Jak2 ed eritropoeisi inefficace in Beta-Talassemia

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SUMMARY

β-Thalassemia, one of the most common congenital anemias, arises from partial or complete lack of β-globin synthesis. β- Thalassemia major, also known as Cooley anemia, is the most severe form of this disease, and is characterized by ineffective rythropoiesis (IE) and extramedullary hematopoiesis (EMH), requiring regular blood transfusions to sustain life. In β-thalassemia intermedia, where a larger amount of β-globin is synthesized, the clinical picture is milder and the patients do not require frequent transfusions. The ineffective production of red blood cells in both forms of the disease has been attributed to erythroid cell death during the maturation process mediated by apoptosis or hemolysis. It was proposed that accumulation of alpha-globin chains leads to the formation of aggregates, which impair erythroid maturation triggering apoptosis.[6-13] Ferrokinetic studies done in 1970 suggested that 60% to 80% of the erythroid precursors in β-thalassemia major die in the marrow or extramedullary sites. However, several observations call into question the view that cell death is the only cause of IE in β-thalassemia. First, the number of apoptotic erythroid cells in thalassemic patients is low compared with that anticipated by ferrokinetic studies.[14,15] In fact, only 15% to 20% of bone marrow (BM) erythroid precursors (CD45+/CD71+) present apoptotic features in aspirates from affected patients.[6,8,16] Second, hemolytic markers in young β-thalassemic patients are normal or only slightly increased, unless the patients suffer from splenomegaly or the liver has been damaged by iron overload or viral infections. Third, the original ferrokinetic studies[18-21] do not exclude that the majority of the iron administered to patients affected by IE could be directly stored by liver parenchymal cells rather than being used by erythroid cells. This would explain the ferrokinetic studies without invoking massive erythroid apoptosis or hemolysis. Given the controversies in the literature over the cause of IE, we have undertaken a detailed investigation of this process in 2 mouse models that mimic β-thalassemia intermedia *(th3/+)* and major *(th3/th3)*. In *th3/+* mice, both the β-*minor* and β-*major* genes have been deleted from one chromosome. Adult *th3/+* mice exhibit hepatosplenomegaly, anemia, and aberrant erythrocyte morphology comparable with that

found in patients affected by β-thalassemia intermedia. Mice completely lacking adult β-globin genes *(th3/th3)* die late in gestation, limiting their utility as a model of β-thalassemia major. To circumvent this problem, we undertook bone marrow transplantation, wherein hematopoietic fetal liver cells (HFLCs) were harvested from *th3/th3* embryos at embryonic day 14.5 (E14.5) and injected into lethally irradiated syngeneic wild-type (wt) adult recipients. Hematologic analyses of engrafted mice performed 6 to 8 weeks after transplantation revealed severe anemia due not to pancytopenia but rather to low red blood cell (RBC) and reticulocyte counts together with massive splenomegaly and extensive EMH. These animals could be rescued and the hematologic parameters, splenomegaly, and EMH normalized by lentiviral-mediated β-globin gene transfer or by blood transfusion, supporting the notion that their phenotype is specifically due to erythroid impairment. In this way, we established the first adult mouse model of β-thalassemia major. The principal regulator of both basal and stress erythropoiesis is erythropoietin (Epo). Interaction of Epo with the Epo receptor (EpoR) induces, through Jak2 and Stat5, multiple signaling pathways designed to prevent apoptosis and to support erythroid mproliferation. The severity of the anemia in *Stat5*-deficient mice correlates with the relative loss of *Bcl-XL* expression. Bcl-XL prevents apoptosis during the final stages of erythroid differentiation rather than at the erythroid colony-forming unit (CFU-E) or proerythroblast stage as shown by several groups. Therefore, up-regulation of *Bcl-XL* mediated by Epo is expected to protect erythroid cells primarily during the final stages of differentiation. Thus, abnormal Epo levels as well as increased synthesis or posttranslational modification of cell cycle–associated proteins could play a crucial role in regulating the proliferation and apoptosis of erythroid cells in β-thalassemia. The present results provide 5 new major findings. First, the mechanism leading to a disproportionate number of proliferating erythroid cells in β-thalassemia is associated with expression of cell cycle–promoting and survival factors that mitigate apoptosis. Second, although our data do not exclude a role for apoptosis in triggering IE, they suggest that controlling maturation of erythroid precursors plays an important role in this process. Third, our observations point to additional factors, intrinsic and/or extrinsic, that limit erythroid differentiation in β-thalassemia. Fourth, we have demonstrated that inhibition of Jak2 has a profound effect both in vitro and in vivo, limiting erythroid cell proliferation and reversing splenomegaly in thalassemic mice. Finally, some of the animal results have been corroborated in human blood and spleen specimens from patients, suggesting that our findings can be extended to the human disease.

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INTRODUCTION

Chapter1

BIOLOGY OF ERYTHROPOIESIS

1.1 Erythroid progenitors cell compartment

Erythropoiesis is the mechanism through which a multipotent stem cell gives rise to a mature red blood cell (RBC). The erythroid progenitor cell compartment, situated functionally between the multipotent stem cell and the morphologically distinguishable erythroid precursor cells, contains a spectrum of cells with a parent-to-progeny relationship, all committed to erythroid differentiation. The first, more primitive class, consists of the burst forming units-erythroid (BFU-E), named for their ability to give rise to multiclustered colonies (erythroid bursts) of hemoglobin-containing cells. BFU-E represent the earliest progenitors committed exclusively to erythroid differentiation and a quiescent reserve, because only 10% to 20% are in cycle any given time. However, once stimulated to proliferate in the presence of the appropriate cytokines, they demonstrate a significant proliferative capacity in vitro, giving rise to colonies of 30,000 to 40,000 cells or more, which become fully hemoglobinized after around 15 days. In contrast to this class of progenitor cells, a second, more differentiated class of progenitors consists of the colony-forming units-erythroid (CFU-E). Most (60-80%) of these progenitors are already in cycle and thus proliferate immediately after initiation of culture to form erythroid colonies within 7 days. Because CFU-E are more differentiated than BFU-E, they require fewer divisions to generate colonies of hemoglobinized cells, and the colonies are small (8-65 cells). Although the two classes of committed erythroid progenitors, BFU-E and CFU-E, appear distinct from each other, in reality progenitor cells constitute a continuum, with graded changes in their properties, such as differential expression of surface markers and sensitivity to different growth factors (Fig1). Only progenitor cells at both ends of the differentiation spectrum have distinct

properties. The earliest cell with the potential to generate hemoglobinized progeny is defined as

	CFU-GEMM (CMP)	BFU-E	CFU-E
General features			
Self-renewal	++	+	0
Differentiation potential	Multipotent	Erythroid Committed	Erythroid Committed
Cycling status % suicide with ³H Thymidine	15-20	30-40	60-80
Cell density (g/mL)	<1.077	<1.077	<1.077
Incidence/10^5 cells	2-5	40-120	200-600
Circulate in blood	+	+	0
Growth Factor Response			
Erythropoietin	+	+	++
Thrombopoietin	+	+	+
KIT ligand	+	+	–
GM-CSF, IL-3	+	+	–
Flt3 ligand	+	0	0
G-CSF, IL-6, IL-1	+	0	0
Insulin, insulin-like growth factor, activin	0	0	+
Tumor necrosis factor-β1	–	–	++
Hyper-IL-6	+	+	+
Receptor/Antigen			
CD34	++	++	–
CD33	+	+	0
c-KIT	++	++	–
HLA-DR (-DP, -DQ)	++	++	+
Erythropoietin receptor	+	+	++
gp130	+	+	+
Tumor necrosis factor receptor	+	+	++
Ep-1[59]	+	+	++
23.6[309]	0	0	+
CD36	0	±	+
Glycophorin A	0	0	+
ABH, iI[†]	0	+	+
Adhesion Molecules			
VLA-4 (CD49d/CD29)	++	++	++
VLA-5 (CD49e/CD29)	+	+	++
CD41	+	+	
CD11a/CD18	+	+	
CD44	+	+	
HCAM[‡]	+	+	
Transcription Factors			
GATA-2	++	+	–
▸GATA-1	+	++	+++
Scl	+	+	+
EKLF	+	+	++
Myb	++	+	–
▸Id1,2	++	+	–

Figure1. Changes in the general properties of erythroid progenitors during differentiation

common myeloid progenitor (CMP) as is capable of giving rise to mature cells of at least another lineage (granulocytic, monocytic or megakaryocytic) in addition to the erythroid. The CMP and the BFU-E have physical and functional properties in common, such as high proliferative potential, low rate of cycling, response to a combination of cytokines, and presence of specific surface antigens (Fig1). In contrast, the latest CFU-E display limited proliferative potential, they cannot self-renew, they lack the cell surface antigens common to all early progenitors, and they are exquisitely sensitive to erythropoietin (EPO). BFU-E display receptors for stem cell factor (C-KIT), EPO (EPOR), thrombopoietin (TPO) (MPL), GM-CSF, IL-3, IL-6 and IL-11 (Clark and Kamen 1987). CFU-E display some of these receptors, but the most important functional difference with BFU-E is the abundance of EPOR and thei dependence on EPO for cell survival. CFU-E, in contrast to BFU-E, cannot survive in vitro even for a few hours in the absence of EPO. Although more than 80% of CFU-E have detectable EPOR (Sawada, Krantz et al. 1988), only a small proportion of BFU-E have receptors (Broudy, Lin et al. 1991) and can terminally differentiate in culture in the presence of EPO alone (Migliaccio, Migliaccio et al. 1988). Direct binding studies show that the number of EPOR peaks at the CFU-E/proerythroblast stage and progressively declines when cells mature further (Sawada, Krantz et al. 1988) , reflecting the decline in the influence of EPO. In addition to the abundance of EPOR, erythroid progenitors are distinguished by the presence of high levels of transferrin receptor (TfR1/CD71) (Sawyer and Krantz 1986). Peak levels of transferring receptors are seen on CFU-E and erythroid precursors, and lower levels are present on reticulocytes (Sawyer and Krantz 1986)

1.2 **Erythroid precursors cell compartment**

The earliest recognizable erythroid cell is the proerythroblast, wich after four to five mitotic divisions and serial morphological changes, gives rise to mature red blood cells. Its progeny include basophilic erythroblasts, which are the earliest daughter cells,, followed by polychromatophilic and orthochromatic erythroblasts. Their morphologic characteristics reflect the accumulation of erythroid-specific proteins (i.e. hemoglobin) and the decline in nuclear activity (Fig.2). After the last mitotic division, the inactive dense nucleus of the orthochromatic erythroblast moves to one side of the cell and is extruded, encased by a thin cytoplasmatic layer. Expelled nuclei are ingested by macrophages, and the resulting enucleated cell is a reticulocyte. It is unlikely that the maturation from proerythroblast to the reticulocyte always adheres to a rigid sequence in which each division is associated with the production of two more differentiated and morphologically distinct daughter cells (i.e. with a basophilic erythroblast giving rise to two polychromatophilic ones). Rather, significant flexibility may be allowed, both in the number and rate of divisions and in the rate of enucleation. Such deviation from the normal orderly maturation sequence may be dictated by the level of erythropoietin. Thus, when there is an acute or chronic demand for red blood cell production (because of blood loss or ineffective erythropoiesis as seen in thalassemia syndromes), the kinetics of formation of new reticulocytes are significantly more rapid. Resulting red blood cells may be larger (i.e with increased mean corpuscular volume, MCV). This has led to the concept of "skipped" divisions (Stohlman, Ebbe et al. 1968). In addition, studies in the mouse and observation in humans have shown that, to accommodate acute of chronic needs in red blood cell production, the progenitor cell compartment undergoes changes in their relative frequencies and recruitment of the spleen or liver, as an additional hematopoietic site. This phenomenon, called Extra-Medullary-Hematopoiesis (EMH) is particularly common in anemias where there is a definciency in red blood cell production, like β-thalassemia.

Mature red blood cells (RBCs) are packed with a very high concentration of hemoglobin, approximately 5 mM in both humans and mice (Chen 2007). Thus, during erythroid differentiation and maturation, it is critical that the 3 components of hemoglobin, α-globin, β-globin, and heme, are made in the 2:2:4 ratio in order to form stable $\alpha_2\beta_2$ hemoglobin complexed with 4 heme molecules. Imbalance of these 3 components can be deleterious since each component is cytotoxic to RBCs and their precursors. The importance of equimolar

concentration of α- and β-globin proteins is best illustrated by the prevalent human red cell disease of thalassemia, and the significance of the globin-heme ratio is well demonstrated by the hypochromic anemia in iron deficiency.
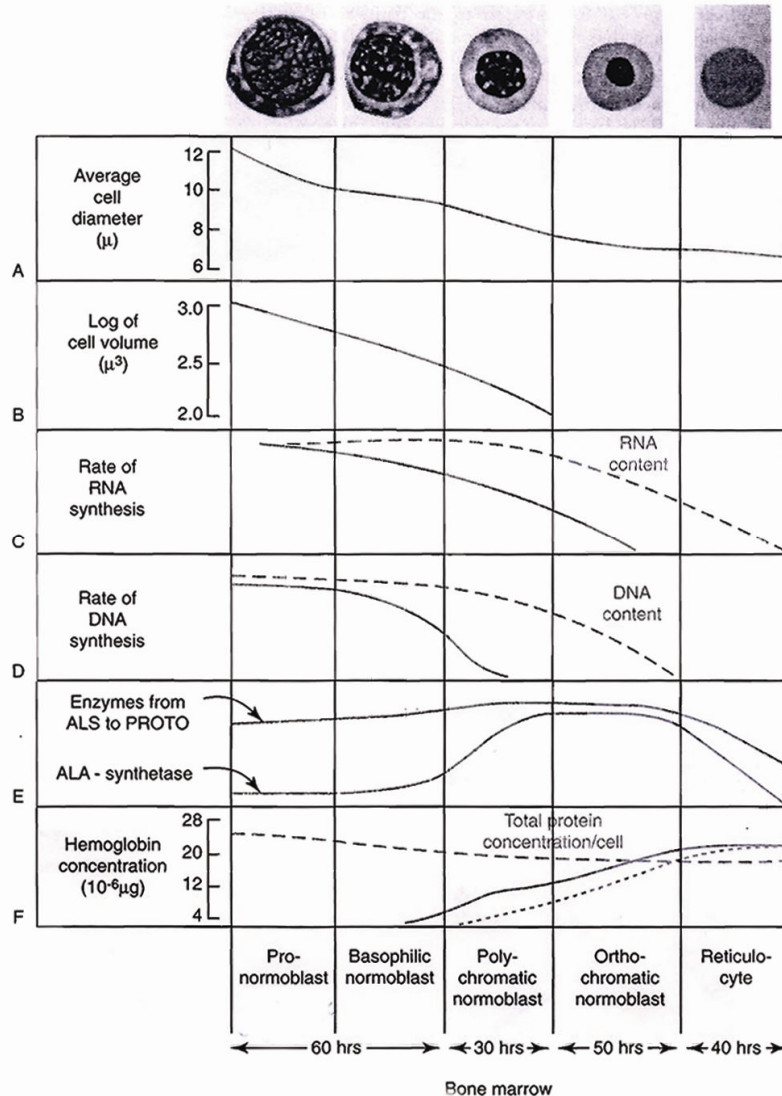
Fig 2. Erythroid maturation sequence. As proliferation parameters (i.e. rates of DNA and RNA synthesis) and cell size decrease, accumulation of erythroid specific proteins (i.e. heme and globin) increases, and the cells adapt their chacteristic morphology

Iron and heme play very important roles in hemoglobin synthesis and erythroid cell differentiation (Ponka 1997). Heme iron accounts for the majority of iron in the human body, and hemoglobin (the most abundant hemoprotein) contains as much as 70% of the total iron content of a healthy adult. In addition to serving as a prosthetic group for hemoglobin, heme also regulates the transcription of globin genes through its binding to the transcriptional factor

Bach1 during erythroid differentiation (Taketani 2005). Therefore the iron uptake is a pivotal process in erythropoiesis, and in erythroid precursors it is exclusively mediated by the transferrin receptor 1 (TfR1 also called CD71), that upon binding to holo- transferrin is internalized and recycled on the surface of the cell. CD71 is found in extreme abundance in erythroid cells (300-800,000/cell) (Iacopetta, Morgan et al. 1982), and this is a reflection not only of the proliferative needs of erythroid cells but also of their extreme requirements for iron uptake for hemoglobin synthesis, as stated above. It is for this reason that CD71 persist in maturing, non-dividing erythroblasts and reticulocytes. CD71 belongs to a large group of receptors that internalize their ligand through receptor mediated endocytosis. This cycle allows for reuse of both the ligand (transferrin) to be resaturated with iron and of the recptor to enter an additional route of endocytosis (Huebers and Finch 1987). CD71 density decrease with maturation , and after the reticulocyte stage, receptors appear to be shed as small lipid vescicles (Huebers and Finch 1987). There is an inverse relationship between receptor density and iron availability. At low-iron conditions, *trans*-acting regulatory proteins (IRP1+2) bind to conserved hairpin structures (iron responsive elelements, [IRE]) in the 3'-untranslated region (UTR) of CD71 mRNA, which selectively stabilizes this mRNA and ensure proper CD71 cell-surface expression and iron uptake (Hentze, Muckenthaler et al. 2004). Upon iron excess, IRP-1 is converted to a cytosolic aconitase catalyzing isomerization citrate to isocitrate (Haile, Rouault et al. 1992), while IRP-2 is degraded by the proteasome (Guo, Phillips et al. 1995). Thus, both proteins no longer bind to IREs, resulting in strongly reduced CD71 mRNA stability, which leads to reduced CD71 surface expression and iron uptake (Koeller, Casey et al. 1989).

Recently two independent studies (Kerenyi, Grebien et al. 2008; Zhu, McLaughlin et al. 2008) show how both CD71 mRNA stability and transcription are regulated by the activation of Stat5 a/b, linking the erythropoietin signaling to iron metabolism. In these works it shown how embryos lacking the expression of Stat5a/b die perinatally by severe microcytic anemia due to decreased expression of CD71 on the surface of erythroid progenitors. The selective deletion od Stat5a/b in the hematopoietic lineage also leads to microcytic hypochromic anemia due to a reduction of 50% in expression of CD 71. Therefore the activation of Stat5 seems crucial and maybe a role in this activation could be played by the EPO-EPOR-JAK2 signaling axis.
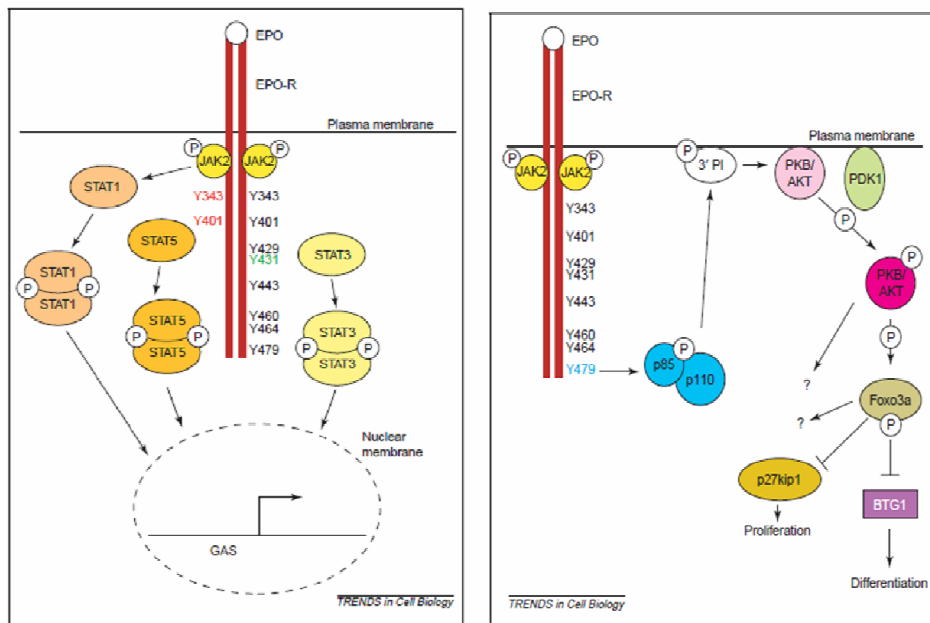

1.3 Erythropoietin

Epo, a 35 kD glycoprotein (D'Andrea, Lodish et al. 1989), is the physiologically obligatory growth factor for erythroid development. It is produced in the kidney by peritubular   cells

(Koury, Bondurant et al. 1988). A heme-containing protein senses oxygen need and then triggers the synthesis of Epo and its release into the bloodstream (Goldberg, Dunning et al. 1988; Ebert and Bunn 1999). According to the prevailing model of hematopoiesis, progenitor cells committed to erythroid differentiation (i.e BFU-E) are generated in a stochastic fashion from pluripotent stem cells (Enver and Greaves 1998). Neither Epo nor other lineage-restricted regulators play any role in determining lineage commitment. According to this model, Epo influences erythroid differentiation by rescuing (from apoptosis) cells that express the erythropoietin receptor (EpoR) and amplifying them further. Autoradiography studies in purified BFU-E populations indicate that EpoR increase as BFU-E mature to CFU-E, with the highest level observed at the CFU-E/proerythroblast boundary (Sawada, Krantz et al. 1988). The BFU-E and CFU-E can be generated *in vitro* (Papayannopoulou, Brice et al. 1993) and *in vivo* in the absence of Epo or EpoR, but their survival and terminal differentiation normally depend on Epo. For CFU-E, Epo seems to stimulate all the biochemical processes characterizing erythroid cells (i.e., heme synthesis, globin synthesis, and synthesis of cytoskeletal proteins). However, the precise role of Epo in these processes has not been delineated. Whether Epo only increases the number of cells engaged in these biochemical changes or directly influences the intracellular mechanism of transcription of erythroid specific proteins is unclear. Indeed, experiments in vitro showing complete maturation of BFU-E in absence of Epo suggest that other factors or combination of factors can influence red blood cell maturation (Sui, Tsuji et al. 1996; Kimura, Sakabe et al. 1997). Whatever the precise mode of Epo action, it directly affects the number of CFU-E and the maturation of their progeny. This control is achieved by influencing CFU-E survival and not the cycling status (Spivak 1986). CFU-E are irrevocably lost after one cycle of DNA synthesis if Epo is not present (Koury and Bondurant 1990). Direct binding studies have shown that as CFU-E amd the proerythroblast mature into reticulocyte, there is a progressive decrease in the number of EpoR (Broudy, Lin et al. 1991). Pure reticulocyte populations show detectable binding to Epo. The maturation-associated decline in the number of EpoR parallels declining influence of Epo on erythroid cells during terminal phase maturation.

1.4 Signal transduction by erythropoietin

Epo binds exclusively to its cognate receptor EpoR, that is expressed in the eryhtroid precursors. EpoR is a transmembrane potein with and extracellular domain responsible for the binding of Epo, a trans-membrane domain and a cytoplasmatic domain that mediates the signal

transduction (Richmond, Chohan et al. 2005). The membrane proximal positive regulatory region of EpoR binds constitutively to Jak2 (Witthuhn, Quelle et al. 1993), a cytoplasmatic tyrosine kinase necessary for erythroid differentiation, since mice lacking the corresponding gene die at early embryonic stage (Neubauer, Cumano et al. 1998). Jak2 is required for appropriate Golgi processing and cell surface expression of EpoR (Huang, Constantinescu et al. 2001) and, once activated by Epo-EpoR binding, initiates several events in EpoR mediated signal transduction. Jak2 initially activates the tyrosine phosphorylation of several tyrosine residues of the cytoplasmatic tail of the EpoR. These phosphorylated tyrosines next serve as docking sites for the binding of other cytoplasmatic effector proteins containing SH2 domains, such as the p85 subunit of PI3 kinase (Damen, Mui et al. 1993), the adaptor protein *Shc* (Barber, Corless et al. 1997) and the STAT transcription factors STAT5 (Damen, Wakao et al. 1995) and STAT3 (Wojchowski, Gregory et al. 1999). Once these proteins have docked on the EpoR, they become tyrosine phsophorylated and engage other downstream signaling events. Three major signaling pathways are activated by Epo: the STAT1, 3 and 5a/b pathway, the PI3K pathway and the MAP kinase pathway (Jelkmann 2004).

Fig.3. Signal transduction by the erythropoietin receptor (Richmond, Chohan et al. 2005)

The first above mentioned pathway, which involves the STAT family proteins, has been investigated in detail. Studies with cell lines showed that Epo activates only three of the seven members of STAT family: STAT1, 3 and 5a/b. In particular activation of STAT1 is dependent on Jak2 but does not require cytoplasmatic tyrosines of EpoR and STAT1 null mice show a

condition of stress erythropoiesis (Halupa, Bailey et al. 2005). Also STAT3 seems to be phosphorylated in response to Epo even if its role in erythropoiesis remains still unclear (Kirito, Nakajima et al. 2002). Of more relevance for the erythropoietic process seems though the phosphorylation of Stat5a/b: upon binding to the phosphor-tyrosines on the cytoplasmatic tail of EpoR, STAT5 is phosphorylated and dimerizes forming a hodimer complex that translocate into the nucleus and activates the expression of several genes involved in cell proliferation and differentatiation such as Pim-1, Citokine Inducible Sh2 Containing Protein (CIS), Mip-1α, Bcl-6 and β-casein (Matsumoto, Masuhara et al. 1997). One of the most important gene which expression is regulated by STAT5 is the antiapoptotic protein Bcl-X$_L$. Studies performed using KO mice for Stat5a/b showed the existence of a pool of immature erythroblasts exhibiting reduced Bcl-X$_L$ levels and undergoing apoptosis at high rate (Socolovsky, Nam et al. 2001). These evidences strongly suggest that antiapoptotic effect of Stat5 is exerted at level of erythroid precursors and is mediated by Bcl-X$_L$.

The second pathway activated by Jak2 is the PI3-kinase/Akt pathway that has an important role in supporting erythropoiesis (Ghaffari, Kitidis et al. 2006). Aky serine-threonine kinase of PKB family is a mjor effector of PI3K playing fundamental roles in the regulation of cell cycle, survival, differentiation and intermediary metabolism ((Brazil, Yang et al. 2004). Akt targets include GSK3, insulin recptor substrate-1, phosphodiesterase-3B, BAD, caspase-9, FOX and NF-kb transcription factors, mTOR, e NOS, Raf, BRCA1 and p21. Of particular interest is the role that PI3K/Akt pathway could play in the regulation of apoptosis. Indeed phosphorylation of BAD by Akt, a proapoptotic factor, inhibits its proapoptotic effects. In absence of phosphorylation, BAD is targeted to the mithocondria where it forms a complex with Bcl-2 or Bcl-X$_L$, and inhibits their antiapoptotic activity. Akt can also activate the transcription of antipoptotic genes through the activation of the transcription factof NFkB (Kane, Shapiro et al. 1999). When bound to its inhibitor IkB, NFkB localizes to the cytoplasm. Akt associates and activates the IkB kinases (IKKs) which phosphorylates IkB, targeting it for degradation by the proteasome. This allows NFkB to translocate to the nucleus and activate transcription of a variety of substrates including antiapoptotic genes such as the inhibitors of apoptosis c-IAP1 and 2. In erythroid cells the rapid Akt phosphorylation and activation in response to Epo has been recently demonstrated to be a regulator of GATA1 functions in orchestrating the full erythroid maturation such as the induction of β-globin, Bcl-X$_L$, and EpoR genes (Gregory, Yu et al. 1999). Another important consequence of Epo-mediated Akt activation is the inhibition of Foxo3a, whose target is p27. Indeed activation of PI3K in primary erythroblasts decreases the expression of p27 (Bouscary, Pene et al. 2003) inducing erythroid proliferation.

The third pathway activated by Epo involves the MAP kinases pathway. MAPK is classified in 4 distinct subfamilies: ERKs (ERK 1 and 2), p38 MPAK, JNK/SAPK and ERK5/BMK1. Between those, ERK ½, SAPK/JNK and p38 are directly activated by Epo, resulting in protection of erythroid cells from apoptosis by the inhibition of caspases (Mori, Uchida et al. 2003).

Previous studies have also suggested that erythropoietin functions synergistically with other multilineage growth factors, such as KIT ligand and IL-3. Epo and KIT ligand function together, resulting in increased erythroid colony cell growth in methylcellulose culture (Arcasoy and Jiang 2005). Recent studies with the EpoR polypeptide suggest a molecular mechanism for such synergy (Wu, Klingmuller et al. 1995). Activation of the KIT receptor by KIT ligand results in transphosphorylation of the EpoR at the cell surface. Also, a direct interaction between the EpoR and the KIT receptor has been demonstrated. Take together, these results suggest that receptor cross-talk at the cell surface may account, at least in part, for the physiologic interaction of some cytokines in controlling hematopoietic growth.

1.5 Molecular control of erythropoiesis

It is widely believed that lineage-specific transcriptional factors are responsible for regulating the expression of erythroid genes during ontogeny and during the course of erythroid differentiation. Stuidies in mice with targeted gene disruption have provided key insights into the complex molecular pathway that regulate hematopoiesis and erythropoiesis in particular (Cantor and Orkin 2002). Because erythropoiesis is the first differentiated lineage in embryonic yolk sac hematopoiesis and the predominant lineage in fetal liver hematopoiesis, factors that affect hematopoiesis in general will disturb erythropoiesis during early stages of development and will lead to lethality at different gestational days, depending on the defect. The time of development in which the disruption of each specific gene manifests its phenotype is used to establish a hierarchical control among the different transcription factors (fig7). The earliest disruption of erythroid differentiation is observed in mice lacking the bHLH factor Tal-1/*SCL*. SCL-null embryos are bloodless and die very early with abrogation of both yolk sac and fetal liver erythropoiesis (Robb, Lyons et al. 1995). Because of the requirement of *SCL* in the formation of the transcription complex wth the nuclear protein Rbtn2/LMO2 and *GATA1*, it is not surprising that targeted disruption of Rbtn2 an LMO2 also produces a bloodless phenotype (Warren, Colledge et al. 1994). Mice lacking GATA2 expression, a member of the GATA family of transcription factors, also exhibit an early and severe quantitative defect in

hematopoiesis that influences all lineages (Tsai, Keller et al. 1994). Other regulatoty factors seems to totally spare embryonic (yolk sac) hematopoiesis and have a specific effect only on fetal liver hemtopoiesis, with death occurring at later days (12.5 p.c.). In this category are the proto-oncogene c-myb and the core-binding factors CBFα2/AML-1 and CBFβ (Okuda, van Deursen et al. 1996; Wang, Stacy et al. 1996; Wang, Stacy et al. 1996).

Of pivotal importance for adul erythropoiesis is the transcription factor GATA1 (Shivdasani and Orkin 1996). The GATA1 protein controls erythroid differentiation at different levels : by controlling, in cooperation with GATA2, the proliferative capacity of erythroid progenitor/precursor cells; the apoptotic rate of the erythroblasts; and the expression of lineage specific genes. These effects are mediated through activation of expression of target genes by bindin to specific sequences, WGATAR, present in the regulatory domains of virtually any erythroid gene, including EpoR and GATA1 itself. Insights into the specificity of GATA1 in erythoid differentiation have been obtained by studies on the organization of the WGATAR sites in erythroid-specific regulatory sequences. A minimal erythroid transcription-activation sequence that consists of a core binding motif flanked by two canonical GATA1 binding sites has been identified. The core binding motif is composed of one SCL and one GATA binding site separated by 10 bp (Cantor and Orkin 2002). Different domains of the GATA1 protein are responsible for binding to the core and flanking elements. In fact, at least three functional domains have been identified in the GATA1 protein: two zinc-finger domains, the aminoterminal (NF) and carboxy-terminal (CF) finger, and an active amino-terminal domain. The NF domain is required for association with FOG-1: the GATA1/FOG1 heterodimeric complex binds to the two flanking sites of the minimal erythroid transcription activation domain. Experimentally induced genetic mutations impairing GATA1/FOG1 interaction in mice lead to impaired megakaryopoiesis and absence of definitive erythropoiesis while primitive erythropoiesis is normal (Chang, Cantor et al. 2002). The CF domain, on the other hand, binds to the GATA site localized in the core of the minimal erythroid transcription sequence 10 bp downstream to the SCL binding site. SCL and GATA1 bind simultaneously to thei respective sites of the core as multimeric complexes formed by SCL/E47/LMO2, on one hand, and by GATA1/LMO2, on the other. The binding of the two complexes to the core is then stabilized by Lbd1 that forms a physical bridge between them. In addition to forming heterodimers with LMO2, CF can also form complexes with two factors essential for myelomonocytic differentiation, Sp1 and Pu.1. Since the GATA1/PU.1 complex is unable to bind DNA, its function might be to establish either an erythroid or a myelo-permissive cellular environment, depending on which of the two factors is expressed at highest concentration (Cantor and

Orkin 2002). The presence of relatively high concentration of GATA1 would favor the formation of GATA1/LMO2 complex, leading to the activation of erythroid-specific genes, while the presence of relatively higher concentrations of PU.1 would lead mainly to the formation of the transcriptionally inactive GATA1/PU.1 complexes.

Transcription Factor	Binding Motif	Role in Hematopoiesis	KO phenotype	Mutations/Human disease
GATA-1	(A/T)GATA(A/G)	↑ erythroid differentiation	• no terminal erythropoiesis • arrest in Mk development (with hyperproliferation)	• X-linked thalassemia/ thrombocytopenia • Leukaemia (Down syndrome)
GATA-2	(A/T)GATA(A/G)	↑ proliferation ↓ differentiation	↓ proliferative expansion of primitive and definitive erythropoiesis; absence of mast cells	
FOG-1	None	GATA-1-cofactor	↓ erythroid maturation; block in megakaryocytopoiesis	
EKLF	CACCC	Promotes terminal erythroid differentiation	severe anemia; β-globin deficiency	β-thalassemia
SCL	CANNTG (E box)	Specification of hematopoiesis	absence of prenatal hematopoiesis. ↓ erythro/Mk in adults	translocation in T-ALL
LMO2	LIM domain		absence of hematopoiesis	T-cell acute lymphocytic leukemia
Myb	(T/C)AAC(G/T)G	↓ definitive erythropoiesis	block in definitive erythropoiesis	
Fli-1	winged helix-turn helix	Inhibition of GATA-1 expression		
BKLF	CACC		myeloproliferative disorder	
SHP-1 (BKLF activated?)				erythroleukemia polycythemia vera
STAT 5	GAS		transient fetal anaemia due to apoptosis of erythroid progenitors; mild anaemia, exacerbated by stress in adult life.	
PU.1	GGAA	↓ erythropoiesis	absence of myelo-monocytic differentiation	
Id		blocks terminal differentiation of all cell types		
IaPI-3 kinase (p85)		↓ proliferation/ differentiation	↓ fetal erythropoiesis perinatal death	
Gfi-1B	zinc finger domain	↑ proliferation (↑ GATA-2)		
Sp3			↓ fetal erythropoiesis perinatal death	
NF-E2	TGAGTCA	Promotes terminal erythroid differentiation in vitro	thrombocytopenia; absence of erythroid abnormalities (?)	

Fig.7. Major transcription factors/Signaling   molecules involved in the control of erythropoiesis

Indeed the lineage-specific action of GATA1 in the regulation of gene expression is achieved through the presence of lineage-specific regulatory sequences in the promoter regions of the target genes. Therefore, the relative concentration of GATA1, as opposed to the levels of few key regulatory partners, may establish a lineage-permissive environment. Further, the existence of lineage-specific sequences in the GATA1 gene itself ensure that such concentrations are achieved only in the right cell. Indeed, although GATA1 is expressed in erythroid, megakaryocytic, mast and eosinophilic cells, its levels of expression differ greatly among the various cell types, with erythroid cells expressing the most.

Another gene of the GATA family important for erythroid differentiation is GATA2. Both GATA1 and GATA2 are expressed early in multipotential progenitors; however, their expression ratios change as the cells differentiate (table), suggesting that the ratio of these two factors may be important at specific stages of erythroid differentiation. Knock-out experiments with both of these genes have showed that. Thus, in contrast to GATA2, expressed at high levels in early cells and affecting expansion of all hematopoietic lineages (Tsai, Keller et al. 1994), GATA1 expression increases as differentiation advances and seems to be the obligatory factor required for survival and terminal differentiation of erythroid cells. In mice with targeted disruption of GATA1, erythropoiesis proceeds only up to the stage of proerythroblast, which die early and fail to mature further (Fujiwara, Browne et al. 1996). Furthermore, transgenic mice with partial loss of function of GATA1 show that erythroid differentiation is dose-dependent with respect to GATA1 (McDevitt, Shivdasani et al. 1997). High levels of GATA1 are necessary to form complexes with its co-factor FOG1 (Tsang, Visvader et al. 1997) and with the other protein described previously during terminal erythroid differentiation.

Another factor with special importance in the erythroid lineage is the CACCC binding protein, designated erythroid Kruppel-like factor (EKLF), which is expressed at all stages of erythropoiesis but binds preferentially to CACCC sites in the β-globin promoter. Mice lacking EKLF die from a thalassemic-like defect, because of severe deficiency of β-globin expression (Nuez, Michalovich et al. 1995).

In summary, the emerging picture is that certain genes, such as SCL, are absolutely required for hematopoietic development, whereas others, such as GATA2, c-Myb, CBF and some downstream signal transducing molecules, such as Jak2 and Stat5, are responsible for expansion and maintenance of a normal pool of fetal liver and adult hematopoietic progenitors. The participation of many of these molecules in multicomponent molecular complexes with

protein/protein and protein/DNA interactions (i.e., LMO2/Lmbd1/SCL/GATA), during the early, proliferative stages of hematopoiesis (Visvader, Mao et al. 1997) may underlie their role in the proliferation and maintenance of immature progenitor/precursor pools in eryhtropoiesis. Other genes such as GATA1, its partner FOG1, and EKLF are necessary to direct high levels of function of erythroid-specific genes in cells already committed to terminal differentiation. Thus, a hierarchical requirement in the expression of specific regulators during early versus late eryhtroid differentiation or during embryonic versus fetal liver/adult erythropoiesis is established.

1.6 Cellular dynamics in stress erythropoiesis

The primary function of the end product of erythropoiesis, the mature red blood cell, is to transport oxygen efficiently through the circulation to the tissues. To achieve this goal, the adult marrow must release approximately 3×10^9 new red blood cells, or reticulocytes, per kilogram body weight per day (Donohue, Reiff et al. 1958). This number of reticulocytes represents $1/100^{th}$ of the total red blood cell mass and is derived from an estimated 5×10^9 erythroid precursors per kilogram (Donohue, Reiff et al. 1958). In addition to maintaining homeostasis (i.e., a stable hematocrit), the erythron must be able to respond quickly and appropriately to increased oxygen demands, either acute (i.e., following red blood cell loss) or chronic (i.e., hemolytic anemias). It is now well established that Epo is responsible both for maintaining normal erythropoiesis and for increasing red blood cell production in response to oxygen needs. The overall marrow response, however, is complex and requires not only the participation of erythroid cells responsive to Epo but also a structurally intact microenvironment and an optimal iron supply within the marrow. Epo stimulation elicits two types of measurable responses: changes in proliferative activity (including improved survival) and changes in maturation rates. Those effects are achieved through an intact Epo-EpoR-Jak2-Stat5 signaling axis. Indeed, transgenic mice that express a truncated knock-in allele of EpoR that preserve though the tyrosine 343, that is the binding site for Stat5, are able to respond to phenihydrazine-induced hemolysis, while mice that express an EpoR without phospho-tyrosine residues are only able to sustain steady state erythropoiesis and fail to recover from phenyhydrazine treatment (Menon, Karur et al. 2006). The first detectable response to increased serum Epo is amplification of CFU-E and erythroid precursors, cells that are extremely sensitive to Epo. Because virtually all of these cells already are cycling, increases in their numbers cannot be achieved by increasing their fraction in cycle. Either additional divisions are involved or new cells are recruited to the CFU-E pool (from a pre-CFU-E pool). Additional divisions of CFU-e or precursor

cells would increase their transit time within the bone marrow and potentially delay the delivery of new red blood cells to the periphery. Because a shortened maturation time has been observed instead and the proliferative potentials of CFU-E and proerythroblasts are finite, high levels of amplification cannot be achieved through this mechanism.. Therefore, such needs are met by influx into the CFU-E and precursor pools of newly differentiating cells from earlier progenitor compartments. Such a surge of newly produced cells has been observed in all previous experiments. A rapid influx of fresh cells was particularly notable in polycythemic mice that were experimentally depleted of CFU-E and erythroid precursors at the time the stimulus was applied. Because of the rapidity of the response (i.e. within 24 hours in the polycythemic animals), it appeared that the orderly progression from BFU-E to CFU-E to proerythroblast had been compressed. Such acceleration of differentiation is possible through shortened intermitotic intervals, fewer mitotic divisions, or differentiation without divisions. This short-circuiting in differentiation requires high serum levels of Epo and adequate numbers of BFU-E. Once CFU-E and precursors are expanded through this mechanism, most persisting erythropoietic demands can be met through this pool without excess input from pre-CFU-E pools. Thus, acute demands for erythropoiesis are met by influx from pre-CFU-E pools through an accelerated differentiaton and maturation sequence. In contrast, chronic demands (i.e. chronic hemolytic anemias) seems to be mainly satisfied through a greatly amplified late erythroid pool and with a minimum distortion in the differentiation sequence (Hillman and Finch 1967). That the kinetics of erythroid differentiation/maturation are different in acute versus chronic marrow regeneration is supported by differing qualitative changes in the newly formed red blood cells. An increase in hemoglobin F (HbF) expression as well as an increase in cells with higher mean corpuscular volumes is seen with an acute response, whereas these are minimal or reduced with chronic responses (Papayannopoulou, Chen et al. 1980). When severe anemia persists from birth onwards, erythroid production can increase up to 10-fold above baseline (Hillman and Finch 1967). This is possible not only because of maximally expanded erythropoietic pools but also because the sites of active eryhtropoiesis may extend to include those that support red blood cell differentiation during fetal life. Thus, although the bone marrow space in axial bones (vertebrae, pelvis, ribs, sternum, clavicles) is sufficient for normal erythropoiesis or to respond to moderate anemia, the femur, humerus, spleen and /or liver, and (rarely) thymus may support red blood cell production in children with congenital hemolytic anemia (e.g. thalassemia major), in a phenomenon called Extra-Medullary Hematopoiesis (EMH).

Additional mechanisms that support the expansion of the erythron in stress conditions have recently been described. The anti-apoptotic effect of Epo on early progenitors has been

shown recently to be mediated at least partially by down-regulation of Fas and Fas-L expression in the eryhtroid progenitors (Liu, Pop et al. 2006). Liu et al. found that in the mouse spleen at steady state conditions, 40-60% of the proerythroblasts express Annexin V. This apparent high apoptosis rate in this particular population of erythroid progenitors was found to be mediated by the cell surface expression of Fas and Fas-L. In conditions of erythropoietic stress, high levels of Epo induce a suppression of those proteins, resulting in an improved survival of proerythroblasts and therefore an overall expansion of the erythron in response to the rise of the levels of Epo. Recently it has been found also that in the mouse the increase in BFU-E cells during stress erythropoiesis occurs in a distinct subset of stress BFU-E progenitors that are unique to the spleen (Lenox, Perry et al. 2005). These cells arise in response to stress through a bone morphogenetic protein 4 and Smad5-dependent mechanism. Unlike bone marrow BFU-E, which reuire bothe Epo and one of SCF, GM-CSF or IL-3 for burst formation, splenic stress BFU-E require only high Epo. The *flexed tail* mouse, characterized by fetal anemia and a deficient stress erythropoiesis, was recently shown to contain a mutation in the Smad5 gene and to fail to generate stress BFU-E.

1.7 Iron metabolism and erythropoiesis

Erythropoiesis and iron metabolism are processes closely linked to each other. The large majority of iron in our body is utilized by the erythropoietic system to generate functionally active hemoglobin molecules, which are harbored in the RBC. It is estimated that 1800 mg of iron in our body is present in RBCs, 300 mg in the bone marrow and 600 mg in the reticuloendothelial macrophages of the spleen (Hentze, Muckenthaler et al. 2004), accounting for more than 60% of total body iron. This massive utilization of iron by the erythropoietic system requires the presence of finely tuned regulatory systems that allow storage, mobilization and traffic of iron and at the same time prevent toxicity due to highly reactive iron ions. It is also important to remember that our body lacks an efficient means of iron excretion. Therefore, its regulation occurs primarily at the site of absorption, duodenal enterocytes. Other important regulatory sites are the liver, where large quantities of iron can be stored in hepatocytes and Kuppfer cells, and the spleen, where macrophages recycle iron from senescent RBCs. All three compartments are of pivotal importance for erythropoiesis since they control the bioavailability of iron to erythroid progenitors, and all of them respond to what has been found to be the master regulator of iron metabolism, the peptide hormone hepcidin. Hepcidin (Krause, Neitz et al. 2000; Park, Valore et al. 2001) is synthesized mainly in the liver, and when released into the

blood stream, binds to ferroportin, the major cellular iron exporter expressed at high levels on the surface of duodenal enterocytes, liver hepatocytes and Kuppfer cells, and splenic macrophages (Abboud and Haile 2000; Donovan, Brownlie et al. 2000; McKie, Marciani et al. 2000; Canonne-Hergaux, Donovan et al. 2006) (Fig5).
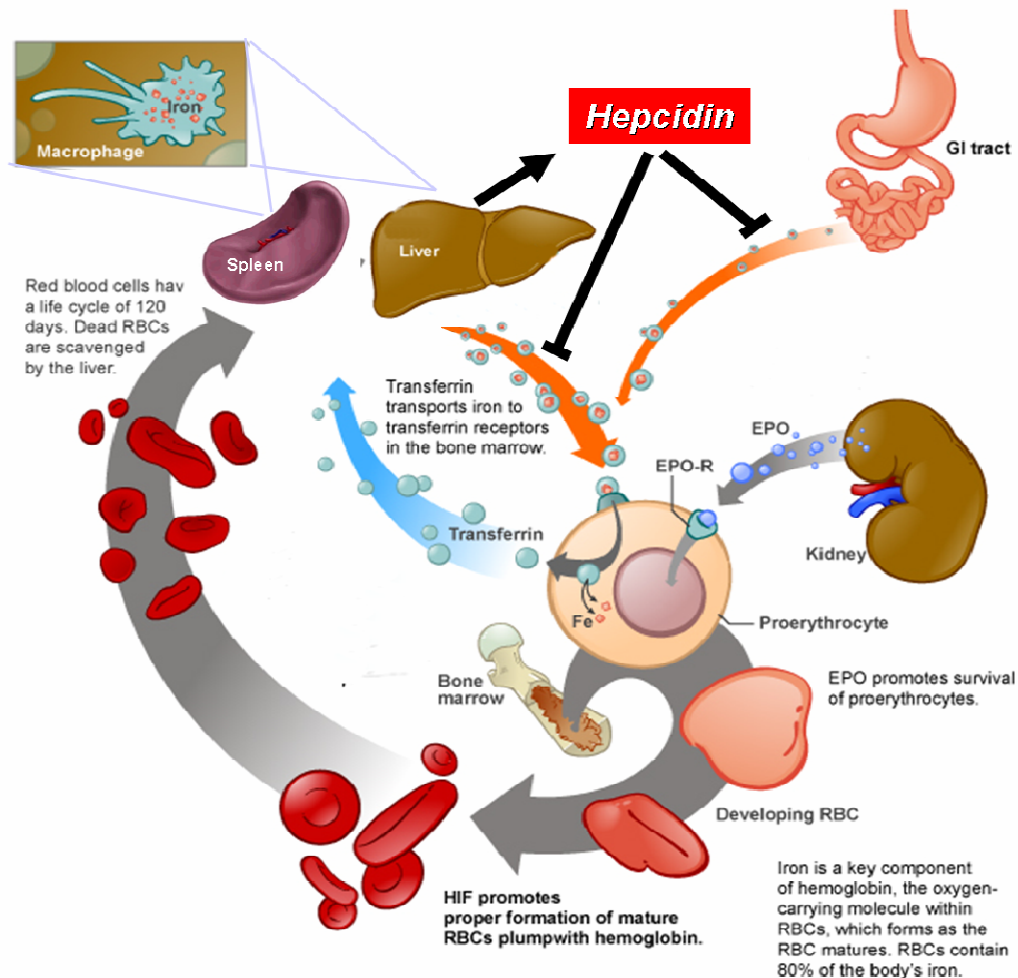

Fig.5 Major pathways of iron traffic between cells and tissues.

The binding of hepcidin to ferroportin induces its internalization and destruction in the cellular proteasome (Nemeth, Tuttle et al. 2004; De Domenico, Ward et al. 2007). Therefore, the main function of hepcidin is to reduce iron absorption from enterocytes, and to limit iron export and trafficking from hepatocytes and splenic macrophages. As expected the action of hepcidin becomes critical in conditions of iron overload, when too much iron is potentially bioavailable and a drastic reduction in its absorption and mobilization is required.

Being the master regulator of iron absorption, it is not surprising that hepcidin expression is strictly regulated, with multiple pathways that respond to iron storage (storage regulator) (Pigeon, Ilyin et al. 2001), hypoxia (hypoxia regulator) (Yoon, Pastore et al. 2006;

Peyssonnaux, Zinkernagel et al. 2007), inflammation (inflammatory regulator) (Nicolas, Chauvet et al. 2002; Nemeth, Valore et al. 2003; Nemeth, Rivera et al. 2004; Wrighting and Andrews 2006), and more importantly erythropoiesis (erythroid regulator) (Nicolas, Chauvet et al. 2002; Pak, Lopez et al. 2006; Vokurka, Krijt et al. 2006). All these systems interact with one another resulting in a very complex and finely tuned regulation of hepcidin expression.

Since the intimate relationship between hepcidin and erythropoiesis exists, it is not surprising that pathologies that involve alteration in the erythropoietic rate, also involve alterations of iron metabolism and hepcidin expression.

1.8 Hepcidin in β-thalassemia

In β-thalassemia the process of IE is accompanied by a massive iron overload, due to an increased rate of iron absorption by the gastrointestinal (GI) tract and to frequent blood transfusions. In this setting two major systems would contribute to hepcidin expression, the 'store regulator' and the 'erythroid regulator'. Due to the high levels of total body iron, the store regulator should act by increasing hepcidin expression thereby avoiding further iron absorption. On the other hand, the erythroid regulator would decrease hepcidin expression in an attempt to compensate for ineffective erythropoiesis. A recent study in mouse models of β-thalassemia intermedia (*th3*/+) and major (*th3*/*th3*) showed that the erythroid regulator did indeed dictate the pattern of iron absorption and distribution according to the degree of IE (Gardenghi, Marongiu et al. 2007). In *th3*/+ mice iron overload and the degree of IE gradually become more severe as the animals aged. Gardenghi and colleagues demonstrated how disregulation of iron absorption in young *th3*/+ mice is due mainly to a dramatic decrease of hepcidin expression in the liver. However, as iron overload progressively increases in older animals, hepcidin is up-regulated, while ferroportin expression is increased in the GI tract in order to maintain high levels of iron absorption to compensate for the IE. In *th3*/*th3* mice, where IE is more pronounced, the increased iron absorbed was not found in hematopoietic organs such as the spleen and bone marrow, but rather in the liver and other non-hematopoietic organs, suggesting that this iron is not utilized by erythroid progenitors, as would be expected according to the model of IE described above where erythroid progenitors display an increased proliferative activity but a decreased differentiation rate, resulting in a limited synthesis of hemoglobin and therefore limited iron uptake. Instead, in *th3*/+ animals where the degree of IE is lower and there is a substantial effective erythropoiesis, an increased iron content is found in the spleen and Kupffer cells of the liver. This leads to the conclusion that the erythroid regulator overrides the store

regulator in *th3*/*th3* mice, resulting in low levels of hepcidin expression and further increasing the iron concentration in the liver. In contrast, in states of relatively mild anemia, iron absorption would be lower and the erythroid organs, spleen and bone marrow, would utilize part of the absorbed iron, as is observed in *th3*/+ animals. The idea that not all the iron absorbed in β-thalassemia is utilized for erythropoiesis has been confirmed by our recent data (Gardenghi et al., in submitted) in *th3*/+ animals kept on a low-iron diet. These animals show a lower iron content compared to counterparts fed a regular diet, but do not display a decrease in hemoglobin levels, suggesting again that an excessive amount of iron is absorbed in thalassemia but is not utilized for the erythropoietic process.

1.9 Control of hepcidin expression by the erythroid regulator

It is becoming clear how the erythroid compartment regulates the levels of hepcidin both in physiologic and pathologic conditions. In β-thalassemia several factors have been identified and studied as candidate hepcidin regulatory proteins including growth differentiation factor 15 (GDF15) (Tanno, Bhanu et al. 2007), and human twisted gastrulation factor (TWSG1) (Tanno, Porayette et al. 2009). Both of these two factors are members of the TGF-beta superfamily, which controls proliferation, differentiation and apoptosis in numerous cells, and are secreted by erythroid precursors. TWSG1 gene expression occurs early during erythroblast maturation contrasting with the more sustained increase in GDF15 expression in more mature hemoglobinized erythroblasts.

GDF15, which is elevated in the sera of thalassemic patients, has the ability to down-regulate the expression of hepcidin *in vitro*, although the mechanism is still unknown. However, thalassemia sera also suppressed hepcidin expression to a lesser degree after immunoprecipitation of GDF15 (Tanno, Bhanu et al. 2007). Moreover, in patients with hematopoietic malignancies who underwent stem cell transplantation, serum hepcidin levels were not correlated with GDF15, indicating that a circulating factor other than GDF15 negatively regulates hepcidin production in such settings (Kanda, Mizumoto et al. 2008). Therefore, the role of GDF15 may be to limit hepcidin synthesis when erythroid precursors undergo cell death, one factor in the development of IE (Libani, Guy et al. 2008), such as occurs in β-thalassemia and refractory anemia with ring sideroblasts (RARS)(Ramirez, Schaad et al. 2009).

TWSG1 has been shown to inhibit the up-regulation of hepcidin by bone morphogenic proteins 2 and 4 (BMP2, BMP4), mediated by Smad phosphorylation**,** in human hepatocytes. On

the other hand, the effect of TWSG1 was not BMP-mediated in murine hepatocytes (Tanno, Porayette et al. 2009). Tanno and colleagues proposed that TWSG1 might act with GDF15 to dysregulate iron homeostasis in thalassemia.

Although these represent new and exciting findings, more studies are required to complete the characterization of these and other factors that regulate hepcidin production in condition of erythroid stress and, equally important, to utilize them for their potential prognostic or therapeutic effect on thalassemic patients

Chapter 2

β-Thalassemia and ineffective erythropoiesis

2.1 Etiology and pathophysiology

The thalassemias are inherited as pathologic alleles of one or more of the globin genes located on chromosome 11. These lesions range from total deletion or rearrangement of the loci to point mutations that impair transcription, processing, or translation of globin mRNA. Thalassemias have been encountered in virtually every ethnic group and geographic location. They are most common in the Mediterranean basin and equatorial or near-equatorial regions of Asia and Africa. The "thalassemia belt" extends along the shores of the Mediterranean and throughout the Arabian Peninsula, Turkey, Iran, India, and southeastern Asia, especially Thailand, Cambodia and Southern China (Weatherall 2001) (figure 6)
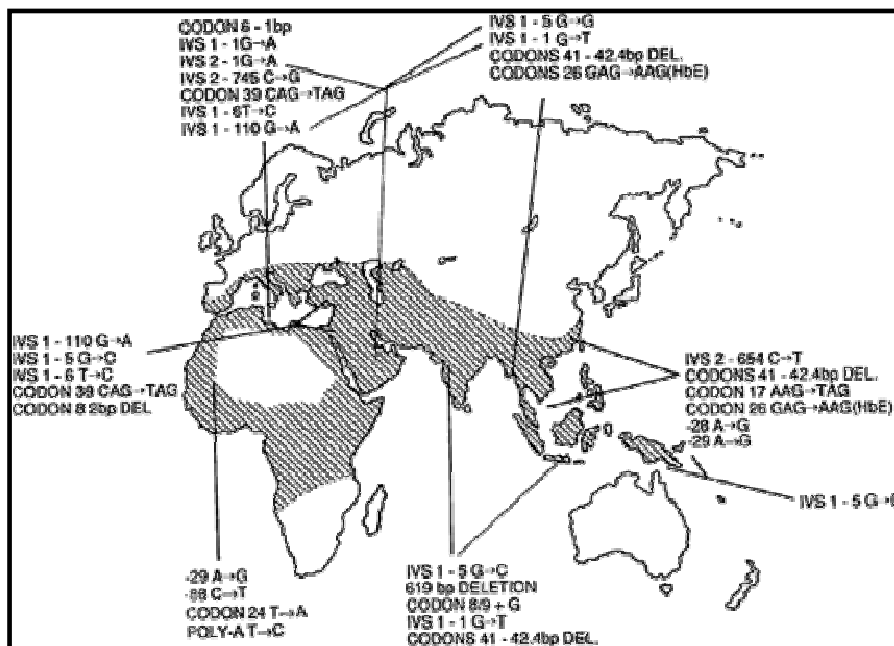

Fig.6 Thalassemia distribution

The prevalence of thalassemia in these regions is in the range of 2.5-15%. Like sickle cell anemia, thalassemia is most common in those areas historically afflicted with endemic malaria. Malaria seems to have conferred slective survival   advantage   to   thalassemia

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heterozygotes, in which infection with the malaria parasite is believed to result in milder disease and less impact on reproductive fitness. Therefore, the gene frequency for thalassemia has become fixed and high in populations exposed to malaria over many centuries.

Many different mutations cause β-thalassemia. They are inherited in a multitude of genetic combinations responsible for a heterogenous group of clinical syndromes. β-thalassemia Major, also known as Cooley's anemia or homozygous β-thalassemia, is a clinically severe disorder that result from the inheritance of two β-thalassemia alleles, one on each copy of chromosome 11. As a consequence of diminished HbA synthesis, the circulating cells are very hyopchromic and abnormally shaped; they contain markedly reduced amounts of hemoglobin. Accumulation of free α-globin chains leads to dEposition of precipitated aggregates of these chains to the detriment of the erythrocyte and its precursors in the bone marrow. The anemia of thalassemia major is so severe that chronic blood transfusions are usually required. The term *β-thalassemia intermedia* is applied to a less severe clinical phenotype in which significant anemia occurs but chronic transfusion therapy is not absolutely required. It usually results from the inheritance of two β-thalassemia mutations, one mild and one severe; the inheritance of two mild mutations; or, occasionally, the inheritance of complex combinations, such as a single β-thalassemia defect and an excess of normal α-globin genes, or two β-thalassemia mutations co-inherited with heterozygous α-thalassemia (in this last form, known as αβ-thalassemia, the α-thalassemia allele reduces the burden of unpaired α-globin chains). Simple heterozygosity for certain forms of β-thalassemic hemoglobinopathies can also be associated with a thalassemia intermedia phenotype, sometimes called dominant β-thalassemia (Weatherall 2001).

Thalassemia minor, also known as β-thalassemia trait or heterozygous β-thalassemia, is caused by the presence of a single β-thalassemia mutation and a normal β-globin gene on the other chromosome. It is characterized by profound mycrocytosis with hypochromia but mild or minimal anemia.

2.2 Molecular Pathology

Forms of β-thalassemia arise from mutations that affect every step in the pathway of globin gene expression: transcription, processing of the mRNA precursor, translation of mature mRNA, and post-translational integrity of the β-polypeptide chain (Schrier 2002). Large deletions removing two or more non-α- genes are found in rare cases, as are smaller partial or total deletions of the β-gene alone. Most types of β-thalassemia are caused by point mutations affecting one or a few bases. Of the more than 175 point mutations causing β-thalassemia, about 15 account for the vast majority of affected   patients, with the remainder responsible for the

disorder in only relatively few patients. It has been determined that five or six mutations usually account for more than 90% of the cases of β-thalassemia in a given ethnic group or geographic area (Fig.6). Several mutations alter the promoter region upstream of the β-globin mRNA-encoding sequence, impairing mRNA synthesis, whereas mutations that derange the sequence used as the signal for the addition of the poly-A tail of the mRNA (polyadenilation signal) have been shown to result in abnormal cleavage and polyadenilation of the nascent mRNA precursor, with resulting reduced accumulation of mature mRNA. Many forms of β-thalassemia are caused by mutations that impair splicing of the mRNA precursor into mature mRNA in the nucleus or that prevent translation of the mRNA in the cytoplasm. The molecular pathology of splicing mutations is complex. Some base substitutions ablate the donor (GT) or acceptor (AG) dinuclotides, which are absolutely required at the intron-exon boundaries for normal splicing, and thereby completely block production of mature functional mRNA. Thus, no β-globin can be synthesized (β^0-thalassemia). Other mutations alter the consensus sequences that surround the GT- and AG-invariant dinucleotides and decreases the efficiency of normal splicing signals by 70% to 95%, resulting in β^+-thalassemia; some consensus mutations even abolish splicing completely, causing β^0-thalassemia. A third type of splicing aberration results from mutations that are not in the immediate vicinity of a normal splicing site. These alter regions within the gene, called cryptic splice sites, which resemble consensus splice sites but do not normally sustain splicing. The mutations activate the site by supplying a critical GT or AG nucleotide or by creating a sufficiently strong consensus signal to stimulate splicing at that site 60% to 100% of the time. The activated cryptic sites generate abnormally spliced, untranslatable mRNA species. Only 10% to 40% of the mRNA precursors are thus spliced at the normal sites, which causes β^+-thalassemia of variable severity. The mutation responsible for the most common form of β-thalassemia among Greeks and Cypriots activates a cryptic site near the 3' end of the first intron (position 110). The determinants that dictate the degree to which each mutation alters splice site use remain largely unknown. Mutations that abolish translation occur at several locations along the mature mRNA and are very common causes of β^0-thalassemia. The most common form of β^0-thalassemia in Sardinians results from a base substitution in the gene that changes the codon encoding the 39th aminoacid of the β-globin chain from CAG, which encodes glutamine to TAG, whose equivalent (UAG) in mRNA specifies termination of translation. A premature termination codon totally abrogates the ability of the mRNA to be translated into normal β-globin. Premature translation termination also results from frameshift mutations (i.e., small insertions or deletions of a few bases, other than multiples of 3, that alter the phase or frame in which the nucleotide sequence is read   during translation). An in-phase termination

premature termination codon is invariably encountered within the next 50 bases downstream from a frameshift.

2.3 Pathophysiology

The biochemical hallmark of β-thalassemia is reduced biosynthesis of the β-globin subunit of HbA ($\alpha_2\beta_2$). In β-thalassemia heterozygotes, β-globin synthesis is about half normal (β/α ratio 0.5-0.7). In homozygotes for β^0-thalassemia, who account for about one third of patients, β-globin synthesis is absent. β-Globin synthesis is reduced from 5% to 30% of normal levels in β^+-thalassemia homozygotes or β^+/β^0-thalassemia compound heterozygotes, who together account for about two thirds of the cases.

Because the synthesis of HbA ($\alpha_2\beta_2$) is markedly reduced or absent, the red blood cells are hypochromic and microcytic. γ-Chain synthesis is partially reactivated, so that the hemoglobin of the patient contains a relatively large proportion of HbF. However, the γ-chains are quantitively insufficient to replace β-chain production. In heterozygotes (β-thalassemia trait), relatively little α-globin accumulation occurs. Output from the single normal β-globin gene supports substantial HbA formation, thus preventing harmful accumulation of excess of α-globin chains. Thus, one encounters hypochromia with microcytosis but relatively little evidence of anemia, hemolysis, or ineffective erythropoiesis. Individuals inheriting two β-thalassemic alles experience a more profound deficit of β-chain production. Little or no HbA is produced; more importantly, the imbalance of β-globin and α-globin is far more severe (Fig.7). The limited capacity of red blood cells to proteolyze the excess of α-globin chains, a capacity that probably exerts a protective effect in heterozygous β-thalassemia, is overwhelmed in homozygotes. Free α-globin accumulates, and unpaired α-chains aggregate and precipitate to form inclusion bodies, which cause oxidative membrane damage within the red blood cell and developing erythroblasts within the bone marrow, a process called Ineffective Erythropoiesis (IE). Consequently, relatively few of the erythroid precursors undergoing eryhtroid maturation in the bone marrow survive long enough to be realesed into the bloodstream as erythrocytes. The occasional erythrocytes that are formed during erythropoiesis bear a burden of inclusion bodies. The reticuloendothelial cells in the spleen, live, and bone marrow remove these abnormal cell prematurely, producing a hemolytic anemia. Defective β-globin synthesis exerts at least three distinct yet interrelated effects on the generation of oxygen.carrying capacity fro the peripheral blood: 1) ineffective erythropoiesis, which impairs production of new red blood cells; 2) hemolytic anemia, which shortens the survival of the few red blood cells produced;

and 3) hypochromia with microcytosis, which reduces the oxygen carrying capacity of those few red blood cells that do survive. In the most severe forms of the disorder, these three factors conspire

to produce a catastrophic anemia, complicated by the effects of hemolysis. The profound deficit in oxygen-carrying capacity of the blood stimulates production of high levels of Epo in attempt to promote compensatory erythroid hyperplasia. Unfortunately, the ability of the marrow to respond positively is markedly impaired by IE. Massive bone marrow expansion does occur, but very few erythrocytes are actually supplied to the circulation. The marrow becomes packed with immature erythroid precursors, which die from their burden of precipitated α-chains before they reach the reticulocyte stage. Profound anemia persists, driving eryhtroid hyperplasia to still higher levels. In some cases, erythropoiesis is so exuberant that masses of extramedullary erythropoietic tissue form in the chest, abdomen or pelvis. Massive bone marrow expansion exerts numerous adverse effects on the growth, development, and function of critical organ systems and creates the characteristic facies caused by maxillary marrow hyperplasia and frontal
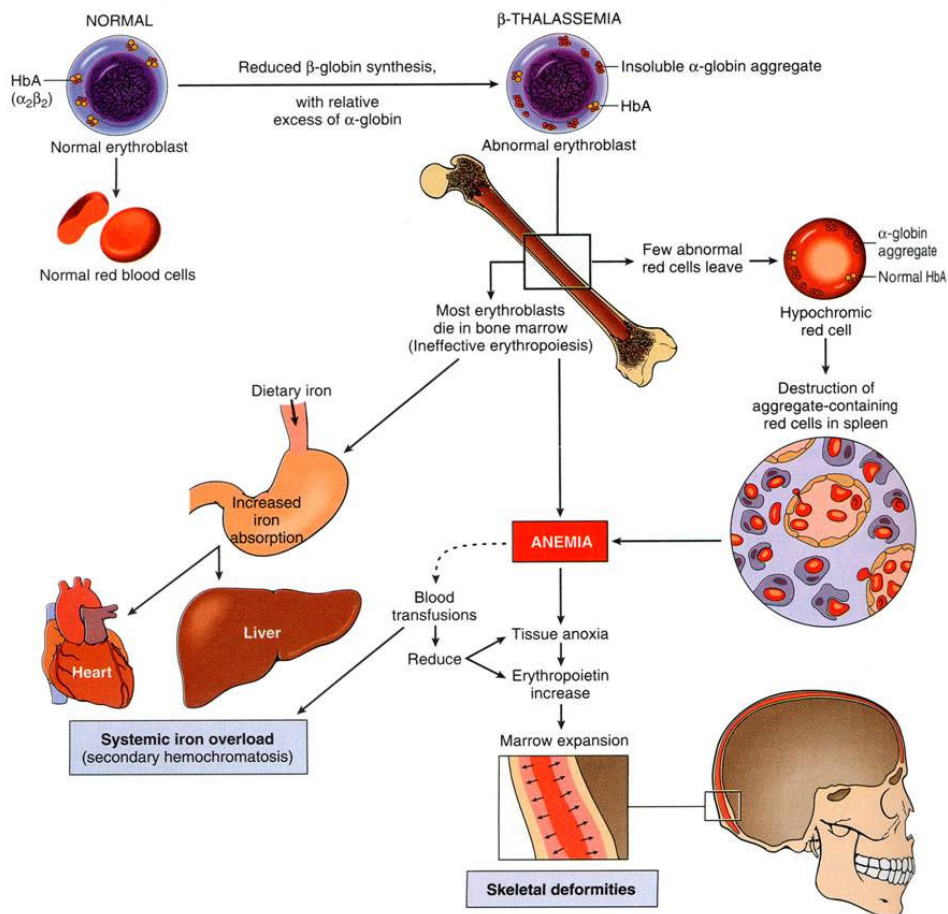

Fig7: Pathophysiology of β-thalassemia. From Robbins et al.

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bossing. IE results in massive splenomegaly and high output congestive heart failure. In untreated cases, death occurs during the first two decades of life. Treatment with red blood cell transfusions sufficient to maintain hemoglobin levels above 9.0 to 10 g/dl improves oxygen delivery, suppresses excessive IE, and prolongs life. Unfortunately a major complication of chronic blood transfusions is iron overload. Each unit of packed red blood cells contains approximately 220 to 250 mg of iron. Based on usual blood requirements in patients with thalassemia major, the rate of transfusion iron accumulation is about 0.30 to 0.60 mg/kg/day.

Humans have no physiologic mechanism to induce significant excretion of iron when levels of the metal are abnormally high, therefore iron overload is the primary cause of morbidity in β-thalassemia patients, leading to the development of liver cirrhosis and cardiotoxicity. So far the chelation therapy with iron chelators such as deferoxamine and deferiprone is the only treatment available to decrease the iron burden. Approximately one half to to two thirds of iron excreted in response to deferoxamine is in the urine, with the reminder in the stool

Untransfused patients affected by β-thalassemia display also iron overload caused by an increased iron absorption due to the down-regulation of the iron absorption regulatory hormone hepcidin (Nemeth and Ganz 2006; Gardenghi, Marongiu et al. 2007; Kearney, Nemeth et al. 2007; Origa, Galanello et al. 2007). Hepcidin physiologically binds to ferroportin on the apical surface of enterocytes, causing its internalization and therefore decreasing iron absorption (Nemeth, Tuttle et al. 2004). In β-thalassemia the low hepcidin allows excessive iron absorption and development of systemic iron overload, similar to hereditary hemochromatosis. The signal causing hepcidin suppression in β-thalassemia appears to be generated by high eryhtropoietic activity and outweighs the effects of the resulting iron overload on hepcidin regulation. GDF15 and TWSG1 are two eryhtroid factors that may contribute to hepcidin suppression in syndromes with IE (Tanno, Bhanu et al. 2007; Tanno, Porayette et al. 2009). Hepcidin diagnostics may be useful for iron-loading anemias to identify the patients at higher risk of iron toxicity due to severely decreased hepcidin levels. Moreover, future hepcidin agonists may be sufficient to prevent the life-threatening iron overload in these patients. The first promising evidence of the beneficial effect of hepcidin comes from the th3/+ mouse model of β-thalassemia in which moderate transgenic expression of hepcidin resulted in lower spleen and liver iron content, decreased IE in the spleen and even improvement of hematological parameters (Gardenghi et al., submitted).

In chronically transfused patients, hepcidin concentrations are much higher than in nontransfused patients, presumably due to both increased iron load and the alleviation of IE.

Interestingly, Origa et al. showed that nontransfused patients have liver iron concentrations similar to those of regularly transfused thalassemia major patients. However, because of the different hepcidin levels, the cellular distribution of iron in the liver differed in these two groups. In nontransfused thalassemia, iron was dEposited in hepatocytes, whereas higher hepcidin levels in transfused patients resulted in macrophage iron loading. As a consequence of this difference in cellular iron distribution, serum ferritin levels were much lower in nontransfused patients, and did not adequately reflect the patients' liver iron load. Considering that high hepcidin shifts iron distribution to macrophages and decreases intestinal iron absorption , it is possible that hepcidin agonists could be useful even in transfused thalassemia patients. Trapping iron in macrophages where it is less toxic may postpone iron dEposition and consequent damage in the parenchyma, but this remains to be investigated.

2.4 Ineffective Erythropoieis in β-thalassemia

The ineffective production of red blood cells in β-thalassemia intermedia and major has been attributed to erythroid cell death during the maturation process mediated by apoptosis or hemolysis. It was proposed that accumulation of α-globin chains leads to the formation of aggregates , which impair eryhtroid maturation triggering apoptosis (Yuan, Angelucci et al. 1993; Yuan, Bunyaratvej et al. 1995; Kuypers, Yuan et al. 1998; Mathias, Fisher et al. 2000; Pootrakul, Sirankapracha et al. 2000; Ellis and Pannell 2001; Schrier 2002; Kong, Zhou et al. 2004) (Fig.8).
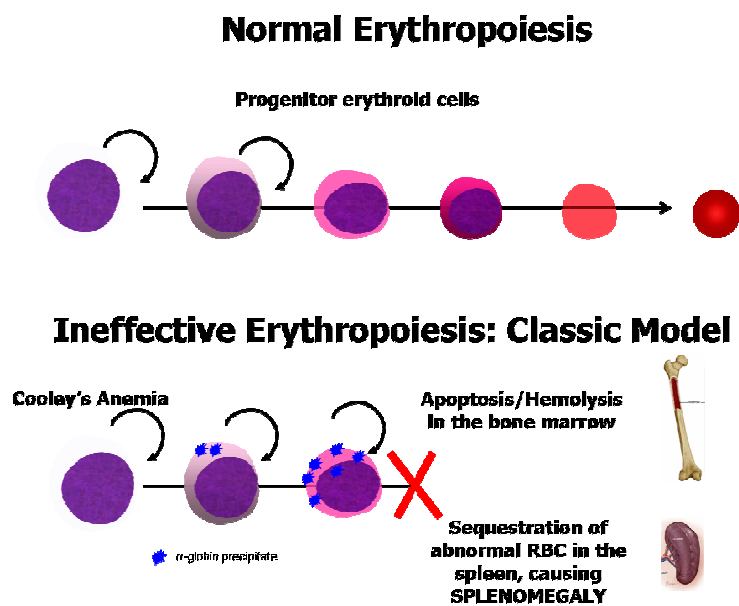

Fig.8: Ineffective Erythropoiesis (IE) in β-thalassemia

Ferrokinetic studies done in 1970 suggested that 60% to 80% of the erythroid precursors in β-thalassemia major die in the marrow or extramedullary sites (Finch, Deubelbeiss et al. 1970). However , several observations call into question the view that cell death is the only cause of IE in β-thalassemia.

The first observation stem from the ferrokinetic studies performed β-thalassemia patients. Those analyses revealed that 75% to 90% of the iron in donor serum, labeled with ^{59}Fe and injected into healthy subjects, appeared in circulating red cells within 7 to 10 days. In some thalassemic patients, however, only 15% of the ^{59}Fe was incorporated into circulating erythrocytes. This discrepancy was attributed to the fact that iron would be sequestered in those organs in which premature destruction of erythroid precursors occurs (Finch, Deubelbeiss et al. 1970). Therefore, in β-thalassemia erythropoietic organs such as the bone marrow (BM) in humans and the BM and spleen in mice would be expected to show the highest iron concentrations. This seems not to be the case, at least in thalassemic mice. Gardenghi et al. showed that IE in *th3/+* and *th3/th3* mice dictates the pattern of iron distribution. Their data show that iron accumulates progressively in the spleen of *th3/+* mice and at a lower pace in the Kupffer cells of the liver, whereas in *th3/th3* mice iron overload occurs rapidly and involves predominantly liver parenchymal cells. The highest liver iron content is observed in *th3/th3* mice that show the lowest Hb levels and the highest degree of IE. In contrast, blood transfusion reduces IE and iron dEposition in the livers of the same animals. Altogether these observations indicate that in conditions of extreme IE, there is relatively little peripheral destruction of red cells and iron accumulates more rapidly in the liver than in the spleen, consistent with the interpretation that iron loading results primarily from increased intestinal absorption (Bannerman, Keusch et al. 1967; Heinrich, Gabbe et al. 1973; Cossu, Toccafondi et al. 1981; Fiorelli, Fargion et al. 1990).

These studies do not exclude that the majority of the iron administered to patients affected by IE could be directly stored by liver parenchymal cells rather than being used by erythroid cells (Adamsky, Weizer et al. 2004; Breda, Gardenghi et al. 2005; Weizer-Stern, Adamsky et al. 2006; Pinto, Ribeiro et al. 2008). This would explain the ferokinetics studies without invoking massive erythroid apoptosis or hemolysis.

Second, the number of apoptotic erythroid cells in thalassemic patients is low compared with that anticipated by ferrokinetics studies (Finch, Deubelbeiss et al. 1970). In fact, only 15% to 20% of bone marrow (BM) erythroid precursors (CD45-/CD71+) present apoptotic features in aspirates from affected patients (Yuan, Angelucci et al. 1993; Centis, Tabellini et al. 2000; Mathias, Fisher et al. 2000).

Third, hemolytic markers in young β- thalassemia patients are normal or only

slightly increased, unless the patients suffer from splenomegaly or the liver has been damaged by iron overload or viral infections (Angelucci, Muretto et al. 2002).

2.5 β-globins in human and mouse

The human β-globin locus is characterized by 5 expressed genes located in a 80 kb region on the chromosome 11 (Fig.9).
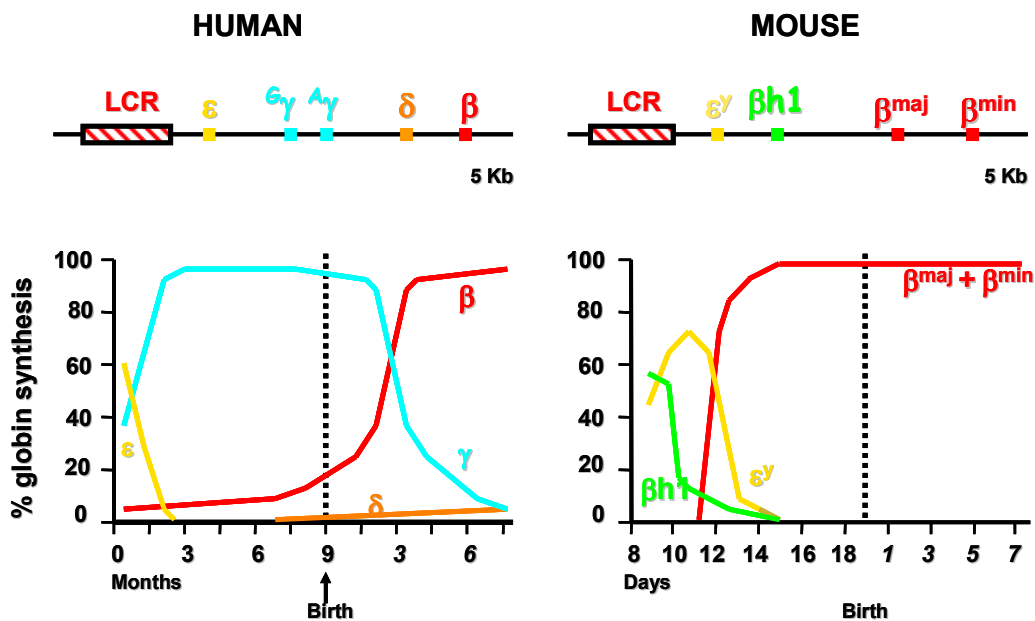

Fig 9. Structure of the human α and β-globin loci

The entire human β-globin gene cluster is organized with the various genes in their order of development expression. The embryonic ε gene is located at the 5', followed by γ^G and γ^A genes and the adult δ and β-globin genes the define the 3' end of the locus. In addition to the promoter elements several other important erythroid specific regulatory sequences have been identified in the globin gene cluster. In the case of β-globin gene cluster the region is marked by 5 hypersensitive sites (named after their sensibility to treatment with DNasi, indicating regions of extremely active and open chromatin): HS1 to HS5 that together form the Locus Control Region (LCR). The most 5' (HS5) does not show tissue specificity while the HS1 through the HS4 are largely erythroid specific and contain a variety of binding sites for erythroid transcription factors. The LCR together with the proximal cis-element transcription factors controls the developmental stage specific expression of genes: from ε to γ, and from γ to β and δ during the

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development from embryo to adult. Very early in gestation there is a switch from ε to γ-globin production. The fetal hemoglobin (HbF) is detected at weeks old embryos, reaches a plateau after 10 weeks and remains high until 2 years after birth when it begins to decrease to 0.5-1% if total hemoglobin in adult red blood cells. The β-globin chains synthesis starts at 30-35 weeks of gestation contributing at this time to 10% of the total hemoglobin. After birth it increases to 97% forming the adult hemoglobin HbA. The other type of adult hemoglobin is the HbA2 formed by δ chains whose sythesis begins at the 7th month of gestation reaching 2-3% in adult life. In mouse the β-globin gene cluster is characterized by two embryonic genes, βh1 and $ε^{γ2}$, and two adult genes called b1 ($β^{major}$) and b2 ($β^{minor}$), but no γ-like genes are present in mice. In mouse the LCR function is associated with 6 HS sites. HS6 represents a minor HS (Bender, Bulger et al. 2000) and contains an high density of potential binding sites for erythroid transcription factors GATA-1 and NF-E2, consistent with several other β-globin LCR HSs. The most interesting difference between human and mouse concerns the embryonic to adult hemoglobin switch. This happens before birth in mice, whereas in humans it occurs 6 months after birth; consequently, mice homozygous for mutations that prevent expression of β-globin genes die perinatally, due to the absent expression of adult hemoglobin.

2.6 *Th3* mouse model of β-thalassemia

Th3 represents a mouse model of β-thalassemia that carries a deletion of both the $β^{major}$ and $β^{minor}$ genes (Ciavatta, Ryan et al. 1995; Yang, Kirby et al. 1995). Mice homozygous for this deletion (*th3/th3*) die late in gestation, while heterozygotes containing one functional copy of the $β^{major}$ and $β^{minor}$ genes (*th3/+)* are viable and thalassemic (Fig.10). The adult *th3/+* mice exhibit the most severe anemia (7-9 g/dl of Hb) and a level of disease severity (hepato-splenomegaly, iron overload, anemia aberrant erythrocyte morphology) similar to that found in patients with β-thalassemia intermedia. In fact, at 8-10 weeks of age these animals are significantly smaller than wild type littermates and their red blood cells show a marked anisocytosis and poikilocytosis; there are many microcytes, occasional polychromatic macrocytes, and frequent target cells. The red cells are generally hypochromic, with variable numbers of teardrop and oval forms, and fragmented cells are seen. Nucleated red blood cells are also present. Precipitated α-globin chains are seen in the red blood cells (Yang, Kirby et al. 1995). A markedly decreased hematocrit, hemoglobin concentration, and red blood cell count are also present. The reticulocytes counts are dramatically increased (up to 21 folds), indicating a state of active erythropoiesis in heterozygous animals.
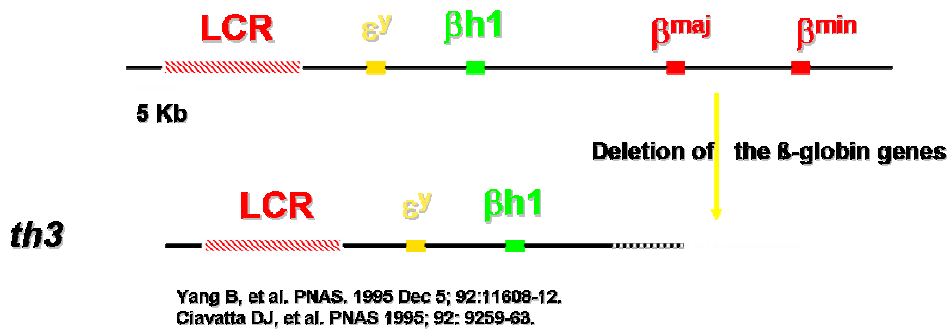
Fig.10 *Th3* **mouse model of β-thalassemia**

2.7 Adult Mouse Model of β⁰-thalassemia

Generation of a mouse model affected by thalassemia major has been hampered by the fact that, in addition to their two adult β-globin genes, mice lack the γ-like globin gene, which is present in humans. Moreover, the embryonic to adult switch happens before birth in mice, where in humans this switch occurs 6 moths after birth. Consequently, mice homozygous for mutations that prevent expression of β-globin genes die perinatally, due to the absent expression of fetal and adult hemoglobin. The lack of an animal model for thalassemia major has limited the full investigation of the physiopathology underlying this disease and has hampered the evaluation of both pharmacological and genetic treatments. For these reasons a new adult mouse model of β⁰-thalassemia has been generated by Rivella and colleagues (Rivella, May et al. 2003). Since mice lacking adult β-globin genes (th3/th3) die late in gestation, they attempted to transfer the hematological defect to adult syngenic C57BL/6 mice. Wild type animals were engrafted, after myeloablation, with null fetal liver cells (FLCs) harvested from 14.5 days post coitum th3/th3 embryos, obtained by intercrossing mice affected by β-thalassemia intermedia (Fig.11). The embryo genotype was identified by Hb electrophoresis prior to transplantation. Unlike mice engrafted with +/+ or th3/+ FLCs, which survive for at leat 9 months, recipients of th3/th3 cells die 7 to 9 weeks after transplantation (Rivella, May et al. 2003). After 6-7 weeks these mice closely mimic the thalassemic features observed in patients affected by β-thalassemia major.
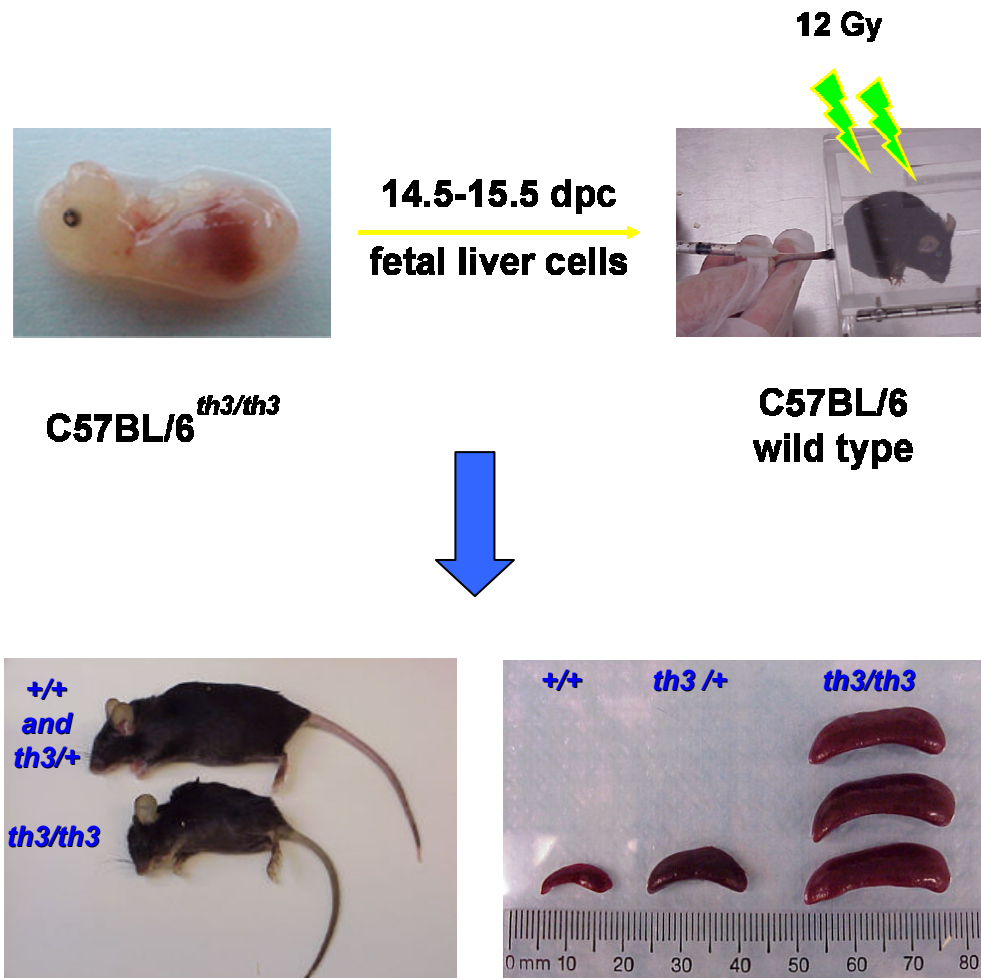
Figure 11. Creation β⁰-thalassemia mice

Hematological analyses performed 6-8 weeks post-transplant in mice engrafted with th3/th3 FLCs reveal severe anemia (2.8±0.8 g/dl of Hg, versus 13.2±1.0 g/dl in +/+ chimeras and 11.1±2.1 g/dl in th3/+ chimeras). Low RBC counts, hematocrit values and reticulocytes counts, further confirm the development of a profound erythroid deficiency. The profound anemia settled in after 50 days, consistent with the clearance rate of the recipient's normal RBCs , eventually leads to death within 60 days. Moreover, these mice present a reduced body weight, massive splenomegaly due to major erythroid hyperplasia, extensive EMH and iron overload. Analyses of murine globin transcripts by primer extension analyses (Rivella, May et al. 2003) also show that murine β-globin expression is very low or undetectable in bone marrow, spleen and blood of mice engrafted with th3/th3 cells. This observation confirms that these chimeras die from a selective erythroid defect due to lack of β-globin expression. This is thus a valis model of β-thalassemia major and can be used to better understand the mechanisms underlying the development of the disease as a first step toward developing definitive gene therapy and pharmacological approaches. The utility of   these mouse models may be significant for the

analyses of all the pathological aspects of β-thalassemia and, as far as the study presented in this thesis, for the study of the mechanisms that trigger ineffective erythropoiesis in the thalassemic syndromes. Recetly this model has been used to describe potential roles of some iron related genes in thalassemic animals who had either received a transfusion or not (Gardenghi, Marongiu et al. 2007). It was shown that organ iron distribution and the mechanism that leads to iron overload differ in th3/th3 and th3/+ mice and that their increased iron concentration is dictated by the different degree of IE, organ iron content and by the relative expression levels of iron related genes. The final picture that emerges from that study was that extreme IE in β-thalassemia major results in more iron being absorbed than that required by red blood cell production, despite an increasing liver iron content. In contrast, iron absorption in β-thalassemia intermedia responds to the relative degree of IE, the body iron content, and other as yet undefined factors that accumulate or appear in aging thalassemic mice and regulate the iron related gene expression.

AIMS OF THE STUDY

Given the controversies in the literature over the causes of ineffective erythropoiesis, we have undertaken a detailed investigation of this process in 2 mouse models that mimic β-thalassemia intermedia (th3/+) and major (th3/th3), analyzing the erythropoietic features in these animals. Therefore the specific aims of this study were to:

- Evaluate the apoptotic and hemolytic processes in the erythroid compartment in th3/+ and th3/th3 mice
- Characterize the expression of genes that could have a role in ineffective erythropoiesis
- Investigate possible mechanisms that sustatain erythroid cell proliferation and protect them from apoptosis, leading to expansion and mobilization of erythroid precursors to extramedullary sites
- Once identified the key players in the onset of ineffective erythropoiesis, find new targeted therapeutic approaches for the amelioration of splenomegaly and iron overload in thalassemic patients

To achieve these aims molecular, cellular biology and animal handling techniques were used in association with morphological examination and cell cycle analysis of erythroid cells in both thalassemic and normal animals.

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MATERIALS AND METHODS

Bone Marrow Transplantation Procedure (BMT) to Generate the Mouse Model of β-Thalassemia

Adult C57BL/6 *Th3/+* female mice were bred with adult C57BL/6 *Th3/+* males (Jackson Laboratories, Bar Harbor, ME, USA) to generate embryos *Th3/Th3*, *Th3/+* and wild type for the mouse β-globin gene. At 14.5-15.5 days post-coitum (dpc or days of gestation), pregnant females were sacrificed by cervical dislocation under anesthesia with isofluorane, and the embryos were collected, euthanized by decapitation, and screened for their genotype for the mouse β-globin gene.

Fresh peripheral blood from every embryos and from the mother was collected from the jugular vein or from cardiac puncture and the samples of red cell lysates were analyzed by cellulose acetate electrophoresis (Helena Laboratories, Beaumont, TX, USA) (**Fig. 1**). Afterward, hemoglobin bands were visualized by Ponceau S staining.
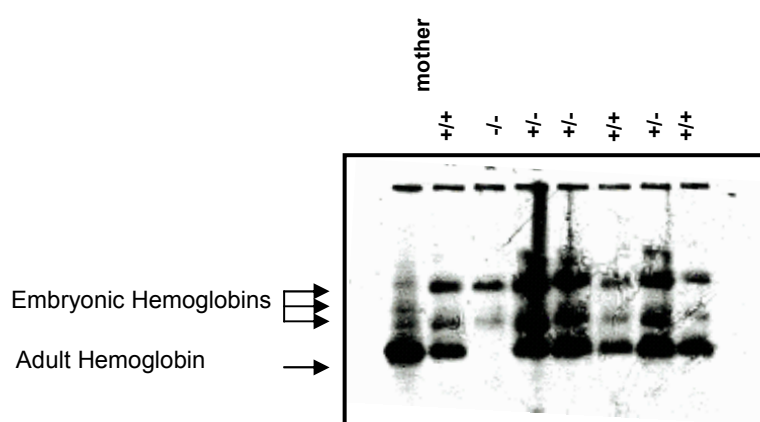
Once determined the genotype of the embryos, their FLCs were harvested, counted and used for BMT.

Recipients: Adult Wild-Type Female Mice.

Recipient mice were maintained on acid water starting a week before transplantation. On the day of transplantation, the hosts were lethally irradiated with a cesium source (950 rads single dose). Successively, between 1 and 2 millions FLCs resuspended in 200 microL of sterile PBS

were administered to each recipient mice by a single intravenous injection, given by the tail vein (no anesthesia necessary). The hosts were maintained on acid water and for at least 4 weeks after the treatment. Moreover, transplanted mice were monitored daily in order to identify any sign of distress.

Blood Transfusion

The life span of mouse RBCs is approximately 60 days and the total blood volume of the mice is approximately 2 mL. This corresponds to a weekly turnover of approximately 250 microL of blood. Starting at 21 days after transplantation, mice were infused weekly via the tail vein with 250 to 300 microL freshly harvested blood from normal healthy C57BL/6 donor mice.

Blood was collected from retro-orbital plexus into acid citrate dextrose (7 volumes of blood for 1 volume of acid citrate dextrose) under isofluorane anesthesia. Hemoglobin levels were measured 1 day before and 3 days after transfusion. The animals received blood transfusions for up to 5 weeks and sacked at 8 weeks after transplantation.

Hematological Studies

Blood samples were obtained by retro-orbital puncture under anesthesia. 50 microL of blood were collected and resuspended, in 1:4 proportion, in 150 microL PBS added with 5% BSA and 10mM EDTA (dilution factor 1:4). Total hemoglobin, hematocrit, and red cell, neutrophil and platelet counts will be measured on Advia 120 Hematology System (Bayer, Tarrytown, NY). All the data were multiplied for the dilution factor to calculate the correct number.

Serum Collection

Serum samples were extracted from mouse whole blood using separator tubes (BD Biosciences, Franklin Lakes, NJ). After blood collection from the retroocular vein the blood was let sediment for 2 hours and then centrifuged at 3000 rpm for 30 minutes. The serum was collected in a new tube and store at -20°C in the dark for further analysis of Epo or bilirubin content.

Serum Erythropoietin Level Quantification

Epo levels were quantified using mouse/rat erythropoietin Quantikine Mouse/rat Immunoassay kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. It is an ELISA assay in which a monoclonal antibody specific for mouse Epo has been pre-coated onto a microplate. Standards, control and samples are pipetted into the wells and any mouse Epo

present is bound by the immobilized antibody. After washing an enzyme-linked monoclonal antibody specific is added to the well.

Mouse sera collected were diluted 2 fold with the Calibrator Diluent RD&Z provided.

50 microl of the Assay Diluent RD1 provided + 50 microL of samples, standard or control were added to each well and incubated for 2 hours at Room Temperature on orbital microplate shaker. After 4 washes with 400 microL of washing buffer 100 microL of Epo Conjugate were added to each well and incubated for 2 hours at Room Temperature on orbital microplate shaker. After further washes 100 microL of Substrate solution provided were added and incubated for 30 minutes. At the end 100 microL of stop solution provided were added and the absorbance was read at 450 nm and corrected automatically with reading at 570 nm. Absorbance data were analyzed with SoftMaxPro 4.8 software using a four parameter logistic (4-PL) curve-fit to calculated the Epo concentration in each sample (pg/mL).

The experiment were performed in triplicate for each animal analyzed.

The level of Epo in each animal was correlated to its hemoglobin value and Pearson correlation test was performed to determine the statistical significance.

Serum Bilirubin and LDH Activity Quantification

For total bilirubin measurement, sera samples were collected as described above. Bilirubin content was measured with a colorimetric assay (Wako Pure Chemicals Industries, LTD, Richmond, VA) based on Vanadate oxidation method. When the sample is mixed with the reagent containing the detergent and the vanadate at pH=3 total bilirubin in the sample is oxidized to biliverdin. This causes the absorbance of yellow specific of bilirubin to decrease. By the measurement of the absorbance before and after the vanadate oxidation it is possible to obtain the bilirubin concentration. Manufacturer's instructions were followed. Briefly: 10 microL of samples were added to 280 microL of buffer and incubated for 5 minutes at Room Temperature. After the absorbance reading to set the blank 70microl of vanadate were added. After 5 minutes incubation absorbance were measured at 450 nm of main wavelength and 546 nm of substrate wavelength and the data were analyzed with SoftMaxPro 4.8 software.

The total bilirubin (mg/dL) content was calculated as:

Total bilirubin: $= (A_{SMP} - A_{BL})/(A_{STD} - A_{BL}) *$ Conc of Standard (mg/dL)

Where:

$A_{SMP}=A_{smp}-k*A_{smp'}$

$A_{BL}=A_{Bl}-k*A_{Bl'}$

$A_{STD}=A_{std}-k*A_{std'}$

K= (Sample volume+reagent volume)/ (Sample volume +Reagent 1 volume +Reagent 2 volumes)

A_{smp}:sample blank absorbance of sample

A_{std} :sample blank absorbance of standard

A_{Bl} sample blank absorbance of blank

$A_{smp'}$:Absorbance of sample at final measuring point

$A_{std'}$:Absorbance of standard at final measuring point

$A_{Bll'}$ Absorbance of reagent at final measuring point

LDH levels were measured in 200 microL of sera from BMT and non BMT animals were collected as described above through NYP-Weill Cornell Central Laboratory using Beckman LX20 system (Beckman Coulter, Inc, Fullerton, CA).

The experiments were performed in triplicate for each animal analyzed.

Fresh Organs Preparation

Organs collected and washed in PBS were fixed in paraformaldehyde and embedded in paraffin with the following procedure: after an overnight soak in 4% paraformaldehyde tissues were washed in PBS 3 times for 10 min each and then underwent to the following passages of tissue dehydration in:

50% ethanol for 30 min

70% ethanol for 30 min to overnight

85% ethanol for 30 min

95% ethanol for 30 min

100% ethanol for 1 hour 2X

After that, organs were transferred twice in histoclear for 1 hour and then in histoclear/paraffin for 45 min in 58^0C vacuum. At the end samples were put in paraffin for 1 hour and finally embedded in boats.

Some samples, after collection and washing with PBS, were soaked in 4% paraformaldeide for 4h to overnight. The day after tissues were washed in 1X PBS 3 times for 10 min each and then soaked in 30% sucrose/PBS for 4h to overnight. Before staining they were embedded in O.C.T. media (Optimum Cutting Temperature Media) on dry ice.

Longitudinal sections (4-microm) were stained with Hematoxylin and Eosin (H&E). Immunohistochemistry was performed on spleen, liver, and bone marrow sections from two months old mice (BMT and non-BMT) using antibodies against Ki-67, Mcm3, cleaved caspase 3 as previously described (Gerdes J, 1991, Kausch I, 2003.. Endl El, 2001, Traut W, 2002).

Immunohistochemistry (IHC)

For immunostaining, rehydrated cells were antigen retrieved and immunostained in indirect simple or double immunohistochemistry, as previously described in publication by G. Cattoretti (Cattoretti G, 1995, and Cattoretti G, 2005).

Briefly, air- dried slides were fixed in acetone (Fisher; 10 minutes at room temperature), dried, fixed in buffered 10% formalin (10 minutes at RT), rinsed in 0,05 mol/L phosphate-buffered saline (PBS), pH 7.5, and fixed in cold methanol (Fisher; 10 minutes at -20°C). The slides were then washed in 0.05 mol/L Tris-buffered saline, pH 7.5,0.01% Tween-20 (TBS) before incubation with a blocking 3% human AB serum followed by overnight exposure to the appropriate primary antibody. Slides were then washed twice in TBS, incubated with a biotin-labelled goat-anti-rabbit antibody (Dako, Carpintera,CA; 1:300) for 45 minutes, washed twice in TBS, and then overlayed with horseradish peroxidase-conjugated avidin (Dako; 1:300, 20 minutes) and washed again. The slides were developed in aminoethyl carbazole (Sigma, St Louis, MO) and counterstained with hematoxylin. Alternative fixation methods (acetone 10 minutes at RT; buffered10% formalin 10 minutes at RT; cold methanol at -20°C for 10 minutes; acetone followed by methanol) were compared with the above-described acetone-formaline-methanol fixation. Double-staining of sections and cytospins was performed by applying the primary mouse monoclonal antibody after the formalin fixation step (45 minutes at RT). The slides were then washed, fixed in methanol, and incubated with the rabbit antibody. Two-color immunohistochemistry was performed with cleaved caspase antibody from Cell Signaling Technology, MIB 1 (from Dr Gerdes J, Borstel, Germany).

TUNEL

Four micron-thick formalin fixed, paraffin embedded sections were de-waxed, re-hydrated and treated for detection of apoptotic cells via the TUNEL method (In situ cell detection kit, fluorescein, ROCHE, Basel, CH) as per manufacturer's instructions.

Briefly, after reydratation samples were permeabilized for 2 minutes on ice, washed and incubated for 60 minutes at 37 °C with enzyme Terminal Transferase (TdT) and labelling solution (Fluorescein dUTP). During this step Tdt catalyzes the attachment of FITC-dUTP to free 3'OH ends in the DNA. After a final washing analyzed at fluorescent microscope**.**

Purification of Erythroid Cells from Spleen

Spleen from wt, *Th3/+* and *Th3/Th3* mice (both non-BMT and BMT) were collected, resuspended in 10 mL of PBS-10% BSA solution, smashed and reduce in single cell suspension with a 0,40 microm strainer.

Cells were counted and incubated on ice for 15 minutes with a cocktail of non-erythroid FITC-conjugated antibodies :

- GR-1,
- MAC-1,
- CD-4,
- CD-8,
- CD-11b,
- CD-49

10 ug each antibody were used (BD PharMingen, San Diego, CA).

After washing with 5mL of degassed microbeads buffer (PBS-1%BSA), the cells were resuspended in the same buffer and incubated with anti-FITC microbeads as per manufacturer's instructions (Miltenyi Biotech, Auburn, CA) for 15 minutes in a 4°C cold room. The cell suspension was placed in a magnetic column and the erythroid eluted cells are kept for different purposes: resuspended in 750 microL of TRIzol® Reagent (Invitrogen, Carisbad, CA, USA) for RNA extraction, flash frozen for protein analysis or stained flow cytometry analysis. Part of the cells are also seeded for *in vitro* culture with Carboxyn Fluorescein Succymidil Ester (CFSE) (Molecular Probes, Eugene, OR) staining.

Primary Splenic Erythroid Cells Cultures and CFSE Staining

Erythroid cell isolated from the spleen of wt, *Th3/+* and *Th3/Th3* mice as described above were centrifuged and resuspended in sterile phosphate buffer saline (PBS). Samples were set aside for flow cytometry analysis prior to CFSE labeling. CFSE was then added to the cells to give a final concentration of 1.25 microM (working stock 5mM in DMSO). After 10 minutes at 37°C further dye up-take was prevented by addition of 5 volumes of cold complete medium and incubated for additional 5 minutes on ice. Cells were washed 4 times in fresh medium and seeded at 10×10^6 cells/mL in Iscove's Modified Dulbecco's Medium (IMDM) with 30% FBS (BioWest LLC, Miami, FL), 1% deionized BSA, 100 IU/mL Penicillin 100ug/mL streptomycin solution (Mediatech, Herndon, VA), mM □-thioglycerol (mTG) (Sigma-Aldrich, St. Louis, MO), and 10 U/mL rHuEpo. Cells were then divided to be cultured in presence or absence of 100 microM Colcemid. All cells were analyzed after 48.

Flow Cytometry and Determination of Absolute Number of Ter119$^+$, CD71$^+$, Ter119$^+$/CD71$^+$ Cells in Spleen and Bone Marrow.

For fluorescence-activated cell sorting (FACS) analysis, spleen cells were collected as described above while BM cells were obtained flushing the femors or the tibia from BMT or non BMT animal with 5 mL of PBS-1% BSA solution. Cells were resuspended in single cell passing through a strainer and counted.

Staining for CD71 and Ter119

1×10^6 cells were used for each staining tube. Cells were washed and incubated on ice for 15 minutes with 0.1 microg of FITC-labeled anti–mouse CD71 and PE-conjugated anti–mouse Ter119 antibodies alone or together (BD PharMingen, San Diego, CA) in PBS-1% BSA. Control samples were incubated with 0.1 microg FITC-labeled anti–mouse Ig of this and PE-conjugated rat IgG2b isotype control antibodies (BD PharMingen, San Diego, CA).

For CFSE experiment, PE-labeled anti–mouse CD71 and APC-conjugated anti–mouse Ter119 (BD PharMingen San Diego, CA) were used at the same concentration. Acquisition was made with FACS Calibur. Linear Scale was used to define the cells population on FSC and SSC plot while logarithmic scale were use to acquire and analyzed the fluorescence of the dyes. Results were analyzed using Flow-Jo software (Tree Star, Ashland, OR). The percentage obtained for the analysis were used to obtain the absolute number of cells positive for Ter119, CD71 or both of them.

In particular for the spleen cells were counted after smashing of the whole organ while the total number of BM cells was determined by the number of cells from a single femur and multiplying this by 16.7, as indicated in "The total marrow mass of the mouse: a simplified method of measurement" by Boggs DR. in Am. J. of Hemat. 16-3 (1984); pag.277-86. The total number in the BM and spleen were then multiplied by the percentage of CD71+/Ter119+ or CD71-/Ter119+ for each mouse. At least three mice per genotype were analyzed.

Annexin-V /7-AAD Staining for Apoptosis Evaluation For evaluating apoptosis, FITC-Annexin-V (BD PharMingen San Diego, CA cat no. 51-65875X) staining was used in combination with 7-Amino-Actinomycin (7-AAD) (BD PharMingen San Diego, and PE-Ter119 (BD PharMingen San Diego, CA) in order to distinguish between the erythroid (Ter119$^+$) population and viable cells (Annexin-V$^-$/7-AAD$^-$) from early apoptotic cells (Annexin-V$^+$/7-AAD$^-$) or late apoptotic and necrotic cells (Annexin-V$^+$/7-AAD$^+$). After the staining with PE-Ter119 as described above cells, were washed twice with cold PBS and resuspended at concentration of $1*10^6$ cells/mL in 1X binding buffer :10mM Hepes/NaOH (ph 7.4)140mM NaCl2.5 mM CaCl$_2$.100 microL of this solution (10*5 cells) were transferred to a 5mL culture tube and 5 microL of FITC-Annexin-V and/or 5microL of 7-AAD solution were added. Cells were incubated for 15 minutes at room temperature .

400 microL of 1X binding was added to each sample and analyzed immediately by flow cytometry. At least 3 experiments were done for each genotype.

Cytospin Preparation

Cytospins from erythroid purified mouse splenic cells were obtained by centrifugation at 400 rpm for 5 min. Slides were dried for 24h in the dark at room temperature and then stored at –80°C before staining with May-Grunwald-Giemsa or antibodies against Ki-67, cleaved caspase 3, or p21. For BrdU detection, cells were obtained from animals injected with BrdU (50 mg/Kg body weight) and animals were sacrificed two hours after injection. Cytospin cells were stained using anti-BrdU antibody (clone BMC 9318; Roche, Indianapolis, IN).

Caspases Activity Determination

Caspase 3/7 and 9 activities were evaluated on fresh cells using the luminescence assay Caspase-Glo[TM] 3/7 and Caspase-Glo[TM] 9 (PROMEGA, Madison, WI) on splenic erythroid cells purified as described above.

HL-60 cell lines incubated for 4h in presence of 50 microM Vinblastine were used as internal control. As positive control Caspase enzyme 3/7 or 9 provided were used The cells were collected, counted and 40000 erythroid beaded cells or Vinblastine treated HL-60 cells were seeded in a microplate to perform the assay.

To each well were added 100 microL of the Caspase-Glow 3/7 or Caspase-Glow 9 reagent provided. After 2 hours incubation the luminescence was measured by the Absorbance reading at 465 nm and data were analyzed with SoftMax Pro 4.8 software using a four parameter standard curve. The absorbance of the medium used to resuspend the cells was subtracted to obtain the net absorbance of each sample. The experiment was performed in triplicate for at least 3 animals for genotype.

Two-Phase Liquid Culture of Human Erythroid Precursors

After informed consent, fresh blood samples were obtained from normal adult and from ☐-thalassemia patients with different genotypes. Cultures were set up according to the two-phase liquid culture approach previously described by Fibach (Fibach 1993 and 1998). Mononuclear cells were isolated by centrifugation on a Ficoll-Hypaque density gradient (Nycomed Pharma, Zurich, Switzerland) and seeded at a density of $5x10^6$ cells/mL in alpha-

minimal essential medium (α-MEM) supplemented with 10% FBS (both from Gibco-Invitrogen, Carlsbad, CA), 1 microg/mL cyclosporin A (Sigma-Aldrich, St.Louis, MO) and 10% conditioned media from the K5637 bladder carcinoma cell line. The cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air, with extra humidity (phase I). After one week, the non-adherent cells were harvested, washed and re-cultured at a density of $2x10^6$ cells/mL in fresh medium composed of α-MEM, 30% FBS, 1% deionized Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, MO), 10^{-5}M β-mercaptoethanol, (Sigma-Aldrich, St. Louis, MO) and 1U/mL human recombinant erythropoietin (r-HuEpo) (Eprex, Cilag AG International, Switzerland) (phase II).

Samples for expression analysis were collected at the end of the culture and stored for RNA extraction.

Cytospins from human erythroid cells at days 0, 3, 6, 9 and 13 of phase II were harvested and spinned down for 10 min at 306*xg* at 4°C.

After washing cells were resuspended in PBS (ph=7.4)/10%BSA at concentration of $1x10^6$ cells/mL. $1*10^5$ cells were centrifuged for 4 minutes, 800 rpm at room temperature to prepare each cytospin. The slides were dried over night at room temperature and stored at –80°C before staining.

Cytospins from both erythroid purified mouse splenic cells and Human erythroid cultures were stained with May Grunwald –Giemsa or by IHC with anti Ki-67 antibody.

Cytospins were incubated in May Grunwald solution for five minutes at room temperature. After washing in 20mM Tris ph 8.2 slides were incubated for 20 minutes in Giemsa Solution, washed in water and let dry before analysis.

Cell Lines Cultures

Murine erythroleukemic cells (MEL), human lymphoblastic cells (Jurkat), murine fibroblasts cell line (NIH-3T3), HeLa cells, human myeloblastic cells (HL-60), human erythroleukemia cells (K562), and human breast cancer cells (MCF-7) were maintained in RPMI media 1640, Dulbecco's Modified Eagle Media (DMEM), Iscove's Modified Dulbecco Media (IMDM), minimal essential media (Eagle) respectively in presence of :

10% fetal bovine serum (FBS) (BioWest LLC, Miami, FL),

1% L-glutamine (Mediatech, Herndon, VA),

100 IU/mL Penicillin

100ug/mL streptomycin solution (Mediatech, Herndon, VA).

Cell Lysates Preparation and Protein Concentration Assay (BCA)

Cells lysates were obtained after harvesting erythroid purified mouse splenic cells (as described above) and resuspending them in RIPA buffer:

150 mM NaCl, 1%Triton X-100,

0.4% deoxycholic acid sodium salt,

5mM EDTA,

50mM Tris-HCl, ph 7.2

proteases inhibitor cocktail (Pierce, Rockford, IL).

For analysis of phosphorylated proteins specific phosphatase inhibitors were added upon lysis at the following concentration:

1 mM NaF

2 mM Na_4O_4V

The organelles, cytoskeleton and membranes were pelleted by centrifugation at 13,000 rpm for 10 minutes at 4°C. and the supernatant was collected and stored at -80°C before the protein concentration assay.

Protein concentration was determined by the BCA^{TM} Protein Assay Kit (Pierce, Rockford, IL) using manufacturer instruction. Briefly, 25 microL of 1:10 diluted samples or standard were loaded on a microplate and added with 200 mL working reagent prepared mixing the buffer A and B provided at the indicated proportions (1:50).

After incubation of 30 min at 37°C, absorbance at 465 nm was read. Standard curve was made using six different dilution of Bovine Serum Albumin (BSA) provided (stock solution 2mg/ml) and four parameters curve was used for the interpolation by SoftMaxPro Software in order to obtain the final concentration of the samples.

Proteic samples were boiled in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA) for 5 min at 100°C, spinned down to remove debrids and stored at -20°C before the running gel.

Western Blot

25 microg of proteins were separated on 7.5%, 10%, 12.5% polyacrylamide gel and proteins were transferred to a polyvinylidene difluoride membrane (PVDF) (Bio-Rad Laboratories,

Hercules, CA). Nonspecific binding was inhibited by incubation in phosphate buffered saline with 0.1% Tween 20 (PBS-T) (Calbiochem, La Jolla, CA) containing 5% non-fat dry milk or 3% BSA/0.5% NaN_3 for 2h at room temperature.

For phosphorylated protein blocking with 3%BSA was preferred because casein could intefere with the detection of target protein.

Primary antibodies against CDK2 (Santa Cruz Biotechnologies, Santa Cruz, CA), Bcl-X_L (BD PharMingen, San Diego, CA), Cyclin A (Santa Cruz Biotechnologies, Santa Cruz, CA), □-actin (Abcam, Cambridge, MA) were diluted at 1:200, 1:200, 1:1500, 1:3000 respectively in PBS-T containing 3% BSA/0.5% NaN_3; antibodies against p53, p21 (Santa Cruz Biotechnologies,INC, Santa Cruz, CA), Stat-5 and pStat-5 (Cell Signaling Technology, Danvers, MA) were diluted at 1:100, 1:1000 and 1:1000 respectively in PBS-T containing 5% non fat dry milk. All of them were incubated overnight at 4°C followed by incubation with respective horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO) for 1 hour at room temperature.

Immunoreactive proteins were detected according to the enhanced chemiluminescent protocol (Amersham Biosciences). Pictures were acquired with KODAK MI software.

RNA Extraction

Tissue samples from splenic erythroid cells were resuspended in 750 µL of TRIzol® Reagent (Invitrogen, Carisbad, CA, USA) Total RNA was isolated from mouse splenic erythroid enriched cell using standard chloroform/phenol extraction and resuspended in 20 microL of DEPC water.

Retrotranscription of RNA

The first step involves inhibition of RNase and DNAse treatment with 1U of DNase I 10U/microL (Roche, Mannheim, Germany) at the following conditions:

20 minutes 37°C

10 minutes 70 °C

After this step1 microg of RNA was used for cDNA synthesis using oligo-dT (SuperScripit™ III First-Strand Synthesis; Invitrogen Carlsbad, CA).

More in details:

1microL of 50 mM Oligo(dT)$_{20}$ and

1microL of 10mM dNTPs mix

were added to the DNase I treated mix for 5 minutes at 65°C.

To perform the polymerization reaction

1microL of 10X buffer,

4 microL of 25 mM MgCl$_2$,

2 microL of 0,1M DTT and

1microL of (200U/microL) SuperScript III enzyme

were added and incubated at the following condition:

50 minutes, 50°C

5 minutes, 85°C.

At the end the RNase H treatment was performed at 37°C for 20 minutes with 1microL of *E. Coli* RNase H (2 U/microL).

Q-PCR

Real-time PCR was performed in duplicates on Light Cycler 9600 (Applied Biosystem, Foster City, CA) using *Taq*Man (*Taq*Man PCR 2X Master mix; Applied Biosystem) for the housekeeping gene GAPDH and SYBR Green (iTaq^{TM} SYBR Green Supemix; Bio-Rad Laboratories, Hercules, CA) for the other genes. Gene specific primers were designed to span intron-exon boundary by Real-Time PCR Primer Design Program (www.genscript.com).

Real time PCR was performed in duplicates using 12,5 ng of cDNA were in a final reaction volume of 12,5 microL made of:

- 6,25 microL SYBR Green Supemix (2X)

- 0,05 microL of each primer (100microM stock, final concentration in the reaction:400nM)

- 2,5 microL of sample (12,5 ng)

- Water up to 12,5 microL

if SYBR Green was used, or:

- 6,25 microL (*Taq*Man PCR 2X Master mix; Applied Biosystem)

- 0,625 of *Taq*Man probe (Applied Biosystem)

- 2,5 microL of sample (12,5 ng)

- Water up to 12,5 microL

if TaqMan probe was used

The following conditions were used as termal cycle:

Cycle 1: 95°C for 10 min and

Cycle 2: 95°C for 15 seconds

60°C for 1 minutes   X 40 cycles

72°C for 30 seconds.

Cycle 3: 72°C for 7 minutes

Melting curve analysis was also performed to check specificity of product in SYBR Green amplification reaction under the standard the following conditions:

95°C for 15 seconds

60°C for 20 seconds

95°C for 15 seconds

Ramp time: 19 minutes and 59 seconds.

Data were collected at the last step.

For each gene primers were set a specific baseline and threshold to perform the analysis. Relative quantification of gene expression between multiple samples was achieved by normalization against endogenous GAPDH using the $2^{-\Box ct}$ formula. The stability and the consistency of the data were evaluated in parallel experiments using $\Box$-actin or S14 as housekeeping control genes. The fold change was also calculated making the ratio between +/+ and *Th3/+* or *Th3/Th3* relative expression

Jak2 inhibitor administration

Different doses of TG101209 (TargeGen, San Diego, CA), a selective inhibitor of Jak2 (Pardanani, Hood et al. 2007) were administered through oral gavage to the mice twice a day for 10 days. After 10 days the animals were sacrificed and the data collected. In one set of experiments the drug treatment was coupled with blood transfusions. In this case

the mice were transfused one week prior the beginning of drug treatment, and then transfused once a week until the end of the experiment.

Phospho-Jak2 analysis

One million cells per genotype were fixed and permeabilized (Fix and Perm Kit; Invitrogen, Grand Island, NY) as per the manufacturer's instructions. Cells were incubated for 30 minutes with 0.05 microg phospho-Jak2 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or with 0.05 microg isotype control (Santa Cruz Biotechnology). The cells were washed twice with 1% BSA–phosphate-buffered saline and then incubated for 30 minutes at room temperature in the dark with 0.05 microg of a secondary antibody (Jackson ImmunoResearch,West Grove, PA). After washing twice, the cells were immediately analyzed using flow cytometry. For the peptide competition assay, 0.05 microg phospho-Jak2 polyclonal antibody was incubated for 2 hours at room temperature with a 5-fold concentration of blocking peptide in Medium B of the Fix and Perm Kit. The peptide-antibody solution was then added to the fixed cells and they were incubated as described in this paragraph.

RESULTS

**Increased numbers of immature and proliferating erythroid cells in β-thalassemia**

Using HFLCs derived from wt, th3/+, and *th3/th3* embryos, we generated groups of mice that underwent transplantation with the same genetic background. As a control for the transplantation procedure, we compared these animals to same-sex *th3/+* and wt mice that had not undergone transplantation, observing no statistical differences in their respective patterns of erythropoiesis (n ≥ 3 per genotype, data not shown). Two months after transplantation, *th3/+* and *th3/th3* mice revealed anemia (Table1) together with splenomegaly, hepatic EMH, and iron overload as shown previously (Boggs 1984; May, Rivella et al. 2002; Rivella and Sadelain 2002; Rivella, May et al. 2003; Gardenghi, Marongiu et al. 2007).

Table 1. Hematologic parameters in β-thalassemia

Hematologic parameter	wt	th3/+	th3/th3
HB level, g/dL	14.4 ± 0.92	8.80 ± 1.10	2.80 ± 0.81
Total RBC count, $10^6/\mu L$	9.60 ± 0.60	7.90 ± 1.10	2.40 ± 0.60
Total reticulocyte count, $10^5/\mu L$	2.80 ± 0.54	22.4 ± 4.64	2.05 ± 0.74
Total BM CD71$^+$/Ter119$^+$, × 10^8	2.58 ± 0.09	4.04 ± 1.68	1.82 ± 0.83
Total BM CD71$^-$/Ter119$^+$, × 10^8	0.73 ± 0.29	0.23 ± 0.08	0.18 ± 0.08
Total spleen CD71$^+$/Ter119$^+$, × 10^8	0.21 ± 0.09	7.58 ± 3.38	14.40 ± 5.04
Total spleen CD71$^-$/Ter119$^+$, × 10^8	0.71 ± 0.43	1.00 ± 0.35	1.17 ± 0.73
Total BM$^+$ spleen CD71$^+$/Ter119$^+$, × 10^8	2.95 ± 0.99	11.80 ± 5.04	15.0 ± 6.13
Total BM$^+$ spleen CD71$^-$/Ter119$^+$, × 10^8	1.51 ± 0.44	1.26 ± 0.39	1.52 ± 0.88
% (CD71$^+$/Ter119$^+$)/(Total Ter119$^+$)	4.97 ± 2.48	58.70 ± 0.94	80.20 ± 4.73

Data are means plus or minus SD.

We also examined the spleen and the BM by fluorescence activated cell sorting (FACS), comparing the fraction of cells in discrete erythroid populations using markers for the transferrin receptor (CD71) and erythroid specificity (Ter119). In thalassemic mice, there was both a relative and absolute expansion of the immature erythroid progenitor cell fraction compared with cells in the final stages of erythroid differentiation and maturation (orthochromatic cells, reticulocytes, and erythrocytes; Figures S2,Table 1). A skewed FACS profile indicative of IE was already visible in *th3/th3* embryos at E16.5, as indicated by the absence of more mature cells (Figure 3 red oval). This indicated that engraftment of HFLCs into healthy adult animals faithfully transferred the genetic defect

leading to IE. Animals that received a transplant of *th3/th3* HFLCs showed low reticulocyte counts and loss of mature erythroid cells (CD71+/Ter119+) by FACS, suggesting that the disappearance of erythroid cells in these animals occurs prior to or simultaneous with the generation of reticulocytes. Cytospins (Figure 1A) of purified erythroid cells (as shown in Figure S1) clearly showed that the thalassemic samples were composed of a homogeneous population characterized by a larger and more immature cells. This findings indicates either elimimation of late-stage erythroid progenitors or an imbalance between cell proliferation and cell differentiation.
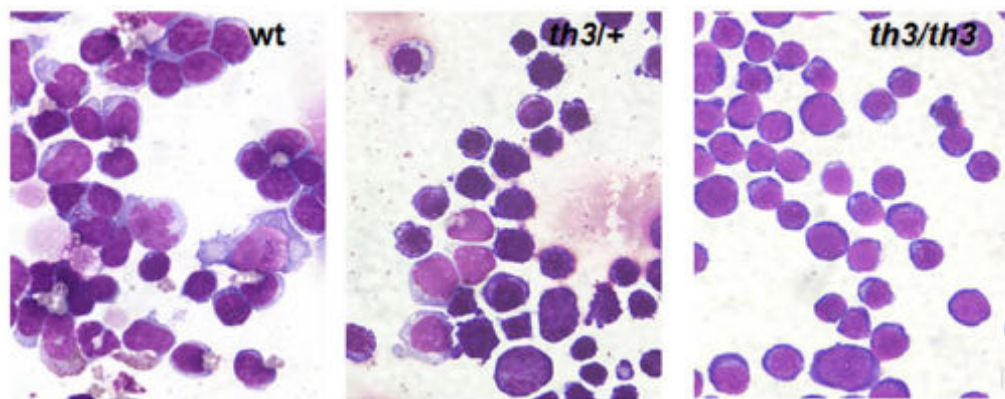
Figure1. In cytospins of purified splenic erythroid cells, distinctive type of cells can be seen representing different stages of maturation. More mature cells are characterized by a smaller size, decreased cytoplasmatic basophilia, and an increase in nuclear pyknosis.

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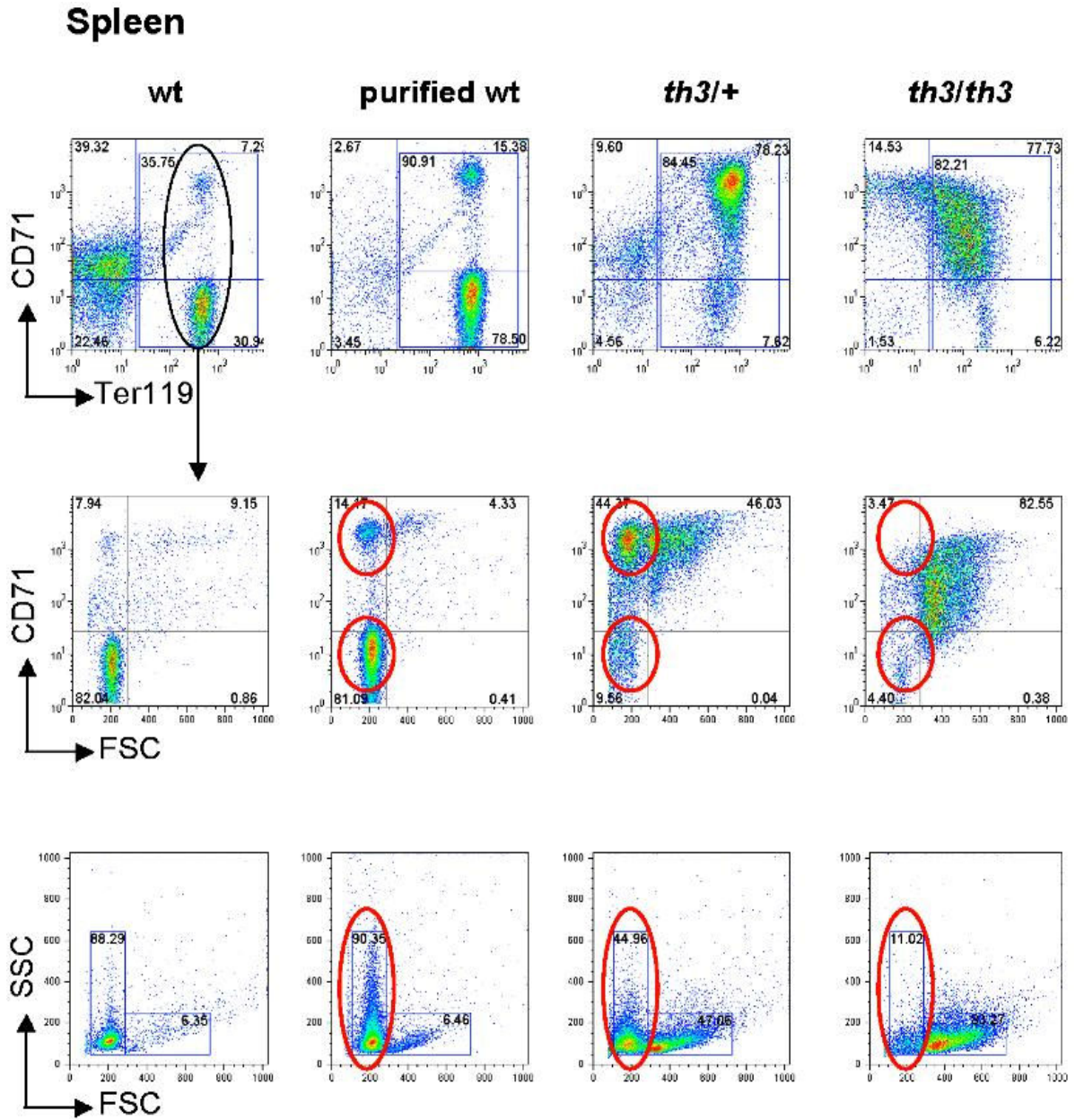
Figures S2. Characterization of the erythroid compartment shows an increase of homogeneous undifferentiated immature cell populations in thalassemic mice

FACS analysis of splenic erythroid populations using CD71 and Ter119 co-staining. CD71^{+}/Ter119^{+} and CD71^{−}/Ter119^{+} staining identifies early (nucleated) and late (non-nucleated) erythroid cells[1]. The FACS profiles were further analyzed as described by Liu et al. (Liu, Pop et al. 2006). In particular, the Ter119^{+} cells (indicated by the black oval) were analyzed by CD71/FSC, showing that the *th3*/+ mice exhibited a reduction of the most mature fractions (orthochromatic and non¬nucleated indicated with red circles in wt mice) and an enrichment of the immature one (indicated with a green circle). The purified population indicates a representative FACS profile of splenocytes enriched in erythroid cell fractions by immunomagnetic lineage negative depletion (as described in the Material and Methods section). More than 95% purity was achieved in all cases.

Apoptosis and hemolysis levels are only slightly increased in β-thalassemia

Historically, IE in thalassemia has been attributed to increased cell death due to apoptosis or hemolysis of erythroid cells during the maturation process. Apoptosis in β-thalassemia has been investigated primarily using the annexin-V assay. We used this assay in our animal studies and observed some differences (Figure 3E), although they were not statistically significant. However, annexin-V also labels mature erythrocytes, and it has been shown that thalassemic cells have an abnormal exposure of phosphatidylserine (Boggs 1984; Kuypers 1998). Therefore, this assay could also recognize the membranes of senescent and damaged erythroid cells that are not necessarily undergoing apoptosis. To evaluate only apoptosis in nucleated erythroid cells, we conducted more specific assays, involving cleaved-caspase-3 (CC3; Figure 3B) and TUNEL on the erythroid tissue specimens (Figure 3C). Both assays exhibited limited qualitative differences between thalassemic and healthy mice, lower than those observed with the annexin-V assay. Only quantitative analyses by CC3 (Figure 3D) staining of purified splenic cells revealed a small increase in the percentage of apoptotic cells in thalassemic mice compared with controls (from less than 1% in wt mice to 4% in *th3/th3* animals). Bilirubin and lactic acid dehydrogenase (LDH) levels, which are elevated if red cells hemolyze, were unchanged or only slightly increased in thalassemic compared with healthy mice (Figure 3F,G). In *th/+* mice, these observations indicated that limited hemolysis was present despite erythrocyte formation. In *th3/th3* erythroid cells, the average amount of alpha-globin transcript was, on average, 3-fold less than that in wt animals (data not shown). Therefore, the low bilirubin and LDH levels in *th3/th3* mic emphasize the limited maturation of their erythroid cells, the erythropoiesis blockade happening before the formation of fully hemoglobinized cells. In summary, the immature morphology exhibited by thalassemic erythroid cells suggests that an altered cell cycle and limited cell differentiation could be responsible for the low levels of apoptosis and hemolysis seen in this disease compared with earlier predictions arising from ferrokinetic measurements (Finch, Deubelbeiss et al. 1970; Pootrakul, Sirankapracha et al. 2000).
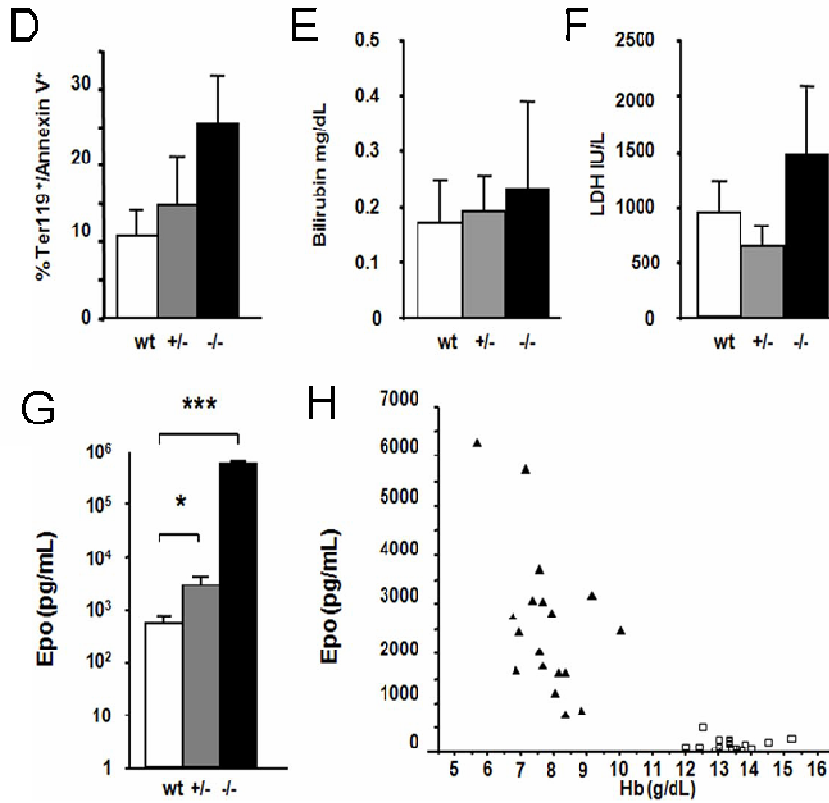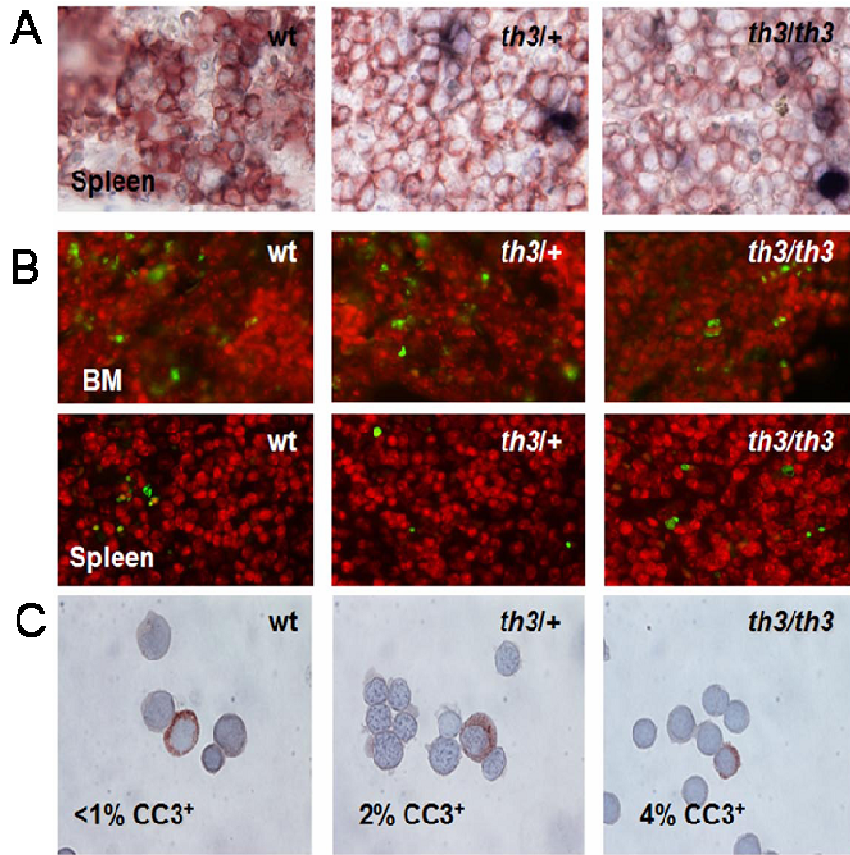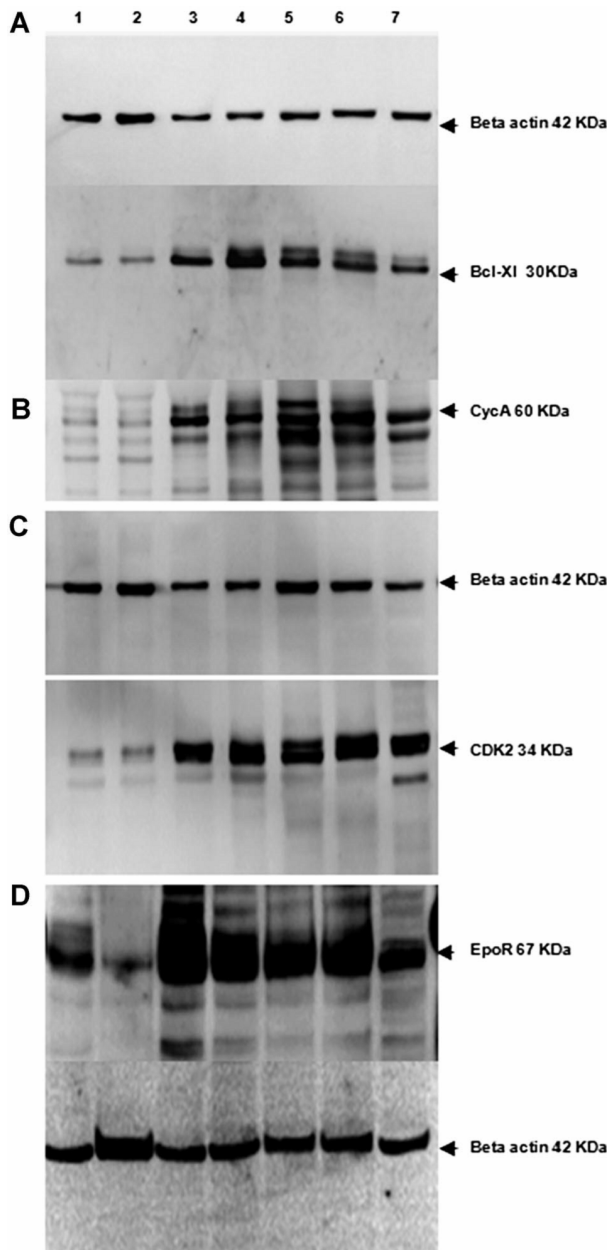
Figure 3

Apoptosis was investigated (A)by CC3 assay on spleen sections; (B) by TUNEL assay on BM and spleen sections; (C) by CC3 assay on purified erythroid cells; and (D) by annexin-V assay on fresh spleen and BM cells (data not shown; n ≥ 3 per genotype). The levels of hemolytic markers (E) bilirubin and (F) LDH were also investigated (n ≥ 6 per genotype). In panels D-H, *th3/+* and *th3/th3* mice are indicated as +/- and-/-, respectively. A nonparametric *t* test was used for statistical analysis. Increased Epo levels inversely correlate with those of hemoglobin in thalassemic mice. Measurements were made of (H) Epo levels in mice 2 months after BMT and (I) Epo and Hb levels in mice up to 1 year of age. In panel H, a nonparametric *t* test was used for statistical analysis; n ≥ 3 per genotype; $P < .037$ (*) and $P < .001$ (***), respectively, for *th3/+* and *th3/th3* mice compared with wt animals. In this panel *th3/+* and *th3/th3* mice are indicated, respectively, as +/- and -/-. In panel H, increased Epo levels inversely correlate with Hb in thalassemic mice. Epo levels were measured in random mice up to 1 year of age or 1 year after BMT in wt (□, n = 17) and *th3/+* (▲, n =18)mice. Pearson r test was used to determine the degree of linear association or the correlation coefficient between the Hb and Epo levels (wt, nonsignificant, $P=.087$; *th3/+*, $P = .027$).

Error bars represent SD.

β-Thalassemia is characterized by an increased number of proliferating eryhtroid cells

Because the Epo levels in thalassemic animals were dramatically increased (Figure 3G, H), we investigated some of the downstream markers related to the cell cycle. High levels of Epo could explain the relatively low levels of apoptosis that we observed in thalassemic animals, since this cytokine prevents apoptosis and induces cell proliferation (Richmond, Chohan et al. 2005). On binding Epo, EpoR undergoes a conformational change that, through Jak2, triggers the signal transducers and activators of the transcription factor Stat5. As a consequence, several proteins involved in the cell cycle and in apoptosis modulation such as Bcl-XL might be up-regulated (Ihle 2001; Rhodes, Kopsombut et al. 2005). Using Western blot analysis, we showed that larger amounts of the proteins EpoR, Bcl-XL, cyclin-dependent kinase 2 (Cdk2), and CycA (Figure 2A-D) were associated with purified *th3/+* and *th3/th3* erythroid cells compared with those from wt controls. These data suggest that an increased number of erythroid cells are proliferating and protected from apoptosis or that genes, which promote the cell cycle and attenuate apoptosis, are up-regulated in the erythroid cells of β-thalassemic mice. To further analyze cell cycling and differentiation, we investigated the number of proliferating cells. Ki-67 can be detected in all active phases of the cell cycle, whereas exit from the active cell cycle leads to a rapid down-regulation of its mRNA and protein expression (Endl, Kausch et al. 2001; Traut, Endl et al. 2002). The Mcm3 protein is expressed both in proliferating cells and in those that have ceased to do so, but are not terminally differentiated (Endl, Kausch et al. 2001). Both Ki-67 and Mcm3 staining gave similar results, showing that a larger number of erythroid cells in the splenic red pulp of *th3/+* and *th3/th3* animals were positive for these 2 markers than in wt mice (Figure 5A-C). Moreover, clonogenic assays also showed an increased number of erythroid progenitor cells in *th3/+* and *th3/th3* mice compared with controls (erythroid burst-forming units [BFU-Es] were increased 1.3- and 4.7-fold, respectively, in BM, and 40- and 600-fold, respectively, in spleen, whereas CFU-Es were increased 4.0- and 5.0-fold in BM, respectively, and 43- and 600-fold, respectively, in spleen; n = 3 per genotype). Similar results were obtained previously in another strain of mice affected by thalassemia intermedia (Popp, Popp et al. 1985; Beauchemin, Blouin et al. 2004). To assess whether Ki-67+ cells were proliferating, and to quantify the percentage and total number of erythroid cells in S-phase, we injected 5-bromo-2-deoxyuridine (BrdU) into healthy and thalassemic mice.

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Our data showed that a large number of BrdU+ cells were also Ki-67+ (Figure 6), and a higher proportion of purified nucleated erythroid cells were in S-phase (BrdU+) in thalassemic compared with healthy mice (22%, 30%, and 45%, respectively, in wt, th3/+, and *th3/th3* mice; Figure 5D). Taken together, these data indicate that there is an increased number of erythroid cells in thalassemia that are proliferating (Ki-67 and BrdU assays) and that are not terminally differentiated (Mcm3 assay).


Figure 4. Increased amount of antiapoptotic and cell cycle–related proteins in purified wt and thalassemic erythroid cells. Representative Western blots performed on cells from wt (lanes 1,2), *th3/+* (lanes 3,4), and *th3/th3* (lanes 5,6) mice, and control cell lines (lane 7) probed with (A) Bcl-XL (control: NIH-3T3 cells), (B) CycA (control: Mel cells); (C) Cdk2 (control: Mel cells); and (D) EpoR (control: K562 cells). The upper band in panel A is described as the deamidated form of the protein. Bcl-XL deamidation has been shown to produce a complete loss of the antiapoptotic function of Bcl-XL (Takehara and Takahashi 2003). Similar ratios of the 2 bands are present in both normal and thalassemic mice. The membrane used for Bcl-XL was reprobed with CycA antibody. Specific antibodies against the phosphorylated and nonphosphorylated forms of the protein were used sequentially. In all cases, the same membranes were reprobed against β-actin as a loading control.
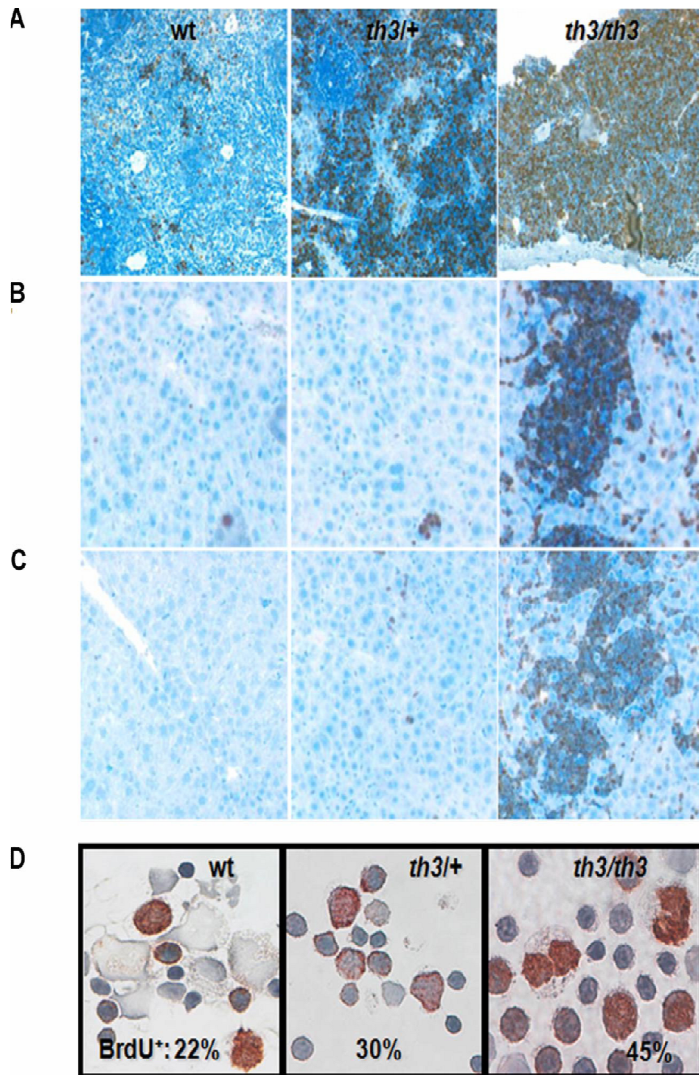
Figure 5. Increased number of cycling and undifferentiated erythroid cells in thalassemic versus healthy mice.
Immunostaining for Ki-67 on spleen (A) and liver (B) specimens and for Mcm3 on liver (C) showed an increased number of cycling and undifferentiated cells in extramedullary sites of thalassemic mice (magnification, 400X). Mcm3 was also probed on spleen sections and the pattern was very similar to that observed for Ki-67 (data not shown). In particular, thalassemic liver sections showed an increased number of proliferating cells in areas associated with EMH. Cyclin-B1 staining (data not shown) confirmed that more proliferating cells are present in the spleens of thalassemic compared with wt mice. (D) Staining and analysis of cytospins of purified splenic erythroid cells after injection of BrdU in vivo showed that there is an increased percentage of cycling erythroid cells in β-thalassemic mice compared with healthy (20%, 30%, and 40% in wt, *th3/+,* and *th3/th3,* respectively; magnification, 400x)
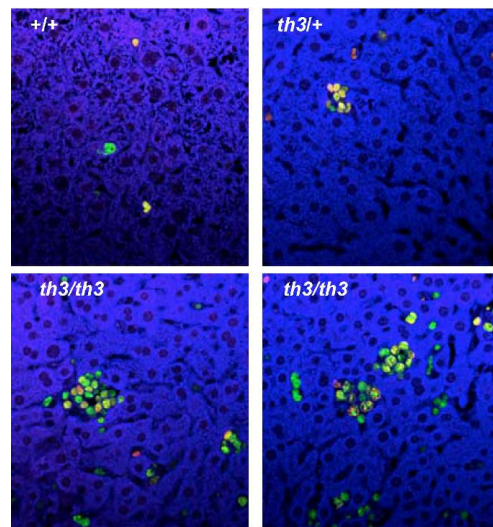
Figure 6. The majority of the Ki-67^{+} cells are also BrdU[+]
In order to confirm that Ki-67^{+} cells were replicating, BrdU[+] was injected in vivo and areas of EMH in liver sections from normal and thalassemic animals were investigated for BrdU and Ki-67 by immunofluorescence. Double positive cells were observed only rarely in +/+ liver sections, while many Ki-67^{+}/BrdU^{+} cells were identified in specimens from thalassemic mice. Ki-67 (green)/BrdU (red)/Toto-3 (blue).

An inhibitor of Jak2 prevented proliferation of thalassemic erythroid cells

Based on the BrdU results achieved in vivo, we sought further evidence that the expansion of the immature erythroid compartment was sustained by a large number of cycling cells in vitro. Purified erythroid cells isolated from the spleens of healthy and thalassemic mice were cultured in the presence of Epo, with and without colcemid, an antimitotic agent. To visualize cell division, we stained the cultured erythroid cells with CFSE. Once the dye is inside the cell, it binds to cytoskeletal proteins and is divided equally between daughter cells. Thus, it is possible to determine whether cells are dividing by monitoring the reduction of CFSE fluorescence (Figure 7A). After 48 hours in culture, wt cells exhibited some differences, depending upon whether they were cultured with or without colcemid, indicating absent or limited cell proliferation (46% ± 9% and 61% ± 12% of the initial cell numbers, respectively, with and without colcemid; n = 4). In contrast, a large proportion of *th3/+* and *th3/th3* cells were able to proliferate over the same time period (Figure 7A), leading to an increase in the total cell number (*th3/+,* 88% ± 18% with colcemid and 132% ± 19% without; *th3/th3,* 72% ± 25% with and 170% ± 22% without; n = 4 each genotype). We then investigated the phosphorylation of Jak2 in normal and thalassemic erythroid cells. This analysis showed that a larger percentage of erythroid cells was positive for phospho-Jak2 (pJak2) in thalassemic compared with healthy mice (Figure 7B, n = 3). Based on these observations, we investigated the effect of Jak2 inhibitors on the erythroid cultures. AG490 and TG101209, inhibitors of Jak2 (Neria, Caramelo et al. 2007; Pardanani, Hood et al. 2007), had the same effect as colcemid, blocking cell proliferation (Figure 7A, only the results for AG490 are shown). The FACS profile and the total number of cells were also similar with colcemid and the Jak2 inhibitors (data not shown). Altogether, these data indicate that the increased number of proliferating cells in β-thalassemia are associated with Jak2-mediated signaling.
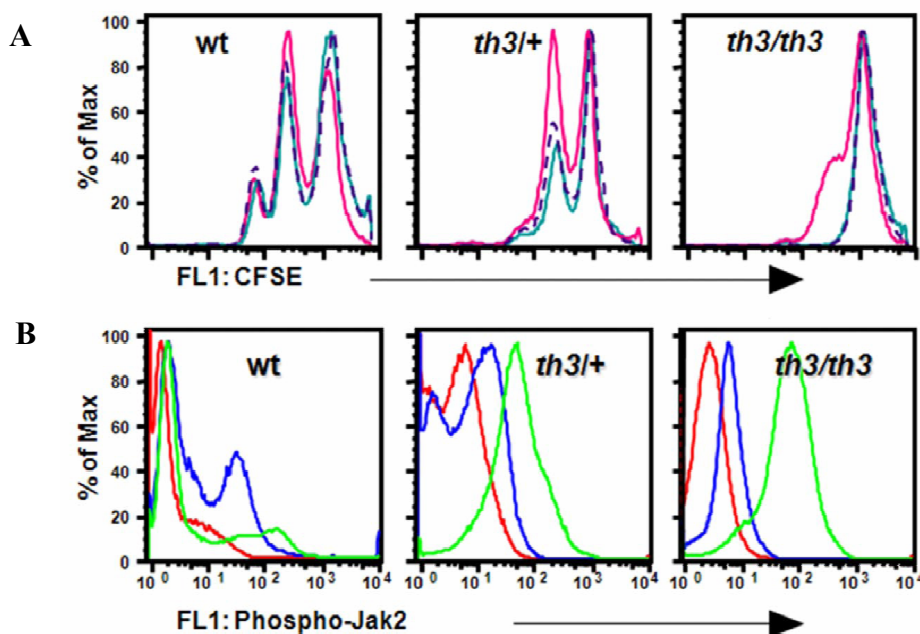
Figure 7. Increased number or erythroid cells in thalassemic versus healthy mice.
(A) FACS analysis of CFSE-treated cells costained with antibodies to CD71 and Ter119. Erythroid cells from wt mice cultured in the presence of colcemid (purple line) orAG490 (blue line) showed little difference from untreated cells (pink line). Staining with 7-AAD, PI, and annexin-V excluded dead or apoptotic cells (n=4 per genotype). After 48 hours, no further cell expansion was observed; instead there is a decline in cell number, indicating that these cells did not have an intrinsic self-sustaining abilityto proliferate under these tissue culture conditions. (B) FACS analysis of freshly purified erythroid cells using an antibody that recognizes the phosphorylated form of Jak2 (green line). The blue line represents the cells stained with the isotype. As a control for the specificity of the antibody, the same cells were stained with the antibody after preincubation with the competitor peptide (red line, n=3 per genotype).

Thalassemic erythroid cells differentiated less than similar immature normal erythroid cells *in vitro*

To determine whether the differences observed in vitro corresponded solely to the relative number of erythroid cells in dissimilar stages of erythroid differentiation in healthy and thalassemic mice, we induced anemia in healthy mice by repeated phlebotomies, decreasing their hemoglobin (Hb) level to less than 40 g/L (4 g/dL). At this point, the mice exhibited splenomegaly similar to that seen in *th3/th3* mice with almost exclusive production of CD71+/Ter119+ cells (Figure 8A,B). Cytospin analysis of these cells showed morphology similar to that observed in *th3/th3* mice, with the majority of cells showing a predominant primitive progenitor morphology and no hemoglobin content, as assayed with tolidine staining (Figure 8C). A larger number of wt cells, however, were enucleated compared with the *th3/+* ones, showing indeed higher Hb content, revealed by the tolidine staining. No enucleated

erythroid cells were detectable in the *th3/th3* specimens, revealing no Hb content and a proerythroblast morphology (Figure 8D). The CFSE profile of wt cells cultured under colcemid conditions was similar to that of cells from *th3/+* mice, corroborating the fact that phlebotomy expanded the immature fraction of erythroid cells (Figure 8D). However, the CFSE profile of erythroid cells derived from phlebotomized wt and *th3/+* mice did not completely overlap that of the *th3/th3* mice, indicating that the rate of erythroid cell differentiation in thalassemia is proportional to the degree of anemia. Although a larger number of normal cells proliferated compared with those from nonphlebotomized wt animals (data not shown), they rapidly differentiated in culture. This observation suggests that thalassemic erythroid cells have an intrinsic ability to limit their differentiation. Therefore, the differences observed *in vivo* are likely to have resulted from an expansion of the immature fraction of the erythroid cells limited in their capacity to differentiate.
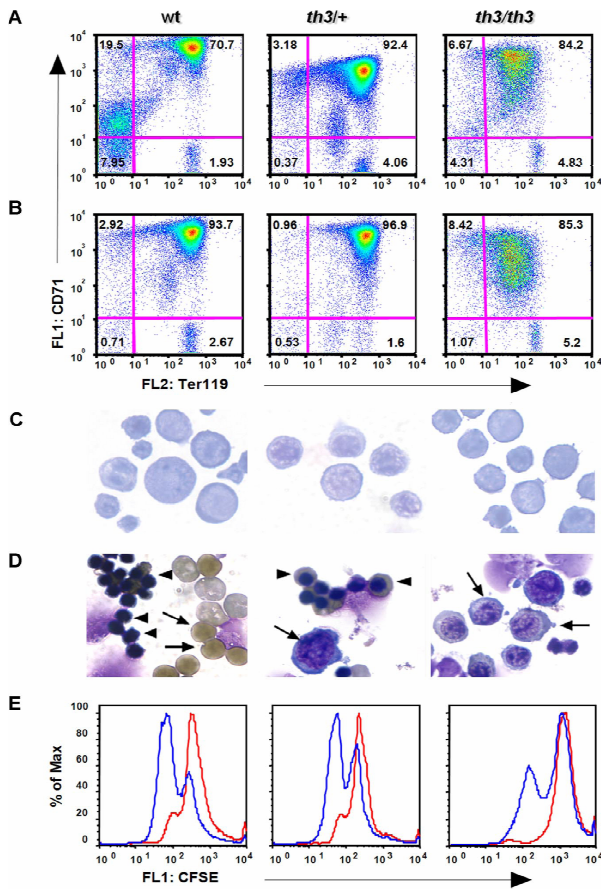
Figure 8. Thalassemic erythroid cells differentiated less than similar immature normal erythroid cells in vitro. (A) FACS analysis of wt, *th3/+,* and *th3/th3* splenic erythroid cells before erythroid cell selection. Wt and *th3/+* mice were phlebotomized. (B) FACS analysis was repeated after selection. Numbers on plots are percentages of total cells in the respective gates. (C) Cytospin analysis at time 0 and (D) after culturing the cells for 48 hours in the presence of Epo. Wt cells are all tolidine positive, with the presence of extruded nuclei (arrowhead), and bright tolidine-positive reticulocytes (arrow). The *th3/+* sample is characterized by the presence of hemoglobinized polychromaticorthochromatic erythroblasts (arrowhead), and some rare proerythroblasts (arrow). In the *th3/th3* sample, only proerythroblasts/early basophilic erythroblasts (arrow) were detectable, with no presence of tolidine-positive or enucleated cells. (E) CFSE analysis of the erythroid populations. Erythroid cells cultured in the presence of colcemid plus Epo (purple line) or Epo alone (blue line).

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**In vivo administration of a jak2 inhibitor reversed splenomegaly**

Based on our accumulated data, we postulated 2 hypotheses. In the first one, relentless phosphorylation of the Jak2 protein by high Epo levels might be sufficient to maintain the erythroid cells in active proliferation, limiting their differentiation. However, this is unlikely to be the right scenario, because constitutively active mutant Jak2 tyrosine kinases, such as Jak2V617F, lead to polycythemia vera rather that IE (Arcasoy, Karayal et al. 2002; Wang, Vandris et al. 2008). Alternatively, we suggest that constitutive activation of the Epo-Jak2 pathway is necessary but not sufficient to cause IE. To discriminate between these 2 alternative hypotheses, we administered TG101209 to cohorts of healthy and thalassemic mice of different ages. We found that 10 and 18 days of treatment were sufficient to dramatically reduce the spleen size in 6- and 12-week-old thalassemic mice (Figure 9). This treatment was associated with a reduced ratio of CD71+/Ter119+ and CD71+/ Ter119+ cells in mice treated with TG101209 compared with placebo (Figure 9). However, these changes were associated with decreasing Hb levels (Figure 9). These observations indicate that the main role of pJak2 is to propel the erythropoietic drive. It might also be a factor in IE, but further experiments are required to completely elucidate its potential role in limiting erythroid cell differentiation in thalassemia. Administration of the Jak2 inhibitor also affected both erythropoiesis and the size of the spleen in young healthy animals, at a time when the erythron is still expanding. On one hand, these observations support the notion that pJak2 has a physiologic role in normal erythropoiesis. On the other, the use of Jak2 inhibitors may have limited effects on normal erythropoiesis of healthy adults while still limiting splenomegaly as in thalassemia patients.
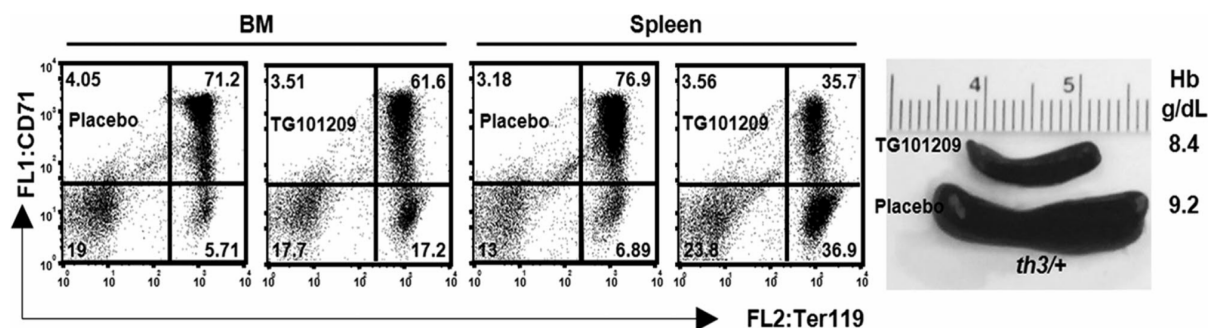

Figure 9. TG101209, a Jak-2 inhibitor, reduced splenomegaly in thalassemic mice. Representative FACS analysis of 6-week-old *th3*/+ mice injected for 10 days with TG101209 or placebo, as indicated. The corresponding spleens and the Hb levels are shown. Numbers on plots are percentages of total cells in the respective gates.

Erythroid cells from thalassemic patients exhibit some of the features observed in thalassemic mice

We also analyzed erythroid cells from the peripheral blood of healthy and thalassemic subjects. Using a 2-phase liquid culture system (Figure 10), we amplified erythroid progenitor cells isolated from the blood specimens.55 Although β-globin mRNA and Hb production were reduced in thalassemic patients as expected (data not shown), the levels of cell cycle–related mRNAs such as *Jak2, Ki-67, CycA, Bcl-XL,* and *EpoR* were significantly higher than normal (Figure 11A). Up-regulation of Ki-67 was confirmed by immunohistochemical analysis of the cells after various times during the second phase (erythroid expansion and differentiation) of the culture system (Figure 11B). In addition, spleen sections derived from control and thalassemic patients undergoing splenectomy were stained with glycophorin C, alpha-1-spectrin, and Ki-6 antibodies to qualitatively evaluate the number of replicating (Ki-67+) erythroid cells. Both the alpha-1-spectrin and glycophorin C markers recognize early erythroid progenitors, whereas glycophorin C also tags late erythroid cells. Figure 11C shows that thalassemic splenic tissue has more red pulp and a greater number of Ki-67+ erythroid cells than that from a healthy patient (traumatic splenic rupture). These last observations confirm the data from the 2-phase liquid culture system and, for the first time, show that a considerable number of erythroid cells in an enlarged thalassemic spleen were actively proliferating despite the patient having received a transfusion.
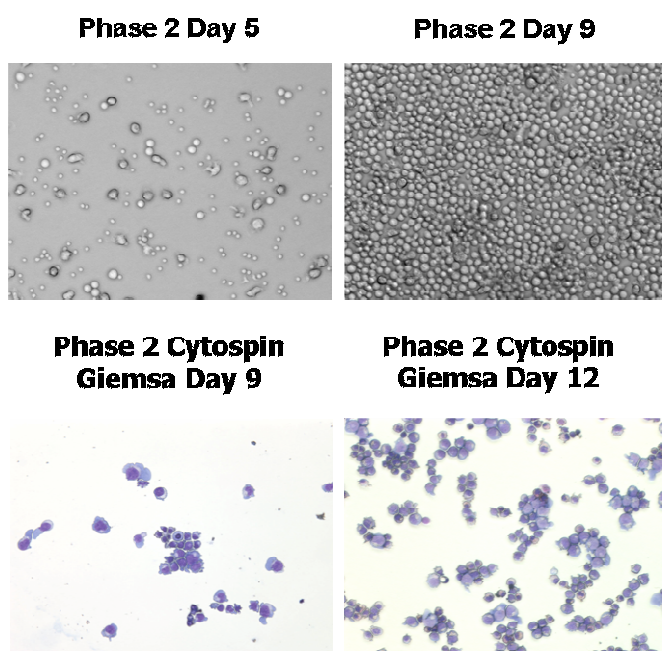

Figure 10. 2-Phase liquid culture. Early erythroid progenitors are collected from peripheral blood. Subsequently they are cultured in medium in the presence of Epo where they grow and eventually differentiate.
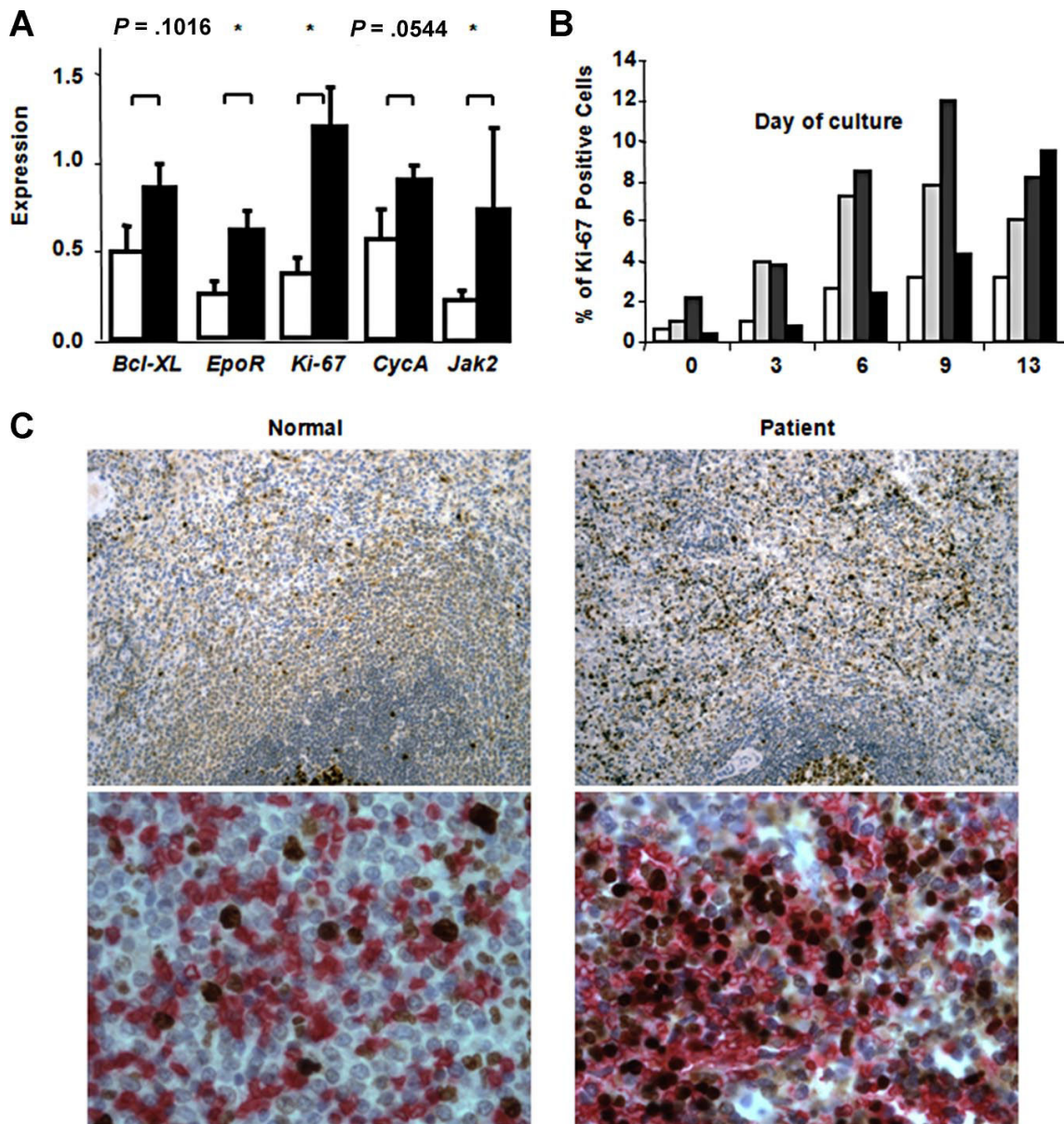
Figure 10. Increased number of proliferating erythroid cells in human thalassemic specimens. (A) At the end of the 2-phase liquid culture, the absolute expression of *Bcl-XL, EpoR, Ki-67, CycA,* and *Jak2* mRNA relative to 14S ribosomal control RNA was quantified in 11 patients (black) and 6 healthy controls (white). An unpaired *t* test was used for statistical analysis. Benzidine staining was used to evaluate the level of erythroid differentiation. Both the amounts of β-globin mRNAs and those of cell cycle–related genes were quantified by quantitative polymerase chain reaction assay. We also performed high performance liquid chromatography (HPLC) to determine variations in the percentages and absolute amounts of adult Hb in treated and non-treated samples (not shown). Error bars represent SD. (B) Time dependence of proliferating erythroid cells from 3 thalassemic patients (light gray, gray, and black) and one control subject (white).Aliquots of cells after various days of culture (second phase), corresponding to different stages of erythroid differentiation, were collected, cytospun, and stained for the proliferative marker Ki-67. Thalassemic patients showed an increased number of proliferating cells. More than 300 cells were counted to obtain the percentage of Ki-67–positive cells in each aliquot. (C) Spleen sections from a healthy subject (traumatic rupture) and a thalassemic patient (transfused thalassemia intermedia) who underwent splenectomy. Top panels: Ki-67 staining (brown; magnification, 100X). Bottom panels: Ki-67 (brown) and a mixture of glycophorin C and alpha-1-spectrin (red; magnification, 400X).

TG101209 reversed splenomegaly and decreased extra-medullary erythropoiesis in transfused *th3/th3* mice

β-thalassemia intermedia (TI) and major (TM) are characterized by Ineffective Erythropoiesis (IE). We hypothesized that the kinase Jak2 plays a major role in IE and splenomegaly. To test this hypothesis we administered a Jak2 inhibitor (TG101209) to mice affected by TI, showing that this treatment was associated with a marked decrease in IE, and a moderate decrease in hemoglobin (Hb) levels (~1 g/dL). This last observation indicates that the use of a Jak2 inhibitor might exacerbate anemia in thalassemia. However, we hypothesized that using standard transfusion to treat TM mice would also adequate to prevent any further anemia caused by Jak2 inhibition while still allowing for decreased splenomegaly. Therefore, we analyzed the erythropoiesis and iron metabolism in TM animals treated with a Jak2 inhibitor and transfused (Figure 11).
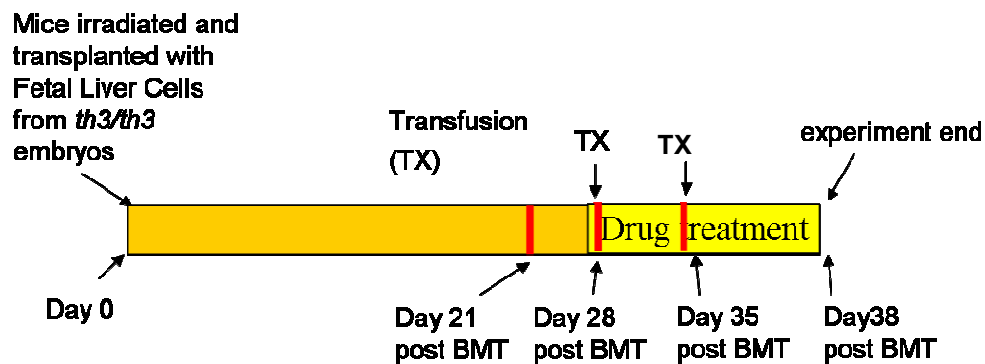
Figure 11. Schematic representation of administration of TG101209 together with blood transfusions

Use of TG101209 in TM mice not only reduced the spleen size dramatically (0.42±0.15 g and 0.19±0.10 g respectively in transfused+placebo (N=4) vs transfused+TG101209 (N=8), P= 0.007) (Fig. 12A and B), but also allowed the mice to maintain higher Hb levels (respectively 7.3±1.1 g/dl vs 9.3±1.2 g/dl, P=0.019) (Fig. 12C). This was likely due to reduced spleen size and limited red cell sequestration. Contrary to TM mice treated with transfusion+placebo, no foci of extra-medullary hematopoiesis were detectable in the parenchema of mice treated with TG101209 (Fig. 12D). Hepcidin (*Hamp1*) expression inversely correlated with the spleen weight, possibly indicating that suppression of IE (due both to blood transfusion and TG101209 administration) had a positive effect on Hamp1 expression. Hb levels also directly correlated with *Hamp1* expression in the same animals. In this case, however, only transfusion played a role in increasing *Hamp1* expression, although TG101209 undoubtedly had a positive effect by reducing the spleen size and thereby indirectly increasing the Hb levels.

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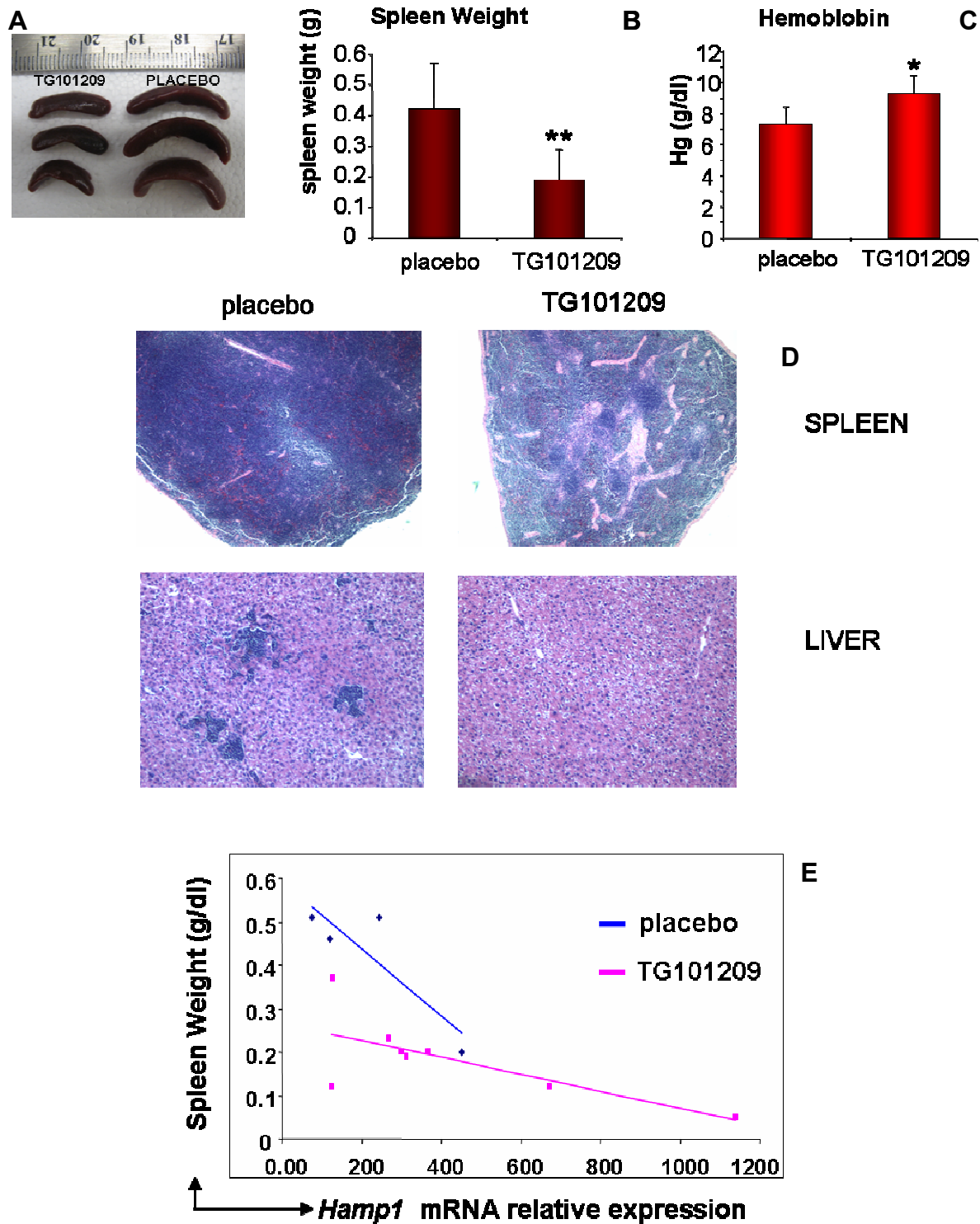
Figure 12. (A) Representative picture of spleens from transfused *th3/th3* mice sacrificed 10 days after TG101209 (N=8) or placebo (N=4) treatment. (B) Plot analysis showing a significant (P<0.01) reduction of spleen weight in TG101209 treated animals compared to the placebo treated mice and increased hemoglobin levels in the drug treated mice (P<0.05) (C). (D) Representative pictures (magnification 40X) of spleen and liver sections from drug treated and placebo treated animals stained with H&E. Spleen from *th3/th3* placebo treated mice show an altered morphology, with loss of typical architecture due to over-expansion of the red pulp. On the other hand, spleens from *th3/th3* TG101209 treated mice show a partially restored morphology, with foci of white pulp and surrounding red pulp. Liver of drug treated animals shows the absence of erythropoietic foci. (E) Negative correlation between hepcidin levels and spleen weight in mice treated with TG101209.

A tailored administration of TG101209 reversed splenomegaly without affecting anemia in th3/+ mice

The suppression of erythropoiesis by blood transfusion limits the extent of our interpretations as it may mask the effect of the Jak2 inhibitor. Therefore we hypothesized that the administration of a tailored and reduced dose of the drug could be effective in reducing the splenomegaly in nontransfused TI mice, without affecting the Hb levels. We also hypothesized that the suppression of erythropoiesis would also lead to increased *Hamp1* expression in the presence of iron overload. Compared to mice treated with placebo (N=5), analysis of TI mice treated with a tailored dose of 100mg/kg/day of the drug (N=11) showed a significant decrease in spleen size (0.18±0.05 g and 0.27±0.05 g, P=0.006 for drug treated mice and placebo treated mice respectively) (Fig. 13B). Of note no significant difference of Hb levels was detectable between the 2 groups (Fig 13A). In the drug treated mice we observed a significant decrease of the immature erythroid cell population (P=0.012) (13D) and amelioration of the architecture of the spleen, with the reappearance of white pulp foci and a significant restoration of the splenic lymphocitic populations (Fig.14). Drug treated mice showed increased levels of *Hamp1* mRNA that inversely correlated with the spleen weight, suggesting a direct feedback between erythropoietic rate and expression of *Hamp1*(Fig.14).
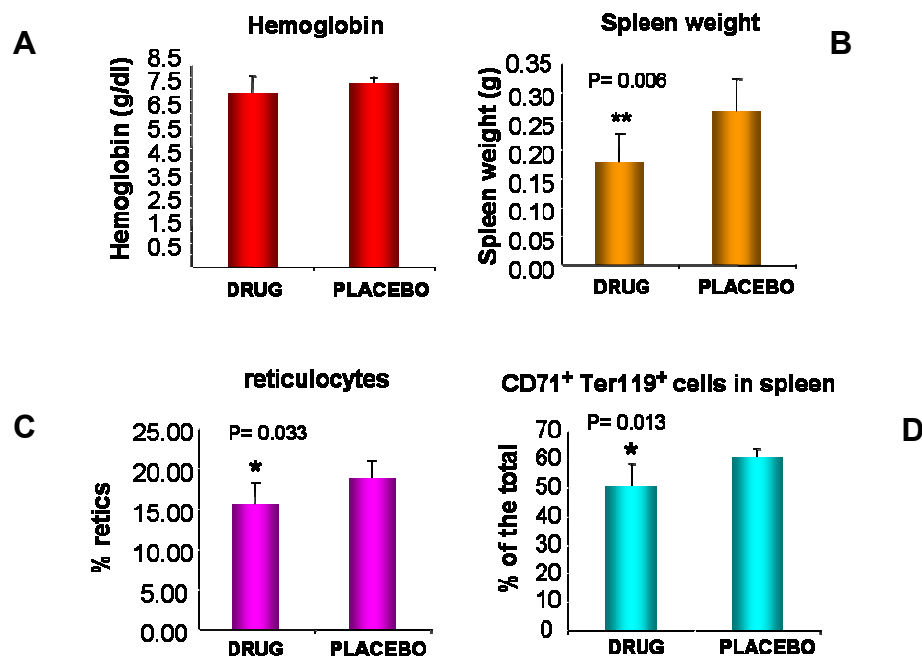
Figure 13. Administration of TG101209 to untransfused th3/+ mice. Without affecting the hemoglobin levels (A) the treatment was efficient in reducing the spleen size (B), the retics count (C) and the percentage of immature erythroid cells in the spleen (D).
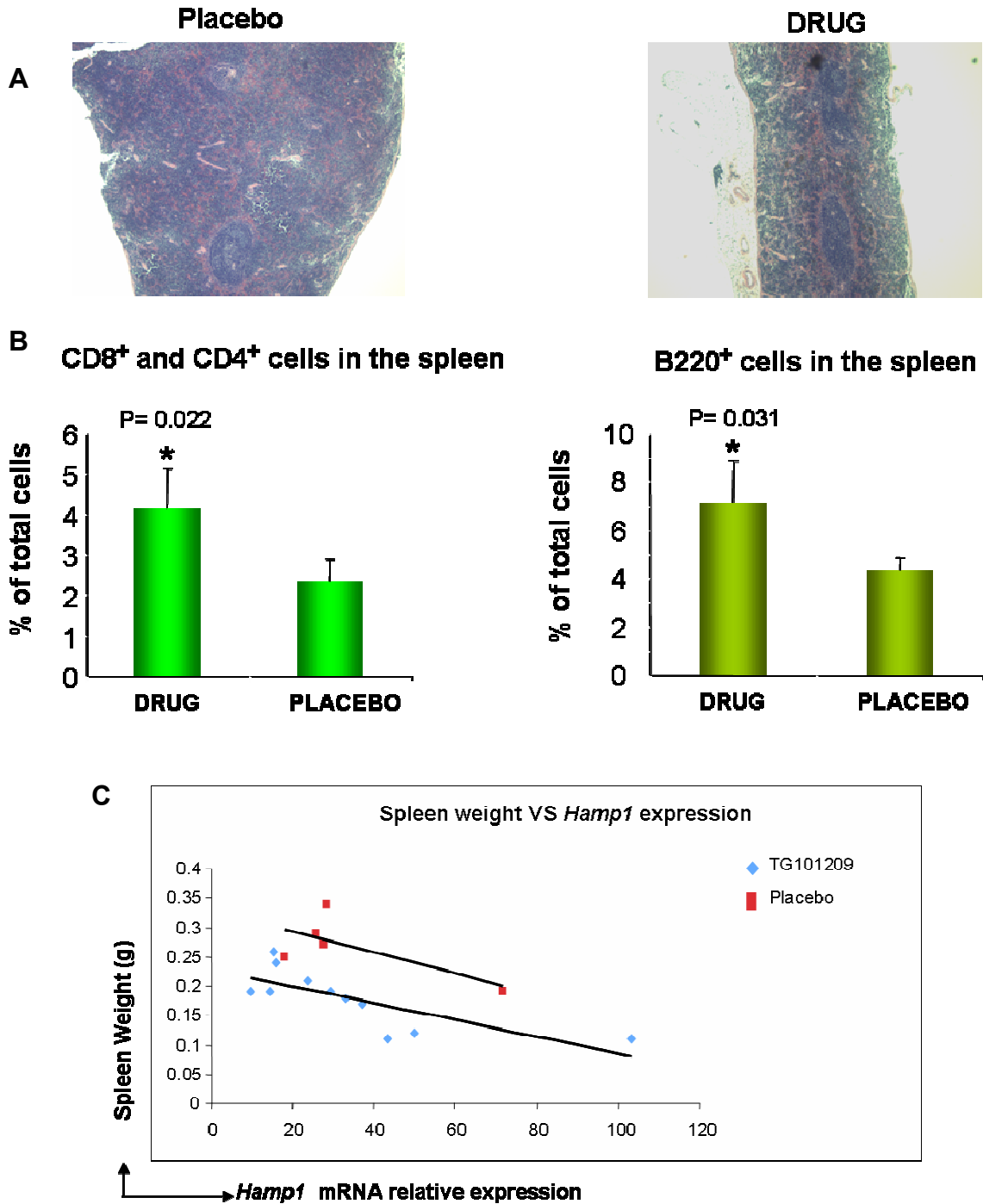
Figure 14. TG101209 treatment was successduful in ameliorating the architecture of the spleen (A) and in re-establishing the lymphocytic population in the spleen (B). The decreased erythropoietic rate induced by the drug resulted in increased hepcidin levels (C)

DISCUSSION

In β-thalassemia, IE has been attributed to increased expansion of late erythroid progenitor cells in combination with hemolysis and accelerated apoptosis. We have observed that bilirubin and LDH levels in thalassemic mice are only slightly increased compared with those in healthy mice. In addition, the percentage of cells undergoing apoptosis, as measured by others (Kean, Brown et al. 2002; Beauchemin, Blouin et al. 2004), including human specimens (Centis, Tabellini et al. 2000), was relatively low compared with the extreme expansion of medullary and extramedullary erythroid progenitors and predicted by the ferrokinetic studies conducted earlier (Finch, Deubelbeiss et al. 1970; Pootrakul, Sirankapracha et al. 2000). The normal distribution of murine erythroid progenitors is approximately 90% in the bone marrow and 10% in the spleen. In thalassemia, however, the spleen expands up to 20-fold and its erythroid content up to 95% of the total cell population, with mice affected by β-thalassemia major exhibiting even higher levels of IE than animals with the intermedia form of the disease. In addition, *th3/th3* mice produce low numbers of reticulocytes. This scenario elicits an "erythroid paradox." What is the fate of erythroid cells produced in β-thalassemia? Although the percentage of erythroid cells undergoing apoptosis in *th3/th3* mice increases 3 to 4 times compared with normal, as evidenced by the CC3 assay, the total number of erythroid progenitors increases dramatically, leading to a large expansion of the erythron just in a few weeks after bone marrow transplantation (BMT). When the fulminant EMH and splenomegaly in these animals reach their peak, the Hb levels are extremely low and the mice die. In mice affected by thalassemia intermedia, erythroid expansion occurs at a slower pace because these animals still have a "relatively efficient" erythropoiesis. They produce up to 10 times more reticulocytes and almost the same number of RBCs as healthy animals. Therefore, in younger mice affected by thalassemia intermedia, hemolytic anemia seems to play a major role, although IE is also a factor as shown by the

increased number of CD71+/Ter119+ cells. Nevertheless, as we showed in our previous study (Gardenghi, Marongiu et al. 2007), the Hb levels in these mice decrease with time, whereas the spleen size, the number of nucleated erythroid cells, and the ratio of liver to spleen iron all increase, resulting in the animals eventually exhibiting some of the features associated with the extreme form of IE observed in *th3/th* mice at 2 months after BMT. At this stage, *th3/+* mice start succumbing to the disease. In the *th3/th3* mice, which show the greatest number of apoptotic cells, the net expansion of the spleen is 15% to 20% per day. This represents an extremely high rate of cell proliferation. These observations call for alternative explanations for the IE in β-thalassemia. The Western blot results cannot distinguish whether up-regulation of genes such as *CycA* and *Cdk2* is intrinsic or simply reflects the increased number of proliferating thalassemic erythroid cells compared with normal. In thalassemic animals, high Epo levels support the former hypothesis, whereas the relative increase of CD71+/Ter119+ cells, the latter one. Probably both mechanisms contribute to the results, as would be expected in stress erythropoiesis. In any case, our data in vitro further corroborate the profoundly different behavior of thalassemic versus normal erythroid cells and indicate that additional as-yet-uncharacterized factors, not associated with stress erythropoiesis, serve to limit cell differentiation in thalassemia. These factors might be responsible for the differences between stress and ineffective erythropoiesis. Considered together, these observations challenge the established notion that IE in β-thalassemia is primarily due to cell death and/or hemolysis. In contrast, we propose a novel model of IE in which limited cell differentiation decreases red cell production as well as apoptosis. High Epo levels might be responsible for preventing apoptosis and enhancing cell proliferation. High levels of Bcl-XL have been shown to protect erythroid progenitor cells from apoptosis, while its disruption leads to severe hemolytic anemia (Ihle 2001; Rhodes, Kopsombut et al. 2005). Therefore, the relative increase in the amount of EpoR, CycA, and Bcl-XL that we observed supports our hypothesis that Epo stimulates proliferation and simultaneously limits apoptosis in thalassemic erythroid cells. Moreover, the observation by Ghaffari et al. (Zhao, Kitidis et al. 2006) that Epo stimulates the synthesis of Bcl-XL through the Jak2/Stat-5 signal transduction pathway rather than alternative Jak2-activated pathways such as Akt strongly suggests that the Epo-EpoR-Jak2 axis plays a major role in this process. In fact, our data show that a larger number of thalassemic erythroid cells exhibited phosphorylation of Jak2 than did normal cells. However, based on our data, we propose a model in which the Epo-Jak2 pathway is necessary but not sufficient to cause IE, thus additional factors are required. In this scenario, the relative strength of these hypothetical factors may determine whether high Epo levels lead to

effective or ineffective erythropoiesis. For instance, the relative amount of iron overload and related reactive oxygen species (ROSs) might determine the behavior of erythroid cells in the presence of high Epo levels (Marinkovic, Zhang et al. 2007).Under these conditions, for instance, high Epo levels might aggravate IE. This would increase the rate of cell proliferation and, concurrently, limit erythrocyte production. In fact, our model predicts that as anemia worsens, Epo levels can increase without leading to higher RBC production. Further studies in *th3*/+ mice might clarify this point, because higher Epo level do not correlate with higher Hb levels in a subset of these animals, but rather with the lowest ones. The most plausible explanation is that a combination of intrinsic and extrinsic factors contributes to this process. One intriguing hypothesis is that the globin chain imbalance or an excess of heme might play a role, providing a signal that prevents cell differentiation, which would otherwise lead to cells with a level of alpha-globin aggregates too toxic for survival. However, under our tissue culture conditions, the cells eventually die even in the presence of Epo. Therefore, diffusible factors such as SCF or cortisol (Zitnik, Peterson et al. 1995; Arcasoy and Jiang 2005), hypoxic conditions, or cell-cell and/or cell-stroma interactions, which were not reproduced in our cultures, might be involved. Turning from the theoretic to the practical, the results achieved with TG101209 in *th3*/+ mice might have a profound impact on the treatment of splenomegaly and in limiting IE-related damage due to excessive iron absorption, including secondary osteoporosis. The slower progression of the disease in mice affected by β-thalassemia intermedia closely mimics that in transfusion independent thalassemic patients who eventually develop splenomegaly with decreasing Hb levels. Although the spleen in these patients likely sequesters sufficient erythrocytes to affect the Hb level, the exact cause of the splenomegaly and characterization of the various splenic populations are unknown. Our data, from both thalassemic mice and patient specimens, shed light on this phenomenon, suggesting that an increased number of proliferating erythroid progenitors accumulate in the spleen under conditions of IE. In such a situation, splenomegaly might arise as the rate of differentiation is further reduced, leading to increased sequestration of erythrocytes progressively exacerbating the process. Transfusion independent β-thalassemia intermedia patients, if affected by splenomegaly, develop a need for blood transfusion therapy and eventually must undergo splenectomy. The use of Jak2 inhibitors in this situation would not be expected to improve anemia, but rather to limit or reduce splenomegaly. It is possible to envision that patients affected by thalassemia intermedia and splenomegaly could be treated temporarily with these compounds to reduce the spleen size and in the presence of blood transfusion to prevent further anemia. If this treatment were successful, higher Hb levels might be expected

in the absence of Jak2 inhibitors, and transfusion support and the need of splenectomy would be obviated. Moreover, even patients affected by β-thalassemia major might benefit from Jak2 inhibitors through reduction of IE and splenomegaly. The discovery of Jak2 as an important mediator of IE and splenomegaly in β-thalassemia suggests that the use of small organic molecules to inhibit Jak2 could be beneficial in reducing IE and splenomegaly. The idea of treating an erythropoietic disorder with an agent that limits erythropoiesis arises because IE in β-thalassemia resemble a leukemic blastic expansion, with immature erythroid progenitors that proliferate abnormally, fail to differentiate and invade other organs (spleen and liver) compromising their function. Ideally, use of a Jak2 inhibitor would target only the rapidly proliferating erythroid progenitors in the spleen, blocking their expansion and therefore allowing shrinkage of this organ by decreasing the presence of red pulp. This in turn would contribute to an amelioration of the spleen architecture and a reduction of RBC sequestration, enhancing their lifespan. Jak2 inhibitors are indeed able to induce a dramatic decrease in spleen size in thalassemic animals with a limited effect on anemia. However, it is important to point out that β-thalassemia major and β-thalassemia intermedia patients who develop splenomegaly require regular blood transfusions and often undergo splenectomy. It is appealing to speculate that thalassemia intermedia patients affected by splenomegaly could be treated temporarily with Jak2 inhibitors so as to reduce the spleen size and, in the presence of blood transfusions, to prevent further anemia. Moreover, the data on transfused and non-transfused *th3/+* and *th3/th3* mice suggest that even patients affected by β-thalassemia major, who may develop splenomegaly and EMH, could benefit from administration of Jak2 inhibitors. In these settings, the use of Jak2 inhibitors would be expected to limit or reduce splenomegaly, thereby preventing or delaying the need for splenectomy and indirectly improving the management of anemia by reducing the rate of blood transfusions. Another important effect of administration of jak2 inhibitors is the increase of hepcidin levels. The strong negative feedback between erythropoietic rate and expression of hepcidin raises an important question as to whether agents that limit IE, such as Jak2 inhibitors, could actually act indirectly as inducers of hepcidin expression. The data on *th3/+* and transfused *th3/th3* mice treated with a Jak2 inhibitor suggest that this may be the case. Animals treated with the drug showed increased levels of hepcidin in the liver compared to animals treated with a placebo, and more importantly the levels of hepcidin negatively correlated with the spleen weight in these animals, suggesting a strong connection between spleen weight, erythropoietic activity and hepcidin expression in β-thalassemia. That Jak2 inhibitors could have a more direct effect on iron metabolism is suggested by a recent study that shows how

expression of transferrin receptor 1 (TfR1) on erythroid cells depends on Jak2-Stat5 signaling (Kerenyi, Grebien et al. 2008). Perhaps the use of Jak2 inhibitors could reduce the expression of TfR1 on erythroid cells and potentially other cell types, limiting iron uptake and reducing the toxicity induced by cellular iron overload. In this scenario, the therapeutic treatment of β-thalassemia patients with Jak2 inhibitors would be ideal as it would target the two major complications of this pathology, IE with its related splenomegaly and the massive iron overload. Our paper emphasizes the proliferative component of IE and introduces the notion that limited cell differentiation also plays a role in this process. Two main consequences stem from this approach, the quest for alternative factors that control erythroid differentiation in IE and a potential clinical role for erythroid inhibitors such as TG101209 in treating thalassemic patients. In addition, we believe that the use of Epo, which is often suggested for patients affected by IE, should be carefully weighed. Depending on the relative strength of the various factors that control erythroid differentiation in each patient affected by IE, Epo may or may not be beneficial. In particular, in those patients in whom Epo would not be beneficial, treatment might exacerbate anemia and EMH, that is, more erythroid precursors would be made with limited production of differentiated erythroid cells. In conclusion, our data challenge the dogma that increased apoptosis is solely responsible for IE. In addition to moderate apoptosis, we propose that IE in β-thalassemia is driven by a large number of proliferating cells and limited differentiation, mimicking tumorlike behavior. This study suggests that use of Jak2 inhibitors has the potential to fundamentally transform the management of this disorder.

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