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Future alternative therapies for β -thalassemia

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Abstract

β -thalassemia is an inherited disorder due to mutations found in the β -globin gene, leading to anemia and requiring sporadic or chronic blood transfusions for survival. Without proper chelation, β -thalassemia results in iron overload. Ineffective erythropoiesis can lead to iron overload even in untransfused patients who are affected by β -thalassemia intermedia. Better understanding of the molecular biologic aspects of this disorder has led to improvements in population screening and prenatal diagnosis, which, in turn, have led to dramatic reductions in the number of children born with β -thalassemia major in the Mediterranean littoral. However, as a consequence of decreases in neonatal and childhood mortality in other geographical areas, β -thalassemia has become a worldwide clinical problem. A number of unsolved pathophysiological issues remain, such as ineffective erythropoiesis, abnormal iron absorption, oxidative stress, splenomegaly and thrombosis. In the last few years, novel studies have the potential to introduce new therapeutic approaches that might reduce these problems and limit the need for blood transfusion.

Keywords

anemia; β -thalassemia; erythropoietin; ferroportin; fetal hemoglobin; gene transfer; hepcidin; ineffective erythropoiesis; iron metabolism; Jak2; lentiviral vector; radicals; reactive oxygen species

β -thalassemia

β -thalassemia, one of the most common congenital hemolytic anemias, is due to partial or complete lack of synthesis of β -globin chains resulting in considerable morbidity and mortality, and is found mainly in the Mediterranean region and South-East Asia. The β -thalassemias are characterized by their clinical severity (phenotype). Patients with Cooley's anemia, also known as β -thalassemia major (β -TM), which is the most severe form of this disease, require many blood transfusions every year and is characterized by ineffective erythropoiesis and extramedullary hematopoiesis (EMH) [1]. Untreated β -TM uniformly is fatal in the first few years of life [1]. In β -thalassemia intermedia (β -TI), where a greater number of β -globin chains

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are synthesized, the clinical picture is milder, and the patients require only infrequent or no transfusions [1,2]. In both β -thalassemias, with time the spleen is enlarged, the hemoglobin level decreases and progressive iron overload occurs from increased GI iron absorption in addition to transfusions [1]. β -thalassemia trait refers to carriers of mutations inherited from only one of the parents; such individuals have microcytosis and hypochromia with either normal hemoglobin or mild anemia [3]. Current disease management includes prenatal diagnosis, transfusion therapy, iron chelation and allogeneic bone marrow transplantation (BMT). The WHO estimates that approximately 50,000–100,000 children are born with symptomatic features of β -thalassemia every year. Despite the prevalence of this disease in the Mediterranean area and South-East Asia, β -thalassemia is an orphan disease [4]. Studies to develop new therapies are rare and not well funded.

β -thalassemia is caused by more than 200 mutations, mostly associated with one or a limited number of nucleotides. The vast majority of β -thalassemias are caused by point mutations within the gene or its immediate flanking sequences and are classified according to the mechanism by which they affect gene regulation: transcription, RNA processing and mRNA translation [1]. These mutations are classified according to whether the corresponding allele results in no (β^0) or low (β^+) β -globin chain synthesis. Mutations that lead to alternative splicing are associated with reduced synthesis of normal β -globin mRNA and protein and are defined β^+ . By contrast, mutations that completely impair β -globin synthesis (e.g., premature termination codons [PTCs]) are defined β^0 . Depending on the association of these different mutations, patients are classified into three principal groups with none, very low or low β -globin production ($\beta^0/0$, $0/+$, $+/+$ respectively). The levels of fetal hemoglobin (HbF) account for a large part of the clinical heterogeneity observed in patients with β -thalassemia. Variation in HbF expression among individuals is an inheritable disease modifier and high HbF (composed from two α - and two γ -chains) levels generally correlate with reduced morbidity and mortality in this disorder, since the γ -globin chains combine with the excess α -chains. Novel discoveries are showing promise for identifying new drugs to reactivate HbF in adult individuals (Table 1) [5–12].

Despite recent discoveries concerning the genetic abnormalities that lead to the β -thalassemia syndrome, a number of unsolved pathophysiological issues remain, such as ineffective erythropoiesis, EMH, abnormal iron absorption, oxidative stress resulting from reactive oxygen species (ROS), bone fragility and endocrine disorders. Novel treatments are emerging that might improve the current practice and address these issues. However, current β -thalassemia therapy is much superior compared with the clinical practice utilized in the past. For this reason any new treatment will need to be compared to ongoing effective treatments before it is utilized in the clinical standard care.

Ineffective erythropoiesis

The hallmark of β -thalassemia is ineffective erythropoiesis. Mutations in the β -globin gene result in decreased or absent production of the β -globin chain and a relative excess of the α -chain. This interferes with erythroid cell maturation, causes premature death (apoptosis) of the red cell precursors and limits red cell production. In the attempt to compensate for the anemia, the erythron expands. The balance between expansion of the pool of erythroid precursors and red cell production is dynamic and many factors can alter this relationship over time, exacerbating the anemia. For instance, damaged erythrocytes are trapped in the spleen, aggravating the anemia and limiting tissue oxygenation. Hypoxia stimulates kidney erythropoietin production and erythroid expansion. Under these circumstances, hepatosplenomegaly develops further, exacerbating the trapping of the red cells and the anemia and contributes to the ineffectiveness of the marrow activity. In addition, extramedullary erythropoietic tissues, primarily in the thorax and the paraspinal regions, can expand, leading

to the characteristic deformities of the skull and face, osteopenia, and demineralization of the bones, which are then prone to fractures. Blood transfusion is the only therapeutic option for severe ineffective erythropoiesis. Repeated transfusions can suppress the endogenous ineffective erythropoiesis. Splenectomy is required in order to prevent a fatal outburst of this organ and to reduce the transfusional regimen.

The principal regulator of erythropoiesis is erythropoietin (Epo), a 34-kDa renal glycoprotein whose receptor, EpoR, is expressed by the earliest erythroid progenitors at the burst forming unit-erythroid (BFU-E) stage [13–15]. EpoR signals for erythroblast formation involve first the activation of Jak2, an essential kinase that preassembles at a conserved EpoR domain [16–19]. Jak2 then mediates phosphorylation of conserved cytoplasmic EpoR tyrosine motifs [16–19]. The resultant EpoR phosphotyrosine sites function as a scaffold for the assembly of signal transduction factors with Src-homology 2 (SH2) and phosphotyrosine-binding domains [17]. Some of the best characterized SH2 domain-containing transcription factors are the signal transducer and activator of transcription factor 5a and 5b (STAT5a and STAT5b). Studies in mice lacking full-length Stat5a or Stat5b, (Stat5^{-/-}), show fetal anemia and decreased stress erythropoiesis due to apoptosis of the early erythroblasts [20,21]. The severity of anemia in Stat5 deficient mice correlates with the loss of expression of the antiapoptotic *Bcl-X_L* gene [20,22,23]. Moreover, the cell surface levels of the transferrin receptor-1 (TfR1) are decreased more than twofold on erythroid cells of Stat5^{-/-} animals, suggesting that Jak2 might also control iron intake in red cells [24]. Compared with normal mice, it has been shown that mice affected by β -TI exhibit a higher number of erythroid cells associated with the expression of cell cycle promoting genes such as *EpoR*, *Jak2*, cyclin-A, *Cdk2* and *Ki-67*, and the antiapoptotic protein *Bcl-X_L* [25]. These cells also differentiated less than the corresponding normal cells *in vitro*. This observation suggests that limited erythroid cell differentiation could also contribute to exacerbate the ineffective erythropoiesis and anemia in β -thalassemia [25,26].

The mice affected by β -TI exhibit a progression of the disease similar to that observed in transfusion-independent thalassemic patients, who eventually develop splenomegaly. The enlarged spleen in both mice and patients sequesters erythrocytes, reducing hemoglobin levels. In addition, studies in mice also suggest that an increased number of erythroid progenitors accumulate in the spleen under conditions of ineffective erythropoiesis and this observation was confirmed in spleen specimens from thalassemic patients [25,26]. When the number of erythroid precursors increase in the spleen, this probably increases both the spleen volume and the sequestration of erythrocytes, further exacerbating the anemia. Another consequence is an additional increase in Epo levels, thus activating the EpoR/Jak2 pathway. It has been proposed that activation of Jak2 in β -thalassemia is associated with limited cell differentiation [25,26]. However, activation of the Epo/EpoR/Jak2 pathway is not likely to be the only cause for the limited erythroid differentiation observed in this disorder. Mutations that lead to constitutive activation of Jak2 in absence of other erythroid defects lead to polycythemia vera rather than ineffective erythropoiesis [27]. Therefore, this model requires that additional genetic mutations or environmental conditions, in addition to the activation of Jak2, need to occur to interfere with erythroid cell differentiation. Unbalanced synthesis of heme or globin chains, iron overload and ROS have been proposed to explain why, in addition to elevated Epo levels and Jak2 activity, the erythroid cells lose some of their ability to differentiate [26]. Under these circumstances, Jak2 might be an important factor in modulating ineffective erythropoiesis. Based on this model, an excessive activity of this kinase under conditions of splenomegaly might exacerbate the anemia. If this is the case, use of Jak2 inhibitors might have a beneficial effect on the spleen size rather than limiting red cell production. In fact, administration of a Jak2 inhibitor to thalassemic mice had a profound effect on splenomegaly and ineffective erythropoiesis with limited effect on anemia (Figure 1) [25].

Transfusion-independent β -TI patients, if affected by splenomegaly, often develop a need for blood transfusion therapy, and many patients eventually require splenectomy. Based on the preclinical data using a Jak2 inhibitor, it is possible to envision that β -TI patients who develop splenomegaly might benefit from using these compounds. Jak2 inhibitors might be utilized temporarily to reduce the spleen size and, in the presence of blood transfusions, to prevent the ongoing severe anemia. Additional data on transfused and nontransfused thalassemic mice suggest that even patients affected by β -thalassemia that require transfusion and develop splenomegaly, might benefit from administration of Jak2 inhibitors [Melchiori *et al.*, Unpublished Data]. The use of Jak2 inhibitors might limit or reduce splenomegaly, thereby preventing or delaying the need for splenectomy and indirectly improving the management of anemia by reducing the rate of blood transfusions.

Jak2 not only activates the erythroid programming. In red cells Jak2 might play an important role in controlling TfR1 synthesis [24]. Therefore, activation of Jak2 might increase iron intake of the erythroid progenitors. In addition, it has been proposed that Jak2 phosphorylates ferroportin, the iron transporter that controls egress of this metal from enterocytes, macrophages and, recently, erythroid precursors [28,29]. Once ferroportin is phosphorylated at specific tyrosine residues, this protein is ubiquitinated and degraded in the late endosome/lysosome [30]. Therefore, Jak2 may play an important role not only in the Epo/EpoR cascade, but also in the pathways that control TfR1 and ferroportin synthesis/turnover, establishing a potential link between erythropoiesis and iron metabolism.

Iron overload & iron chelation

Iron is essential for the growth and survival of most organisms. In mammals, iron is essential for many functions, including cell proliferation, DNA synthesis and oxygen transport action. The majority of this element is incorporated into red cells to form hemoglobin, which is fundamental for oxygen delivery. Therefore, iron metabolism and erythropoiesis are closely related and understanding of the mechanisms by which they cooperate with and regulate each other is of crucial importance for the understanding of vital physiological functions.

Iron balance must be carefully regulated to provide iron as needed, while avoiding the toxicity associated with excessive iron concentrations. In β -thalassemia, red blood cell (RBC) transfusions are a life-saving treatment. However, every unit of transfused blood contains 200–250 mg of iron, and the human body has no mechanism to actively excrete excess iron. Therefore, cumulative iron overload is an inevitable consequence of chronic transfusion therapy [31]. Iron overload, if not prevented or adequately treated, is fatal in both transfused and nontransfused patients. Therefore, management of tissue iron overload through iron chelation is a primary focus of β -thalassemia. Iron not bound to transferrin, also termed nontransferrin-bound iron (NTBI), is a catalyst in the formation of oxygen free radicals via the Fenton reaction and consequently damages the heart, endocrine organs and liver [32,33]. Excess of NTBI can lead to myocyte damage, arrhythmias and congestive heart failure, the main causes of death in patients who have β -thalassemia [34–36].

For chelation therapy to be effective, an agent must bind and remove iron from the body at a rate that is either equal to the rate of transfusional iron input (maintenance therapy) or greater than that rate (reduction therapy). In β -TM, the iron input is on average approximately 0.5 mg/kg/day [31]. Deferoxamine (DFO), the current reference standard iron chelator, has been used clinically for many years. Iron chelation therapy with DFO has dramatically improved the outlook in β -thalassemia. Parenteral DFO reduces tissue iron stores, prevents iron-induced organ damage, and reduces morbidity and mortality, with little serious toxicity. However, the burden of prolonged subcutaneous portable pump infusions, high cost and patient noncompliance have prompted the development of new methods of administration and new

formulations of DFO as well as oral iron chelators. For this reason, more convenient oral iron chelators were developed. Currently, two oral iron chelators are available, deferiprone (DFP; Apotex, Toronto, Canada; L1, CP20, Ferriprox[®] and Kelfer[®]) and deferasirox (DFR, Novartis Pharma, ICL670, Exjade[®]). With DFP and DFR, the only oral iron chelators studied in large long-term clinical trials, compliance and quality of life were improved [31]. Combinations of two iron chelators (such as parenteral DFO plus oral DFP) have been shown to produce additive and synergistic effects, explained by the shuttle hypothesis. Iron bound to a 'shuttle' – an oral agent that mobilizes tissue iron – is exchanged in the bloodstream with a 'sink', such as parenteral DFO and excreted via the kidneys, while the shuttle is reutilized [37]. Therefore, combination therapy may produce enhanced iron excretion, target specific iron compartments, minimize side effects, increase treatment options, improve compliance, and facilitate individualization of therapy. Better understanding of the kinetics of iron metabolism, iron overload and chelation should improve therapeutic strategies [37].

Labile plasma iron (LPI) is the nontransferrin-bound component of plasma iron detected in iron overload disorders by virtue of its redox activity and accessibility to chelators. LPI is a potential source of cellular iron overload and ensuing oxidative damage, but it can also serve as both a diagnostic marker of systemic iron overload and a target of iron chelation. Various chelation regimens in terms of their ability to control daily LPI levels were measured in β -TM patients treated with different iron chelators (DFO, DFP, DFR and combination of DFP and DFO). This analysis showed that the proportion of patients whose LPI levels were successfully maintained within baseline levels ($<0.45 \mu\text{M}$) over 24 h varied with treatment type. In particular, prevention of daily LPI reappearance was achievable in most of the patients treated with DFR or sequentially with DFP and DFO; however, only in less than half the patients treated with either DFP or DFO alone [38]. This study ultimately suggested that regular daily monitoring of LPI can contribute significantly to achieving optimal and individualized chelation treatments in β -TM.

Iron overload & hepcidin

Ferroportin, the iron exporter on the basolateral surface of absorptive intestinal enterocytes, hepatocytes, placental cells and macrophages, releases iron into the plasma [39,40]. Release of iron is regulated by hepcidin, an iron-responsive peptide [41–44]. Hepcidin, a cysteine-rich 25-amino acid peptide produced by hepatocytes and secreted into the bloodstream, derives from the C-terminus of an 84-amino acid prepropeptide, originally isolated from urine and blood ultrafiltrate [45]. Increased plasma iron from macrophage recycling of aged RBC or from intestinal absorption of iron stimulates hepatocytes to produce more hepcidin [40], which in turn controls the concentration of ferroportin on cell surfaces [46]. Degradation of ferroportin interrupts cellular iron export at two primary sites, the intestinal epithelium and tissue macrophage. Circulating iron is mostly derived from the recovery of iron from senescent RBC, through phagocytosis by tissue macrophages, particularly in the spleen. The cells are lysed, and hemoglobin is catabolized, presumably by heme oxygenase, to liberate iron. Ferroportin is critical for macrophage iron export. Thus, different hepcidin levels can change the rate of iron absorbed from the enterocytes and the ratio between iron stored and released from the macrophages. Low levels of hepcidin can lead to increased intestinal absorption and iron overload resulting from excessive absorption of dietary iron [47,48]. Hyperabsorption of dietary iron results in hyperferremia and tissue iron deposition. This is accompanied by a paucity of iron in intestinal epithelial cells and tissue macrophages.

In β -TI patients who do not require transfusions, iron absorption studies show that the rate of iron loading from the GI tract is approximately three- to four-times greater than normal, varying between 2 and 5 g/year and depending on the severity of erythroid expansion [49,50]. Regular transfusions may double this rate of iron accumulation. Increased gastrointestinal iron

absorption may also play a role in transfused β -TM patients, increasing when hemoglobin levels decrease [50]. Patients affected by β -TM analyzed before and after transfusion therapy showed that, in the absence of transfusion, iron absorption was elevated, whereas it was decreased or normalized after transfusion therapy. After demonstration that hepcidin synthesis was low or relatively low in mouse models of β -thalassemia, similar results were subsequently observed in thalassemic patients [51–55].

In normal individuals liver hepcidin mRNA levels correlated with hemoglobin concentration and inversely correlated with the serum transferrin receptor, erythropoietin and NTBI. By contrast, in β -thalassemic patients these parameters did not correlate with indices of iron load, and urinary hepcidin levels were disproportionately suppressed with regard to iron burden [51]. Urinary hepcidin was suppressed relative to the degree of iron burden, whereas transfusion led to its increase [56]. Therefore, there is a clear correlation between the degree of anemia, iron absorption and iron overload. This study and previous investigations led to the conclusion that iron absorption in humans is regulated by the combined influences of hypoxia, the body's iron stores [41,42,46] and the erythropoietic demand for iron [44,57].

After phlebotomy, hemolysis or Epo administration, hepcidin production is decreased, indicating that the increased erythropoiesis exerts its effect by suppressing production of this peptide [44,58,59]. Administration of inhibitors of erythropoiesis after phlebotomy were utilized to disassociate the effects of anemia, hypoxia and Epo from those of increased erythropoiesis and iron use [60]. Phlebotomized control mice developed anemia, tissue hypoxia, increased Epo, increased erythropoiesis, decreased serum iron and decreased hepatic hepcidin mRNA levels. When erythropoietic inhibitors were administered in conjunction with phlebotomy, serum and tissue iron levels rose dramatically as did those of hepcidin mRNA, even though the mice were anemic. These experiments suggest that the dominant regulators of hepcidin during increased erythropoiesis include a signal arising from the erythropoietic activity. Two erythroid regulators have been proposed, GDF15 and twisted gastrulation (TWSG1) [61,62]. Compared with controls, both GDF15 and TWSG1 are elevated in the serum of β -thalassemic patients and suppress hepcidin expression *in vitro* [62]. GDF15 and TWSG1 are members of the TGF- β superfamily of proteins, which are known to control proliferation, differentiation and apoptosis in numerous cell types. However, GDF15 is not elevated after stem cell transplantation in patients with hematopoietic malignancies [63]. This suggests that GDF15 is not produced by the proliferating erythroid precursors, but rather by the erythroid precursors that undergo premature cell death, as in β -thalassemia or refractory anemia with ring-sideroblasts [64]. Therefore, the role of GDF15 may be to limit hepcidin synthesis when the erythroid precursors undergo cell death, one factor in the development of ineffective erythropoiesis [25].

In contrast to GDF15, the highest expression level of TWSG1 was detected at early stages of erythroblast differentiation before hemoglobinization of the cells. Therefore, TWGS1 probably functions as a marker of increased erythropoiesis [61]. The combined effect of GDF15 and TWSG1 might therefore repress hepcidin expression at higher levels in condition of increased erythropoiesis and erythroid cell death, dysregulating iron homeostasis in β -thalassemia syndrome. Other factors have been proposed to control iron absorption under conditions of stress erythropoiesis. Several studies have emphasized the role of hypoxia and Epo [65–68]. Together, these studies suggest a more general role for the hypoxia pathway in coordinating iron metabolism and oxygen transport by the simultaneous coordinated expression of hepcidin, ferroportin and Epo. These studies might also indicate that several factors acting synergistically lead to hepcidin downregulation when increased erythropoiesis, apoptosis of red cell precursors and hypoxia exist simultaneously. This was illustrated by using mouse models of β -thalassemia, in which the effect of ineffective erythropoiesis on hepcidin synthesis was investigated. The first studies indicated that both mice affected by β -TM and β -TI had,

respectively, low or relatively low hepcidin expression levels compared with the amount of iron overload [53–55,69]. A more extensive analysis indicated that, while in 1-year-old β -TI mice, hepcidin expression was similar to or higher than that of control mice, it was rather the upregulation of ferroportin in the duodenum that caused iron overload [57]. In transfused mice hepcidin expression augmentation was probably due to the combined effect of reduced anemia, increased iron content, and suppression of erythropoiesis [57].

Analysis of these mice also indicated that while the concentration and distribution of iron in the liver, spleen and kidney increased over time, the hemoglobin levels decreased. This indicates that in β -thalassemia, more iron is absorbed than required for erythropoiesis. Thus, it is possible that reducing iron intake by increased levels of hepcidin might have a beneficial effect in limiting iron overload with little or no effect on anemia (Figure 1). However, since hepcidin targets ferroportin, which is also expressed on the surface of macrophages, increased concentration of hepcidin may block iron egress from these cells. This could reduce the efficiency of iron recycling. Therefore, although increased concentrations of hepcidin might reduce iron absorption in β -thalassemia, a careful analysis will need to be done to evaluate if this might exacerbate the anemia, especially in patients affected by β -TI that have increased iron absorption but do not require blood transfusion.

Hypercoagulable state

The improvement in the medical treatment of patients with β -TM and β -TI result in the significant prolongation of their survival [36]. However, at the same time, new complications are becoming more frequent and deserve more attention. One of these complications is a hypercoagulable state resulting in thromboembolic phenomena. Several factors contribute to the development of a hypercoagulable state. The most important one is due to the denaturation of the excess α - or β -globin chains to heme and globin in the severe forms of α - (hemoglobin H [HbH] disease) or β -TM and β -TI [70]. The free iron that releases from heme, which can be measured as labile cellular iron (LCI), is a catalyst in the formation of ROS with the consequent oxidation of polyunsaturated fatty acids (PUFAs) that can be measured following an increase in malondialdehyde (MDA) and exposure of phosphatidylserine (PS) residues as a result of the 'flip flop' from the internal to the external part of the cell lipid membrane [71,72]. The exposure of PS residues activates the prothrombinase complex to form thrombin, which is a major factor in thrombus formation and endothelial perturbation [70,73]. In addition, it has been suggested that ROS might repress hepcidin expression and that anti-oxidants might be used to prevent this phenomena [74].

Another factor which contributes to the hypercoagulable state is chronic endogenous platelet activation, demonstrated by increased platelet aggregation, shortening of platelet survival and increase in urinary metabolites, such as thromboxane A₂ (TXA₂) and prostacyclin (PGI₂) which already exists from early childhood [70]. Platelet adhesion under flow condition, the primary event in thrombus formation, is increased in thalassemic patients, mainly in those who had already experienced a thromboembolic event [75]. Moreover, flow cytometry studies demonstrated the presence of platelets with the activation marker CD62P (P-selectin) and CD63 [76]. Last but not least, there is an increase in the number of platelets by more than 500,000/cumm in splenectomized patients, particularly in patients with β -TI. There is also evidence for elevated levels of endothelial adhesion proteins resulting in adherence of RBC to endothelial cells, mainly in sickle cell anemia, but also in β -thalassemia [77]. Another finding in patients with β -thalassemia from different ethnic backgrounds is the low levels of the coagulation inhibitors, proteins C and S, which are probably acquired at an early age, and also can contribute to development of a hypercoagulable state [78].

Following these data, it is no wonder that there are many reports of both venous and arterial thrombosis in many organs of patients with the severe forms of β -thalassemia (mainly in those with β -TI) who are splenectomized and have a platelet count of more than 500,000/cumm [79]. They include deep vein thrombosis, pulmonary embolism and thrombosis and cerebrovascular accidents (CVA), particularly in older patients [80]. At this point there are still no clear guidelines on whether, and how to, prevent thromboembolic complications in β -thalassemia because no controlled studies have been reported so far. However, it seems that to patients at risk, particularly those with β -TI and splenectomized with platelet counts greater than 500,000/cumm, treatment with antiplatelet aggregants, such as aspirin, should be recommended. To this group of patients, a diagnostic MRI of the brain should be considered after the age of 20 years in order to rule out asymptomatic ischemic brain damage. Obviously, a well-controlled prospective study is indicated to determine whether preventive anticoagulant and/or antiplatelet aggregants are indicated since these patients are at higher risk to develop thromboembolic phenomena.

Bone marrow transplantation, genetic & cellular therapies

Current disease management of β -thalassemia consists of prenatal diagnosis, transfusion therapy or allogeneic BMT [37,81,82]. Only the latter is potentially curative [83]. The first successful BMT of β -thalassemia was reported in 1982 [84]. Consequently, several centers have utilized this approach as definitive therapy [85–87]. The most extensive experience in treating β -thalassemia patients with BMT is that of Lucarelli and coworkers in Italy [85]. Established protocols can lead to a high success of β -thalassemia-free survival, although the transplant-related mortality is still significant and the chronic graft-versus-host disease is still a potential long-term complication of allogeneic hematopoietic stem cell (HSC) transplantation [86,88]. In addition, availability of allogeneic bone marrow is subject to finding a donor with an identical HLA. On average, only one in four siblings is HLA identical. However, development of new techniques to improve the management of graft-versus-host disease, to perform BMT from unrelated donors and cord blood stem cells may expand the pool of potential donors in the near future [89]. In particular, unrelated cord blood transplantation (CBT) for β -thalassemia is an emerging alternative therapy [90,91]. CBT has become apparent as a therapeutic option for a number of nonmalignant diseases affecting the bone marrow and leading to clinical manifestations most likely affecting distant organs [91–98]. Donor umbilical cord blood is also attractive for patients with nonmalignant diseases since offers the advantages for the recipient of finding faster an available donor, to tolerate one to two HLA mismatches and low incidence of acute graft-versus-host disease.

In addition, patients with severe β -thalassemia might also benefit from new genetic and cellular approaches. From this prospective, β -thalassemia is an excellent candidate disease for genetically based therapies in autologous HSCs [99–101]. Alternatively, somatic cells reprogrammed to induced pluripotent stem cells might also provide a possible new approach to treat β -thalassemia (Figure 2) [102,103].

Treatment of β -thalassemia, sickle cell anemia and other diseases through gene therapy is actively studied in murine and primate models [104–112]. The original studies showed that lentiviral mediated human β -globin gene transfer can rescue mice affected by β -TI and β -TM [104,113,114]. The β -TI mouse models (*th3/+*) utilized in these studies is characterized by the deletion of both the β^{minor} and β^{major} genes in heterozygosity [115]. Adult *th3/+* mice have a degree of disease severity (hepatosplenomegaly, anemia, aberrant erythrocyte morphology) comparable to that of patients affected by β -TI. Efficient transfer of the human β -globin gene into murine HSCs of these animals was achieved with a lentiviral vector harboring large segments (3.2 kb) of the β -globin locus control region (LCR) and promoter. This recombinant lentivirus was termed TNS9. *Th3/+* mice recipients of TNS9 transduced bone marrow cells

demonstrated amelioration of red cell pathology (anisocytosis and poikilocytosis) and significantly increased hemoglobin levels (from 8–9 g/dl to 11–13 g/dl). The massive splenomegaly found in chimeras engrafted with control *th3/+* bone marrow was not observed in TNS9-treated animals.

Mice completely lacking adult β -globin genes (*th3/th3*) die late in gestation, limiting their utilization as a model for Cooley's anemia [115]. For this reason, adult animals affected by Cooley's anemia were generated by transplantation of hematopoietic fetal liver cells harvested from *th3/th3* embryos at E14.5 into lethally irradiated syngeneic adult recipients [104]. Hematological analyses of engrafted mice performed 6–8 weeks post-transplant revealed severe anemia due not to pancytopenia but rather to low RBC and reticulocyte counts together with massive splenomegaly and extensive EMH [104,114]. These animals could be rescued using TNS9 or by blood transfusions, supporting the notion that their phenotype is due specifically to erythroid impairment [57,104]. In addition, successful transfer of globin genes into hematopoietic cells of humans has been demonstrated and is encouraging [106,116]. These, and many other relevant studies, support the use of lentiviral vectors for the cure of β -thalassemia and additional approaches have been undertaken to further improve this therapeutic strategy [105,117–124].

Another promising approach for the treatment of genetic disorders due to PTCs is the use of drugs to force stop codon readthrough and, therefore, protection from nonsense-mediated mRNA decay (NMD) [125]. Aminoglycoside antibiotics have been tested on mRNAs carrying premature stop codons, showing that these drugs decrease the accuracy in the codon–anticodon base pairing, inducing a ribosomal read through of premature termination codon. These findings have introduced new hopes for the development of a pharmacologic approach to treat thalassemic patients carrying the $\beta 0$ –39 mutation, which introduces a PTC in codon 39 of the β -globin gene and is one of the most frequent thalassemic mutations in the Mediterranean littoral [1]. To test whether aminoglycosides and analogous molecules can restore β -globin protein synthesis, human erythroid cells (K562) carrying a lentiviral construct containing the $\beta 0$ –39 globin gene were produced [126]. Treatment of these clones with geneticin (G418) and other aminoglycosides restored the production of β -globin [126]. More importantly, after fluorescence-activated cell sorting and high-performance liquid chromatography (HPLC) analyses, G418 was also demonstrated to correct the biological function of the $\beta 0$ –39 globin mRNA in erythroid precursor cells from $\beta 0$ –39 homozygous β -thalassemia patients [127]. This study strongly suggests that ribosomal readthrough should be considered a novel approach for treatment of $\beta 0$ thalassemia caused by premature stop codon mutations and NMD.

Although defective β -globin gene expression and β -globin deficiency can be attributed to almost 200 thalassemic mutations, only ten mutations are responsible for the majority of cases worldwide. Of these, some of the most frequent cause aberrant splicing of intron 1 (IVS1–110, IVS1–6 and IVS1–5) or intron 2 (IVS2–654 and IVS2–745) of the human β -globin gene [128,129]. A promising and elegant approach to treating thalassemic patients carrying these kind of mutations is based on the use of small nuclear (sn) RNA and splice-switching oligonucleotides that can restore the corrected splicing, therefore re-establishing synthesis of the normal protein [130–139]. These mutations activate aberrant splice sites, leading to incorrectly spliced mRNAs, even though the correct splice sites remain undamaged and potentially functional. Therefore blocking the aberrant splice sites with antisense oligonucleotides might force the splicing machinery to reselect the existing correct splice sites. Expression of antisense sequences targeted to the aberrant splice sites in thalassemic pre-mRNA has been successful, restoring the correct splicing pattern and ultimately restoring hemoglobin synthesis [133,140]. The therapeutic value of this system was demonstrated in HSCs and erythroid progenitor cells from a patient with IVS2–745/IVS2–1 β -thalassemia. At 12 days after transduction of the patient cells with a lentiviral vector that express a snRNA

targeting the mutant RNA, the levels of correctly spliced β -globin mRNA and adult hemoglobin were approximately 25-fold over background [137]. A similar approach was utilized to restore the correct splicing pattern in a mouse model of IVS2–654 β -thalassemia. This was achieved by a systemic delivery *in vivo* of a splice-switching oligonucleotide, a morpholino oligomer conjugated with an arginine-rich peptide. Repaired β -globin mRNA restored significant amounts of hemoglobin in the peripheral blood of the IVS2–654 mouse, improving the number and quality of erythroid cells [139]. These studies support this approach for the cure of β -thalassemic patients carrying alternative splicing associated mutations.

Another approach that demonstrates great potential is the use of stem cells, which have the capacity to differentiate into various cell types. This unique characteristic suggests the possibility of producing large quantities of cells of a particular type. Their use might lead to the development of cell replacement therapies [102,141–143]. Induced pluripotent stem (iPS) cells have been generated from mouse and human somatic cells by introducing *Oct3/4* and *Sox2* with either *Klf4* and *c-Myc* or *Nanog* and *Lin28* genes using retroviruses and lentiviruses [102,144–146]. More recently, iPS cells have been generated using excisable vectors, nonintegrating vectors and transient transfection approaches [147–151]. iPS cells have been made from a variety of tissues, including skin fibroblasts, hepatocytes, stomach, testicular and neural cells [152–155]. The reprogramming of somatic cells into iPS cells opens a new approach of treating β -thalassemia. Ye and coworkers have shown that iPS cells can be generated from cells derived from skin fibroblasts, amniotic fluid or chorionic villus sampling of patients with β -thalassemia [156]. Subsequently, the iPS cells were differentiated into hematopoietic cells that synthesized hemoglobin. Therefore, in the future, the mutation in the β -globin gene of these iPS cells could be corrected by gene targeting and the cells differentiated into HSCs to be returned to the patient [138,156]. Indeed, sickle cell anemia was cured in this manner in a mouse model [143]. However, there are some obstacles that need to be overcome before iPS treatment of β -thalassemia will be utilized. One of the most pressing problems is elimination of the transcription factors when they are no longer required. Second, it is necessary to reestablish the correct re-programming so that the iPS cells do not develop in tumors. One potential approach to solving the first obstacle is to activate or replace the expression of the transcription factors that are able to reprogram adult cells to iPS using small molecules, which would offer an alternative to retroviral integration that may disturb gene functions. This approach was successfully utilized by Lyssiotis and coworkers, showing that a compound that functionally replaces the reprogramming factor *Klf4* was able, in combination with *Oct4*, *Sox2* and *c-Myc*, to generate iPS cells [157]. Therefore, the identification of additional molecules that could replace the use of introducing *Oct4*, *Sox2*, and *c-Myc* into the patient cells may help to bring the iPS cell technology to clinical application.

Expert commentary

Although the human β -globin gene was one of the first genes ever cloned, β -thalassemia is currently a pressing medical problem and only limited therapeutic options are available for patients affected by this disorder. In the last few years new discoveries and techniques are rapidly leading to novel therapeutic approaches that could completely change the way we treat and cure this disorder.

Five-year view

Pharmacological approaches are being devised to increase the expression of HbF, to limit iron overload and splenomegaly. Gene therapy and reprogramming of adult cells to stem cells might offer an alternative to allogeneic bone marrow transplantation. Altogether, these new approaches might improve the regimen to prevent iron overload and limit or eliminate completely the need for blood transfusion.

Key issues

- Thalassemic patients require sporadic or chronic blood transfusion for survival.
- Patients with severe forms of β -thalassemia suffer from iron overload and develop splenomegaly.
- Iron overload is currently treated with iron chelators.
- Hepcidin, the iron regulator, is not adequately produced and consequently thalassemic patients have increased iron absorption. Administration of hepcidin or agonists of this peptide could offer additional support to decrease the severity of iron overload.
- Splenomegaly, which contributes to the severity of the anemia, necessitates removal of the spleen (splenectomy).
- Use of Jak2 inhibitors might limit or reverse the expansion of the erythron in the spleen, offering an alternative to splenectomy and consequently preserve this organ and its important function.
- In β -thalassemia, other major complications that might develop are thromboembolic events. Use of antioxidants, anticoagulants and preservation of the spleen might limit or prevent this complication.
- Transplantation using allogeneic hematopoietic stem cells is the only current available cure for β -thalassemia. Limited donors, the incidence of graft-versus-host disease and other risk factors associated with the overall health of the patient are the current limitations for this approach.
- New techniques to isolate hematopoietic stem cells and new conditioning regimens of the patients might overcome these problems.
- Gene therapy might also offer an alternative to allogeneic bone marrow transplantation since it would utilize corrected autologous hematopoietic stem cells.
- Reprogramming of adult cells to stem cells and their genetic correction may offer an alternative source of autologous hematopoietic stem cells for transplantation.
- Pharmacological approaches are being devised to increase the expression of fetal hemoglobin or correct the mutated β -globin mRNA. These, and the aforementioned approaches, if successful, would prevent or limit the need for blood transfusion.

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Figure 1. List of potential beneficial effects using (A) Jak2 inhibitors or (B) agonist of the hepcidin protein

Figure 2. Gene therapy and stem cell therapy approach

Mediated, respectively, by (A) gene transfer into hematopoietic stem cells and (B) reprogramming of adult cells to stem cells.

iPS: Induced pluripotent stem cell.

Table 1
List of potential therapies to improve the management of β -thalassemia

Problem	Potential therapy
Anemia	Reactivation of fetal hemoglobin Correction of the aberrant splicing Ribosomal readthrough for premature termination codons Gene therapy Generation of induced pluripotent stem cells and correction of the genetic defect(s)
Iron overload	Iron chelation supported by hepcidin agonists
Splenomegaly	Use of Jak2 inhibitors
Thrombosis	Antioxidants and/or anticoagulants