

β -thalassemias: paradigmatic diseases for scientific discoveries and development of innovative therapies

Stefano Rivella

Department of Pediatrics Hematology-Oncology Department of Cell and Developmental Biology Weill Cornell Medical College
New York, NY, USA

ABSTRACT

β -thalassemias are monogenic disorders characterized by defective synthesis of the β -globin chain, one of the major components of adult hemoglobin. A large number of mutations in the β -globin gene or its regulatory elements have been associated with β -thalassemias. Due to the complexity of the regulation of the β -globin gene and the role of red cells in many physiological processes, patients can manifest a large spectrum of phenotypes, and clinical requirements vary from patient to patient. It is important to consider the major differences in the light of potential novel therapeutics. This review summarizes the main discoveries and mechanisms associated with the synthesis of β -globin and abnormal erythropoiesis, as well as current and novel therapies.

The complex phenotype of β -thalassemias

β -thalassemias are monogenic disorders characterized by reduced or no synthesis of the β -globin chain, one of the major components of adult hemoglobin (HbA). Several hundred mutations in the β -globin gene or regulatory elements have been associated with β -thalassemias.¹ Homozygous or compound heterozygous mutations in the β -globin gene or promoter impair the production of β -globins. This results in the relative overproduction of α -globins and formation of insoluble hemichromes. The hemichromes damage cell membranes, while their heme component leads to the formation of noxious reactive oxygen species (ROS) and increased oxidative stress.^{2,3} Altogether, this impairs erythropoiesis, triggers erythroid apoptosis and, in turn, leads to anemia.^{2,3} Due to the complexity of the regulation of the β -globin gene and the role of red cells in many physiological processes, patients can manifest a large spectrum of phenotypes.^{4,5} As clinical requirements vary from patient to patient, it is appropriate to emphasize the major differences in the light of potential novel therapeutics.

Patients suffering the most severe form, indicated as β -thalassemia major, require chronic blood transfusion for survival. The excess of iron from the blood transfusion requires intense iron chelation to prevent an increase in plasma iron levels and formation of non-transferrin bound iron (NTBI). NTBI can increase cellular iron concentration, disrupt iron homeostasis and trigger harmful ROS formation leading to tissue iron overload and organ damage.^{6,8} Some additional pressing issues are osteoporosis and parenchymal damage in several different tissues, predominantly in the liver, heart and endocrine organs.⁴ Patients associated with a milder phenotype, as in β -thalassemia intermedia or non-transfusion dependent thalassemia (NTDT), produce comparatively higher levels of hemoglobin and might require only sporadic transfusions.⁵ However, these patients exhibit increased iron absorption and NTBI leading to severe iron overload and clin-

ical sequelae.⁹ In addition, they are more prone to thrombotic-related complications than patients affected by β -thalassemia major.^{10,11} Furthermore, the phenotype of these patients might also change over time, as NTDT patients often become transfusion dependent.

Historically many investigators have focused on understanding the mechanisms controlling β -globin gene expression and the consequences of the thalassaemic mutations on red cell production and, in turn, on physiological processes affected by hypoxia and abnormal erythropoiesis. In addition, many scientists and clinicians have attempted or are currently trying to translate scientific discoveries into new therapeutics, with the aim of improving the clinical care and quality of life of these patients.

The first part of this review will summarize the main discoveries and mechanisms associated with the synthesis of β -globin and abnormal erythropoiesis. The second part will provide a brief overview of the current treatments. And finally, the third and more extensive section of the review will discuss some of the novel therapies that are under development, bearing in mind the requirements for patients with more or less severe phenotypes.

Globin synthesis, erythropoiesis and iron metabolism: a complex ménage à trois

β -globin, Locus Control Region and switching

The human β -globin gene is mapped on chromosome 11, along with the ϵ -, γ - and δ -globin genes.¹² The β -globin gene was one of the first to be cloned and the corresponding protein crystallized.^{13,14} The β -globin gene has also been much used to study RNA transcription and processing, while mutations in the β -globin gene have provided invaluable information to further characterize these processes and associated mechanisms, such as nonsense-mediated RNA decay.¹⁵⁻¹⁸ In addition, mutations in the β -globin gene have been closely

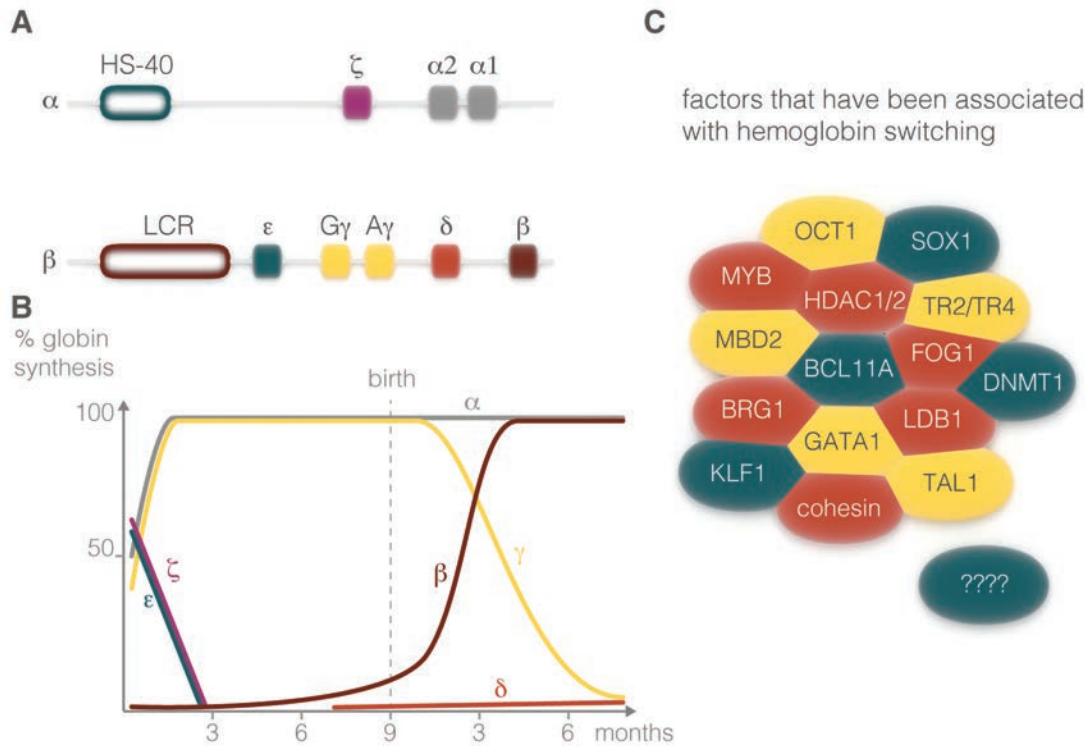


Figure 1. Representation of the genomic structure of the α - and β -globin loci. (A) Genes are indicated. LCR: locus control region (not to scale). (B) Relative expression of the globin genes during development. (C) Graphic representation of some of the candidate proteins involved in the regulation of the switching between fetal to adult hemoglobin.

correlated with the selective pressure triggered by the presence of the malaria parasite.¹⁹ The expression of these genes during development is regulated by several transcription factors (discussed below) and a genomic region in cis to the globin genes, indicated as Locus Control Region (LCR) (Figure 1A). The LCR has historically been characterized by clinical observations and by additional genetic approaches. Deletions of this region in humans are associated with forms of β -thalassemia,²⁰⁻²² where the β -globin gene, despite absence of mutations, was inactivated.^{23,24} Further characterization of this region, by the use of transgenic animals, indicated that the LCR is absolutely required for high level of expression of the β -globin gene in erythroid cells.²⁵⁻²⁸ The single genetic components of the LCR were identified as hypersensitive sites (HS) to the DNase I in the chromatin of erythroid cells.²⁹ The chromatin at the individual HSs is composed of arrays of multiple ubiquitous and lineage-specific transcription factor-binding sites (discussed further below).³⁰ The LCR activates the genes at the β -globin locus by folding and looping the HSs of the LCR to the appropriate promoter (Figure 2A). This creates a close association between this “holocomplex”, made of LCR-bound transcription proteins and co-activators, and the promoter of the adjacent gene, enhancing its transcription.²⁷ In addition, the sequential looping of the LCR is also responsible for the switch between embryonic, fetal (HbF) and HbA (Figure 2A).³¹⁻³⁴

The pattern of expression of the β -globin gene has also been a subject of intense investigations since the switching between HbF and HbA represents an important biological phenomenon and an exemplary model to understand how gene expression is regulated during development. In

humans, the switching between the expression of γ -globin and β -globin gene occurs in the first three months after birth (Figure 1B).³⁵ It has been shown that hereditary persistence of HbF is beneficial in individuals that concurrently inherit mutations in β -globin.³⁶ Therefore, characterization of this process could lead to the development of new reagents or strategies to reactivate production of HbF, with potential therapeutic effects not only in β -thalassemia, but also in sickle cell anemia.³⁷⁻⁷⁹

Transcription factors such as GATA binding protein 1 (GATA1), Friend of Gata1 (FOG1), B-cell lymphoma/leukemia 11A (BCL11A), Krueppel-like factor 1 (KLF1) and LIM domain binding 1 (LDB1) represent some of the most important proteins required for proper globin gene activation and switching (Figure 1C).⁴⁰ In the last few years, it has become evident that activation of the globin genes depends on the co-ordinated function of the LCR and these transcription factors. In particular, directly or indirectly, these factors contribute to establish LCR-enhancer proximity through chromatin looping,⁴¹⁻⁴⁵ activating globin gene expression (Figure 2A).⁴⁶

BCL11A is a zinc finger transcription factor and repressor of γ -globin expression in humans.^{47,48} In adult erythroid cells, BCL11A occupies several regions within the human β -globin cluster, including the LCR and the ϵ -globin gene.⁴³ Knockdown of BCL11A in human definitive erythroblasts results in increased expression of HbF.⁴⁹ Furthermore, in transgenic mice affected by sickle cell disease, inactivation of Bcl11A corrected the hematologic and pathological defects of this disorder through HbF induction.⁵⁰ As a consequence, BCL11A is considered an excellent target for reactivation of HbF in patients with β -hemoglo-

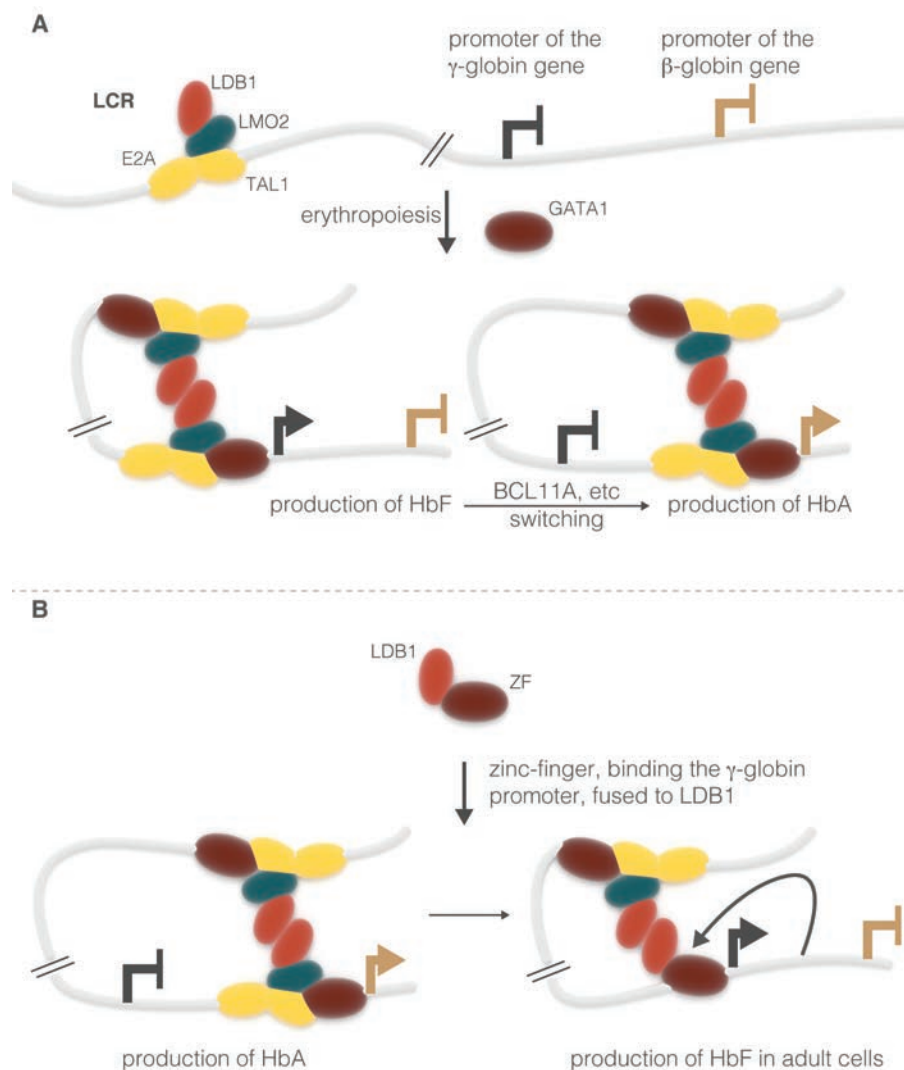


Figure 2. Illustration of the looping model and mechanism of HbF reactivation mediated by ZF-Ldb1. (A) In erythroid cell, the chromatin at the LCR is composed of arrays of multiple ubiquitous and lineage-specific transcription factor-binding sites, forming a holocomplex. This holocomplex loops on promoters at the β -globin locus determining the activation of the corresponding γ or β -globin gene. (A) ZF-Ldb1 is made by fusing Ldb1 to a specific zinc finger protein that recognizes a sequence in the γ -globin promoter. When ZF-Ldb1 is expressed in erythroid cells, this forces the holocomplex to move from the β -globin promoter and loop on the γ -globin promoter, determining the reactivation of HbF in adult cells.

binopathies. KLF1 is an erythroid-specific transcription factor essential for β -globin expression, definitive erythropoiesis, and switching HbF to HbA.⁵¹ Klf1 null mice die in utero due to failure of β -globin gene activation and aberrant erythropoiesis during fetal development.⁵² However, reduced expression of KLF1 in human erythroblasts is associated with cell viability and differentiation, reduced expression of BCL11A and increased γ - to β -globin ratio.⁵³ In fact, in patients, reduced synthesis of KLF1 is associated with survival and increased HbF synthesis; it can also result in an amelioration of the β -thalassemic phenotype.^{54,55} Therefore, KLF1 expression is also considered an excellent target for activating HbF in individuals with sickle cell disease and β -thalassemia.

LDB1 is a non-DNA-binding protein with a 200-amino acid N-terminal domain required for its dimerization or multimerization *in vitro*.⁵⁶⁻⁶⁹ In erythroid cells, LDB1 interacts with LIM domain only 2 (LMO2) and the DNA-binding partners GATA1 and T-cell acute lymphocytic leukemia 1 (TAL1).^{45,60} Importantly, genome-wide localization studies suggest that regulation of gene expression requiring Tal1 and Gata1 in mouse erythroid cells are exe-

cuted in concert with Ldb1.⁶¹⁻⁶⁵ In particular, the important role of Ldb1 in globin gene regulation has been emphasized by the observation that this protein is able to reactivate the silenced mouse embryonic globin and the human γ -globin genes when fused to an artificial zinc finger tethering Ldb1 onto their promoters (Figure 2B).^{32,34} It has been shown that this artificial zinc finger-Ldb1 fusion protein is able to force the LCR-holocomplex to loop onto the promoter recognized by the zinc finger moiety.^{32,34} This repositioning of the LCR is sufficient to re-activate the expression of otherwise silenced globin genes.^{32,34} This activity supports the model of the LCR-promoter looping mechanism and underscored the importance of LDB1 in the transcription of the genes at the β -globin cluster.

Erythropoiesis

Erythropoiesis involves the process of proliferation and differentiation of new red blood cells from erythroid progenitors, which at steady-state conditions primarily occurs in the bone marrow (BM). The key player, erythropoietin (EPO), primarily produced in the kidney in adults, regu-

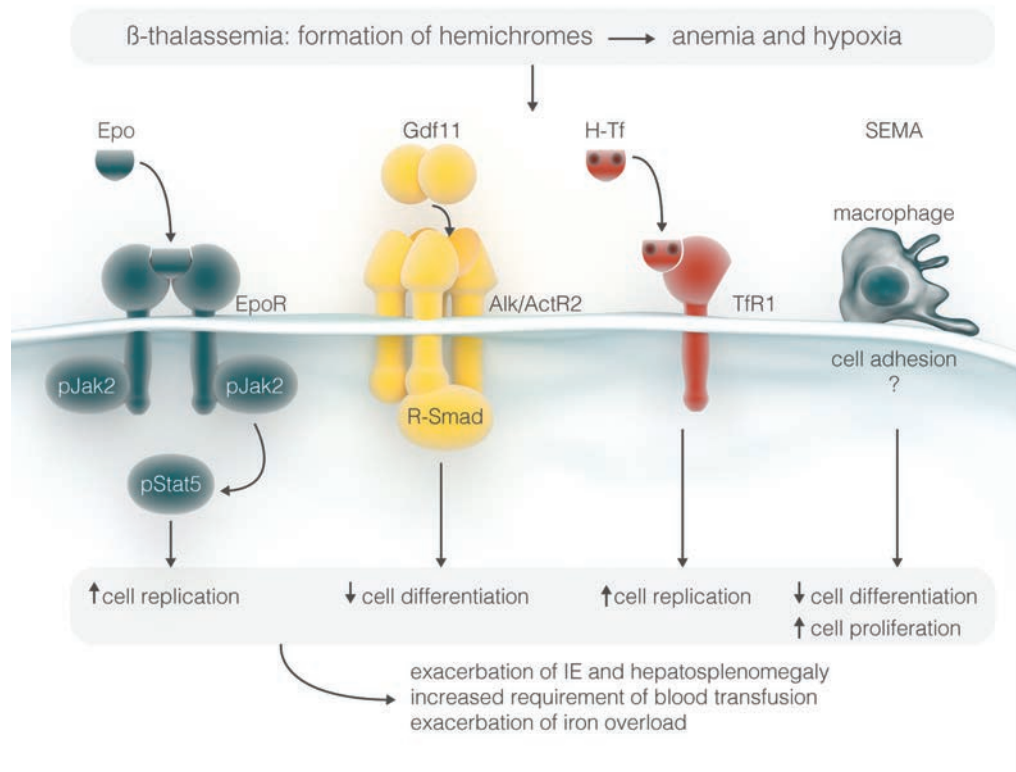


Figure 3. Representation of some of the pathways regulating steady state and stress erythropoiesis and their relationship to ineffective erythropoiesis in β-thalassemia. In this disorder, the imbalanced synthesis between α- and β-globin chains leads to formation of hemichromes, ROS formation and apoptosis of the late stage erythroid progenitors. This leads to anemia and hypoxia. As a consequence, Epo and Gdf1 synthesis are increased, leading to activation of the Jak2/Stat5 and R-Smad pathways respectively, thus altering the proliferation and differentiation of the erythroid progenitors. Furthermore, increased iron absorption and stress erythropoiesis macrophage activity (SEMA) also negatively influence ineffective erythropoiesis by supporting the proliferation of erythroid progenitors. Altogether, activation of these pathways leads to increased proliferation and reduced maturation of the erythroid progenitors, exacerbating the ineffective erythropoiesis. The question mark indicates molecules and related pathways that have not yet been identified.

lates the erythropoietic activity in response to cellular hypoxia and activation of hypoxia inducible factors (HIF).⁶⁶ The erythroid progenitor cells were identified by their colony-forming potential in vitro. These are the burst-forming unit-erythroid (BFUe) colonies, each one consisting of approximately 500 cells,^{67,68} and subsequent colony-forming unit-erythroid (CFUe), containing 8-32 cells.^{69,70} CFUe-derived erythroid cells progressively mature to red cells through a process of differentiation, which likely requires 3-5 divisions.⁷¹ The different stages of differentiation post CFUe were identified as proerythroblasts, basophilic, polychromatic, orthochromatic erythroblasts, reticulocytes and red blood cells.⁷¹⁻⁷³

Upon the binding of erythropoietin to EPO receptor (EPOR), the tyrosine kinase/Janus kinase 2 (JAK2) is phosphorylated, which in turn activates multiple signal transduction pathways crucial in erythropoiesis (Figure 3). One such pathway consists of activation of Signal Transducer and Activator of Transcription 5 (Stat5) and downstream antiapoptotic B-cell lymphoma-extra large (BclxL) protein.⁷⁴ The relative levels of BclxL and proapoptotic Bim protein during erythropoiesis modulate cell survival.⁷⁵ Similarly, Epo signaling also modulated survival by controlling the expression level of the death receptor Fas and

its ligand (FasL) on early erythroblasts.⁷⁵⁻⁷⁷ In erythroid cells, Irf2 can bind iron responsive element (IRE) on several transcripts, and depending on the exact position of the complementary IRE, it stimulates expression of genes associated with cellular iron uptake, such as Tfr1, or limit expression of those associated with iron storage, such as ferritin.⁷⁸ In particular, Epo, through Stat5, controls expression of Irf2 in erythroid cells, linking accelerated cellular activity with erythroid iron intake. Interestingly, also proteins that are associated with the iron sensing complex(es) in the liver play a role in erythroid cells. For instance, transferrin receptor 2 (Tfr2) and High Ferum/iron (Hfe) are genes that control iron metabolism in the liver and are mutated in hemochromatosis.⁷⁹ In erythroid cells, it has been recently shown that Tfr2 adjusts erythrocyte production according to iron availability, likely by modulating erythroblast Epo sensitivity, while Hfe has been involved in modulation of erythroid iron homeostasis.^{80,81}

When steady-state erythropoiesis is insufficient to provide adequate levels of oxygenation, such as in hypoxic conditions or severe blood loss, the production of red cells is increased through a mechanism indicated as stress erythropoiesis (SE). Switching from steady state to SE depends on the production of Epo and additional factors,

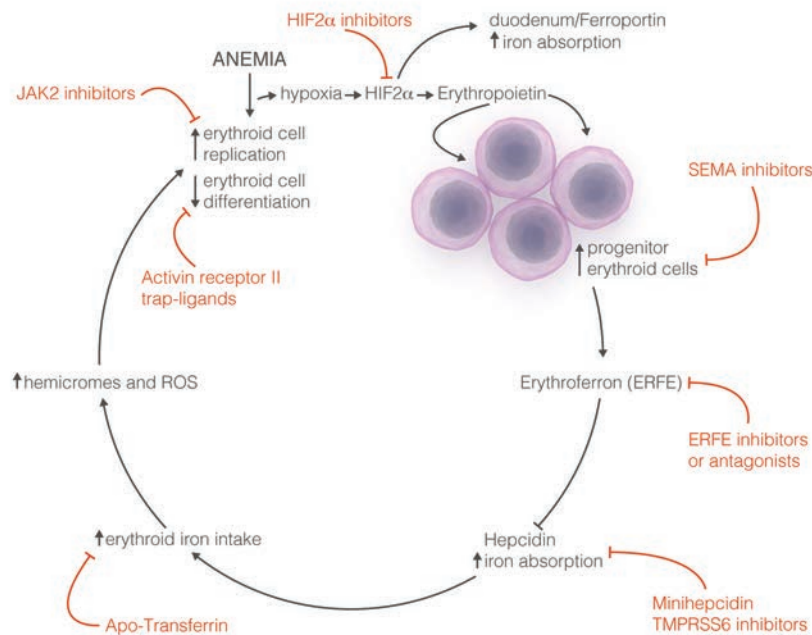


Figure 4. Illustration of the relationship between anemia, hypoxia, Epo, erythropoiesis and iron metabolism in β -thalassemia. Hypoxia, through HIF2 α , contributes to augmented iron absorption by increasing expression of Fpn, Dmt1 and DcytB in the duodenum. Epo, ROS and Growth differentiation factor 11 (Gdf11) alter erythropoiesis, increasing cell proliferation and decreasing cell maturation, contributing to the extramedullary hematopoiesis. As the number of erythroid progenitors increases, more ERFE and less hepcidin are produced, leading to increased iron absorption and increased Tf-sat. Altogether, these modifications contribute to the pathophysiology of β -thalassemia, and exacerbate the ineffective erythropoiesis and iron overload over time. The diagram also shows potential targets and therapeutics that might benefit β -thalassemia, as discussed in the text.

such as bone morphogenetic protein 4 (Bmp4), iron intake and the microenvironment. Nevertheless, Epo-induced pathways still play a major role in activating SE.^{82,83} Increased Epo levels are associated with further induction of BclxL and suppression of Bim and Fas-FasL, with a net increase in the number of erythoid progenitors surviving and proliferating.^{82,83} In addition, the downstream transcription factor Stat5 can increase erythoid iron intake through Irp2-mediated increased Tfr1 translation. In SE, however, additional proteins and mechanisms are required that do not seem to be essential to steady-state erythropoiesis. It has been shown that in SE some of the signals that regulate this process are Hedgehog, Bmp4, stem cell factor and hypoxia.⁸⁴⁻⁸⁶ The Bmp4-dependent SE pathway plays a key role in the recovery from acute anemia.⁸⁷ Bmp4 induces, through Smad5 signaling, the proliferation of stress erythroid progenitors, which are phenotypically different from steady-state progenitors.⁸⁸

In addition, macrophages are emerging as erythropoietin-complementary regulators of erythroid development, particularly under stress conditions. In fact, macrophages contribute decisively to recovery from induced anemia, as well as the pathological progression of polycythemia vera and β -thalassemia, by modulating erythroid proliferation and differentiation, through a stress erythropoiesis macrophage-supporting activity (SEMA) (Figure 3).⁸⁹ SEMA might require support from the expression of many adhesion molecules on erythrocyte progenitors, which likely allow the erythroid progenitors to receive support from the macrophages and, possibly, the microenvironment. These adhesion molecules might include α 4, α 5 and β 1 integrins, CD44, Lu, Icam-4, Vcam1, Emp and Swap70.⁹⁰⁻⁹³ These proteins might also be present on macrophages and are potentially responsible for various adhesive homotypic and heterotypic interactions within the erythropoietic niche, namely the erythroblastic island.⁹¹

These erythroblastic islands provide survival, proliferation and differentiation signals at early stages of erythropoiesis. It has been postulated that several pathways are triggered by these interactions, including those characterized by the activity of Phosphatidylinositol-4,5-bisphosphate 3-kinase/RAC- α serine/threonine-protein kinase (Pi3k/Akt) and BclxL, which regulate survival,^{94,95} while Focal Adhesion Kinase 1 (Fak1) and mitogen-activated protein/extracellular-regulated kinases (Mapk/Erk) modulate proliferation.⁹⁶⁻⁹⁹ Similarly, stem cell factor (Scf) and its receptor, c-Kit, have an important role in the expansion of stress erythroid progenitors via Erk and Akt, which is enhanced by the concomitant activation of the glucocorticoid receptor (GR).¹⁰⁰ Additional factors that might contribute to SE are the RNA-binding protein ZFP36L2,¹⁰¹ the Notch receptor 2,¹⁰² the anti-inflammatory polymeric immunoglobulin A1 (pIgA1)¹⁰³ and dexamethasone.¹⁰⁴ In particular, dexamethasone, in addition to inducing proliferation of proerythroblasts,¹⁰⁵ stimulates expansion of these cells indirectly by supporting the activity of macrophages.¹⁰⁵

Iron metabolism

There is a close connection between erythropoiesis and iron metabolism.¹⁰⁶ In fact, the process of red cell hemoglobinization and synthesis requires harmonization with erythroid iron intake, heme production and, overall, iron metabolism. Therefore, it is not surprising that iron availability affects erythropoiesis, likely through the IRP/IRE system, as observed in iron deficiency. In addition, as erythropoiesis needs to be increased under hypoxia conditions, iron metabolism and absorption are stimulated.

The hormone that controls iron absorption is hepcidin. Hepcidin is synthesized in the liver and secreted in the bloodstream. Hepcidin in the serum targets ferroportin (FPN), the only known iron exporter.¹⁰⁷⁻¹⁰⁹ Upon binding of FPN, this protein is internalized and degraded, preventing

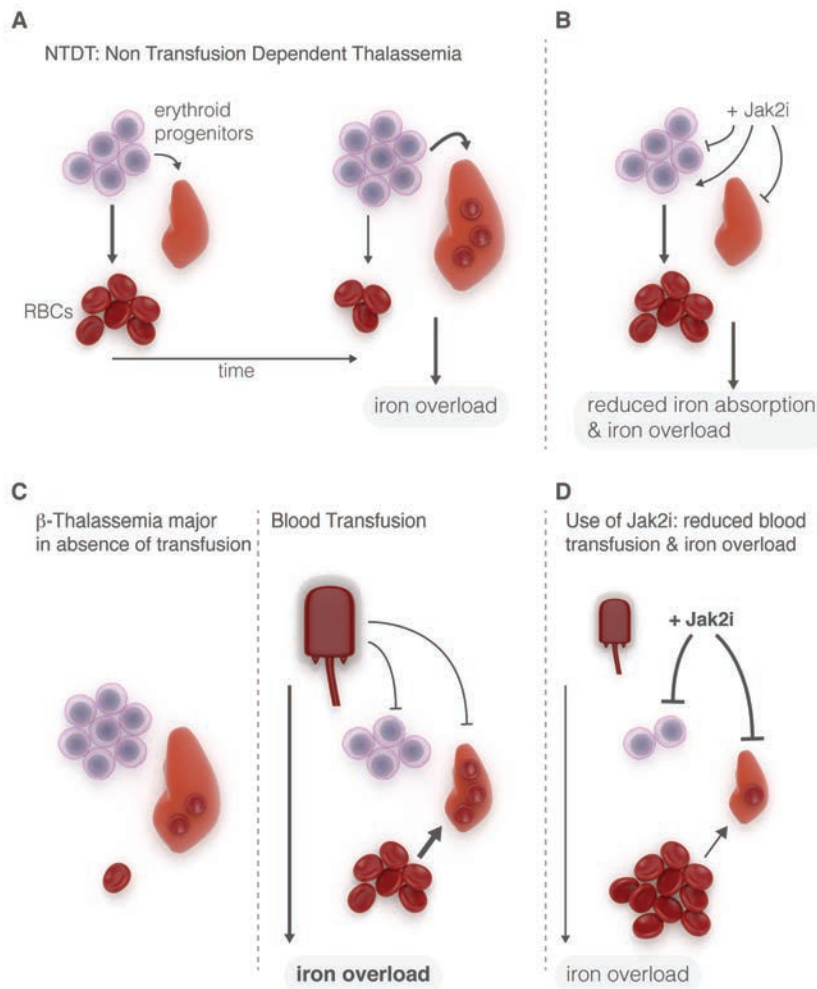


Figure 5. Potential use of JAK2 inhibitors in β -thalassemia. (A) In NTDT, the underlying chronic stress erythropoiesis exacerbates the anemia and the hepatosplenomegaly over time. This leads to increased sequestration of RBCs and further worsening of the ineffective erythropoiesis and iron overload. (B) Administration of a JAK2 inhibitor for a short period of time might decrease the number of erythroid progenitors, reversing the hepatosplenomegaly and decreasing iron absorption, with no or limited side effects. If administration of the JAK2 inhibitor is associated with reduced production of RBC, some blood transfusion could be provided to NTDT patients during administration of the drug. (C) In β -thalassemia major, splenomegaly might ensue over time, limiting the number of RBC transfused in circulation. (D) Administration of a JAK2 inhibitor might decrease the number of erythroid progenitors and reverse the hepatosplenomegaly. In turn, this might reduce the number of RBC sequestered by the spleen and, in turn, the requirement for blood transfusion, and ameliorate the management of iron overload.

iron egress.¹⁰⁹ Hepcidin synthesis is controlled by Tf-sat and iron storage, inflammation and erythropoiesis' demand. Fpn is expressed mainly on enterocytes, on macrophages and hepatocytes.¹¹⁰ Therefore, the relative abundance of hepcidin in the bloodstream and Fpn on the cellular membranes controls iron absorption in the duodenum, iron recycling in the reticuloendothelial system and iron storage in the liver.¹¹⁰

Two molecules that take a major role in controlling both erythropoiesis and iron metabolism are hypoxia inducible factor-2 α (Hif2 α) and Irp1. Hif2 α is a transcription factor that orchestrates the response to hypoxia, including Epo synthesis.¹¹¹ Hif2 α not only stimulates Epo production, but also the transcription of the divalent metal transporter 1 (Dmt1), apical ferric reductase, duodenal cytochrome B (DcytB) and Fpn in the enterocytes.¹¹² Therefore, under conditions of hypoxia, both erythropoiesis and iron absorption are increased by, respectively, elevated levels of Epo and augmented activities of duodenal Dmt1, DcytB and Fpn. Irp1 operates as either an IRE/RNA-binding protein in conditions of low intracellular iron, or a cytosolic aconitase in iron-repleted cells.¹¹¹ IRP1, as an RNA-binding protein, reduces HIF2 α mRNA translation. In fact, Irp1^{-/-} mice exhibit features of Hif2 α overexpression and hyperproduction of Epo, while Irp1 constitutive transgenic mice show defects in erythroid differentiation that can be

attributed to decreased Hif2 α expression.¹¹³⁻¹¹⁵ These observations indicate that Irp1 acts as an iron and oxygen sensor, linking iron metabolism with erythropoiesis via EPO. In iron deficiency, Irp1 suppresses HIF2 α and Epo expression to reduced iron availability, consistently with iron-restricted erythropoiesis.¹¹¹ In contrast, under iron-replete conditions, unconstrained HIF2 α mRNA translation increases Epo levels and erythropoiesis, as a homeostatic adaptation to the deficit of oxygen.¹¹¹

It has been postulated that an erythroid factor communicates to the liver the need of iron for the incoming red cells. This factor would be produced by erythroid cells, especially under condition of SE, and its function would be to suppress hepcidin synthesis in the liver. A variety of erythroid factors have been proposed, such as growth differentiation factor 15 (GDF15), twisted-gastrulation 1 (Twsg1) and Erythroferone (Erfe).¹¹⁶⁻¹¹⁸ However, only this last factor is increased in both animals affected by physiological-induced SE (following Epo administration) or chronic-SE, like β -thalassemia.¹¹⁸ Erfe is a member of the tumor necrosis factor (Tnf)-related protein family and is produced, and presumably secreted, by nucleated erythroid cells in response to Epo. Erfe-KO mice fail to suppress hepcidin following phlebotomy and show a delay in recovery from the anemia. Erfe expression is also significantly augmented in mice affected by thalassemia inter-

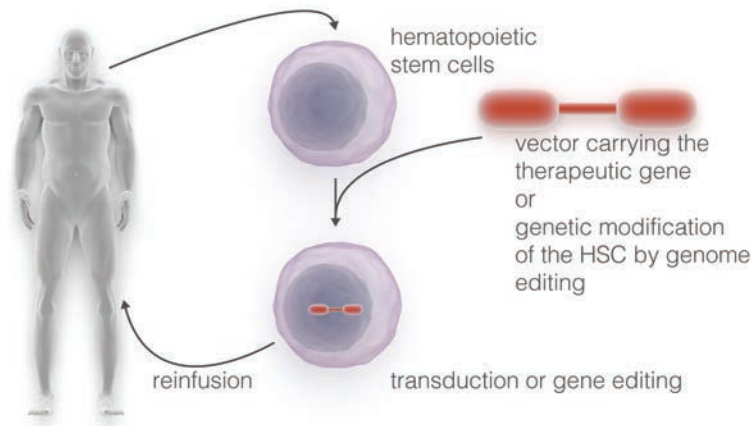


Figure 6. Schematic representation of gene therapy/gene editing approach for the cure of hemoglobinopathies. In this approach, HSC are harvested from the patient and modified by transduction (gene addition) or homologous DNA recombination (HDR). Following partial or full myeloablation, the genetically modified HSC are then re-infused in the bone marrow of the patient.

media, contributing to the suppression of hepcidin and the systemic iron overload.¹¹⁸

Novel potential therapies for β -thalassemia patients

Mouse models of β -thalassemia and ineffective erythropoiesis

Features associated with the phenotype of β -thalassemia in humans are well reproduced in mouse models indicated as $Hbb^{th1/th1}$ and $Hbb^{th3/+}$.¹¹⁹⁻¹²¹ The $Hbb^{th1/th1}$ mice were generated by a homozygous deletion of the β -major mouse globin gene, whereas $Hbb^{th3/+}$ mice present a heterozygous deletion of both the β -major and β -minor globin genes *in cis*.¹¹⁹⁻¹²¹ In these two models, the animals show a phenotype very similar to that observed in patients affected by NTDT,¹²²⁻¹²⁴ such as splenomegaly and iron overload in absence of transfusion. An additional transplantable model showing features of β -thalassemia major has been generated by transplanting fetal liver cells from $Hbb^{th3/th3}$ embryos into wild-type mice.¹²⁵ These animals exhibit features associated with this disorder, such as profound anemia, need for chronic blood transfusion for survival, and rapid iron overload.¹²³

In particular, use of these mouse models has led to further characterization of the ineffective erythropoiesis (IE) in β -thalassemia.^{89,124} Although in β -thalassemia apoptosis of erythroid progenitors and decreased life span of erythrocytes are the primary cause of anemia, the inefficient oxygen-carrying ability of the abnormal red cells cause a chronic state of hypoxia, which, in turn, stimulates erythropoietic activity, resulting in chronic SE. This lasting effort on red blood cell production has many counterproductive effects. The increased EPO levels, together with formation of reactive oxygen species in erythroid cells, are responsible for increasing proliferation and decreasing the differentiation (or maturation) of erythroid progenitors. This not only exacerbates IE over time, leading to hepatosplenomegaly, but also increases iron absorption, in a vicious circle that over time worsens the patient's phenotype (Figure 4).¹²⁶ These animals have provided important information to widen understanding of the relationship between hepcidin and iron overload in this disorder as well. In fact, $Hbb^{th3/+}$ mice were the first to show a cor-

relation between relative low hepcidin mRNA levels in the liver and iron overload in this disorder.^{127,128}

Altogether, these new notions, together with the use of the β -thalassemic mouse models, have been utilized to identify new drugs or strategies that are currently under development or in clinical trial; these will be described in the next sections. Another class of compounds that might also benefit β -thalassemia are fetal hemoglobin inducers; these will not be discussed in this manuscript but were recently summarized in several excellent reviews.^{37,129,130}

JAK2 inhibitors

In animals affected by β -thalassemia, it has been shown that elevated Epo production is associated with high levels of Jak2 phosphorylation, in a sort of physiological gain of function of this phosphokinase.¹²⁴ This leads to a significant increase in the number of erythroid progenitors, contributing to extramedullary hematopoiesis. Based on this observation, it has been proposed that acute administration of a Jak2 inhibitor (JAK2i) could reverse the splenomegaly in this disorder, avoiding the need for splenectomy. This treatment has been shown to be effective in $Hbb^{th3/+}$ mice, as splenomegaly was reversed with a limited decrease in red cell production. As Jak2i limits erythropoiesis, it might also reduce the production of the erythroid factor(s), partially reversing the suppression on hepcidin and limiting iron absorption (Figure 5A and B). Since erythroid progenitors have been documented in the spleens of patients affected by β -thalassemia,¹²⁴ this approach could potentially work also in humans as an alternative to splenectomy.¹³¹ Furthermore, the use of JAK2i might also be extended to patients that require blood transfusion. In this case, as JAK2i decreases the splenomegaly and the amount of blood sequestered by this organ, this might also reduce the amount of blood required per transfusion or the rate of blood transfusion (Figure 5C and D). Several JAK2i have already been developed, showing significant and beneficial results in myelofibrosis and JAK2-related polycythemia vera,^{132,133} a disease associated with chronic SE.⁸⁹ However, trials with Jak2 inhibitors in myeloproliferative disorders have also shown several side effects, among them thrombocytopenia and anemia.¹³⁴ This could be especially important for NTDT patients, in whom reduced splenomegaly due to the administration of a Jak2i might also decrease RBC pro-

duction. However, in pre-clinical thalassemic models, splenomegaly was reversed in less than two weeks and no side effects were observed (such as thrombocytopenia), except for a minor reduction in RBC production.¹²⁴ Therefore, once splenomegaly has been reversed, administration of the Jak2i could be discontinued. In addition, blood transfusion could be provided to NTDT patients during administration of the Jak2i. As a result, the side effects may be prevented or reduced compared to those observed in patients treated chronically with these agents.

Activin receptor-II trap ligands

GDF11 is a member of the bone morphogenetic protein (BMP) family and the TGF-beta superfamily and a ligand of the Activin receptor-II trap ligands A and IIB (ActRIIA and ActRIIB).¹³⁵ These form complexes with additional receptors that regulate gene expression primarily by activating the SMAD2/3 subfamily of intracellular effectors.¹³⁶ GDF11 is involved in development and, in adults, it has been involved with rejuvenation of stem cells found in the skeletal muscle and brain of aged mice.¹³⁷⁻¹³⁹ ActRIIA and ActRIIB are recognized by several ligands, including GDF11, and have been involved in a variety of physiological functions, including bone homeostasis and age-related bone loss.¹⁴⁰ The trap ligand ACE-011 was made by fusing the extracellular domain of ActRIIA to the Fc domain of human IgG1.¹⁴¹ The goal was to reduce the binding of ligand(s) to the membrane-associated cellular receptor ActRIIA, interfere with the downstream signaling cascades, and prevent osteoporosis.¹⁴¹ Interestingly, and unexpectedly, in a phase I clinical trial in postmenopausal women to treat osteoporosis, ACE-011 increased hematocrit levels.¹⁴¹ The observation triggered further investigation into this, and another trap ligand targeting ActRIIB (ACE-536), in mouse models of myelodysplastic syndromes (MDS) as well as β-thalassemia, showed a significant improvement of the anemia.¹⁴²⁻¹⁴⁴ In both these disorders it has been suggested that the mechanism of action of these drugs is mediated by targeting Gdf11, which in turn decreases Smad2/3 activation in erythroid progenitors, and ultimately improves erythroid maturation and RBC production.¹⁴²⁻¹⁴⁴ In addition, in Hbb^{th1/th1} mice, it has been shown that oxidative stress, through the Gdf11 ligand (Figure 3), also decreases apoptosis through overactivation of the Fas-Fas ligand pathway.^{126,127} As mentioned previously, both decreased apoptotic rate and maturation of early erythroid precursors leads to exacerbation of IE, splenomegaly, and increased iron absorption.^{117,128,129} Furthermore, these compounds also target the aberrant metabolism that leads to premature osteoporosis in this disorder, improving bone structure in these mice. Clinical trials with these agents are underway, showing amelioration of the anemia in NTDT patients and a potential reduction of the transfusion regimen in patients affected by β-thalassemia major.¹⁴⁵

Minihepcidin

Longitudinal analyses of Hbb^{th3/+} mice indicate that hemoglobin levels decrease over time, while the concentration of iron in the liver, spleen, and kidneys increases. Furthermore, excessive organ iron content is associated with low levels of hepcidin. Individuals affected by NTDT develop systemic iron overload from increased dietary iron absorption, associated with inappropriately low hepcidin.^{146,147} Significantly, progressive iron overload is the

most salient and ultimately fatal complication of β-thalassemia.⁴ Based on these observations, it has been postulated that more iron is absorbed in β-thalassemia than is required for erythropoiesis, and that increasing the concentration of hepcidin might be therapeutic, limiting iron overload. This hypothesis has been proved by generating Hbb^{th3/+} mice over-expressing hepcidin.¹⁴⁸ In fact, these animals showed decreased organ iron content. Furthermore, decreased iron absorption was associated with decreased transferrin-saturation (Tf-sat), which, in turn, decreased erythroid iron intake, heme synthesis and formation of insoluble membrane-bound globins, as well as reactive oxygen species. Altogether, moderate overexpression of hepcidin ameliorated iron overload and also increased the lifespan of RBC, reversed IE and splenomegaly, and increased total hemoglobin levels.

Therefore, by limiting the availability of iron to erythroid precursors, hepcidin agonists might improve the efficiency of erythropoiesis and the survival of the resulting reticulocytes and erythrocytes, by decreasing the formation of hemichromes. Minihepcidins (MH) are short peptide mimetics (9 AA long) that are sufficient to induce Fpn degradation in reporter cells.

In vivo, these compounds lowered serum iron levels and were efficacious in ameliorating the iron overload in animals affected by Hfe- and Hamp-related hemochromatosis.^{149,150} Furthermore, use of these compounds significantly reduced iron overload and erythroid cell damage in Hbb^{th5/1} mice, which in turn led to reduced IE, reticulocyte count, spleen size, and improved anemia.¹⁵¹

Tmprss6 inhibitors

Matriptase-2, or Transmembrane protease serine 6 (TMPRSS6), is a transmembrane serine protease that attenuates hepcidin expression.¹⁵²⁻¹⁵⁶ The fundamental role of TMPRSS6 on hepcidin expression is underscored by the observation that patients and mice with mutations in this gene are affected by iron-refractory iron deficiency anemia (IRIDA).^{154,157} Interestingly, lack of Tmprss6 in Hbb^{th3/+} mice significantly improved iron overload and anemia, corroborating the notion that increased hepcidin activity could be beneficial in this disorder.¹⁵⁸ In fact, in Hbb^{th3/+} mice, use of both antisense oligonucleotide (Tmprss6-ASO) and RNA interference (Tmprss6-siRNA) can reduce the synthesis of transmembrane serine protease Tmprss6 by degrading the corresponding mRNA. This led to increased hepcidin expression, decreased Tf-sat and reduction of hemichrome formation and apoptosis in erythroid cells. These animals also exhibited lower Epo levels, a significant amelioration of IE and splenomegaly, and an increase in total hemoglobin levels. Altogether, these data suggest that Tmprss6-ASOs or -siRNA molecules could be beneficial in individuals with β-thalassemia.^{159,160}

Administration of Apo-transferrin

Based on the notion that decreased Tf-sat can be beneficial in β-thalassemia, it has been shown that administration of Apo-transferrin (apo-Tf) can decrease erythroid iron intake, significantly improving the phenotype of Hbb^{th1/th1} mice.¹²²⁻¹²⁴ In particular, apo-transferrin administration normalized labile plasma iron concentrations, normalized RBC survival, and increased hemoglobin production together with decreased reticulocytosis, Epo synthesis and splenomegaly. These results suggest that Tf therapy might be beneficial in patients affected by β-thalassemia.

HIF2 α inhibitors

As mentioned previously, the relative level of expression of hepcidin in the liver and Fpn in the duodenum dictates iron absorption. Fpn is elevated in enterocytes of Hbb^{th3/+} mice, likely contributing to the increased iron absorption observed in these animals.¹²³ In addition, Fpn, divalent metal transporter 1 (Dmt1) and apical ferric reductase duodenal cytochrome B (DcytB) in the duodenum are regulated by hypoxia and intracellular iron concentration.^{112,115,161,162} It has been shown that expression of the Dmt1, DcytB and Fpn are increased in the duodenum of Hbb^{th3/+} mice as a consequence of hypoxia and Hif2 α stabilization and activity.^{112,162} In fact, Hbb^{th3/+} mice showed improvement in tissue-iron levels and anemia following genetic ablation of intestinal Hif2 α .¹¹² This observation suggests that duodenal HIF2 α might represent a novel therapeutic target in β -thalassemia to improve the anemia as well as the iron overload.

Gene therapy

The only established and definitive curative option for β -thalassemia is allogeneic bone marrow transplantation. However, this approach is limited by the scarcity of matched donors and the significant risk of graft-versus-host disease after transplantation of the donor cells (Figure 6). Gene therapy may offer an alternative approach to cure patients with severe β -thalassemia,¹⁶³ as autologous hematopoietic stem cells (HSC) are isolated, genetically modified and returned to the same patient. Over recent years, the techniques and tools to achieve transfer of a curative β -globin gene using lentiviral vectors have been significantly improved and have proved to be curative in several animal models for β -thalassemia.^{163,164} As a result, clinical trials are in progress and several patients seemed to have been successfully treated with this approach.⁶⁵⁻¹⁶⁷ These encouraging results are now invigorating the field of gene transfer and cellular therapies. Even with the ability of current vectors to improve the hemoglobin synthesis in patients affected by hemoglobinopathies, additional efforts are now focusing on improving the ability of these vectors to express curative hemoglobin levels with a reduced number of gene integrations per cell. Reducing integrations minimizes the chance of oncogenic random integration and limits the level of myeloablation required for these patients to receive the corrected HSCs. In order to improve this approach, additional strategies are being explored. For instance, new elements that induce fetal hemoglobin expression by forcing LCR- γ -promoter looping, such as the zinc finger-Ldb1 fusion protein, are being investigated for curative purposes (see " β -globin, LCR and switching"). Studies aimed at characterizing and including insulator elements into viral vectors to reduce genome toxicity are being actively pursued.¹⁶⁸ In addition, new technologies to genetically modify HSCs and induce pluripotent stem cells by genome editing are also being explored (see "Genome editing").

Genome editing

Mutations that lead to increased levels of HbF can profoundly improve the phenotype of patients with hemoglobinopathies.³⁸ For this reason, drugs that could increase synthesis of HbF are being actively investigated, as reviewed elsewhere.^{38,129,130} The transcriptional factor BCL11A has been recognized to be one of most important

factors in controlling the switch from HbF to HbA. After birth, as the level of BCL11A increases, the level of HbF decreases, while that of HbA increases.^{47,48} Therefore, targeting BCL11A represents a very attractive option to increase the synthesis of HbF. However, BCL11A is considered a very challenging protein to target due to the fact that it is a transcriptional factor (i.e. it interacts with many other proteins) and also plays an essential role in many different body tissues. However, additional studies indicated that suppression of BCL11A only in erythroid cells might be achieved by deletion of a specific erythroid enhancer, so that the expression of BCL11A would only be limited in these cells and not in other hematopoietic lineages.¹⁶⁹ Use of a zinc finger genome-editing technology might be able to knock out the erythroid enhancer of BCL11A in HSC of patients affected by β -thalassemia (<http://investor.sangamo.com/releasedetail.cfm?ReleaseID=818108>). If successful, engraftment of these cells following myeloablation may enable the permanent production of therapeutic fetal hemoglobin, reducing the excess of α -globin chains in RBCs, and improving the phenotype of these patients.

Potential combinatorial therapies

β -thalassemia is associated with a large spectrum of phenotypes, based on the different genotypes and quality of care that patients have received during their lifetime. If many of these compounds and genetic strategies prove to be safe and efficacious, identifying the best therapeutic approach for each patient will represent a positive, but challenging task for clinicians. In addition, some of these new drugs might benefit from combinatorial therapies. For instance, use of TMPRSS6 inhibitors, apo-Tf and MH can benefit from the use of iron chelators that accelerate the removal of iron from the liver.^{170,171} Similarly, acute use of JAK2i might rapidly revert splenomegaly, while the subsequent use of ARII-trap ligands, apo-TF, TMPRSS6 inhibitors or MH might prevent the reoccurrence of the enlargement of the spleen while improving anemia and iron overload. Again, administration of MH, apo-TF or TMPRSS6 inhibitors might be beneficial to the use of ARII-trap ligands if the latter drugs prove to be suboptimal in preventing excessive iron absorption and formation of NTBI.

The genetic strategies for β -thalassemia appear to be potentially curative. However, once again, due to the phenotypic variability of this disorder, some patients might produce increased but suboptimal levels of red cells and hemoglobin after gene transfer or editing. Therefore, some of these patients might also benefit from administration of ARII-trap ligands, apo-TF, TMPRSS6 inhibitors, MH or fetal hemoglobin inducers,^{172,173} shifting their management from transfusion-dependent to -independent approaches.

Conclusion

In conclusion, challenging old paradigms associated with ineffective erythropoiesis and improving gene therapy strategies have led, and will continue to lead, to scientific discoveries and new therapeutics. If future studies and clinical trials prove these to be efficacious and safe, these novel therapeutic approaches could potentially revolutionize the clinical management of β -thalassemia, with a good chance of improving the quality of life and survival of many patients.

Funding

This work is supported by the NIH grants NIDDK-R01DK090554, NIDDK-R01 DK095112, NHLBI-R01 HL102449, UE FP7 THALAMOSS Project (THALassaemia Modular Stratification System for personalized therapy of beta-thalassemia), The Child Reach Foundation, Isis Pharmaceuticals, Bayer Healthcare and and Merganser Biotech (to S. Rivella).

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Giardine B, Borg J, Viennas E, et al. Updates of the HbVar database of human hemoglobin variants and thalassemia mutations. *Nucleic Acids Res.* 2014;42(Database issue):D1063-1069.
2. Shinar E, Rachmilewitz EA. Oxidative denaturation of red blood cells in thalassemia. *Semin Hematol.* 1990;27(1):70-82.
3. Yuan J, Kannan R, Shinar E, et al. Isolation, characterization, and immunoprecipitation studies of immune complexes from membranes of beta-thalassemic erythrocytes. *Blood.* 1992;79(11):3007-3013.
4. Ginzburg Y, Rivella S. beta-thalassemia: a model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism. *Blood.* 2011;118(16):4321-4330.
5. Musallam KM, Rivella S, Vichinsky E, Rachmilewitz EA. Non-transfusion-dependent thalassemias. *Haematologica.* 2013;98(6):833-844.
6. Hershko C, Graham G, Bates GW, Rachmilewitz EA. Non-specific serum iron in thalassaemia: an abnormal serum iron fraction of potential toxicity. *Br J Haematol.* 1973;40(2):255-263.
7. Breuer W, Hershko C, Cabantchik ZI. The importance of non-transferrin bound iron in disorders of iron metabolism. *Transfus Sci.* 2000;23(3):185-192.
8. Brissot P, Ropert M, Le Lan C, Loreal O. Non-transferrin bound iron: a key role in iron overload and iron toxicity. *Biochim Biophys Acta.* 2012;1820(3):403-410.
9. Musallam KM, Cappellini MD, Wood JC, Taher AT. Iron overload in non-transfusion-dependent thalassemia: a clinical perspective. *Blood Rev.* 2012;26 Suppl 1:S16-9.
10. Cappellini MD, Poggiali E, Taher AT, Musallam KM. Hypercoagulability in beta-thalassemia: a status quo. *Expert Rev Hematol.* 2012;5(5):505-511; quiz 12.
11. Musallam KM, Taher AT, Karimi M, Rachmilewitz EA. Cerebral infarction in beta-thalassemia intermedia: breaking the silence. *Thromb Res.* 2012;130(5):695-702.
12. Deisseroth A, Nienhuis A, Lawrence J, Giles R, Turner P, Ruddle FH. Chromosomal localization of human beta globin gene on human chromosome 11 in somatic cell hybrids. *Proc Natl Acad Sci USA.* 1973;75(3):1456-1460.
13. Maniatis T, Kee SG, Efstratiadis A, Kafatos FC. Amplification and characterization of a beta-globin gene synthesized in vitro. *Cell.* 1976;8(2):163-182.
14. Edsall JT. Hemoglobin and the origins of the concept of allosterism. *Fed Proc.* 1980;39(2):226-235.
15. Maquat LE, Kinniburgh AJ, Beach LR, et al. Processing of human beta-globin mRNA precursor to mRNA is defective in three patients with beta+ thalassemia. *Proc Natl Acad Sci USA.* 1980;77(7):4287-4291.
16. Neu-Yilik G, Gehring NH, Thermann R, Frede U, Hentze MW, Kulozik AE. Splicing and 3' end formation in the definition of nonsense-mediated decay-competent human beta-globin mRNPs. *EMBO J.* 2001;20(3):532-540.
17. Nathan DG, Lodish HF, Kan YW, Housman D. Beta thalassemia and translation of globin messenger RNA. *Proc Natl Acad Sci USA.* 1971;68(10):2514-2518.
18. Hahn CK, Lowrey CH. Induction of fetal hemoglobin through enhanced translation efficiency of gamma-globin mRNA. *Blood.* 2014;124(17):2730-2734.
19. Weatherall DJ, Williams TN, Allen SJ, O'Donnell A. The population genetics and dynamics of the thalassemias. *Hematol Oncol Clin North Am.* 2010;24(6):1021-1031.
20. Curtin P, Pirastu M, Kan YW, Gobert-Jones JA, Stephens AD, Lehmann H. A distant gene deletion affects beta-globin gene function in an atypical gamma delta beta-thalassemia. *J Clin Invest.* 1985;76(4):1554-1558.
21. Driscoll MC, Dobkin CS, Alter BP. Gamma delta beta-thalassemia due to a de novo mutation deleting the 5' beta-globin gene activation-region hypersensitive sites. *Proc Natl Acad Sci USA.* 1989;86(19):7470-7474.
22. Van der Ploeg LH, Konings A, Oort M, Roos D, Bernini L, Flavell RA. gamma-beta-Thalassaemia studies showing that deletion of the gamma- and delta-genes influences beta-globin gene expression in man. *Nature.* 1980;283(5748):637-642.
23. Kioussis D, Vanin E, deLange T, Flavell RA, Grosveld FG. Beta-globin gene inactivation by DNA methylation in gamma beta-thalassaemia. *Nature.* 1983;306(5944):662-666.
24. Forrester WC, Epner E, Driscoll MC, et al. A deletion of the human beta-globin locus activation region causes a major alteration in chromatin structure and replication across the entire beta-globin locus. *Genes Dev.* 1990;4(10):1637-1649.
25. Caterina JJ, Ryan TM, Pawlik KM, et al. Human beta-globin locus control region: analysis of the 5' DNase I hypersensitive site HS 2 in transgenic mice. *Proc Natl Acad Sci USA.* 1991;88(5):1626-1630.
26. Fraser P, Pruzina S, Antoniou M, Grosveld F. Each hypersensitive site of the human beta-globin locus control region confers a different developmental pattern of expression on the globin genes. *Genes Dev.* 1993;7(1):106-113.
27. Li Q, Peterson KR, Fang X, Stamatoyannopoulos G. Locus control regions. *Blood.* 2002;100(9):3077-3086.
28. Kim A, Dean A. Chromatin loop formation in the beta-globin locus and its role in globin gene transcription. *Mol Cells.* 2012;34(1):1-5.
29. Grosveld F, van Assendelft GB, Greaves DR, Kollias G. Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell.* 1987;51(6):975-985.
30. Wijgerde M, Grosveld F, Fraser P. Transcription complex stability and chromatin dynamics in vivo. *Nature.* 1995;377(6546):209-213.
31. Lloyd JA, Krakowsky JM, Crable SC, Lingrel JB. Human gamma- to beta-globin gene switching using a mini construct in transgenic mice. *Mol Cell Biol.* 1992;12(4):1561-1567.
32. Deng W, Lee J, Wang H, et al. Controlling long-range genomic interactions at a native locus by targeted tethering of a looping factor. *Cell.* 2012;149(6):1233-1244.
33. Behringer RR, Ryan TM, Palmiter RD, Brinster RL, Townes TM. Human gamma- to beta-globin gene switching in transgenic mice. *Genes Dev.* 1990;4(3):380-389.
34. Deng W, Rupon JW, Krivega I, et al. Reactivation of developmentally silenced globin genes by forced chromatin looping. *Cell.* 2014;158(4):849-860.
35. Trimbom T, Gribnau J, Grosveld F, Fraser P. Mechanisms of developmental control of transcription in the murine alpha- and beta-globin loci. *Genes Dev.* 1999;13(1):112-124.
36. Conley CL, Weatherall DJ, Richardson SN, Shepard MK, Charache S. Hereditary persistence of fetal hemoglobin: a study of 79 affected persons in 15 Negro families in Baltimore. *Blood.* 1963;21:261-281.
37. Perrine SP, Pace BS, Faller DV. Targeted fetal hemoglobin induction for treatment of beta hemoglobinopathies. *Hematol Oncol Clin North Am.* 2014;28(2):233-248.
38. Thein SL, Menzel S. Discovering the genetics underlying foetal haemoglobin production in adults. *Br J Haematol.* 2009;145(4):455-467.
39. Sankaran VG, Xu J, Orkin SH. Advances in the understanding of haemoglobin switching. *Br J Haematol.* 2010;149(2):181-194.
40. Sankaran VG. Targeted therapeutic strategies for fetal hemoglobin induction. *Hematology Am Soc Hematol Educ Program.* 2011;2011:459-465.
41. Dekker J. Gene regulation in the third dimension. *Science.* 2008;319(5871):1793-1794.
42. Splinter E, de Laat W. The complex transcription regulatory landscape of our genome: control in three dimensions. *EMBO J.* 2011;30(21):4345-4355.
43. Drissen R, Palstra RJ, Gillemans N, et al. The active spatial organization of the beta-globin locus requires the transcription factor EKLF. *Genes Dev.* 2004;18(20):2485-2490.
44. Vakoc CR, Letting DL, Gheldof N, et al. Proximity among distant regulatory elements at the beta-globin locus requires GATA-1 and FOG-1. *Mol Cell.* 2005;17(3):453-462.
45. Song SH, Kim A, Ragozcy T, Bender MA, Groudine M, Dean A. Multiple functions of Ldb1 required for beta-globin activation during erythroid differentiation. *Blood.* 2010;116(13):2356-2364.
46. Deng W, Blobel GA. Manipulating nuclear architecture. *Curr Opin Genet Dev.* 2014;25:1-7.
47. Menzel S, Garner C, Gut I, et al. A QTL influencing F cell production maps to a gene

- encoding a zinc-finger protein on chromosome 2p15. *Nat Genet.* 2007;39(10):1197-1199.
48. Uda M, Galanello R, Sanna S, et al. Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of beta-thalassemia. *Proc Natl Acad Sci USA.* 2008;105(5):1620-1625.
 49. Sankaran VG, Menne TF, Xu J, et al. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. *Science.* 2008;322(5909):1839-1842.
 50. Xu J, Peng C, Sankaran VG, et al. Correction of sickle cell disease in adult mice by interference with fetal hemoglobin silencing. *Science.* 2011;334(6058):993-996.
 51. Miller IJ, Bieker JJ. A novel, erythroid cell-specific murine transcription factor that binds to the CACCC element and is related to the Kruppel family of nuclear proteins. *Mol Cell Biol.* 1993;13(5):2776-2786.
 52. Nuez B, Michalovich D, Bygrave A, Ploemacher R, Grosfeld F. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. *Nature.* 1995;375(6529):316-318.
 53. Borg J, Papadopoulos P, Georgitsi M, et al. Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. *Nat Genet.* 2010;42(9):801-805.
 54. Satta S, Perseu L, Moi P, et al. Compound heterozygosity for KLF1 mutations associated with remarkable increase of fetal hemoglobin and red cell protoporphyrin. *Haematologica.* 2011;96(5):767-770.
 55. Liu D, Zhang X, Yu L, et al. KLF1 mutations are relatively more common in a thalassemia endemic region and ameliorate the severity of beta-thalassemia. *Blood.* 2014;124(5):803-811.
 56. Breen JJ, Agulnick AD, Westphal H, Dawid IB. Interactions between LIM domains and the LIM domain-binding protein Ldb1. *J Biol Chem.* 1998;273(8):4712-4717.
 57. Jurata LW, Pfaff SL, Gill GN. The nuclear LIM domain interactor NLI mediates homo- and heterodimerization of LIM domain transcription factors. *J Biol Chem.* 1998;273(6):3152-157.
 58. Xu Z, Huang S, Chang LS, Agulnick AD, Brandt SJ. Identification of a TAL1 target gene reveals a positive role for the LIM domain-binding protein Ldb1 in erythroid gene expression and differentiation. *Mol Cell Biol.* 2003;23(21):7585-7599.
 59. Cross AJ, Jeffries CM, Trewella J, Matthews JM. LIM domain binding proteins 1 and 2 have different oligomeric states. *J Mol Biol.* 2010;399(1):133-144.
 60. Wadman IA, Osada H, Grutz GG, et al. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *EMBO J.* 1997;16(11):3145-3157.
 61. Fujiwara T, O'Geen H, Keles S, et al. Discovering hematopoietic mechanisms through genome-wide analysis of GATA factor chromatin occupancy. *Mol Cell.* 2009;36(4):667-681.
 62. Tripic T, Deng W, Cheng Y, et al. SCL and associated proteins distinguish active from repressive GATA transcription factor complexes. *Blood.* 2009;113(10):2191-2201.
 63. Yu M, Riva L, Xie H, et al. Insights into GATA-1-mediated gene activation versus repression via genome-wide chromatin occupancy analysis. *Mol Cell.* 2009;36(4):682-695.
 64. Soler E, Andrieu-Soler C, de Boer E, et al. The genome-wide dynamics of the binding of Ldb1 complexes during erythroid differentiation. *Genes Dev.* 2010;24(3):277-289.
 65. Li L, Freudenberg J, Cui K, et al. Ldb1-nucleated transcription complexes function as primary mediators of global erythroid gene activation. *Blood.* 2013;121(22):4575-4585.
 66. Haase VH. Regulation of erythropoiesis by hypoxia-inducible factors. *Blood Rev.* 2013;27(1):41-53.
 67. Iscove NN, Sieber F. Erythroid progenitors in mouse bone marrow detected by macroscopic colony formation in culture. *Exp Hematol.* 1975;3(1):32-43.
 68. Heath DS, Axelrad AA, McLeod DL, Shreeve MM. Separation of the erythropoietin-responsive progenitors BFU-E and CFU-E in mouse bone marrow by unit gravity sedimentation. *Blood.* 1976;47(5):777-792.
 69. Stephenson JR, Axelrad AA, McLeod DL, Shreeve MM. Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. *Proc Natl Acad Sci USA.* 1971;68(7):1542-1546.
 70. Li J, Hale J, Bhagia P, et al. Isolation and transcriptome analyses of human erythroid progenitors: BFU-E and CFU-E. *Blood.* 2014;124(24):3636-3645.
 71. Hu J, Liu J, Xue F, et al. Isolation and functional characterization of human erythroblasts at distinct stages: implications for understanding of normal and disordered erythropoiesis in vivo. *Blood.* 2013;121(16):3246-3253.
 72. Koulis M, Pop R, Porpiglia E, Shearstone JR, Hidalgo D, Socolovsky M. Identification and analysis of mouse erythroid progenitors using the CD71/TER119 flow-cytometric assay. *J Vis Exp.* 2011(54):pii:2809.
 73. Chen K, Liu J, Heck S, Chasis JA, An X, Mohandas N. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. *Proc Natl Acad Sci USA.* 2009;106(41):17413-17418.
 74. Socolovsky M. Ineffective erythropoiesis in Stat5a^{-/-}5b^{-/-} mice due to decreased survival of early erythroblasts. *Blood.* 2001;98(12):3261-3273.
 75. Koulis M, Porpiglia E, Porpiglia PA, et al. Contrasting dynamic responses in vivo of the Bcl-xL and Bim erythropoietic survival pathways. *Blood.* 2012;119(5):1228-1239.
 76. Socolovsky M, Murrell M, Liu Y, Pop R, Porpiglia E, Levchenko A. Negative autoregulation by FAS mediates robust fetal erythropoiesis. *PLoS Biol.* 2007;5(10):e252.
 77. Koulis M, Liu Y, Hallstrom K, Socolovsky M. Negative autoregulation by Fas stabilizes adult erythropoiesis and accelerates its stress response. *PLoS One.* 2011;6(7):e21192.
 78. Anderson Sheila A, Nizzi CP, Chang Y-I, et al. The IRP1-HIF-2 Axis Coordinates Iron and Oxygen Sensing with Erythropoiesis and Iron Absorption. *Cell Metab.* 2013;17(2):282-290.
 79. Camaschella C. Iron and hepcidin: a story of recycling and balance. *Hematology Am Soc Hematol Educ Program.* 2013;2013:1-8.
 80. Ramos P, Guy E, Chen N, et al. Enhanced erythropoiesis in Hfe-KO mice indicates a role for Hfe in the modulation of erythroid iron homeostasis. *Blood.* 2011;117(4):1379-1389.
 81. Nai A, Lidonnici MR, Rausa M, et al. The second transferrin receptor regulates red blood cell production in mice. *Blood.* 2015;125(7):1170-1179.
 82. Goodell MA, Porpiglia E, Hidalgo D, Koulis M, Tzafirri AR, Socolovsky M. Stat5 Signaling Specifies Basal versus Stress Erythropoietic Responses through Distinct Binary and Graded Dynamic Modalities. *PLoS Biol.* 2012;10(8):e1001383.
 83. Peslak SA, Wenger J, Bemis JC, et al. EPO-mediated expansion of late-stage erythroid progenitors in the bone marrow initiates recovery from sublethal radiation stress. *Blood.* 2012;120(12):2501-2511.
 84. Perry JM, Harandi OF, Paulson RF. BMP4, SCF, and hypoxia cooperatively regulate the expansion of murine stress erythroid progenitors. *Blood.* 2007;109(10):4494-4502.
 85. Perry JM, Harandi OF, Porayette P, Hegde S, Kannan AK, Paulson RF. Maintenance of the BMP4-dependent stress erythropoiesis pathway in the murine spleen requires hedgehog signaling. *Blood.* 2009;113(4):911-918.
 86. Harandi OF, Hegde S, Wu DC, McKeone D, Paulson RF. Murine erythroid short-term radioprotection requires a BMP4-dependent, self-renewing population of stress erythroid progenitors. *J Clin Invest.* 2010;120(12):4507-4519.
 87. Chow A, Huggins M, Ahmed J, et al. CD169(+) macrophages provide a niche promoting erythropoiesis under homeostasis and stress. *Nat Med.* 2013;19(4):429-436.
 88. Lenox LE, Perry JM, Paulson RF. BMP4 and Madh5 regulate the erythroid response to acute anemia. *Blood.* 2005;105(7):2741-2748.
 89. Ramos P, Casu C, Gardenghi S, et al. Macrophages support pathological erythropoiesis in polycythemia vera and beta-thalassemia. *Nat Med.* 2013;19(4):437-445.
 90. Chasis JA. Erythroblastic islands: specialized microenvironmental niches for erythropoiesis. *Curr Opin Hematol.* 2006;13(3):137-141.
 91. Chasis JA, Mohandas N. Erythroblastic islands: niches for erythropoiesis. *Blood.* 2008;112(3):470-478.
 92. Mohandas N, Chasis JA. The erythroid niche: molecular processes occurring within erythroblastic islands. *Transfus Clin Biol.* 2010;17(3):110-111.
 93. Ripich T, Jessberger R. SWAP-70 regulates erythropoiesis by controlling alpha4 integrin. *Haematologica.* 2011;96(12):1743-1752.
 94. Kovach NL, Lin N, Yednock T, Harlan JM, Broudy VC. Stem cell factor modulates avidity of alpha 4 beta 1 and alpha 5 beta 1 integrins expressed on hematopoietic cell lines. *Blood.* 1995;85(1):159-167.
 95. Eshghi S, Vogelezang MG, Hynes RO, Griffith LG, Lodish HF. Alpha4beta1 integrin and erythropoietin mediate temporally distinct steps in erythropoiesis: integrins in red cell development. *J Cell Biol.* 2007;177(5):871-880.
 96. Paulson RF, Shi L, Wu DC. Stress erythropoiesis: new signals and new stress progenitor cells. *Curr Opin Hematol.* 2011;18(3):139-145.
 97. Ulyanova T, Jiang Y, Padilla S, Nakamoto B, Papayannopoulou T. Combinatorial and distinct roles of alpha and alpha integrins in stress erythropoiesis in mice. *Blood.* 2011;117(3):975-985.
 98. Kapur R, Cooper R, Zhang L, Williams DA. Cross-talk between alpha(4)beta(1)/alpha(5)beta(1) and c-Kit results in opposing effect on growth and survival of hematopoietic cells via the activation of focal adhesion kinase, mitogen-activated protein kinase, and Akt signaling pathways. *Blood.* 2001;97(7):1975-1981.
 99. Vemula S, Ramdas B, Hanneman P, Martin J, Beggs HE, Kapur R. Essential role for focal adhesion kinase in regulating stress hematopoiesis. *Blood.* 2010;116(20):4103-4115.
 100. Varricchio L, Tirelli V, Masselli E, et al. The Expression of the Glucocorticoid Receptor in Human Erythroblasts is Uniquely Regulated by KIT Ligand: Implications for Stress Erythropoiesis. *Stem Cells Dev.*

- 2012;21(15):2852-2865.
101. Zhang L, Frak L, Rayon-Estrada V, et al. ZFP36L2 is required for self-renewal of early burst-forming unit erythroid progenitors. *Nature*. 2013;499(7456):92-96.
 102. Oh P, Lobry C, Gao J, et al. In Vivo Mapping of Notch Pathway Activity in Normal and Stress Hematopoiesis. *Cell Stem Cell*. 2013;13(2):190-204.
 103. Coulon S, Dussiot M, Grapton D, et al. Polymeric IgA1 controls erythroblast proliferation and accelerates erythropoiesis recovery in anemia. *Nat Med*. 2011;17(11):1456-1465.
 104. Stellacci E, Di Noia A, Di Baldassarre A, Migliaccio G, Battistini A, Migliaccio AR. Interaction between the glucocorticoid and erythropoietin receptors in human erythroid cells. *Exp Hematol*. 2009;37(5):559-572.
 105. Falchi M, Varricchio L, Martelli F, et al. Dexamethasone targeted directly to macrophages induces macrophage niches that promote erythroid expansion. *Haematologica*. 2015;100(2):178-187.
 106. Kerenyi MA, Grebien F, Gehart H, et al. Stat5 regulates cellular iron uptake of erythroid cells via IRP-2 and TfR-1. *Blood*. 2008;112(9):3878-3888.
 107. Park CH, Valore EV, Waring AJ, Ganz T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *J Biol Chem*. 2001;276(11):7806-7810.
 108. Nicolas G, Bennoun M, Devaux I, et al. Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice. *Proc Natl Acad Sci USA*. 2001;98(15):8780-8785.
 109. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science*. 2004;306(5704):2090-2093.
 110. Ganz T. Systemic iron homeostasis. *Physiol Rev*. 2013;93(4):1721-1741.
 111. Wilkinson N, Pantopoulos K. The IRP/IRE system in vivo: insights from mouse models. *Front Pharmacol*. 2014;5:176.
 112. Anderson ER, Taylor M, Xue X, et al. Intestinal HIF2alpha promotes tissue-iron accumulation in disorders of iron overload with anemia. *Proc Natl Acad Sci USA*. 2013;110(50):E4922-4930.
 113. Anderson SA, Nizzi CP, Chang YI, et al. The IRP1-HIF-2alpha axis coordinates iron and oxygen sensing with erythropoiesis and iron absorption. *Cell Metab*. 2013;17(2):282-290.
 114. Ghosh MC, Zhang DL, Jeong SY, et al. Deletion of iron regulatory protein 1 causes polycythemia and pulmonary hypertension in mice through translational derepression of HIF2alpha. *Cell Metab*. 2013;17(2):271-281.
 115. Wilkinson N, Pantopoulos K. IRP1 regulates erythropoiesis and systemic iron homeostasis by controlling HIF2alpha mRNA translation. *Blood*. 2013;122(9):1658-1668.
 116. Tanno T, Porayette P, Sripichai O, et al. Identification of TWSG1 as a second novel erythroid regulator of hepcidin expression in murine and human cells. *Blood*. 2009;114(1):181-186.
 117. Tanno T, Bhanu NV, Oneal PA, et al. High levels of GDF15 in thalassemia suppress expression of the iron regulatory protein hepcidin. *Nat Med*. 2007;13(9):1096-1101.
 118. Kautz L, Jung G, Valore EV, Rivella S, Nemeth E, Ganz T. Identification of erythropoietin as an erythroid regulator of iron metabolism. *Nat Genet*. 2014;46(7):678-684.
 119. Ciavatta DJ, Ryan TM, Farmer SC, Townes TM. Mouse model of human beta zero thalassemia: targeted deletion of the mouse beta major- and beta minor-globin genes in embryonic stem cells. *Proc Natl Acad Sci USA*. 1995;92(20):9259-9263.
 120. Yang B, Kirby S, Lewis J, Detloff PJ, Maeda N, Smithies O. A mouse model for beta 0-thalassemia. *Proc Natl Acad Sci USA*. 1995;92(25):11608-11612.
 121. Skow LC, Burkhardt BA, Johnson FM, et al. A mouse model for beta-thalassemia. *Cell*. 1983;34(3):1043-1052.
 122. Li H, Rybicki AC, Suzuka SM, et al. Transferrin therapy ameliorates disease in beta-thalassemic mice. *Nat Med*. 2010;16(2):177-182.
 123. Gardenghi S, Marongiu MF, Ramos P, et al. Ineffective erythropoiesis in beta-thalassemia is characterized by increased iron absorption mediated by down-regulation of hepcidin and up-regulation of ferroportin. *Blood*. 2007;109(11):5027-5035.
 124. Libani IV, Guy EC, Melchiori L, et al. Decreased differentiation of erythroid cells exacerbates ineffective erythropoiesis in beta-thalassemia. *Blood*. 2008;112(3):875-885.
 125. Rivella S, May C, Chadburn A, Riviere I, Sadelain M. A novel murine model of Cooley anemia and its rescue by lentiviral-mediated human beta-globin gene transfer. *Blood*. 2003;101(8):2932-2939.
 126. Breda L, Rivella S. Modulators of erythropoiesis: emerging therapies for hemoglobinopathies and disorders of red cell production. *Hematol Oncol Clin North Am*. 2014;28(2):375-386.
 127. Adamsky K, Weizer O, Amariglio N, et al. Decreased hepcidin mRNA expression in thalassemic mice. *Br J Haematol*. 2004;124(1):123-124.
 128. Weizer-Stern O, Adamsky K, Amariglio N, et al. mRNA expression of iron regulatory genes in beta-thalassemia intermedia and beta-thalassemia major mouse models. *Am J Hematol*. 2006;81(7):479-483.
 129. Musallam KM, Taher AT, Cappellini MD, Sankaran VG. Clinical experience with fetal hemoglobin induction therapy in patients with beta-thalassemia. *Blood*. 2013;121(12):2199-2212; quiz 372.
 130. Thein SL. The emerging role of fetal hemoglobin induction in non-transfusion-dependent thalassemia. *Blood Rev*. 2012;26 Suppl 1:S35-39.
 131. Melchiori L, Gardenghi S, Rivella S. beta-Thalassemia: Hijacking Ineffective Erythropoiesis and Iron Overload. *Adv Hematol*. 2010;2010:938640.
 132. Savona MR. Are we altering the natural history of primary myelofibrosis? *Leukemia Res*. 2014;38(9):1004-1012.
 133. Pasquier F, Cabagnols X, Secardin L, Plo I, Vainchenker W. Myeloproliferative Neoplasms: JAK2 Signaling Pathway as a Central Target for Therapy. *Clin Lymphoma Myeloma Leuk*. 2014;14(Suppl):S23-S35.
 134. Meyer SC, Keller MD, Woods BA, et al. Genetic studies reveal an unexpected negative regulatory role for Jak2 in thrombopoiesis. *Blood*. 2014;124(14):2280-2284.
 135. Oh SP, Yeo CY, Lee Y, Schrewe H, Whitman M, Li E. Activin type IIA and IIB receptors mediate Gdf11 signaling in axial vertebral patterning. *Genes Dev*. 2002;16(21):2749-2754.
 136. Zhou L, Nguyen AN, Sohal D, et al. Inhibition of the TGF-beta receptor I kinase promotes hematopoiesis in MDS. *Blood*. 2008;112(8):3434-3443.
 137. Sinha M, Jang YC, Oh J, et al. Restoring systemic GDF11 levels reverses age-related dysfunction in mouse skeletal muscle. *Science*. 2014;344(6184):649-652.
 138. Andersen RE, Lim DA. An ingredient for the elixir of youth. *Cell Res*. 2014;24(12):1381-1382.
 139. Szumska D, Pielek G, Essalmani R, et al. VACTERL/caudal regression/Currarino syndrome-like malformations in mice with mutation in the proprotein convertase Pcsk5. *Genes Dev*. 2008;22(11):1465-1477.
 140. Lotinun S, Pearsall RS, Horne WC, Baron R. Activin receptor signaling: a potential therapeutic target for osteoporosis. *Curr Mol Pharmacol*. 2012;5(2):195-204.
 141. Raje N, Vallet S. Sotatercept, a soluble activin receptor type 2A IgG-Fc fusion protein for the treatment of anemia and bone loss. *Curr Opin Mol Ther*. 2010;12(5):586-597.
 142. Suragani RN, Cawley SM, Li R, et al. Modified activin receptor IIB ligand trap mitigates ineffective erythropoiesis and disease complications in murine beta-thalassemia. *Blood*. 2014;123(25):3864-3872.
 143. Dussiot M, Maciel TT, Fricot A, et al. An activin receptor IIA ligand trap corrects ineffective erythropoiesis in beta-thalassemia. *Nat Med*. 2014;20(4):398-407.
 144. Suragani RN, Cadena SM, Cawley SM, et al. Transforming growth factor-beta superfamily ligand trap ACE-536 corrects anemia by promoting late-stage erythropoiesis. *Nat Med*. 2014;20(4):408-414.
 145. Piga AG, Perrotta S, Melpignano A, et al. ACE-536 Increases Hemoglobin and Decreases Transfusion Burden and Serum Ferritin in Adults with Beta-Thalassemia: Preliminary Results from a Phase 2 Study. *Blood*. 2014;124(21):abs. 53.
 146. Origa R, Galanello R. Pathophysiology of beta thalassaemia. *Pediatr Endocrinol Rev*. 2011;8 Suppl 2:263-270.
 147. Origa R, Galanello R, Ganz T, et al. Liver iron concentrations and urinary hepcidin in beta-thalassemia. *Haematologica*. 2007;92(5):583-588.
 148. Gardenghi S, Ramos P, Marongiu MF, et al. Hepcidin as a therapeutic tool to limit iron overload and improve anemia in beta-thalassemic mice. *J Clin Invest*. 2010;120(12):4466-4477.
 149. Ramos E, Ruchala P, Goodnough JB, et al. Minihepcidins prevent iron overload in a hepcidin-deficient mouse model of severe hemochromatosis. *Blood*. 2012;120(18):3829-3836.
 150. Preza GC, Ruchala P, Pinon R, et al. Minihepcidins are rationally designed small peptides that mimic hepcidin activity in mice and may be useful for the treatment of iron overload. *J Clin Invest*. 2011;121(12):4880-4888.
 151. Casu C, Oikonomidou R, Nemeth E, Ganz T, MacDonald B, Rivella S. Use of Minihepcidins As a "Medical Phlebotomy" in the Treatment of Polycythemia Vera. *Blood*. 2014;124(21):abs. 3231.
 152. Du X, She E, Gelbart T, et al. The serine protease TMPRSS6 is required to sense iron deficiency. *Science*. 2008;320(5879):1088-1092.
 153. Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C. The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemouvelin. *Cell Metab*. 2008;8(6):502-511.
 154. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nat Genet*. 2008;40(5):569-571.
 155. Maxson JE, Chen J, Enns CA, Zhang AS. Matriptase-2- and proprotein convertase-cleaved forms of hemouvelin have different roles in the down-regulation of hepcidin expression. *J Biol Chem*. 2010;285(50):39021-39028.
 156. Finberg KE, Whittlesey RL, Fleming MD, Andrews NC. Down-regulation of Bmp/Smad signaling by Tmprss6 is required for maintenance of systemic iron homeostasis. *Blood*. 2010;115(18):3817-3826.

157. Huang FW, Pinkus JL, Pinkus GS, Fleming MD, Andrews NC. A mouse model of juvenile hemochromatosis. *J Clin Invest.* 2005;115(8):2187-2191.
158. Nai A, Pagani A, Mandelli G, et al. Deletion of *Tmprss6* attenuates the phenotype in a mouse model of beta-thalassemia. *Blood.* 2012;119(21):5021-5029.
159. Guo S, Casu C, Gardenghi S, et al. Reducing *Tmprss6* ameliorates hemochromatosis and beta-thalassemia in mice. *J Clin Invest.* 2013;123(4):1531-1541.
160. Schmidt PJ, Toudjarska I, Sendamarai AK, et al. An RNAi therapeutic targeting *Tmprss6* decreases iron overload in *Hfe*^{-/-} mice and ameliorates anemia and iron overload in murine beta-thalassemia intermedia. *Blood.* 2013;121(7):1200-1208.
161. Taylor M, Qu A, Anderson ER, et al. Hypoxia-inducible factor-2alpha mediates the adaptive increase of intestinal ferroportin during iron deficiency in mice. *Gastroenterology.* 2011;140(7):2044-2055.
162. Mastrogiannaki M, Matak P, Peyssonnaud C. The gut in iron homeostasis: role of HIF-2 under normal and pathological conditions. *Blood.* 2013;122(6):885-892.
163. Dong A, Rivella S, Breda L. Gene therapy for hemoglobinopathies: progress and challenges. *Transl Res.* 2013;161(4):293-306.
164. Breda L, Casu C, Gardenghi S, et al. Therapeutic hemoglobin levels after gene transfer in beta-thalassemia mice and in hematopoietic cells of beta-thalassemia and sickle cells disease patients. *PloS One.* 2012;7(3):e32345.
165. Payen E, Leboulch P. Advances in stem cell transplantation and gene therapy in the beta-hemoglobinopathies. *Hematology Am Soc Hematol Educ Program.* 2012;2012:276-283.
166. Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMG2 activation after gene therapy of human beta-thalassaemia. *Nature.* 2010; 467(7313):318-322.
167. Boulad F, Wang X, Qu J, et al. Safe mobilization of CD34+ cells in adults with beta-thalassemia and validation of effective globin gene transfer for clinical investigation. *Blood.* 2014;123(10):1483-1486.
168. Liu M, Maurano MT, Wang H, et al. Genomic discovery of potent chromatin insulators for human gene therapy. *Nat Biotechnol.* 2015;33(2):198-203.
169. Bauer DE, Kamran SC, Lessard S, et al. An erythroid enhancer of *BCL11A* subject to genetic variation determines fetal hemoglobin level. *Science.* 2013;342(6155):253-257.
170. Schmidt PJ, Racie T, Westerman M, Fitzgerald K, Butler JS, Fleming MD. Combination therapy with a *Tmprss6* RNAi-therapeutic and the oral iron chelator deferiprone additively diminishes secondary iron overload in a mouse model of beta-thalassemia intermedia. *Am J Hematol.* 2015 Jan 2. [Epub ahead of print]
171. Casu C, Aghajan M, Oikonomidou R, Guo S, Monia BP, Rivella S. Combination of *Tmprss6*-ASO and the Iron Chelator Deferiprone Improves Erythropoiesis and Reduces Iron Overload in a Mouse Model of Beta-Thalassemia. *Blood.* 2014; 124(21):abs. 4024.
172. Breda L, Rivella S, Zuccato C, Gambari R. Combining gene therapy and fetal hemoglobin induction for treatment of beta-thalassemia. *Exp Rev Hematol.* 2013;6(3):255-264.
173. Zuccato C, Breda L, Salvatori F, et al. A combined approach for beta-thalassemia based on gene therapy-mediated adult hemoglobin (HbA) production and fetal hemoglobin (HbF) induction. *Ann Hematol.* 2012;91(8):1201-1213.