelity DNA Polymerase (Finnzymes, Espoo, Finland), and cloned into the pGL2 reporter vector (Promega, Fitchburg, WI). The 70-nt SOCS3 region (nt -2727 to nt -2678) containing the double Sox6 binding site (either wild type and mutated), was cloned (SacI-NheI restriction sites) upstream to the SOCS3 promoter. Amplified DNAs were sequenced to avoid undesired mutations. All primers used are listed in the supplemental material section.

Lentiviral vector production

Viral stocks stocks pseudotyped with the VSV.G envelope were produced by transient cotransfection of 4 plasmids in 293T cells and titered on HeLa cells as previously described (Follenzi and Naldini, 2002). Cells supernatant was collected, ultracentrifugated at 50,000x g for 2 h at RT and the pellet was resuspended in PBS 1% and stored at –80°C. Viral titers were determined by transduction of HEL cells with serial dilutions of the vector stocks and transduction efficiency was evaluated by scoring GFP transgene expression by FACS analysis.

Cell cultures and transduction

K562 and HEL cells were cultured in RPMI medium supplemented by 10% Fetal Bovine Serum, PenStrep and L-glutamine. Transduction (four independent experiments) was performed overnight, with a multiplicity of infection (MOI) of 30.

$CD34^+$ cells were purified from cord blood by positive selection from mononuclear cells (Migliaccio et al., 2002), using anti-CD34 microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. $CD34^+$ cells were plated at a concentration of $0.5\text{-}1x10^6$ cells/ml and prestimulated for 30 hours in CellGro medium (Cell Genix, Freiburg, Germany) supplemented with 300 ng/ml human stem cell factor (hSCF), 300 ng/ml human Flt-3-ligand (hFlt3-l), 100 ng/ml human thrombopoietin (TPO) and 60 ng/ml human IL-3 (all PeproTech, Rocky Hill, NJ) on plates coated with retronectin (Takara Shuzo, Shiga, Japan). Transduction was performed overnight, with a MOI

of 100. Erythroblasts (d6) derived from cord blood $CD34^+$ cells were transduced overnight at MOI 50. The following day cells were washed and grown in suspension as erythroid culture.

$CD34^+$ cells were cultured for 2 weeks in StemSpan (Stem Cell Technologies, Vancouver, Canada) containing 20% of fetal bovine serum (FBS, Hyclone, Logan, UT) and supplemented with hSCF (10 ng/ml), human erythropoietin (EPO, 1 U/ml), hIL-3 (1 ng/ml), 10^{-6} M dexamethasone (Sigma Aldrich, St Louis, MO), and 10^{-6}M β-estradiol (Sigma), according to a single phase protocol, as modified from ref. 24. $CD34^+$ cells were seeded at a concentration of 10^5 cells/ml and diluted over time to maintain the concentration at 1–2 x 10^6 cells/ml. The progression toward erythroid differentiation was evaluated by staining with PE-conjugated anti-CD235 (GlycophorinA, GpA) (Dako, Carpinteria, CA) and FACS analysis. Morphological analysis and differential counting was performed on cytospins by MayGrünwald-Giemsa staining and microscope inspection.

CFU assay for human progenitors

$CD34^+$ cells were plated at a density of 1000 cells/ml in methylcellulose medium containing hSCF, hGM-CSF, hIL3 and hEPO (GF H4434 Stem Cell Technologies, Vancouver, Canada). After 2 weeks, BFU-E, CFU-GM, and CFU-GEMM colonies were counted, and single colonies (20–30 for each experiment) were isolated. DNA and RNA were extracted by TRIzol REAGENT (Invitrogen, Carlsbad, CA) for molecular analysis.

Chromatin Immunoprecipitation (ChIP) assay

$1x10^6$ K562 or $1x10^7$ primary cells for each Immunoprecipitation reaction were fixed with 0.4% formaldehyde for 10 minutes at room temperature, and chromatin was sonicated to a size of about 500 bp. Immunoprecipitation was performed after overnight incubation with anti-FLAG antibody (Sigma, F-7425), and subsequent incubation with protein A agarose (Upstate). Immunoprecipitated DNA was then analysed by amplifying an equivalent of DNA from 10^4 K562 or 10^5 primary erythroblasts. Primers are listed in Supplemental materials.

RNA isolation and RT-PCR

Total RNA from $1x10^5$ erythroid cells was purified with TRI-Reagent (Applied Biosystems, Foster City,CA), treated with RQ1 DNase (Promega) for 30 min at 37°C and retrotranscribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem cat n°4368814). Negative control reactions (RT^-) gave no signal. Real time analysis was performed using ABI Prism 7500 (Applied Biosystems). Primers were designed to amplify 100-150bp amplicons. Specific PCR product accumulation was monitored by SYBR Green dye fluorescence in 25-µl reaction volume. Dissociation curves confirmed the homogeneity of PCR products. All primers are listed in supplemental materials section.

Western blot

Total and nuclear extracts from K562 cells were prepared according to standard protocols (Schreiber et al., 1989) and proteins were subjected to

SDS-PAGE separation and blotting. The Sox6FLAG protein was detected by the use of the anti-FLAG antibody (Sigma F7425). Protein loading was checked by reprobing filters with an anti beta-actin antibody (Sigma). Antibodies binding was detected by using appropriate horseradish peroxidise-conjugated IgG and revealed by ECL (LiteAblot, Euroclone).

Transfection experiments

1,5 x 10^5 exponentially growing K562 cells were transfected in 0,5 ml of Opti-MEM medium (Invitrogen), using 2µl of Lipofectamine 2000 (Invitrogen), 800 ng of the reporter plasmid, and increasing amounts (from 0,2 to 1 µg) of the Sox6 expression plasmid (pCMV-Sox6Tag4B), per well. The pCMV-Tag4B empty vector was added to each transfection at the concentration required to equalize the total amount of DNA transfected in each reaction. After 24 h, total cellular extracts were prepared and Luciferase activity was measured according to the Promega Luciferase reporter system protocol. Transfections were repeated in triplicate with three independent plasmid preparations.

Electrophoretic mobility shift assay (EMSA)

^{32}P-labeled DNA probes were incubated with 5-10 µg of total or nuclear extracts, for 20 min at 15°C in a buffer containing 5% glycerol, 50 mM NaCl, 20 mM Tris, pH 7.9, 0.5 mM EDTA, 5 mM MgCl, 1 mM dithiothreitol (DTT), 100 ng/µl poly(dG-dC), and 50 ng/µl bovine serum albumin (BSA) in a 15-µl final volume. The reaction mixture was loaded onto a 8% polyacrylamide gel (29:1 acrylamide-bisacrylamide ratio) and

run at 4°C at 150 V for 3 h. Nuclear extracts were prepared according to standard protocols (Bose et al., 2006; Schreiber et al., 1989). The antibodies used were: anti-FLAG, (Sigma F7425); anti-GATA1 (SantaCruz N6, sc-265).

Flow cytometric analysis and FACS

PE-conjugated anti-pSTAT5 (pY694) (BD Becton Dickinson, San Jose, CA) was used at 5 μg/mL. Intracellular staining was done according to the manufacturer's instructions, using BD™ Phosflow Fix Buffer I. Propidium Iodide and AnnexinV staining were done using Annexin V Apoptosis Detection Kit from Santa Cruz Biotechnology. Flow cytometry data were acquired on a FACSCalibur (Becton Dickinson) and analyzed with FlowJo software (version 8.4.5; Tree Star Inc.). K562 electroporated with pTween SOCS3-IRES-GFP expressing plasmid were FACS sorted for GFP expression 48 hours after transfection. Cell sorting was performed on a MoFlo (DAKO-Cytomation) cell sorter and the purity obtained was >95%.

Hemoglobin quantitation

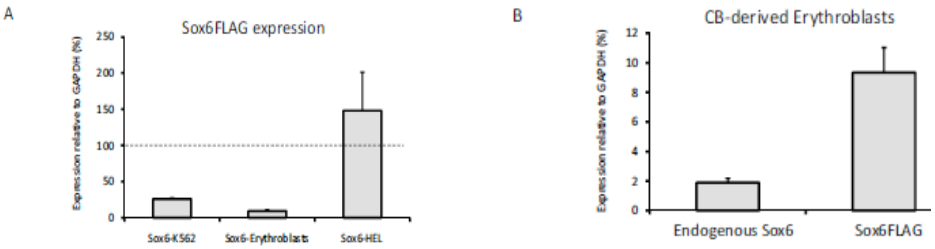
Total hemoglobin was quantitated by using the Human Hemoglobin-ELISA Quantitation Set (Bethyl Laboratories Inc., Cat. No. E80-135), according to the Manifactures's instruction. Hemin induction was obtained by growing K562 cells in the presence of 50 μM Hemin for four days.

RESULTS

SOX6 overexpression strongly induces erythroid differentiation in K562 cells

To get insight into the role of Sox6 in human erythropoiesis, we first overexpressed Sox6 by lentiviral transduction of the human erythroleukemic cell line K562.

K562 cells were transduced with a vector containing a Sox6cDNA-FLAG expression cassette upstream to an IRES-GFP element (Sox6-GFP), and in parallel with a control Empty Vector (EV-GFP) lacking Sox6 cDNA (Figure 1A). The efficiency of transduction, assayed by monitoring GFP expression, was similar for both vectors (between 80 and 90%, data not shown). The expression of the exogenous Sox6 was verified by RT-PCR, Western blot, Figure 1B) and quantitated by Real Time PCR (Supplementary Figure 1).



Supplementary Figure 1 (A) **Real Time quantitation of Sox6 over-expression** in K562 cells, $CD34^{+}$-derived primary erythroblasts and HEL cells, relative to GAPDH expression (set equal to 100%) (B) **Expression of endogenous Sox6 and of transduced Sox6 Sox6Flag) in primary erythroid cells.** Histograms show the relative levels of expression (mean +/-SEM of at least 3 indipendent experiments) compared to GAPDH. considered as 1.

72h after transduction, K562 cells overexpressing Sox6 show a profound phenotypic change: Sox6-K562 form a red pellet upon centrifugation, indicating the accumulation of haemoglobin, also revealed by the increased number of O-Dianisidine positive cells on cytospin preparations (Figure 1C-D). ELISA detection reveals a significant increase of total hemoglobin content in cells overexpressing Sox6 (from about 5ng/10^5 cells in EV-K562 to about 22ng/10^5 cells in Sox6-K562) although their hemoglobin content is lower than that of cells treated with Hemin (about 75ng/10^5 cells) (Figure 1C, right panel).

Moreover, FACS analysis shows a significant accumulation of a highly $CD235^+$ (GlycophorinA) cell population (R6 in figure 1E). To better characterize the effect of Sox6 at molecular level, we analysed the expression of a series of genes known to vary during erythroid maturation, comparing EV-K562 with Sox6-K562 cells by Real Time-PCR (Figure 1F).

As expected on the basis of the observed increased hemoglobinization, among genes whose expression is strongly increased upon Sox6 overexpression there are globin genes (see below, detailed analysis), together with genes encoding key enzymes of the heme biosynthetic pathway (ALAS-E, FECH). In these same cells, the appearance of a more mature erythroid phenotype, is mirrored by a reduced expression of the "megakaryocytic genes" GPIIB and GPIIIA.

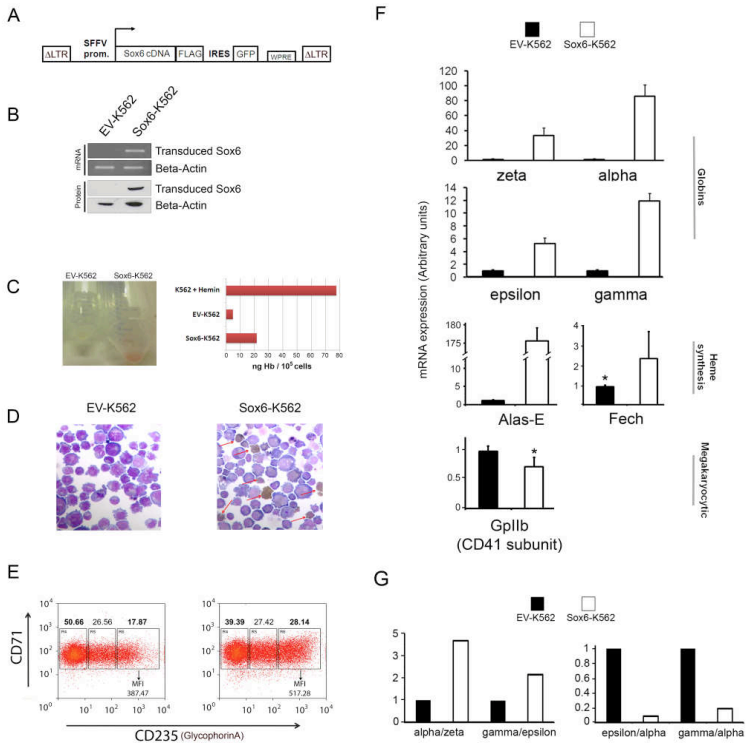


Figure 1. Sox6 overexpression in K562 cells (A) Schematic representation of the lentiviral vector used. (B) The expression of the transduced Sox6 was assayed at mRNA and protein level. Upper panel: RT-PCR with primers detecting the exogenous, vector-derived Sox6 transcript (see Mat&Met for details). Beta Actin primers were used as control. Lower panel: Western blot with the anti Flag antibody detects the exogenous Sox6 protein. The anti-Beta actin antibody was used to normalize for protein loading. **Phenotipic changes of K562 cells upon Sox6 overexpression (C-F).** Sox6-K562 cells (right panels), when compared with EV-K562 cells (left panels), show: (C) a reddish pellet indicating the accumulation of Hemoglobin chains, quantitated by ELISA in the right panel; (D) an increased number of O-Dianisidine positive cells -brown staining- indicating hemoglobin accumulation; (E) an increased CD235 (GlycophorinA) positivity (compare cells in R6 gate: 28.14%, MFI of 517.28 in Sox6-K562 versus 17.87% , MFI of 384.47 in EV-K562, FACS analysis); (F) an increased expression of "erythroid genes" -globins, heme biosynthesis pathway-, accompanied by a downregulation of "megacaryocitic genes" –GPIIB, Real Time-PCR analysis. Histograms show the relative levels of expression (mean +/- SEM of at least 3 indipendent experiments) compared to GAPDH, considered as1. **(G) All globins chains normally expressed in K562 are upregulated in Sox6-K562 cells but the ratios of their expression level change significantly** Left panel: fold increase of γ/ε and α/ζ ratios in EV-K562 (set equal to 1) versus Sox6-K562 cells. Right panel: fold decrease of ε/α and γ/α ratios EV-K562 (set equal to 1) versus Sox6-K562 cells.

mRNA from genes encoding the major transcription factors responsible for the erythroid cell type specification, i.e. GATA1, GATA2, EKLF, and p45-NFE2, do not change significantly, suggesting that the observed phenotype is not mediated by a Sox6 effect on these transcription factors expression (not shown).

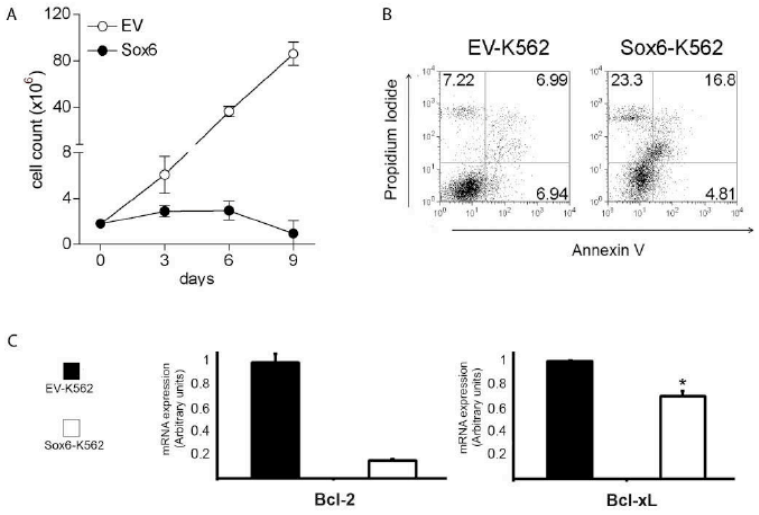


Figure 2. Sox6-K562 cells stop growing and undergo apoptosis (A) 1 x 10^6 exponentially growing K562 cells were transduced at day0 either with Sox6 or the EV vector , washed and replated in fresh medium 24h after transduction. Sox6-transduced cells stop growing 3 days after transduction and the culture die within day 9. Error bars refer to 3 indipendents experiments. (B) 72h after transduction, FACS analysis was performed using anti Annexin antibody and Propidium Iodide (PI) staining to evaluate apoptosis. In Sox6-K562 cells, 16.8% cells are $AnnexinV^+PI^+$, while only 7% of EV-K562 cells are double positive for the same markers. (C) Real Time-PCR on Bcl-2 and Bcl-xL expression, 72h after transduction. Histograms show the relative levels of expression (mean +/- SEM of at least 3 indipendent experiments) compared to GAPDH, considered as 1. *the difference is statistically significative ($p<0.05$).

Since Sox6 has been proposed to repress the ε and γ embryonic globin genes[11-14], we carefully analysed by Real Time PCR the relative changes in globin genes transcription upon Sox6 overexpression, considering

Since Sox6 has been proposed to repress the ε and γ embryonic globin genes[11-14], we carefully analysed by Real Time PCR the relative changes in globin genes transcription upon Sox6 overexpression, considering GAPDH as internal standard. As shown in figure 1F, both β-like genes (ε and γ) and α-like genes (ζ and α), normally expressed by K562 cells, are -in terms of absolute amount of transcript-induced by Sox6 overexpression. The internal ratio between the two α-like genes (α/ζ) and between the two β-like genes (γ/ε) is shifted, in Sox6-K562 cells, in favour of α and γ expression (α/ζ 3.68 fold and γ/ε 2.19 fold increase, respectively).

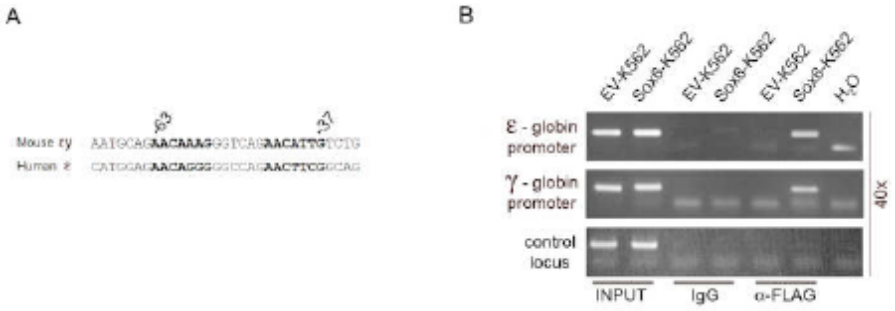


Figure 2. ChIP analysis confirms the ability of Sox6 to bind the human ε and γ-globin promoters (A) Sequence comparison of the double Sox6 binding sites within εγ mouse and human ε–globin promoters. Nucleotides positions are relative to the start site. (B) The anti-FLAG antibody or rabbit IgG were used to immunoprecipitate chromatin from EV-K562 or Sox6-K562 cells. Lanes 1 and 2: input chromatins. Lanes 3 and 4: normal rabbit IgG. Lane 5 and 6: anti-FLAG antibody (that recognizes the Sox6FLAG transduced protein) Lane7: Water. 40x: PCR cycles number.

The increased γ/ε ratio indicates that ε-globin is less strongly induced than γ, in agreement with the known repressor role of Sox6 on the ε-globin gene (Yi et al., 2006). However, ε/α and γ/α ratios are both decreased in Sox6-K562 vs EV-K562, suggesting a Sox6 repressive effect also on γ-globin, although less evident than that on ε (the ε/α ratio is almost 10 fold reduced, while the γ/α ratio is reduced by 5 fold (Figure 1G). On this basis, we performed Chromatin Immunoprecipitation to test whether Sox6 binds to the human ε-globin promoter (where a double Sox6 binding site is partially conserved between mouse and man (Supplementary Figure 2A) and to the γ globin genes promoter regions. By using an anti-FLAG antibody that recognizes the vector-derived Sox6-FLAG protein, we demonstrated that Sox6 is indeed able to bind to the human ε and γ globin gene promoters *in vivo* (Supplementary Figure 2B).

The enhanced erythroid differentiation of Sox6-K562 cells, is accompanied by a marked reduction of proliferation, with complete exhaustion of the culture within day 9 after transduction (Figure 2A).
FACS analysis, carried out 72h after transduction, shows an increased number of AnnexinV positive cells (34.6% in Sox6-K562 vs 8.0 % in EV-K562), accompanied by an increased Propidium Iodide uptake (45.8% vs 8.4%), suggesting increased apoptosis (Figure 2B). in addition, Real Time PCR analysis reveals a decreased expression the two antiapoptotic genes Bcl-2 and Bcl-xL (Figure 2C). Together these data suggest that Sox6 overexpression induces a strong erythroid

differentiation in K562 cells, accompanied by a dramatic reduction of cell proliferation.

Sox6 overexpression enhances and anticipates erythroid terminal differentiation of CD34$^+$ derived erythroid cultures

Because of the profound effect on K562 induced by Sox6 overexpression, we moved to an *in vitro* model of human erythroid cells differentiation, starting from cord-blood derived CD34$^+$ cells. In this culture, erythroid progenitors first expanded to the erythroblast stage (d0-d8) and then undergo terminal differentiation (d9-d14). Real time PCR analysis shows that Sox6 expression is absent in the most immature progenitors (d0 to d6), starts to be detectable at the beginning of the erythroblast differentiation phase (d8, corresponding to about 40% of GpA$^+$ cells), reaches a peak around d12 (about 80% of GpA$^+$ cells), and finally decline at the end of the culture (d14, >80% of GpA$^+$ cells) (Figure 3A). On the basis of this expression pattern, we transduced the culture at d6, immediately prior to the onset endogenous Sox6 expression and we subsequently carried out the analysis on samples taken at d8, d10, d12 and d14. The evaluation of the percentage of GFP$^+$ cells by FACS shows that cells were transduced with similar efficiency by EV-GFP and Sox6-GFP vectors (about 85%). The experiment was repeated in triplicate and the result of a representative experiment is shown and discussed below.

Erythroid maturation was evaluated by measuring the proportion of GFP$^+$GpA$^+$ cells by FACS. In control EV-erythroblasts, GFP$^+$GpA$^+$ cells are about 70% at d10 and remain essentially stable until d14 (Figure 3B,

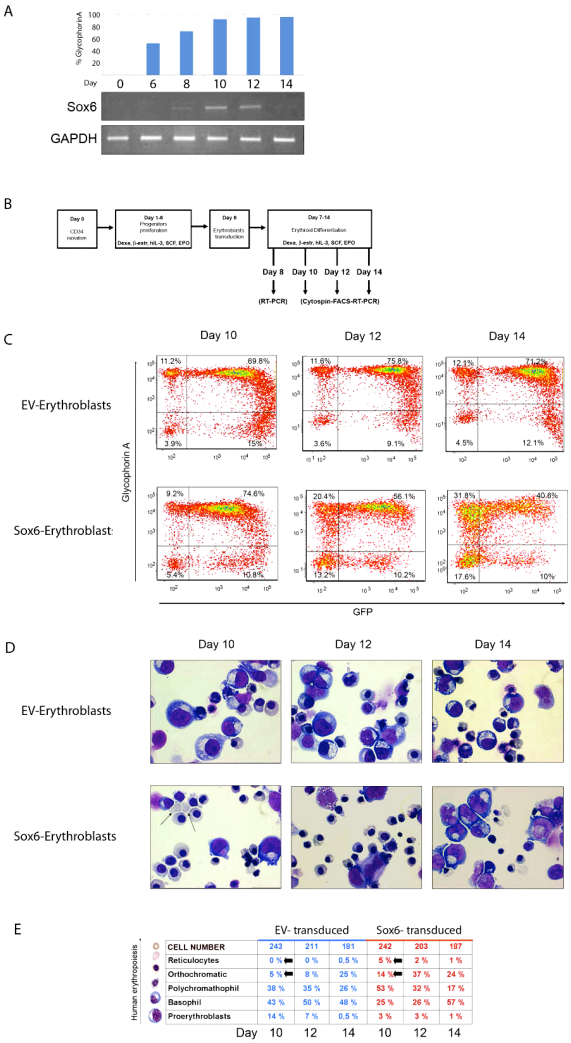
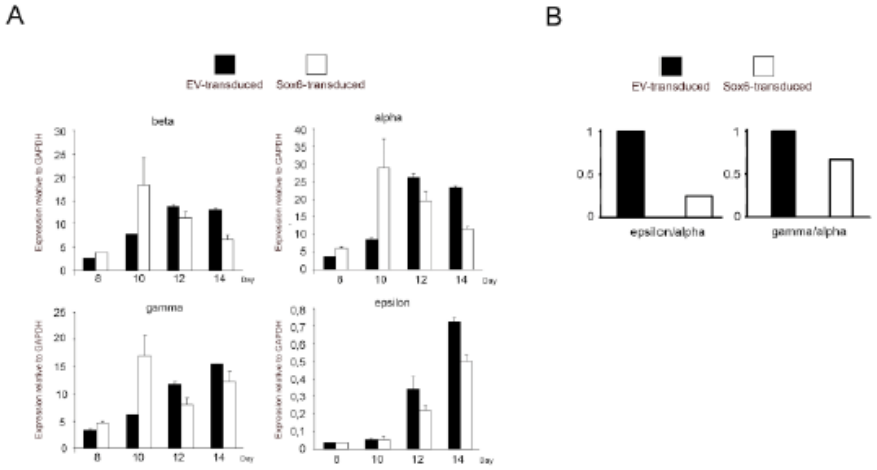


Figure 3. Sox6 enhances erythroid differentiation in Cord Blood-derived cell cultures (A) Upper panel: percentage of Gpa^{+} cells , estimated by FACS analysis at the different days of the culture. Lower panel: semi quantitative RT-PCR at the same days. Upper gel: Sox6 expression; Lower gel: GAPDH. (B) Experimental outline of the transduction experiments. (C) FACS analysis on erythroblasts transduced either with the Empty Vector (EV-erythroblasts) or with the Sox6 overexpressing vector (Sox6-erythroblasts): x axis, GFP expression; Y axis, GpA expression. (D) May Grünwald-Giemsa staining on cytospin preparations of the same samples as above. (E) Differential counts on cells from the same cells as in D. A total number of more than 200 cells were scored for each samples.

upper panels). By contrast, Sox6-erythroblasts reach the peak of GFP^+GpA^+ double positivity at d10 (74,6%) and then decline to 56.1% at d12 and to 40.6% at d14, suggesting a progressive loss of transduced cells (Figure 3B, lower panels).

To better correlate these data with erythroid maturation, cells from the same samples as above, were cytospun, stained with May-Grunwald/Giemsa and differentially counted to score the relative number of cells at the different stages of erythroid maturation (Figure 3C-D). At d10, in Sox6-transduced cells, many polychromatic and orthocromatic erythroblasts, usually appearing at later days in control cultures, are already present (Figure 4C-D). More strikingly, 3 to 5% of reticulocytes are scored, while in the control culture a maximum of 0,5-1% is observed at the end of the culture (d14). In parallel with the increased number of more mature cells (polychromatic and orthochromatic erythroblasts, and reticulocytes), a decrease of more immature cells (proeryhtroblasts and basophilic erythroblasts) is observed in Sox6 overexpressing cells at d10 (25+3=28% *vs* 43+14=57%). Finally, the distribution of the different cell types between the two cultures returns equal at d14, when GFP^+ (Sox6 overexpressing) cells reach the minimum of their contribution to the culture (Figure 3B-D). The analysis of the expression of globin genes ($\alpha, \varepsilon, \gamma, \beta$) in transduced and control cells demonstrates that Sox6 transduction greatly stimulates α, β and γ, but not ε expression, likey reflecting the accelerated maturation described above (Supplemental figure 3A).



Supplemental Figure 3. (A) **Sox6 overexpression anticipates and induces the expression of the α, β and γ but not of the ε globin genes in cord blood derived erythroid culture**. Real time PCR quantification of globins mRNA expression level on samples taken at d8, d10, d12, d14 of the culture. Histograms represent the mean expression level (relative to GAPDH expression); standard deviations refer to 3 independent experiments. Please note the different scale on the y axis: ε expression is marginal and does not increase upon Sox6 overexpression. (A) **The ε/α and γ/α ratios (set equal to1 in untransduced cells) are decreased in Sox6-transduced cells.**

Interestingly, in cells overexpressing Sox6, the ε/α and γ/α ratios are decreased, as previously observed in K562 cells, supporting the notion that, beside the general induction of maturation, Sox6 specifically concurs to repress ε and γ is genes transcription.

Sox6 induces early loss of CD34$^+$ progenitor cells

In a second set of experiments we directly transduced CD34$^+$ cells with either the control or the Sox6 vector as above, and then a portion of transduced cells was either placed in the unilineage erythroid culture or

plated in methylcellulose medium for colony forming unit (CFU) assay (exp. n = 3). The proportion of GFP$^+$ cells in the Sox6 transduced culture progressively declines from about 30% at d4 to 3% by d14 (Figure 4B).

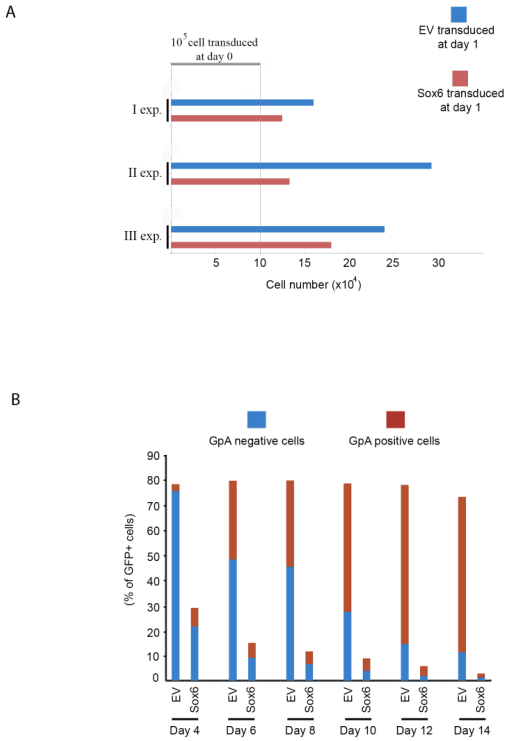


Figure 4. Sox6 transduction of CB-derived CD34$^+$ cells causes early loss of progenitor cells (A) CD34$^+$ cells transduced with Sox6 grow slower than EV-transduced cells. 10^5 freshly purified CD34$^+$ cells were transduced and counted 24h after transduction in three independent experiments. Blue bars: EV-transduced cells; Red bars: Sox6-transduced cells. (B) FACS analysis on CD34$^+$ transduced cells at different time points after transduction. X axis: days after infection, y axis: percentage of GFP$^+$ cells. Different colours in the histogram columns represent the proportion of GFP$^+$ cells which are GpA$^-$ (blue) or Gpa$^+$ (red), respectively.

This is in strong contrast with the high level of GFP^+ cells (80-90%) transduced with the control vector, which remains constant during erythroid maturation (d14). The low level of GFP positivity in Sox6-transduced cells, suggests an early loss of cells overexpressing Sox6 as also indicated by a slow proliferation kinetic of these cells in the first 24h after transduction (Figure 4A). Of interest, the proportion of Sox6-transduced cells that are GPA^+ is already very high (34%, red) at d4, in contrast to the very low GPA positivity (3,7%) of EV-transduced cells, suggesting that cells surviving Sox6 overexpression undergo accelerated erythroid differentiation (Figure 4B).

Finally, the same transduced cells were also seeded in methylcellulose and no GFP^+ colonies, analyzed either by FACS and by RT-PCR analysis, were scored at d14, indicating the loss of Sox6-transduced progenitors and/or of their progeny.

SOCS3 is an early Sox6 target gene

The above results prompted us to search for Sox6 direct targets possibly responsible for the Sox6 induced erythroid differentiation. To this aim we carried out a genome-wide search for evolutionarily conserved potential Sox6 binding sites by using the TFBScluster software (Donaldson and Gottgens, 2006), taking the ε-globin Sox6 binding site as a model. The *in silico*-identified candidate targets (more than 800, data not shown), were filtered by selecting genes whose expression is known to be enriched in erythroid cells, on the basis of literature and of DNA microarray data

comparing the expression profile of three distinct cells populations FACS sorted from E11.5-E13.5 mouse fetal liver: pluripotent hematopoietic progenitors c-kit^{+}/TER119^{-}; erythroid committed early progenitors c-kit^{+}/TER119^{+}; more differentiated erythroblasts c-kit^{-}/TER119^{++} cells (Cantù et al., 2010 and unpublished results). Among the remaining genes (Cantù et al., 2010), we focused on a double Sox6 consensus sequence lying 2700nt upstream to the Suppressor of Cytokine Signalling 3 (SOCS3) gene start site (Figure 6A). SOCS3 is involved in the negative regulation of cytokines signalling, including Epo (Fried, 2009; Richmond et al., 2005) and IGF-1 (Akahane et al., 1987; Merchav et al., 1988), both of which regulate erythroid growth (Correa and Axelrad, 1991; Emanuelli et al., 2000).

To functionally validate the Sox6 site found upstream the SOCS3 gene, we set up EMSA experiments using as a probe an oligonucleotide containing either the wt double Sox6 consensus sequence (wt) or a mutated version (mut) (Figure 5B). Nuclear extracts from Sox6-K562 and EV-K562 were used.

As shown in figure 5B, Sox6 binds to its consensus sequence in a specific manner. The band generated by the Sox6-Flag protein is correctly supershifetd by the anti-Flag antibody (lane 2, Figure 5B). In competition experiments show that increasing amounts of the wt oligonucleotide (lanes 4-5) and of a known Sox6 consensus sequence (Col2a1, lanes 8-9) efficiently compete for the Sox6 band whereas, the mutated oligonucleotide (lanes 6-7) does not.

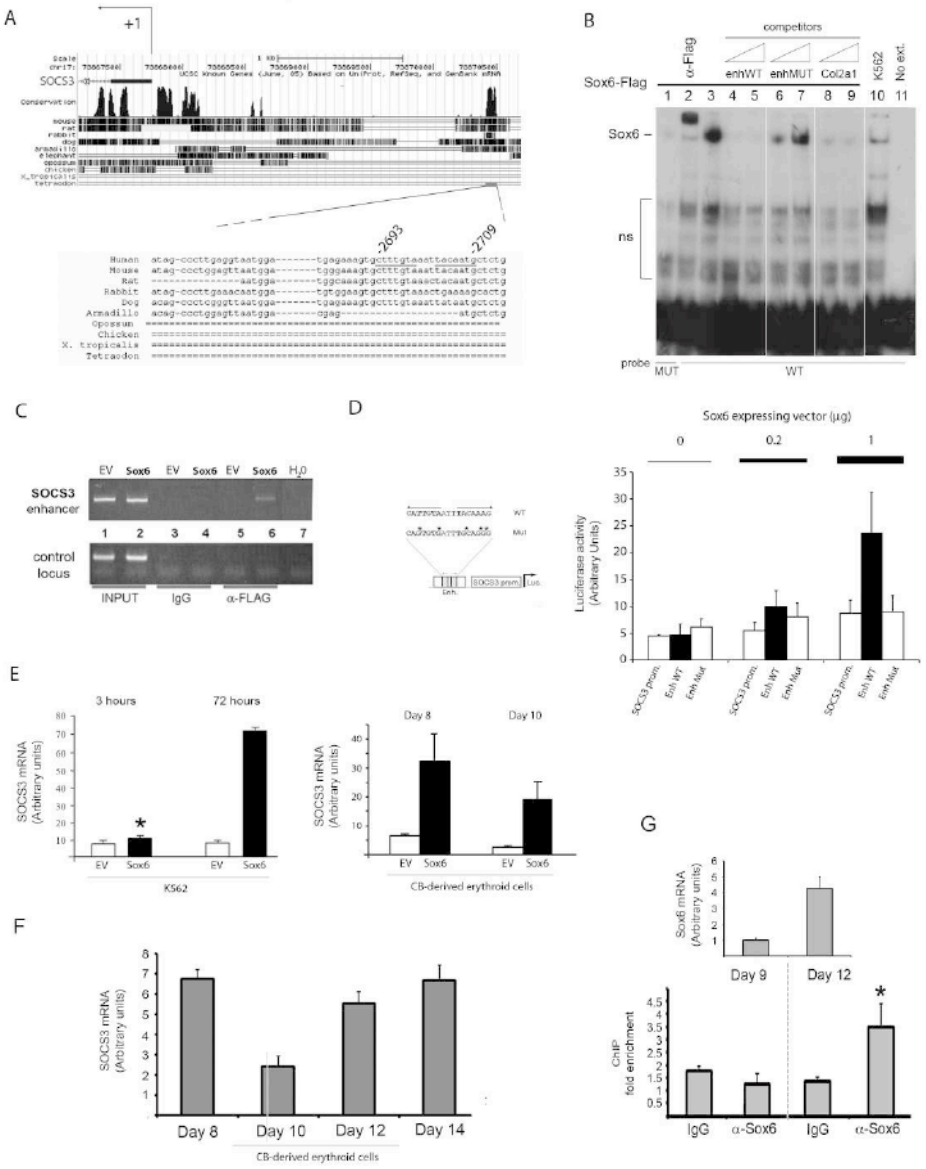


Figure 5. SOCS3 gene is an early direct target of Sox6 (A) mapping of the SOCS3 conserved region containing the double Sox6 binding site. UCSC map indicates a block of conservation of about 100nt, centred on the Sox6 site.

The double site if fully conserved in rat, mouse and man. (B) Sox6 binds *in vitro* to the SOCS3 enhancer in EMSA. Nuclear extracts from Sox6-K562 (lanes 1-9) or from EV-K562 (lane10) were incubated with either the wt (lanes 2-10) or the mutated (lane1) SOCS3 labelled probes. The retarded band generated by the Sox6-Flag protein is supershifted by the antiFlag antibody (lane2) and competed either by the SOCS3 oligo itself (lanes 4-5) and by a known Sox6 consensus (lanes 8-9, Col2a1[48]). The SOCS3 probe mutated in the Sox6 consensus fails to give any binding (lanes 1) and to compete for the Sox6 band (lanes 6-7). The wt probe alone (lane 11) gives no bands. Ns: not specific binding. (C) ChIP on chromatins from transduced K562 cells: the Socs3 enhancer element is Immunoprecipitated by the anti-Flag antibody (that recognize the Sox6 transduced protein), but not by the corresponding preimmune serum (IgG). Upper panel: Socs3 region; lower panel: GAPDH locus, as negative control. Lanes 1-2: input chromatins; Lanes 3-4: IgG; lanes 5-6: anti-Flag antibody; lane 7: water. EV: empty vector; Sox6: Sox6 vector. (D) The SOCS3 enhancer containing the Sox6 double site is activated by Sox6 in cotransfection experiments in K562 cells. A 70nt. fragment containing either the wt (EnhWT) or the mutated Sox6 double site (EnhMut) was cloned upstream to the -393 +12 region corresponding to the SOCS3 minimal promoter (SOCS3prom.). Mutations in the Sox6 consensus (left panel) are the same as in B, proved to abolish Sox6 binding in EMSA. Right panel: all constructs were cotransfected in K562 cells together with increasing amounts (0.2 μgrs and 1 μgrs) of a Sox6 expressing plasmid. The EnhWT construct is activated in a dose dependent manner by the addition of the cotransfected Sox6 plasmid (black columns), while the corresponding mutated element, EnhMut, is insensitive to Sox6 cotransfection (right white columns). (E) Real Time quantification of SOCS3 expression upon Sox6 transduction in K562 (3h and 72h after transduction) and primary Cord Blood derived erythroblasts (48h and 96h after transduction). (F) SOCS3 expression expression during $CD34^{+}$-derived erythroblasts terminal maturation, Real Time PCR. (G) Upper panel. Real time quantification of Sox6 expression at d9 and d12. Lower panel: ChIP performed on chromatins from enhancer. *p=<0.05. IgG antibodies were used as a control.

Finally, nuclear extracts from K562 cells transduced with the control vector generate a weak Sox6 band due to the endogenous Sox6 protein (lane 10) and the probe alone, in the absence of nuclear extracts, fails to give any retarded band (lane 11).

To further test the ability of Sox6 to bind to this element, we performed Chromatin Immunoprecipitation experiments (ChIP) on K562 cells

transduced with the Sox6-vector, using an antiFlag antibody (Figure 6C). The antiFlag antibody specifically immunoprecipitates the SOCS3 enhancer element in cells overexpressing Sox6 (lane 6, upper panel). No bands were detected performing ChIP with control IgG (lanes 3-4).

We then cloned a region of 70 nt surrounding the Sox6 binding sites (either the wt or mutated within the Sox consensus), upstream to the SOCS3 promoter (He et al., 2003) in a luciferase reporter plasmid, that we co-transfected in K562 cells together with a Sox6 expressing plasmid. While the wt SOCS3 element is activated by Sox6, the same element mutated within the Sox6 consensus sequence (same mutation proved to abolish Sox6 binding in EMSA) is insensitive to Sox6 co-transfection (Figure 5D).

Finally, Real Time PCR carried out on Sox6-K562 cells 3h and 72h after transduction revealed a strong early induction of SOCS3 mRNA upon Sox6 overexpression (Figure 5E).

In untransduced $CD34^+$-derived erythroid cultures, the profile of expression of SOCS3 shows a progressive increase in the last days of the culture (From day10, where its expression is at minimum, to day 14) (Figure 5F). to correlate this pattern with the binding of Sox6 to the SOCS3 enhancer *in vivo*, we carried out a ChIP on chromatins from primary cells at day9 and day12. As shown in Figure 5G lower panel, whereas at d9 no significant enrichment is observed, at d12 (corresponding to the peak of Sox6 expression, upper panel) the anti-Sox6 antibody does immunoprecipitate the SOCS3 enhancer, suggesting that this element is recognized *in vivo* by Sox6 when Sox6 accumulates at

late stages of erythroid differentiation. In agreement with this, when Sox6 is overexpressed in $CD34^+$-derivederythroblasts at d6 it subsequentely induces SOCS3 mRNA expression (Real Time PCR at d8 and d10, Figure 5E, right panel).

These data confirm the ability of Sox6 to bind *in vitro* and *in vivo* to the SOCS3 conserved element and to transactivate it in a dose dependent manner in transfection experiments, thus confirming that SOCS3 is a direct Sox6 target.

SOCS3 overexpression mediates growth arrest K562 and primary cells.

To evaluate to what extent the Sox6-dependent SOCS3 induction could recapitulate the phenotype induced by Sox6 overexpression, we overexpressed SOCS3 in K562 cells. To this end, we transfected K562 cells with a SOCS3-IRES-GFP vector by electroporation and 48 after transfection, we sorted GFP^+ from GFP^- cells and we tested them for SOCS3 overexepression by Real Time PCR. Eight days after cell sorting, GFP^+ cells die with a kinetic resembling to that observed upon Sox6 overexpression (Figure 6B). However, 72 hours after transfection, mRNA levels of α, γ and ε globin genes, chosen as prototypic erythroid markers, are comparable in GFP^+ and GFP^- cells (Figure 6A) suggesting that additional Sox6 targets are required to fully recapitulate the Sox6-induced phenotype.

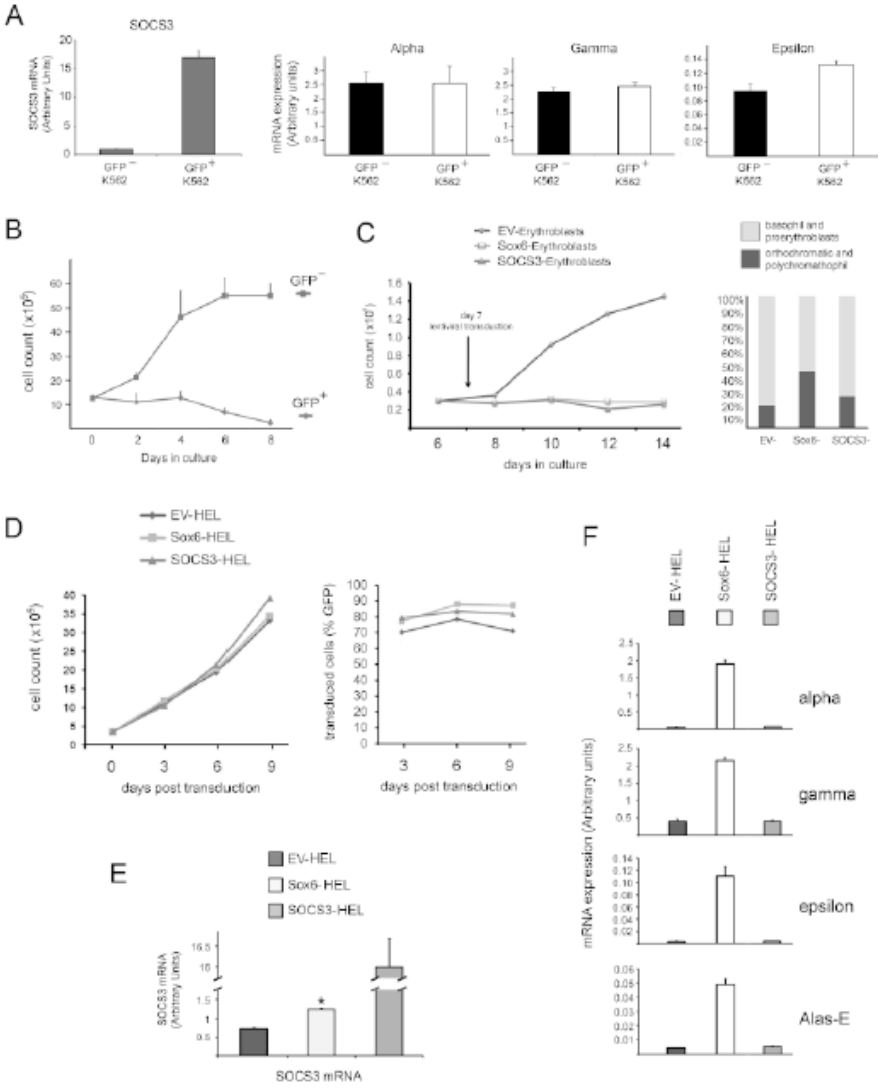


Figure 6. SOCS3 is a relevant Sox6 target (A) SOCS3 overexpression in K562: a SOCS3-IRES-GFP plasmid was transfected in K562 cells and 48 hours after transfection GFP^+ cells were FACS sorted and seeded in parallel with GFP^- cells, and the increased expression of SOCS3 in GFP^+ cells was tested by Real Time PCR. The increase of SOCS3 is not associated with K562 differentiation, as shown by Real-Time PCR on alpha, gamma and epsilon globin transcripts. (B) GFP^+ K562 (SOCS3-overexpressing) stop proliferating with kinetic similar to that observed upon Sox6 overexpression (Figure 3A), y axis: number of cells, x axis: days after sorting.

(C) **SOCS3 overexpression in CD34$^+$-derived erythoblasts.** Left panel: growth curve of cultures infected with the empy vector (diamonds), the Sox6 overexpressing vector (squares) or the SOCS3 overexpressionvector (triangles). y axis: number of cells, x axis: days in culture (all culture were transduced at d6, with similar efficiency). Left panel: differential cells counts. The different colours in the colomns represent the proportion of mature (orthocromatic + polychromatophils, O+P) and immature (basophils+proerythroblasts) cells, respectively. EV- transduced cells: O+P= 43%; SOCS3 transduced cells: O+P= 24%. (D-F) **Trabsduction experiments in HEL cells.** (D) Neither Sox6 or SOCS3 overexpression alter cell proliferation. Growth curve (left) and percentage of GFP$^+$ cells (right) of HEL cells transduced with the emptycontrol vector (EV-HEL), the Sox6 overexpressing vector (Sox6-HEL) and the SOCS3 overexpressing vector (SOCS3-HEL). (E)Quantitation by Real Time PCR of SOCS3 expression in HEL cells transduced as above, relative to GAPDH. (F) Real Time PCR: only Sox6 activates erythroid genes transcription.

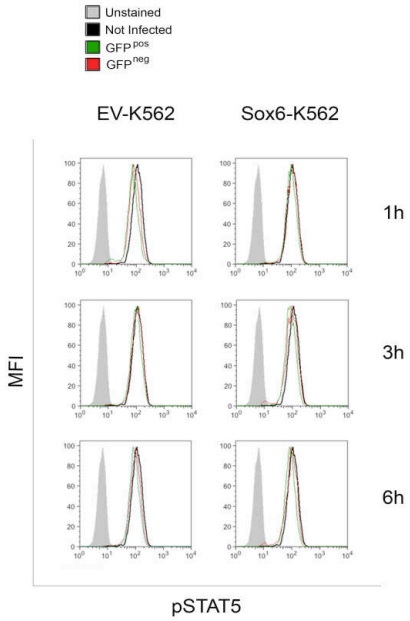
To confirm these data in primary cells, we transduced CD34$^+$-derived erythroblasts (at day6, as for Sox6) with a SOCS3-IRES-GFP overexpressing vector. As in K562 cells, SOCS3 overexpression strongly decreases cell proliferation (Figure 6C, right panel) but, in contrast to Sox6, does not strongly decreases cell proliferation (Figure 6C, right panel) . Infact, differential cells counts at day10 show that whereas Sox6-transduced cultures have an increased number of more mature cells at the expenses of more immature populations, in SOCS3 transduced cultures the distribution of mature versus immature cells is similar to that of the control culture. This data confirms in primary cells that Sox6-induced SOCS3 activation is responsible for the reduced cell growth but not for erythroid differentiation.

This observation raises the prediction that Sox6 overexpression, if achieved in a context devoid of SOCS3 functional contribution, would

lead to increased erythroid gene expression, without any change in the cell growth.

To verify our prediction we took advantage of the HEL cell line. These cells carry the myeloproliferative disorder-associated JAK2V617F mutation, which makes them insensitive to SOCS3 (Quentmeier et al., 2006). Sox6 overexpression in these cells (at a level comparable to that obtained in K562 and primary cells, Supplementary figure 1A) does not alter cell growth (Figure 6D) but significantly increases erythroid specific gene expression, as demonstrated by Real Time PCR quantitation of some prototypical erythroid genes (Figure 6F). complemental to this data, SOCS3 overexpression in HEL cells neither induces a block in cell proliferation nor erythroid specific gene expression (Figure 6D-F). togheter these experiments confirms the role of SOCS3 as the main Sox6 target controlling cell proliferation.

SOCS3 has inhibitory functions on several cytokines signalling, including the Epo-Jak-STAT and the IGF-1R pathways, both of which are required for survival, proliferation and differentiation of committed erythroid progenitor cells (Sasaki et al., 2000; Usenko et al., 2007).In particular, K562 cells are known to depend for their proliferation on autocrine IGF-1 signalling induced by Bcr/abl (Lakshmikuttyamma et al., 2008).



Supplemental Figure 4. Stat5 phosphorylation does not change upon Sox6 overexpression. STAT5 phosphorylation was monitored by FACS analysis at 1, 3 and 6 hours after Sox6 transduction in K562, in parallel with GFP expression. x axis: Mean Fluorescence Intensity, y axis: Phospho STAT5. Left panels: EV-K562; Right panels, Sox6-K562. Gray peaks: unstained cells. Black peaks: uninfected K562. Red peaks: GFP^- cells, not expressing the transduced vectors. Green peaks: GFP^+ cells, expressing the transduced vector (note that GFP positivity coincides with Sox6 expression thanks to the bicistronic Sox6-IRES-GFP cassette).

Whereas STAT5 phosphorylation is not altered by Sox6 overexpression (Supplementary Figure 4), IGF-1 mRNA expression is reduced in K562 cells overexpressing either Sox6 or SOCS3 (Figure 7A).

Administration to Sox6-K562 cells of recombinat IGF-1 (20, 100 and 200 ng/ml) does not rescue the Sox6-induced inhibition of cell proliferation (not shown) as expected on the hypothesis that this effect could be a

consequence of the Sox6-mediated SOCS3 activation (which acts downstream to IGF-1R).

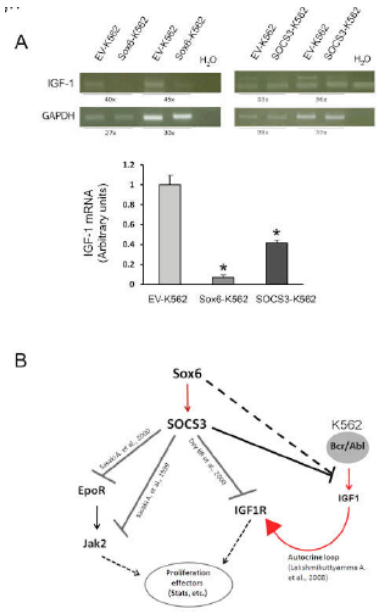


Figure 7. SOCS3 (left panel) and Sox6 (right panel) overexpression is accompanied by a strong repression of IGF-1 transcription. Left panel: RT-PCR on cDNA from GFP^+ and GFP^- obtained after SOCS3 electroporation and cell sorting. Right panel: RT-PCR on cDNA from EV-K562 and Sox6-K562 cells. PCR cycles for IGF-1 and GAPDH amplifications are indicated below the figure. (D) Proposed model for the Sox6 induced growth arrest mediated by SOCS3: SOCS3 interferes with Epo/Jak/Stat pathway by binding EpoR and Jak2[34], and with IGF-1 signalling by binding IGF-1R.[44] K562 proliferation is sustained by an autocrine IGF-1 signalling loop activated by Bcr/Abl.[36] The SOCS3-mediated inhibition of IGF-1 transcription might be responsible for K562 decreased proliferation and increased apoptosis downstream to Sox6.

DISCUSSION

Sox6 is emerging as an important gene controlling different steps of erythropoiesis, ranging form cell differentiation to the control of globin genes expression (Cohen-Barak et al., 2007; Dumitriu et al., 2006b {Yi, 2006 #12; Sankaran et al.; Sripichai et al., 2009; Xu et al.). To get insight to its role in human erythroid cells we overexpressed Sox6 by lentiviral vector-mediated transduction in the erythroleukemic cell line K562 and in primary erythroid cultures derived from cord blood purified $CD34^+$ cells. In both cell types, Sox6 enforced expression drives a significant erythroid terminal maturation. Among Sox6 direct target genes we found the Suppressor of Cytokine Signalling (SOCS3), whose overexpression partially recapitulates the Sox6-induced phenotype, possibly interfering with the IGF-1 signalling.

K562 cells overexpressing Sox6 stop growing within 72h after transduction and undergo terminal erythroid differentiation, as shown by a strong induction of the transcription of several erythroid specific genes, including heme-synthesis enzymes and globin chains. Of note, K562 cells are normally able to differentiate only upon induction with high concentration of chemical inducers, such as hemin (Charnay and Maniatis, 1983; Rowley et al., 1992). Similar results were obtained in *in vitro* cultures, from $CD34^+$ human Cord Blood cells (Figure 3). In these erythroid cultures, the early peak of Sox6 expression driven by the lentiviral vector, causes a strong acceleration of differentiation, leading to

the appearance at day 10 of culture of enucleated cells which are absent in the control culture. This result confirms the ability of Sox6 to accelerate and boost erythroid terminal differentiation of human progenitors. The molecular mechanism of Sox6 action remains to be elucidated in terms of targets and interactors: Sox6 acts either as activator and as repressor of transcription, exerting an architectural role on chromatin organization (Lefebvre). Further studies are required to better study these aspects of Sox6 activity.

Sox6 was recently reported to repress embryonic globin genes, possibly in cooperation with BCL11A (Xu et al.; Yi et al., 2006) and its variation in expression has been proposed to play a role in HbF repression in human erythroid progenitors (Sripichai et al., 2009). In these cells, knockdown of Sox6 leads only to a modest induction of HbF, which is greatly increased by the combined knockdown of BCL11A, suggesting that although these two proteins cooperate to silence γ genes, BCL11A has a preeminent role (Xu et al.). We show that Sox6 strongly stimulates the transcription -in absolute terms- of all globins genes normally expressed by K562 cells, including γ and ε-globin (Figure 1). This result is apparently in conflict with the above reports. However we suggest that Sox6 overexpression has two independent effects on globin synthesis: on one hand, by inducing cell differentiation, it increases the overall expression of globins genes in general; on the other hand it negatively modulate ε and γ-globin transcription, relatively to other globin genes. In fact, the ε/α and γ/α ratios are reduced by 10 and 5 times respectively in

Sox6-K562 when compared with EV-K562. This is supported by the observation that both promoters are occupied in vivo in ChIP experiments by Sox6 (Supplementary Figure 2).

We demonstrate that SOCS3 is an early direct Sox6 target and that SOCS3 overexpression partially recapitulates the Sox6 effect of cell growth arrest in K562 and primary cells, whereas it does not affect proliferation in HEL cells that are insensitive to SOCS3 (Hookham et al., 2007) (Figure 6D-F). SOCS3 is involved in the downregulation of different signaling pathways, and its deregulation in hematopoiesis has has been implicated in the ethiopathogenesis of myeloproliferative disorders; (Baker et al., 2006 {Hookham, 2007 #43; Fourouclas et al., 2008; Usenko et al., 2007).

Among pathways controlled by SOCS3, there are EPO/JAK2/STAT5 and IGF-1/IGF-1R, both of which are required for erythroid progenitor cells survival (Dey et al., 2000; Sasaki et al., 2000). Our experiments show the reduction of IGF-1 mRNA as a Sox6 downstream effect in K562 cells, where Bcr/Abl promotes autocrine IGF-1 signalling thus stimulating cell proliferation and protecting cells from apoptosis (Usenko et al., 2007). Of note, autocrine or paracrine IGF-1 signalling is required in many stages of hematopoisis and its inappropriate activation is an important event in leukemia (Usenko et al., 2007; Lakshmikuttyamma et al., 2008).

In K562 cells, IGF-1R signalling stimulates proliferation and protects cells from apoptosis downstream to bcr/abl signalling, which promotes autocrine IGF-1 signalling. Expression of IGF-1 in hematopoietic cells

(Majka et al., 2001) suggests that autocrine or paracrine IGF-1 signaling is important in sustaining the IGF-1 pathway, which acts as a powerful inhibitor of apoptosis and as survival factor for hematopoietic cells and whose alteration is an important event in leukemia (Lakshmikuttyamma et al., 2008; Usenko et al., 2007).

Together these data indicate that SOCS3 activation elicited by Sox6 might be a relevant event in normal and pathological erythroid differentiation. On this basis, the fine mapping of the molecular mechanisms downstream to the Sox6-SOCS3 induction will be crucial to understanding differentiation not only in erythropoieis but also in other processes where Sox6 plays a central role in cell commitment and differentiation.

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FOOTNOTES

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CHAPTER 3

OVER-EXPRESSION OF AHSP IN THALASSEMIC BONE MARROW-DERIVED CD34^{+} CELLS IS NOT EFFECTIVE IN AMELIORATING THE THALASSEMIC PHENOTYPE.

(manuscript in preparation)

OVER-EXPRESSION OF AHSP IN BONE MARROW-DERIVED CD34^{+} CELLS IS NOT EFFECTIVE IN AMELIORATING THE THALASSEMIC PHENOTYPE.

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Running title: Over-expression of AHSP

ABSTRACT

During erythroid development, hemoglobin synthesis is exquisitely coordinated by an homeostatic mechanism to produce a balanced level of α and β subunits, to avoid excess of either chain and their consequent cytotoxic effect on erythroid cells. β-thalassemia is a genetic anemia characterized by a reduced or absent synthesis of β-globin chains. As a consequence, free α-chains accumulate in the cell generating reactive oxygen species (ROS) that cause cellular damages and decrease erythrocytes life span (ineffective erythropoiesis). Several evidences show that Alpha Hemoglobin Stabilizing Protein (AHSP) is a molecular chaperone that binds free α-globin chain and stabilizes its structure to limit the citotoxic properties. AHSP is required for normal erythropoiesis. Ahsp-null mice exhibit shortened erythrocyte half-life with globin precipitates and ineffective erythropoiesis. AHSP-null erythrocytes contain Hb precipitates, exhibit signs of oxidative damage and short life span. Loss of AHSP exacerbates β-thalassemia in mice, indicating that AHSP has a specific role to neutralize the effect of free α-chains. Here, we over-expressed AHSP in experimental model of β-thalassemia to validate the hypothesis that by stabilizing the excess of α-globin chains, AHSP would ameliorate the thalassemic phenotype. We use a viral-mediated gene transfer to deliver AHSP in $CD34^+$ cells then differentiated in erythroid liquid culture. Increase level of AHSP in thalassemic erythroid precursors does not have an impact on disease pathophysiology: ROS production is not decreased and the apoptosis level does not change.

In conclusion, AHSP over-expression is not an effective strategy to ameliorate the damaging effects of free α-chains that accumulates in thalassemic cells. This is, most likely, due to the fact that the cells already express high level of AHSP that is sufficient to neutralize free α-globins avoiding their accumulation and citotoxic effects. On the other hand, other factors, like the iron overload and heme accumulation might have critical effects to determine the oxidative stress in the thalassemic erythroid precursors.

Abbreviations

SCF = Stem Cell Factor

EPO = Erythropoietin

IL-3 = Interleukin-3

Dexa = Dexamethasone

β-estr = β-estradiol

FBS = Fetal Bovine Serum

HbF = Fetal Hemoglobin

HbA = Adult Hemoglobin

GpA = Glycophorin A

CD45 = pan-leucocyte antigen CD45

AHSP = Alpha Hemoglonin Stabilizing Protein

ROS = Reactive Oxigen Species

NT = Not Transduced (cells)

d = day

INTRODUCTION

Hemoglobin is the major carrier of oxygen in adult mammalian red blood cells. It is a heterotetramer composed of two protein subunits, α- and β-globin. In order to produce a balanced level of α and β subunits to avoid excess of either chain and minimize their consequent cytotoxic effect on erythroid cells, there are many homeostatic mechanism regulating hemoglobin production but unfortunately are still not completely defined. In particular how α- and β-globin are balanced in erythroid precursors is largely unknown and there are same pathologic conditions related to the production of α- and β-chains. The importance of globin balance is dramatically illustrated by the β-thalassemia (Kihm et al., 2002), a genetic anemia characterized by reduced (β^{+}) or absent (β^{0}) synthesis of β-globin chains. In thalassemic cells the free α-globin chains accumulate and generate reactive oxygen species (ROS) (Rifkind et al., 2003) that damage cellular proteins, lipids and nucleic acids and form large insoluble precipitates causing membrane damage (Bank, 1968; Brunori et al., 1975; Joshi et al., 1983; Nathan and Gunn, 1966). Together, these effects are responsible for premature destruction of erythroid precursors (ineffective erythropoiesis) and increase of apoptosis because of a shortened life span of circulating erythrocytes. Many evidence suggest that erythroid cells produce a variety of molecular chaperone to bind and stabilize otherwise toxic proteins during or after their synthesis. Recent characterization of the Alpha-Hemoglobin Stabilizing Protein (AHSP)

has generated interest in understanding their role in normal and pathologic conditions. Alpha hemoglobin stabilizing protein (AHSP), also called erythroid differentiation related factor (EDRF) and erythroid associated factor (ERAF), is a molecular chaperone that binds free α-globin chains and stabilizes its structure prior to incorporation into Hemoglobin A (HbA) (Ellis, 1993; Ellis and Hemmingsen, 1989; Gell et al., 2002; Hartl and Martin, 1995; Kihm et al., 2002; Shaeffer, 1967; Tavill et al., 1968; Tavill et al., 1967) to limit their citotoxic properties providing a potential compensatory mechanism through erythroid precursors neutralize the deleterious effect of free α-globin. In support of this idea, it has been show that AHSP, in solution, specifically binds to the α-chain but not to the β-chain or tetrameric hemoglobin A (HbA) (Kihm et al., 2002). Consistent with a role for AHSP in regulating coordinated globin expression, gene targeting studies in mice showed that ablation of AHSP function leads to erytrocyte abnormalities that also are observed in β-thalassemia, such as increased amount of apoptotic erythroblasts, elevated reticulocyte levels and erythroid hyperplasia in bone marrow (Baudin-Creuza et al., 2004; Kong et al., 2004). Loss of AHSP exacerbates β-thalassemia in mice (Kong et al., 2004), indicating that AHSP is required for normal Hb production and erythrocyte function. $AHSP^{-/-}$ erythrocytes exhibit high Hb inclusion body content, evidence of oxidative damage and short life span. $AHSP^{+/-}$ erythrocytes are normal in number, appearance and life span, but contain increase levels of α-globin precipitate (Baudin-Creuza et al., 2004).

Given these properties of AHSP, we reasoned that its over-expression might have a specific role in thalassemic cells by binding the excess of α-chains and by inhibit ROS production. Using a gene therapy approach, we transduced with by a lentiviral vector bone marrow derived $CD34^+$ cells and then differentiate them in vitro towards the erythroid lineage. The different stages of erythropoisis were confirmed by morphologic and flow cytometric analysis. We obtained increased level of AHSP in thalassemic erythroid precursors but the analysis of erythroid cells at different time points of maturation suggests that over-expression of AHSP is not sufficient to ameliorate β-thalassemic phenotype. This is probably because the high AHSP levels are already sufficient to stabilize the nascent α-globins thought its turnover. Moreover, intracellular proteolysis system in thalassemic erythroid precursors could be activated by the excess of AHSP or AHSP-α-Hb complex consequent to the absence or reduction of β-globin chains.

EXPERIMENTAL PROCEDURES

Human subjects

Human healthy donor samples

$CD34^+$ progenitor cells from human bone marrow of healthy control individuals were purchased from Lonza Inc (Walkersville, MD)

Human thalassemic samples

Pretransplantation marrow samples of β-thalassemia major patients, undergone bone marrow withdrawal for diagnosis, were obtained following prior consent of patients, undisclosed in accordance with the Italian privacy laws.

Plasmids preparation

The hAHSP gene was amplified by PCR from plasmid provided by Prof. Mitchell J. Weiss, Children's Hospital of Philadelphia and The University of Pennsylvania, Philadelphia, USA. The primers are (F5'-TAAATGCA CTGACCTCCCAC-3' and R5'-CTAGCTAGCCTACATCTATAGCTT GGCAC-3').

Subsequently, the PCR fragment was subcloned in TOPO TA Cloning (Invitrogen), digested with NheI and SwaI and cloned into the pRRLSIN18-βEV1-EGFP vector (NheI-SwaI) to produce the pRRLSIN18-β-prom-hAHSP, by replacing the EV1-EGFP. The pRRLSIN18-βEV1-EGFP vector was kindly provided by Michel

Antoniou, Division of Genetics & Molecular Medicine, King's College of London and was used as control for the experiments. In this vector, the hAHSP gene is under the control of the β-globin promoter, (position -265/+50 with respect to the transcriptional start site), HS2 and HS3 LCR elements, and utilizes the poly A signal of the β-globin gene.

Lentiviral vector production

Viral stocks stocks pseudotyped with the vescicular stomatitis G protein (VSV.G) were prepared by transint co-transfection of 293T cells using a three-plasmid system (the pRRLSIN18-β-prom-hAHSP transfer vector, the pCMVΔR8.74 encoding Gag, Pol, Tat and Rev, and the pMD.G plasmid encoding VSV-G) (Follenzi and Naldini, 2002). Moreover, a forth plasmid expressing Rev protein under the control of the RSV promoter (pRSV.Rev) was added in order to increase the level of unspliced viral RNA to be packaged. Transfection of DNA was performd using calcium phosphate precipitation and following 30 hours of incubation, the virus was concentrated 350-fold from viral supernatant by ultracentrifugation. Viral titers were determined by transduction of HEL cells with serial dilution of the vector stock followed by quantitative PCR (qPCR) after three weeks of culture to allow diluition of unintegrated vector below detection level. Genomic DNA was isolated using the QIAmp DNA mini Kit (Quiangen). Vector copy number (VCN) was measured by qPCR, using primer and a probe specific for the RRE region: forward primer 5'- TGAAAGCGAAAGGGAAACCA-3', reverse

primer 5'- CCGTGCGCGCTTCAG-3' AND PROBE 5'-*VIC*-AGCTCTCTCGACCGCAGGACTCGGC-*MGB*-3'.

Cell cultures and transduction of human CD34$^+$ cells

Mononuclear cells (MNC) were isolated from BM of thalassemia major patients by FICOLL density separation. CD34$^+$ cells were purified by positive selection from mononuclear cells[23], using anti-CD34 microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. CD34$^+$ cells were plated at a concentration of 0.5-1x10^6 cells/ml and prestimulated for 24-30 hours in CellGro medium (Cell Genix, Freiburg, Germany) supplemented with 300 ng/ml human stem cell factor (hSCF), 300 ng/ml human Flt-3-ligand (hFlt3-l), 100 ng/ml human thrombopoietin (TPO) and 60 ng/ml human IL-3 (all PeproTech, Rocky Hill, NJ) on plates coated with retronectin (Takara Shuzo, Shiga, Japan). Transduction was performed overnight, with a MOI of 50. The following day cells were washed and portion of transduced cells were either placed in unilineage erythroid culture or plated in methylcellulose medium for colony forming assay. The percentage of transduced cells was determined by vector specific PCR after 14 days of clonogenic assay.

Erythroid liquid culture

Cells were cultured in StemSpan medium (Stem Cell Technologies, Vancouver, Canada) for 15 days. The expansion procedure comprised two steps. In the first step (day 0-7) CD34$^+$ cells were seeded at a

concentration of 10^5 cells/ml and cultured in the presence of 20% (H20-50) or 5% (H5-10) of fetal bovine serum (FBS, Hyclone, Logan, UT), human Stem Cell Factor (hSCF) 50 (H20-50) or 10 ng/ml (H5-10), human erythropoietin (EPO) 1 U/ml, human Interleukin-3 (hIL-3) 1 ng/ml, 10^{-6} M dexamethasone (Dexa, Sigma Aldrich, St Louis, MO), and 10^{-6}M β-estradiol (β-estr, Sigma). In the second step (d8-d11) the cells were resuspended at a concentration of 10^6 cells/ml in Stem Span medium supplemented with 10% (H20-50) and 2,5% (H5-10) of FBS and EPO, 2 U/ml. In the third step (d11-d15) the cells were resuspended at a concentration of 10^6 cells/ml in Stem Span medium supplemented with 10% (H20-50) and 2,5% (H5-10) of FBS. Cultures were maintained at 37 °C in 5% CO_2 and diluted over time to maintain the concentration at 1-2x10^6 cells/ml. The progression toward erythroid differentiation during the culture was monitored by FACS analysis for the expression at different time points of the pan-leukocyte antigen CD45 and the CD235 (GlycophorinA, GpA) using a FITC-conjugated anti-CD45 and a PE-conjugated anti-GpA antibodies. Staining was performed on 1x10^5 cells in 100 μl of PBS containing 1% FBS and 2 μl of a mix of anti-Glycophorin A-PE (DakoCytomation), anti-CD45-FITC (CALTAG Laboratories) antibodies.

Morphological analysis was performed by cytospin, MayGrünwald-Giemsa staining and microscope inspection.

ROS assay

For detection of intracellular ROS, $2x10^5$ cells are washed and diluted with PBS and incubated at 4 °C for 15 minutes with DCFH-DA 2',7'-Dichlorofluorescin Diacetate (DCF, Sigma-Aldrich) previously dissolved in EtOH at a final concentration of 20 μM. Before the incubation, the cells are stimulated or not stimulated with H_2O_2 100 μM for 15 minutes at 4 °C to obtain a positive control. The cells were, at the end, resuspended in PBS and analyzed by flow cytometry.

CFU assay for human progenitors

Transduced thalassemic or normal $CD34^+$ cells were washed with PBS and plated at a density of 1000 cells/ml in methylcellulose medium containing hSCF, hGM-CSF, hIL3 and hEPO (GF H4434 Stem Cell Technologies, Vancouver, Canada) and supporting the growth of human colonies. After two weeks, BFU-E, CFU-GM, and CFU-GEMM colonies were counted, and single colonies (20–30 for each experiment) were isolated, washed and pelleted. DNA extraction was performed (Charge Switch Forensic DNA Purification Kit, Invitrogen) for analysis of transduction efficiency by specific PCR for pRRLSIN18-β-prom-hAHSP. It was performed with primers annealing the vector HS2-HS3 sequence (forward primer HS2F 5'-GTTGGAGGATACCCATTCTCTATCT-3' and reverse primer HS3R 5'-TGGGTCAGTGGTCTCAATGTAGCA-3'; PRIMM, Italy) amplified for 1 cycle at 94°C X 10', 30 cycles at 94°C X 1', 60°C X 1', 72° X 1' and 1 cycle at 72°C X 10 minutes.

Analysis of hemoglobin production

Detection of human HbA and HbF by intracellular staining and FACS analysis

The proportion of erythroid cells expressing human hemoglobin A (HbA) and fetal hemoglobin (HbF) was assessed by flow cytometry. Cells were fixed and permeabilized with BD Cytofix/Cytoperm solution (BD Biosciences Pharmingen), washed and stained with a multimix of FITC-conjugated anti-human HbA (Perkin Elmer) and PE-conjugated anti-human HbF antibody (BD Pharmigen) for 30 minutes at 4°C prior to FACS analysis.

Western blot

Total protein extracts from erythroid cells at different stages of maturation were prepared according to standard protocols (Schreiber et al., 1989) and proteins were subjected to SDS-PAGE separation and blotting. The AHSP protein was detected by the use of an anti-AHSP antibody (kindly provided by M.Weiss, Philadelphia). Protein loading was checked by reprobing filters with an anti-GAPDH antibody (Sigma). Antibodies binding was detected by using appropriate horseradish peroxidase-conjugated IgG and revealed by ECL (Amersham, GE Healthcare).

RESULTS

The AHSP level is similar in normal and thalassemic erythroid cells

To get insight into the role of AHSP in human erythropoiesis, we first evaluated the AHSP level in normal and thalassemic cells during the erythropoiesis. We used an *in vitro* model of human erythroid cells differentiation, starting from bone marrow derived $CD34^+$ cells. In this culture, erythroid progenitors are first expanded to the erythroblast stage (d0-d8) and then induced to terminal differentiation (d8-d15). Erythroid maturation was evaluated by measuring the proportion of GpA^+ (Glycophorin A, CD235) cells by FACS analysis at different stages of maturation.

In a recent publication, Varricchio et al. assert an increase of AHSP at mRNA level in thalassemic erythroid cells compared to normal cells (Varricchio et al.). They hypothetize a correlation of this expression with the level of free α-chains that is higher in thalassemic cells because of the lack of β-chains. We demonstrate that at different time points of erythroid maturation (d6, d8, d10, d12, d14) there is no difference between normal and thalassemic erythroid cells in the AHSP at protein level (Fig. 1). The only difference is the expression of AHSP at day6. In order to explain the observed difference with a delayed differentiation of erythroid thalassemic cells compared to the normal ones, we performed phenotypic analysis of cultured cells. We evaluated by FACS analysis the GpA and pan-leukocyte antigen CD45 (CD45) expression and we found a

difference in the proportion of erythroid thalassemic cells expressing GpA and CD45 compared to erythroid normal cells at d6 (9% *vs* 30% of GpA^+ and 99% *vs* 88% of CD45) (Fig.2). This difference confirms that the different level of AHSP at day 6 is a consequence of the delay in maturation of thalassemic cells.

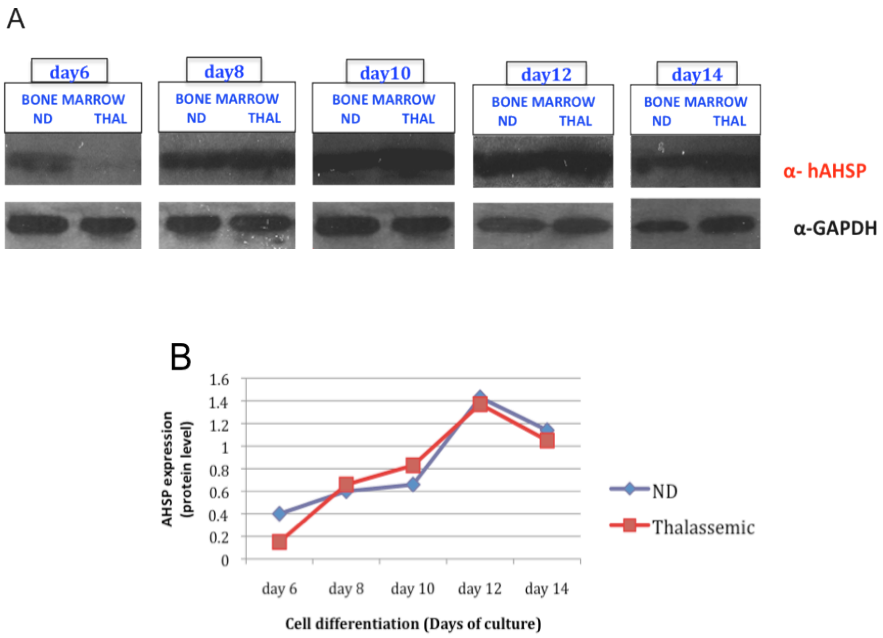

Figure 1. Comparison of AHSP expression in normal versus thalassemic human erythroid cells. There are comparable levels of AHSP in normal and thalassemic eryhtroid cells derived from bone-marrow $CD34^+$ and differentiated in the in vitro culture system described in the text. **A.** Western blot analysis, at different time points of the erythroid culture (d6, d8, d10, d12, d14) of total protein extracts hybridized with anti-AHSP and anti-GAPDH antibodies. **B.**Quantitative analysis of AHSP expression in thalassemic (red line) and normal (blu line) cells evaluated by normalizing for the GAPDH expression level show that there are no difference in the AHSP protein level between normal and thalassemic erythroid cells. The only difference, evident at day6, is a consequence of the delay in differentiation of thalassemic cells (see text for detailes)

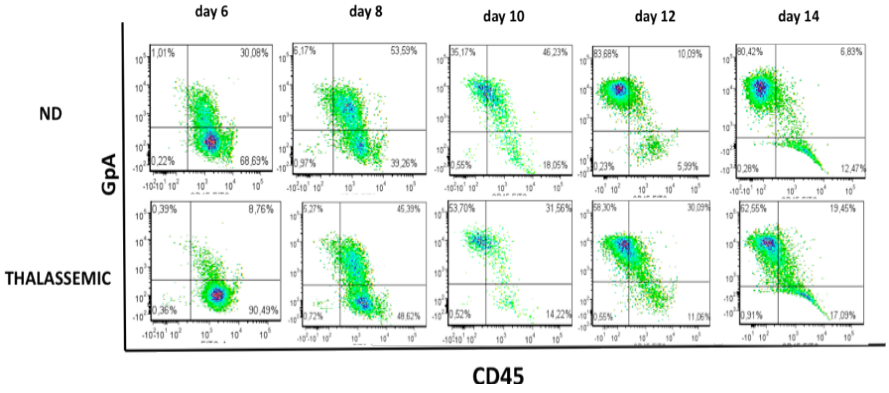

Figure 2. Evaluation of differentiation in normal versus thalassemic human erythroid cells. FACS analysis to evaluate the diffentiation level of erythroid cells during the stages of maturation show that the percentage of GpA^+ cells, at day6, is lower in erythroid thalassemic cells compared to normal cells (9% *vs* 30%). The analysis of CD45 expression confirms the delay in the differentiation: as expected, the percentage of $CD45^+$ cells, at the same day, is higher in thalassemic (99%) cells than in the normal (88%).

The AHSP level is increased in normal and thalassemic erythroid cells after the transduction with AHSP-LV

In order to investigate the role of the AHSP over-expression, we cloned the AHSP gene in a lentiviral vector. THE AHSP (cointaining a part of exon 1, the exon 2, the exon 3 and the two introns) was amplified by PCR from plasmid provided by Prof. Mitchell J. Weiss, Philadelphia, USA. Subsequently, the PCR fragment was subcloned in TOPO TA Cloning (Invitrogen), digested with NheI and SwaI and cloned into the pRRLSIN18-βEV1-EGFP (EGFP-LV) vector (NheI-SwaI) to produce the pRRLSIN18-β-prom-hAHSP (AHSP-LV), by replacing the EV1-EGFP. The pRRLSIN18-βEV1-EGFP (EGFP-LV) vector was kindly provided

by Michel Antoniou, King's College of London and was used as control for the experiments. In the new vector, the hAHSP gene is under the control of the β-globin promoter, (position -265/+50 with respect to the transcriptional start site), HS2 and HS3 LCR elements, and utilizes the poly A signal of the β-globin gene.

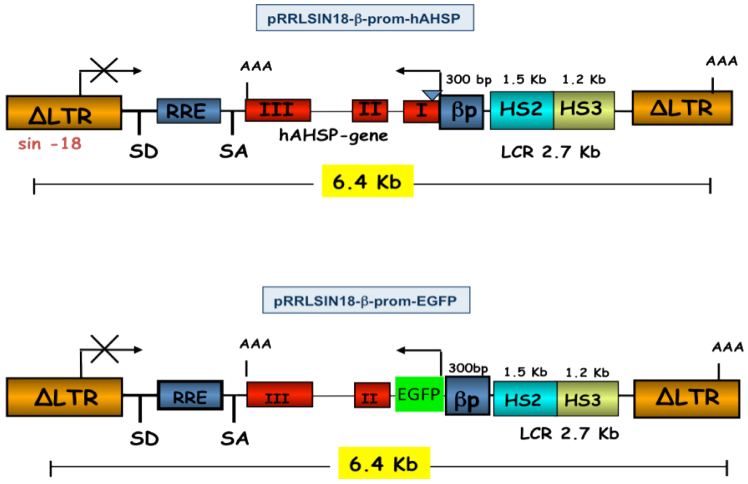

Figure 3. The pRRLSIN18-β-prom-hAHSP lentiviral vector. AHSP and the EGFF are in opposite direction, down-stream the β-globin promoter with the minimal LCR (HS2-HS3).

By using the two described vectors (AHSP-LV and EGFP-LV as control) we pre-activated and transduced the human Bone Marrow (BM)-derived $CD34^+$ cells. After the transduction, the cells were either grown in liquid culture and were plated to carry out the clonogenic assay. After 14 days,

DNA was extracted from BFU-E and PCR analysis was performed to evaluate the percentage of transduction, that was estimated 85% in normal erythroid cells and 45% in thalassemic cells (data not shown). The cells grown in the liquide erythroid culture were analyzed at different time points of maturation (d7, d10, d14).

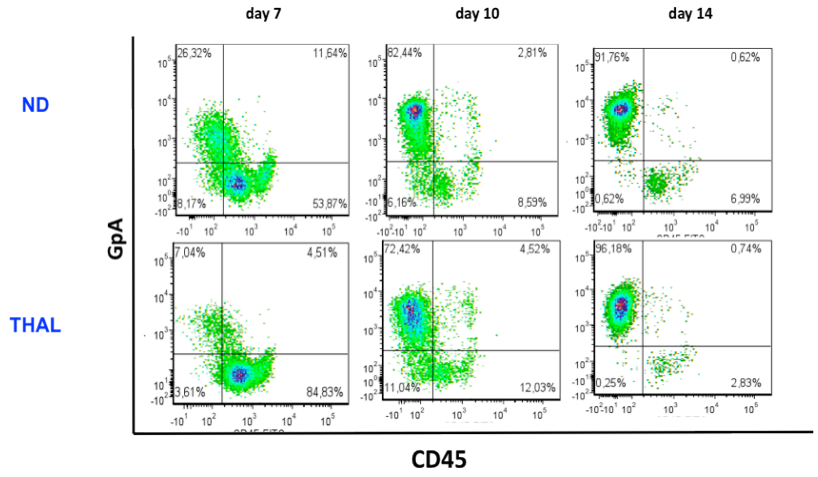

Figure 4. Comparison of differentiation level in normal vs thalassemic cultured erythroid cells. FACS analysis was perfermed to evaluate the diffentiation level of cultured cells at different time points of erythroid maturation. The percentage of GpA^+ cells at d7 is lower in erythroid thalassemic cells compared to normal cells (11% *vs* 37%): thalassemic cells start later to differentiate. The analysis of CD45 expression (89% in thalassemic cells and 65% in normal cells) confirms the delay in the differentiation of thalassemic erytrhoid cells. At d10 the percentage of GpA^+ cells in the normal erythroid population is 85% while in thalassemic erythroid population is 76%. The difference in the differentiation level disappeared at d14: both in normal and thalassemic cells the percentage of GpA^+ cells is more than 90%.

Western Blot analysis shows that AHSP expression starts in normal cells at the beginning of the erythroblast differentiation phase, d7 (corresponding to about 37% of GpA^+ cells) while in thalassemic cells, there is no detectable AHSP (corresponding to about 9% of GpA^+ cells) (Fig. 5). Probably, the difference in AHSP expression in normal versus thalassemic cells at day7 is due to the difference of maturation level (37% *vs* 11%, Fig. 4). The AHSP expression was evident during the other stages of maturation at d10 (85% of GpA^+ cells in normal erythroid cells *vs* 76% in thalassemic erythroid cells) and d14 (more than 90% of GpA^+ cells both in normal and thalassemic cells, Fig.4 and Fig.5). The fold increase in AHSP expression over the endogenous level was calculated by comparing cells transduced by pRRLSIN18-β-prom-hAHSP and cells transduced by the control vector pRRLSIN18-βEV-1-EGFP. We compared the level of AHSP in normal and thalassemic erythroid cells transduced with the AHSP-LV and those transduced with the EGFP-LV at different time points of erythroid culture (d7, d10, d14). The level of AHSP at each time points was calculated normalizing for the expression of the GAPDH. There is an increase in the level of AHSP in transduced normal (from 1,9 to 5,2 fold, Fig. 5A) and thalassemic (from 1,3 to 3,7 fold, Fig. 5B) erythroid cells. Then, in order to evaluate the exact increase of AHSP we normalized the values obtained of AHSP for the percentage of transduced cells (calculated by PCR analysis on DNA of the BFUs). The effective values of AHSP were calculated and we obtained an increse from 2,2 to 6,1 fold in normal erythroid cells (Fig. 5A) and from 2,8 to 7,9 fold in thalassemic erythroid cells (Fig. 5B).

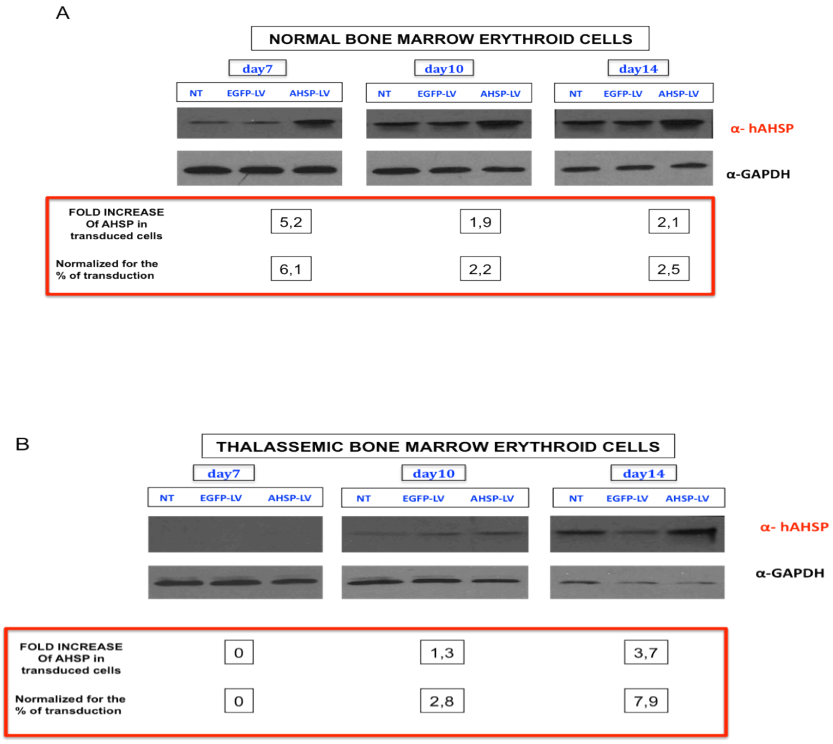

Figure 5. Over-expression of AHSP at protein level. Western Blot analysis show increased levels of AHSP in erythroid cells at different stages of erythroid maturation (d7, d10, d14) in normal erythroid cells (**A**) or thalassemic erythroid cells (**B**). Both in A and B, the fold increase of AHSP in the transduced cells (first line in the box) was calculated by comparing the AHSP level in the cells transduced by pRRLSIN18-β-prom-hAHSP (AHSP-LV) and the control vector pRRLSIN18-βEV-1-EGFP (EGFP-LV). The second line in the box show the values obtained by normalizing for the percentage of transduction calculated by PCR analysis on BFUs. **A.** Normal erythroid cells. At d7, d10 and d14 there is an increase of AHSP at protein level from 1,9 to 5,2 fold that reach high levels (from 2,2 to 6,1 fold) when the values are normalized for the percentage of transduction of cells. **B**. Thalassemic eryhtroid cells. At d10 there is an increase of AHSP at protein level from 1,3 fold to 2,8 when the value is normalized for the percentage of transduction. At day 14 the increase of AHSP level is 3,7 and 7,9 fold when the values are normalized.

ROS production and apoptosis level does not change in thalassemic erythroid cells over-expressing AHSP

In order to investigate if the increased level of AHSP-expression is able to ameliorate the thalassemic phenotype, FACS analysis was performed to compare the apoptosis level and the Reactive Oxigen Species (ROS) production in cells over-expressing AHSP and control cells not transduced (NT). Apoptosis was evaluated by comparing the percentage of Annexin$^+$ cells in thalassemic NT and AHSP-LV transduced cells. The experiments were performed on the erythroid cells derived from the erythroid culture of thalassemic BM-derived cells. At the same stages of erythroid maturation (d7, d10, d14) that we assessed the over-expression of AHSP by Western Blot we performed the FACS analysis to evaluate the percentage of Annexin$^+$ cells in transduced cells and control NT cells. There are similar percentage of Annexin$^+$ cells in the cells over-expressing AHSP compared to the control NT at d7 (1,9% *vs* 1,4%), d10 (3,8% *vs* 6,1%) and d14 (11% *vs* 9%) (Fig. 6 A).

ROS levels were measures by using the DCF as a probe to evaluate intercellular oxidative stress. The cells are incubated with the profluorescent, lipophilic DCFH-DA 2',7'- Dichlorofluorescin Diacetate which can diffuse through the cell membrane. Inside, the acetate groups of the DCFH-DA are cleaved by cellular esterases and the resulting modified DCF cannot leave the cells. In the cell cytoplasm, the reaction of the DCF with ROS results in the fluorescent molecule DCF, detectable to the flow cytometry (emission 488-530nm). The emitted fluorescence of

the DCF is directly proportional to the concentration of intracellular ROS levels. In this experiment, erythroid cells were incubated with the DCF for 15 minutes, are washed and resuspended in PBS and then analyzed by flow cytometry.

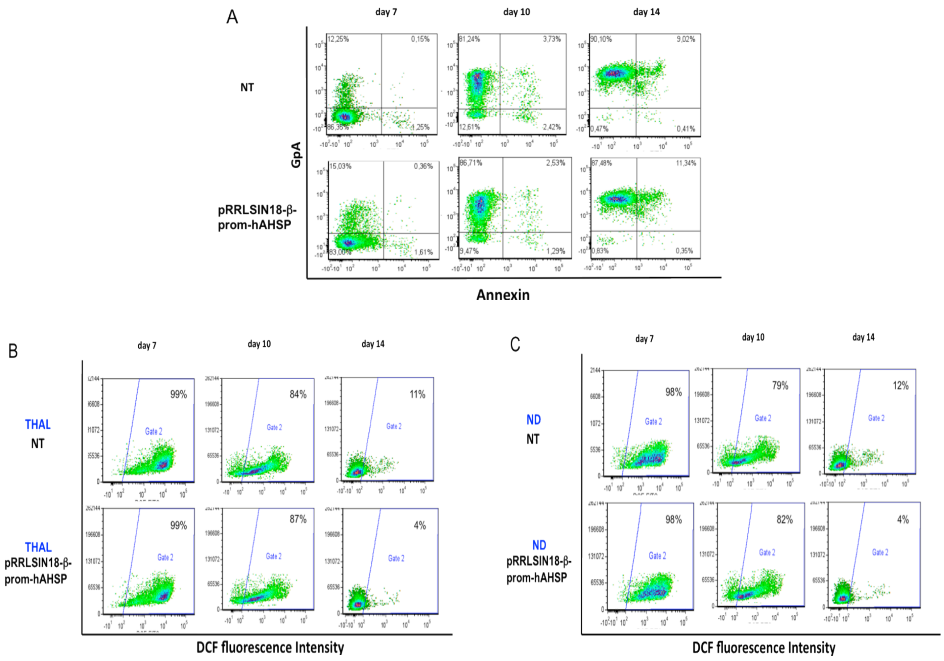

Figure 6. Apoptosis level and ROS production not change in thalassemic eryhtroid cells over-expressig AHSP. FACS analysis on thalassemic erythroid cells at different time points of maturation. **A.** The Annexin$^+$ cells are comparable in thalassemic cells over-expressing or not AHSP at day 7 (1,9% *vs* 1,4%), d10 (3,8% *vs* 6%) e d14 (11,7% *vs* 9,5%). **B.** The ROS production is not reduced in cells over-expressing AHSP. The level is similar in transduced cells compared to the control: day 7 (98% *vs* 98%), day 10 (79% *vs* 82%) and day14 (12% *vs* 4%). **C.** There are no difference as expected, in the ROS levels in normal NT cells compared to the normal AHSP over-expressing cells. ROS levels are comparable in normal and thalassemic erythroid cells at each point of erythroid maturation (d7,d10 ,d14)

The levels of ROS production are similar in the thalassemic NT and in AHSP-LV transduced cells at d7 (98 % *vs* 98%), d10 (82% *vs* 79%) and d14 (4% vs 12%) (Fig. 6B). When we looked at the temporal development of the ROS (Fig. 6 B and C) we can see that the ROS production is similar at each point of erythroid maturation (d7,d10,d14) both in normal and thalassemic cells. At d7, in particular, the oxidative stress is higher than in the later stages of erythroid maturation. This could be due to the general situation of the erythroid cell at this time point: many events occur in the erythroid precursors (high levels of iron up-take, the heme group formation, the hemoglobin synthesis) that could be responsible for the high levels of ROS at this stage of maturation.

Optimization of the *in vitro* erythroid culture.

In our standard protocol of erythroid culture (named H20-50) the $CD34^+$ cells are cultured in StemSpan medium (Stem Cell Technologies, Vancouver, Canada) supplemented with 20% of fetal bovine serum (FBS, Hyclone, Logan, UT), Stem Cell Factor (SCF, 50 ng/ml), erythropoietin (EPO, 1 IU/ml), Interleukin-3 (IL-3, 1 ng/ml), 10^{-6} M dexamethasone (Dexa, Sigma Aldrich, St Louis, MO), and 10^{-6} M β-estradiol (Sigma). FACS analysis of fetal haemoglobin (HbF)-positive cells revealed that the reactivation of gamma-globin synthesis in this culture condition was high. We explained this results as a response of erythroid cells to the exposition at the cytokines and the FBS present in the medium. Because of the induced synthesis of gamma globin, the amount of free α-chains is reduced and thalassemic cells are able to overcome the apoptotic block in

differentiation previously described as a landmark of these types of cultures (Sripichai et al., 2009). For this reason, we optimized our culture protocol in order to reduce the HbF induction without compromising the growth and differentiation of the erythroid population. There are previous publications reporting the capacity of the SCF (Aerbajinai et al., 2009) and bovine serum (Constantoulakis et al., 1990) to induce HbF.

We set up a new protocol, named H5-10 (see material and methods) in which the erythroid progenitors are cultured in the presence of lower level of FBS (5% *vs* 10%) and SCF (10 ng/ml *vs* 50 ng/ml) compared to the standard protocol and we observed a significant decrease of HbF^+ cells (H20-50, 16 ± 5% vs H5-10, 63 ± 25%, Fig. 7A). In this condition the differentiation process, measured as percentage of GpA^+ cells (Fig. 7B, d14) and proportion of cells in different late stages of erythropoiesis (Fig. 7C, d14), does not change. Moreover, the differentiation kinetic of erythroid cells cultured in the new conditions (H5-10) is accelerated. At day 7 the GpA^+ cells are 67% in the H5-10 protocol compared to the 44% obtained with the H20-50 protocol (Fig. 7B, d7). At the same days of the FACS analysis, cells were cytospun, stained with May-Grunwald/Giemsa and analyzed by microscopy. The analysis confirms that erythroid cells are more differentiated when the FBS and the SCF are reduced: at d7, the erythroid cells cultured following the H5-10 protocol (Fig. 7C) are predominantly policromatophilic erythroblasts while those cultured in H20-50 protocol are mainly at the basophilic and pro-erythroblast stages (Fig. 7C).

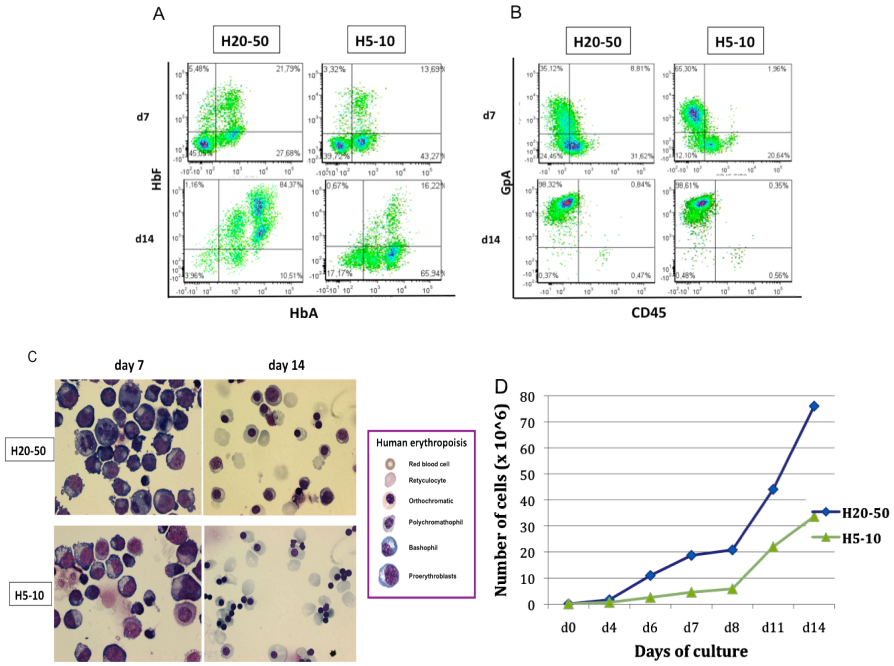

Figure 7. Evaluation of CD34$^+$ cells differentiation level in two different erytroid culture conditions. H20-50 is a protocol of erythroid differentiation in which the FBS level is 20% and the SCF concentration 50 ng/ml. H5-10 is a new tested protocol of erythroid culture in which the FBS is reduced from 20% to 5% and the SCF from 50 ng/ml to 10 ng/ml. **A.** FACS analysis at d14 show a decrease in the percentage of HbF$^+$ cells in the new culture condition (H5-10) compared the standard protocol (H20-50), 16 % *vs* 65%. At the same day the HbA level is high in the two culture conditions: H5-10 82% and H20-50, 94%. **B.** FACS analysis to evaluate the differentiation level of erythroid cells during the stages of maturation show comparable level of GpA and CD45, 98% of GpA$^+$ cells at d14 and 0,5% of CD45$^+$ cells in both conditions of culture. **C.** Morphologic analysis at d14 show that there is any difference between erythroid cells cultured in the two different culture conditions. At d7 the cells are more differentiated in the H5-10 (polychromathopilic stage) than in the H20-50 (bashophilic and proerythroblast stages) because of the low levels of FBS and SCF accellerate the differentiation process. This is confirmed also by the FACS analysis at d7 in which GpA$^+$ cells are 67% in the H5-10 protocol and 42% in H20-50 protocol. **C.** The preliferation kinetics of the erythroid cells cultured with the H20-50 protocol (blu line) and H5-10 protocol (green line) show that the number of cells is decreased using low levels of FBS and SCF but at the end are likewise a consistent and sufficient number.

DISCUSSION

AHSP is an important molecular chaperone for alpha globin chain and we investigated its role in the amelioration of the thalassemic phenotype. We developed a lentiviral vector to over-express the human AHSP in primary erythroid cultures derived from bone marrow purified $CD34^+$ cells, then differentiated toward the erythroid lineage. Significative increase of AHSP at protein level has been obtained but there is not consistent amelioration of the phenotype, evaluated as ROS production and apoptosis level in the erythroid cells at different time points of maturation.

Several lines of evidence suggest that AHSP provides a mechanism by which erythroid cells are equipped to regulate globin chains balance. AHSP is a small erythroid-specific protein that binds α-globin, stabilizes its structure and limits pro-oxidant activity (Feng et al., 2004; Feng et al., 2005; Gell et al., 2002; Kihm et al., 2002; Kong et al., 2004). It is considerated a potential modifier gene for β-thalassemia because by binding α-chains, it is able to limit their toxicity. We reasoned that because in β-thalassemic erythroid cells, where there is an excess of free α-chains, the over-expression of AHSP, by acting as a buffer for the α-chain excess, could ameliorate the pathology. We expected a reduction of ROS production, and as a consequence a decrease of citotoxic effects resulting in a reduced apoptosis in the erythroid population over-expressing AHSP.

The results show that the increased level of AHSP is not sufficient to mend the problems due to the excess of the free α-globin in thalassemic cells. In these cells, while the β-globin production is impaired, the level of α-globin chains is normal. It is possible that the endogenous AHSP is sufficient in the balance of the reaction between α-globin and AHSP. It is known that one molecule of AHSP is sufficient to stabilize 50 molecules of α-globin and there is a very fast turnover of AHSP. Recently, Brillet and collegues asserted that high levels of AHSP are necessary during the normal erythroid differentiation to satisfy the high flux of α-globin production and assure the stability of the binding AHSP-α-globin to complete the correct folding of the nascent chains. Because of this, the authors conclude that AHSP seems to be present in erythroid cells in a great excess (over 100-fold) relative to the requirement (Brillet et al.).

The confirmation of the high levels of AHSP in thalassemic cells derived from a study of Kong et al., that compares thalassemic mice bred with heterozygous or homozygous mutants of AHSP (Kong et al., 2004). In thalassemic mice in which the AHSP gene is completely ablated, erythrocytes have an increase in the ROS level and evidence of oxidative damage, hemoglobin precipitates and short half-life (Kong et al., 2004). On the contrary, the AHSP heterozygous state had no significant effect in thalassemic cells. AHSP haploinsufficiency does not worsen the thalassemic phenotype, indicating that half amount of the molecular chaperone is sufficient to maintain erythroid cells in the same condition in which AHSP is present at normal level. So, high levels of AHSP are present in the normal erythroid cells (Brillet et al.) and also in thalassemic

cells (Kong et al., 2004) and these elevated endogenous levels could be sufficient to well stabilize the pool of α-globin in excess also in thalassemic cells. Therefore, there are two hypothesis to explain why the AHSP over-expression does not have a relevant impact in thalassemic cells: the imbalance between α-globin and its molecular chaperone is not affected and, most importantly, the level of AHSP is already generally very high in erythroid cells.

Another important issue that could explain the lack of effect of AHSP over-expression in thalassemic cells is that the imbalance between α- and β-chain lead to a change in the association kinetic of α-globin and AHSP, because the increased level of free α-chains and AHSP generate an higher dissociation rate of α-globin from AHSP. As a consequence, the overall contact time of the nascent α-chain to AHSP is not enough to complete the necessary protein folding of the α-chain, important to reach a stable structure and the iron oxidation. Therefore, the α-Hb bound to the AHSP exposes likewise a molecule of Fe^{3+} implicated in redox reaction and hence many complexes that generate ROS and Heinz Bodies precipitates in thalassemic cells compared to normal erythroid cells. Recently, it has been indicated that iron and heme could be the main factors responsible for oxidative stress and could have a central role in determining the pathological thalassemic condition (Fibach et al., 2010; Gardenghi et al., 2010).

In conclusion, it is likely that many factors, some probably not completely known, are involved in the thalassemic cells contributing to their phenotype. Further investigation are necessary to understand the

molecular mechanisms that are implicated in the regulation of the HbA formation in order to understand the negative and positive feed-back that regulate the α-Hb and β-Hb production in respect to the AHSP, heme, porphyrins and iron.

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Chapter 5

FINAL DISCUSSION: MOLECULAR AND CLINICAL CONSIDERATIONS

Summary

The aims of this thesis are to study regulatory and structural molecules whose function is crucial for erythroid differentiation, regulation of the globin switching process and formation of hemoglobin. In particular we focused on Sox6 and Alpha Globin Stabilizing Protein (AHSP). In the first part of the work, we focused our attention on Sox6. We demonstrated that Sox6 enforced expression induces terminal erythroid maturation in the erythroleukemic cell line K562 and in primary cultures of $CD34^+$ progenitors derived from human cord blood. We then aimed to unravel the molecular mechanisms underlying Sox6 function, by searching its direct target binding sites on DNA. Among them we found a possible direct regulation of Sox6 on SOCS3 transcription, a gene involved in the regulation of the progression through the cell cycle.

In the second part of the work, we focused our attention on a molecular chaperone, the Alpha Hemoglobin Stabilizing Protein (AHSP), to investigate their role in thalassemic cells. Normally, in erythroid cells AHSP binds and stabilizes the α-globin to complete the folding and avoid the citotoxic consequence of free α-globin accumulation. Since in the thalassemic cells there is a reduced or absent synthesis of β-globin chain and a consequent increase of α-globin, we hypothesized that the over-expression of AHSP, by stabilizing the pool of free α-chains, might ameliorate the thalassemic phenotype

5A. THE IMPORTANCE OF SOX6 IN THE ERYTHROID DIFFERENTIATION.

Sox6 a member of Sry related HMG box family of transcription factors that have an important role in several tissues. Sox6 play important functions in the developing central nervous system (Hamada-Kanazawa et al., 2004a), in cartilage and in muscle (Hagiwara et al., 2000; Han and Lefebvre, 2008; Ikeda et al., 2004; Smits et al., 2001). Despite these reports, no data are available so far about its molecular mechanisms of action. Moreover, Sox6 have a specific role in erythroid differentiation and globin genes regulation. Mice lacking of Sox6 have an impairment of the definitive erythropoiesis (Dumitriu et al., 2006a). We discovered that Sox6 overexpression has a strong effect in erytholeukemic K562 cells: they start to differentiate and transcribe several erythroid specific genes, including heme-synthesis enzymes and globin chains (Chapter 2, figure 1 C-F). Normally, K562 cells differentiate only upon induction with high concentration of chemical inducers, such as hemin (ferriprotoporphyrin IX) or chemotherapeutic drugs (Charnay and Maniatis, 1983; Rowley et al., 1992). It is really interesting that a single gene, Sox6, is able to activate erythroid differentiation in K562, in the total absence of any other chemical stimulus.

This result of erythroid differentiation mediated by Sox6 was confirmed in primary cells, human $CD34^{+}$- cord blood derived than differentiated toward an erythroid lineage in vitro (Chapter 2, figure 4).

Sox6 normally starts to be expressed in erythroid progenitors, at the erythroblast stage (day 8 of our erythroid culture system), and reach a

peak in more differentiated precursors (day 10 and 12). The over-expression of Sox6 by Sox6-lentiviral transduction at day 6 of the erythroid culture produces an "artificial peak" of Sox6 expression. As consequence we observed an accelerated kinetic of maturation, higher percentage of differentiated cells at the same day of culture by compared to the normal (cells transduced with a lentiviral vector expressing the EGFP reporter) and, interestingly, the appearance of completely enucleated cells already at day 10 of the culture that are absent, at this stage, in the control (Chapter 2, figure 4). This result confirm the ability of Sox6 to accelerate the erythroid differentiation in a more physiologic model, the erythroid liquid culture suggesting that Sox6 is an important regulator of the erythropoietic differentiation program.

5A.1. The role of Sox6 during the globin switching

Sox6 strongly stimulates the transcription of all the globins in K562 cells. We analysed by Real time PCR the relative changes of globin genes transcription upon Sox6 overexpression and found that both β-like genes (ε and γ) and α-like gene (ζ and α) normally expressed by K562 cells, are induced by Sox6 overexpression. Of interest, the γ/ε transcripts ratio is induced from 2,5 to 5,5 comparing to the control cells not over-expressing Sox6. It was recently reported that Sox6 specifically repress εy globin expression in mouse by direct binding on its promoter (Yi Z. et al., 2006) and, in the same way, directly silences the embryonic εy-globin gene in definitive erythroid cells (Cohen-Barak et al., 2007; Yi et al., 2006). Moreover, Sox6 cooperates with BCL11A in silencing γ-globin,

possibly via direct physical interaction (Xu et al.). Our experiment show that the level of ε globin gene expression results, in terms of absolute amount of transcript, increase and Chromatin Immunopecipitation demonstrate that Sox6 is able to bind to the human epsilon promoter. This result seems in conflict with the above reports and might reflect two independent effects on globin synthesis: on one hand, by inducing cell differentiation, it increases the overall expression of globins genes in general; on the other hand it negatively modulate ε and γ-globin transcription, relatively to other globin genes. Taken together, these results suggest that Sox6 is a general activator of the erythroid specific transcriptional program but has a marginal role in the human globin switching process. It is interesting to investigate if Sox6 plays a specific role into the ε- to γ-globin switch in the early phase of human embryonic development.

5A.2 Sox6 and SOCS3 as target.

Sox6 activation of globin transcription does not seem to be a direct effect. Sox6, as a member of group D of Sox factors, does not have a trans-activation domain, and interact with other partners as Sox5 and Sox9 in the cartilage system (Lefebvre et al., 2007), or by the HDAC recruitment on the cyclin D1 promoter (Iguchi et al., 2007). Moreover, the majority of described Sox6 binding sites are paired HMG box consensi (Guth and Wegner, 2008; Kiefer, 2007; Lefebvre et al., 2007). In confirmation of this, the εy promoter, the only know example of erythroid Sox6 target, is composed of two binding sequences, with an opposite orientation (Yi et

al., 2006). To find similar consensi through the human genome, we took advantage of the web tool TFBS cluster (Transcription Factor Binding Site - http://hscl.cimr.cam.ac.uk/TFBScluster-) which allows to identify conserved patterns of binding sites present in evolutionary conserved regulatory regions in the mammalian genomes (Donaldson and Gottgens, 2007). The software found 875 double Sox consensus sequences, in mouse-man conserved regions, interspersed in the human genome. Of course, every single target found is of potential interest, but we started to analyse the 56 consensi found within 10 Kbp in 5'position of a known gene, where generally reside most of the regulatory sequences that regulates the transcription of a gene. This approach revealed us 7 genes of extreme interest: among them, in particular, we found SOCS3 that have the double Sox consensus very close to the transcription start site, and within an highly conserved region among mammalian genomes. Subsequentely, EMSA and ChIP experiment demonstrate the capability of Sox6 to bind this region of SOCS3. Therefore, SOCS3 is directly regulated by Sox6 and the binding could be responsible for the effect in differentiation consequent to the Sox6 over-expression.

5A.3 Sox6 block of the progression through the cell cycle

The enhancement of differentiation in erythroid progenitors cells upon Sox6 overexpression, is accompanied with a strong reduction in their proliferation rate: K562 cells, despite their leukemic nature stop growing and die in culture 10 days after Sox6 transduction; $CD34^+$ progenitors, transduced by Sox6-expressing lentiviral vector show early cell death,

probably because of a block in the cell divisions program similar to that observed in K562.

Among genes involved in cell cycle, SOCS3 is the only one whose expression levels significantly change upon Sox6 transduction: its increase in K562 cells is evident already 3 hours after transduction. Moreover, we demonstrate that SOCS3 is an early direct Sox6 target and that SOCS3 overexpression partially recapitulates the Sox6 effect of cell growth arrest. The Sox consensus found within the SOCS3 locus is located 2.7 Kbp upstream to the transcription start site in a very well conserved short region of nearly 120 nucleotides. Results obtained using the luciferase reporter gene assay, suggest that Sox6 directly regulates SOCS3 transcription in a dose dependent manner. Among pathways controlled by SOCS3, we investigated on two that are required for erythroid progenitor cells survival: EPO/JAK2/STAT5 and IGF-1/IGF-1R (Dey et al., 2000; Sasaki et al., 2000).

We hypothesized that SOCS3 could be able to block the Epo-Stat5 signalling transduction, by binding Janus Kinase 2 (Jak2), preventing its phosphorilation and the further Stat5 activation (Ingley et al., 2004). As a consequence it blocks the proliferation of K562 cells and hemopoietic progenitors in culture. We checked for the phosphorilation level of Stat5 after Sox6 tranduction, and we found that there is not difference compared to the control (cells transduced with the empty vector). Then, we moved to investigate if the IGF-1/IGF-1R pathway mediates the Sox6 effect of cell growth arrest and, interestingly, we found that IGF-1 mRNA is reduced as a Sox6 downstream effect, both in K562 cells and in

erythroid cultures. It is known that the downregulation of the IGF gene interferes with the cell cycle progression and the apoptosis downstream the bcr/abl signaling, which promotes autocrine IGF-1 signalling (Lakshmikuttyamma et al., 2009). IGF-1 signaling is important in sustaining the IGF-1 pathway, which acts as a powerful inhibitor of apoptosis and as survival factor for hematopoietic cells and whose alteration is an important event in leukemia (Lakshmikuttyamma et al., 2009; Usenko et al., 2007). This is really important because SOCS3 is involved in the downregulation of different signaling pathways, and its deregulation in hematopoiesis has been implicated in the ethiopathogenesis of myeloproliferative disorders (Usenko et al., 2007; Fourouclass et al., 2008; Baker et al., 2006; Hookham et al., 2007).

5A.4. The clinical relevance of Sox6

Sickle cell anemia and β-thalassemia are two genetic diseases that affect millions of people in the world (Vichinsky, 2007). The only cure is the bone marrow trasplantation when a compatible donator is available. Even though recent progresses in medical treatment extended life expectancy of the patients, a definitive cure for the hemoglobin disorders is not yet available (Cunningham, 2008). Therefore, it is interesting to improve understanding of the molecular basis of the disease to provide clues for potential molecular targets.

Several medical observations started few decades ago, demonstrate that the maintenance of γ-globin expression through adult life is able to significantly ameliorate the clinical conditions of thalassemic patients

(Forget, 1998). Sox6 seems to act as an activator of γ-globin in erythroid precursors and could be an interesting target to design specific strategy in the context of the fetal hemoglobin reactivation.

Moreover, because of a strong induction of differentiation by Sox6 in erythroid precursor cells, it could be interesting to elucidate the molecular mechanisms by which Sox6 acts. This is important because the direct activation of SOCS3 by Sox6 in the hemopoietic tissue, and probably, in other tissues where Sox6 is expressed (Cohen-Barak et al., 2001), could be implicated in the cell cycle progression. Molecular elucidation of SOCS3 regulation is a support in the research concerning tumors from different tissues treatment: its mis-regulation is the cause of numerous proliferative disorders in humans affecting lung and liver (Baltayiannis et al., 2008) and the digestive tract (Isomoto, 2009). Specifically, in the hematopoietic system, SOCS3 is involved in the aberrant signaling transduction of myeloproliferative neoplastic disorders (Kota et al., 2008), and in the molecular mechanism causing Polycythemia Vera (Chen and Prchal, 2006). Very recently, it has been very recently shown how the overexpression of SOCS3 is able to revert the molecular defect of Polycythemia Vera cells, correcting the hypersensitivity to IGF molecule, that leads to erythocytes precursors overgrowth (Usenko et al., 2007). The implication of IGF-1 in this context, support our data of IGF-1 mRNA reduction as a downstream effect of Sox6 over-expression. The IGF-1/IGF-1R pathway is known to interfere with the cell cycle progression downstream the bcr/abl signaling (Lakshmikuttyamma et al., 2009). In particular this pathway acts as a powerful inhibitor of apoptosis

and as survival factor for hematopoietic cells, and its alteration is an important event in leukemia (Lakshmikuttyamma et al., 2009; Usenko et al., 2007). The strong increase of SOCS3 and the decrease of IGF-1 mRNA transcript in Sox6 overexpressing cells motivates further studies to elucidate the mechanism of action in order to clarify their importance as possible targets of specific malignancy in which they are found alterated.

5B. AHSP AND THE HEMOGLOBIN FORMATION

Alpha Hemoglobin Stabilizing Protein (AHSP) is a molecular chaperone that has a specific role in erythroid cells. During erythropoiesis, erythrocyte precursors produce abundant α- and β-chains, which assemble each other to form hemoglobin A (HbA). Hemoglobin A is the major blood oxygen carrier in adult mammals and is a heterotetramer of α- and β-subunits, each bound to heme (Dickerson et al., 1983; Geis, 1983; Perutz, 1981). The process of hemoglobin assembly is coordinated by a homeostatic mechanism to avoid imbalance between α- and β-chain production and the consequent citotoxic effects. In particular free α-Hb (α-globin plus heme, or holo-α-globin) is a potent oxidant, catalyzing the production of ROS, which damage erythroid precursors (Brunore et al., 1975). In addition, α-Hb is inherently unstable and tends to precipitate, lodging in the cell membrane, or degrade, releasing toxic heme, porphyrins, iron, and apo-α-globin polypeptide (α-globin without heme) leading to apoptosis of eythroid precursors.

It is known that erythroid precursors contain a small pool of excess free α-Hb with no deleterious effects (Grayzel et al., 1976; Shaeffer et al., 1976) because adaptative mechanism stabilizes these free α-chains and limit their toxicity. Proteolytic system recognizes and eliminates misfolded and/or potentially toxic proteins generally in all cell types. In erythroid cells there are specific pathways to degrade excess free α-Hb but the molecular mechanism is not well defined (Shaeffer, 1983; Shaeffer and Cohen, 1998). Moreover, most cells contain molecular chaperones that bind, stabilize, and fold potentially toxic protein

intermediates during their normal synthesis or upon pathologic accumulation and, in particular in erythroid cells, has been recently discovered the AHSP. It is a small, abundant protein that binds multiple forms of α-chains and stabilizes the newly translated α-chains to complete the folding process and then incorporate in the HbA (Kihm et al., 2002). The binding of AHSP is specific for α-globin: *in vitro* studies using purified proteins showed that binds α-Hb, but not β-Hb or HbA (Baudin-Creuza et al., 2004; Kihm et al., 2002) and, in confirmation, crystallographic analysis revealed that AHSP and β-Hb have overlapping binding sites on α-Hb (Feng et al., 2004).

Moreover, of interest, under normal physiologic concentration of β-Hb, AHSP does not interfere with HbA synthesis but the importance of this molecular chaperone is evident in the $AHSP^{-/-}$ mice. They exhibit haemolytic anemia with increased levels of ROS and Hb precipitates. These observations are important to validate the role of this molecular chaperone in Hb homeostasis (Kong et al., 2004).

5B.1. AHSP role in thalassemic cells

β-Thalassemia is a genetic anemia characterized by a reduced or absent synthesis of β-globin chains. As a consequence free α-globin accumulates in erythroid precursors and generates ROS and Hb precipitate that damages cellular proteins, lipids and nucleic acids and forms large insoluble precipitates causing membrane damage and increase of apoptosis. In this context, AHSP have a prominent role: by binding to α-globin, avoids the excess of the free α-chain that accumulates and their

consequent cytotoxic effect on erythroid cells. As mentioned above, loss of AHSP exacerbates β-thalassemia by increasing ROS production and Hb precipitation in a murine model (Kong et al., 2004). This suggests that AHSP have a much greater role in thalassemic cells where α-Hb accumulates for the absence or reduction of the β-Hb. We reasoned that if inhibiting the toxicities of excessive free α-Hb is the major role of AHSP, and especially in thalassemic cells, the over-expression of this chaperone could relieve the phenotype in these cells by stabilizing the excess of α-Hb. We over-expressed AHSP in thalassemic cells and we expected a reduction in the ROS production and in percentage of apoptotic cells. Over-expression of AHSP failed to ameliorate thalassemic phenotype suggesting that the cellular mechanism of erythroid precursors is more complicated and that other factors and regulatory elements could be implicated in the process of hemoglobin synthesis. Moreover, in normal and thalassemic cells, normally, the levels of AHSP are already very high. A very recent study of Brillet and colleagues show that in erythroid cells AHSP is expressed over 100-fold relative to the requirements (Brillet et al., 2010). This is a crucial point in order to explain the failed effect of the AHSP over-expression to ameliorate the thalassemic phenotype. However, further investigation are necessary to understand the molecular mechanisms that are implicated in the regulation of the HbA formation in order to understand the negative and positive feed-back that regulates the α-Hb and β-Hb production in respect to the AHSP, heme, porphyrins, iron.

5B.2. The clinical relevance of AHSP

Recent findings on AHSP indicate that this molecular chaperone protects against α-Hb-associated toxicities. This is interesting for the potential implications for human disease to design compounds that mimic the function of AHSP or define a minimal active domain to generate stable, cell-permeable synthetic peptides that stabilized free α-globin as new pharmachologic strategies for adjuvant treatments. So far, our results indicates that the candidate AHSP is not able to modulate the thalassemic phenotype but further investigations could identify new candidates or factors associate to AHSP that have a positive impact to treat the symptoms related to the β-thalassemia and ameliorate the phenotype of patients.

The formation of hemoglobin is a fascinating process but the mechanisms and causal relationships that underlie these associations are not completely clarify and need further investigations. It is important to consider that in thalassemic cells the high proportion of free α-chains decrease the overall contact time of the nascent α-chain to AHSP. As result, the association with α-chain is not enough to complete the necessary protein folding important to reach a stable structure. As a consequence, the AHSP-α-Hb complex remains unstable and exposes likewise a molecule of Fe^{3+} responsible for redox reaction. The knowledge of the structure of the AHSP and the understanding its their structural changes could be important to design molecule that by physical interaction and/or induction of structural alterations, block the capacity of the AHSP-α-Hb complex to generate ROS and the consequent damages.

Moreover, to correlate the importance of AHSP to the hemoglobin formation, basic research examining AHSP gene regulation is necessary to elucidate the potential involvement of this chaperone in the stabilization of α-globin, the globins synthesis, iron regulation and heme formation. It is important to consider that each of the elements that take part to the process of hemoglobin formation is correlated to each other and could be a possible target candidate for a treatment.

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