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

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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.[3] However, these patients exhibit increased iron absorption and NTBI leading to severe iron overload and clinical 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 thalassemic 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

 doi:10.3324/haematol.2014.114827
Manuscript received on January 16, 2015. Manuscript accepted on February 20, 2015.
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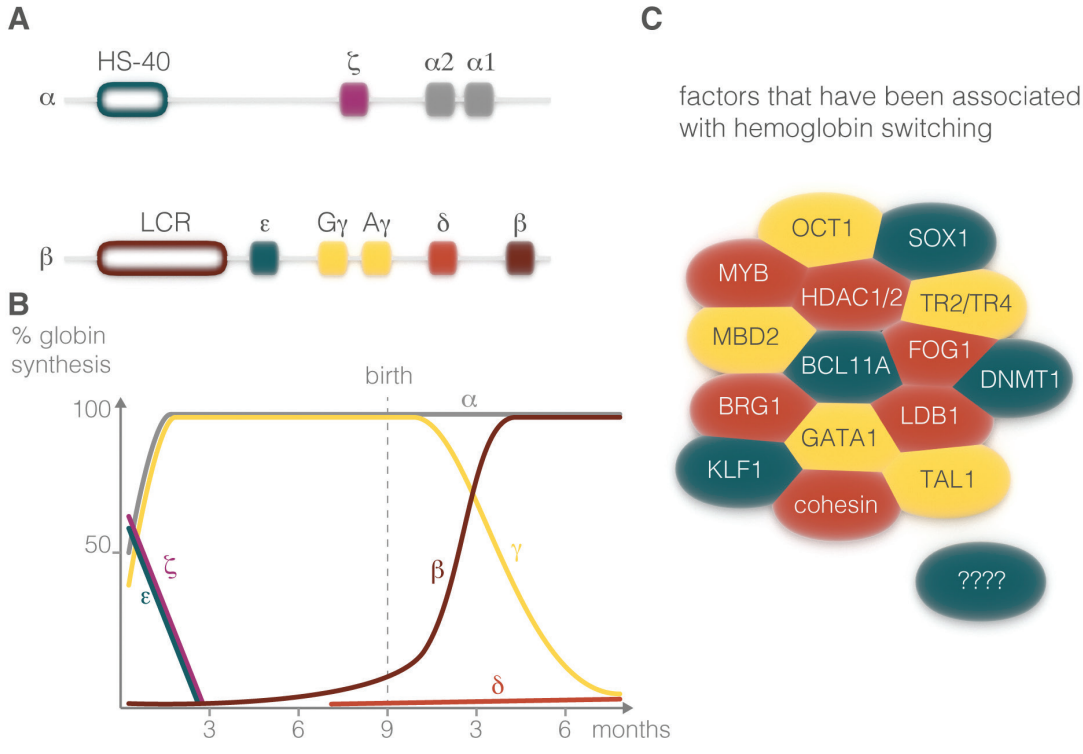

Figure 1. Representation of the genomic structure of the α- and β-globin loci. (A) Genes are indicated. LCR: locus control region (not to scale). (B) Relative expression of the globin genes during development. (C) Graphic representation of some of the candidate proteins involved in the regulation of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

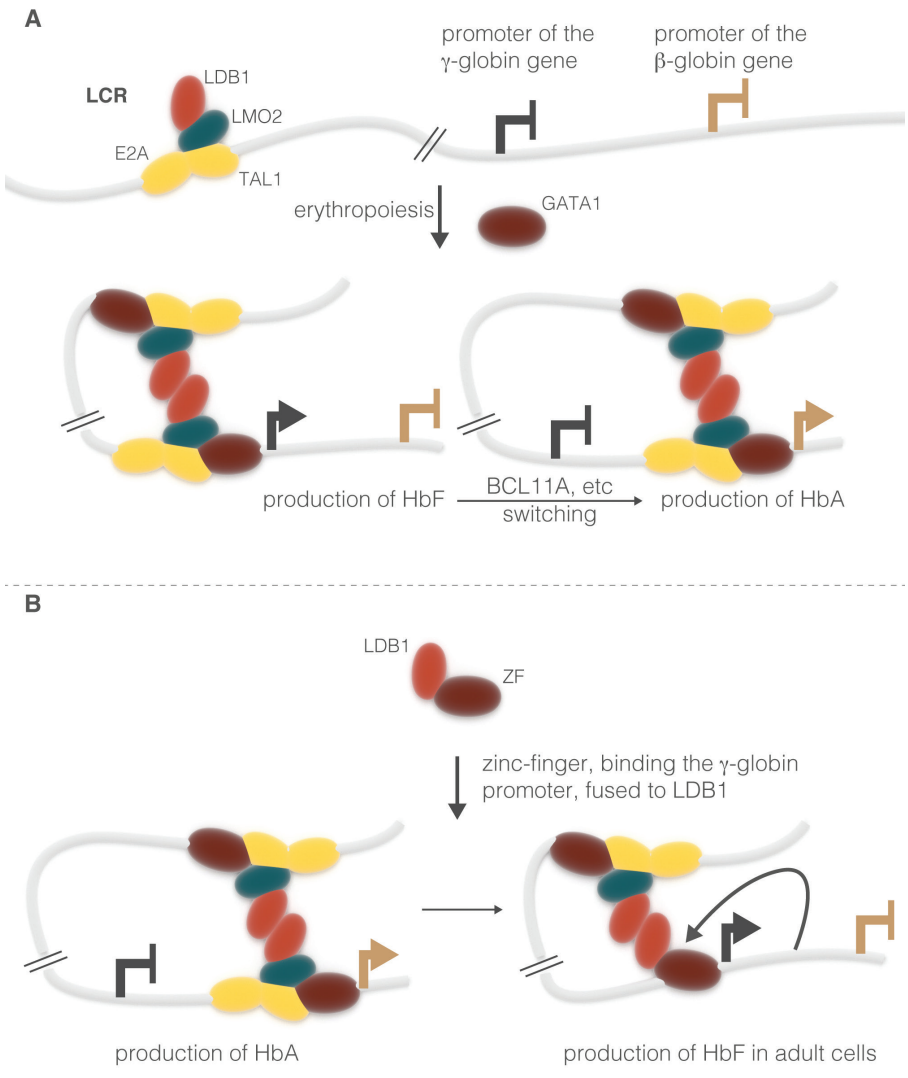

Figure 2. Illustration of the looping model and mechanism of HbF reactivation mediated by ZF-Ldb1. (A) In erythroid cell, the chromatin at the LCR is composed of arrays of multiple ubiquitous and lineage-specific transcription factor-binding sites, forming a holocomplex. This holocomplex loops on promoters 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 executed 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-

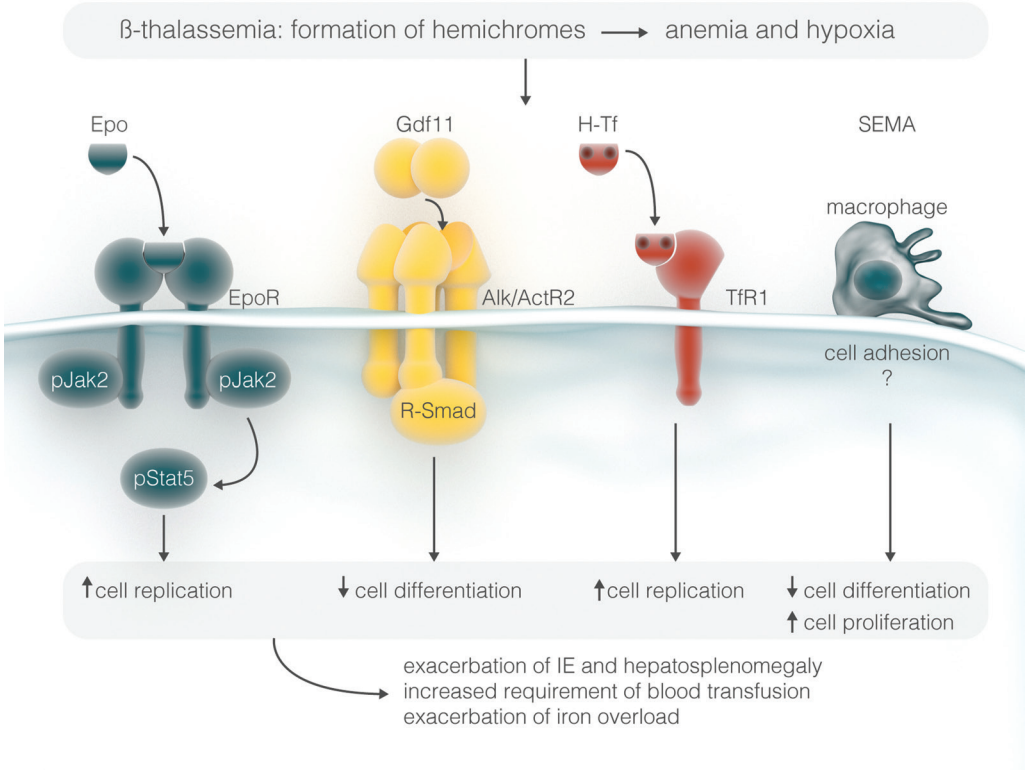

Figure 3. Representation of some of the pathways regulating steady state and stress erythropoiesis and their relationship to ineffective erythropoiesis in β-thalassemia. In this disorder, the imbalanced synthesis between α- and β-globin chains leads to formation of hemichromes, ROS formation and apoptosis 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 erythoid 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, Irp2 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 Irp2 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,

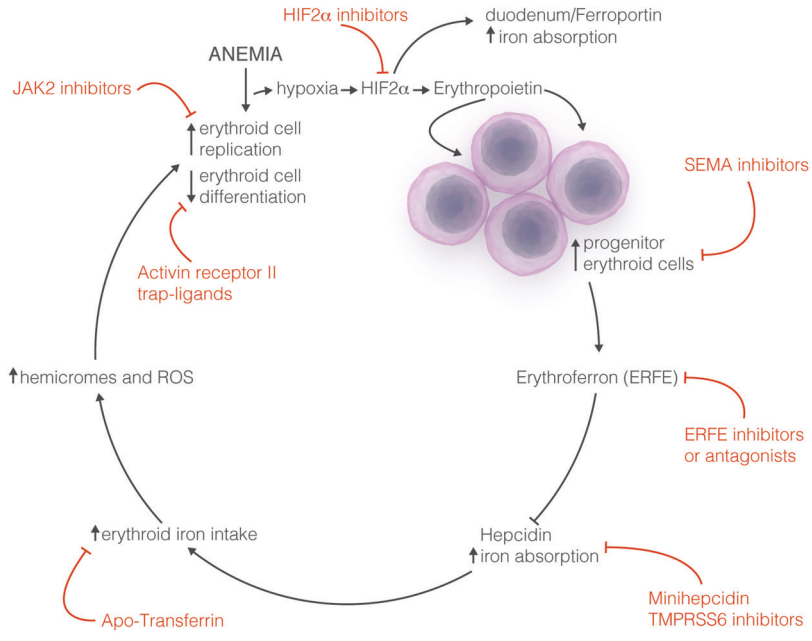

Figure 4. Illustration of the relationship between anemia, hypoxia, Epo, erythropoiesis and iron metabolism in β-thalassemia. Hypoxia, through HIF2a, contributes to augmented iron absorption by increasing expression of Fpn, Dmt1 and DcytB in the duodenum. Epo, ROS and Growth differentiation factor 11 (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 ß-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 α4, α5 and β1 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-alpha 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

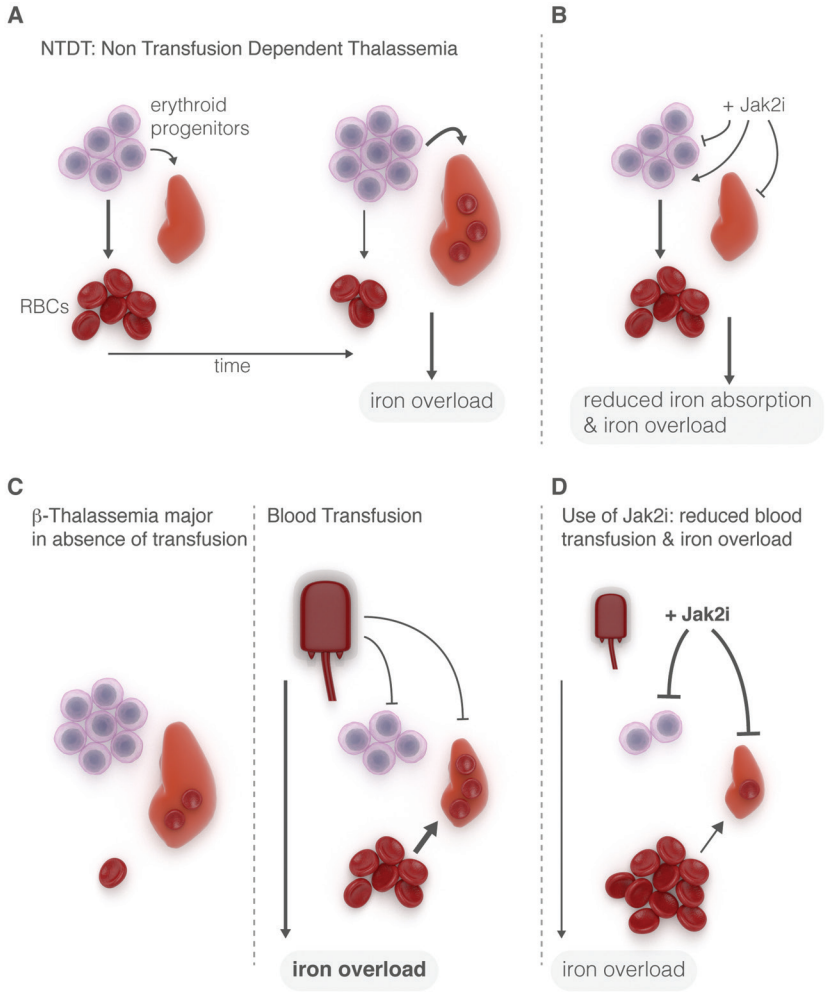

Figure 5. Potential use of JAK2 inhbitors in β-thalassemia. (A) In NTDT, the underlining chronic stress erythropoiesis exacerbates the anemia and the hepatosplenomegaly over time. This leads to increased sequestration of RBCs and further worsening of the ineffective erythropoiesis and iron overload. (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 inhbitor 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-2α (Hif2α) and Irp1. Hif2α is a transcription factor that orchestrates the response to hypoxia, including Epo synthesis.[111] Hif2α 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 DMmt1, 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 HIF2α mRNA translation. In fact, Irp1$^{-/-}$ mice exhibit features of Hif2α overexpression and hyperproduction of Epo, while Irp1 constitutive transgenic mice show defects in erythroid differentiation that can be attributed to decreased Hif2α 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 HIF2α and Epo expression to reduced iron availability, consistently with iron-restricted erythropoiesis.[111] In contrast, under iron-replete conditions, unconstrained HIF2α 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 Erythroferrone (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-

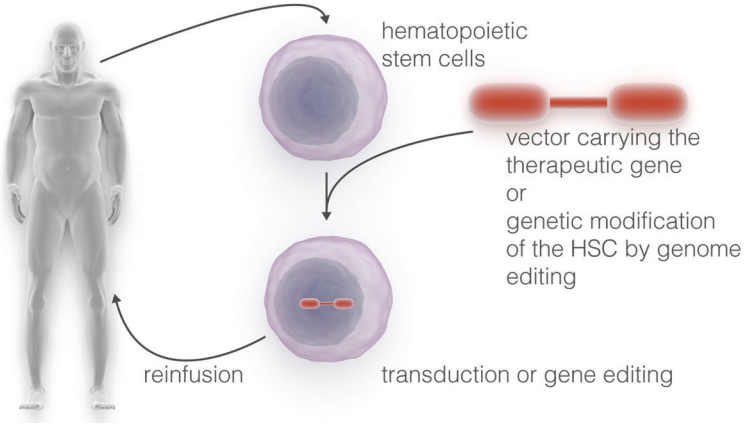

Figure 6. Schematic representation of gene therapy/gene editing approach for the cure of hemoglobinopathies. In this approach, HSC are harvested form the patient and modified by transduction (gene addition) or homologous DNA recombination (HDR). Following partial or full myeloablation, the genetically 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 erythoid 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 correlation 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 erythoid 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 $Hbb^{th3/1}$ mice, which in turn led to reduced IE, reticulocyte count, spleen size, and improved anemia.[151]

Tmprsss6 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.

HIF2α 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 dudodenum 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 Hif2α stabilization and activity.[112,162] In fact, $Hbb^{th3/+}$ mice showed improvement in tissue-iron levels and anemia following genetic ablation of intestinal Hif2α.[112] This observation suggests that duodenal HIF2α 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 ß-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.

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