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"The transcription factor SOX6 induces differentiation and cell cycle withdrawal in BCR-ABL$^+$ and JAK2V617F$^+$ cellular model systems of leukemia"

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A Giovanna e Carlo,

a Elisabetta " il mio raggio di sole".

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GENERAL INTRODUCTION

1.1 HEMATOPOIESIS

Hematopoiesis is the process by which self-renewing stem cells (HSCs) generate progeny that differentiate into all lineages of the blood: eythroid, myeloid and lymphoid lineages (Orkin and Zon 2008; Seita and Weissman, 2010). This gives rise to at least eight different blood cell types: erythrocytes, platelets, granulocytes, macrophages, dendritic cells, T cells, natural killer cells and B cells.

In humans, mature erythrocytes or red blood cells (RBCs) are flexible biconcave disks, lacking cell nucleus and most organelles. RBCs, the most abundant cell type in blood, are rich in hemoglobin, an iron-containing biomolecule that can bind oxygen and carbon dioxide and is responsible for blood's red color. platelets are rod-shaped cell fragments derived from the differentiation of large cells, denominated megakaryocytes, and have an essential role in blood coagulation. lymphocytes, divided into B- and T-lymphocytes, are cells of the immune system involved in defending the body against both infections and foreign material. They perform this task by either activating other cells that eliminate infected cells (T cells and NK cells), or producing soluble immunoglobulines (antibodies) that promote the identification and elimination of foreign agents (B cells) (Janeway, 2001). Granulocytes, distinguished by the presence of cytoplasmic granules, are divided in neutrophils, eosinophils and basophils, and represent the innate immune system, with a role in the inflammatory response and phagocytosis. Macrophages and mast cells are not found in circulation but within tissues. Macrophages arise from circulating monocytes that infiltrate the damaged tissues crossing the endothelium of blood vessels, where they undergo terminal differentiation. They are responsible for the phagocytosis of dead cells, cellular debris and foreign substances. Mast cells originate

from unidentified progenitors of the hematopoietic tissue in the in the bone marrow. These progenitors migrate through the blood into other body sites, particularly into the connective tissue, where they differentiate into mature mast cells. They are involved in wound healing and protection against pathogens, as well as in allergy, and anaphylaxes (Janeway, 2001). Dendritic cells also arise from monocytes, and besides having roles similar to macrophages, they can also stimulate additional immune responses via antigen presentation to lymphocytes.

1.1.1 ONTOGENY OF THE MAMMALIAN HEMATOPOIETIC SYSTEM

The hematopoietic system is one of the first major tissues to form, initially to supply the growing embryo with oxygen and later to permit the remodeling of the hematopoietic tissue into the adult form (Moore, 2004; Palis, 2008).

Throughout life, hematopoietic cells undergo a very high turnover, requiring constant production of new cells. Differentiated hematopoietic cells are continually moving throughout the organism both within the bloodstream (e.g. red cells) and within tissues (e.g. macrophages). However, specific anatomic locations are the sites for the production of new blood cells (Ottersbach, 2010). Hematopoietic stem cells (HSC) precursors are specified from mesoderm and , to mature into functional HSCs, they undergo self-renewing divisions to generate a pool of HSCs. In vertebrates, HSCs are generated in a variety of sites that change during development (Mikkola, 2006), as the anatomy of the embryo changes during organogenesis. In mammals, the sequential sites of hematopoiesis include the yolk sac, an area surrounding the dorsal aorta termed the aorta-gonad mesonephros (AGM) region, the fetal liver, and finally the bone marrow (Fig. 1.1). The placenta has been more recently recognized as an

additional hematopoietic site during the AGM to fetal liver transition period (Orkin, 2008).

The initial wave of blood production in the mammalian yolk sac is termed ‘‘primitive”. The primary function for primitive hematopoiesis is the production of red blood cells that facilitate tissue oxygenation as the embryo undergoes rapid growth. In the yolk sac, from a network of mesoderm cells organized in homogeneous clusters, peripheral cells start to acquire the morphology and markers of endothelial cells, whereas internal cells simultaneously disappear to open the first vessel lumens. Clumps of primitive mesodermal cells remaining adherent to the newly formed vascular endothelium – called blood islands – are at the origin of extra-embryonic hematopoiesis. Since both endothelial and hematopoietic cells develop from the same clusters of mesoderm, the existence of a precursor common to both lineages was postulated: the hemangioblast (Tavian, 2005).

The transient primitive hematopoietic systems is followed by “definitive” hematopoiesis” that is characterized by the production of all blood cell types. At this stage, the region of embryonic hematopoiesis is the Aorta-Gonad Mesonephros (AGM) region (Medvinsky, 1996). AGM forms haemogenic endothelial cells in the ventral wall of the aorta, form which Hematopoietic Stem Cells (HSCs) start to bud, therefore being the site for the beginning of definitive hematopoiesis. The embryonic origin of definitive hematopoiesis is still a controversial topic and different hematopoietic sites has been proposed to start this phase of development (Medvinsky, 1996; Delassus, 1996). In particular, significant numbers of HSCs are also found in the mouse placenta (Gekas, 2005). Thus, the relative contribution of each of the above mentioned sites to the final pool of adult HSCs remains largely unknown. Subsequently, definitive hematopoiesis

consists in the colonization of fetal liver (at approximately E10.5 in mouse), thymus, spleen, and ultimately bone marrow (Fig. 1.1) (Galloway, 2003; McGrath, 2008).

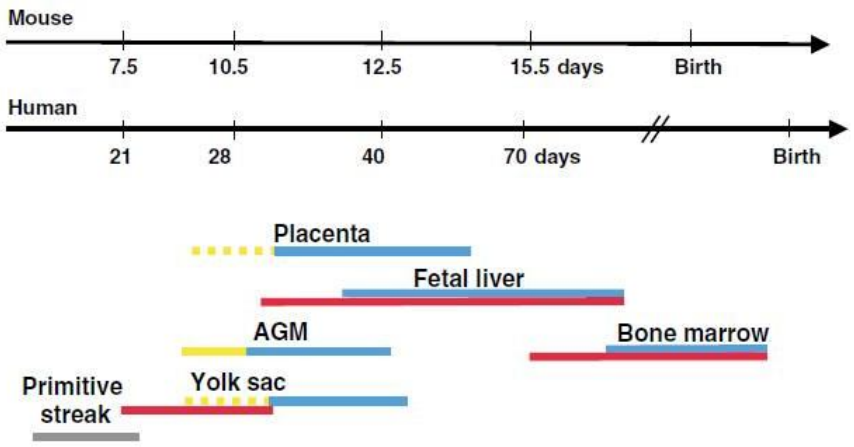


***Fig. 1.1: Human major anatomical sites of hematopoiesis during development**. Different mouse/human hematopoietic sites and representation of the period during which they are active. Gray bars: mesoderm; red bars: active hematopoietic differentiation; yellow bars: HSC genesis; blue bars: presence of functional adult-type HSCs. Broken yellow bars for yolk sac and placenta indicate that de novo HSC genesis has not been experimentally proven. (Figure from Mikkola and Orkin 2006).*

The compartmentalization of hematopoiesis into multiple sites likely involves different inductive signals from the microenvironments to support the development of undifferentiated HSCs in a compartment, while concomitantly generating mature blood cells in a different location. In particular**,** the initial HSC pool that emerges from hemogenic sites must expand to establish an adequate supply of HSCs for postnatal life. Consequently, early fetal HSCs are largely cycling and undergo symmetric cell divisions, in which both daughter cells retain self-renewal ability and multipotency, resulting in a net expansion of HSCs (Lessard, 2004).

1.1.2 THE HEMATOPOIESIS SYSTEM HYERARCHY

Since Till and McCulloch demonstrated the existence of HSCs with the ability to regenerate all types of blood lineages (Till and McCulloch, 1961), intermediated progenitors were then identified on the basis of their expression of specific surface markers (by flow cytometry) and of their differentiation potential in transplantation experiments in mice (Chao, 2008). Initially, HSCs from murine bone marrow (BM) were enriched only by size and density, then stem cells and progenitor cell populations were prospectively isolated and characterized by cell surface phenotype and fluorescence-activated cell sorting (FACS) (Weissman, 2008). FACS integrates the physical properties of the cells and the ability to label them with fluorochrome-conjugated antibodies specific to cell surface antigens distinctly expressed by certain cell types, enabling the phenotypic but not the functional characterization of the tested cells. Since HSCs are exceptionally rare cells (estimated 1 in $3x10^6$ human bone marrow cells; 1 in $1x10^4$ mouse bone marrow cells) (Wang, 1997) and are morphologically indistinguishable from their descendants, HSC identity could only be assessed retrospectively by *in vitro* and *in vivo* functional assays. Hence, the two fundamental assays to characterize HSCs and their progeny, have been colony assays and the *in vivo* transplantation of purified cells population into lethally irradiated adult mice (Dzierzak and Medvinsky, 1995).

These studies led to the "standard" model of hematopoiesis. According to this model, hematopoiesis occurs through a stepwise process, each step generating cells with an increasingly restricted differentiation potential (Fig. 1.2). The first division of HSC leads to one daughter cell keeping stem cell characteristics and the other daughter cell differentiate into the so-called multipotent progenitors (MPPs). These cells retain the ability to give rise to

all different blood cell but have lost the capacity to self-renew. MPPs can differentiate into either common lymphoid progenitor (CLP) (Kondo, 1997) or common myeloid progenitors (CMP)(Akashi, 2000). CLPs are restricted to the lymphoid lineage and will produce B cells, T cells and natural killer cells. CMP will give rise to megakaryocyte/erythroid progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs). Subsequently, GMPs produce the committed precursors of mast cells, granulocytes and macrophages and of a specific group of myeloid derived dendritic cells, whereas MEPs produce the committed precursors of red blood cells and megakaryocytes.

At each branch point, progenitors display a progressively more restricted lineage- potential. The progressive restriction of differentiation potential is orchestrated by the expression of unique combinations of transcription factors (TFs). TFs act together with chromatin-remodeling and modifying co-factors to establish a given lineage-specific gene expression program (Akashi 2000; Akashi, 2003). This is obtained by the onset of the expression of lineage-specific genetic programs, as well as by the concomitant suppression of programs associated with early multipotential states and of alternative lineages.

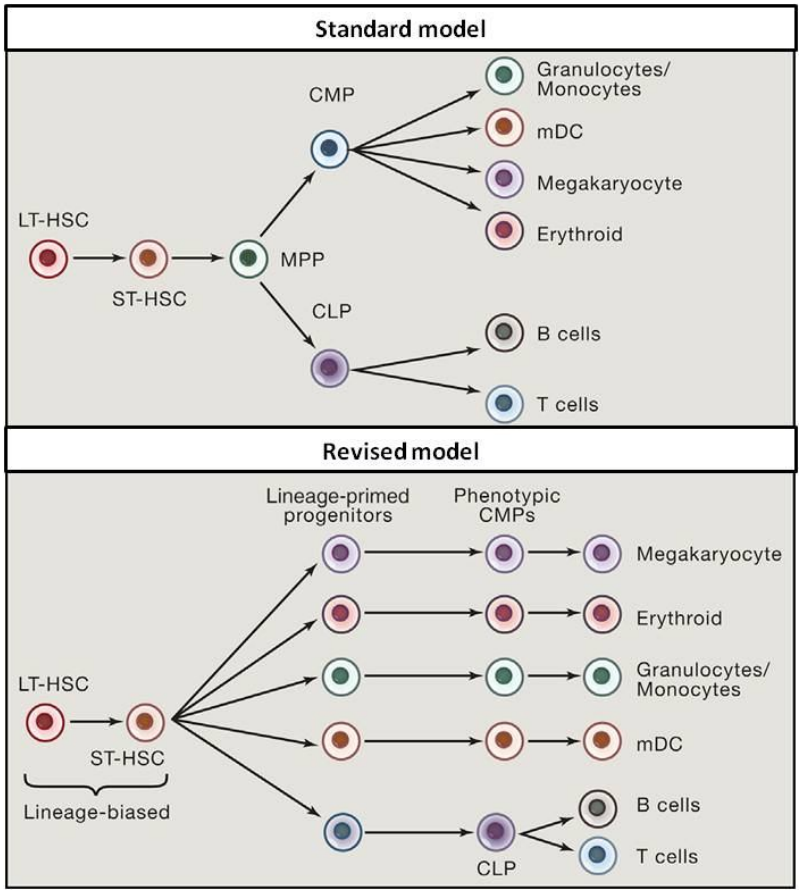


Fig. 1.2: Lineage Determination in Hematopoiesis. *The "standard" model of hematopoiesis based on the assumption that HSCs commit to a lymphoid or myeloid fate and progressively narrow their differentiation potential. The "revised model" postulating the early commitment of HScs towards individual cell fates. (Figure modified from Mercier, 2015).*

Until now, the multiple cell types comprising blood have been thought to emerge from progenitors with a progressively narrower lineage options. New data suggest that lineage fate can might may be determined earlier than it was previously thought.

Paul et al. using single-cell transcriptional profiling and functional assays, found that myeloid progenitors commit very early to differentiation toward distinct blood lineages. Analyzing RNA-sequencing data of 2,730 single lineage-negative, Sca-1, c-Kit^{+} myeloid progenitors, they found that

individual cells cluster into lineage-specific differentiation programs (Paul, 2015). Contrary to previous thought, very few progenitors express multiple transcription factors regulating different fates. Another group with a different approach obtain analogue results. They sorted bulk populations of hematopoietic progenitors, labeled them ex vivo with a library of lentiviral barcodes, and transplanted them into mice to track their fate in vivo. They found that very few CMPs generated both erythroid and myeloid cells. Interestingly, by applying the same experimental design to immunophenotypically defined HSCs and multipotent progenitors (MPPs), they found that HSCs were capable of multi-lineage reconstitution (erythroid, myeloid, and dendritic cells in their assay), while MPPs showed an intermediate phenotype: some, but not all, committed to a single lineage (Perièr, 2015).

The overall picture emerging from these experiments, suggests that the common myeloid progenitors (CMP) are not an homogeneous population, as previously thought, but rather an heterogeneous mixture of progenitors already committed -transcriptionally and functionally- to a specific lineage (Fig. 1.2).

These studies suggest that early progenitor blood cells are pre-programmed with strong biases to make specific lineage types and that making one lineage type is not inconsistent with having stem-cell-like self-renewing capability. This ‘‘hard-wiring’’ of cell lineage fate may, in principle, extend up to the stem cell itself, implying that stem/progenitor cell pools may not be a collection of equipotent cells but, rather, a complex mix of cells with different potential (Mercier, 2015). If this is true, an important implication is that in malignancies, cells may not need to ‘‘re-acquire’’ the ability to self-renew, needed to become a disease-producing malignant clone.

The hematopoietic system in vertebrates requires the continuous production of new cells to maintain homeostatic conditions, since mature cells have limited lifespan. This requires a fine balanced and coordinated equilibrium between different decisions: quiescence versus proliferation, self-renewal versus differentiation, survival versus death, migration from the bone marrow niche into circulation. The molecular circuits underlying HSC and progenitors fate choice must enable the hematopoietic system to both maintain homeostasis and to respond to stress conditions in response to external stimuli. These processes are regulated by a combination of intrinsic factor (lineage determining transcription factors and their epigenetic regulators) and extrinsic regulator (such as cytokines, hormone, growth factors). Even if specific lineages can be instructed by cytokines, this does not preclude the existence of additional cell-intrinsic lineage choice mechanisms that could lead to lineage choice in the absence of cell-extrinsic instructive signals (Rieger, 2012).

Lineage-specific transcription factors (likely working in conjunction with general transcription factors) play essential roles in this process. The function of many transcription factors is highly conserved from mouse to human, and their mutations cause severe lineage abnormalities and blood tumor (Cantor and Orkin, 2002; Rosenbauer and Tenen, 2007).

In the past, many findings demonstrated the ability of lineage-specific transcription factors to drive the differentiation of cells into a specific lineage. In some case TFs were proven to be able to "reprogram" cells already committed to alternative lineages (transdifferentiation). For example, ectopic expression of GATA1 in multipotent cells or cells committed to other than the megakaryocyte–erythrocyte lineages (primary bipotent progenitors, which normally display only neutrophil and monocyte

differentiation *in vitro)* enforces the differentiation into erythroid and megakaryocytic cells (Heyworth, 2002). Other examples are C/EBPα and PU.1, essential for the generation of granulocyte-macrophage progenitors. C/EBPα expression in committed lymphoid cells (B and T cells) instructs the differentiation into macrophages (Xie, 2004; Laiosa, 2006) and PU.1 ectopic expression induces committed T cells to transdifferentiate into myeloid cells (Laiosa, 2006).

1.2 THE SOX FAMILY

SOX genes encode a family of transcription factors that belong to a super-family of genes characterized by the presence of the HMG (high mobility group) box, a DNA binding domain highly conserved throughout eukaryotic species.

SOX genes are defined as the ones containing the HMG box of SRY (sex determining region on the Y chromosome), the gene involved in sex determination. Twenty SOX genes are present in humans and mice. The SOX family is divided into 8 subgroups, A–H, with two B subgroups, B1 and B2, accordingly to their homology within the HMG domain and in other structural motifs (Bowles, 2000)(Fig. 1.3).

SOX proteins within the same group share a high degree of identity (generally 70–95%) both within and outside the HMG box, whereas SOX proteins from different groups share partial identity (≥46%) within the HMG box domain and almost none outside this domain. Most SOX genes have one to three exons and give rise to a single protein product. Instead, the SoxD and SoxH genes are split into multiple exons and give rise to several splice variants with different properties (Lefebvre, 2007).

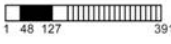
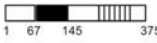
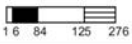
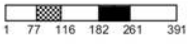
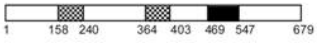
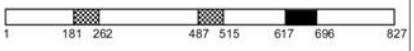
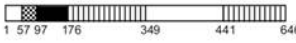
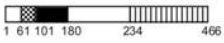
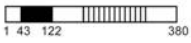
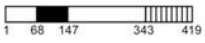
Group	Gene	Locus	Schematic	References
A	*Sry*	YC3		Gubbay et al., 1992 Dubin et al., 1995
B1	*Sox1*	8 A1-A2		Collignon et al., 1996 Kamachi et al., 1999
	Sox2	3 A2-B		Collignon et al., 1996 Kamachi et al., 1999
	Sox3	X A7.3-B		Collignon et al., 1996
B2	*Sox14*	9 E3.3		Hargrave et al., 2000
	Sox21	14 E4		Uchikawa et al., 1999
C	*Sox4*	13 A3-A5		van de Wetering et al., 1993
	Sox11	12 A3		Kuhlbrodt et al., 1998
	Sox12	2 G3		NCBI - CAM23207
D	*Sox5*	6 G3		Denny et al., 1992 Lefebvre et al., 1998
	L-Sox5	6 G3		Lefebvre et al., 1998 Hiroaka et al., 1998
	Sox6	7 F1		Lefebvre et al., 1998 Takamatsu et al. 1995 Connor et al., 1995
	Sox13	1 E4		Kido et al., 1998
E	*Sox8*	17 A3		Schepers et al., 2000
	Sox9	11 E2		Sudbeck et al., 1996 Wright et al., 1995
	Sox10	15 E1		Pusch et al., 1998 Kuhlbrodt et al., 1998
F	*Sox7*	14 C3		Taniguchi et al., 1999 Takash et al., 2001
	Sox17	1 A1		Kanai et al., 1996
	Sox18	2 H4		Dunn et al., 1995 Hosking et al., 2001
G	*Sox15*	11 B3		Beranger et al., 2000
H	*Sox30*	11 B1.1		Osaki et al., 1999

Fig. 1.3: ***Classification, gene chromosomal location (locus), and structural organization of mouse Sox proteins.*** *Sox proteins are schematized as boxes, with the position of the first and last amino acid residues and boundaries of functional domains indicated. Internal boxes are shown for functional domains: HMG box domain (closed), transactivation domain (vertical stripes), transrepression domain (horizontal stripes), and dimerization domain (checkers)(From Lefebrvre, 2007).*

The consensus motif for SOX proteins has been defined as the hexameric sequence 5'-WWCAAW-3', where W indicates A or T (Bowles, 2000). Preferences for nucleotides adjacent to the core consensus, dictated by the HMG box, likely allow SOX proteins of the same group to target the same DNA sites *in vivo*, whereas SOX proteins of other groups either target other sites or compete to each other for the same sites (Harley, 1994; Mertin, 1999; Lefebvre, 2007).

Co-operation and competition mechanisms exist *in vivo:* SOX5/SOX6 (belonging to the SoxD group) cooperate with SOX9 (SoxE) in activating chondrocyte-specific genes (Lefebvre, 1998). Instead, SOX5 and SOX6 compete with SOX9 and SOX10 (both SOXE factors) for the binding to the oligodendrocyte-specific Myelin Protein Zero (MPZ) and Myelin Basic Protein (MBP) promoters (Lefebvre, 1998).

Several SOX proteins have been shown to bind *in vivo* to DNA sequences that only partially match the *in vitro*-defined SOX consensus. Since imperfect SOX binding sites are abundantly found on DNA, it is clear that DNA sequence is not the only factor that directs SOX proteins to their target sites *in vivo*.

SOX proteins bend DNA upon binding. In support of the notion that DNA bending is an essential Sox function, SRY and SOX2 mutations that selectively interfere with DNA bending, and not with DNA binding cause a disease phenotype in humans (Pontiggia, 1994; Scaffidi, 2001). The DNA bending angle induced by SOX proteins varies from as little as 30° up to as much as 110°, which has led to their qualification as "floppy" proteins (Weiss, 2001).

While most other types of DNA-binding proteins contact the DNA major groove and only induce minor changes of the DNA conformation, the HMG domain binds DNA in the minor groove, intercalate amino acid side chains

between DNA base pairs, and induces a significant bending of the DNA helix. Hence, upon SOX proteins binding to DNA, the minor groove is widened and the major groove compressed. HMG domain proteins are thus unique in their ability to alter the conformation of DNA and to increase its accessibility and plasticity (Lefebvre, 2007).

1.2.1 SOX6

The **SOX6** transcription factor is a member of the SoxD group. The members of the SoxD (SOX5, SOX6 and SOX13) group are characterized by the presence of a leucine zipper (LZ) and glutamine-rich (Q-box) domains located in the N-terminal half of the protein (Wegner, 1999; Kamachi, 2000). In SOX6, the currently identified functional domains include one DNA binding domain (the HMG box), and two coiled-coil domains, where the LZ and Q-boxes are located (Connor, 1995; Lefebvre, 1998). The more N-terminus coiled-coil domain is involved in protein-protein interactions with multiple proteins (Lefebvre, 1998; Yamashita, 2000; Cohen- Barak, 2003; Iguchi, 2007; Ohe, 2009), as it is, although to a lesser extent, for the SOX6 HMG box (Iguchi, 2007). Despite this dual function, i.e. activation and repression, SOX6 lacks any conventional transactivation or transrepression domain.

Thus, SOX6 ability to activate and repress gene expression, depends on its interaction with different partners that allows the recognition of slightly different target sequences (Kamachi, 2000; Kiefer, 2007; Lefebvre, 2007).

The functional versatility of SOX6 makes it a very important player in the differentiation of many cell types throughout development (Hagiwara, 2011).

SOX6 was originally isolated form adult mouse testis (Connor, 1995) but it is also required for the development of the central nervous system (Stolt,

2006), for chondrogenesis (Lefebvre, 1998), and for cardiac and skeletal muscle formation (Hagiwara, 2000; Hagiwara, 2005).

SOX6 promotes cardiomyocyte differentiation, an event associated with the down-regulation the L-type Ca21 alpha1c gene, by binding to eight consensus Sox recognition sites lying within the Ca21 alpha1c promoter (Cohen-Barak, 2003). The Prtb (proline-rich transcript of the brain) transcription factor, which interacts with SOX6, represses the activity of the alpha1c gene promoter, as SOX6 does. However, the repression of the same promoter is abolished when Prtb and SOX6 are co-expressed, suggesting that Prtb and SOX6 antagonize each other. Skeletal muscle development consists of two successive waves of myogenesis, embryonic and fetal, generating slow and fast fibers, respectively. SOX6 mutant mice show increased slow fiber type-specific gene expression and reduced fast fiber gene expression. In fact, SOX6 represses the transcription of slow fiber type-specific genes, typified by the myosin heavy chain beta (MyHCb), slow isoform (Hagiwara, 2005).

As for the mechanism of SOX6-mediated repression, on some targets, SOX6 recruits CtBP2 as corepressor, (Murakami, 2001). On other targets, such as on the insulin II gene. SOX6 interacts with PDX1 to elicit repression (Iguchi, 2005). Instead, on the cyclin D1 promoter , SOX6 interacts with beta-catenin and the histone deacetylase HDAC1 (Iguchi, 2007).

1.2.2 ROLE OF SOX6 IN ERYTHROPOIESIS

SOX6 plays a crucial role in erythropoiesis. It is expressed in the fetal liver and bone marrow, and also in purified adult definitive erythroblasts (Dumitriu, 2006; Yi, 2006; Cantù, 2010; Xu, 2010). SOX6 stimulates erythroid cell survival, proliferation and terminal maturation during definitive murine erythropoiesis (Dumitriu, 2006).

Mice carrying the chromosomal inversion p100H, disrupting the SOX6 locus, have the same gross phenotype as SOX6-null mice: pups develop cardiac and skeletal myopathy, they are anemic and present defective red blood cells. Pups lacking SOX6 compensate for anemia by elevating the serum level of erythropoietin and progressively enlarging their erythropoietic tissues. SOX6 indeed is necessary for efficient erythropoiesis in adult mice, under both basal and stress conditions (Dumitriu, 2010). The erythroid-specific inactivation of SOX6 (obtained through a conditional knock-out, by using a CRE recombinase knocked-in the EPO receptor locus) causes the same phenotype, demonstrating a cell autonomous role SOX6 role in erythroid cells (Dumitriu, 2006). SOX6 potentiates the ability of erythropoietin signaling to promote proerythroblast survival and has an effect additive to that of erythropoietin in stimulating proerythroblast and erythroblast proliferation. Moreover, SOX6 facilitates critical aspects of erythroblast and reticulocyte maturation, including hemoglobinization, cell condensation and enucleation, ensuring erythrocyte cytoskeleton long-term stability (Dumitriu, 2006). SOX6 overexpression in both $CD34^+$ cells purified from cord blood causes cell cycle withdrawal and terminal maturation, together with a general increase of several erythroid specific genes, including globins (Cantù, 2011).

Beside its role as a general globins activator, SOX6 also plays an important role in the developmentally regulated globin genes expression (hemoglobin Switching). The hemoglobin switching consists in the transition between embryonic to fetal and fetal to adult globin genes expression (Sankaran, 2010). The expression of the embryonic globin genes is limited to primitive red blood cells produced in the yolk sac and becomes silenced in the fetal liver, where fetal definitive erythroblasts are generated (Trimborn, 1999). It has been shown that SOX6 plays a significant role in silencing the

transcription of the mouse embryonic β-like globin gene εy (Yi, 2006). This effect is cell autonomous, as shown by the substantial increase in εy expression in SOX6-null hematopoietic stem cells engrafted into adult mouse bone marrow (Cohen-Barak, 2007). In addition, SOX6 takes part in the suppression of the human fetal γ gene in adult definitive red blood cells, in cooperation with the BCL11A protein (Xu, 2010), a known transcriptional repressor (Liu, 2006). Genome-wide association studies (GWAS) of human fetal globin (HbF) led to advance in our understanding of the globin gene switching in erythropoiesis (Sankaran, 2008; Sankaran, 2010).

The regulation of SOX6 expression in erythroid cells is mediated by both extracellular signals and subsequent intracellular feedback mechanisms. First, different cytokine conditions can alter SOX6 expression levels in human erythroid cell cultures (Sripichai, 2009). Second, it has been shown that in differentiating erythroblast cultures, SOX6 protein acts as repressor of its one transcription by binding to its own promoter (Cantù, 2010).

Furthermore, SOX6 was bound in vitro and in vivo to an evolutionarily conserved regulatory SOCS3 element, which induced transcriptional activation (Cantù, 2011). SOCS3 overexpression in K562 cells and in primary erythroid cells recapitulated the growth inhibition induced by the overexpression of SOX6, therefore SOCS3 is a relevant SOX6 effector.

1.3 THE SOCS FAMILY

The **SOCS** (Suppressor Of Cytokine Signaling) genes are also named Cytokine-inducible SH2 protein (CIS). Both these aliases refer to their main activity. In fact, they are induced through cytokine signalling (in particular upon STAT proteins activation) and they act as negative regulators of the cytokine JAK–STAT pathway, thus mediating a negative feedback mechanism of regulation. There are eight proteins members of the SOCS family (CIS and SOCS1–SOCS7), sharing a central SH2 domain, an amino-terminal domain of variable length and sequence, and a carboxy-terminal 40- amino21 acid module, known as SOCS box (Fig. 1.4).

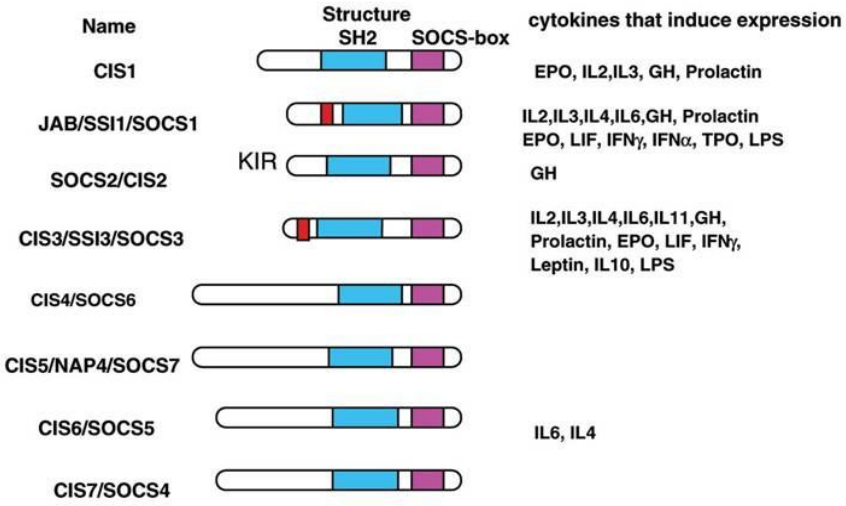


Fig. 1.4: Structures of suppressor of cytokine signaling (SOCS) family molecules*. CIS, Src homology 2 (SH2)-containing protein; EPO, erythropoietin; JAB, JAK (Janus family kinase)-binding protein; KIR, kinase inhibitory region; NAP4, Nck/Ash-binding protein 4; SSI-1, STAT (signal transducer and activator of transcription)-induced STAT inhibitor-1 (Figure from Yoshimura, 2005).*

SOCS1 and SOCS3 have a kinase inhibitory region (KIR), which mediates the suppression of JAK tyrosine kinase activity (Fig. 1.4). The SOCS box interacts with Elongins B and C, Cullin-5 or Cullin-2, Rbx-1 and E2, therefore functioning as E3 ubiquitin ligases and mediating the degradation of proteins associated through their N-terminal regions (Yoshimura, 2005).

SOCS knock-out in mouse revealed that these proteins are involved in several physio/pathological processes. SOCS-$1^{-/-}$ mice die within 3 weeks after birth because of severe lymphopenia, activation of peripheral T cells, degeneration and necrosis of the liver, macrophage infiltration of major organs. This mice also suffer from mild anemia and present an excess of nucleated erythroid cells in the spleen (Alexander, 1999; Marine, 1999). $SOCS2^{-/-}$ mice show a 40% increase in body weight and an enhanced growth hormone-induced STAT5 activation. The double $SOCS^{-/-}$ and $STAT5^{-/-}$ reverts the phenotype. Growth promotion by growth hormone (GH) is dependent on its induction of IGF1 (insulin etc), whereas SOCS2-deficient mice do not exhibit an increase in serum IGF1 (Metcalf, 2000). SOCS2 therefore appears to play an essential physiological role in the regulation of growth, possibly due to its ability to modulate growth hormone and/or IGF1 signaling (Krebs, 2011). $SOCS5^{-/-}$ mice show reduction in IL-4-induced activation of STAT6 and thus reduced Th2 polarization. SOCS5 is dispensable for lymphocyte production and function (Brender, 2004). $SOCS7^{-/-}$ mice show hydrocephalus, characterized by cranial distortion, dilation of the ventricular system, reduced thickness of the cerebral cortex and disorganization of the subcommissural organ (Krebs, 2004). Finally,SOCS6 deficient mice are normal, with the exception that they weight 10% less than wild-type littermates (Krebs, 2002).

1.3.1 SOCS3

SOCS3 is induced by a broad spectrum of cytokines including interleukin IL-10 (Ito, 1999), leptin (Bjorbaek, 1998), ciliary neurotrophic factor (CNTF) (Bjorbaek, 1999) and leukemia inhibitory factor (LIF) (Starr, 1997), as well as by factors such as lipopolysaccharide (Stoiber, 1999). As with the other SOCS proteins, overexpression of SOCS3 can suppress responses to a number of cytokines including growth hormone (GH) (Hansen, 1999), erythropoietin (EPO) (Sasaki, 1999), LIF (Nicholson, 1999), CNTF (Bjorbaek, 1999) and leptin (Bjorbaek, 1998). In the case of cytokines that utilize glycoprotein 130 (gp130, transmembrane protein important for signal transduction following cytokine engagement), inhibition is associated with the binding to receptor sites of tyrosine phosphorylation.

The potential physiological roles of SOCS3 have also been examined in SOCS3 KO mice by Marine and colleagues. and by Roberts and colleagues. In both studies, disruption of the SOCS3 gene resulted in an embryonic lethality, although the timing of embryonic death was different.

In the first study, was suggested that SOCS3 plays an important role in erythropoiesis on the basis of its expression pattern. In fact, SOCS3 mRNA is present at low levels in all adult mice tissues, but its expression is very high in fetal livers, during fetal liver erythropoiesis, which is characterized by the dramatic expansion of the early erythroid lineage cells (Marine, 1999). Moreover, enforced expression of SOCS3 *in vivo* resulted in embryonic death and the absence of liver erythropoiesis. Together, these data suggest that constitutive SOCS3 expression completely blocks fetal liver erythropoiesis. Surprisingly, knocking out the gene for SOCS3 in mice also results in embryonic lethality (Marine, 1999; Roberts, 2001). The only obvious pathology associated with the homozygous deletion observed by Marine et al. (Marine, 1999) is a marked erythropoiesis leading to a

complete loss of normal liver architecture and erythemia throughout the body. SOCS3-deficient liver hematopoietic stem cells could functionally reconstitute lethally irradiated wild-type adult mice, without any pathological excessive proliferation, although these cells have an enhanced responsiveness to cytokines, as shown by the increased *in vitro* proliferative capacity of progenitors. The authors of this study therefore proposed that SOCS3 deficiency per se does not affect bone marrow erythropoiesis but rather SOCS3 is a critical negative regulator of fetal liver erythropoiesis. The large number of immature red blood cells found at peripheral sites in the SOCS3 null embryo, can be explained as a direct consequence of increased liver production or as a consequence of a new acquired ability of peripheral sites to produce erythrocytes in the absence of SOCS3. Alternatively, erythroid progenitors that leave the fetal liver could maintain a proliferative capacity because of deregulated EPO response, which would normally be controlled by SOCS3 induction. It was therefore concluded that SOCS3 is essential in EPO regulation, a model supported by the occurrence of anemia in transgenic mice constitutively expressing SOCS3 (Marine, 1999).

In contrast, Roberts et al. found no evidence of excess erythroid cell production, neither they found any abnormality in hematopoietic progenitor frequencies or any increased responsiveness of erythroid progenitor cells to cytokine stimulation in SOCS3 deficient mice. Instead, they observed severe defects in the placental development and proposed that the lethality in SOCS-3 null mice results from placental insufficiency. Successively, Takahashi et al., demonstrated by tetraploid rescue that the embryonic lethality of SOCS3-null mice is due to placental defects. In this view, the lack of erythrocytosis in the rescued embryos would be a consequence of placental insufficiency and not a cell autonomous defect of SOCS3 deficient cells. Finally, Takahashi and colleagues suggested that

hypoxia, caused by the placental failure, could result in an increased production of erythropoietin and thus in erythrocytosis.
The reason of the conflicting results of these knockout studies remains unclear.

More recent studies demonstrated that SOCS3 binds directly both to EPO and to IGF1 receptor, thus inhibiting cell proliferation by blocking STAT5 activation (Sasaki, 2000; Dey, 2000).
SOCS3 is known to play an important role in the EPO signaling. SOCS3 binds directly to the EPO receptor (Tyr401) as well to JAK2 and inhibits EPO-dependent proliferation and STAT5 activation (Sasaki, 2000). The over-expression of STAT5, which also binds to Tyr401, reduces the binding of SOCS3 to the EPO receptor and thus inhibits the effect of SOCS3 on EPO signaling.
In situ hybridization experiments on E12.5 embryos show that SOCS3 is highly expressed in fetal liver and co-localize with Ter119 (Marine, 1999). Ter119 is expressed on the surface of all erythroid lineage committed cells (Ikuta, 1990) depending on EPO for survival and differentiation (Wu, 1995b). Thus, SOCS3 is activated by EPO signaling itself. Embryos at the stage of 4–12 cells constitutively expressing SOCS3 in virtually all stages of haematopoiesis show complete absence of fetal liver erythropoiesis at E12.5. The embryonic lethality phenotype is strikingly similar to that seen in embryos deficient in either JAK2 or the EPO receptor.
Furthermore, SOCS3 deficiency have been linked to the neoplastic mechanism for polycythemia vera (PV), an acquired myeloproliferative disorder (MPD) characterized by a pronounced increase of erythroid cells (Usenko, 2007). During erythropoiesis, IGF1R signaling controls cellular growth, differentiation and inhibition of apoptosis. In PV, erythroid

progenitor cells are independent of EPO, but hypersensitive to IGF1. The cause of this hypersensitivity could be a deregulation in negative feedback control of cytokine activity, likely involving mutation in the SOCS genes. In agreement with this hypothesis, SOCS3 overexpression in PV cells results in a specific IGF1 reduction and in a reversal of PV erythroid overgrowth (both in the presence or absence of IGF1).

IGF1 signaling is also involved in chronic myelogenous leukemia (CML) caused by BCR-ABL translocation. In CML, the BCR-ABL fusion protein induces autocrine IGF1 signaling, concurring to cellular overgrowth (Lakshmikuttyamma, 2008).

The crucial inhibitory function of SOCS3 on EPO and IGF1 (Dey, 2000) signaling represents a particularly intriguing aspect of erythroid survival and differentiation.

1.4 LEUKEMIA AND MYELOPROLIFERATIVE DISORDERS

Leukemia is a bone marrow disorder resulting in the accumulation of progenitor cells within either the myeloid or lymphoid lineages. Leukemias can be chronic – characterized by an excess number of progenitor cells or "blasts" that undergo normal differentiation – or acute – in which hematopoietic progenitors are blocked in their differentiation (Gilliland, 1998).

Myeloproliferative disorders (MPDs) are a class of stem cell–derived hematological malignancies characterized by overproduction of one or more differentiated myeloid lineages (erythroid, megakaryocytic or granulocytic cells) (Silvennoinen, 2015). In 1951, Dameshek recognized that these disorders are caused by hyperproliferation of multiple hematopoietic

lineages in the bone marrow. He proposed the existence of a relationship between chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF). In this perspective, MPDs may represent a continuum of related syndromes (Dameshek, 1951). With the discovery of Philadelphia (Ph) chromosome (BCR-ABL+) (Nowell, 1960), classic MPDs were further classified into Ph positive MPD (CML) and Ph negative MPD (PV, ET and IMF).

1.4.1 THE PHILADELPIA POSITIVE LEUKEMIAS: CHRONIC MYELOID LEUKEMIA AND ACUTE LYMPHOBLASTIC LEUKEMIA.

Chromosomal translocations, caused by the breakage and fusion of at least two distinct chromosomes, account for approximately 50% of all leukemias. The first chromosomal rearrangement was described in 1960, through the discovery of a "small aberrant chromosome 22", named later Philadelphia (Ph), that was found to be associated with about 95% of Chronic Myeloid Leukemia (CML) cases (Nowell, 1960). It was also found in 5% of children and 15-30% of adults with acute lymphoblastic leukemia (ALL) (Kurzrock, 1988; Specchia, 1995).

The Philadelphia chromosome translocation t(9;22) leads to an exchange of DNA between chromosomes 9 and 22, which is responsible for the formation of the oncogenic fusion protein BCR-ABL. The BCR-ABL oncoprotein, a constitutively activated tyrosine kinase, recruits and activates several pathways transducing intracellular signals, which ultimately lead to abnormal cellular adhesion, enhanced proliferation and inhibition of apoptosis (Deininger, 2000). The BCR-ABL oncoprotein has been identified in patients in three forms (P190, P210 and P230), with distinct molecular weights (Gilliland, 1998; Ren, 2005) (Fig. 1.5).

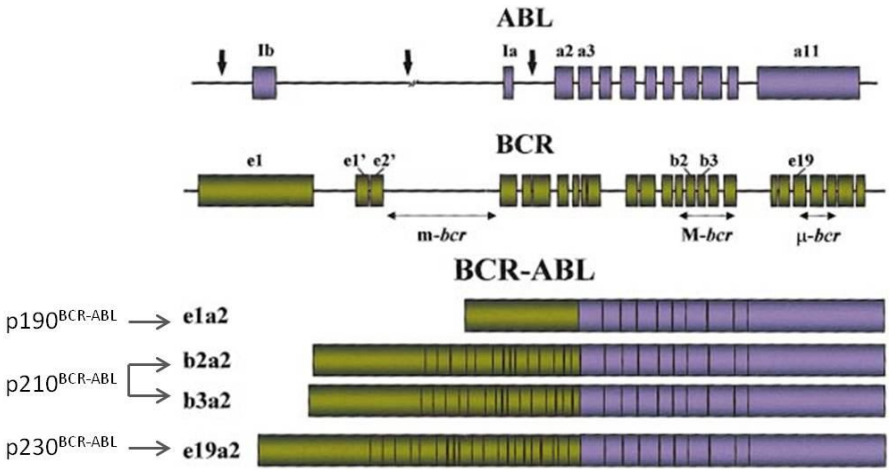


Fig. 1.4: Locations of the breakpoints in the ABL and BCR genes and structure of the chimeric mRNAs derived from the various breaks. *The Ph chromosome is a shortened chromosome 22 that results from the translocation of 3' (toward the telomere) ABL segments on chromosome 9 to 5' BCR segments on chromosome 22. In most cases breakpoints (arrowheads) in the ABL gene are located in the 5' end (toward the centromere) of exon a2. Various breakpoint locations have been identified along the BCR gene on chromosome 22. Depending on which breakpoints are involved, differently sized segments from BCR are fused with the 3' sequences of the ABL gene. This results in fusion messenger RNA molecules (e1a2, b2a2, b3a2, and e19a2) of different lengths that are translated into different chimeric protein products (p190, p210 and p230) with variable molecular weights. m-bcr: minor breakpoint cluster region, M-bcr: major breakpoint cluster region, and μ-bcr: micro breakpoint cluster region. (Figure modified from Deininger, 2000).*

The breakpoints within the ABL gene can occur anywhere over a large area at its 5' end, either upstream to the first alternative exon Ib, downstream to the second alternative exon Ia, or, more frequently, between the two (Melo, 1999)(Fig. 1.4). In contrast, breakpoints within BCR localize in three regions, called breakpoint cluster regions (bcr). Most CML patients harbor the P210 form of BCR-ABL, which is generated through a breakpoint within the major breakpoint cluster region (M-bcr) between exons 12 and 16 (or exon b1-b5) of BCR (Groffen, 1984; Melo, 1997). Because of alternative splicing, fusion transcripts with either b2a2 or b3a2 junctions can be formed, generating the 210 kDa chimeric protein. The smaller P190 form of BCR-ABL is frequently associated with B cell acute lymphoid leukemia (B-

ALL) (Chan, 1987) and is rarely found in CML patients (Melo, 1994). Within the BCR gene, the breakpoint falls within the minor breakpoint cluster region (m-bcr) between the alternative BCR exons e2' and e2. The resultant e1a2 mRNA is translated into a 190 kDa protein.

The largest transcript, BCR-ABL P230, includes the largest portion of BCR, corresponding to exons 1 to 19. The breakpoint can be found downstream to the M-bcr region, between exons 19 and 20 at the micro breakpoint cluster region (μ-bcr) (Verstovsek, 2002). The P230 form of BCR-ABL was originally associated with neutrophilic chronic myeloid leukemia (CML-N) (Fig. 1.5).

Human ABL is a ubiquitously expressed 145 kDa protein with two isoforms. ABL, also called ABL1, is a member of the ABL subfamily of tyrosine kinases that also includes the closely related member ARG, also called ABL2 (Colicelli, 2010; Kruh, 1986). In hematopoietic cells, steady state levels of ABL decrease along with myeloid maturation (Wetzler, 1993). ABL is a cytoplasmic and nuclear tyrosine kinase (Sirvent, 2008). In normal conditions, it is regulated through multiple intramolecular interactions that keep the kinase domain in an inactive conformation and by phosphorylation on tyrosine and serine/threonine residues (Hantschel, 2004). Normal ABL phosphorylation is tightly controlled (Van Etten, 1999), probably by motifs present on its in N-terminal portion. In the N-terminal, SH3 and SH2 domains of ABL regulate the tyrosine kinase domain, through their binding to effector proteins (Mayer, 1994) and by directly interaction with the kinase domain in the self-imposed inactive configuration (Nagar, 2006, Smith, 2003). The loss of this region (as occurs in the formation of BCR-ABL) results in high constitutive kinase enzymatic activity, a key factor in the oncogenic potential of transforming ABL proteins. Tyrosine kinase

enzymatic activity is central to cellular signaling and growth and its constitutive activation has been associated with transformation in several systems.

The 160 kDa BCR protein, like ABL, is ubiquitously expressed (Laneuville, 1995). The BCR gene is a complicated molecule with many different functional motifs. The first exon of BCR gene is included in all known BCR-ABL oncogenic fusion proteins (Muller, 1991; Arlinghaus, 1998). This exon contains a serine and threonine kinase enzymatic activity mediating both auto-phosphorylation as well as the phosphorylation of its substrates.

The fusion protein BCR-ABL has a constitutive tyrosine kinase activity, at least for two reasons: first, the N-terminal negative regulatory domain of ABL (Wang, 1988) is no more present; second it is replaced by BCR sequences that include an amino terminal coiled-coil (CC) tetramerization domain of BCR (Tauchi, 1997). BCR-induced BCR-ABL tetramerization allows reciprocal trans-phosphorylation of ABL Y412 in the activation loop of the catalytic domain. This modification stabilizes a conformation compatible with substrate binding and full catalytic activity, therefore leading to hyperactivation. This tyrosine kinase activity is essential for cell transformation by the BCR-ABL fusion protein, whereas the serine/threonine kinase activity of BCR is dispensable (Pendergast, 1993) or even inhibited by BCR-ABL, the through phosphorylation of tyrosine 360 (Liu, 1996). BCR-ABL oligomers function as docking scaffold for proteins containing SH2 and PTB domains, like GRB2 and CRKL (Hantschel, 2012), which form complexes with other proteins (notably SOS/GAB2 and CRK/CBL) and trigger the activation of a large number of cell signaling pathways. Amongst them, the RAS/MEK/ERK, the JNK and PI3K/AKT kinase pathways have been shown to play a pivotal role in transformation

(Sawyers, 1995; Raitano, 1995; Skorski, 1997). Another fundamental target is the transcription factor STAT5, which can be activated by BCR-ABL either directly or indirectly -through JAK2-and is essential not only for leukemia initiation, but also its maintenance (Shuai, 1996; Hoelbl, 2010). Hence, through the activation of these multiple downstream pathways, BCR-ABL induces resistance to apoptosis, growth factors independent proliferation and altered adhesion, each contributing to transformation. Moreover, BCR-ABL stimulates the formation of oxygen reactive species and enhances spontaneous DNA damage in leukemic cells (Skorski, 2008). In addition, BCR-ABL compromises the fidelity of DNA repair mechanisms, facilitating the accumulation of additional alterations, further leading to disease progression and drug resistance. BCR-ABL expression can also affect cytoskeletal structure, contributing to the altered adhesion and migration observed in leukemia cells (Salgia, 1997).

The Philadelphia translocation is critical for the pathogenesis of chronic myelogenous leukemia (CML) and of a subset of acute leukemias (ALL). CML is a myeloproliferative disease defined by a tri-phasic clinical course. The "chronic phase" is characterized by the expansion of terminally differentiated myeloid cells in both bone morrow and blood. The disease then progresses thought an "accelerated phase", where additional mutations are acquired. These mutations allow the disease to advance to the "blast crisis phase" resembling acute leukemia, in which myeloid blasts display a block in differentiation (O'Hare, 2012). In USA, CML is diagnosed at a rate of 1 to 2 cases per 100,000 people per year, accounting for 15% of all adult leukemias, and 2 to 3 % of childhood leukemias (Lakshmaiah, 2012).

BCR-ABL is the hallmark of CML. This oncogenic fusion protein can also initiate B-ALL. Ph^+ B-ALL account for 30% of adult and 2%-3% of childhood acute lymphoblastic leukemias (Fielding, 2010). BCR-ABL P190 is found in the majority of Ph^+ B-ALL cases in adults (50 to 80%) and children (90%), with BCR-ABL P210 accounting for other cases (Kantarjian, 1991).

Historically, Ph^+ B-ALL has been considered an aggressive disease with a poor prognosis. Even with intensive chemotherapy treatment, long-term survival rates were less than 10% (Fielding, 2010). Until recently, the only potentially curative option has been allogenic bone marrow transplantation. With the introduction of imatinib and other BCR-ABL-specific inhibitors, the prognosis for Ph^+ B-ALL has considerably improved. Imatinib alone proved to be sufficient to induce hemotologic and cytogenetic responses in initial studies, but this was only in 20% of patients (Druker, 2001) and many of these relapsed were due to imatinib resistance. Overall, the response in Ph^+ B-ALL was disappointing in comparison to that observed in CML (Ottoman, 2002). Therapies that combine second-generation inhibitors such as dasatinib or nilotinib along with conventional chemotherapeutic drugs have led to complete remission in almost all patients, and three year survival rates have risen to 50%. Ph^+ B-ALL patients do not respond to imatinib treatment as well as CML patients in blast crisis, despite the fact that BCR-ABL expression drives both diseases. This is possibly due to additional mutations that are believed to synergize with BCR-ABL in Ph^+ B-ALL. Focusing on B-ALL, beside CDKN2A or PAX5 deletions, both occurring in about 50% of cases, IKZF1 deletions are found in more than 80% of patients (Mullighan, 2008; Dupuis, 2013).

1.4.2 THE PHILADEPHIA NEGATIVE MYELOPROLIFERATIVE DISORDERS

Myeloproliferative disorders (MPDs) are a class of stem cell–derived hematological malignancies that result in overexpansion of mature myeloid cells. Two main classes of MPDs are recognized by the World Health Organization: chronic myelogenous leukemia (CML), characterized by the Philadelphia translocation; the Philadelphia negative MPDs, polycythemia vera (PV), essential thrombocythemia (ET), and idiopathic myelofibrosis (IMF) (Vardiman, 2002). Less common disorders, such as chronic neutrophilic leukemia and chronic eosinophilic leukemia/hypereosinophilic syndrome, are also considered chronic myeloid disorders but they are grouped separately as "atypical MPD" (Tefferi, 2006). Clinical manifestations of these disorders include splenomegaly, thrombosis, bone marrow fibrosis, extramedullary hematopoiesis, and leukemic transformation observed in the bone marrow. The latter manifests as increased and clustered megakaryocytes in ET and PV, increased granulopoiesis in PV and IMF and increased erythropoiesis in PV (Michiels, 2007). CML, PV, ET, and IMF have significant clinical and biological phenotypical overlap and were thus considered as related diseases (Dameshek, 1951). In 1960, cytogenetic characterization of CML discovered the Ph chromosome (Nowell, 1960). Lacking obvious cytogenetic differences in the remaining three diseases, multiple groups analyzed bone marrow samples from PV and ET patients. Interestingly, these cells exhibit similar cytokine hypersensitivity to erythropoietin (Krantz, 1968; Ward, 1974) and growth of erythroid progenitors in the absence of exogenous erythropoietin, a phenomenon referred to as "endogenous erythroid colonies" (EEC) (Prchal, 1974). Clonality studies revealed the origin of these diseases in a multipotent hematopoitic stem cell (Adamson, 1976; Fialkow,

1981). Inhibition of endogenous erythroid colonies (EEC) with imatinib mesylate (a tyrosine kinase inhibitor that also inhibits the kinase activity of ABL, KIT and PDGFR)(Oehler,2004) and with the JAK2 inhibitor AG490 (Ugo,2004) suggested a tyrosine kinase phosphorylation as driving event causing EEC proliferation. Because ET can transform into PV and PV into MFI, this pattern of clinical evolution strongly suggest a common etiological factor involved in signaling through different receptors of myeloid lineage in all three MPDs.

Only 11 years ago, essentially nothing was known about the molecular pathogenesis of MDPs. In 2005, four independent groups described a somatic mutation involving a protein tyrosine kinase in a significant number of patients with MPDs (James, 2005; Levine, 2005; Baxter, 2005; Kralovics, 2005). This G to T mutation causes a valine to phenylalanine substitution at position 617 (V617F) within the auto inhibitory JH2 (JAK homology 2) domain of non-receptor tyrosine kinase JAK2. The JAK2V617F mutation results in the abrogation of auto inhibition, leading to constitutive activation of JAK2. The occurrence of the mutation in MPDs was found to be approximately 95% in PV, 50% in ET, and 50% in IMF (Levine, 2005). The overexpression of JAK2V617F in different cell lines abrogates their growth factor dependence; its retrovirally-mediated overexpression in mouse models leads to an MPD phenotype resembling human PV (Lacout, 2006;. Wernig, 2006; Bumm, 2006; Zaleskas, 2006). More recently, other mutations in JAK2 have been found in PV (JAK2 exon 12 mutations) (Scott, 2007) of MDPs (will be discuss later).

The JAK-STAT signaling pathway

The cytokine receptors of type I (as receptors for EPO, TPO, GM-CSF and for several interleukin receptors) lack a cytoplasmatic tyrosine kinase activity and are dependent for signaling on Janus Kinases (JAK) and downstream activation of Signal Transducers and Activators of Transcription (STAT) proteins. The JAK family comprises four different members JAK1, JAK2, JAK3 and TYK2. These tyrosine kinases consist of the JAK homology 1 (JH1) domain with kinase activity, the JH2 pseudo-kinase domain (which is catalytically inactive and has an autoregulatory function) and the JH3-JH7 domain, containing the protein interaction FERM domain. The binding of the FERM domain to a transmembrane receptor leads to a series of conformational changes in the JAK protein, upon ligand binding, which induce phosphorylation and activation of the JAK tyrosine kinase, allowing further downstream signaling through mainly STAT proteins (Ihle,2007; Kaushansky, 2005) (Fig. 1.6).

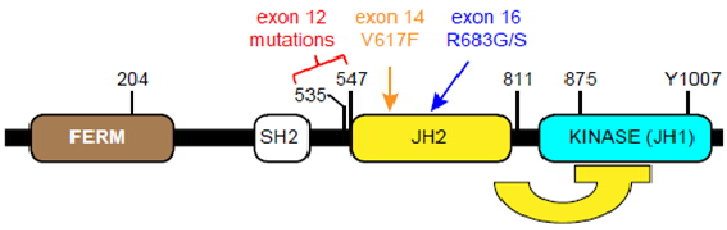


Fig. 1.6: Domain structure of the JAK2 protein. *Numbers indicate amino acid positions within the JAK2 protein. Arrows indicate the position of the most frequently mutated regions. The auto-inhibitory effect of the JH2 domain is indicated in yellow. FERM = N-terminal Band 4.1, ezrin, radixin, moesin domain; JH1 and JH2 = JAK homology 1 and 2 domains; SH2 = Src homology 2 domain. (Figure from Skoda, 2015).*

These proteins serves as signal transducers and activators of transcription, thus regulation the expression of their target genes. Several growth factors

(EPO, TPO, G-CSF, GM-CSF, SCF, IL-3 IGF-1) essential for normal hematopoiesis, all use JAK2 for signaling, mainly acting on STAT3 and STAT5 (Murray, 2007; Ihle, 2007) as downstream effectors.

The regulation of JAK signaling is tightly controlled by several mechanisms to prevent excessive signaling or over-activation, which lead to autoimmune disorders and malignant transformation. Mechanisms controlling cytokine signaling include: direct de-phosphorylation by Src homology tyrosine phosphatases (SHP-1); inhibition by suppressors of cytokine signaling (SOCS) family members through direct binding to JAK2 on tyrosine 1007 (Feng, 1997); competition for STAT binding sites and binding to negative regulators of STAT gene transcription such as protein inhibitors of activated STAT (PIAS) (Khwaja, 2006).

The JAK2V617F mutation

The JAK kinases normally function through their association with cytokine receptors that lack intrinsic kinase activity. The specificity of different cytokine receptors for one or more different JAK kinases accounts in part for their differential effects on signal transduction.

Genetic deletion of JAK2 results in embryonic lethality due to a lack of definitive erythropoiesis, and JAK2 deficient haematopoietic progenitors do not respond to erythropoietin (EPO) stimulation. These data demonstrate that JAK2 is the only JAK kinase responsible for EPO receptor (EPOR) signaling (Parganas,1998). The JAK2V617F mutant protein has constitutive kinase activity (Zhao, 2005), and when expressed *in vitro, it is* constitutively phosphorylated (Levine, 2005).

Expression of JAK2V617F confers cytokine hypersensitivity and cytokine-independent growth to haematopoietic cells, a characteristic feature of haematopoietic colonies grown from patients with PV (Prchal, 1987).

JAK2V617F-mediated transformation to cytokine-independent growth is most efficient in haematopoietic cells that co-express the EPOR, the thrombopoietin receptor (MPL), or the granulocyte colony-stimulating factor receptor (GCSFR)(Lu, 2005). Unlike most cytokine receptors, EPOR, MPL and GCSFR are homodimeric type I cytokine receptors expressed on cells of the erythroid, megakaryocytic and granulocytic lineages, respectively. Although these data do not exclude the possibility that JAK2V617F interacts with non-homodimeric haematopoietic cytokine receptors, the JAK2V617F-mediated transformation requires the interaction with a cytokine receptor scaffold (Levine, 2007). In addition, the preference of the JAK2V617F allele for proliferative syndromes involving the erythroid, megakaryocytic and granulocytic lineages might in part be explained by the differential cytokine receptor expression during differentiation of these cell types.

In vitro studies demonstrate that JAK2V617F activates multiple downstream signaling proteins (Levine, 2005; Lu, 2005), including the STAT family of transcription factors, the mitogen activated protein kinase (MAPK) signaling pathway and the phosphotidylinositol 3-kinase (PI3K)–Akt signaling pathway, all crucial to cell survival and proliferation.

Several lines of evidence suggest that activation of the STAT family of transcription factors is important in JAK2V617F-mediated transformation.

First, expression of either constitutively active STAT5 or its anti-apoptotic target gene BCL-XL in human haematopoietic progenitors results in EPO-independent colony formation (Garcon, 2006), a hallmark of human PV. Moreover, STAT3 activation and BCL-XL overexpression are observed in most PV patient samples (Roder, 2001; Silva, 1998). These data imply that STAT pathway activation is important in JAK2V617F-mediated transformation, but do not explain whether STAT pathway activation is

necessary and/or sufficient for JAK2V617F-mediated transformation. Murine bone marrow transplantation (BMT) assays using *Stat5a*;*Stat5b*-deficient mice have been used to show that STAT5 is required for haematopoietic transformation by the constitutively active TEL–JAK2 fusion tyrosine kinase (Schwaller,2000).

In addition, the activation of signaling by the JAK2V617F kinase might in part be due to escape from negative-feedback mechanisms important in attenuating JAK2 signaling. JAK activity is negatively regulated by the SOCS family of proteins, which normally bind to the JAK kinases and result in their degradation. In particular, SOCS1 and SOCS3 have been shown to bind to JAK2 and to inhibit JAK2 catalytic activity (Nicholson,1999; Sasaki,2000). The overexpression of SOCS1 results in abrogation of *in vitro* and *in vivo* transformation by TEL–JAK2 (Frantsve, 2001). Although expression of SOCS1 results in JAK2 and JAK2V617F degradation and inhibition of kinase activity, the expression of SOCS3 paradoxically results in increased JAK2V617F protein stability, in increased SOCS3 and JAK2V617F phosphorylation (Hookham, 2007). These data demonstrate that regulation of JAK2 kinase activity by SOCS3 is altered in the context of the V617F substitution, and suggest the possibility that therapeutic inhibition of SOCS3 might selectively attenuate JAK2V617F, but not wild-type JAK2 signaling.

The JAK2V617F mutation is present in haematopoietic cells but not in germline DNA of patients with MPDs (Levine, 2005; Kralovics, 2005), demonstrating that JAK2V617F is a somatic mutation acquired in the haematopoietic compartment. In addition, the JAK2V617F allele can occasionally be present in different haematopoietic compartments (Ishii, 2006; Delhommeau, 2007), including B and T lymphoid cells. These findings suggest that the mutation might occur in a pluripotent haematopoietic cell.

Indeed, the JAK2V617F allele has recently been identified in the haematopoietic stem cell (HSC) compartment in patients with PV (Jamieson, 2006). These data are in agreement with the hypothesis that the self-renewing properties of HSCs are required for the MPD phenotype and that activated tyrosine kinases can transform HSCs but not myeloid progenitors that lack the capacity for self-renewal (Huntly, 2004).

In the initial reports describing the JAK2V617F allele in PV, ET and PMF, it was noted that although most patients with MPD are heterozygous for JAK2V617F, a subset of patients, most commonly with PV, are homozygous for the JAK2V617F allele (Levien,2005; Baxter,2005; Kralovics,2005; James, 2005). In these patients, the homozygosity for JAK2V617F results from mitotic recombination and duplication of the mutant MPD allele, a mechanism known as "acquired uniparental disomy (UPD)". UPD involving the chromosomal locus 9p24, that includes JAK2, had previously been noted in PV (Kralovics, 2002; Kralovics, 2005). Homozygous JAK2V617F mutant erythroid colonies can be grown from almost all patients with PV, suggesting that UPD at the JAK2 locus is an early event in the pathogenesis of PV. By contrast, homozygous JAK2V617F mutations are rarely observed in ET, and haematopoietic colonies grown from ET patients are most commonly wild type or heterozygous with respect to JAK2. These data suggest that there are important genetic differences between PV and ET, and that the duplication of JAK2V617F is a crucial event in the pathogenesis of PV.

The mutant allele "dosage theory" postulates that high JAK2V617F expression is correlated with PV whereas its low level expression develops in ET phenotype.

Although the discovery of the JAK2V617F mutation provided new insights into the molecular pathology of MPDs, yet the implication of the same

mutation in three correlated but distinct phenotypes (ET, PV and PMF) remains an enigma. In addition to the "mutant allele dosage theory", several theories have been proposed to explain JAK2V617F pleiotropy, including cytokine receptor signaling alterations and the role of additional genetic mutations.

Other mutations found in Myeloproliferative Disorders

The identification of the V617F JAK2 mutation has prompted new studies aiming to identify other genetic alterations involved in MPDs pathogenesis. In PV, JAK2 V617F is found in 95% of patients and JAK2 mutations in exon 12 mutations in 4%. In ET and PMF, 60% of patients harbor JAK2 V617F, 20% to 25% calreticulin (CARL) mutations, 5% thrombopoietin receptor (MPL) mutations, and 5% to 10% of patients lack mutations in any of these genes (Klampfl, 2013; Nangalia, 2013; Pikman, 2006). These mutations in JAK2, MPL, and CARL are driver mutations, as they all activate the JAK2-STAT pathway, that is the common denominator of MDPs. However in addition to the "phenotypic driver mutations" that are directly linked to hyperproliferation of hematopoietic cells, there is a list of recurrent somatic mutations in several genes (TET2, ASXL1, DNMT3A, CBL, LNK, IDH1/2, IKF1, EZH2, TP53, SRSF2), encoding transcriptional and epigenetic regulators and signaling proteins (Cazzola, 2014; Skoda, 2015). These additional mutations modulate disease progression and can also occur as a primary mutation, but it is now convincingly demonstrated that MPDs can be initiated from a single JAK2 V617F hematopoietic stem cell (Lundberg, 2014).

1.5 SCOPE OF THE THESIS

Leukemias and myelodisplasias are characterized by a block in differentiation leading to an excess of proliferating immature cells. Since SOX6 is a potent inducer of growth arrest and differentiation, we investigated the effect of its ectopic expression in different model systems of leukemia, to enforce these cells to overcome their block in differentiation. The final goal of our research is to characterize in depth SOX6-dependent signaling networks. This could, in principle, identify potential diagnostic signatures/pharmacological targets relevant to enforce cell cycle withdrawal and differentiation in leukemic and/or myelodisplastic cells.

In **Chapter 2**, I used leukemic model systems that varies for their degrees of differentiation/maturation and to the presence/absence of specific genetic lesions. In these cells, I ectopically overexpressed SOX6 both in BCR-ABL positive cell lines (megakaryoblastic MEG.01 and lymphoblastic SUPB15) and in BCR-ABL negative cell lines carrying the JAK2V617F mutation (erythroleukemic HEL cells; thrombocythemic cell lines UKE-1 and SET-2).
I evaluated the effect of SOX6 overexpression on cell proliferation, cell cycle, cellular viability and differentiation.

In **Chapter 3,** I explored the impact of SOX6 ectopic expression in B-ALL BCR-ABL GFP^+ capability to engraft and generate leukemia in C57BL/6J recipient mice and to interfere with the onset/maintenance of leukemia.

In **Chapter 4,** I describe my contribution to other projects carried out in my laboratory exploring other aspects of the role of SOX6 in erythropoiesis. with a particular focus on the identification of SOX6 protein partners and on

SOX6 role in the differential regulation of globins genes during development.

In **Chapter 5,** all the results presented in this thesis are discussed as well as the future perspectives of this research in molecular and translational medicine.

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CHAPTER 2:

SOX6 DOWNSTREAM PATHWAYS CONTROLLING CELL PROLIFERATION VERSUS DIFFERENTIATION IN BCR-ABL^{+} AND JAK2V617F^{+} CELLS

2.1 INTRODUCTION

In order to discover new genes involved in the regulation of erythroid differentiation and of the hemoglobin switching process, we analyzed the global expression profile of purified mouse fetal liver cell populations isolated at different days of embryonic development (E11.5, E12.5, E13.5) and at different stages of maturation. Cells were FACS sorted on the basis of their expression of c-Kit (Stem Cell Factor tyrosine-kinase receptor), that characterizes early hematopoietic progenitors) and of Ter119 (a protein associated to glycophorin A), that defines more mature erythroid cells.

The acquired data may reveal new key regulators of the haemoglobin switching as may help to decipher the gene expression dynamics of cells during differentiation. Amongst genes increasing their expression level, while the erythroid cells differentiate, there is SOX6, a member of the Sox (Sry-type HMG box) family.

SOX6 is a transcription factor (TF) belonging to the Sry-related HMG-box TFs family, which are known to be fundamental for the proper development of several tissues.

In the hematopoietic system SOX6 sustains cell survival, proliferation and terminal maturation during definitive murine erythropoiesis. In fact, in the murine model, SOX6 knock out leads to a strong impairment of definitive erythropoiesis (Dumitriu, 2006). In our laboratory we explored the role of SOX6 in human erythropoiesis by its overexpression in the K562 cell line (erythroleukemic BCR-ABL$^+$ cells from a Chronic Myeloid Leukemia in blast crisis) and in *ex vivo* human cord blood cultures (Cantu, 2011). In these cellular systems SOX6 overexpression enhances erythroid differentiation. Indeed, upon SOX6 overexpression, several erythroid specific transcripts are greatly increased, including mRNAs encoding for enzymes controlling

the heme-biosynthetic pathway and globins chains. Of note, the expression level of major transcriptional regulators of eryhtopoiesis GATA1, GATA2, EKLF of NF-E2 p45 is unchanged upon SOX6 overexpression. This result strongly suggests that SOX6 acts both as a general positive regulator of erythroid maturation and as a specific regulator of globin genes.

SOX6 overexpression in K562 enforces terminal maturation, with cells dying in about ten days after SOX6 transduction, despite their erythroleukemic origin. This last effect is mediated by SOCS3 (Suppressor Of Cytokines Signalling 3) that is a direct, transcriptionally activated, target of SOX6 (Cantù, 2011).

SOCS3 is induced by a variety of cytokines (i.e. GM-CSF, IL-3, TPO and EPO), growth factors and hormones in different cell lines and in primary mouse bone marrow and fetal liver cells. SOCS3 negatively regulates cell growth by inhibiting cytokine receptors signaling. In particular, SOCS3 represses two pathways important for erythropoiesis: the IGF1-IGF1R pathway, in response to insulin-like growth factor 1 (IGF1) (Dey, 2000) and the EPO-JAK-STAT pathway, in response to erythropoietin (Sasaki, 2000). The inhibitory function of SOCS3 in EPO and IGF1 signaling represents a key mechanism to control erythroid survival and differentiation. Indeed, SOCS3 gene targeted disruption in mice results in embryonic lethality at the E12.5 due to erythrocytosis (Marine,1999). In these animals, the proliferative capacity of erythroid progenitors is greatly increased. These results are in line with the observation of a strong hypermethylation of SOCS3 in a cohort of patients with myeloproliferative disorders (MPDs) (Fourouclas, 2008). Moreover, SOCS3 deficiency have been linked to the neoplastic mechanism for polycythemia vera (PV), an acquired myeloproliferative disorder characterized by a pronounced increase of erythroid cells (Usenko, 2007).

During erythropoiesis IGF1R signaling controls cellular growth, differentiation and inhibition of apoptosis. In PV, erythroid progenitor cells are independent from EPO, but hypersensitive to IGF1. The cause of this hypersensitivity could be a deregulation in negative feedback control of cytokine activity, likely involving mutation in the SOCS genes. In agreement with this hypothesis, SOCS3 overexpression in PV cells results in a specific IGF1 reduction and in a reversal of PV erythroid overgrowth (both in presence or absence of IGF1)(Usenko, 2007).

IGF1 signaling is also involved in chronic myelogenous leukemia (CML) caused by the BCR-ABL translocation. Indeed, in CML, the BCR-ABL fusion protein induces autocrine IGF1 signaling, through Hemopoietic Cell Kinase (Hck) belonging to the Src family and through the Signal transducer and activator of transcription 5B (Stat5B), both concurring to cellular overgrowth (Lakshmikuttyamma, 2008).

In K562 cells, proliferation depends on autocrine IGF1 signalling, induced by BCR-ABL (Usenko, 2007; Lakshmikuttyamma, 2008). In the same cells overexpressing either SOX6 or SOCS3 we observed a reduction in IGF1 expression, suggesting that SOX6 indeed plays a pivotal role in blocking cell proliferation through SOCS3 up-regulation and by inhibiting the BCR-ABL-dependent IGF1 signaling (Cantù, 2011).

Leukemias and myelodisplasias are characterized by incomplete differentiation leading to an excess of unrestrained proliferating immature cells. SOX6 has been described by us and others as a potent inducer of proliferation arrest and differentiation, possibly acting on JAK2-STAT and IGF1-IGF1R pathways. On this basis, I investigated the effect of SOX6 overexpression in different model systems of leukemia in order to characterized in depth SOX6 dependent signaling networks. The final goal

of this study is the identification of potential diagnostic signatures/pharmacological targets relevant to enforce cell cycle withdrawal and differentiation in leukemic and/or myelodisplastic cells.

As a first approach, I used leukemic model systems that vary for their degrees of differentiation/maturation and for the presence/absence of specific genetic lesions, i.e. the BCR-ABL fusion oncogene and the JAK2V617F mutation.

To this end, I ectopically overexpressed SOX6 in:

- two BCR-ABL^{+} cell lines: the megakaryoblastic MEG.01 celll line and the lymphoblastic SUPB15cell line;
- three BCR-ABL^{-} cell lines, carrying the JAK2V617F mutation : the erythroleukemic HEL cell line and the thrombocythemic cell lines UKE-1 and SET-2.

2.2 EXPERIMENTAL PROCEDURES

Constructs

The SOX6 murine cDNA was kindly provided by Prof. Michiko Hamada-Kanazawa, Kobe-Gakuin University, Japan. The SOX6 cDNA was cloned in frame with a 3' FLAG epitope to generate a SOX6-FLAG cassette in which SOX6 cDNA lacks the 49 C-ter aminoacids still retaining its biological proprieties (Hamada-Kanazawa, 2004). 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), (Demaison, 2002) lentiviral vector, a kind gift from Prof. Tariq Enver, UCL, London.

The human SOCS3 cDNA was kindly provided by Dr M. G. Francipane, University of Palermo, Italy (Francipane, 2009). SOCS3 cDNA was cloned in pTWEEN lentivector upstream to the IRES-GFP cassette.

The two packaging plasmids psPAX2 and pMD-VSVG were used to produce Lentiviral pseudo-particles in 293T cells (www.lentiweb.com).

Cell lines

Cell line used in this study are briefly described below.

MEG.01: human megakaryoblastic leukemic cell line derived from the bone marrow of a 55-year-old man with BCR-ABL$^+$ CML at the stage of megakaryoblastic crisis. Non-adherent and adherent cells, rounded and square, were grown in RPMI medium (Lonza) supplemented with 20% of FBS (Fetal Bovine Serum) (Sigma), penicillin/streptomycin (100μg/ml), 4mM L-glutamine (EuroClone), at an optimal concentration of $0,5x10^6$ cells/mL, in a humidified 5% CO_2 atmosphere at 37°C.

SUPB15: Human B cell precursor leukemia, established from the bone marrow of a 9-year-old boy with acute lymphoblastic leukemia (B-ALL) in second relapse . it is described to carry the ALL-variant (m-bcr) of the BCR-ABL fusion gene (e1-a2). SUPB15 were grown in suspension, in RPMI medium (Lonza) supplemented with 10% of FBS (Usa Origin Sigma), penicillin/streptomycin (100μg/ml), 4mM L-glutamine (EuroClone), at an optimal concentration of 0,5-1x10^6 cells/mL, in a humidified 5% CO_2 atmosphere at 37°C.

HEL: Human Erythroleukemic cell line derived from a patient with erythroleukemia that co-expresses differentiation markers for both erythroid and megakaryocytic lineage. HEL cells can undergo differentiation along various hematopoietic pathways depending on the stimulus. In particular, these cells have been use to study erythrocyte and megakaryocyte development (Hong, 1996). An additional characteristic of these cells is that they carry the myeloproliferative disorder associated mutation JAK2V617F (Quentemeier, 2006). HEL were grown in suspension, in RPMI medium (Lonza) supplemented with 10% of FBS (Fetal Bovine Serum) (Sigma), penicillin/streptomycin (100μg/ml), 4mM L-glutamine (EuroClone), at an optimal concentration of 0,25-0,5x10^6 cells/mL, in a humidified 5% CO_2 atmosphere at 37°C.

SET2: human essential thrombocythemic cells established from the peripheral blood of a 71-year-old woman with essential thrombocythemia at megakaryoblastic leukemic transformation in 1995. Cells were described to carry the JAK2 V617F mutation (6 mutated alleles and 1 wild type allele) (Quentmeier, 2006). SET2 cells were grown in suspension, in RPMI medium (Lonza) supplemented with 20% of FBS (Fetal Bovine Serum) (Sigma), penicillin/streptomycin (100μg/ml), 4mM L-glutamine (EuroClone), at an

optimal concentration of $0,5x10^6$ cells/mL, in a humidified 5% CO2 atmosphere at 37°C.

UKE1: human essential thrombocythemic cells established from a patient with essential thrombocytosis transformed into acute leukemia. UKE1 cells were grown in suspension in IMDM medium (EuroClone) supplemented with 10% FBS (Fetal Bovine Serum) (Sigma) and 10% Horse Serum (Invitrogen), 1uM di HydroCortisone (Sigma), penicillin/streptomycin (100μg/ml), 4mM L-glutamine (EuroClone), at an optimal concentration of $0,5x10^6$ cells/mL, in a humidified 5% CO_2 atmosphere at 37°C.

HEK 293T: The 293T cell line derived from human embryonic kidney and contains the SV40 T-antigen. It is used for lentiviral production. This cells were grown in adhesion, in DMEM medium (EuroClone) supplemented with 10% of FBS (Fetal Bovine Serum) (Sigma), penicillin/streptomycin (100μg/ml), 4mM L-glutamine (EuroClone) in a humidified 5% CO_2 atmosphere at 37°C.

Lentiviral production and transduction

Exponentially growing HEK-293T cells were transfected with jetPEI™ (Polyplus-Transfection) with the above vectors plus the packaging plasmids psPAX2 and pMD-VSVG to produce the lentiviral pseudo-particles. 72h after transfection, the supernatant with recombinant viruses was collected, filtered (0.45μm) and centrifuged at 20,000g for 8 hours at 4°C. The viral pellet was re-suspended in 1xPBS and aliquoted at -80°C. Lentiviruses were titrated on HEK-293T cells by measuring the percentage of $eGFP^+$ cells by Flow Cytometry.

The transduction of all the cell lines used in this study was performed overnight with a multiplicity of infection (MOI) of 30.

RNA isolation and Real Time PCR

Total RNA from >10^6 of all different cell lines and experimental condition were purified with TRIzol Reagent (Applied Biosystem), treated with RQ1 DNase (Promega) for 30 min at 37°C and retrotranscribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem). Negative control reactions (without Reverse Transcriptase) gave no PCR amplification. Real time analysis was performed using ABI Prism 7500, (Applied Biosystems). Primers were designed to amplify 100 to 200bp amplicons, spanning an exon-exon junction when possible, on the basis of sequences from the Ensembl database (http://www.ensembl.org). Samples from each experiment were analyzed in triplicate. Specific PCR product accumulation was monitored by SYBR Green dye fluorescence in 12-µl reaction volume. Dissociation curves confirmed the homogeneity of PCR products.

All primers used are listed in the table below:

PRIMERS	**SEQUENCE**
EPO Fw	CTGTATCATGGACCACCTCGG
EPO Rw	TGAAGCACAGAAGCTCTTCGG
GAPDH Fw	ACGGATTTGGTCGTATTGGG
GAPDH Rw	TGATTTTGGAGGGATCTCGC
ALASE Fw	CAACATCTCAGGCACCAGTA
ALASE Rw	CTCCACTGTTACGGATACCT
α globin Fw	GAGGCCCTGGAGAGGATGTTCC
α globin Rw	ACAGCGCGTTGGGCATGTCGTC
γ globin Fw	CTTCAAGCTCCTGGGAAATGT
γ globin Rw	GCAGAATAAAGCCTACCTTGAAAG
ε globin Fw	GCCTGTGGAGCAAGATGAAT
ε globin Fw	GCGGGCTTGAGGTTGT
GPIIb Fw	CTCCACAACAATGGCCCTGG
GPIIb Rw	CTTGAGAGGGTTGACAGGAG
BCLxL Fw	GAATGACCACCTAGAGCCTTGG
BCLxL Rw	TGTTCCCATAGAGTTCCACAAAAG
BCL2 Fw	ATGTGTGTGGAGAGCGTCAACC

BCL2 Rw	TGAGCAGAGTCTTCAGAGACAGCC
IGF1 Fw	ATGCTCTTCAGTTCGTGTGTG
IGF1 Rw	GCACTCCCTCTACTTGCGTTC
SOX6 Fw	GAGGCAGTTCTTTACTGTGG
SOX6 (endogenous) Rw	CCGCCATCTGTCTTCATAC
SOX6 (flag) Rw	CTTATCGTCGTCATCCTTGTA
SOCS3 Fw	GGAGACTTCGATTCGGGACC
SOCS3 Rw	GAAACTTGCTGTGGGTGACC
PU1 Fw	AGCTCAGATGAGGAGGAGG
PU1 Rw	CAGGTCCAACAGGAACTGG
PAX5 Fw	GTCAGCAAAATTCTTGGCAGG
PAX5 Rw	CACGGTGTCATTGTCACACAC
EBF1 Fw	GGCGTGAATTCGTTCAGTGG
EBF1 Rw	GCTCGTGGTGACGGAGTTAT
BLNK1 Fw	GCTTCAAAAGATGGTCCATGA
BLNK1 Rw	GACCACTGCTCCTCTTCGTC
CYCLIN D1 Fw	CCTCGGTGTCCTACTTCAAA
CYCLIN D1 Rw	GGGATGGTCTCCTTCATCTT
MPL Fw	TCGCTGCCACTTCAAGTCAC
MPL Rw	CAGTCCTGATGGCCTTCTCC
FLI1 Fw	AATGGATCCAGGGAGTCTCC
FLI1 Rw	CCTTGCCATCCATGTTCTGG

Whole-cell protein extracts

Cells were harvested and centrifuged at 640g for 5 min at 4ºC. The pellet washed three times in ice-cold 1X PBS (phosphate buffered saline, pH 7.4) and gently resuspended in RIPA buffers (20mM TrisHCl ph 7.4; 150mM NaCl; 5mM EDTA; 0,3%Triton) supplemtented with protease inhibitors (complete EDTA-free, Roche). Cells were lysed 30 minutes on ice. After centrifugation at 13000 rpm 5 minutes at 4ºC, the supernatant was retrieved.

Immunoblotting analysis

To confirm the presence of SOX6 overexpression, whole protein extracts (30-µg/lane) were resolved by SDS/PAGE in a 10% gel and blotted onto Hybond ECL Nitrocellulose membrane (GE healthcare life science-Amersham) by "wet blotting." carried out under constant voltage at 100V for 90-120 min at 4ºC (Transblot apparatus, Biorad). Membranes were blocked for 1h at room temperature with Milk 5% in TBS-T 1X (Tris Buffered Saline, pH 7.6 and 0,1% Tween 20 by Sigma) and incubated with the appropriate primary antibody diluted in Milk 5% TBS-T overnight at 4ºC. Membranes were washed for three times with TBS-T and incubated with the secondary antibody (diluted in Milk 5%). In particular, the Streptavidin-HRP (Cell signaling) antibody was incubated for 1h at room temperature (no secondary antibody was required). Antibodies binding was detected by using appropriate horseradish peroxidase-conjugated IgG and revealed by ECL (Millipore).

Apoptosis Assay

Apoptosis assays were carried out using AnnexinV (AnnV) and 7 aminoactinomycin D (7AAD) (BD Biosciences). Cells were washed twice with cold PBS 1X (phosphate buffered saline, pH 7.4) and then resuspend in 1X Binding Buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) with 5 µl of PE Annexin V and 5 µl 7-AAD in a final volume of 100µL. The cells were gently vortexed and incubated for 15 min at RT (25°C) in the dark. After that the samples were diluited with 400 µl of 1X Binding Buffer and analyzed by flow cytometry within 1 hr. (Becton-Dickinson FACS Calibur). Data were analyzed with Summit Software v4.3.

Propidium Iodide Staininig for cell cycle analysis

Cells were washed twice with cold PBS 1X (phosphate buffered saline, pH 7.4), resuspend in 1% paraformaldehyde for 45 minutes. Then added the same volume of PBS1X withTriton 0,2% (Sigma) for 15' minutes. Centrifuge the cells and resupend in 250 µl of PBS with propidium iodide (PI) 1mg/mL and RNAse 100 µg/mL, vortex for 5 seconds and incubate at room temperature for at least 1 hour in the dark. Samples were then analyzed by flow cytometry (Becton-Dickinson FACS Calibur) and data were analyzed with Summit Software v4.3.

Statistics

Statistical analyses of obtained data were performed with GraphPad Prism (version 6.0; GraphPad Software, Inc.). The data are expressed as mean ± standard error of n=3 or more determinations. Statistics was performed using a paired, two-tailed Student t-test.

2.3 RESULTS AND DISCUSSION

2.3.1 SOX6 OVEREXPRESSION IN BCR-ABL$^+$CELLS SUPPORTS THE ERYTHROID PROGRAM AT THE EXPANSES OF ALTERNATIVE LINEAGES AND ARRESTS CELLULAR PROLIFERATION

SOX6 overexpression induces erythroid differentiation and apoptosis in the megakaryoblastic cell line MEG.01

MEG.01 is a megakaryoblastic cell line established from the bone marrow of a patient with blast crisis of BCR-ABL$^+$ chronic myelogenous leukemia (Ogura, 1985). This cell line is a recognized model of megakaryocytic cell type, although it retains some erythroid potential.

MEG.01 cells were transduced with a SOX6 overexpressing vector (SOX6-MEG.01) or with the corresponding empty vector (EV-MEG.01), as control. The efficiency of transduction was monitored by Flow Cytometry (FC) analysis: SOX6-MEG.01 and EV-MEG.01 were transduced at comparable levels (Fig. 2.1 a-b). In the same experiments, the presence of exogenous SOX6 was verified both by RT-qPCR and by Western Blot (Fig. 2.1 c).

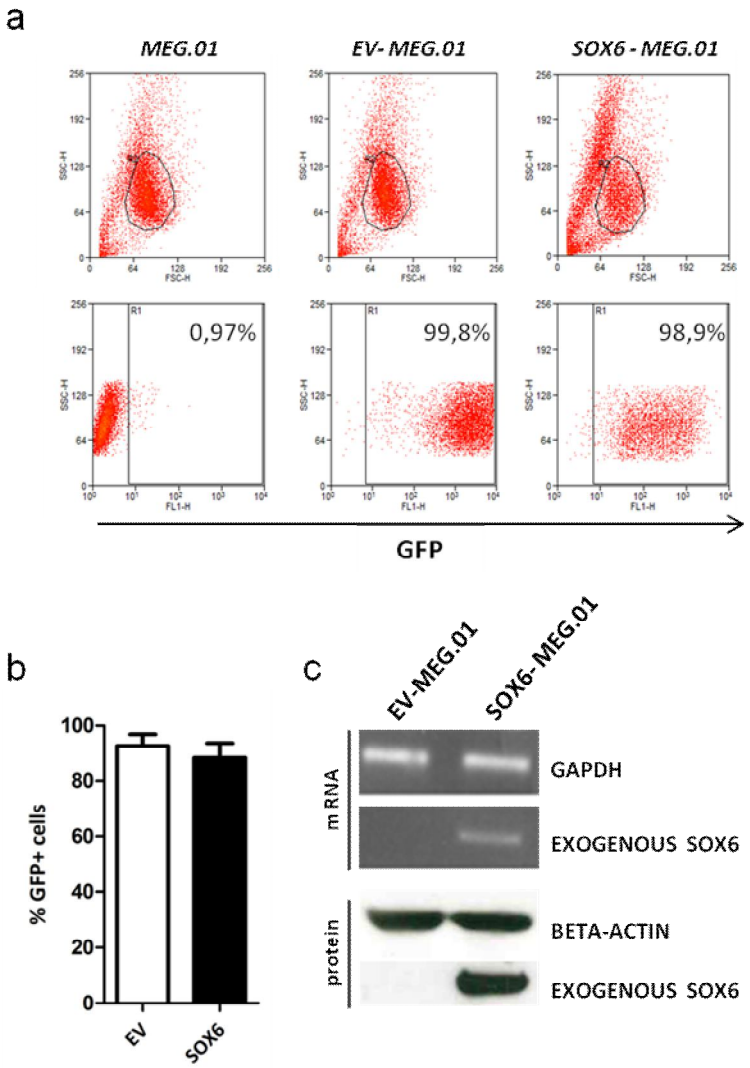

Fig. 2.1: Transduction experiments in MEG.01 cells. a. *The efficiency of transduction was assayed by FC analysis 3 days after the infection with the EV-GFP and SOX6-GFP viral particles. Representative FC plots are shown: untransduced MEG.01 cells; EV-MEG.01 and SOX6-MEG.01. Percentages of GFP$^+$ cells are represented within the plots.* ***b.*** *The histogram shows the percentages of GFP$^+$ cells (error bars: SEM; n≥3).* ***c.*** *The expression level of exogenous SOX6 was assessed at mRNA and protein level 3 days after infection. Upper panel: semiquantitative RT-PCR performed by using primers detecting the exogenous, vector-derived, SOX6 transcript. hGAPDH primers were used as internal control of transcripts abundance. Lower panel: Western Blot performed by using an anti-FLAG antibody that detects the exogenous Sox6 protein. Anti-Beta-Actin antibody was used as loading control.*

Interestingly, despite MEG.01 preferentially differentiate in megakaryocytes, SOX6-MEG.01 are enforced to express erythroid lineage affiliated genes such as globins (α and γ globin genes are here shown as representative globin genes), ALAS-E (aminolevulinate synthase, a gene encoding key enzyme of the heme biosynthetic pathway). Indeed, these genes are significantly upregulated in SOX6 overexpressing cells (Fig. 2.2 a: α and γ= 3.4 average fold increase; ALAS-E= 8.5 fold increase, respectively).
This increase parallels a decreased expression of megakaryocytic lineage affiliated genes, such as GPIIb (a subunit of the integrins complex found on platelets) and RUNX1 (Runt-related transcription factor 1) (Fig. 2.2 a: GPIIb = 0.3 and RUNX1= 0.4 average fold decrease, respectively) (Fig. 2.2 a).
Taken together, these data point to SOX6 as inducer of erythroid identity. Indeed, it is capable of repressing a megakaryocytic-lineage specific genes (here represented by GPIIb and RUNX1) and of promoting the enforcement of the erythroid program.

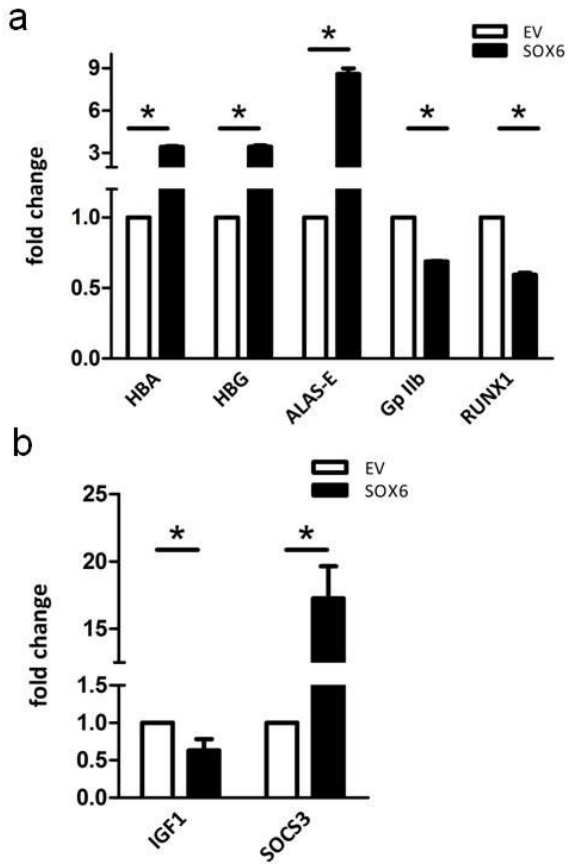

Fig. 2.2: RT-qPCR analysis in SOX6-MEG.01 and EV-MEG.01 cells. a. *RTqPCR shows up-regulation of α and γ globins and of ALAS-E expression and down-regulation of GpIIb and RUNX1 expression, 72h after Sox6 transduction.* ***b.*** *RTqPCR shows decreased expression of IGF1 and an increase of SOCS3expression, 72h after transduction. Histograms show fold change, as compared to the control (EV-MEG.01). hGAPDH expression was used to normalize data (error bars: SEM; n≥3; * P≤0.05).*

In parallel with the skewing toward the erythroid lineage, SOX6-MEG.01 cells show a marked and significant reduction in proliferation, with complete exhaustion of the cultures by day 9 after transduction, as demonstrated by growth curves (Fig. 2.3 a) and by the progressive decrease of the percentage of SOX6-transduced GFP^+ cells in SOX6-MEG.01 cultures (Fig. 2.3 b).

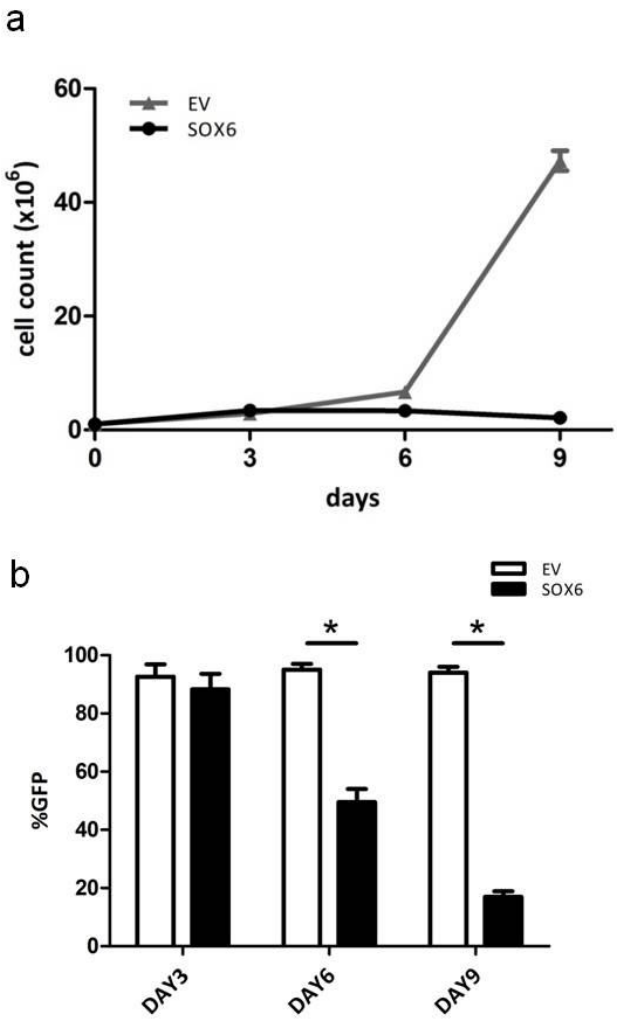

Fig. 2.3: SOX6-MEG.01 cells stop growing upon SOX6 overexpression. a. *1x10^6 exponentially growing MEG.01 cells were transduced at day 0 with viral particles carrying either SOX6-GFP or the EV-GFP lentiviral vectors. SOX6-GFP transduced cells stop growing 3 days after transduction and the culture is exhausted within day 9 (Error bars: SEM; n=3).* ***b.*** *Histogram represents the percentage of GFP$^+$ cells at different time points during the culture (Error bars: SEM; n=3; * P≤0.05).*

The progressive decline of the SOX6-MEG.01 culture observed in Fig. 2.3, could be caused by an increased cell death or by a block in cell cycle progression.

To clarify this point, SOX6-MEG.01 were stained with Annexin V (AnnV) and 7-aminoactinomycin D (7AAD) and analyzed by FC. This set of experiments demonstrated that, starting from 72 hours after transduction, SOX6-MEG.01 cells show an increase in early-apoptotic ($AnnV^+7AAD^-$ cells) and apoptotic ($AnnV^+7AAD^+$ cells) populations, when compared to the control (EV-MEG.01) (Fig. 2.4 a).

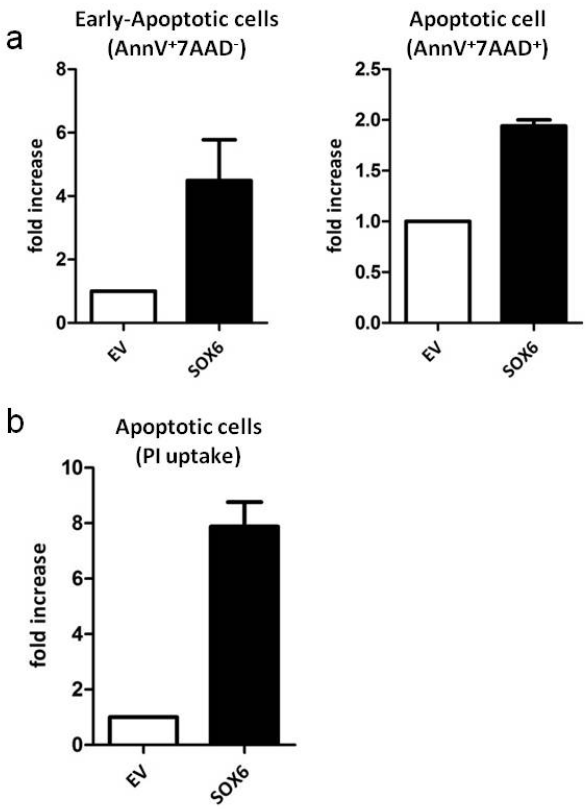

Fig. 2.4: SOX6-MEG.01 have more pre-apoptotic and apoptotic cells compared to the control EV-MEG.01. **a.** *The histograms show the average fold increase of early apoptotic ($AnnV^+7AAD^-$) and apoptotic ($AnnV^+7AAD^+$) populations in SOX6-MEG.01 cells when compared to the control, as assessed by FC analysis (Error bars: SEM; n=2).* ***b.*** *Propidium Iodide uptake (PI) as per fold increase in SOX6-MEG.01 cells, as compared to the control. (Error bars: SEM; n=2).*

SOX6-MEG.01 cells show a significant decrease in IGF-1 expression, coupled with a strong upregulation of SOCS3 expression (Fig. 2.2. b). Together, these results may underlie an alteration in the cell cycle progression of MEG.01 cells overexpressing SOX6. To test this hypothesis, we set up a cell cycle analysis based on the measure of Propidium Iodide (PI) uptake. This analysis confirmed the presence of necrotic cells in the SOX6-MEG.01 population, when compared to the relative control (Fig. 2.4 b), but failed to reveal any significant change in cell cycle phases distribution (Fig. 2.5).

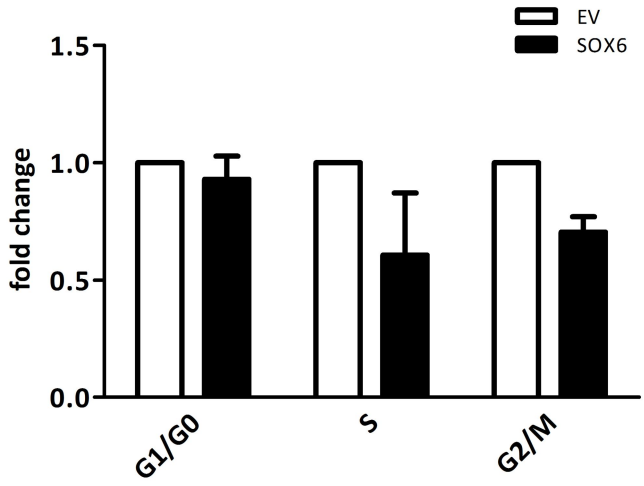

Fig. 2.5: Cell cycle analysis of SOX6-MEG.01 and EV-MEG.01. *FC analysis was performed 3 days after infection with the EV-GFP and SOX6-GFP viral particles, respectively. Histogram bars show the fold change variation in SOX6-MEG.01 cell cycle phases compared to the respective control (error bars: SEM; n=2).*

Taken together these data suggest the exhaustion of SOX6-MEG.01 cells in culture is due to an increased apoptosis more than to a block in cell cycle progression.

SOX6 overexpression alters the expression of genes essential for lymphocytic B cell identity and induces block in the lymphoblastic cell line SUPB15.

To study the relationship between SOX6 effects and the BCR-ABL translocation in a non-erythroid context, we overexpressed it in a lymphoid cell line. The SUPB15 cell line was derived from the bone marrow of an 8-year-old child with acute lymphoblastic leukemia of B-cell precursors, carrying the P190 BCR-ABL fusion gene (Naumovski, 1988).

SUPB15 cells were transduced with the SOX6 overexpressing vector (SOX6-SUPB15) or with the corresponding empty vector (EV-SUPB15). The efficiency of transduction was monitored by FC analysis: SOX6-SUPB15 and EV-SUPB15 were transduced at comparable levels in all the performed experiments (Fig. 2.6 b).

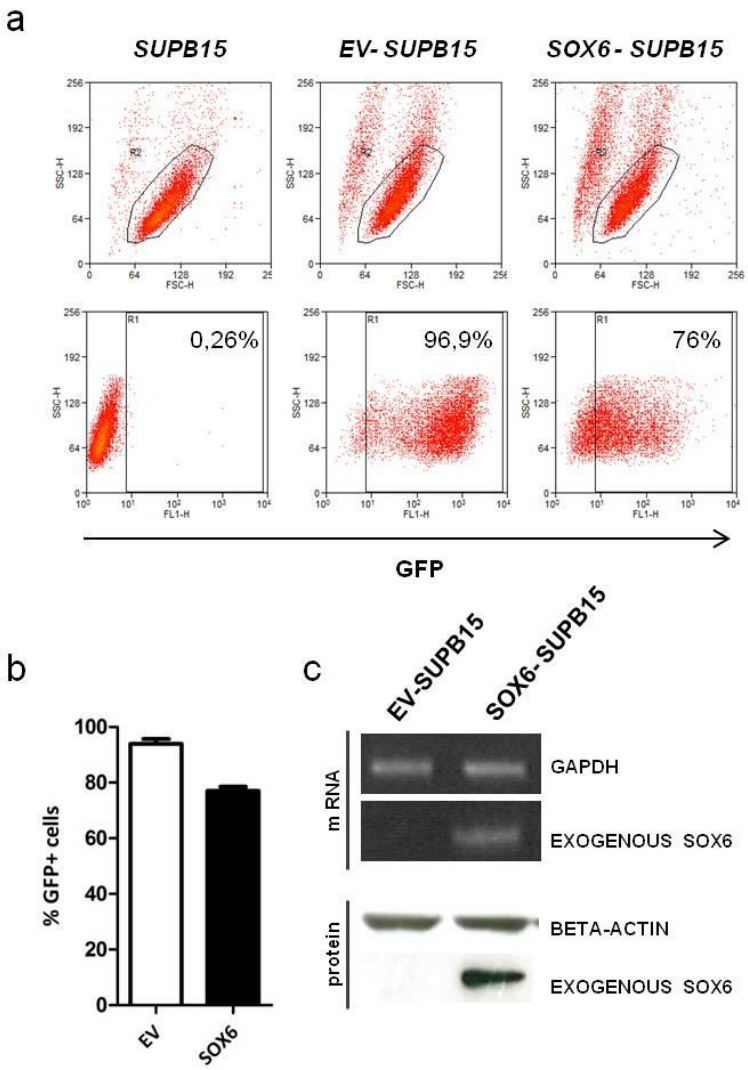

Fig. 2.6: Transduction experiments in SUPB15 cells. a. *The efficiency of transduction was assayed by FC analysis 3 days after infection with the EV-GFP and SOX6-GFP viral particles. Representative FC plots are shown: untransduced SUPB15; EV-SUPB15 and SOX6-SUPB15. Percentages of GFP$^+$ cells are represented within the plots.* ***b.*** *The histogram shows the percentages of GFP$^+$ cells (error bars: SEM; n≥3).* ***c.*** *The expression level of exogenous SOX6 was assessed at mRNA and protein level 3 days after transduction. Upper panel: semiquantitative PCR performed by using primers detecting the exogenous, vector-derived, SOX6 transcript (see Experimental procedure for details). hGAPDH primers were used as internal control for transcripts abundance. Lower panel: Western Blot performed by using an anti-FLAG antibody that detects the exogenous Sox6 protein. Anti-Beta-Actin antibody was used as loading control.*

After 3 days of transduction, SOX6–SUPB15 cells show a decreased expression of genes essential for lymphocytic identity and differentiation, such as PU1, PAX5, EBF1 and BLNK (Fig. 2.7).

In particular, PU1 is required for Common Lymphoid Progenitors (CLP) to become lymphoid restricted. Moreover, by up-regulating the expression of the IL-7 receptor (IL-7R), it has a role in the V(D)J recombination during lymphocyte development (Sitnicka, 2003; Dias, 2005). In SOX6–SUPB15, PU1 transcript is marginally, but significantly, decreased (≈20% decrease compared to the control, EV–SUPB15) (Fig. 2.7).

EBF1 (Early B cell factor 1) has a direct role in specifying the B cell-specific gene expression program and, together with others transcription factor such as AIOLOS, SOX4, BCL11A, E2A and IKAROS, forms a network that controls early B cell lymphopoiesis (Scott, 1994; Dekoter, 2002).

EBF1 and E2A, in turn, induce PAX5 (Paired box protein 5) also known as BSAP (B-cell lineage specific activator). PAX5 promotes B cell commitment by repressing lineage-inappropriate gene expression and reinforcing B cell specific gene expression (Nutt, 1999).

In SOX6–SUPB15, both PAX5 and EBF1 transcripts are significantly decreased (≈50% and 40% decrease compared to the control, respectively) (Fig. 2.7).

Finally, the BLNK gene, encoding for a cytoplasmic linker or adaptor protein that plays a critical role in B cell differentiation (Schebesta, 2007), is significantly decreased in SOX6–SUPB15 (≈30% decrease compared to the control) (Fig. 2.7).

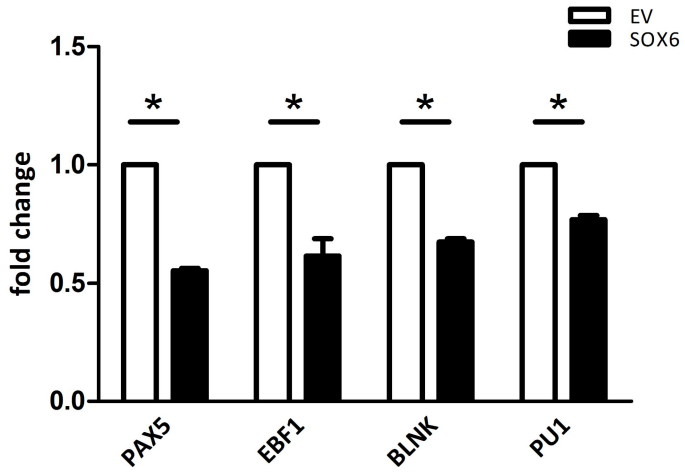

Fig. 2.7: RT-qPCR analysis on SOX6-SUPB15 and EV-SUPB15. *RT-qPCR shows decreased expression of PAX5, EBF1, BLNK, PU1 72h after transduction. Histograms show the fold change compared to the control (EV-SUPB15). hGAPDH was used to normalize data (error bars:SEM; n≥3; * P≤0.05).*

These data confirm that the ectopic expression of SOX6 is capable of altering the early onset of a lineage specific differentiation program in B cells, which are hierarchically very distant from erythroid cells. Although SOX6 overexpression in SUPB15 is not sufficient to trigger the expression of genes important for the onset of erythropoiesis (such as GATA1, NF-E2 p45, KLF1, globins; data not shown), it is capable of interfering with the expression of B lineage specific markers as shown by the decrease of PU1, PAX5, EBF1 and BLNK gene expression.
Taken together, these data reveal that SOX6 is able to repress non erythroid lineages, although it is able to induce a transdifferentiation precess.

With respect to cellular proliferation, SOX6–SUPB15, as demonstrated by the growth curve in Fig. 2.8, when compared to that of control EV–SUPB15 cells, show a decreased proliferation. This explains the observed

progressive loss of transduced GFP$^+$ cells within SOX6–SUPB15 population (Fig. 2.8 a-b).

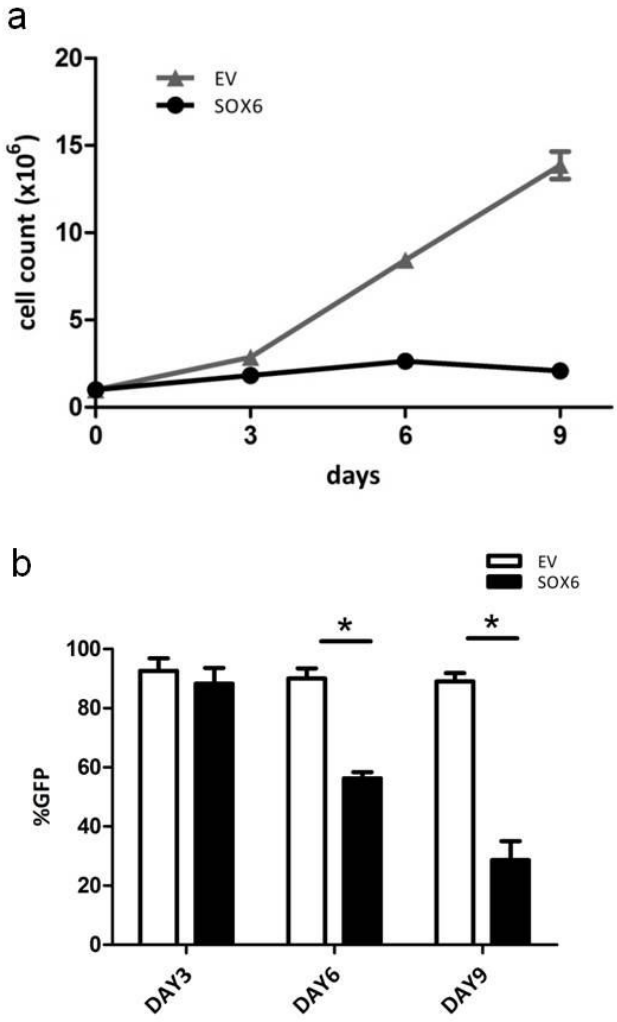

Fig. 2.8: SOX6-SUPB15 cells stop growing upon SOX6 overexpression. a. *1x10^6 exponentially growing SUPB15 cells were transduced at day 0 with viral particles carrying either SOX6-GFP or the EV-GFP lentiviral vectors. SOX6-GFP transduced cells stop growing 3 days after transduction and the culture die within day 9 (Error bars: SEM; n=3; * P≤0.05).* ***b.*** *Histograms represent the GFP$^+$ cells percentages at different time points during the culture (Error bars: SEM; n=3;* P≤0.05).*

SOX6-SUPB15 cells show increased levels of both early-apoptotic and apoptotic cells (Fig. 2.9), as demonstrated by Flow Cytometry analysis, carried out 72 hours after transduction.

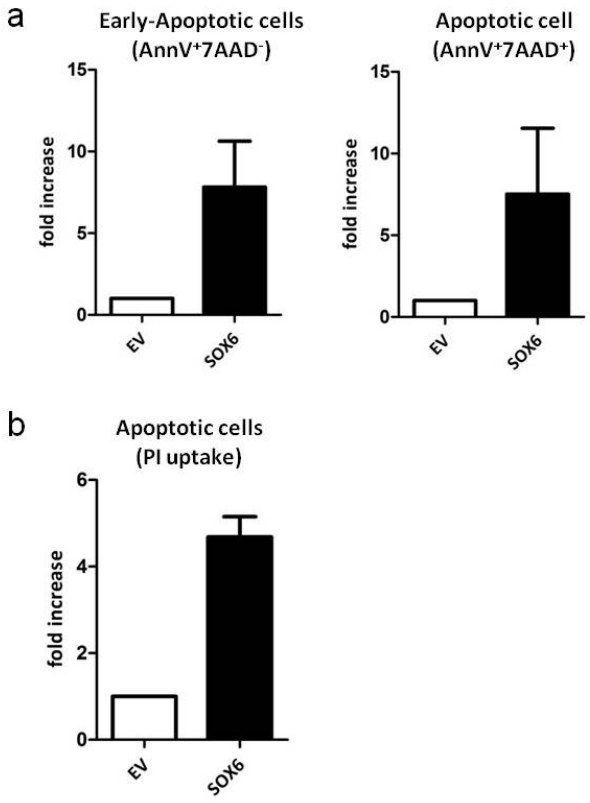

Fig.2.9: SOX6-SUPB15 cells have a high percentage of pre-apoptotic and apoptotic cells, when compared to the control EV-SUPB15 cells. a. *Histograms show the average fold increase of early apoptotic (AnnV+7AAD-) and apoptotic (AnnV+7AAD+) cell populations in SOX6-SUPB15 cells, as compared to the control, as assessed by FC analysis (Error bars: SEM; n=2).* ***b.*** *The histogram represents the Propidium Iodide uptake (PI) as per fold increase of SOX6-SUPB15 cells compared to the control. (Error bars: SEM; n=2).*

Furthermore, SOX6-overexpressing cells show a significant down-regulation of BCL2 (B cell like) expression. BCL2 encodes for an important anti-apoptotic protein (Fig. 2.10 b). Moreover, cell cycle analysis demonstrates

an alteration of cell cycle progression in all the different phases and further confirms an accumulation of apoptotic/necrotic cells (Fig. 2.10 a).

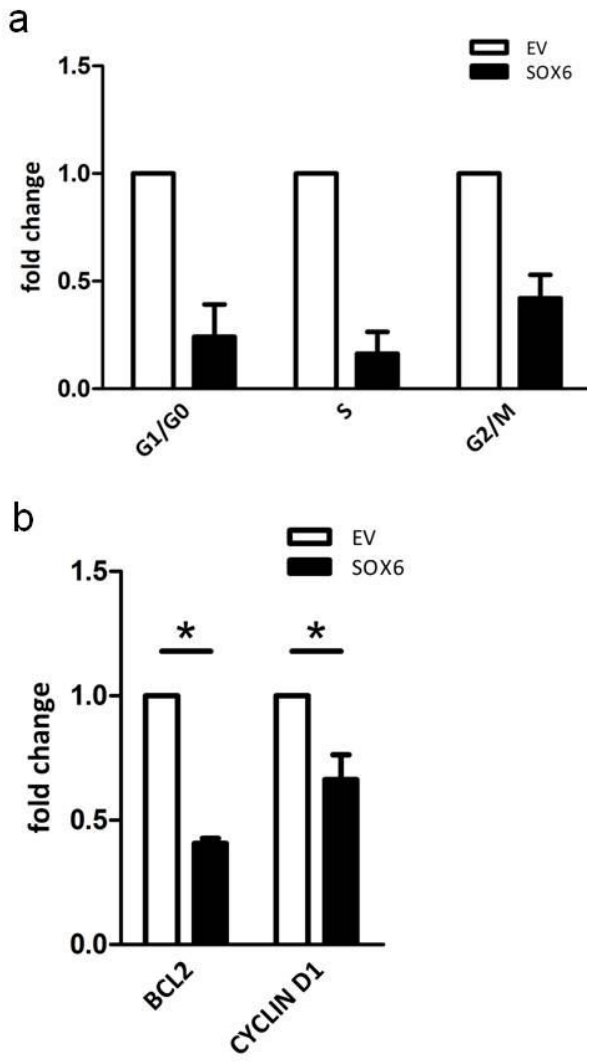

Fig 2.10: Cell cycle and RT-qPCR analysis of SOX6-SUPB15 and EV-SUPB15. a. *FC analysis of cell cycle was performed 3 days after infection with EV-GFP and SOX6-GFP viral particles, respectively. Histogram bars show the fold change variation in SOX6-SUPB15 cell cycle phases compared to the respective control (error bars: SEM; n=2).* ***b.*** *RTqPCR shows decreased expression of BCL2 and CyclinD1 72h after transduction. Histograms show the fold change compared to control EV-SUPB15 cells. hGAPDH expression was used to normalize the data obtained (error bars:SEM; n≥3; * P≤0.05).*

SUPB15 cells are a lymphoid cell line, where SOCS3, IGF1 and IGF1R are not expressed (data not shown). To provide an alternative explanation of the phenotype observed upon SOX6 transduction, we analyzed the expression level of factors important for cell proliferation, such as CyclinD1, ecoded by the CCND1 gene. CCND1 expression level is significantly decreased in presence of ectopic expression of SOX6 (Fig. 2.10 b), confirming that SOX6 may interfere with multiple pathways important for cell cycle progression.
Taken together these data suggest the exhaustion of SOX6-SUPB15 cells in culture triggers an apoptosis program and a block in cell cycle progression, possibly mediated by CyclinD1 downregulation.

In the BCR-ABL$^+$ cell lines tested in this study, the strongest phenotype observed upon SOX6 overexpression is the upregulation of genes related to the erythroid lineage-specific differentiation program both in megakaryocytic MEG.01 cells and SUPB15 B cells, coupled with the suppression of genes specifying for alternative lineages, such as RUNX1 in MEG.01 and PAX5 in SUPB15.
In BCR-ABL$^+$ cells, SOX6 overexpression affects viability. Indeed, both cell lines characterized in this study showed an increase of apoptosis upon SOX6 overexpression. The exhaustion of cell culture is evident as a marked decrease in SOX6$^+$ cells. Our preliminary results suggest that high SOX6 levels also concur to the alteration of the cell cycle, particularly in B cells.
Taken together, these results suggest SOX6 has a pivotal role in erythroid terminal differentiation and tend to suppress alternative lineage specification. This conflict between alternative lineages specification may trigger cell fate decision towards cellular death.

2.3.2 SOX6 OVEREXPRESSION IN JAK2V617F$^+$ CELL LINES

The SOCS3 regulatory region contains an enhancer containing a double SOX6 binding site. The SOX6 binding to this regulatory element activates SOCS3 expression (Cantù, 2011). These data suggest that the SOX6-mediated arrest in cell growth is at least partly mediated by SOCS3. If this is true, it should be possible to uncouple the two main effects downstream to SOX6 overexpression, i.e. the enhancement of erythroid terminal differentiation and the arrest in cells growth.

To clarify this issue, I took advantage of different myeloid cell lines sharing a common mutation: the JAKV617F allele. This mutation affects the JAK2 kinase, which becomes constitutively activated and overcomes the repression elicited by SOCS (Hookham, 2007). Therefore, in our hypothesis, SOX6 overexpression in cell lines harboring this mutation should lead to erythroid differentiation, but should not affect cell growth.

SOX6 induces differentiation but not cell cycle arrest in the HEL (JAK2V617F$^+$) cell line.

To test the above hypothesis I transduced HEL cells with a SOX6-GFP lentiviral vector and I used the same cloning strategy to produce a lentiviral vector for SOCS3 overexpression (SOCS3-GFP). HEL cells were transduced with the SOX6 overexpressing vector (SOX6-HEL) or the SOCS3 overexpressing vector (SOCS3-HEL) and with the corresponding empty vector (EV-HEL) as a control. The efficiency of transduction was monitored by FC analysis: SOX6-HEL or SOCS3-HEL and EV-HEL were transduced at comparable levels (Fig. 2.11 a-b; 2.12 a-b). The presence of exogenous SOX6 and SOCS3 was verified both by RT-PCR and Western Blot (Fig. 2.11 c; Fig. 2.12 c).

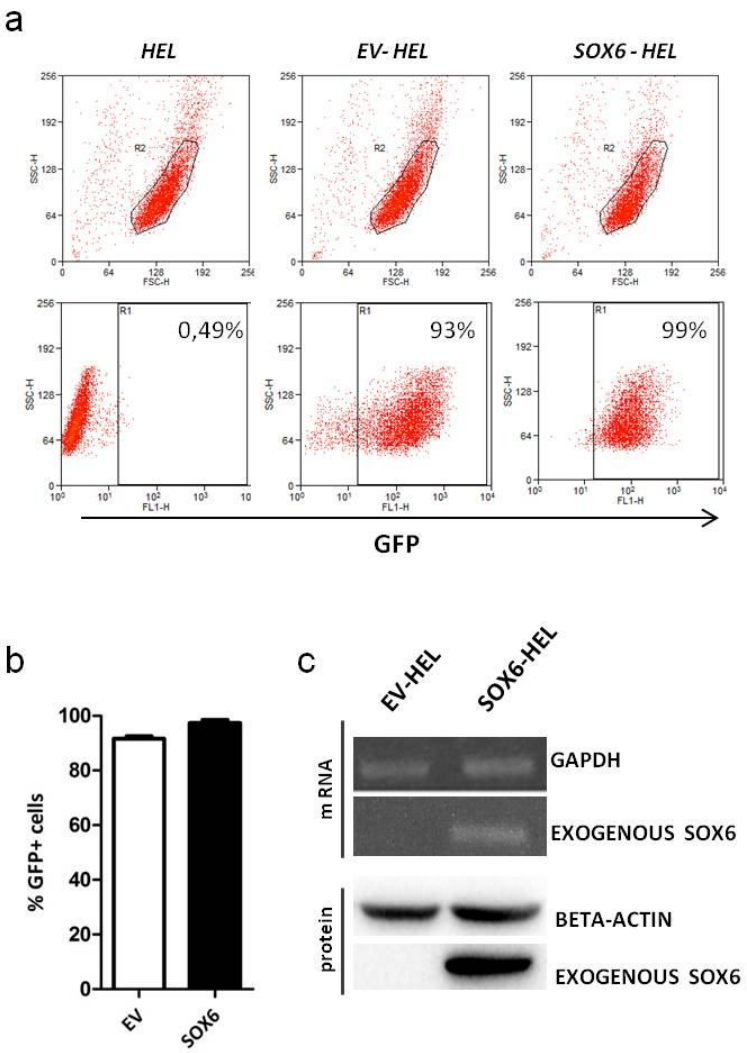

Fig. 2.11: SOX& transduction experiments in HELcells. a. *The efficiency of transduction was assayed by FC analysis 3 days after transduction with the EV-GFP and SOX6-GFP viral particles. Representative FC plots are shown: untransduced HEL; EV-HEL and SOX6-HEL. Percentages of GFP$^+$ cells are represented within the plots.* ***b.*** *The histogram shows the percentages of GFP$^+$ cells (error bars: SEM; n≥3)* ***c.*** *The expression level of exogenous SOX6 was assessed at mRNA and protein level, 72 h after transduction. Upper panel: semiquantitative RT-PCR performed by using primers detecting the exogenous, vector-derived, SOX6 transcript (see experimental procedure for details). hGAPDH primers were used as internal control of transcripts abundance. Lower panel: Western Blot performed by using an anti-FLAG antibody that detects the exogenous Sox6 protein. Anti-Beta-Actin antibody was used as loading control.*

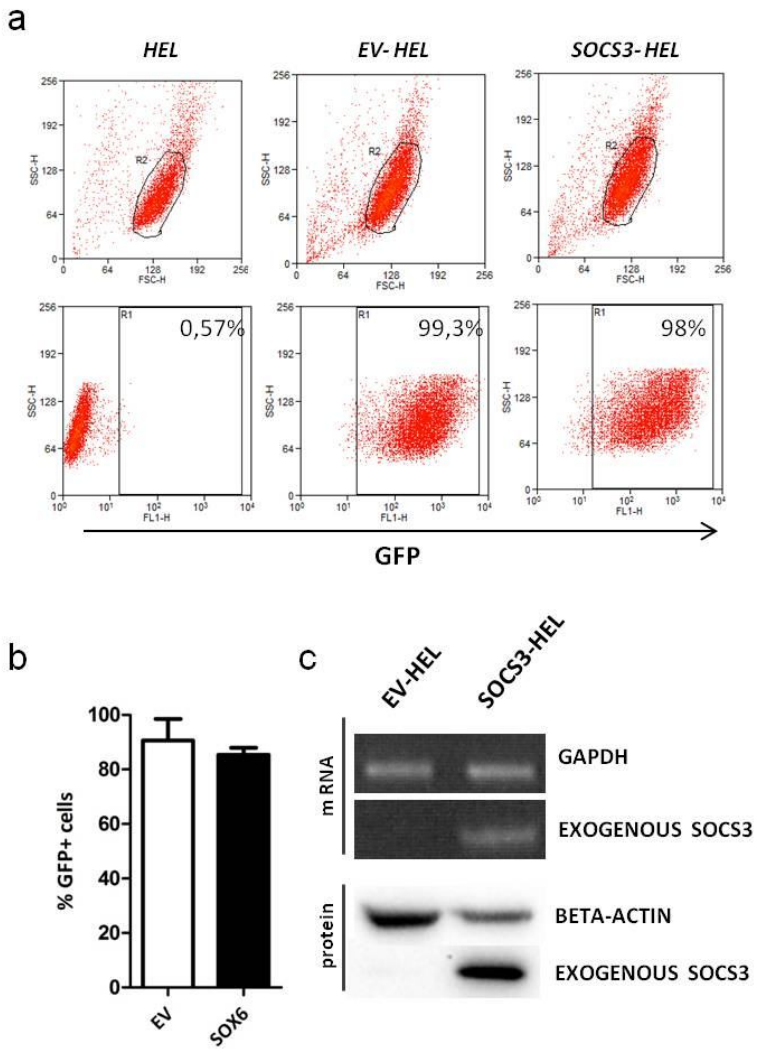

Fig. 2.12: SOCS3 transduction experiments in HEL cells. a. *The efficiency of transduction was assayed by FC analysis 3 days after infection with the EV-GFP and SOCS3-GFP viral particles. Representative FC plots are shown: untransduced HEL; EV-HEL and SOCS3-HEL. Percentages of GFP$^+$ cells are represented within the plots.* ***b.*** *The histogram shows the percentages of GFP$^+$ cells (error bars: SEM; n≥3).* ***c.*** *The expression level of exogenous SOCS3 was assessed at mRNA and protein level 3 days after infection. Upper panel: semiquantitative PCR performed by using primers detecting the exogenous, vector-derived, SOCS3 transcript (see experimental procedure for details). hGAPDH primers were used as internal control of transcripts abundance. Lower panel: Western Blot performed by using an anti-FLAG antibody that detects the exogenous SOCS3 protein. Anti-Beta-Actin antibody was used as loading control.*

HEL cells carry eight copies of the allele JAK2V617F which is typically associated to the myeloproliferative disorder (Quentemeier, 2006). This JAK2 mutation makes HEL cells insensitive to SOCS3 inhibition (Hookham, 2007).

As predicted by my hypothesis, SOX6 overexpression in HEL cells does not result in an alteration of cell growth (Fig.2.13), but it induces erythroid differentiation, as shown by the increased expression of erythroid genes such as globins (α, γ, ε) and ALAS-E (Fig. 2.15 a). To further confirm this finding, I overexpressed SOCS3 in HEL cells. As expected, neither differentiation (Fig. 2.15 b) nor cell proliferation were affected (Fig. 2.14).

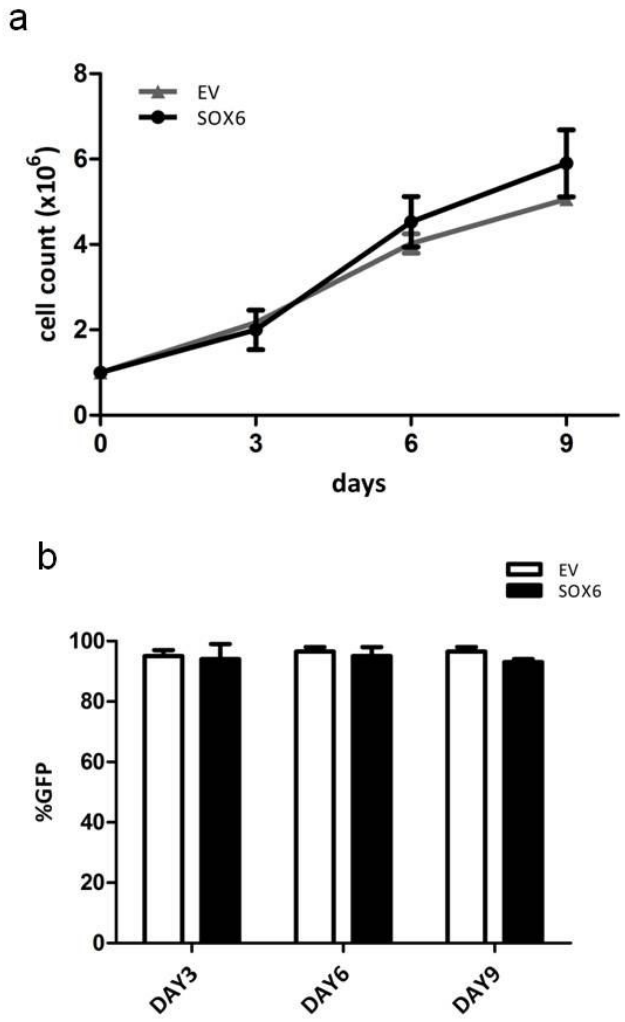

Fig. 2.13 Growth rate is unaffected in SOX6-HEL cells. a. *$1x10^6$ exponentially growing HEL cells were transduced at day 0 with viral particles carrying either SOX6-GFP or the EV-GFP lentiviral vectors. SOX6-GFP transduced cells do not stop growing after transduction (Error bars: SEM; n=3).* ***b.*** *Histogram represents the GFP^+ cells percentages at different time points during the culture (Error bars: SEM; n=3).*

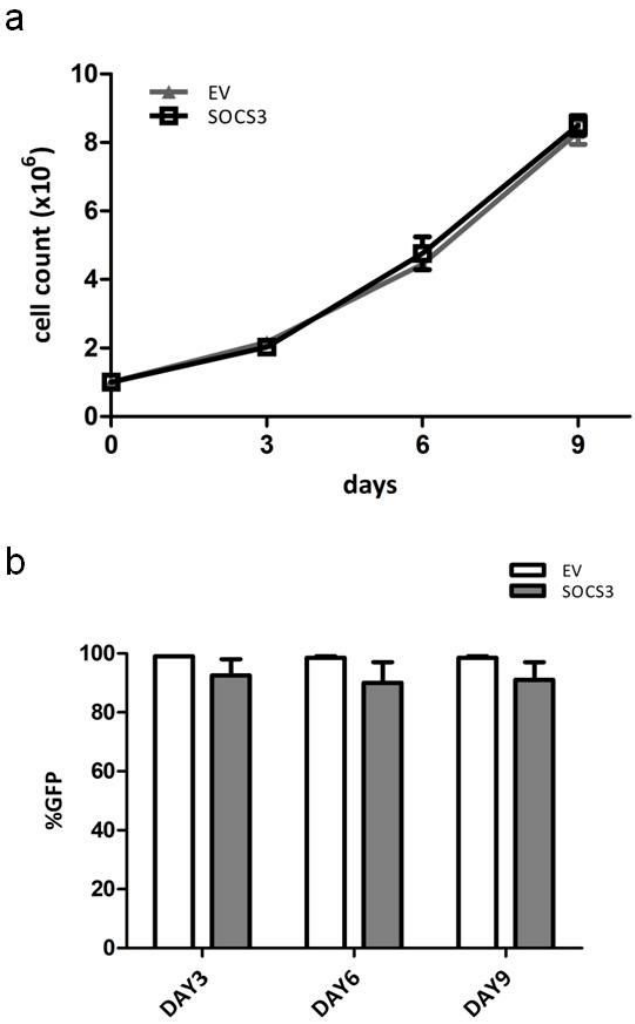

Fig. 2.14: Growth rate is unaffected in SOCS3-HEL cells. a. *$1x10^6$ exponentially growing HEL cells were transduced at day 0 with viral particles carrying either SOCS3-GFP or the EV-GFP lentiviral vectors. SOCS3-GFP transduced cells do not stop growing after transduction (Error bars: SEM; n=3).* ***b.*** *Histogram represents the GFP^+ cells percentage at different time points during the culture (Error bars: SEM; n=3).*

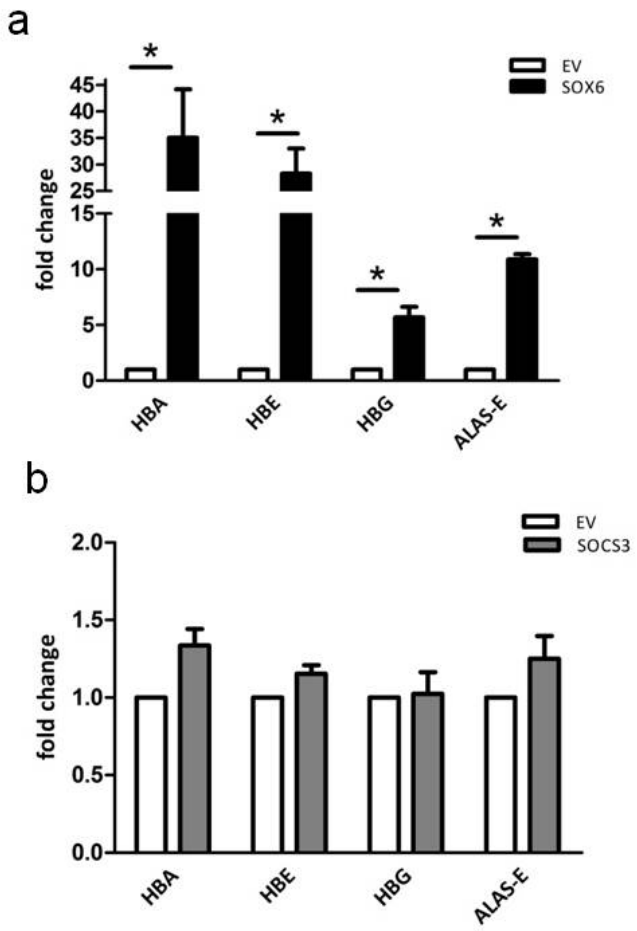

Fig. 2.15: RT-qPCR analysis on SOX6-HEL or SOCS3 -HEL and EV-HEL cells. a. *RTqPCR shows up-regulation of globins (α, γ, ε) and ALAS-E expression, 72h after transduction in SOX6-HEL.* ***b.*** *No significant variation on the expression of the same genesi s observed 72h upon SOCS3 transduction. Histograms show the fold change compared to the control (EV-HEL). hGAPDH expression was used to normalize data (error bars:SEM; n≥3; * P≤0.05).*

In a second set of experiments, I assessed whether SOX6 (or SOCS3) overexpression could trigger apoptosis in cells carrying the JAK2V617F mutation.

Both SOX6 and SOCS3 overexpression does not reduce the expression level of two important anti-apoptotic genes, BCL2 and BCLXL, as assayed by RTqPCR. Moreover, I could not detect significant changes in the rate of early–apoptotic and apoptotic cells, by using AnnV/7AAD staining (data not shown). Finally, cell cycle analysis, assayed by measuring Propidium Iodide intake, indicates that, in SOX6-HEL cells, the proportion of cells in S phase is decreased when compared to the control (Fig. 2.16).

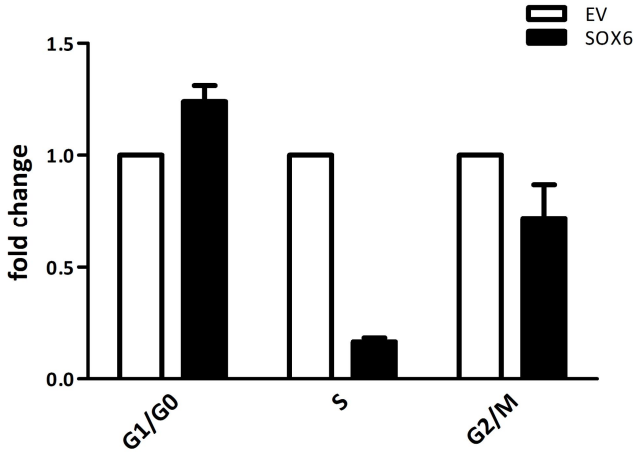

Fig. 2.16: Cell cycle analysis of SOX6-HEL and EV-HEL. *FC analysis was performed 3 days after infection with EV-GFP and SOX6-GFP viral particles, respectively. Histogram bars show the fold change variation of SOX6-HEL cells distribution in the different cycle phases, when compared to the respective control (error bars: SEM; n=2).*

Taken together, these data confirm that SOX6 overexpression leads to erythroid differentiation and that this effect is independent from its direct target SOCS3. Furthermore, this data suggests that SOX6 may trigger cell death through SOCS3. Indeed, in the presence of the JAK2V617F mutation, SOX6 and SOCS3 fail to induce apoptosis.

It is important to note that SOX6 overexpression leads to a change in the cell cycle profile of HEL cells and this may be linked to the enhancement of erythroid terminal differentiation. Indeed, during the last steps of this process, S phase progression is gradually reduced (Pop, 2010).

HEL cells (BCR-ABL$^-$) are not known to depend on IGF1 for their growth and do not express IGF1. Indeed, in SOX6 overexpressing HEL cells there are no significant changes in the expression of IGF1 receptor (not shown), further excluding an involvement of this pathway downstream to SOX6. On the other hand, in contrast to what happens in K562 and MEG.01 cell lines, we observed a significant reduction in EPO receptor (EPOR) expression in SOX6–HEL (Fig. 2.17).

This last observation leads to the hypothesis that in the absence of BCR-ABL, the IGF1 pathway is not perturbed by SOX6-mediated SOCS3 up-regulation (Fig. 2.17) and that in HEL cells SOCS3 elicits its effect on EPO pathway.

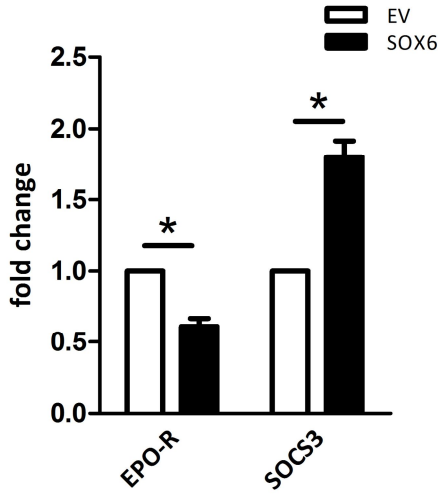

Fig. 2.17: RTqPCR analysis on SOX6-HEL and EV-HEL cells. a. *RTqPCR analysis shows a decreased expression of EPO-R and an up-regulation of SOCS3 expression, 72h after transduction. Histograms show the fold change compared to the control (EV-HEL). hGAPDH expression was used to normalize data (error bars:SEM; n≥3; * P≤0.05.).*

SOX6 ectopic expression in SET2 and UKE1 cell lines (JAK2V617F$^+$) enforces megakaryoblastic cells toward an erythroid fate and blocks their cell proliferation.

The JAK2V617F mutation has a diagnostic and pathogenic role in BCR-ABL negative myeloproliferative chronic disorders since different levels of JAK2V617F are associated with different clinical phenotypes (Levine, 2007). Cells carrying this mutation can escape the inhibitory control of SOCS3 (Hookham, 2007), which is a direct transcriptional target of SOX6. In our experiments, SOX6 overexpression in HEL cells, which carry 8 copies of the JAK2V617F allele, did not decrease cell growth but it induced significant erythroid differentiation.

To understand whether the phenotype observed in the above experiments is related to the JAK2V617F copy number, I overexpressed SOX6 in other two cell lines carrying the mutated allele and representative of two myeloproliferative disorders: SET2 (6 mutated alleles, 1 wild type allele) and UKE1 (2 mutated alleles). These cell lines were established from the peripheral blood of patients with essential thrombocythemia at the stage of megakaryoblastic leukemic transformation (Quentmeier, 2006). Essential thrombocytemia is MPD and most of the cases are associated with mutations that activate JAK2 (Levine, 2007).

I transduced SET2 and UKE1 with SOX6-GFP lentiviral vector used in the previous experiments. As in previous experiments, the percentage of GFP positivity, that reflects the efficiency of transduction, was assessed by FC analysis. SET2 and UKE1 infected with SOX6 or with the empty vector were transduced at comparable levels (Fig. 2.18 a-b; Fig. 2.19 a-b). Again, the expression of the exogenous SOX6 was further confirmed at mRNA and protein level (exogenous protein being detected by anti-FLAG antibody) (Fig. 2.18 c; Fig. 2.19 c).

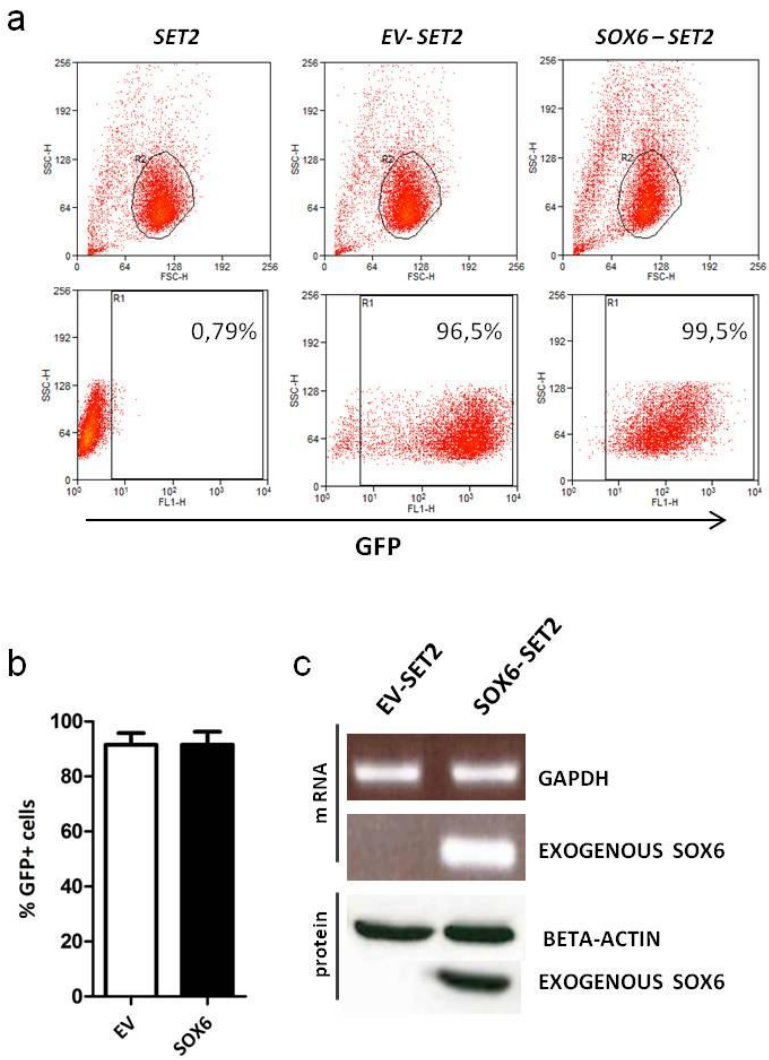

Fig. 2.18: Transduction experiments in SET2 cells. a. *The efficiency of transduction was assayed by FC analysis 3 days after infection with the EV-GFP and SOX6-GFP viral particles. Representative FC plots are shown: untransduced SET; EV-SET2 and SOX6-SET2. Percentages of GFP$^+$ cells are represented within the plots.* ***b.*** *The histogram shows the percentages of GFP$^+$ cells (error bars: SEM; n≥3).* ***c.*** *The expression level of exogenous SOX6 was assessed at mRNA and protein level 3 days after infection. Upper panel: semiquantitative RT-PCR performed by using primers detecting the exogenous, vector-derived, SOX6 transcript (see experimental procedure for details). hGAPDH primers were used as internal control. Lower panel: Western Blot performed by using an anti-FLAG antibody that detects the exogenous SOX6 protein. Anti-Beta-Actin antibody was used as loading control.*

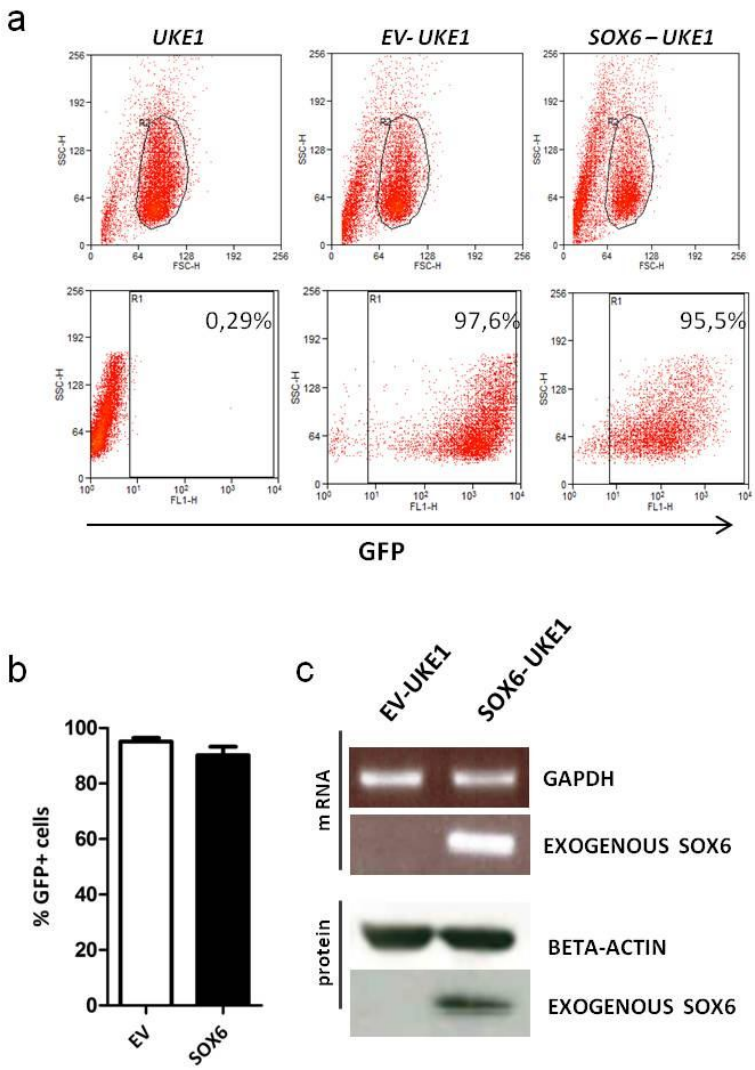

Fig. 2.19: Transduction experiments in UKE1 cells. a. *The efficiency of transduction was assayed by FC analysis 3 days after infection with the EV-GFP and SOX6-GFP viral particles. Representative FC plots are shown: untransduced UKE1; EV-UKE1 and SOX6-UKE1. Percentages of GFP$^+$ cells are represented within the plots.* ***b.*** *The histogram shows the percentages of GFP$^+$ cells (error bars: SEM; n≥3).* ***c.*** *The expression level of exogenous SOX6 was assessed at mRNA and protein level, 72h after infection. Upper panel: semiquantitative RT-PCR performed by using primers detecting the exogenous, vector-derived, SOX6 transcript (see experimental procedure for details). hGAPDH primers were used as internal. Lower panel: Western Blot performed by using an anti-FLAG antibody that detects the exogenous SOX6 protein. Anti-Beta-Actin antibody was used as loading control.*

In these cell lines, SOX6 overexpression determines a significant up-regulation of erythroid markers (α, γ, ε globins and ALAS-E) and also of SOCS3 overexpression and induces a concomitant down-regulation of megakaryocytic markers (GPIIb, RUNX1,FLI1,MPL), as assessed by RTqPCR (Fig. 2.20 a-b).

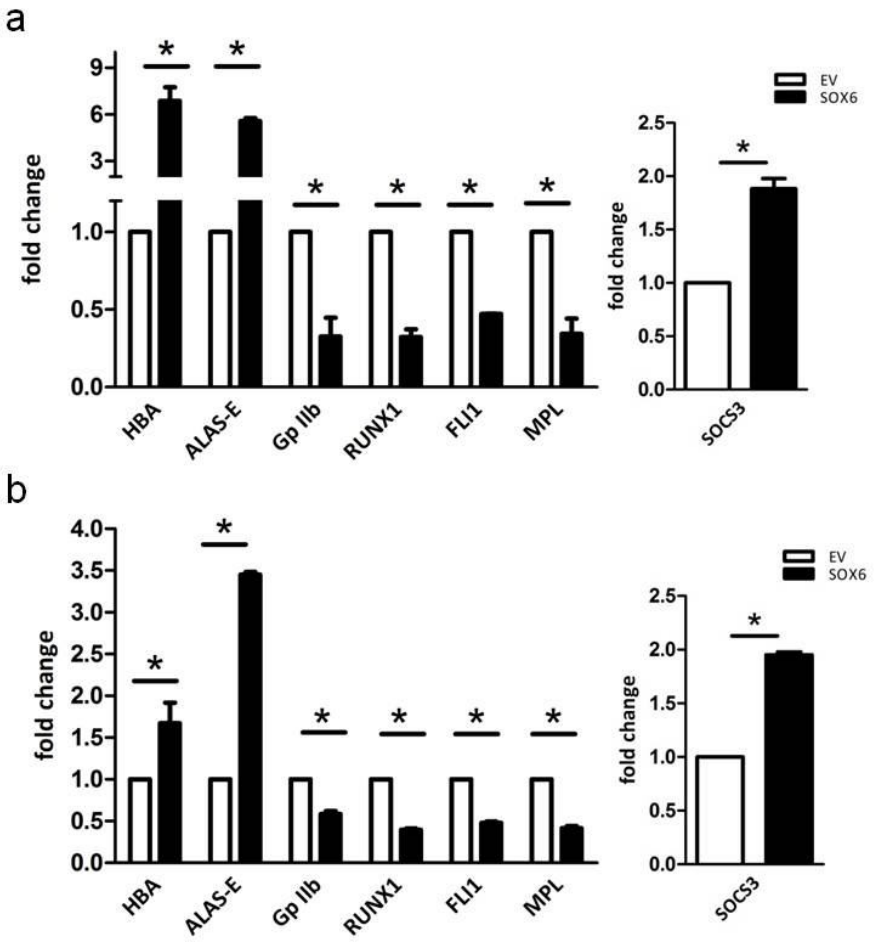

Fig. 2.20: RT-qPCR analysis on SOX6-SET2 (a) and SOX6-UKE1 (b) cells. *RTqPCR analysis on erythroid, alternative lineage markers and SOCS3 was performed as in previous figures. hGAPDH was used to normalize data (error bars: SEM; n≥3; * P≤0.05.)*

FLI1 and RUNX1 are lineage-specific transcription factors with a central role in megakaryocytes production (MKs). RUNX1 is highly expressed during MKs differentiation from MEP (megakaryocyte/erythroid progenitor), but it is downregulated during early phases of erythroid differentiation (Irvin, 2004); FLI1 activates many MKs specific-genes and represses the activity of erythroid factors by interfering with their binding to the DNA (Athanasoius, 2000). MPL is the gene encoding for the thrombopoietin receptor (a growth factor necessary for megakaryocytes proliferation and maturation) and GPIIb is a subunit of the integrin complex on platelets.

Taken together, these data confirm that SOX6 overexpression enhances the erythroid differentiation program at the expenses of the megakaryocytic one.

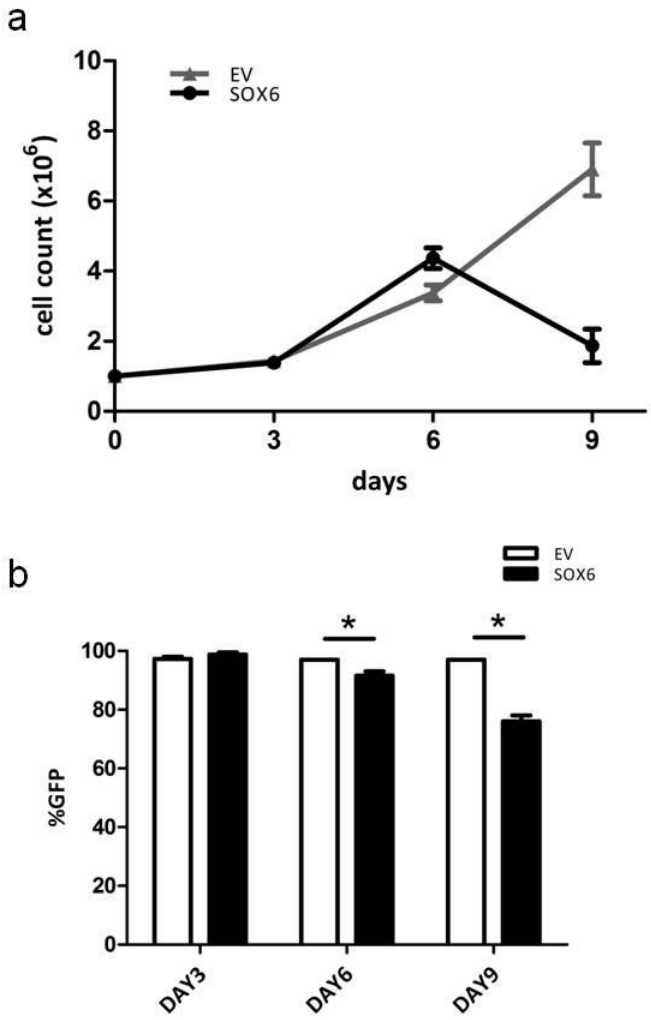

Fig. 2.21: SOX6-SET2 cells stop growing between day 6 and day9 after transduction. a. *$1x10^6$ exponentially growing SET2 cells were transduced at day 0 with viral particles carrying either SOX6-GFP or the EV-GFP lentiviral vectors. SOX6-GFP transduced cells stop growing 3 days after transduction and the culture die within day 9 (Error bars: SEM; n=3; * P≤0.05).* ***b.*** *Histogram represents the GFP positive cells percentage at different time points during the culture (Error bars: SEM; n=3; * P≤0.05).*

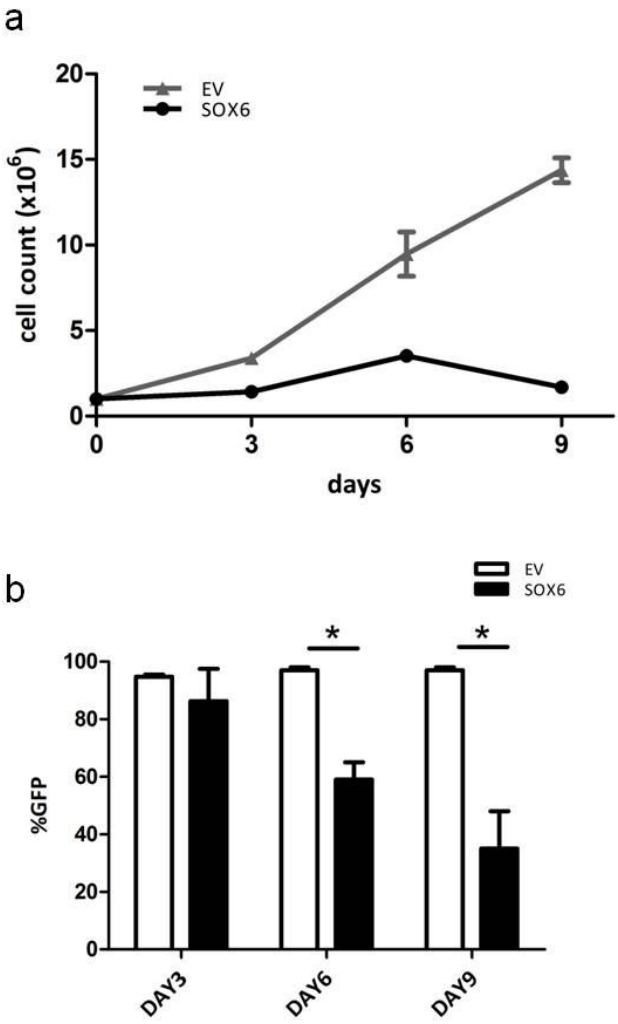

Fig.2.22: SOX6-UKE1 cells stop growing after SOX6 transduction. a. *$1x10^6$ exponentially growing UKE1 cells were transduced at day 0 with viral particles carrying either SOX6-GFP or the EV-GFP lentiviral vectors. SOX6-GFP transduced cells stop growing 3 days after transduction and the culture die within day 9 (Error bars: SEM; n=3).* ***b.*** *Histogram represents the GFP^+ cells percentage at different time points during the culture (Error bars: SEM; n=3; * P≤0.05).*

In SET2 and UKE1 cells, SOX6 transduction induces an arrest in cells growth (Fig.2.21; Fig.2.22). However, growth curves kinetics are different between the two cell lines, with SOX6-UKE1 culture being extinguished earlier than that of SOX6-SET2. Furthermore, the analysis of the rate of early–apoptotic and apoptotic cells, by using AnnV/7AAD staining as well as PI staining, revelas a difference (Fig. 2.23; Fig. 2.25), correlated with the JAK2V617F allele copy number. In fact, whereas HEL cells (8 copies of the JAK2V617F allele) do not show an increase in early–apoptotic and apoptotic sub populations, SET2 (6 copies of JAK2V617F allele and one wild type allele) and UKE1 (2 copies of JAK2V617F allele) do it, although at different extent. This difference is also reflected in the growth rate, where the decreased expansion is dependent on the JAK2V617F copy number: the more alleles are present in a given cell line, the higher is the capability to expand even upon SOX6 overexpression.

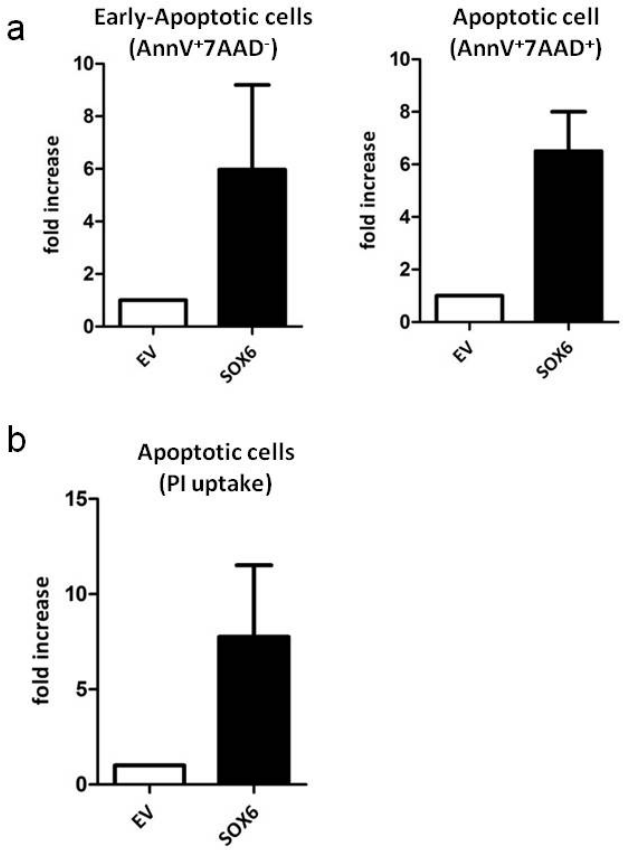

Fig. 2.23: SOX6-SET2 cells present an increased percetage of pre-apoptotic and apoptotic cells compared to the control EV-SET2 cells. ***a.*** *Histogram shows fold increase of early apoptotic (AnnV+7AAD-) and apoptotic (AnnV+7AAD+) populations in SOX6-SET2 cells compared to the control, as assessed by Flow Cytometry analysis (Error bars: SEM; n=2).* ***b.*** *Histogram represents the Propidium Iodide uptake (PI) as per fold increase of SOX6-SET2 cells, compared to the control. (Error bars: SEM; n=2).*

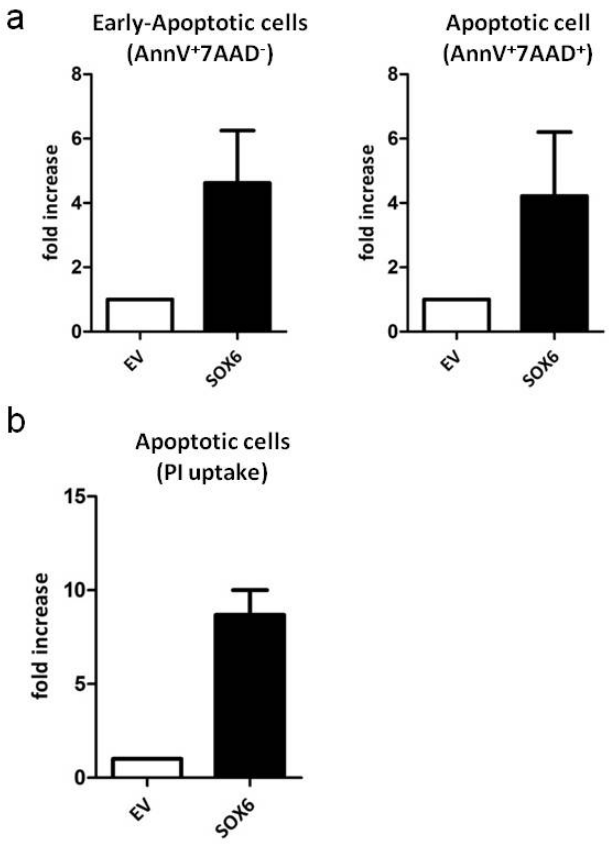

Fig. 2.24: SOX6-UKE1cells show an increasd percentage of pre-apoptotic and apoptotic cells ***a.*** *Histogram shows fold increase of early apoptotic (AnnV+7AAD-) and apoptotic (AnnV+7AAD+) populations in SOX6-UKE1 cells, as compared to the control, (Error bars: SEM; n=2).* ***b.*** *Histogram represents the Propidium Iodide uptake (PI) as per fold increase, in SOX6-UKE1 cells,as compared to the control. (Error bars: SEM; n=2).*

Finally, cell cycle analysis indicated that in SOX6-SET2 cells the percentage of cells in S phase is decreased. The same trend was not detected inUKE1 (Fig. 2.25 a-b). We propose that this difference (85% in HEL cells, 55% in SET2 cells and 8% in UKE1, respectively) depends on the number of the JAK2V617F mutated alleles.

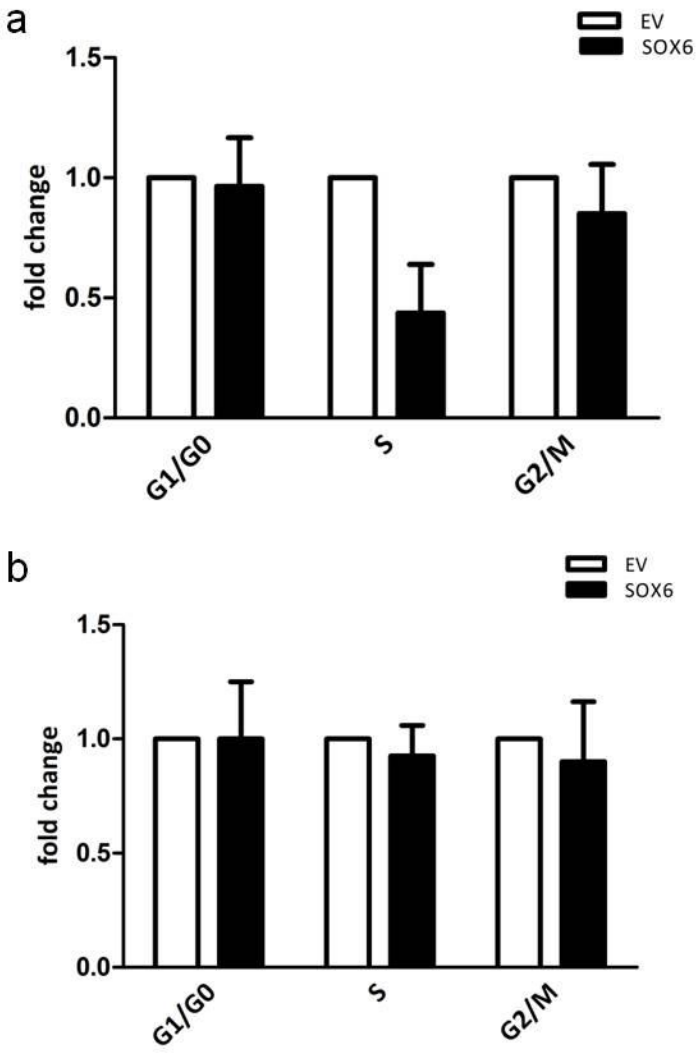

Fig. 2.25: Cell cycle analysis of SOX6-SET2 and SOX6-UKE1cells, as compared to their control. *FC analysis was performed 3 days after infection with the EV-GFP and SOX6-GFP viral particles, respectively.* ***a.*** *Histogram bars show the fold change variation in SOX6-SET2 cell cycle phases compared to the respective control (error bars: SEM; n=2).* ***b.*** *Histogram bars show the fold change variation in SOX6-UKE1 cell cycle phases compared to the respective control (error bars: SEM; n=2).*

Taken together, these data further strengthen the hypothesis that SOX6 promotes erythroid differentiation, independently from its effector SOCS3. Instead, SOX6 triggers cell death through SOCS3 in a dose-dependent manner. Indeed, the presence of high copy number of JAK2V617F mutation, conferring resistance to SOCS3, makes HEL cells insensitive to apoptosis induced by SOX6. Accordingly, when the copy number of JAK2V617F mutation is reduced (SET2 and UKE1 cells) there is a proportional increase in apoptosis triggered by SOX6.

It is interesting to note that SOX6 overexpression leads to a change in the cell cycle profile of JAK2V617F^{+} cells. The decrease of S phase population is again proportional to the number of JAK2V617F alleles, possibly linked to an increased erythroid terminal differentiation.

Conclusions

In all the cell lines tested so far, SOX6 overexpression tends to force the erythroid program, simultaneously repressing alternative lineage-specific differentiation programs.

Thus, cell lines with an erythroid and megakaryocytic potential (i.e. K562, and HEL) are pushed towards an erythroid fate. Cell lines with an almost exclusive megakaryocytic potential (i.e. MEG.01, UKE1, SET2) show a decrease of all the megakaryocytic lineage-affiliated genes and an increase of the erythroid ones. Interestingly, SUPB15 cells (a lymphoid cell line) show a decrease in all the lymphoid lineage-affiliated genes studied, but can not switch to a fully erythroid program. Therefore, it may be possible that SOX6 is sufficient to repress differentiating program alternative to the erythroid one, and it is essential to activate erythroid lineage-affiliated genes in cells that are hierarchically already compatible with an erythroid specification.

Of note, by taking advantage of cell lines carrying a different number of JAK2V617F^{+} alleles, it was possible to study in detail the SOX6 downstream effects on cell cycle withdrawal, enlightening the relevance of SOCS3 as SOX6 target.

In fact, the different JAK2V617F allele copy number seems to dictate a graded resistance to SOCS3 (Fig. 2.26).

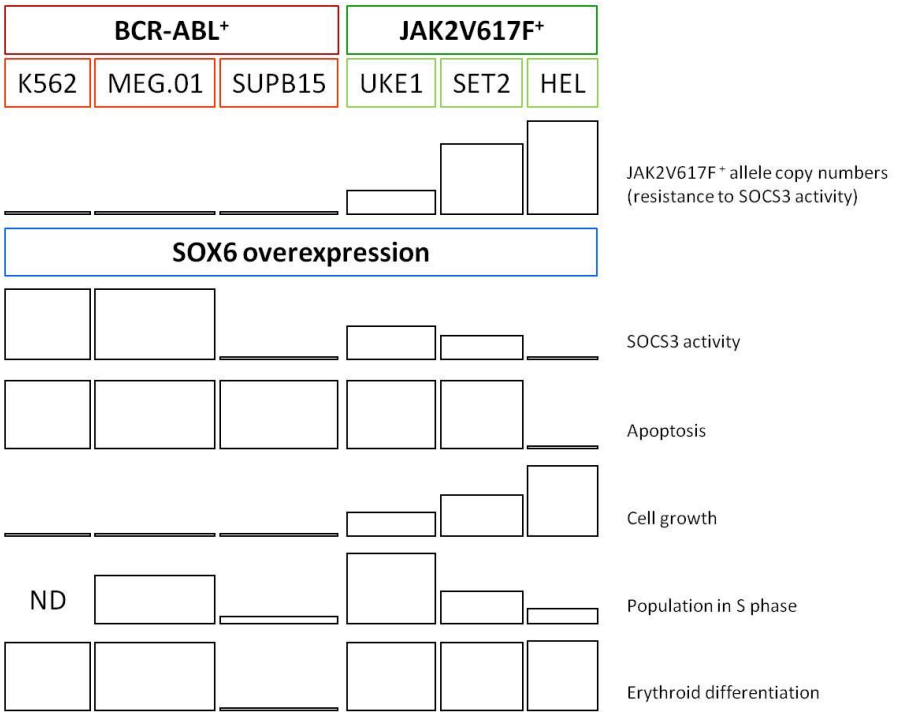

Fig. 2.26: Summary of the the phenotypic changes induced by SOX6 overespression in the cell lines studied in Chapter 2 .

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CHAPTER 3:

SOX6 ECTOPIC EXPRESSION IN BCR-ABL INDUCED ACUTE LYMPHOBLASTIC LEUKEMIC CELLS: IN VITRO AND IN VIVO STUDIES

3.1 INTRODUCTION

Precursor B-cell acute lymphoblastic leukemia (B-ALL) is an aggressive hematopoietic neoplasm characterized by recurrent chromosomal abnormalities. One subtype of B ALL is characterized by the presence of the Philadelphia chromosome (Ph), formed by the t(9,22) (q34;q11.2) translocation, which creates the fusion protein BCR–ABL, a constitutively active tyrosine kinase. BCR-ABL$^+$ B-ALL is the most common molecular subtype of ALL (Acute Lymphoblastic Leukemia) in adults, comprising ~20% of cases (Moorman, 2007). Despite the addition of tyrosine kinase inhibitors in the treatment regimen for BCR-ABL$^+$ B-ALL, prognosis for adults with this disease remains poor, highlighting the need of improved treatments (Fielding, 2014). Recent studies using single nucleotide polymorphism (SNP) arrays have shown that BCR-ABL$^+$ B-ALL is characterized by frequent deletions of key lymphoid transcription factors. For example, IKZF1, which encodes the transcription factor Ikaros, is deleted in >80% of BCR-ABL$^+$ B-ALL cases, and PAX5, a key transcription factor in B-cell development, is deleted in >50% of BCR–ABL$^+$ B-ALL cases (Mullighan, 2008; Mullighan, 2007). Murine models have shown that both loss of PAX5 and partial loss of Ikaros lead to B-cell maturation arrest at the pro–B-cell stage (Nutt, 1999; Kirsetter, 2002). The high frequency of loss of these genes in BCR-ABL$^+$ B-ALL suggests that B-cell maturation arrest is critical to the pathogenesis of this disease. Interestingly, although unable to form mature B cells, both PAX5-deficient and Ikaros-deficient pro-B cells retain the capacity to lineage reprogramming (sometimes referred to as “transdifferentiate”) into cells of the myeloid lineage, specifically macrophages, upon exposure to macrophage-colony stimulating factor (M-CSF) (Nutt, 1999; Reynaud, 2008).

Animal models of BCR-ABL$^+$ leukemias have provided important new knowledge about the molecular patho-physiology of this disease, and answered questions that are difficult or impossible to address using BCR-ABL$^+$ cell lines or primary Ph$^+$ leukemic samples from patients.

Indeed, the murine model is capable of precisely recapitulating BCR-ABL$^+$ leukemias characteristic (Van Etten, 2002).

To achieve that, two commonly approaches are used: the generation of mutant mice by traditional transgenic or knock-out/knock-in methods and, the retroviral/lentiviral bone marrow transduction and transplantation (BMT). BMT consists in the introduction of leukemic oncogenes directly into murine bone marrow cells that are then injected into syngeneic mice (Kennedy, 2008). This technique was first applied to generate mouse models of leukemias caused by the BCR-ABL fusion protein (Daley, 1990). This allowed the production of accurate and quantitative models of human CML and B-ALL.

However species-specific differences do exist in the mechanism of malignant transformation, therefore caution should be exercised when extrapolating results from mouse model to the human situation (Rivera, 2008). Taking this caveat into account, 'humanized' mouse models, in which various types of human cells and tissues are engrafted, are considered extremely useful in basic and applied human disease research (Ito, 2012). The discoveries of nude and severe combined immunodeficiency (SCID) mice were a key advance in the development of immunodeficient mice for xenotransplantation (Issacson, 1962; Bosma, 1983). In particular, the last 10 years, remarkable progress has been achieved in the generation of humanized mouse models by using NOD/SCID/γc^{null} and Rag1/2^{null} γc^{null} mice. These models have been of great relevance for the study of molecular mechanism underlying human hematological diseases (Ito, 2012). Thus ,

retrovral-/lentiviral-mediated transduction of primary human hematopoietic cells followed by their transplantation *in vivo* has emerged as a feasible approach to study the process of human leukemogenesis.

In humans, there is evidence that three different BCR-ABL fusion proteins (P190, P210, P230) are associated with distinct forms of leukemia.
P210 BCR-ABL is found in hematopoietic cells from patients with chronic myeloid leukemia in stable phase and in acute lymphoid and myeloid leukemias (Chen, 1988; Schaefer-Rego, 1988), although some patients with CML diagnosed in blast crisis have been described. P190, in contrast, is commonly found in Ph-positive acute B lymphoid leukemia (Chan, 1987), occasionally in acute myeloid leukemia, and very rarely in CML (Kurzrock, 1987). P230 is associated with neutrophilic CML and CML (Emilia, 1997, Briz, 1997; Mittre, 1997)
In the murine model, all three forms of BCR-ABL are equally capable of inducing chronic myeloid leukemia (CML), but only P190 induces B lymphoid leukemia (Shaoguang, 1999). For this reason, the BMT model system is very useful for studying the molecular pathophysiology of Ph-positive leukemias, such as for testing the functional role of the different BCR-ABL domains, for investigating the physiological effects of BCR-ABL expression in primary hematopoietic cells and for determining signaling pathways relevant to leukemogenesis.

In the experiment presented in chapter two, we observed that the ectopic expression of SOX6 in different model systems of leukemia leads to a blocks in cell proliferation in BCR-ABL$^+$ cell lines, including the lymphoblastic cell line SUPB15.

In SUPB15, SOX6 down-regulates genes essential for B-cells identity, such as PAX5 and its downstream effectors, although it is not sufficient to activate the erythroid differentiation program.

Given these observations, I took advantage of the P190 BCR-ABL GFP^+ induced B Acute Lymphoblasc Leukemic cells (B-ALL) to explore the impact of SOX6 ectopic expression in this type of leukemias.

3.2 EXPERIMENTAL PROCEDURES

Constructs

The SOX6 murine cDNA was kindly provided by Prof. Michiko Hamada-Kanazawa, Kobe-Gakuin University, Japan. The SOX6 cDNA was cloned in frame with a 3' FLAG epitope to generate a SOX6-FLAG cassette, flanked by two BAMHI sites. This SOX6-FLAG cassette was then cloned immediately upstream to the IRES ΔNGFR cassette (blunted BamHI site) of the pHR SIN BX IR/EMW (derived from pHR SIN CSGW), (Demaison, 2002) lentiviral vector, a kind gift from Prof. Tariq Enver, UCL, London.

The two packaging plasmids psPAX2 and pMD-VSVG were used to produce Lentiviral pseudo-particles in 293T cells (www.lentiweb.com).

Cell lines

B-ALL BCR-ABL GFP$^+$: cell line (a gift from Prof. Ghysdael, Paris Diderot University) established from Cdkn2a$^{-/-}$ (knock out for Cyclin-Dependent Kinase Inhibitor 2a) murine bone marrow cells infected with a retrovirus (pMIG) expressing a BCR-ABL P190-GFP cassette.

HEK 293T: The 293T cell line derived from human embryonic kidney and contains the SV40 T-antigen. It was used for lentiviral production. This cells were grown in adhesion, in DMEM medium (EuroClone) supplemented with 10% of FBS (Fetal Bovine Serum) (Sigma), penicillin/streptomycin (100μg/ml), 4mM L-glutamine (EuroClone) in a humidified 5% CO_2 atmosphere at 37°C.

Lentiviral production and transduction

Exponentially growing HEK-293T cells were transfected with jetPEI™ (Polyplus-Transfection) with the above vectors plus the packaging plasmids psPAX2 and pMD-VSVG to produce the lentiviral pseudo-particles. 72h after transfection, the supernatant with recombinant viruses was collected, filtered (0.45μm) and centrifuged at 20,000g for 8 hours at 4°C. The viral pellet was re-suspended in 1xPBS and aliquoted at -80°C. Lentiviruses were titrated on HEK-293T cells by measuring the percentage of $eGFP^+$ cells by Flow Cytometry.

The transduction of B-ALL BCR-ABL GFP^+ cells was performed overnight with a multiplicity of infection (MOI) of 30.

RNA isolation and Real Time PCR

Total RNA from $>10^6$ of B ALL cells, bone marrow and spleen of all the different experimental conditions were purified with TRIzol Reagent (Applied Biosystem), treated with RQ1 DNase (Promega) for 30 min at 37°C and retrotranscribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem). Negative control reactions (without Reverse Transcriptase) gave no PCR amplification. Real time analysis was performed using ABI Prism 7500, (Applied Biosystems). Primers were designed to amplify 100 to 200bp amplicons, spanning an exon-exon junction when possible, on the basis of sequences from the Ensembl database (http://www.ensembl.org). Samples from each experiment were analyzed in triplicate. Specific PCR product accumulation was monitored by SYBR Green dye fluorescence in 12-μl reaction volume. Dissociation curves confirmed the homogeneity of PCR products.

All primers used are listed in the table below:

PRIMERS	SEQUENCE
B220 FW	GTCCAAACTTCTGGCCTTTG
B220 RW	GATTCAGTGGTGCGAGCGA
GATA1 FW	CTACCTTGTCAATGCCTGTG
GATA1 RW	GAGCTTGAAATAGAGGCCGC
GAPDH Fw	TGTGTCCGTCGTGGATCTGA
GAPDH Rw	CCTGCTTCACCACCTTCTTGA
GpA Fw	TCAACTTATCACACGGCCCC
GpA Rw	ATAATCCCTGCCATCACGCC
PAX5 Fw	ACTCCATCAGTGGCATCCTG
PAX5 Rw	TGCTGCTGTGTGAACAGGTC

Whole-cell protein extracts

Cells were harvested and centrifuged at 640g for 5 min at 4ºC. The pellet washed three times in ice-cold 1X PBS (phosphate buffered saline, pH 7.4) and gently resuspended in RIPA buffers (20mM TrisHCl ph 7.4; 150mM NaCl; 5mM EDTA; 0,3%Triton) supplemtented with protease inhibitors (complete EDTA-free, Roche). Cells were lysed 30 minutes on ice. After centrifugation at 13000 rpm 5 minutes at 4ºC, the supernatant was retrieved.

Immunoblotting analysis

To confirm the presence of SOX6 overexpression, whole protein extracts (30-µg/lane) were resolved by SDS/PAGE in a 10% gel and blotted onto Hybond ECL Nitrocellulose membrane (GE healthcare life science-Amersham) by "wet blotting." carried out under constant voltage at 100V for 90-120 min at 4ºC (Transblot apparatus, Biorad). Membranes were blocked for 1h at room temperature with Milk 5% in TBS-T 1X (Tris Buffered Saline, pH 7.6 and 0,1% Tween 20 by Sigma) and incubated with the appropriate primary antibody diluted in Milk 5% TBS-T overnight at 4ºC. Membranes were washed for three times with TBS-T and incubated with

the secondary antibody (diluted in Milk 5%). In particular, the Streptavidin-HRP (Cell signaling) antibody was incubated for 1h at room temperature (no secondary antibody was required). Antibodies binding was detected by using appropriate horseradish peroxidase-conjugated IgG and revealed by ECL (Millipore).

Flow Cytometry

Transduced B-ALL BCR-ABL GFP^+ cells were washed, fixed in 1% Paraformaldehyde and stained with PE-antiNGFR conjugated antibody (Biolegend) for 15 min at 4ºC. The cells were analyzed by flow cytometer (Becton-Dickinson FACS Calibur). and data were analyzed with Summit Software v4.3.

Apoptosis Assay

Cells were washed twice with cold PBS 1X (phosphate buffered saline, pH 7.4) and then resuspend in 1X Binding Buffer (0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) with 5 µl of 7-AAD (BD Biosciences)in a final volume of 100µL. The cells were gently vortexed and incubated for 15 min at RT (25°C) in the dark. After that the samples were diluited with 400 µl of 1X Binding Buffer and analyzed by flow cytometry within 1 hr. (Becton-Dickinson FACS Calibur). Data were analyzed with Summit Software v4.3.

Statistics

Statistical analyses of obtained data were performed with GraphPad Prism (version 6.0; GraphPad Software, Inc.). The data are expressed as mean ± standard error of n=3 or more determinations. Statistics was performed usin a paired or upaired, two-tailed Student t-test.

3.3 RESULTS AND DISCUSSION

3.3.1 SOX6 OVEREXPRESSION IN BCR-ABL INDUCED B CELL ACUTE LYMPHOBLASTIC LEUKEMIC CELLS (B-ALL)

To test whether SOX6 might interfere with the maintenance of leukemia in an *ex vivo* model system, I took advantage of the transformed murine B-ALL BCR-ABL GFP^{+} cell line. This cell line (a gift from Prof. Ghysdael, Paris Diderot University) has been established from Cdkn2a$^{-/-}$ (knock out for Cyclin-Dependent Kinase Inhibitor 2a) murine bone marrow cells infected with a retrovirus expressing a BCR-ABL P190-GFP cassette. These cells retain proliferation capability both in the presence/absence of stroma and, when injected in mice, induce leukemia within 8-18 days, with infiltrations in lymphonodes, liver and bone marrow.

In the construct used to generate the above cells, the BCR-ABL oncogene is placed upstream to an IRES-GFP cassette, thus the resulting cells express GFP. For this reason, to monitor the efficiency of SOX6 overexpression in these cells, I generated a different vector carrying a bicistronic SOX6-ΔNGFR cassette (Fig. 3.1).

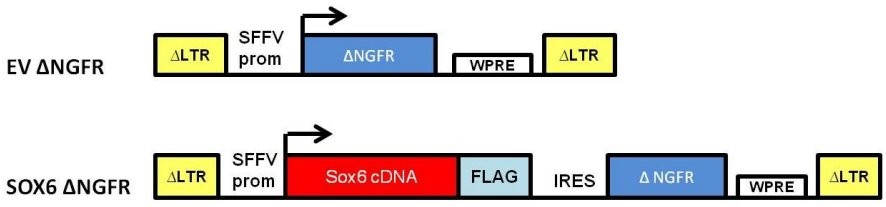

***Fig. 3.1**: **Schematic representation of the EV-ΔNGFR and SOX6-ΔNGFR lentiviral expression vectors.** LTR: Long Terminal Repeats; SFFV: Spleen Focus Forming Virus; ΔNGFR: deleted Nerve Growth Factor Receptor; IRES: internal ribosome entry site; WPRE: Woodchuck Hepatitis virus Posttranscriptional Regulatory Element.*

B ALL BCR-ABL GFP$^+$ cells were infected with the SOX6 lentiviral particles (SOX6-BALL) or with the corresponding empty vector (EV-BALL) as a control. The percentage of ΔNGFR positivity, that reflects the efficiency of transduction, was assessed by FC analysis using an anti-ΔNGFR conjugated with phycoerythrin (PE).

B-ALL cells were transduced at comparable levels with both vectors in all the experiments (Fig. 3.2 a-b). Representative FC plots are shown in Fig. 3.2. The expression of the exogenous SOX6 was detected both at RNA and protein level (exogenous protein being detected by using an anti-FLAG antibody) (Fig. 3.2 c).

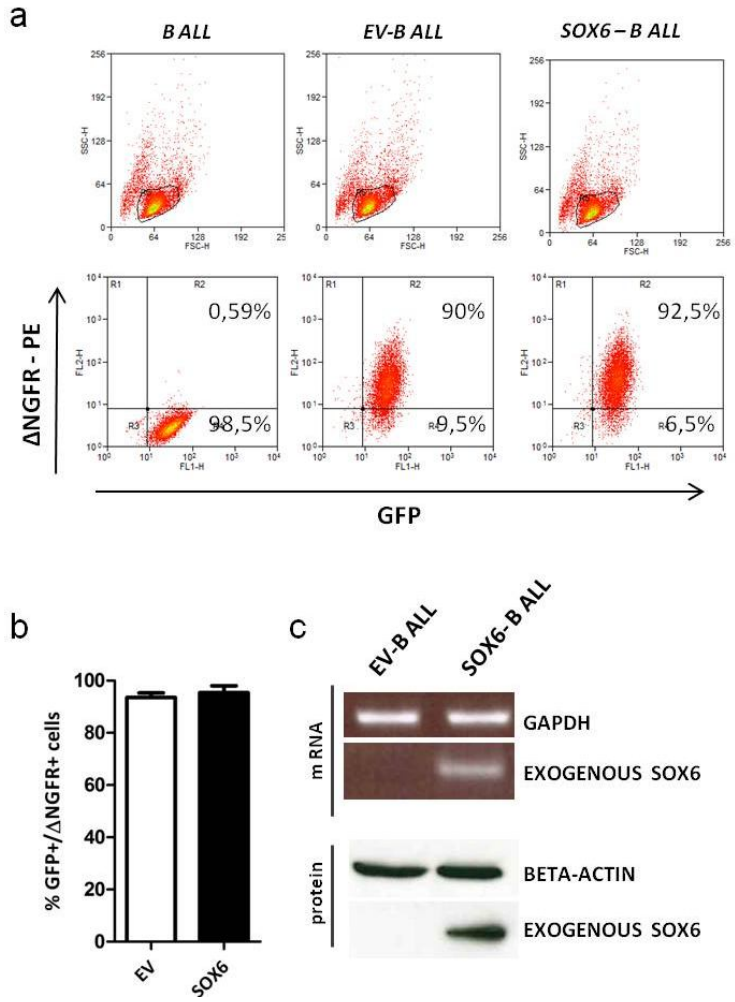

Fig. 3.2: Transduction experiments in B-ALL cells*. a.** *The efficiency of transduction was assayed by Flow Cytometry analysis 3 days after infection with EV-ΔNGFR and SOX6-ΔNGFR viral particles. Representative FCs are shown: untransduced B-ALL; EV-BALL and SOX6-BALL. Percentages of GFP$^+$, and double positive GFP$^+$/ΔNGFR$^+$ cells are represented within the plots.* ***b. *The histogram shows the percentage of GFP$^+$/ΔNGFR$^+$ cells (error bars: SEM; n≥3).* ***c.*** *The expression level of exogenous SOX6 was assessed at mRNA and protein level 3 days after infection. Upper panel: semiquantitative RT-PCR performed by using primers detecting the exogenous, vector-derived, SOX6 transcript. hGAPDH primers were used as internal control. Lower panel: Western blot performed by using an anti-FLAG antibody that detects the exogenous SOX6 protein. Anti-Beta-Actin antibody was used as loading control.*

The effects of the ectopic expression of SOX6 were evaluated by assessing cell growth, cells viability and the transcriptional profile of selected molecular markers.

As shown in the growth curve (Fig. 3.3 a-b), SOX6 overexpression induces a decrease in cell proliferation between day 3 and day 9 of culture. This effect is accompanied by a reduced cell viability in SOX6-BALL. Indeed, in cells overexpressing SOX6 there is an increase (1,6 fold) of $7AAD^+$ cells, when compared to the control, EV-BALL (Fig. 3.4).

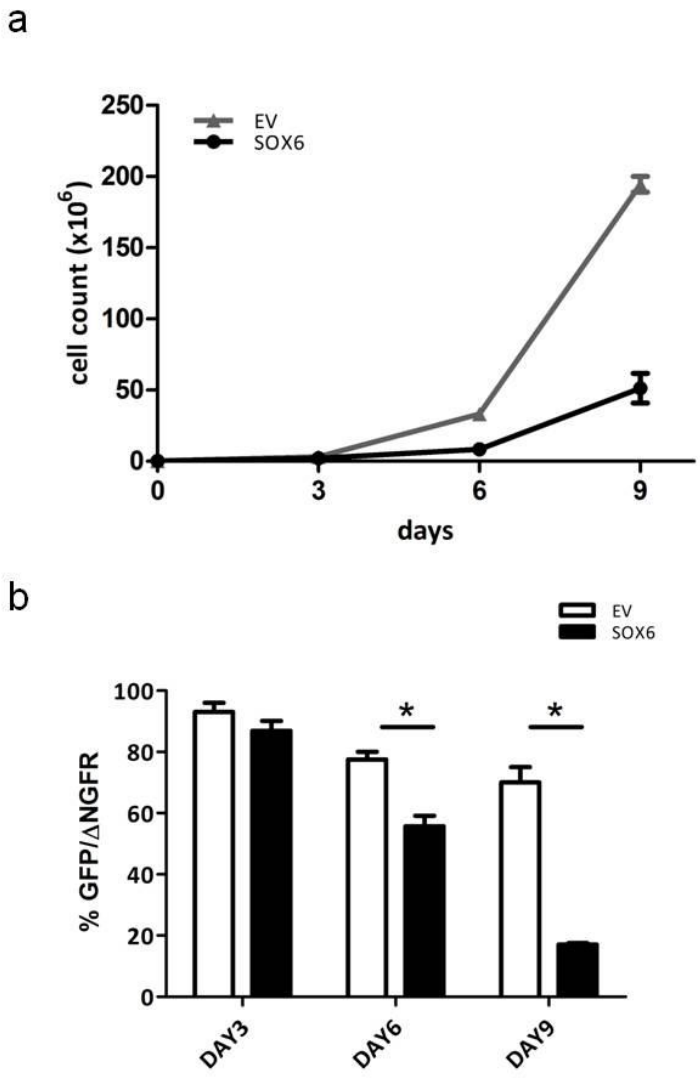

Fig. 3.3: SOX6-BALL cells show a decreased cell proliferation rate. a. *$1x10^6$ exponentially growing B-ALL cells were transduced at day 0 with viral particles carrying either EV-ΔNGFR or SOX6-ΔNGFR lentiviral vectors.(Error bars: SEM; n=3).* ***b.*** *Histogram represents the $GFP^+/\Delta NGFR^+$ percentage of double positive cells at different time points during the culture (Error bars: SEM; n=3).*

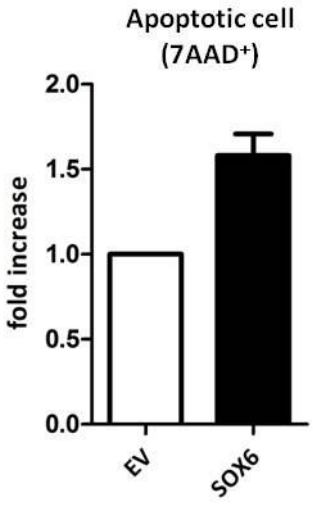

Fig. 3.4: Increased apoptosis in SOX6-BALL vs control EV-BALL cells. *Histogram represents the fold increase in the 7AAD$^+$ population in SOX6-BALL compared to the control, as assessed by FC analysis (Error bars: SEM; n=2).*

The ectopic expression of SOX6 in B ALL decreases the expression of the B cell key regulator PAX5 and of the surface marker of B cells B220. The master erythroid regulator was increased as Glycophorin A, but only at a very low level (Fig. 3.5).

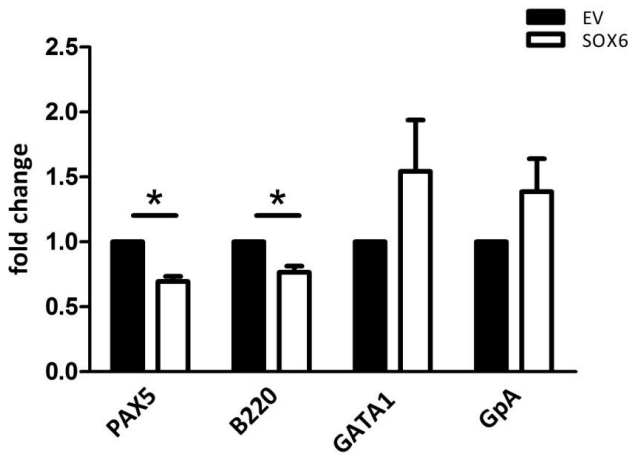

Fig. 3.5: RT-qPCR analysis on SOX6-BALL and EV-BALL. *RTqPCR shows decreased expression of PAX5 and B220 72h after transduction. Histograms show the fold change compared to the control control EV-BALL. hGAPDH expression was used to normalize data (error bars: SEM; n≥3 * P≤0.05).*

3.1.2 EFFECTS OF THE INJECTION OF TRANSFORMED B-ALL GFP$^+$ CELLS OVEREXPRESSING SOX6 IN C57BL/6J MICE

In vitro, the overexpression of SOX6 in B-ALL BCR-ABL GFP$^+$ cells affects cell proliferation rate (Fig. 3.3) and induces cell death (Fig. 3.4). To test whether this is true also *in vivo*, I took advantage of B-ALL BCR-ABL GFP$^+$ cells. These cells, when injected in mouse, are capable of generating leukemia within 8-18 days (Heisterkamp, 1990).

I transduced B-ALL BCR-ABL GFP$^+$ cells with SOX6-ΔNGFR overexpressing vector (SOX6-BALL) and the corresponding empty vector EV-ΔNGFR (EV-BALL). 24 hours later, I injected intravenously the transduced cells in C57BL/6J recipient mice (Fig. 3.6).

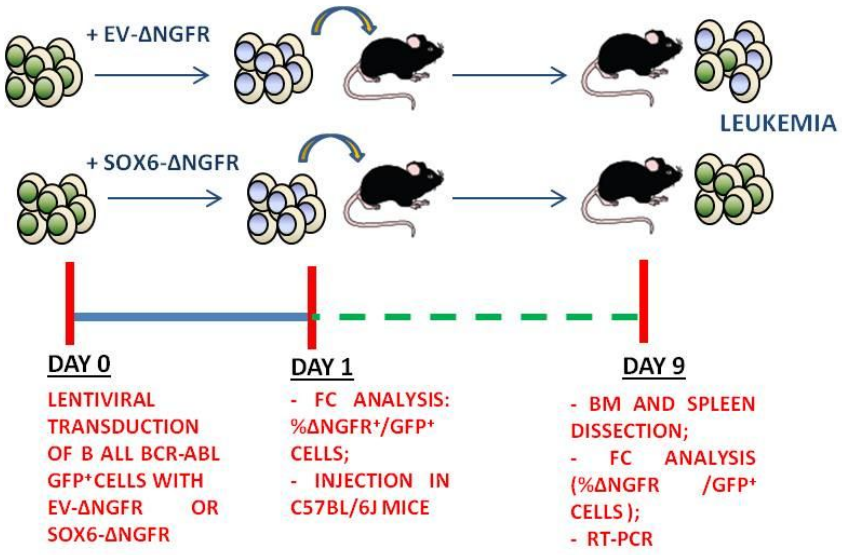

Fig. 3.6: Schematic representation of the experimental procedure.

To assess the efficiency of transduction of the B-ALL GFP$^+$ cells, FC analysis was performed prior to the injection in recipient mice.

Double positive GFP$^+$/ΔNGFR PE$^+$ cells were 94,5% (EV-BALL) and 98% (SOX6-BALL), respectively (Fig. 3.7 a-b; Fig. 3.9 a). It is important to note that in the experiment, there was a small fraction of untransduced B-ALL GFP$^+$ cells (EV-BALL 5% vs SOX6-BALL 1%) , which retain their tumorigenic capability (Fig. 3.9 b). Therefore, I could analyze the effect of SOX6 ectopic expression in B-ALL GFP$^+$ cells by comparing SOX6-BALL and EV-BALL injected recipients, and by comparing the evolution of SOX6-B ALL and B-ALL GFP$^+$ cell populations within the same recipient mouse.

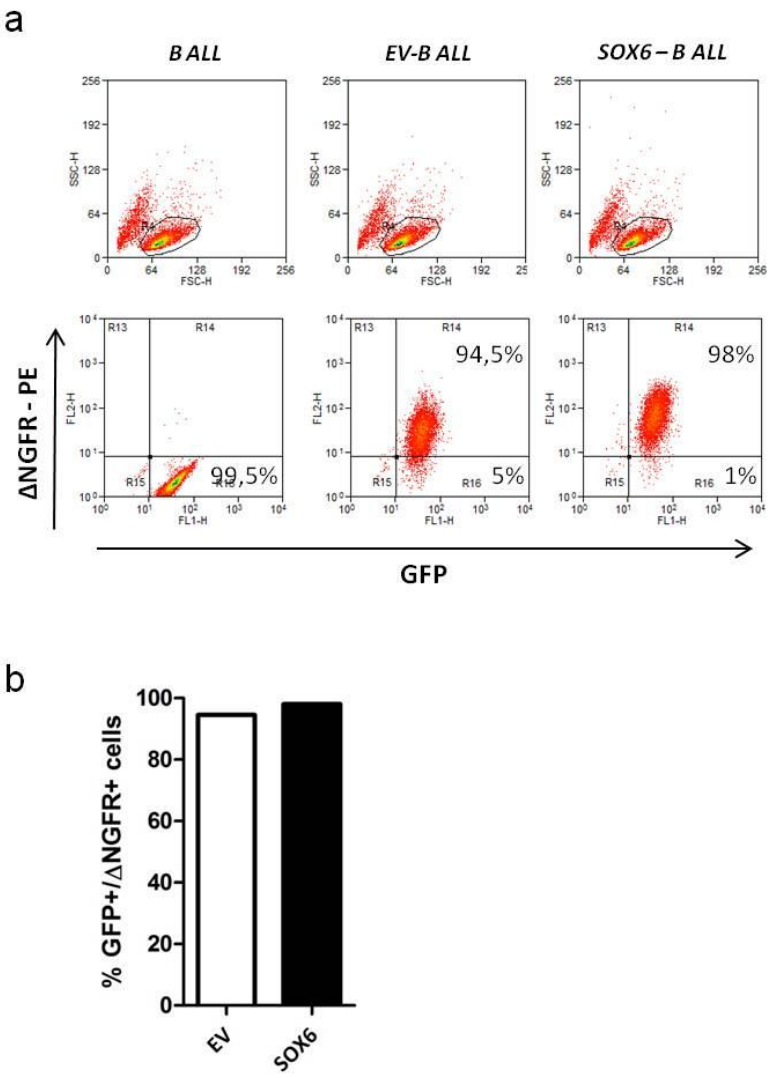

Fig. 3.7: Transduction efficiency of EV-BALL and SOX6-BALL 24h after infection. a. *The efficiency of transduction was assayed by FC analysis 24 hours after infection with EV-ΔNGFR and SOX6-ΔNGFR viral particles. Representative FC are shown: untransduced B-ALL; EV-BALL and SOX6-BALL. Percentages of GFP$^+$ and double positive GFP$^+$/ΔNGFR$^+$ cells are shown within the plots.* ***b.*** *The histogram shows the percentages of GFP$^+$/ΔNGFR$^+$ cells.*

After controlling the transduction efficiency, I injected three recipient mice with 1x10^6 SOX6-BALL cells each, and 3 recipient mice with 1x10^6 EV-BALL cells each, respectively.

I then scored daily mice for their behavior and health conditions. 9 days after the injection one of the 3 mice injected with EV-BALL was in sufferance (breathing and movements impairment), the other two showed milder symptoms, whereas SOX6-BALL injected mice did not show any sufferance. Day 9 was considered as the end point of the experiment, as defined by the protocol, in agreement with our experimental protocol approved by the Italian Ministry of Health.

Compared with a non-injected mouse, the spleens of all the transplanted mice were enlarged, anemic and in some cases necrotic (Fig. 3.8 a). They shared the characteristic of a typical leukemic murine spleen. The weight and size of spleens from mice injected with SOX6-BALL were comparable to the controls injected with the Empty Vector (Fig. 3.8 b-c), in line with the expected development of leukemia due to the B-ALL GFP^+ injection.

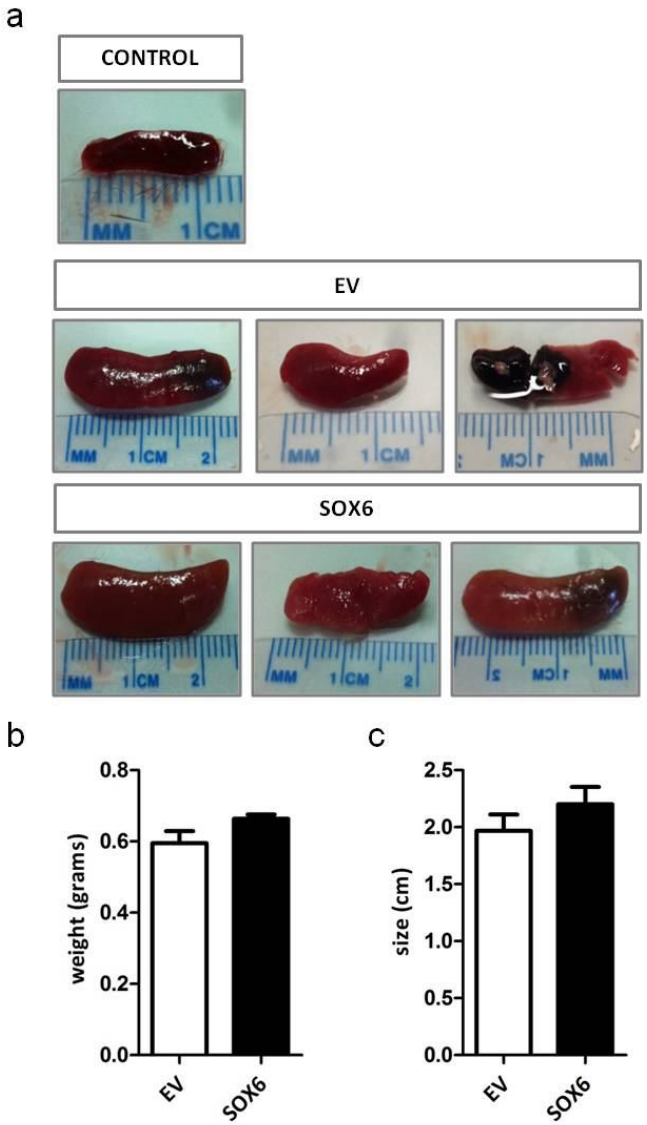

Fig. 3.8: Leukemia development in recipient mice receiving EV-BALL or SOX6-BALL transduced cells. a. *Spleens of mice transplanted with B ALL BCR-ABL GFP$^+$ cells transduced with EV-ΔNGFR (EV) and SOX6-ΔNGFR (SOX6) 9 days after injection.* ***b.*** *Histogram shows the weight in grams (error bars:SEM; n=3 for each condition).* ***c.*** *Histogram shows the spleen size in cm (error bars: SEM; n=3 for each condition).*

However, whereas $GFP^+/\Delta NGFR^+$ cells where present in spleens and bone marrows of EV-BALL-injected mice, they were absent in SOX6-BALL-injected ones (Fig. 3.10; Fig. 3.11).

This result indicates that SOX6-BALL cells are unable to engraft in recipient mice, thus confirming their growth/survival impairment, as expected on the basis of the previously described *in vitro* studies.

It is important to note that $GFP^+/\Delta NGFR^+$ cells decreased also in EV-BALL-injected mice, but this decrease is smaller compared to the one observed for SOX6-BALL-injected mice (10 fold in EV vs 1000 fold in SOX6 both in bone marrow that in spleen) (Fig. 3.9 a). This particular phenomenon may be due to a reduced engrafting capability of transduced cells, that could make them more susceptible to cellular death.

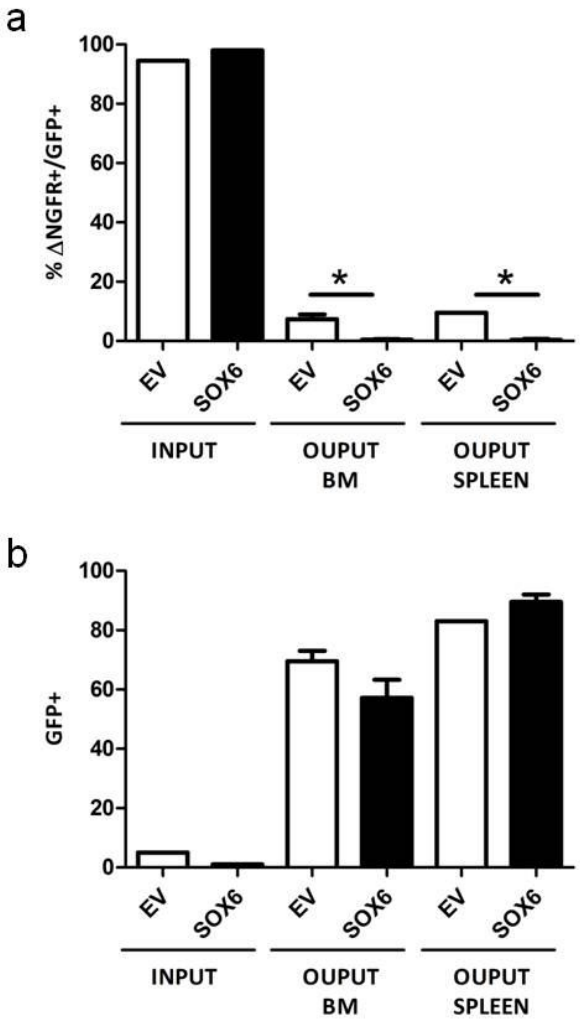

Fig. 2.9: SOX6-BALL cells are unable to engraft in recipient mice. a. *Histogram shows double positive GFP$^+$/ΔNGFR$^+$ cells. INPUT EV-BALL and INPUT SOX6-BALL; OUTPUT BM-EV and BM-SOX6; OUTPUT SPLEEN-EV AND SPLEEN-SOX6.* ***b.*** *Histogram shows double positive GFP$^+$/ΔNGFR$^+$ cells and GFP$^+$ cells: INPUT EV-BALL and INPUT SOX6-B ALL; OUTPUT BM-EV and BM-SOX6; OUTPUT SPLEEN-EV and SPLEEN-SOX6. (error bars:SEM; n≥3 * P≤0.05).*

Finally, GFP^+ cells are present in both EV-BALL and SOX6-BALL-injected mice as shown in the representative FC plot in Fig. 3.10 and Fig. 3.11. These cells likely represent the residual un-transduced B-ALL BCR-ABL GFP^+ (Fig. 2.9 b). In fact, as demonstrated by RTqPCR analysis, they do not express exogenous SOX6 RNA (data not shown). Because these cells have a very high proliferation rate, they could efficiently engraft and give rise to leukemia in all recipient mice.

Taken together, these results show that SOX6-BALL cells are not capable of engrafting in the murine model and that the leukemic phenotype is due to the residual un-transduced B-ALL BCR-ABL GFP^+, which were sufficient to give rise to leukemia in all C57BL/6J recipient mice.

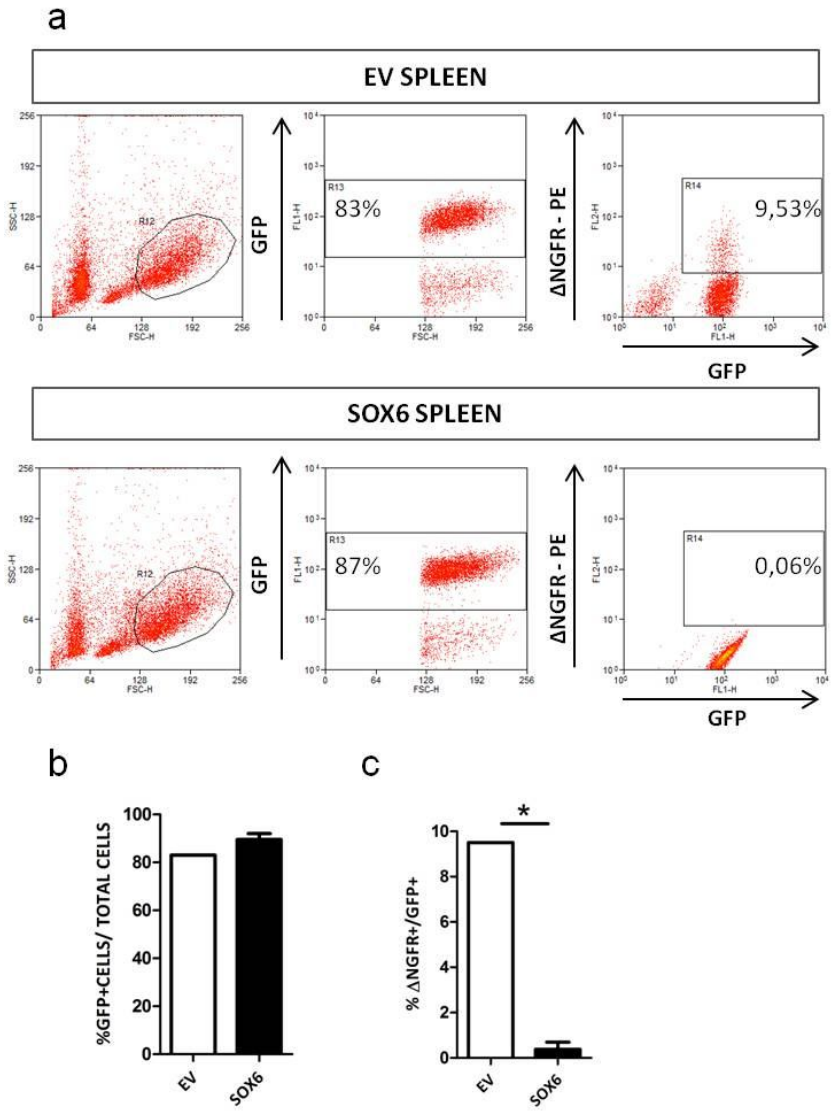

***Fig. 2.10: FC analysis of spleen cells derived from EV and SOX6 injected mice**. **a.** Representative FC plots obtained by staining cells with an anti-ΔNGFR PE antibody to assess the presence of single positive GFP$^+$ cells and double positive ΔNGFR PE$^+$/GFP$^+$ B ALL from EV spleen and SOX6 spleen. GFP$^+$: EV 83% vs SOX6 87 %; GFP+/ΔNGFR+: EV 9,53 % vs SOX6 0,06% **b.** Histogram represents the % of GFP$^+$ cells on total spleen cells**. c.** Histogram represents the % of ΔNGFR$^+$/GFP$^+$ cells on total spleen cells. (Error bars: SEM; n=3, * P≤0.05).*

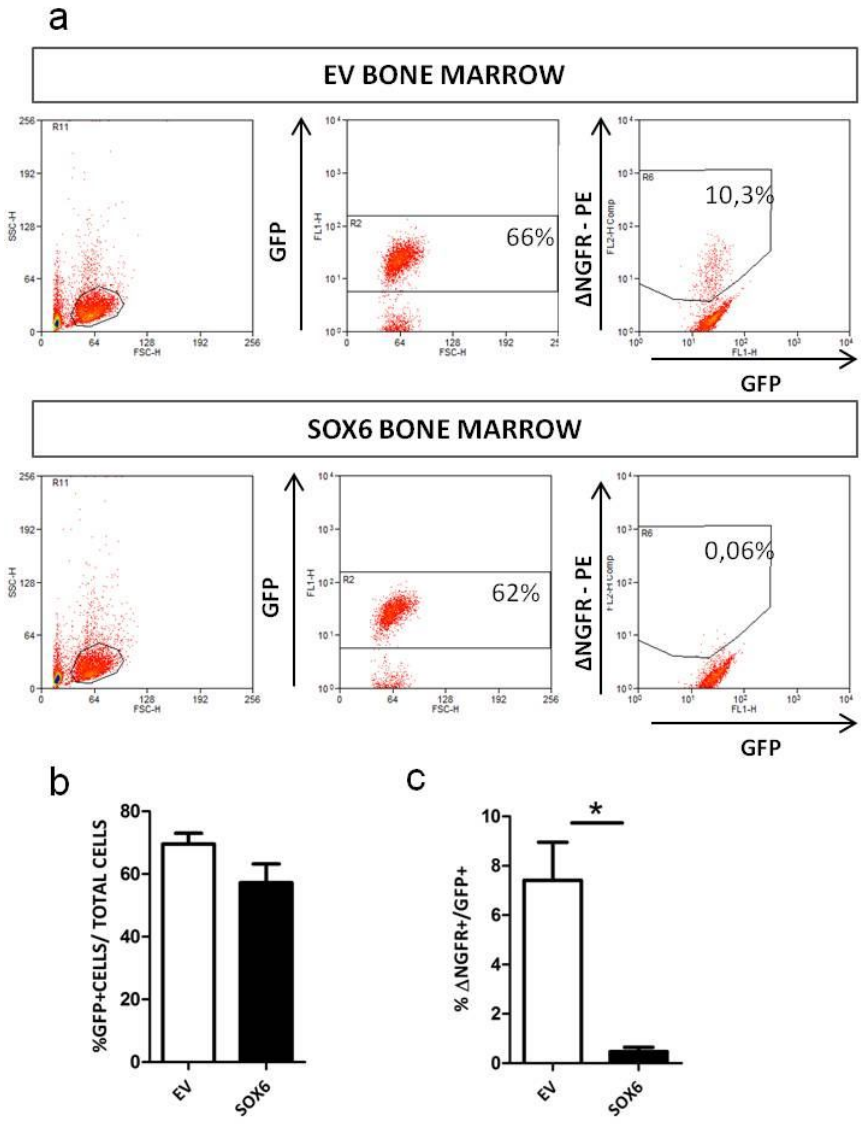

Fig. 2.11: FC analysis of bone marrow cells derived from EV and SOX6 injected mice. a. *Representative FC plots obtained by staining cells with anti-ΔNGFR PE antibody to assess the presence of single positive GFP$^+$ cells and double positive ΔNGFR PE+/GFP+ BALL from EV bone marrow and SOX6 bone marrow. GFP$^+$: EV 66% vs SOX6 62 %; GFP+/ΔNGFR+: EV 10,3% vs SOX6 0,06%.* ***b.*** *Histogram represents the % of GFP$^+$ cells on total bone marrow cells.* ***c.*** *Histogram represents the % of ΔNGFR$^+$/GFP$^+$ cells on total bone marrow cells. (Error bars: SEM; n=3; * P≤0.05).*

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CHAPTER 4:

OTHER ASPECTS OF SOX6 FUNCTION IN ERYTHROID DIFFERENTIATION

4.1 INTRODUCTION

Erythropoiesis, the process through which HSCs give rise to mature red blood cells (RBCs) or erythrocytes is a multifaceted process comprising two distinct aspects:

i) developmentally regulated events, leading to the production of erythrocytes from different hemogenic/hematopoietic progenitors residing in different anatomical sites

ii) the production of mature RBCs, orchestrated by the combined effects of microenvironment, growth factors and a tightly regulated network of nuclear factors promoting the survival, proliferation and differentiation of erythroid progenitors expressing the specific genes (such as globins genes) required for erythrocytes function.

Within erythrocytes, hemoglobin mediates the transport of oxygen and carbon dioxide. Hemoglobin consists of two α-like and two β-like globin chains coordinated by heme. The human α -like and β-like globin loci, located on chromosomes 16 and 11, respectively, encode these protein chains (fig. 4.1 A). During development, different α- and β-globin genes are expressed to produce the developmental stage-specific hemoglobin molecule that meet the oxygen demand of the organism at the different times. The human α-globin locus is composed of α1, α2, and ζ-globing genes, while the human β-globin locus consists of five functional β-like globin genes and an upstream regulatory element, the locus control region (LCR), which contains five DNase I-hypersensitive sites (HSs) (Grosveld, 1987; Forrester, 1987). These genes are expressed in a tissue and developmental specific order and are spatially arranged in the order of their expression during ontogeny, 5'-ε-Gγ-Aγ-δ-β-3'.

During human development, two switches take place: the embryonic to fetal switch and the fetal to adult switch. The ε-globin gene is expressed during the first six weeks of gestation in primitive, nucleated erythroid cells of the yolk sac. The first switch leads to the activation of Gγ- and Aγ-globin genes expression in the definitive hematopoietic cells of the fetal liver. In parallel with γ-globin gene activation, the ε-globin gene is concomitantly silenced. During the second switch, occurring around birth, the β-globin gene and, to a lesser extent (≈1-2%), the δ-globin gene are activated in bone marrow. The onset of the expression of the adult β-globin gene coincides with the silencing of γ-globin genes.

The evolution of specific globin genes expressed during the fetal period is a recent event, which took place 35 to 55 million years ago during primate evolution (Stamatoyannopoulos, 2005). Hence, unlike humans and old world monkeys, most species have only one switch, from embryonic to definitive globins expression, occurring early in development.

In mouse, for example, the β-like globin genes cluster (εY, βh1, βmajor, βminor) is located on chromosome 7 and the α-like globin cluster (x, α1, α2) is on chromosome 11 (Fig.4.1 B). The embryonic εY and βh1 genes are expressed during the embryonic life and they are substituted by the adult βmajor and βminor genes between embryonic days E11.5–E13.5, (Fantoni, 1969). Intriguingly, in the early embryo, βh1 is expressed at higher level than εy, suggesting that, at this stage of development, globins expression may not strictly correlate with the order of genes on the chromosome (Kingsley, 2006).

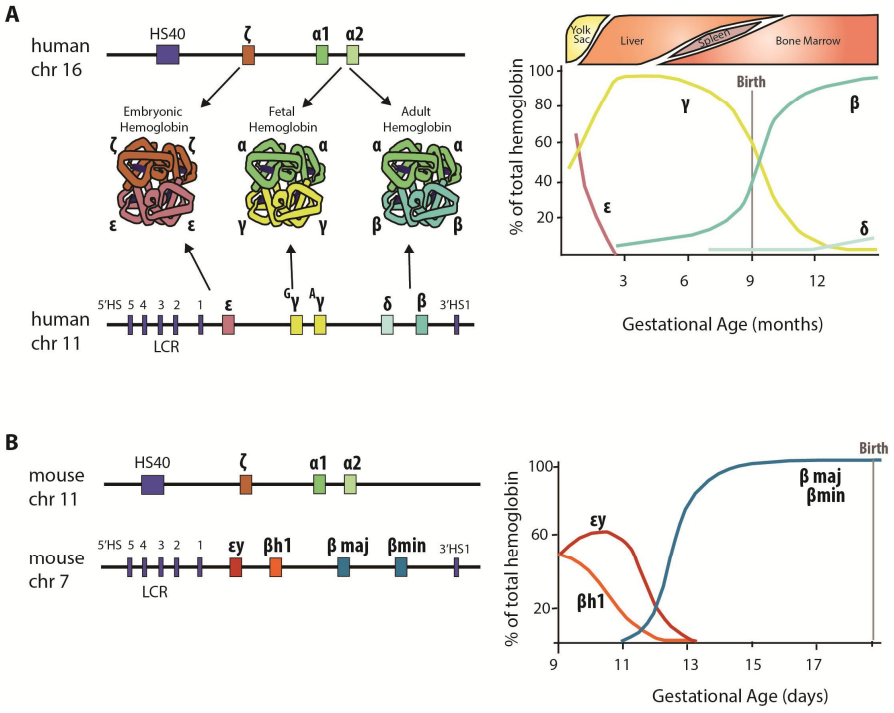

Fig. 4.1: The Hemoglobin Switching. Left: *schematic representation of human (**A**) and murine (**B**) globin loci. The α-globin locus is composed by an upstream Dnase hypersensitive site (purple boxes, HS40) and three α-like globins: ζ, α1 and α1 both in human and mice. The β-like globin locus consists of β-like globin genes (colored boxes), upstream DNaseI hypersensitive sites 5'HS1 to 5 (purple boxes) within the locus control regions (LCR), and downstream 3'HS1. Between the human α- and β-globin loci the main hemoglobins expressed during development are illustrated: embyionic globin (ζ2ε2; HbE Gower-1); fetal hemoglobin (α2γ2, HbF) and adult hemoglobin (α2β2, HbA). Other hemoglobins not depicted in this picture are: Hemoglobin Portland (ζ2,γ2), HbEGrower-2 (α2ε2), Hemoglobin A2 (α2δ2, HbA2).* ***Right:*** *developmental switching of the β-like globin gene expression in human (**A**) and mouse (**B**). Above the graph for the human locus the shifting sites of hematopoiesis are indicated. (Figure from Noordermeer 2008, modified by I. Cantù).*

Although mouse does not possess fetal-specific globin genes, mice transgenic for the human β-globin locus linked to a LCR element express it "appropriately" during development: they start to transcribe fetal γ-globin gene during the fetal life around E10.5 and silence it around E16 (Strouboulis, 1992), the developmental window corresponding to the time

of mouse globins switching. This suggests that humans and mouse primitive erythroblasts undergo similar differentiation mechanisms. For this reason mouse is a considered a good model for studying the human globin switching.

The importance of unraveling the molecular mechanisms of the fetal to adult switch in humans is accentuated by its clinical relevance, because of the high incidence of β-hemoglobin disorders (Stamatoyannopoulos, 2005). β-hemoglobinopathies represent the most common inherited genetic disorders, characterized by qualitative or quantitative defect in the production of adult β-globin. The major outcome of such diseases is an imbalance between α- and β-globins polypeptides, leading to precipitation of excess unbound globins in red cells and thus in the damage of erythrocytes. The most common types of β-hemoglobin disorders are β-thalassemias and sickle cell disease (SCD).

In a group of genetic benign conditions called hereditary persistence of fetal hemoglobin (HPFH), the expression of γ-globin persists at high levels in the adult. Individuals with compound heterozygosity for sickle cell disease or β-thalassemia and HPFH mutations are largely asymptomatic (Weatherall, 2001). These data suggest that the ability to reactivate γ-globin expression in the adult could have a therapeutical effect in patients with β-hemoglobinopathies. Thus, the understanding of the molecular mechanism of the switching from γ- to β-globin is the basis to develop new therapeutical approaches to treat β-hemoglobinopathies (Akinsheye, 2011; Bank, 2006; Stamatoyannopoulos, 2012; Stamatoyannopoulos, 2005).

Several TFs concur to the differential regulation of fetal vs adult globins and are thus candidate targets for treatment aimed to reactivate γ-globin (Zhu, 2012; Xu, 2013; Zhou, 2010). Amongst them, there is SOX6, which is known

to cooperate with Bcl11a, so far considered the major γ-globin repressor in the adult.

In this chapter I present others projects to which I've participated during my PhD on the SOX6 function in erythropoiesis and on its role in the differential regulation of globins genes. These projects aimed to dentify SOX6 interacting proteins (paragraph 4.3), its relationship with other TFs relevant for globin genes regulation (paragraph 4.4) and the set up of a high-throughout, high-content platform for the screening of genes/drugs altering the γ versus β expression (paragraph 4.5).

4.2 EXPERIMENTAL PROCEDURES

Constructs

The construct expressing the *Escherichia coli* BirA biotin-protein ligase (containing 3' HA epitope) was kindly given by Prof. John Strouboulis (de Boer, 2003). The SOX6 murine cDNA (obtained from Prof. Michiko Hamada-Kanazawa, Japan) was cloned in frame with a 3' FLAG epitope to generate a SOX6-FLAG cassette. 3' to the FLAG epitope, a sequence coding the 23 amino acids necessary for the biotin-tag was cloned to generate the bioSox6-FLAG cassette, flanked by two BglII sites. This cassette was then cloned immediately upstream to the IRES - Emerald GFP cassette (blunted BamHI site) of the CSI emerald derived from pHR SIN CSGW, (Demaison, 2002) lentiviral vector, a kind gift from Prof. Tariq Enver, UCL, London. In this vector, the SFFV promoter drives the expression of the exogenous cDNA, highly active in hematopoietic cells. The COUP-TFII murine cDNA (obtained from Prof. Michiko Hamada-Kanazawa, Japan) was cloned in an IRES-ΔNGFR (deleted Nerve Growth Factor Receptor) cassette site of the CSI lentiviral vector (a kind gift from Prof. Tariq Enver), under the control of the spleen focus-forming virus (SFFV) promoter. The two packaging plasmids psPAX2 and pMD-VSVG were used to produce Lentiviral pseudo-particles in HEK 293T cells (www.lentiweb.com).

Lentiviral vector production

Exponentially growing HEK-293T cells were transfected with jetPEI™ (Polyplus-Transfection) with the above vectors plus the packaging plasmids psPAX2 and pMD-VSVG to produce the lentiviral pseudo-particles (www.lentiweb.com). 72h after transfection, the supernatant with recombinant viruses was collected, filtered (0.45μm) and centrifuged at 20,000g for 8 hours at 4°C. The viral pellet was re-suspended in 1xPBS and

aliquoted at -80°C. Lentiviruses were titrated on HEK-293T cells by measuring the percentage of $eGFP^+$ cells by Flow Cytometry.

Cell Cultures and transduction

β-K562 and HEL stably expressing the biotin ligase BirA were cultured in RPMI 1640 medium (Lonza) supplemented with 10% heat inactivated fetal bovine serum (Sigma), L-glutamine (Euroclone), antibiotics Penicillin-Streptomycin 100U/100ug/ml (Euroclone) in a humidified 5% CO_2 atmosphere at 37°C. In the cells stably expressing BirA the medium was supplied with 3μg/ml of puromycin (Sigma). Transduction was performed overnight with a multiplicity of infection (MOI) of 30.

Flow Cytometry

Transduced β-K562 cells were washed, fixed in 4% paraformaldehyde and stained with PE-anti NGFR conjugated antibody (Biolegend) for 30 minutes at 4ºC and analyzed by flow cytometer (Becton-Dickinson FACS Calibur). Data were analyzed with Summit Software v4.3.

RNA isolation and Real Time PCR

Total RNA from $>10^6$ cells (from HEL-BirA cells, β-K562 cells, from mouse fetal liver E11.5, E12.5 and E13.5 dpc) was purified with TRIzol Reagent (Applied Biosystem), treated with RQ1 DNase (Promega) for 30 min at 37°C and retrotranscribed (High Capacity cDNA Reverse Transcription Kit, Applied Biosystem). Negative control reactions (without Reverse Transcriptase) gave no PCR amplification. Real time analysis was performed using ABI Prism 7500, (Applied Biosystems). Primers were designed to amplify 100 to 200bp amplicons, spanning an exon-exon junction when possible, on the basis of sequences from the Ensembl database (http://www.ensembl.org). Samples from each experiment were analyzed

in triplicate. Specific PCR product accumulation was monitored by SYBR Green dye fluorescence in 12-µl reaction volume. Dissociation curves confirmed the homogeneity of PCR products. All primers used are listed in the table below:

Human:

PRIMERS	SEQUENCE
GAPDH Fw	ACGGATTTGGTCGTATTGGG
GAPDH Rw	TGATTTTGGAGGGATCTCGC
α GLOBIN Fw	GAGGCCCTGGAGAGGATGTTCC
α GLOBIN Rw	ACAGCGCGTTGGGCATGTCGTC
γ globin Fw	CTTCAAGCTCCTGGGAAATGT
γ globin Rw	GCAGAATAAAGCCTACCTTGAAAG
ε globin Fw	GCCTGTGGAGCAAGATGAAT
ε globin Fw	GCGGGCTTGAGGTTGT
β globin Fw	TACATTTGCTTCTGACACAAC
β globin Rw	ACAGATCCCCAAAGGAC

Mouse:

PRIMERS	SEQUENCE
Hbb-y Fw	GGAGAGTCCATTAAGAACCTAGACAA
Hbb-y Rw	CTGTGAATTCATTGCCGAAGTGAC
β maj/min Fw	ATGGCCTGAATCACTTGGAC
β maj/min Rw	ACGATCATATTGCCCAGGAG
bh1 Fw	TGGACAACCTCAAGGAGACC
bh1 Rw	ACCTCTGGGGTGAATTCCTT
GAPDH Fw	TGTGTCCGTCGTGGATCTGA
GAPDH Rw	CCTGCTTCACCACCTTCTTGA
SOX6 Fw	TTCCTCCTGCATGGAAAAAC
SOX6 Rw	GATGCTGCCAGCTTTTTCTG
COUP-TFII Fw	AAGCAAGCCACCTCTCCATT
COUP-TFII Rw	GGTGTTGATCACTGCCCTCT

Nuclear extracts

Cells were harvested and centrifuged at 640g for 15 min at 4ºC . The pellet washed three times in ice-cold 1X PBS (phosphate buffered saline, pH 7.4) and gently resuspended in ice-cold NP-40 lysis buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM $MgCl_2$, 0.5% v/v NP-40, supplemented with protease inhibitors (complete EDTA-free, Roche) added just before use). Cells were lysed by incubation on a rotating wheel for 10 min at 4ºC and nuclei were then centrifuged at 3300g. Nuclei were resuspended in nuclear lysis buffer (10mM HEPES-KOH pH 7.9, 100mM KCl, 3mM $MgCl_2$, 0,1 mM EDTA, 20% glycerol and protease inhibitors, as above). Nuclear proteins were extracted by drop-wise addition of 4M KCl with gentle agitation on ice, until the final salt concentration was approx. 350-400mM. Nuclear lysis and protein extraction were then allowed to proceed by incubation on a rotating wheel for 40 min at 4ºC, followed by centrifugation. The supernatant, which correspond to the soluble nuclear extract fraction, was retained.

Statistics

Statistical analyses of obtained data were performed with GraphPad Prism (version 6.0; GraphPad Software, Inc.). The data are expressed as mean ± standard error of n=3 or more determinations. Statistics was performed using a paired, two-tailed Student t-test.

Immunoblotting analysis

To confirm for the presence of SOX6 overexpression and *in vivo* biotinylation, nuclear extracts (30-50µg/lane) were resolved by SDS/PAGE in a 7% gel and blotted onto Hybond ECL Nitrocellulose membrane (GE healthcare life science - Amersham) by "wet blotting." carried out under constant voltage at 100V for 90-120 min at 4ºC (Transblot apparatus, Biorad). Membranes were blocked for 1-2h at room temperature with Milk

5% in TBS-T 1X (Tris Buffered Saline, pH 7.6 and 0,1% Tween 20 by Sigma) and incubated with the appropriate primary antibody diluted in Milk 3% TBS-T 1X overnight at 4ºC. Membranes were washed for three times with TBS-T 1X and incubated with the secondary antibody (diluted in Milk 3%).
The antibodies used were: anti-FLAG antibody (abcam ab125243); anti-Sox6 (abcam ab64956); anti-MTA1 (Santa Cruz sc17779); anti-HDAC1 (Santa Cruz sc81598); anti-BCL11a (all isoforms) (Novus, Ctip1 Antibody [NB600-258]), anti-COUP-TFII (abcam H7147); anti-CDK13 (anti-CDC2L5 Bethyl laboratories). Protein loading control was checked with an anti-CPSF73 homemade antibody (kindly given from Prof. Silvia Barabino laboratory). In particular, the Streptavidin-HRP (Cell signaling) antibody was incubated for 2h at room temperature (no secondary antibody was required).Antibodies binding was detected by using appropriate horseradish peroxidase-conjugated IgG and revealed by ECL (Millipore).

Benzonase treatment

Nuclear extracts were diluted by adding 3 volumes of HENG buffer (0 mM KCl, 10 mM HEPES pH=9, 1.5 mM MgCl2, 0.25 mM EDTA, 20 % glycerol, PMSF or PI) and 25U of Benzonase (Viscolase nuclease, A&A Biotechnology) per mg of nuclear extract were added. The concentration of Mg^{+2} was adjusted to 2mM. Diluted lysates were incubated with Benzonase for 2-4 hours at 4ºC on a rotating wheel. DNA digestion and removal was checked on gel on a phenol-extracted aliquot.

Binding to Streptavidin beads

Paramagnetic streptavidin beads (Dynabeads M-280, Dynal (Life Technologies) 50 µl per 1 mg of protein), were washed three times with PBS at room temperature and blocked with HENG/BSA (Sigma-Aldrich) 200µg/ml (10 mM HEPES pH 9, 1.5 mM MgCl2, 0.25 mM EDTA, 20 %

glycerol, PMSF) for 1h at room temperature under rotation. The binding was carried out with Nuclear Extracts diluted in 150 mM KCl and 0.3 % NP40), overnight on a rotating wheel. After 5 washes with wash HENG solution (HENG buffer with 300mM KCl) and 2 washes with PBS 1x at room temperature, the bound material was eluted by boiling for 10 min in Laemmli protein simple loading buffer (62.5mM Tris–HCl pH6.8, 25% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue) and analyzed by immunoblotting.

Immunoprecipitations

Nuclear extracts were precleaned at 4ºC using protein G sepharose beads and affinity purified IgG (mouse- Santa Cruz, CA, SC-2025). Anti-FLAG M2 affinity gel (A220 – Sigma) was used to immunoprecipitate the proteins. Immunoprecipitations were performed at 4ºC for 3 hours. Washes were carried out at room temperature in PBS 1X (phosphate buffered saline, pH 7.4). Bound material was eluted by boiling in 1x Laemmli protein simple loading buffer (62.5mM Tris–HCl pH6.8, 25% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue) and analyzed by immunoblotting.

Mass-Spectrometry

Proteins eluted from streptavidin beads were resolved by SDS-PAGE, and gel lanes were cut into slices by using an automatic gel slicer and subjected to in-gel trypsinization, (Shevchenko, 1996). Bound proteins were treated with trypsin on the beads after resuspending in 50 mM ammonium bicarbonate and adding trypsin (sequencing grade; Promega) to approximately 60 ng/mg of total protein, followed by overnight incubation at 37°C (Rybak, 2005). The supernatant containing the trypsin-treated peptides was then recovered by magnetically removing the beads. Peptides

released by in-gel or on-bead trypsinization were analyzed by nano-LC-MS/MS performed on either a CapLC system (Waters, Manchester, United Kingdom) coupled to a Q-ToF Ultima mass spectrometer (Waters), operating in positive mode and equipped with a Z-spray source, or on a 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ-Orbitrap or LTQ-FT-MS mass spectrometer (both from Thermo Scientific) operating in positive mode and equipped with a nanospray source. Peptides were trapped and separated on a Jupiter C_{18} reversed-phase column (Phenomenex) using a linear gradient from 0 to 80% medium B (where medium A = 0.1 M acetic acid and medium B = 80% [vol/vol] acetonitrile, 0.1 M acetic acid) using a splitter. The column eluate was directly sprayed into the electrospray ionization source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the peptides was performed in data-dependent mode.

Data analysis and protein identification

Peak lists were automatically created from raw data files by using ProteinLynx Global Server software (version 2.0; Waters, Manchester, United Kingdom) for Q-ToF spectra and Mascot Distiller software (version 2.0; MatrixScience, London, United Kingdom) for LTQ-Orbitrap and LTQ-FT-MS spectra. The Mascot search algorithm was used for searching the National Center for Biotechnology Information (NCBI) database. The Mascot score cutoff value for a positive hit was set to 65. Individual peptide MS/MS spectra with Mowse scores below 40 were checked manually and either interpreted as valid identifications or discarded. Identified proteins listed as NCBI database entries were screened to identify proteins that were also identified in mass spectrometry experiments from control BirA-expressing cells (De Boer, 2003). These were removed as background binding proteins.

The remaining proteins were classified according to gene ontology criteria using the Panther software (www.pantherdb.org) and were then grouped according to their molecular function.

4.3 IDENTIFICATION OF SOX6 INTERACTING MULTI PROTEIN COMPLEXES IN ERYTHROLEUKEMIC HEL CELLS USING A BIOTIN TAGGING APPROACH

Font Monclus I., et al. manuscript in preparation.

4.3.1 INTRODUCTION

SOX6 has the ability to both activate and repress gene expression, depending on its interactions and on its target sequences (Kamachi, 2000; Kiefer, 2007; Lefebrvre, 2007). Despite its dual function of activator and repressor, SOX6 lacks any conventional transactivation or transrepression domain. This evidence suggests that it must partner with other proteins to exert its function.

To identify SOX6-interacting proteins, we set up a proteomic Tag-affinity screening by using a metabolic biotin tagging approach (De Boer, 2003; Rodriguez, 2005) in HEL cells (human erythroleukemia), stably expressing BirA (a bacterial biotin ligase).

In HEL cells, we overexpressed a Biotin-tag SOX6 cassette. Biotinylated SOX6 can be then purified by using Streptavidin-coated beads and proteins co-purified with SOX6 can be analyzed by mass spectrometry (fig. 4.2). The biotin–streptavidin binding offers several advantages: its high affinity allows to purify proteins under high stringent conditions, highly reducing the possible background. Moreover, only very few proteins are naturally biotinylated, and this reduces the chances to immunoprecipitate proteins not specifically interacting with the protein of interest.

Mapping SOX6 partners can help to elucidate the different aspects of SOX6 biology in erythroid cells, i.e.:

i) SOX6 effectors responsible for SOX6-induced cell cycle withdrawal, described in my thesis in chapter two and chapter three.

ii) the basis of SOX6 ability to differentially regulate (either activate or repress) its targets genes -including globins- in erythroid cells.

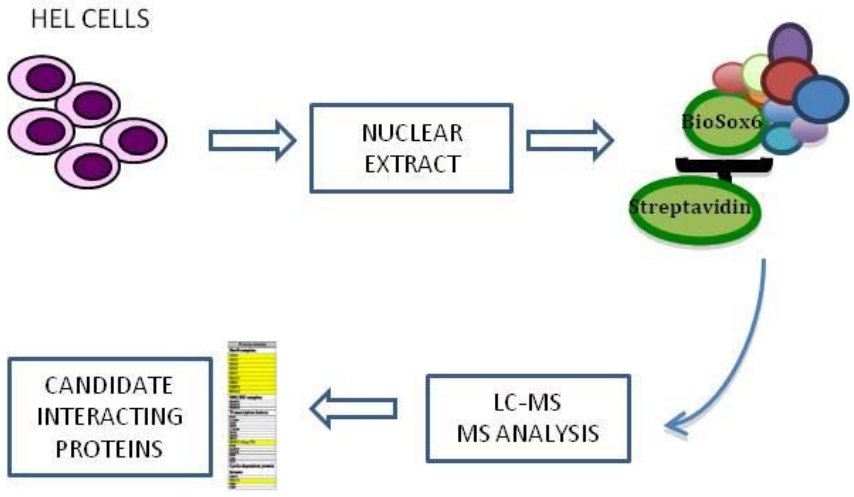

Fig. 4.2: Biotinylation strategy. *Single step- streptavidin affinity purification followed by tandem Mass Spectrometry peptide sequencing. As a final result, a list of putative candidates interactors of SOX6 is generated. Schematic diagram of the experimental approach used.*

4.3.2 RESULTS AND DISCUSSION

Pull down and identification of SOX6-containing multiprotein complexes

Large-scale nuclear extracts from HEL cells overexpressing biotinylated SOX6 (bioSOX6-FLAG) were used to perform single step streptavidin affinity-tag purification. Co-purified nuclear proteins were then fractionated by using SDS/PAGE followed by liquid chromatography and analyzed by tandem Mass Spectrometry peptide sequencing (strategy described on

Figure 4.1). The Mass Spectrometry was performed by Dr. Demmers at the Proteomics Center in Erasmus MC, Rotterdam.

As a negative control, we also performed in parallel a similar pull down in cells expressing BirA and a negative control, the empty vector, to be considered as background for the Mass Spectrometry peptides sequencing. Two biological replicates of the Mass Spectrometry sequencing analysis were performed on HEL cells overexpressing bioSOX6-FLAG.

Filtering criteria and identification of SOX6 candidate interactors

From the list of the potential SOX6 interacting candidates, we filtered out several proteins previously identified as a common biotinylated background proteins, such as carboxylases and their interacting enzymes, factors involved in mRNA processing and ribosomal proteins (De Boer et al., 2003).

As a final output of the Mass Spectrometry analysis carried out in duplicate in HEL cells we obtained a list of 839 putative interacting proteins. However, some of these proteins were also present in the negative control. In order to select for proteins specifically interacting with the bioSox6-FLAG, we used a software based on a Venn diagram (http://bioinfogp.cnb.csic.es/tools/venny/) to select the proteins specifically interacting with bioSOX6-FLAG. We found that between those 839 proteins only 258 were present in the bioSox6-FLAG pull down.

The proteins identified from the Venn diagram to be specifically pulled down by bioSox6-FLAG were then classified according to the molecular function and the biological process, as defined by the Gene Ontology Consortium (www.geneontology.org). Following GO analysis, proteins were classified using the Panther software (www.pantherdb.org). According to GO and Panther analyses, most of the SOX6 interacting proteins are relevant for chromatin binding (nucleic acid binding, chromatin binding and

protein binding), catalytic activity (hydrolase, transferase and enzyme regulator activity) and also for structural functions (in particular proteins with a role in the cytoskeleton modulation). Among them, we decided to exclude proteins with catalytic activity because they are common background (27,7% of the analyzed proteins) (De Boer, 2003). A positive selection of candidates was done on the basis of data from literature, pointing to a role of these proteins in hematopoiesis or erythropoiesis.

SOX6 candidate interactors

In the Mass Spectrometry readout, we identified several peptides of SOX6 present in cells overexpressing bioSOX6-FLAG but absent in HEL-BirA cells, which proves that the pull down experiment was successfully working. Numerous previously described proteins with a role in erythroid development were also identified as candidate of SOX6 interacting proteins: among them i) known erythroid TFs, such us COUP-TFII, GATA1, GATA2 and Gfi1b. ii) chromatin remodeling factors belonging to the NuRD (Nucleosome remodeling and deacetylase) and to the LSD1/CoRest (lysine specific demethylase 1 and repressor element-1 TF corepressor 1) complexes.

However, novel corepressors or coactivators were identified as possible SOX6 interactors during erythroid development: BCOR (Bcl-6 co-repressor), FACT complex, SWI/SNF and PcG. Furthermore, among these novel potential interactors, we found particularly interesting several cyclin dependent kinases (CDKs), such us CDK11, CDK12, CDK13 and CycK. These kinases are not fully related to cell cycle control, but they also have a role in mRNA splicing, genome stability maintenance and DNA damage response (DDR). However, nowadays only CDK13 has been found related with

hematopoiesis, but little is known about its specific function (Lapidot-Lifson Y,1992).

To validate the interaction between some of the novel SOX6 partners, we performed co-immunoprecipitation analyses/pull down by using streptavidin beads followed by immunoblotting detection in human erythroleukemic cells overexpressing and non-overexpressing SOX6 (Fig. 4.3).

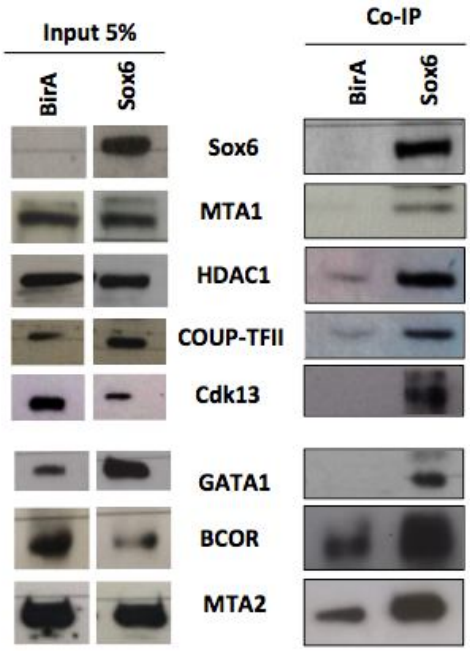

Figure 4.3. Identification of SOX6 interacting proteins. *Co-precipitation of SOX6 interacting proteins by using streptavidin beads to confirm the result obtained by Mass Spectrometry. 5% of the total nuclear lysate was used as input (from I.F. Monclus thesis, manuscript in preparation).*

4.4 CHARACTERIZATION OF THE ROLE OF SOX6 IN THE SPECIFIC REGULATION OF DIFFERENTIAL EXPRESSION OF GLOBIN GENES

Elangovan S., et al. manuscript in preparation.

4.4.1 INTRODUCTION

Several transcription factors are essential for erythroid commitment to occur and for differential globin s expression during development: their absence is associated with a wide spectrum of phenotypes ranging from mild anemia to death due to a complete failure of erythropoiesis.

The approach described in 4.3 aimed to identify SOX6 interactors in erythroid cells. Amongst putative SOX6 interacting proteins, we focused on TFs with a known function in the regulation of the globin genes (COUP-TFII, GATA1, GATA2 and Gif-1b). In particular, we focused our study on the transcription factor COUP-TFII.

COUP-TFII is a TF that binds to direct repeats elements (RE) AGGTCA (Cooney,1992). It binds in vitro to the ε- and γ-globin promoters, where it recognizes to the double CCAAT box region, by interfering with NF-Y binding and possibly repressing ε- and γ-globin expression (Liberati, 1998; Ronchi, 1995). It was thought that COUP-TFII might contribute to the HPFH phenotype because several HPFH mutations mapped within the double CCAAT box region of the γ-globin promoter cause the perturbation of COUP-TFII binding in vitro (Liberati, 2001). Moreover, an *ex vivo* model of primary human erythroblasts differentiation showed that the administration of Stem Cell Factor (SCF) reduces COUP-TFII expression at the mRNA level and protein level and increases γ-globin transcription (Aerbajinai, 2009). A recent study proponed that NF-Y recruits and stabilizes

the binding of BCL11a (B-Cell CLL/Lymphoma 11A or also known as COUP-TF-Interacting Protein 1) with COUP-TFII on the proximal γ-globin promoter region forming the repression complex silencing γ-globin expression (Zhu, 2012).

We focused our study on SOX6 and COUP-TFII because both of them have been implicated in the repression of ε- and /or γ-globin expression, with the aim of elucidating their role in the regulation of embryonic genes and thus in the globins switching. Here, I will describe the results of SOX6 and COUP-TFII overexpression in a clone of K562 cells also expressing β globin (β-K562).

4.4.2 RESULTS AND DISCUSSION

COUP-TFII interacts with SOX6 in HEL cells

The mass spectrometry analysis of SOX6 interactors (described in 4.1) in human erythroleukemic HEL cells, identified several COUP-TFII peptides. In order to validate the interaction between SOX6 and COUP-TFII, we transduced HEL-BirA cells with a lentivirus carrying a biotin-tagged Sox6 cDNA expression cassette (bioSOX6-FLAG). Three days after transduction, we performed a nuclear proteins extraction, followed by the pull down of Sox6 interacting proteins by using streptavidin beads. We then confirmed by Western Blot that COUP-TFII physically interacts with SOX6 in HEL-BirA cells (Fig. 4.4).

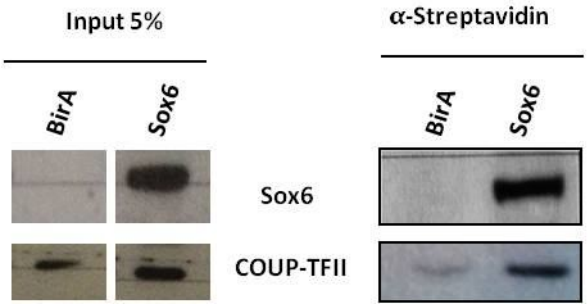

Fig. 4.4: Physical interaction between SOX6 and COUP-TFII in HEL cells. *Co-immunoprecipitation of the bioSOX6 and COUP-TFII proteins from HEL cells transduced and non-transduced with bioSOX6-FLAG. Immunoprecipitation was done using the biotin-streptavidin approach. Co-purified proteins were analyzed by western blot by using anti-SOX6 and anti-COUP-TFII antibodies. 5% of the total nuclear lysate was used as input. (from I.F. Monclus thesis, manuscript in preparation).*

Profiling COUP-TFII and SOX6 expression during mouse embryonic development

To gain insight into the switching process, we analyzed in mouse fetal liver (FL), at days E11.5, E12.5 and E13.5, the transcription levels and the protein levels of SOX6 and COUP-TFII. At these developmental stages, the fetal liver is almost exclusively an erythropoietic organ and the E11.5-13.5 interval corresponds to the developmental window in which hemoglobin switching takes places.

The expression level of SOX6 increases, whereas the expression level of COUP-TFII decreases during the hemoglobin switching period (Fig. 4.5 b). To validate their differential expression at the protein level, we performed Western Blot analysis by using anti-SOX6 and anti-COUP-TFII antibodies. SOX6 almost absent at day E11.5, starts to accumulate from day E12.5 onwards, whereas COUP-TFII progressively declines (Fig. 4.5 a).

Of note, at the RNA levels, the profile of COUP-TFII expression follows the decline of embryonic Hbb-bh1/Hbb-y globins, whereas the increase of SOX6 parallels the progressive accumulation of adult Hbb-b1/2 globins (Fig. 4.6).

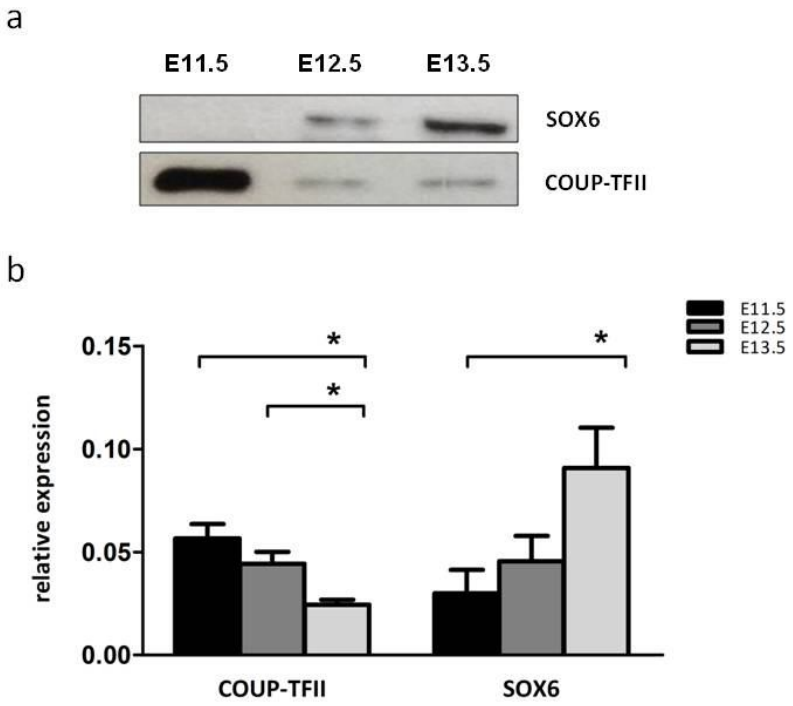

Fig. 4.5: Expression profile of SOX6 and COUP-TFII during the hemoglobin switching in mouse fetal liver cells. a. *Western blot analysis on purified nuclear extracts from the same mouse primary fetal liver cells confirms the expression of both transcription factor at the protein level, by using anti-SOX6 and anti-COUP-TFII antibodies. Anti-CPSF73 was used as a loading control.* ***b.*** *RT-qPCR analysis of the mRNA expression of SOX6 and COUP-TFII during mouse embryonic development (E11.5-E13.5). Histograms show the expression profiles of SOX6 and COUP-TFII relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH);* (Error bars: SEM; *n≥4,; *=P<0.05; **=P<0.01; ***=P<0.001).*

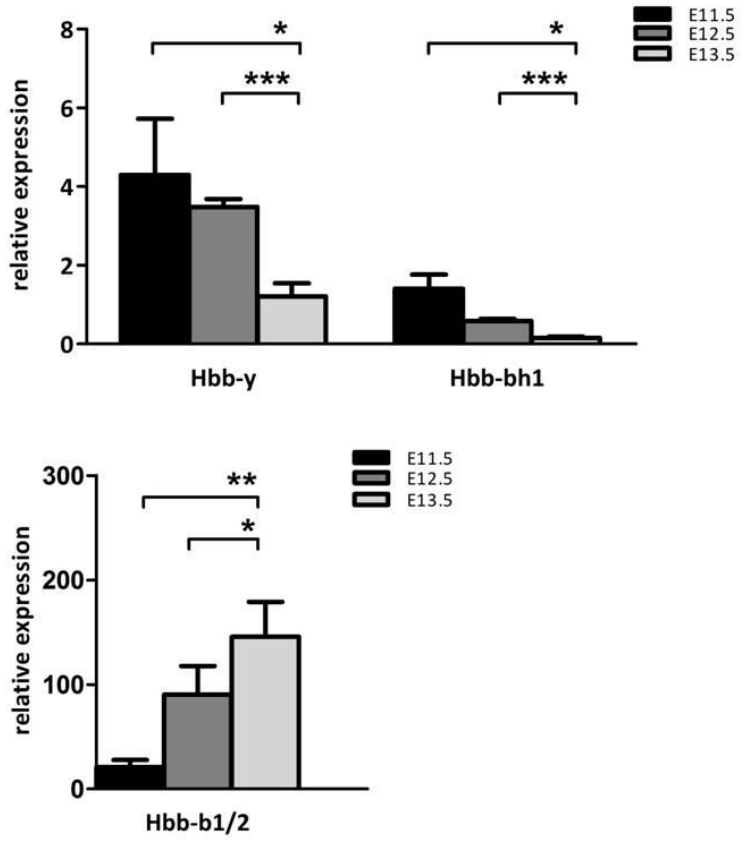

Fig. 4.6: Expression profile of globins during the hemoglobin switching developmental window in mouse fetal liver cells. *RT-qPCR analysis of the mRNA expression of globins during mouse embryonic development (E11.5-E13.5). Histograms show the expression profiles of globins relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH);* (Error bars: SEM; *n≥4,; *=P<0.05; **=P<0.01; ***=P<0.001).*

Overexpression of SOX6 and COUP-TFII in β-K562 cells

The above data suggest that changes in SOX6 and COUP-TFII levels could play a role in mouse globin gene switching. To assess whether this is also true for human globin expression, we took advantage of a K562 subclone, recently characterized in our laboratory (β-K562), which expresses β- in addition to α-, ε- and γ-globin (Durlak, 2015). β-K562 are a good model to study the effect of transcription factors on the regulation of γ- to β-globin

expression ratio. We thus transduced β-K562 cells with COUP-TFII and SOX6 expressing lentiviruses to characterize their role in globins gene regulation.

In agreement with previous results obtained in our lab in the parental K562 cells (Cantu, 2011), the overexpression of SOX6 in the β-K562 cells enhances their terminal maturation. This is accompanied by an increase in the expression level of all the globins genes (α, ε, β and γ) due to a general differentiation effect (Fig. 4.7 a). The largest increase elicited by SOX6 overexpression is that of β-globin expression (8.5x), whereas the smallest increase is observed on γ-globin expression (3.2x). When setting γ+β=1, the β/(γ+β) ratio increases from 0,2 in the EV-β-K562 to 0,7 upon SOX6 overexpression in β-K562 (SOX6-β-K562). In contrast, COUP-TFII overexpression does not induce any characteristic phenotypic change of the erythroid differentiation, as assessed by unchanged levels of CD71 and TER119 analyzed by FACS and RT-qPCR (data not shown). However, it induces an increase in relative levels of ε- and γ-globin genes transcription when compared to those of cells transduced with the corresponding empty vector (EV-β-K562) (Fig. 4.7 b). When setting γ+β=1, the β/(γ+β) ratio decreases from 0,2 in EV-β-K562 to 0,08 in COUP-TFII-β-K562. No changes were observed in the levels of β-globin gene, whereas there is a reduction in the α-globin gene expression levels (by approximately 40% of reduction). Overall, the overexpression of COUP-TFII increases γ-globin expression. This effect is against the previous described role of COUP-TFII, which was proposed as a repressor of γ-globin gene (Liberati, 2001; Zhu, 2012). As the overexpression of COUP-TFII has no typical erythroid phenotypic change, it might be possible that COUP-TFII plays a specific role in the hemoglobin genes regulation.

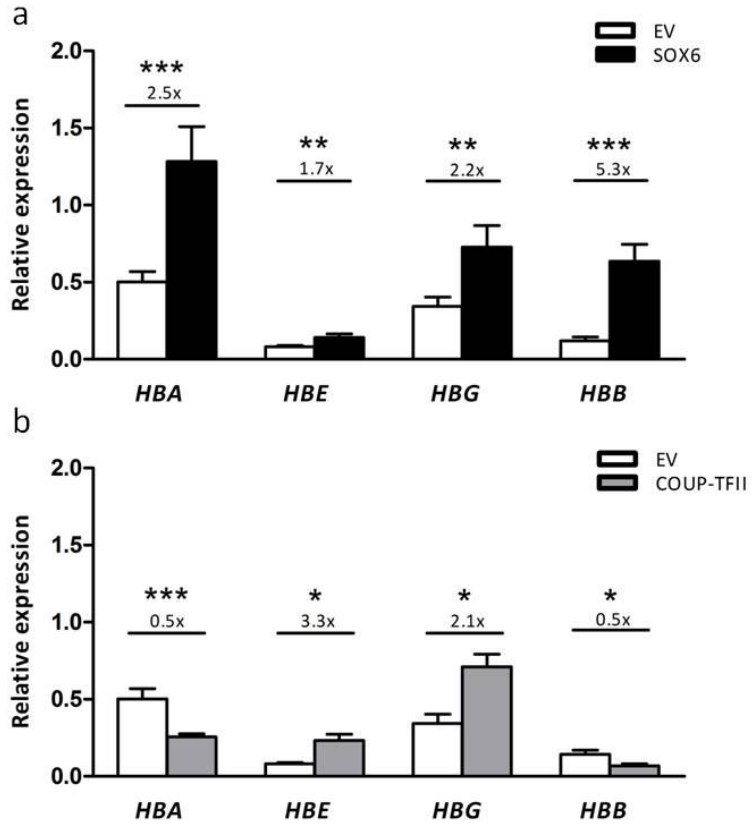

Fig. 4.7: Globins genes expression upon SOX6 and COUP-TFII overexpression. ***a.*** *RT-qPCR analysis of all globins on EV- δ-K562 and SOX6- δ-K562 cells, 72h after transduction. Histograms show expression levels relative to GAPDH (n=4; P<0,05).* ***b.*** *RT-qPCR analysis of all globins on EV- δ-K562 and COUP-TFII- δ-K562 cells, 72h after transduction. Histograms show expression levels relative to GAPDH (error bars: SEM; n=4; *=P<0.05; **=P<0.01; ***=P<0.001). In all experiments >95% cells were infected as assessed by FC analysis and the overexpression of SOX6 and COUP-TFII was confirmed by Western blot (data not shown).*

These data suggest that SOX6 and COUP-TFII have an opposite role on γ- and β-globin expression. SOX6 mainly increases β-globin, whereas COUP-TFII increases γ-globin. To better elucidate whether SOX6 and COUP-TFII could directly contribute to the γ- to β-globin switching, we co-transduced both transcription factors in β-K562 cells in order to analyze globins expression upon simultaneous overexpression of different concentration of

SOX6 and COUP-TFII. These conditions should mimic the changes of COUP-TFII and SOX6 expression observed during the Hemoglobin Switching (Fig. 4.5).

Simultaneous co-transduction of β-K562 cells with SOX6 and COUP-TFII

To set up the experiment of SOX6 and COUP-TFII co-transduction we cloned SOX6 in a vector containing an IRES-eGFP cassette and COUP-TFII in the same vector carrying an IRES-ΔNGFR cassette. The use of the two different reporter cassettes allows us to independently assess the level of transduction of SOX6 and COUP-TFII in co-transduction experiments. The corresponding empty vector (EV) containing either the IRES-eGFP or the IRES- ΔNGFR cassettes were used as controls. Cells were transduced with equal Multiplicity Of Infection (MOI) of both SOX6-eGFP and COUP-TFII-ΔNGFR lentiviruses and with different relative concentrations to mimic the changes in COUP-TFII and SOX6 relative abundance observed during the switching. In particular, we kept one transcription factor at fixed levels and we increased the concentration of the other one by increasing the MOI of the corresponding expression vector. The efficiency of transduction was monitored 72 hours after transduction by flow cytometry (the efficiency of transduction was higher than 90% in all the experiments, data not shown) and the actual level of the two proteins, at the different MOI used, was verified by Western Blot for each experiment (data not shown).

First, against a background of a fixed level of overexpressed SOX6, we progressively reduced COUP-TFII by reducing the MOI of the lentiviral particles produced from the corresponding vector. In a second series of experiments, we increased the relative level of SOX6 against a background of a fixed level of overexpressed COUP-TFII . Tipping the balance towards a high level of SOX6, either by reducing COUP-TFII or by increasing SOX6,

promotes the expression of adult globins (α and especially β), that are normally repressed by the overexpression of COUP-TFII alone (Fig. 4.8 a). Conversely, COUP-TFII overexpression induces ε- and γ-globin and the progressive increase of SOX6 is not capable of overcoming the activation of ε and γ elicited by COUP-TFII alone (Fig. 4.8 a). Thus, in β-K562, the overexpression of COUP-TFII ensures the transcription of embryonic/fetal genes even in the presence of high level of ectopically expressed SOX6. Instead, COUP-TFII reduction relative to SOX6, promotes the expression of adult globins. In terms of relative γ to β ratio, the increase of SOX6 and the decrease of COUP-TFII results in an overall more adult "switched" phenotype (Fig. 4.8 b).

The data above point to a functionally competitive role of these two transcription factors and suggest that the decline of COUP-TFII during the switching and the parallel rise of SOX6 could contribute to the fetal/adult globins switching. Moreover, these results are in agreement with the previous results where both SOX6 and COUP-TFII showed an opposite expression profile during erythroid embryonic development in mouse fetal liver cells from E11.5 to E13.5, where SOX6 was upregulated and COUP-TFII silenced (Fig. 4.5).

These results show the ability of COUP-TFII to upregulate γ-globin expression in β-K562 cells. For this reason in our laboratory we will investigate in more detail its molecular mechanism. In fact, the above results indicate that COUP-TFII is a candidate target for the reactivation of γ-globin in patients with β-hemoglobinopathies, such as β-thalassemia o Sickle Cell Disease (SCD).

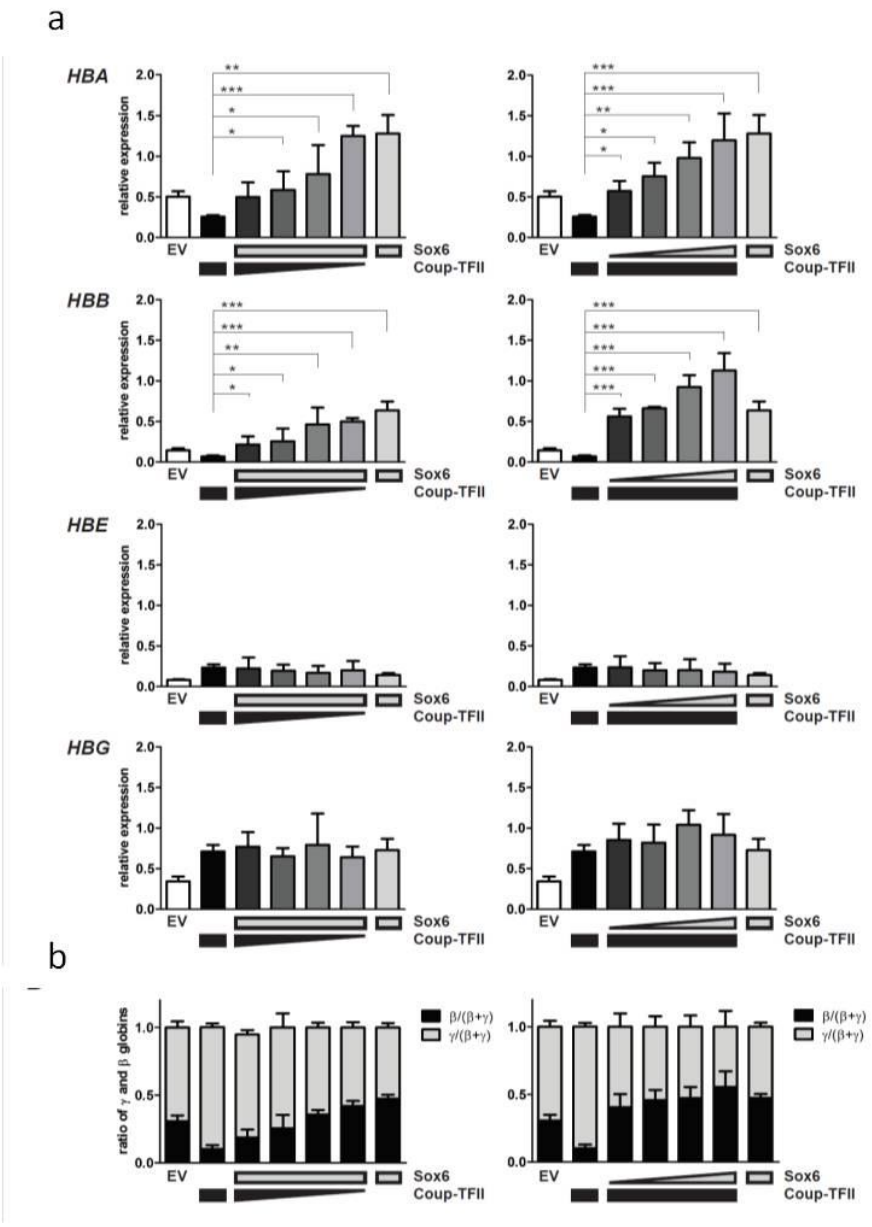

Fig. 4.8: The relative level of Coup-TFII and Sox6 modulates the γ- to ϐ-globin ratio in ϐ-K562 cells. ***a.*** *RTqPCR analysis of individual globin genes upon single or double overexpression of SOX6 and COUP-TFII. Histograms show globin expression level relative to GAPDH (n≥2, error bars: SEM; *=P<0.05; **=P<0.01; ***=P<0.001). Triangles: increasing/decreasing concentrations of SOX6 or COUP-TFII; rectangles: fixed concentrations of the protein. Grey: SOX6. Black: COUP-TFII.* ***b.*** *Corresponding γ- to ϐ-globin ratio (γ+ϐ=1).*

In order to confirm the role of COUP-TFII as γ-globin activator in human cells, we moved to a better model where the levels of γ-globin gene have been already mostly or completely silenced. Primary erythroid cultures form $CD34^+$ cells purified from peripheral blood can be established from both healthy donors and β-thalassemic patients. These results are described in a manuscript in preparation:

"**The transcription factor Coup-TFII specifically activates embryonic/fetal globin gene expression", Elangovan S.,** Fugazza C., Marini M.G., **Barbarani G.** , Giolitto S., Font Monclus I., Marongiu M. F., Manunza L., Strouboulis J., Ottolenghi S., Moi P. and Ronchi A.

4.5 A MULTIPLEX HIGH CONTENT ASSAY FOR QAUNTIFICATION THE GAMMA AND BETA GLOBIN CONTENT AT SINGLE CELL LEVEL

Published by **Durlak M**., Fugazza C., Elangovan S, Marini M.G., Marongiu M.F., Paolo M., Fraietta I., Cappella P., **Barbarani G**., Font-Monclus I., Mauri M., Ottolenghi S., Gasparri F., Ronchi A. PLOS ONE, October 28, 2015.

In collaboration with Dr. Fabio Gasparri and Dr. Marta Durlak from Nerviano Medical Sciences (NMS), we developed a high content screening platform based on multiplex imaging on a subclone of the K562 cell line, to identify new potentially therapeutic drugs to reactivates γ globin expression (Durlak, 2015). Taking advantage of this novel platform, we applied it to carry out a high-content screening for several of the most promising partners of SOX6 identified in the Affinity-Tag purification approach described above.

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RESEARCH ARTICLE

A Novel High-Content Immunofluorescence Assay as a Tool to Identify at the Single Cell Level γ-Globin Inducing Compounds

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Abstract

The identification of drugs capable of reactivating γ-globin to ameliorate β-thalassemia and Sickle Cell anemia is still a challenge, as available γ-globin inducers still have limited clinical indications. High-throughput screenings (HTS) aimed to identify new potentially therapeutic drugs require suitable first-step-screening methods combining the possibility to detect variation in the γ/β globin ratio with the robustness of a cell line. We took advantage of a K562 cell line variant expressing β-globin (β-K562) to set up a new multiplexed high-content immunofluorescence assay for the quantification of γ- and β-globin content at single-cell level. The assay was validated by using the known globin inducers hemin, hydroxyurea and butyric acid and further tested in a pilot screening that confirmed HDACs as targets for γ-globin induction (as proved by siRNA-mediated HDAC3 knockdown and by treatment with HDACs inhibitors entinostat and dacinostat) and identified Heme-oxygenases as novel candidate targets for γ-globin induction. Indeed, Heme-oxygenase2 siRNA knockdown as well as its inhibition by Tin protoporphyrin-IX (TinPPIX) greatly increased γ-globin expression. This result is particularly interesting as several metalloporphyrins have already been developed for clinical uses and could be tested (alone or in combination with other drugs) to improve pharmacological γ-globin reactivation for the treatment of β-hemoglobinopathies.

Introduction

Sickle cell anemia (SCA) and β-thalassemia are among the commonest inherited diseases in humans, with more than 300,000 affected children born every year and with an estimated worldwide population of tens of millions patients suffering from these disorders [1]. The number of these patients is increasing because of the decreased mortality from nutrition problems

Project (Marie Curie Actions of the European Union's Seventh Framework Programme FP7/2007-2013/ under REA grant agreement n°28961). The present research is the result of a collaborative project and MD is the Marie Curie fellow appointed to Nerviano Medical Sciences S.r.l. within this network. PC was a NMS employee at the time the research was performed (present address: FlowMetric Europe srl, Parco Tecnologico Padano, Via A. Einstein 26900, Lodi, Italy).

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and infections in the developing countries [2–4]. SCA is caused by a missense mutation within the adult β-globin chain. Hemoglobin tetramers bearing this altered β chain (HbS) tend to polymerize within the Red Cell, under hypoxic conditions, conferring the typical sickle shape, leading to cell lysis, small vessel occlusion, pain crises and organ damage. In β-thalassemia, the reduced synthesis of β chains causes unbalanced accumulation of α-globin that precipitates, resulting in ineffective erythropoiesis and anemia [5]. Coinheritance of Hereditary Persistence of Fetal Hemoglobin (HPFH), a condition where the expression of the fetal *HBG1/2* is maintained postnatally, can ameliorate β-globinopathies, by reducing sickle hemoglobin polymers in SCA and the α/non-α chain imbalance in β-thalassemia[6]. This observation led to the intensive search for fetal hemoglobin (HbF) inducers that could mimic the beneficial effects observed in HPFH[7–9]. Genome-wide association studies identified three major gene loci (Xmn1-HBG2, HBS1L-MYB and BCL11A) accounting for the majority of inherited HbF variance[10] but their exploitation as therapeutic targets is still distant. Another line of research focused on the development of drugs acting on γ-globin regulatory molecules: different classes of drugs (cytotoxic agents, HDAC inhibitors, DNA methyl transferase inhibitors) have been tested as HbF inducers but, despite the enormous effort in this direction and some encouraging results on some patients, no universal effective drugs have been found so far. Among them, hydroxyurea (HU) has been approved by the FDA for the treatment of SCA and has been recently considered for β-thalassemia, but its efficacy varies among patients. Indeed, about half of the patients do not reach therapeutic levels of HbF at HU doses of acceptable toxicity[11,12]. Other agents, such as short-chain fatty acids (Butyrate and its derivatives), 5-azacytabine, Decitabine and Tranylcypromine act on the epigenetic regulation of HbF, by inhibiting histones deacetylation or methylation of the *HBG1/2*, but their efficacy is still limited to a minority of patients[13–16].

These observations point to the strong need to identify new compounds stimulating γ-globin expression.

With this goal in mind, we set up a high-content screening platform based on multiplexed imaging on a variant K562 cell line (β-K562) spontaneously expressing significant levels of β-globin. Simultaneous analysis of DNA content, adult hemoglobin HbA ($\alpha_2\beta_2$) and fetal hemoglobin HbF ($\alpha_2\gamma_2$) resulted in a robust and sensitive assay, capable of detecting changes at the single cell level in hemoglobinization and in γ/β ratio in response to drugs, as proved by the response of β-K562 to the known γ-globin inducers hemin, hydroxyurea and butyric acid and to two additional HDAC inhibitors: entinostat and dacinostat.

The method was further validated by transfecting β-K562 with a panel of 70 siRNAs. Among them, we identified HMOX2, coding for Heme-oxygenase2 (HO-2), as a gene whose knockdown greatly increases γ globin levels, both in terms of percentage of expressing cells and of γ-globin accumulation per cell. Tin Protoporphyrin IX, a prototypical compound inhibiting HO-2, induced selective γ-globin accumulation in β-K562, suggesting that Heme-oxygenases could be a promising pharmacological target to ameliorate the α/β chains unbalance in β-hemoglobinopathies.

Materials and Methods

Cell lines and chemical treatments

ECACC-K562 (European Collection of Cell Cultures) and β-K562 (a kind gift of Prof. G. Ferrari, HSR, Milano) were grown in standard conditions[17]. β-K562 were originally purchased from ATCC (CCL-243™). Doubling times were calculated on cells growing in exponential phase. β-K562 authentication was obtained by short tandem repeat fingerprinting (AmpFlSTR Identifiler Plus PCR Amplification kit -Applied Biosystems-), as described in [18]. For

chemical treatments, 5 x 10^4 cells were exposed to increasing doses in 24-well plates. After four days, cells were analyzed by RTqPCR or high-content analysis. All experiments were performed in triplicate (at least two technical replicates per experiment). Chemicals and antibodies are listed in S1 Table.

siRNA oligonucleotide transfections

β-K562 cells were transfected with siRNA oligonucleotides (H-Silencer Select Druggable Genome siRNA Library V4, Ambion). A siRNA oligo targeting the proteasome subunit PSMC3 and a non-targeting oligo (siNTO) were used as positive and negative controls for transfection (siRNA sequences are listed in S2 Table). At least two siRNAs oligonucleotides per gene were transfected by using lipofectamine® RNAiMAX (Invitrogen), as described in S1 File.

Immunofluorescence and high-content analysis

Cells were collected and fixed in 3.7% paraformaldehyde for 20' at RT, washed and permeabilized in staining buffer (PBS with 0.05% v/v Triton® X-100 and 1% w/v powdered milk) for 30'. After washing in PBS, cells were incubated overnight at 4°C in staining buffer containing the appropriate antibodies and 1μg/ml Hoechst 33342. After washing, cells were resuspended in PBS and transferred to 96-well CELLSTAR®, Black/μClear® plates (Greiner Bio-One). Plates were spun for 5' at 2g to facilitate cell attachment, sealed and analyzed with the ArrayScan VTI high-content screening reader (Thermo-Fisher Scientific). At least 600 cells were acquired in each well with a 20x magnification in three fluorescence channels (blue, green and red). The Molecular Translocation Bioapplication was used to determine the cell count per field, the nuclear area and intensity (based on the Hoechst staining in the blue channel) and the cytoplasmatic fluorescence intensity of β (green) and γ (red) globins. For simultaneous globins/GlycophorinA staining, APC-anti-CD235 antibody was added for 2 hours to cells already stained for globins.

Confocal microscopy

K562 and β-K562 cells, stained as above, were transferred to a microscope glass slide and mounted with Mowiol (Sigma-Aldrich). Microphotographs were acquired with a confocal Zeiss microscope LSM710.

Flow cytometry

10^6 cells were washed, fixed and permeabilized for 10' on ice, then washed and incubated in PBS+1% milk for 20'. After washing, cells were stained overnight at 4°C in PBS+1% milk containing the appropriate antibodies. After washing, cells were analyzed with FACSCalibur (Becton Dickinson).

RNA Isolation and RT-PCR

Total RNA from 10^6 cells was extracted with TRI Reagent (Applied Biosystems), treated with RQ1 DNase (Promega) for 30' at 37°C and retrotranscribed (Applied Biosystems). Negative control reactions (RT⁻) gave no signal. Real time analysis was performed using ABI Prism 7500, (Applied Biosystems). Primers are listed in S3 Table.

Statistical analysis

Each experiment was statistically analyzed using a paired, two tailed Student-*t*-test.

Results

Identification and characterization of a K562 variant subclone expressing β-globin

K562 are probably the most extensively used cellular model of a human "erythroid" cell. This line was established in 1975[19] from a patient with chronic myelogenous leukemia (CML) in blast crisis. K562 have been widely used to study the molecular regulation of embryonic and fetal globin genes and to assess the therapeutic potential of differentiation-inducing drugs[20]. However, the major limitation for their use in the study of the differential γ/β regulation is their "fetal-like" pattern of globin genes expression, since they exclusively express embryonic (HbGower-2, α2ε2) and fetal (HbF, α2γ2) hemoglobin[21,22].

While characterizing different K562 subclones, we came across a variant clone expressing the adult *HBB*, that we named β-K562. These cells are morphologically similar to ECACC (European Collection of Cell Culture) K562 cells, here considered as "prototypical" K562 cells (not shown). Moreover, K562 and β-K562 have a similar doubling time (S1A Fig) and are equally sensitive to drugs known to inhibit K562 proliferation: imatinib mesylate and dasatinib, two tyrosine kinase inhibitors targeting the bcr/abl fusion protein and doxorubicin, a DNA intercalating agent (S1B Fig). In addition, β-K562 fingerprinting characterization by using the STR AmpFlSTR Identifiler Plus, gave a profile substantially corresponding to K562 (Identity score[18] of 89.2%, not shown). Despite their *bona fide* "K562-like" profile, β-K562 do express the adult β-globin chain, as assessed by flow cytometry (FCM) analysis (S1C Fig).

Based on this observation, we reasoned that the β-K562 subclone could be used to set up an immunofluorescence high-throughput, high-content screening platform to search for new genes/drugs modulating hemoglobinization and, in particular, the γ/β ratio.

Development of a multiplexed high-content assay for the quantification of γ- and β-globin content in β-K562 at the single-cell level

$5x10^4$ K562 or β-K562 were seeded in 24-well plates. Nuclei were stained with Hoechst-33342; γ- and β-globins were immunostained by using specific PE-anti γ and FITC-anti β-globin antibodies, respectively (S1D Fig). Cells were subsequently analyzed with an Array Scan VTI reader (Thermo-Fisher Scientific) and data were acquired and processed as shown in Fig 1A and 1B to obtain an automated and quantitative fluorescence imaging at a single cell level. The intensity of the staining is automatically converted in the corresponding intensity of colors: blue for Hoechst, green for β-globin and red for γ-globin.

The detection threshold for the scoring of single γ^+-, β^+- and double $\beta^+\gamma^+$-cells was defined by using cells stained with the respective isotype controls (PE-IgG_1 and FITC-IgG_1, an example is shown in S1E Fig). When signals from the three single channels are merged (Fig 1A), the double expression of γ plus β results in an orange/yellow color of different intensity, depending on the amount of γ and β chains (see cells 3 and 6 in Fig 1B). This analysis allows measuring of both the percentage of single-positive (γ^+ or β^+) and of double positive ($\gamma^+\beta^+$) cells in each field. Moreover, the signal intensity per cell uncovers the intrinsic heterogeneity within the cell population. In a standard experiment, data are acquired from a minimum of 500 cells and plotted to give an immediate visual image of cell distribution with respect to globin accumulation per cell, as in Fig 1A, where a representative experiment is shown. The majority of K562 cells are γ^+ (54.1% $\gamma^+\beta^-$ + 3.1% $\gamma^+\beta^+$), the remaining being mostly $\gamma^-\beta^-$, with just a few marginally β^+ cells (3.1% $\gamma^+\beta^+$+0.7% $\gamma^-\beta^+$). In contrast, about 57% of β-K562 cells are γ^+ (38.3% $\gamma^+\beta^-$ + 18.7% $\gamma^+\beta^+$) and about 22% of cells are positive for β staining (18.7% $\gamma^+\beta^+$ + 3.6% $\gamma^-\beta^+$). Moreover, the Mean Fluorescence Intensity (MFI, Y axis) of γ^+ cells is higher than in β-K562 (Fig 1A and S1C Fig). The

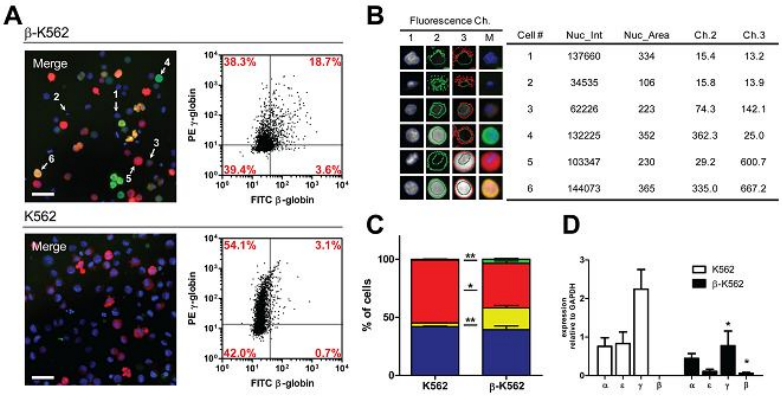

Fig 1. Analysis of γ/β globin levels by immunofluorescence and automated image capture. A) Image acquisition and analysis for β-K562 and K562. Merged signals of DNA (Hoechst-33342), β-globin and γ-globin are read in channel 1 (Ch1), channel 2 (Ch2) and channel 3 (Ch3), respectively (see also S1D Fig). Bar = 50μm. The intensity value of signals is automatically assigned by the instrument and converted into a corresponding intensity of color. The relative scatter plots show the distribution of double $\gamma^-\beta^-$ negative, single $\gamma^+\beta^-$ positive, single $\gamma^-\beta^+$ positive and double $\gamma^+\beta^+$ positive cells (x axis: FITC-β-globin; y axis: PE-γ-globin). Numbers within plots refer to the averaged percentage of cells within each population from three independent experiments (n = 3). The relative st.errors are shown in panel C: *p<0,05; ** p<0,01; ***p<0,001. B) Quantitative fluorescence imaging of single cells: cells numbered from 1 to 6 in panel A are taken as an example of $\gamma^-\beta^-$ double negative (1 and 2), single $\gamma^+\beta^-$ positive (5), single $\gamma^-\beta^+$ positive (4) and $\gamma^+\beta^+$ double positive (3 and 6). C) Statistical analysis (n = 3): $\gamma^-\beta^-$ cells; red: $\gamma^+\beta^-$ cells; yellow: $\gamma^+\beta^+$ cells; green: $\gamma^-\beta^+$ cells. D) RTqPCR on α, ε, γ- and β-globins. Histograms show the relative levels of expression normalized on glyceraldehyde-3-phosphate dehydrogenase (GAPDH). n≥3, statistical analysis: *p<0,05; **p<0,01; ***p<0,001.

doi:10.1371/journal.pone.0141083.g001

expression of β-globin in β-K562 was further confirmed by RTqPCR (Fig 1D). Of interest, an intrinsic heterogeneity of the culture is present also for β signal: the large majority of β^+ β -K562 cells also co-express γ-globin (18.7% of total cell population, corresponding to ≈ 85% of β^+ cells, Fig 1C), whereas only few cells appear to be completely "switched" to β expression (3.6% of total cell population, corresponding to≈ 15% of β^+ cells, Fig 1C). Overall, these data confirm that β-K562 express β-globin and provide additional information on the population heterogeneity.

Validation of γ/β globin high-content assay by using the known hemoglobin inducers hydroxyurea and butyric acid

To test the sensitivity of the method in detecting changes of γ- and β-globin levels, we treated β-K562 cells with the known Hemoglobin inducers, hydroxyurea (HU) and butyric acid (BA). We measured the response of β-K562 cells at different pharmacological concentrations of these drugs (S2 Fig) and we analyzed the same cell samples by both RTqPCR and immunofluorescence. Fig 2 summarizes data relative to drug concentrations most commonly used in the literature to induce hemoglobinization in K562.

Both inducers, as expected, increase the hemoglobin content, reducing the number of double negative cells (Fig 2A and 2B). hydroxyurea induces both γ and β chains accumulation, as demonstrated by the increased percentage of $\gamma^+\beta^+$ double positive cells. Instead, butyric acid especially increases the percentage of $\gamma^+\beta^-$ cells, suggesting a different mechanism of action for

these compounds (Fig 2B). In addition to increasing the number of globins-positive cells, these drugs also strongly increase the proportion of highly fluorescent cells (S2B Fig) acting predominantly on γ-globin accumulation versus β-globin.

At the mRNA level, both HU and BA stimulate γ expression by about 6–7 fold relative to untreated cells, whereas β-globin expression is essentially unchanged (Fig 2C). Finally, α-globin is moderately induced by both drugs, with BA eliciting the strongest increase (about four times). Overall, RTqPCR data confirm the effects observed at the protein levels, indicating the reliability of the assay.

The visual microscopy analysis can provide further information on additional parameters at the single cell level, such as nuclear morphology (Fig 2D) and/or the expression of specific markers of interest. As an example, the quadruple staining of β-globin, γ-globin, nuclei and GlycophorinA shows increased levels of GlycophorinA in β-K562 exposed to HU (Fig 2D).

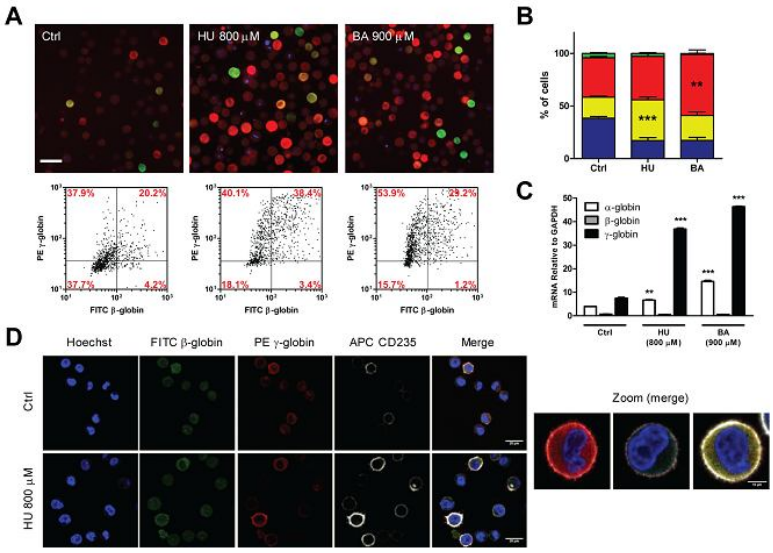

Fig 2. High-content analysis of compound-induced changes in globins accumulation. β-K562 cells were treated with 800μM hydroxyurea and 900μM butyric acid (n = 3, a representative experiment is shown here) and the same cells were analyzed in parallel by immunofluorescence and by RTqPCR 4 days after the addition of the drugs. A) Immunofluorescence images (Bar = 50μm) and relative scatter plots. Data from three independent experiments are presented and statistically analyzed (B) as in Fig 1. C) RTqPCR on α-, γ- and β- globins. Histograms show the relative levels of expression relative to GAPDH. D) Confocal analysis of β-K562 cells untreated or treated with HU as in panel A and subjected to a quadruple staining with Hoechst (blue), anti β- (green), anti γ-globin (red) and anti-CD235a (white). Magnification: 20x. Right panel: 40x magnification of individual cells γ⁺CD235a⁺ or β⁺CD235a⁺ double positive and γ⁺β⁺CD235a⁺ triple positive, respectively.

doi:10.1371/journal.pone.0141083.g002

The transfection of a panel of siRNAs confirms the effectiveness of the immunomicroscopy platform to identify genes affecting hemoglobin synthesis and to test the efficacy of their modulators

We then tested the efficacy of our method in identifying genes that could affect hemoglobinization and/or γ/β ratio by siRNA transfection. Firstly, we set up different types of controls: i) as a negative control we transfected a non-targeting oligo (siNTO); ii) as a positive transfection control we targeted the proteasome 26S subunit, ATPase3 (siPSMC3), the knockdown of which should severely affect cell growth; iii) we knocked-down γ-globin (siHBG1) and β-globin (siHBB) obtaining almost complete ablation of the respective signals (S3A Fig).

To test the sensitivity in capturing changes in γ/β ratio, we knocked down HDAC3, reproducing the γ-globin promoter de-repression induced by treatment with butyric acid and its derivatives [23]. In HDAC3kd cells, we observed a marked increase in both γ- and β-globins, suggesting a broad mechanism of transcriptional de-repression (Fig 3A and 3B). In line with this, the cells treatment with HDAC inhibitors entinostat (MS-275) -an inhibitor of HDAC1 and HDAC3- and dacinostat (LAQ-824) induced γ-globin accumulation in a dose response manner (Fig 3C and 3D and S3B and S3C Fig). These results further confirm HDAC as targets for γ-globin reactivation and identify two additional inhibitors–in addition to BA (Fig 2)-, as potential therapeutic agents activating γ-globin.

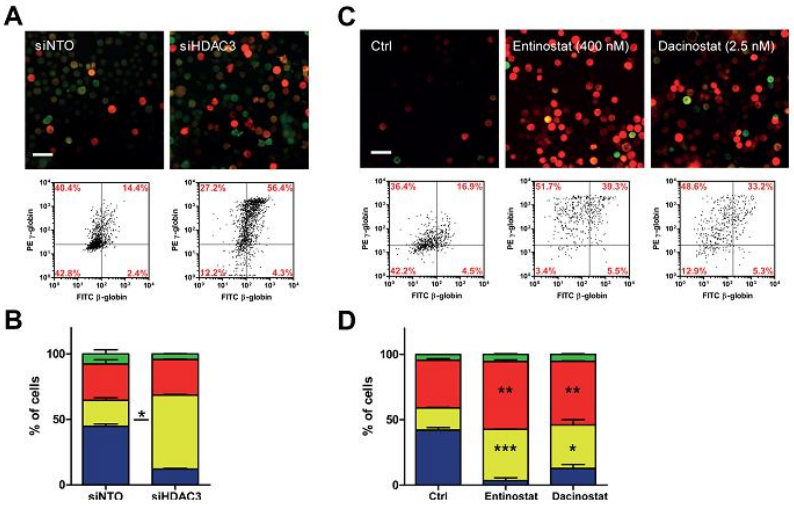

Fig 3. High-content γ/β globin analysis as readout of siRNA screening in β-K562 confirms HDAC as targets for γ-globin activation. A) Cells were transfected with a non-targeting oligo (siNTO) as negative control and with a siRNA directed to HDAC3. Two siRNAs were tested, with two technical replicates. C) β-K562 treated with two different HDAC inhibitors: entinostat and dacinostat (see also S3 Fig). A and C) Immunofluorescence images (Bar = 50μm) and relative scatter plots. Data from three independent experiments are presented and statistically analyzed (B and D) as in Fig 1.

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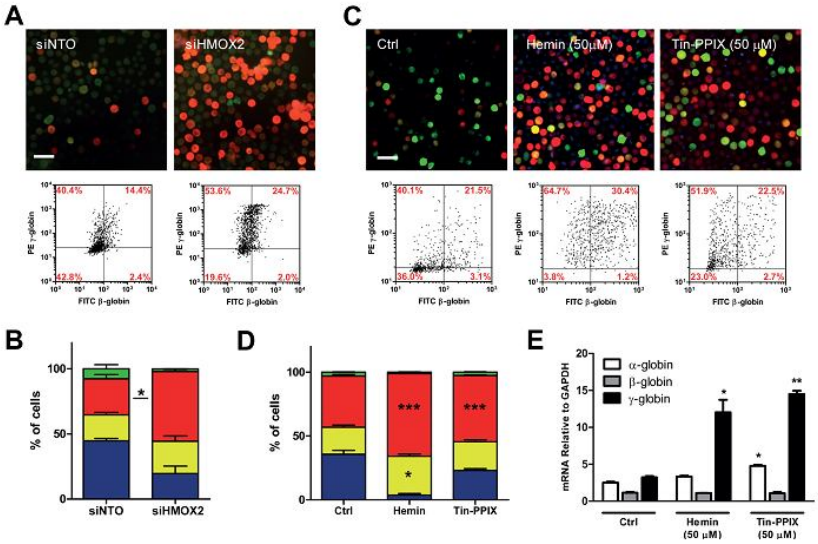

Fig 4. HMOX2 siRNA-mediated knockdown and hemin or Tin-PPIX treatment have similar effects on β-K562 hemoglobinization levels. A) Cells were transfected with a non-targeting oligo (siNTO) as negative control and with a siRNA directed to HMOX2. Two siRNAs (see also S4A Fig) were tested, with two technical replicates. C) Cells were treated with 50μM of either hemin or Tin-PPIX. A, C) Immunofluorescence images (Bar = 50μm) and relative scatter plots. Data from three independent experiments are presented and statistically analyzed (B and D) as in Fig 1. E) RTqPCR on α-, γ- and β-globins from cells treated with hemin or Tin-PPIX. Histograms show levels of globins expression relative to GAPDH (n = 3).

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Is Heme-oxygenase a potential target for γ-globin induction?

Given the above results, we undertook a pilot transfection screening on β-K562 with a panel of 70 siRNAs from the Ambion V4 library (S4 Table). Amongst them, siRNAs targeting the Heme-oxygenase (HO) coding gene HMOX2 (S4A Fig) gave a striking increase in globins with a prevalent accumulation of highly γ-globin expressing cells (Fig 4A and 4B). Heme-oxygenase catalyzes the conversion of heme to biliverdin (that, in turn, is immediately converted into bilirubin), iron and carbon monoxide. Both hemin, and Heme-oxygenase work on heme pool homeostasis, albeit in an opposite way, the first by replenishing the heme pool and the latter by promoting its degradation[24]. We then reasoned that the effect of the pharmacological inhibition of HO could result in globins stimulation similar to that known to be elicited by hemin (and possibly by the HMOX-2 knockdown shown in Fig 4). To test this hypothesis, we performed a dose/response treatment for hemin and Tin protoporphyrin IX (Tin-PPIX), here considered as prototypical HO inhibitor (S4B Fig). Fig 4C and 4D shows a striking increase in the percentage of γ-globin expressing cells and in γ-globin accumulation obtained upon Tin-PPIX treatment. Interestingly, whereas hemin treatment significantly increases the percentage of

double $\gamma^+\beta^+$ positive cells, Tin-PPIX seems to have a more selective activity on γ, as confirmed by the MFI values (S4C Fig). Instead, at the mRNA level, the effect of hemin and Tin-PPIX are very similar (at equal concentrations), suggesting that post-transcriptional effects may play additional roles in balancing the α/non-α ratio.

Discussion

We present a reliable and sensitive assay based on the unique property of β-K562 that express both γ and β genes to perform a first step high-throughput screening (HTS) to identify genes/drugs influencing the γ/β globin ratio. This overcomes the major limitation of the available human erythroid cell lines to study hemoglobin switching, i.e. their exclusive expression of embryonic/fetal genes. Indeed, HTS approaches published so far are almost exclusively based on cell lines transfected with a variety of reporters, under the control of γ and β globins promoters in the context of artificial genes/genomic arrangements[25],[26]. Recently established IPs-derived immortalized cell lines [27] represent a very promising tool for similar studies, but we feel that the easiness of growth and manipulation of β-K562 still represent a valuable advantage. Moreover, the growth factors independence of β-K562 prevents the possibility that globins expression could be influenced by subtle changes in growth conditions, and/or by other manipulations required to establish hES/hIPs.

More physiological models for these studies are also available: mice carrying artificial chromosome constructs encompassing the entire human β-locus, some of them containing knock-in fluorescent reporters under the control of globins promoters[28–30]. The advantage of such models with respect to cell lines is counterbalanced by their reduced manageability making them unsuitable for first-step HTS.

β-K562 cells are a valid tool for first-step screening because of their spontaneous expression of both γ- and β-globins from the intact β-locus (Fig 1). The presence of $\gamma^+\beta^+$ double positive cells and the plasticity in modulating γ and β expression suggest that in β-K562 the chromatin environment at the β locus is overall relatively accessible, making these cells particularly sensitive in detecting possible drugs/siRNA effects on γ activation. Regarding this issue, it is important to note that also in normal adult individuals there is a low proportion of HbF-positive cells–a few percent-, and immature cells in adults are known to transiently express γ-globin [31,32]. This suggests that a permissive environment for γ-globin expression may be present, although transiently, also in adult human cells, and could be modulated by drug treatment.

Specific antibodies allow a reliable picture of the final readout of interest, i.e. the amount of β- and γ-globin protein upon different drugs treatments/genes manipulations at the single cell level, providing hints about the heterogeneity of the response. In parallel, RTqPCR provides information on the differential regulation (transcription/RNA processing and stability versus translation) of globin expression (as well as on any other gene of interest) elicited by drugs/treatments (Figs 2–4) and modulators (siRNA targeting of HDAC3 and HMOX2, Figs 3 and 4).

The microscopy analysis can be further implemented to simultaneously analyze changes in multiple cellular parameters, such as morphology and/or expression of specific markers (Fig 3E), thus allowing the detection of possible effects of the tested drugs/treatments on different cellular processes. Importantly, the globin expression analysis is performed at the single cell level. This latter aspect is of particular relevance since the response to pharmacological agents that increase HbF is expected to involve either the accumulation of γ-globin in each single cell (due to transcriptional and/or translational effects) or the selection of subsets of erythroid differentiating "responder" cells, on the basis of a pre-existing heterogeneity.

β-K562 cells, allowed an efficient automated transfection-based screening, that led to the confirmation of HDACs as "druggable" targets for γ reactivation[16] (Fig 3) and to the identification of Heme-oxygenases (Fig 4) as possible novel target.

Regarding HDACs, we tested the effect on β-K562 of three different inhibitors: butyric acid (BA), a well known γ-inducing agent, entinostat (MS-275) and dacinostat (LAQ-824). Dacinostat has been considered for myeloid leukemia treatment because it promotes apoptosis in CML and AML cells [33]. Entinostat, an inhibitor of HDAC1 and HDAC3, is an inducer of cell differentiation in AML cells, not associated with apoptosis induction[34–36]. Whereas BA appears to specifically lead to γ-globin expression, entinostat and dacinostat lead to an increase in both γ- and β-globins, suggesting a different selectivity profile of these three compounds.

Concerning Heme-oxygenases, our study identified them as a novel class of potential druggable targets to reactivate γ-globin. Our results show that drug competitive inhibition of Heme-oxygenases by protoporphyrins (and thus presumably by other derivatives) is able to induce a substantial increase in γ-globin accumulation. In erythroid cells, the main function of heme is to serve as the oxygen-carrying moiety in hemoglobin (Hb), and heme biosynthesis is thus strictly coordinated with globin accumulation along with erythroid differentiation and maturation[37]. Heme, when present at high concentrations in the globin-unbound state (as in β-thalassemia), inactivates the heme-regulated eukaryotic initiation factor eIF2α-kinase (Heme Responsive Inhibitor, HRI), converting it into an inactive form. As active HRI inhibits the eIF2α translation initiation factor, excess heme increases globin synthesis, allowing a better balance between heme and globin chains[38]. Therefore, Heme-oxygenase inhibition, by increasing heme levels, is expected to favor globin synthesis. In addition, heme catabolism by oxidation is linked to generation of biologically active molecules, such as iron, biliverdin, CO and NO[24,37]. The observation that both heme addition and inhibition of HO increase globin expression in β-K562 is consistent with the expected role of heme in relieving translational inhibition by HRI; however, the increase of globin mRNA levels, particularly of γ-globin (mRNA and protein) is not easily explained only by a simple effect on globin mRNA translation. It is possible that the translational effect of HRI also operates on other factors, for example transcription or chromatin factors regulating the *HBG1/2*. Previously, studies of HMOX1 deficiency demonstrated a clear effect on both stress and steady state erythropoiesis[39–42], but less is known about the possible specific role of the constitutive HMOX2 (the most abundant isoform expressed by K562[43]) in erythroid cells[37,44]. Of interest, a polymorphism in the HMOX1 gene was associated with high levels of fetal hemoglobin in Brazilian patients with sickle cell anemia[45].

Both HO-1 and HO-2 are sensitive (albeit to a different extent) to the competitive inhibition elicited by different Metalloporphyrins and the availability of different drugs with different selectivity make HOs an attractive target for pharmacological inhibition. This result is of particular interest because different Metalloporphyrins have been developed and tested in clinics [46]. Our results suggest that their use, as single agent or in association with other known and FDA-approved HbF inducers, such as HU, should be explored as a promising tool to improve the α/non α globin chain imbalance in β-hemoglobinopathies. To address this question we are currently studying the effect of different Metalloporphyrins in mice carrying a complete human HBB locus transgene e and in *ex-vivo* cultures from thalassemic patients.

Supporting Information

S1 Fig. Characterization of the β-K562 subclone by comparison with ECAAC-K562. A) Growth curves (n = 2). B) Response (IC_{50}) to imatinib mesylate, dasatinib and doxorubicin ($n \geq 3$). C) FCM analysis: cells were stained with anti γ- and anti β-globin antibodies and with the corresponding isotype controls and read in FL-1 (FITC, green channel) or in FL-2 (PE, red channel). A representative experiment is shown. **Immunofluorescence setup.** D) In the immunofluorescence analysis, nuclei were stained with Hoechst-33342; HbF and HbA were immunostained by using specific anti γ- and anti β-globin antibodies and signals were acquired in

three single channels: blue for Hoechst (Ch1), green for β-globin (Ch2) and red for γ-globin (Ch3), respectively, and then merged for analysis (Merge). E) Images acquired in the single channels for representative isotype controls and relative scatter plots. Bar = 50μm.
(TIF)

S2 Fig. Dose/response of β-K562 to Hydroxyurea and Butyric Acid. A) Representative ArrayScan pictures of β-K562 cells treated with increasing doses of HU and BA (n≥3). Bar = 50μm. B) Fluorescence intensity plots to better visualize the changes in mean fluorescence intensity (MFI) of stained cells upon drugs treatment. Y axis: number of events (cells); X axis: fluorescence intensity for β-globin signal (upper panels) or γ-globin signal (lower panels), respectively. Green/Red curves: treated cells. Black curve: untreated cells. The vertical dotted line within each panel corresponds to the threshold set in Fig 2A. The MFI and the percentage of positive cells (%) are indicated within each panel.
(TIF)

S3 Fig. HDAC3 siRNA-mediated knockdown and HDAC inhibitors treatment in β-K562 confirm HDACs as targets for γ-globin activation. A) Cells were transfected with a non-targeting oligo (siNTO) as negative control and with a siRNA directed to PSMC3 as positive transfection control. As further control, siRNAs targeting γ- and β-globins greatly reduced the corresponding globins chains. For each gene, two siRNAs were tested, with two technical replicates (immunofluorescence images of representative experiments are shown). Bar = 50μm. Scatter plots are provided for each immunofluorescence image (n = 2). B) Representative ArrayScan pictures of β-K562 cells treated with increasing doses of entinostat and dacinostat. C) MFI plots as in S2 Fig.
(TIF)

S4 Fig. Hemin and Tin-PPIX have similar effects on β-K562 hemoglobinization levels. A) HMOX2 knockdown: RTqPCR on cells transfected with a non targeting oligo (siNTO) and with two independent siRNA directed to HMOX2. B) Representative ArrayScan pictures of β-K562 cells treated with increasing doses of Hemin and or Tin-PPIX. Bar = 50μm. C) MFI plots as in S2 Fig.
(TIF)

S1 File. siRNAs oligonucleotide detailed transfection method.
(PDF)

S1 Table. Chemicals and antibodies.
(PDF)

S2 Table. List of siRNA oligos.
(PDF)

S3 Table. List of primers used for RTqPCR.
(PDF)

S4 Table. List of genes tested by siRNA-mediated knockdown and selected from the Ambion-library.
(PDF)

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Author Contributions

Conceived and designed the experiments: FG AER. Performed the experiments: MD CF SE MGM MFM IF PC GB IFM MM. Analyzed the data: MD CF PM FG AER. Contributed reagents/materials/analysis tools: PC MM. Wrote the paper: PM SO FG AER.

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CHAPTER 5:

FINAL DISCUSSION. MOLECULAR AND CLINICAL CONSIDERATIONS

5.1 WHY IT IS IMPORTANT TO STUDY SOX6?

SOX6 is a transcription factor belonging to the Sry-related HMG-box TFs family. It controls terminal differentiation and lineage specification of many cell types by mediating cell cycle withdrawal and activation of lineage specific genes (Hagiwara, 2011). Its role in the hematopoietic system has been thoroughly explored: SOX6 sustains cell survival, proliferation and terminal maturation during definitive murine erythropoiesis (Dumitriu, 2006).

A report published by our laboratory demonstrated that SOX6 is a powerful inducer of terminal erythroid differentiation. Its overexpression induced significant erythroid differentiation in the human erythroleukemic BCR-ABL$^+$ K562 cell line and in primary erythroid cultures from human cord blood CD34$^+$ cells. Furthermore, SOX6 is a potent inducer of growth arrest in leukemic BCR-ABL$^+$ K562 cells (Cantù, 2011). Taken together, these data hint to the EPO-JAK-STAT and IGF1-IGF1R pathways as possible SOX6 effectors (Dey, 2000; Sasaki, 2000), since K562 rely on these two pathway for their growth (Lakshmikuttyamma, 2008).

SOX6 is therefore a potent inducer of cell proliferation arrest and differentiation in erythropoiesis as it does in different cell types (such as: chondrocytes, myocytes, cardiocytes, erythrocytes, olygodendrocytes, corical interneuron) (Lefebvre, 1998; Han, 2008; Ikeda, 2004; Hagiwara, 2000; Cohen-Barak O., 2001; Dumitriu, 2006; Cantù, 2011; Hamada-Kanazawa, 2004a, Hamada-Kanazawa, 2004b).

SOX6 has been proposed to have a tumor suppression function in various human malignancies, including chronic myeloid leukemia (Cantù, 2011), esophageal squamous cell carcinoma (Qui, 2011) and hepatocellular

carcinoma (Xie, 2012; Guo, 2013). It is also frequently downregualted in different human malignancies (Xie, 2012; Guo, 2013; Qui, 2011). Although SOX6 has also been porposed as a prognostic marker and potential therapeutic target in some malignancies, the molecular mechanism underlying SOX6 tumor suppression activity remains unclear. A better knowledge of SOX6 biological pathways associated with cancer development and progression can provide opportunities to develop targeted therapeutics for cancer treatment, with higher specificity, efficiency and safety.

Thus, having a broader picture of SOX6-dependent downstream signaling mediating cell cycle withdrawal and cell fate specification might reveal important targets relevant for cell cycle progression in hematopoietic cells and possibly in other cell types.

To this aim in my work, I investigated the effect of SOX6 ectopic expression in different model systems of leukemia (BCR-ABL$^+$ and JAK2V617F$^+$), to test whether high SOX6 levels can force these cells to resume differentiation and overcome their pathological condition.

5.2 SOX6 OVEREXPRESSION ENHANCES ERYTHROID DIFFERENTIATION PROGRAM, SIMULTANEOUSLY REPRESSING ALTERNATIVE LINEAGE-SPECIFIC DIFFERENTIATION PROGRAMS.

SOX6 overexpression enhances erythroid differentiation in bipotent megakaryoblastic and erythroleukemic cells (K562 and HEL) and enforces megakaryoblastic cells towards an erythroid fate (MEG.01, SET2 and UKE1) (chapter 2: fig. 2.7; fig. 2.15; fig. 2.20) .

Thus, in cells with both erythroid and megakaryocytic potential, SOX6 overexpression is capable of repressing the megakaryocytic lineage specific program (assessed in this study by the ability to transcriptionally

activate/repress key regulators) and of promoting the onset of an erythroid program. Indeed, genes such as globins (α and γ) and ALAS-E are activated in presence of SOX6, whereas genes such as MPL, GPIIb, FLI1, RUNX1, affiliated to a megakaryocytic lineage choice, are downregulated for all the cell lines analyzed in this study (chapter2: fig. 2.7; fig. 2.15; fig. 2.20).

It is surprising that a single gene, is able to activate many aspects of erythroid differentiation, in the absence of any other chemical stimuli (such as hemin) and without inducing changes in already known major transcription factors involved in erythropoiesis specification. (i.e. GATA1, EKLF) (chapter2: fig. 2.7; fig. 2.15; fig. 2.20). This is surprising taking into account that SOX6 has been so far considered as a "late" erythroid transcription factor and that its knock out in the erythroid lineage has a relatively mild effect (Dumitriu, 2006 a, Dumitriu, 2006 b).

Unexpectedly, SOX6 overexpression in a lymphoblastic cell line (SUPB15) is capable of downregulating genes essential for B-cells identity (such as PAX5, EBF1, BLNK, PU1) whereas it is not sufficient to activate an erythroid program (chapter 2: fig. 2.7).

Together these data indicate that SOX6 is sufficient to repress differentiating program alternative to the erythroid one but it is able to activate erythroid lineage-affiliated genes only in cells that are hierarchically already compatible with an erythroid specification, as MEG.01.

Given these results, i.e. the ability of SOX6 to interfere with the expression of key lymphoid genes (PU1, PAX5, EBF1, BLNK) (chapter 2: fig. 2.7; chapter 3: fig. 3.5) it will be of interest to test whether in a Pre-B cells $PAX5^{-/-}$ background SOX6 overexpression is able to trigger erythroid differentiation. This could be possible since PAX5 promotes B cell commitment by

repressing lineage-inappropriate gene expression and reinforcing B cell specific gene expression (Nutt, 1999).

Busslinger and his colleagues demonstrated that pro-B cells derived from $PAX5^{-/-}$ mice develop into multiple other tissues including macrophages, osteoclasts, dendritic cells, granulocytes, and natural killer cells after culture in appropriate growth factors (Nutt, 1999). However, restoration of PAX5 expression in these cells restricts them to the B-cell lineage implying that PAX5 represses these alternate lineage choices under normal conditions.

In the case of SUPB15 cells, the "conflict" induced by the simultaneous expression of genes normally expressed in alternative lineages may be responsible of cell fate decision towards cellular death.

For all these reasons understanding the mechanism by which SOX6 can interfere with specific lineage program and how this is coupled with cell death in leukemic cells could reveal interesting potential therapeutic drugguble target.

5.3. SOX6 ROLE IN CELL CYCLE WITHDRAWAL

Another interesting aspect of SOX6 is its ability to induce cell cycle withdrawal in leukemic cells carrying different genetic lesions: the BCR-ABL fusion protein and the JAK2V617F mutation.

In fact, SOX6 overexpression blocks proliferation in all the tested $BCR\text{-}ABL^{+}$ cell lines, irrespective to their lineage commitment (erythroleukemic K562, megakaryoblastic MEG.01, lymphoblastic SUPB15 and B-ALL BCR-ABL GFP^{+}) (chapter 2: fig. 2.3; fig. 2.8; chapter 3: fig. 3.3), although through different mechanisms.

In MEG.01 the exhaustion of the cultures of $SOX6^{+}$ cells is mainly due to apoptosis more than a block in cell cycle progression (chapter 2: fig. 2.4;

fig. 2.5). instead, in SUPB15 we can observe an alteration of the cell cycle in all different phases (chapter2: fig. 2.8) and an accumulation of apoptotic/necrotic cells (chapter2: fig. 2.9). In both cases, SOX6 overexpression lead to cell cycle withdrawal ad apoptosis. For MEG.01 probably this effect could be mediate by SOCS3 and by its ability to interfere with signal transduction pathway (Dey, 2000; Sasaki, 2000) altered in BCR-ABL$^+$ cells. Indeed, MEG.01 and K562 cells overexpressing SOX6 (or SOCS3) display a reduction in IGF1 expression (chapter 2: fig. 2.2.), suggesting that SOX6 plays a pivotal role in blocking cell proliferation probably through SOCS3 regulation (fig.5.1). This result is of particular interest since BCR-ABL is known to transforms a variety of cytokine-dependent cell lines to factor independence, possibly through its ability to induce expression of cytokines such as IL-3, G-CSF , GM-CSF, M-CSF, IL-1b and VEGF (Lakshmikuttyamma, 2008). In K562 cell, BCR-ABL regulates IGF1 expression and inhibition of IGF1R reduces viability, proliferation and apoptosis, suggesting a crosstalk between IGF1R and BCR-ABL signaling (Lakshmikuttyamma, 2008; Cantù, 2011).

SUPB15 are a lymphoid cell line where SOCS3, IGF1 and IGF1R are not expressed. The arrest of cell proliferation, upon SOX6 transduction in this cellular context is likely due to apoptosis and to a block in cell cycle progression, possibly triggered by CyclinD1 downregulation (chapter2: fig. 2.9; fig. 2.10).

Taking advantage of JAK2V617F cell lines, we tried to uncouple the two main effect downstream of SOX6 overexpression: erythroid differentiation versus cellular growth arrest. Cells carrying this mutation can escape the inhibitory control imposed by SOCS3, which is a direct SOX6 target (Hookham, 2007, Cantu', 2011).

By using three JAK2V617F$^+$ cell lines (two thrombocythemic: UKE1 and SET2, one erythroleukemic: HEL) we were able to demonstrate that the ability of SOX6 to induces erythroid differentiation does not rely on SOCS3 activation (chapter 3: fig. 2.15; fig. 2.20). Instead, SOCS3 is very important in regulating cell cycle withdrawal and apoptosis (fig. 5.1). In fact, the presence of high copy number of the JAK2V617F allele, confers resistance to SOCS3 and makes HEL cells insensitive to apoptosis. However, in the presence of a lower copy number of the JAK2V617F allele, as in SET2 and UKE1 cells, there is a proportional increase in the rate of apoptosis (chapter 2: fig. 2.23; fig. 2.24) induced by SOX6. This difference is also reflected in the cellular growth rate: the higher is the JAK2V617F copy number allele,the higher is the capability to expand even upon SOX6 overexpression (chapter 2: fig. 2. 21; fig. 2.22).

Thus, the presence of the JAK2V617F allele in multiple copy mirrors a graded resistance to SOCS3. It is also interesting to note that SOX6 overexpression leads to a change in the cell cycle profile of JAK2V617F$^+$ cells. The decrease of the percentage of cells in S phase in the different cell types is again proportional to the number of JAK2V617F alleles (85% in HEL cells, 55% in SET2 cells and 8% in UKE1, respectively). This effect is possibly linked to an increased erythroid terminal differentiation (chapter 2: fig. 2.16; fig. 2.25). In fact, it is known that terminal erythropoiesis is associated with a progressive reduction of S phase (Pop, 2010). The reduced effect on S phase reduction elicited by SOX6 in SET2 and UKE1 cells could also reflect their “non erythroid” nature.

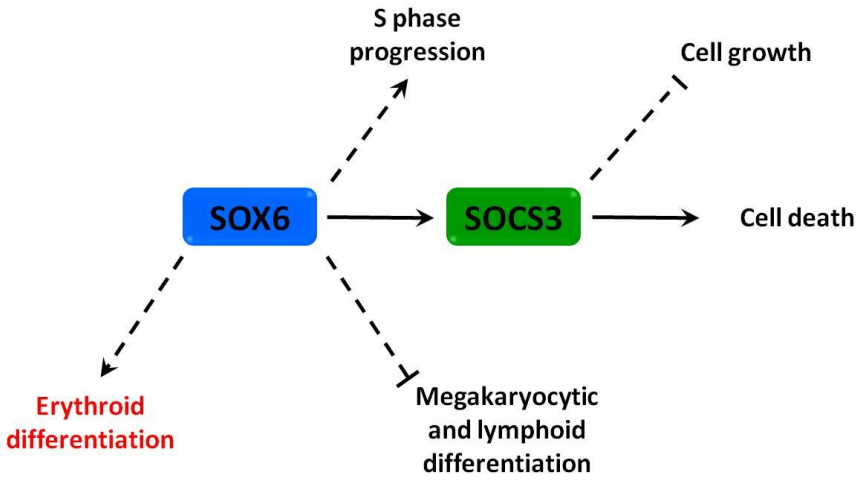

Fig. 5.1: Diagram showing the effects of SOX6 overexpression observed in different cellular model systems.

Malignant transformation leads to a block in differentiation at a distinct stage, which result in the accumulation of immature myeloid or lymphoblastic leukemic cells. Several observations suggest the strategy of inducing malignant cells to overcome their block of differentiation and to enter the apoptotic pathways as an elegant alternative to killing cancer cells by cytotoxic therapies (Nowak, 2009). Tumor cells are blocked at early or intermediate stages of differentiation. Agents able to induce them to reenter their normal differentiation program can lead to loss of tumorigenicity (Leszczyniecka, 2001).

To date, such "differentiation therapy" has only been used routinely in a subtype of acute myeloid leukemia, namely, in acute promyelocytic leukemia (APL). Currently, >95% of patients with APL can expect to be cured by differentiation therapy alone without the use of cytotoxic chemotherapy (Lo-Coco, 2013). In contrast, no differentiating therapies have proven effective in the treatment of B cell Acute Lymphoblastic Leukemia (B-ALL).

We know that forcing malignant B-ALL cells to differentiate into mature, B cells may not be possible because requires the coordinated expression of multiple genes (Nutt, 2007). PAX5 deficient pro-B cells retain the capacity to "reprogram" into cells of the myeloid lineage (Nutt, 1999; Reynaud, 2008). However, similarly to mouse PAX5$^{-/-}$ pro-B cells, the primary human B-ALL cells might be poised to reprogram to the myeloid lineage. Recent studies using single polymorphism (SNP) arrays have shown that in B-ALL the BCR-ABL positivity is associated with high frequency of PAX5 deletions. Furthermore, the expression of receptors for myeloid cytokines by B ALL cells has been described (Munoz, 2001) and myeloid reprogramming of PAX5$^{-/-}$ cells can be triggered by exposure to myeloid cytokines.

Many studies strongly support the concept that the levels of certain transcription factors are an important determinant of the proliferation versus differentiation decision in leukemia and in other types of malignant cells (Rosembauer 2007; Papetti, 2007; Liu, 2014). The restoration of PAX5 itself in B-ALL PAX5$^{-/-}$ cells causes rapid cell cycle exit and disables their leukemia-initiating capacity.

5.4 SOX6 INTERFERES WITH THE ONSET OF LEUKEMIA IN MURINE MODEL MIMICKING B-ALL BCR-ABL^{+}.

In chapter 3, I took advantage of a clone of P190 BCR-ABL GFP^{+} induced B Acute Lymphoblastic Leukemic cells (B-ALL) to explore the impact of SOX6 ectopic expression in this type of leukemia in *vitro* and in *vivo*.

The theoretical basis of this experiments are the results of obtained on SUPB15 cells, showing that SOX6 has an impact on B-ALL BCR-ABL^{+} cell identity: it decreases the expression of PAX5 and of the surface marker of B cells B220 (chapter 3: fig. 3.5). In parallel, cellular viability (chapter 3: fig 3.3; fig. 3.4 is reduced. I was able to confirm these results in *vivo.* In fact,

experiments in chapter 3: paragraph 3.3.2 show that B-ALL BCR-ABL$^+$cells overexpressing SOX6 are unable to engraf in the murine model.

In *in vivo* experiments, I considered two aspects downstream to SOX6 ectopic expression: i) the onset of the leukemic phenotype in the injected recipients mice (SOX6-injected mice vs EV-injected mice); ii) the competition generated by the simultaneously injection of un-transduced B-ALL GFP$^+$ and of transduced SOX6-BALL or EV-BALL within the same recipient mouse.

Related to the first point, leukemia was induced not only in SOX6 injected mice but also in their respective controls (chapter3: fig. 3.8 a-b). This result was expected and it is due to the residual amount of un-transduced B-ALL GFP$^+$ cells within the injected cells (5% in EV-BALL vs 1% in SOX6 B.-ALL) (chapter 3: fig. 3.7; fig. 2.9 b). These residual un-transduced GFP$^+$ cells are sufficient to give rise to leukemia in all the recipient mice. This observation is consistent with the results obtained in the laboratory of prof Ghysdael (Institute Curie, Paris), who kindly provided us the B-ALL BCR-ABL GFP$^+$ cells. In fact, in serial dilution experiments, they observed that 100 B-ALL BCR-ABL GFP$^+$ cells are enough to induce leukemia in the injected mice within 18 days.

However, if we consider the number of GFP$^+$/ΔNGFR$^+$ cells in the spleen and in the bone marrow (chapter 3: fig. 2.9b), SOX6-BALL cells are substantially unable to engraft into recipient mice, being the percentage of GFP$^+$/ΔNGFR$^+$ cells expressing SOX6 almost equal to zero. GFP$^+$/ΔNGFR$^+$ cells in mice injected with cells transduced with the empty vector also tend to decrease, possibly because to some toxic effect due to the viral transduction. However, the decline of SOX6-BALL injected cells is hundred times higher than in the control (chapter 3: fig. 2.10; fig. 2.11), supporting the notion that SOX6 suppress cell growth and engraftment.

As a future development of this line of research, I will try to set up an analog approach on human models of leukemogenesis. This approach is feasible but it requires the optimization of many technical aspects.

From the experiments of many groups, it is clear that primary leukemic cells (especially B-ALL blasts) are very fragile (Biagi, 2001) and require serum-free suspension-culture systems for their growth (Cox, 2004). Another important caveat is the great variability in cell survival between leukemic cells. Furthermore, primary cells are very sensitive to transduction conditions to recipient mice genetic background. The nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mouse strain, which lacks functional mature B and T cells, has been shown to be particularly receptive to engraftment of normal and malignant human hematopoietic cells (Shultz, 2007). The NOD/SCID model of B-ALL retains the phenotypic and genotypic characteristics of the original patient sample and provides an accurate representation of the human disease (Cox, 2004). Fortunately, the development of biologic assays that permit the investigation of human B-ALL cells properties *in vitro* and *in vivo* should make it possible to characterize the effect of ectopic expression of SOX6 also in primary cells.

5.5 SOX6 INTERACTORS

SOX6 has the ability to both activate and repress gene expression, depending on its interactions and on its target sequences (Kamachi, 2000; Kiefer, 2007; Lefebvre, 2007). Despite its dual function of activator and repressor, SOX6 lacks any conventional transactivation or transrepression domain (Hagiwara, 2011). This evidence suggests that SOX6 must partner with other proteins to exert its function. This characteristic results in a great SOX6 functional versatility in so many cellular contexts.

We undertook the identification of SOX6 partners in erythroid cells to elucidate the different aspects of SOX6 biology in this cell type (chapter 4.1). Amongst SOX6 partners, we focused our attention on the transcription factor COUP-TFII. In fact, both TFs have been described to have a role in the hemoglobin switching (Yi, 2006; Xu, 2010; Liberati, 2001). Our study elucidated the interplay between SOX6 and its interactor COUP-TFII in the regulation of globin genes expression.

The mRNA and protein analysis of the expression pattern of both TFs in mouse fetal liver cells during embryonic development at the E11.5, E12.5 and E13.5 stages, revealed that the two TFs have an opposite pattern of expression: whereas SOX6 expression increases, COUP-TFII decreases during development and they are simultaneously present at similar levels only at E12.5 (chapter4: fig. 4.5). To mimic this situation we overexpressed these two factors β-K562 cells, a subclone of cells expressing both γ and β globins. In these cells, the modulation of the relative levels of COUP-TFII and SOX6 suggest that COUP-TFII promotes ε- and γ- globin transcription at the expense of α and β adult genes. In the same cells, forced expression of SOX6 induces all globins (as expected on the basis of its role as a general inducer of erythroid differentiation) but especially β, confirming a potential functional antagonisms of these two proteins (chapter 4: fig. 4.7; fig. 4.8). These studies, together with others results (Elangovan et al., manuscript in preparation), elucidate the ability of COUP-TFII to partially overcome the γ-globin gene repression imposed by the adult erythroid environment (in which known repressors such as BCL11A-XL and SOX6 are present) and suggest an alternative therapeutic strategy in view of γ reactivation.

In this perspective, the identification of possible COUP-TFII activators might provide an important contribution to develop new therapies for β-hemoglobinopathies.

Because of the high potential of SOX6 acting in different cellular contests, the identification of the protein interactors of SOX6 in erytroleukemic HEL cells might reveal also interesting candidate responsible for SOX6-induced cell cycle withdrawal. Related to this aspect, the affinity tag-purification of SOX6 interactors, identified several peptides of different Cyclin dependent kinases (CycK, CDK11, CDK12 and CDK13) that could be implicated in erythropoiesis regulation.

By combining all the different approaches described above we are trying to identify SOX6 partners/targets that could be responsible for the growth arrest/differentiation elicited by SOX6. The candidate genes/proteins will be tested for their de-regulation upon SOX6 overexpression in the cells of interest.

5.6 APPLICATIONS OF HIGH-THROUGHPUT PLATFORM

In paragraph 4.5 (chapter 4), in Durlak et al., we describe a high content screening platform based on multiplex imaging to identify new potentially therapeutic drugs/genes able to reactivates γ globin expression (Durlak, 2015). Taking advantage of this novel platform, we applied it to carry out a high-content screening on several of the most promising SOX6 partners identified in the Affinity-Tag purification approach. The goal is to discover new genes affecting hemoglobinization and the γ/ β globin expression ratio. The set platform is also suitable to identify molecules/drugs able to interfere with proliferation and survival of leukemic cells. The terminal goal is the development of more targeted and specific therapies for leukemia carrying specific genetic lesions.

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