

## 2017 Clinical trials update in new treatments of $\beta$ -thalassemia

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The underlying basis of  $\beta$ -thalassemia pathology is the diminished  $\beta$ -globin synthesis leading to  $\alpha$ -globin accumulation and premature apoptotic destruction of erythroblasts, causing oxidative stress-induced ineffective erythropoiesis, bone marrow hyperplasia, splenomegaly, and increased intestinal iron absorption with progressive iron overload. Better understanding of the molecular mechanisms underlying this disease led to the recognition of new targets with potential therapeutic utility. Agents such as JAK2 inhibitors and TGF- $\beta$  ligand traps that reduce the ineffective erythropoiesis process are already being tested in clinical trials with promising results. Other agents that aim to reduce oxidative stress (activators of Foxo3, HRI-eIF2aP, Prx2, Hsp70, and PK anti-oxidant systems and inhibitors of HO-1) and to decrease iron overload (hepcidin agonists, erythroferrone inhibitors and exogenous transferrin) are also under experimental investigation. Significant progress has also been made in the area of allogeneic hematopoietic stem cell transplantation with several ongoing clinical trials examining new condition regimens as well as different donor selection and stem cell source options. Gene therapy has reached a critical point and phase 1 clinical trials have recently been launched to examine the effectiveness and especially long term safety. Epigenetic manipulation and genomic editing of the  $\gamma$ - or  $\beta$ -globin gene are novel and promising experimental gene therapy approaches for  $\beta$ -thalassemia giving hope for cure for this chronic disease. This review outlines the key points of the molecular mechanisms underlying  $\beta$ -thalassemia in relation to the development of new therapies and an update is given both at the pre-clinical and clinical level.

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### ■ Introduction

Beta-thalassemia ( $\beta$ -thalassemia) is an autosomal recessive hereditary hemoglobinopathy that affects the production of the  $\beta$ -globin chains of the hemoglobin. The defect in the  $\beta$ -globin gene on chromosome 11 leads to impaired production of  $\beta$ -globin chains and accumulation of excess  $\alpha$ -globin chains that form insoluble hemichromes. These iron-containing bodies generate the production of reactive oxygen species (ROS) that oxidize cell membrane proteins and lipids. The oxidative damage leads to cell lysis and premature apoptosis of the erythroblasts and to inhibition of the final stage of erythroid differentiation and maturation. Altogether, this process leads to ineffective erythropoiesis (Fig. 1) [1,2].

Ineffective erythropoiesis in combination with peripheral hemolysis leads to anemia, hypoxia and reactive increase of erythropoietin (EPO) production with subsequent hyperplasia of the bone marrow and extramedullary hematopoiesis with hepatosplenomegaly. EPO acts through its receptor (EPOR) via multiple signaling pathways mainly the JAK2/STAT5 (Janus kinase 2/signal transducer and activator of transcription5) as well as the p13K/AKT/mTOR (phosphoinositide 3-kinase inhibitor/AKT/mammalian target of rapamycin) and RAS-MAPK (rat sarcoma/mitogen-activated protein kinases) system [3]. Increased, yet inefficient activity of erythroblasts induces the release of factors, such as GDF15 (growth and differentiating factor 15), TWSG1 (twisted gastrulation protein homolog 1), HIF (hypoxia-inducible factor), and ERFE (erythroferrone) which inhibit hepcidin, the major regulator of iron homeostasis. Low hepcidin levels promote intestinal iron absorption and release from macrophages, leading to progressive tissue iron overload of the parenchymal cells. Free iron hinders erythropoiesis creating a vicious cycle between inefficient erythropoiesis and increased iron absorption and release [4–9] (Fig. 1).

Beta-thalassemia major ( $\beta$ -TM) occurs when two defective  $\beta$ -globin genes result in severe decrease in  $\beta$ -globin production with life-long transfusion-dependent anemia. Patients who are homozygous or double heterozygotes for mutations in the  $\beta$ -globin gene that lead to mild or moderate decrease in  $\beta$ -globin production have  $\beta$ -thalassemia intermedia ( $\beta$ -TI), and display varying degrees of anemia, requiring occasional transfusions.  $\beta$ -TI covers the spectrum between heterozygous  $\beta$ -thalassemia and  $\beta$ -TM [10].

Given the clinical diversity, medical management of  $\beta$ -thalassemia is tailored to each patient's profile and is mainly focused on the amelioration of anemia, control of iron overload and its consequences. Chelating agents, such as desferrioxamine, deferiprone and deferasirox are used for the treatment of iron overload [11–13]. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is the only definitive cure for transfusion dependent young patients before the development of iron-related tissue damage [14].

In parallel, effort has been put on the development of novel therapies for the treatment of anemia and iron overload that are based on the correction of the pathophysiological mechanisms of the disease. In order to restore the balance between the  $\alpha$ - and non- $\alpha$ -chains of hemoglobin, agents that induce the production of  $\gamma$ -chains, such as hydroxycarbamide, short-chain fatty acids, 5-azacytidine and thalidomide, have been tested

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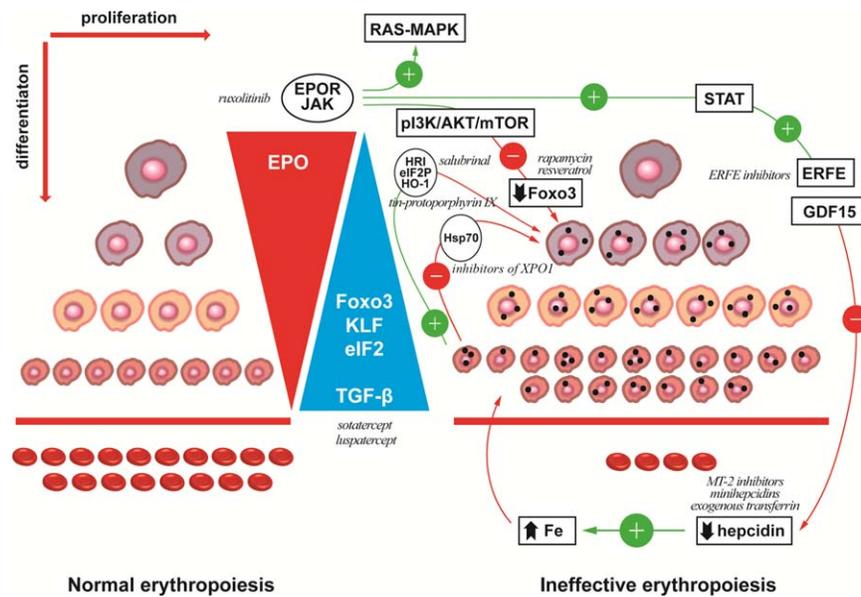
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**Figure 1.** Normal and ineffective erythropoiesis. Normal erythropoiesis: The first phase of colony forming unit-erythroid (CFU-E) differentiation is highly erythropoietin (EPO)-dependent, whereas later stages are no longer dependent on EPO. In contrast, factors such as transforming growth factor- $\beta$  (TGF- $\beta$ ) family, Krüppel-like factor 1 (KLF1), eukaryotic initiation factor 2 (eIF2) and Forkhead box O3 (Foxo3) are mostly expressed at the final stages and are crucial for the normal termination of erythropoiesis. Ineffective erythropoiesis: In  $\beta$ -thalassemia the intracellular accumulation of insoluble hemichromes promotes oxidative stress, cell membrane damage and apoptosis of the erythrocyte progenitor cells. Ineffective erythropoiesis combined with peripheral hemolysis lead to anemia, hypoxia and increased- through Janus kinase 2/signal transducer and activator of transcription (JAK2/STAT) pathway - EPO production with subsequent expansion of early erythroid precursors and erythroid progenitors which produce factors such as growth and differentiating factor 15 (GDF15) and erythroferone (ERFE). These factors inhibit the production of hepcidin, the major regulator of iron hemostasis, and increase the intestinal absorption and macrophage release of iron contributing to tissue iron overload. The presence of excess iron in the bone marrow has a negative effect on erythropoiesis generating a vicious cycle between ineffective erythropoiesis and iron overload. The oxidative stress activates the HRI-eIF2-ATF4 (heme-regulated kinase-eIF2-activating transcription factor 4) pathway in order to inhibit protein synthesis, including that of  $\alpha$ -globin. HO-1 (heme oxygenase-1) expression increases in response to stress and could possibly increase the iron release from free heme and contribute to tissue damage. The presence of hemichromes inactivates the Hsp70 (heat shock protein 70) system, which is involved in protein folding and cell protection from stress and this contributes to the end-stage maturation arrest and apoptosis. JAK2/STAT5 pathway: During the binding of EPO to EPOR, dimerization of the superficial regions of the receptor is caused and triggers the autophosphorylation and activation of JAK2 kinase, the phosphorylation of EPO receptor (EPOR) tyrosine residues which lead to the activation of STAT transcriptional factors, and the initiation of RAS-MAPK (rat sarcoma/mitogen-activated protein kinases) and PI3K/AKT/mTOR (phosphoinositide 3-kinase inhibitor/AKT/mammalian target of rapamycin) pathways. The PI3K/AKT/mTOR pathway inactivates the antioxidant factor Foxo3 contributing to oxidative damage in late erythroblasts. In italics are the relevant novel agents that are in preclinical or clinical development. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

and showed partial responses but without long term beneficial results [15–18]. Induction of erythropoiesis has also been attempted by administering recombinant human EPO with varying results [19,20].

Over the last few years, a better understanding of the mechanism of ineffective erythropoiesis, iron overload and the transition of fetal hemoglobin (HbF) to adult hemoglobin (HbA) has resulted in the emergence of promising novel therapeutic agents that are being tested both in preclinical and clinical level. Furthermore, amelioration of allo-HSCT techniques regarding the conditioning regimen, donor selection and hematopoietic stem cell source are currently under evaluation in clinical trials. The effectiveness and long term safety of gene therapy with  $\beta$ -globin addition is also under investigation and new experimental gene therapy methods, such as genome editing, have recently emerged. The aim of this review is to present the novel therapeutic approaches that are on preclinical development and to provide a clinical trial update on the emerging treatments on  $\beta$ -thalassemia.

## ■ New Therapeutic Approaches in Preclinical Development For $\beta$ -Thalassemia (Table I)

### Modulators of erythropoiesis

On the basis of the extended knowledge on the field of murine erythropoiesis, several agents have recently emerged and tested, especially in models of  $\beta$ -TI mice. Although promising, the findings of

these studies need careful interpretation, given the well documented variations in globin gene composition and erythroid differentiation between human and mouse [21].

**Foxo3 activation.** Foxo3 (forkhead box O3) is a transcription factor that protects the cell from oxidative stress by upregulating antioxidant enzymes. During the early stages of erythropoiesis, Foxo3 is phosphorylated by proteins of the EPOR-pi3K/AKT/mTOR signaling pathway and is translocated out of the nucleus where it remains inactivated. In the late phase of erythropoiesis, the increased production of hemoglobin and the presence of iron lead to the production of ROS, which induce oxidative stress and could potentially block the normal termination of the process. At this point, Foxo3 is relocated in the nucleus and its activation leads to the production of antioxidants that neutralize ROS and allow the uneventful ending of erythropoiesis [22,23].

In  $\beta$ -TI mice, Foxo3 has been found to be downregulated due to persistent activation of EPOR-pi3K/AKT/mTOR pathway. The inactivation of Foxo3 leads to oxidative damage in late erythroblasts and plays a significant role in the process of ineffective erythropoiesis (Fig. 1) [24]. Activation of Foxo3 could be beneficial in improving anemia in  $\beta$ -thalassemia.

The use of rapamycin, an mTOR inhibitor, has been tested in  $\beta$ -TI mice and remarkably improved erythroid cell maturation,  $\beta$ -globin production and anemia through Foxo3 activation [24]. In another study, rapamycin increased  $\gamma$ -globin mRNA expression and HbF production in cultured erythroblasts from  $\beta$ -TI patients [25]. Another Foxo3 activating agent is resveratrol (3,5,4'-trihydroxy-trans-stilbene), a non-flavonoid polyphenol that up-regulates antioxidant enzymes.

TABLE I. Novel Therapeutic Approaches in Preclinical Development for  $\beta$ -Thalassemia

Drug	Mechanism	Material	Reference	Institution/Developer
Rapamycin	mTOR inhibitor Antioxidant effect Foxo3 activation	Erythroid progenitors from $\beta$ -TI patients	Zhang X, et al. American journal of Hematology 2014;89:954-963 27	Icahn School of Medicine at Mount Sinai, New York
		Thalassemic (th3/+) mice study	Pecoraro A, et al. Hemoglobin 2015;39:225-229 34	Dipartimento di Oncologia ed Ematologia, "Ospedali Riuniti Villa Sofia-Cervello", Palermo, Italy
Resveratrol	Antioxidant effect Foxo3 activation	Thalassemic (th3/+) mice study	Franco SS, et al. Haematologica 2014;99:267-275 35	Dept. of Medicine, University of Verona, Verona, Italy
Salubrinol	Inhibition of eIF2aP dephosphorylation Hemichromes reduction HbF induction	Erythroid progenitors from $\beta$ thalassemic (th3/+) mice	Suragani RN, et al. Blood 2012;119:5276-5284 39	Harvard-MIT Division of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA
		Differentiating human CD34+ cells	Hahn CK, Lowrey CH. Blood 2014;124:2730-2734 41	Department of Pharmacology and Toxicology, Geisel School of Medicine at Dartmouth, Hanover, NH
PEP1-Prx2 fusion protein	Activation of Erk signaling pathway	(Prx2-/-) mouse model	Mattè A, et al. 57th American Society of Hematology (ASH) Annual Meeting; 2015 # 406 44	Department of Medicine, University of Verona, Italy
tin-protoporphyrin IX	HO-1 inhibitor, free heme reduction	Thalassemic (th3/+) mice study	Santos DG, et al. 57th ASH Annual Meeting; 2015 # 337346	Lady Davis Institute for Medical Research, Montreal, Canada
Leptomycin B KPT 251	XPO1 inhibitors Improvement of erythroid terminal differentiation	Erythroid progenitors from $\beta$ -TM patients	Guillem F, et al. 57th ASH Annual Meeting; 2015 # 2368 50	Laboratoire INSERM, Paris, France
AG-348	Pyruvate kinase activation	Thalassemic (th3/+) mice study	Mattè A, et al. 21st EHA Congress; 2016 # S135 53	Department of Medicine, University of Verona, Italy
Exogenous transferrin	TfR1 down-regulation Improvement of erythroid terminal differentiation and hepcidin production	Thalassemic (th3/+) mice study	Li H, et al. 57th ASH Annual Meeting; 2015 # 754 70	New York Blood Center and Weill Cornell Medical College, New York, US
ISIS-TMPRSS6-LRx	Anti-TMPRSS6 antisense oligonucleotide hepcidin up-regulation	Thalassemic (th3/+) mice study, normal monkeys	Guo S, et al. 57th ASH Annual Meeting; 2015 # 753 67	Isis Pharmaceuticals
Minihepcidins	Hepcidin up-regulation	Thalassemic (th3/+) mice	Casu C, et al. 55th ASH Annual Meeting; 2013 # 431 69	Weill Cornell Medical College, New York, NY
ERFE-deficiency	Hepcidin up-regulation	Thalassemic (th3/+) mice	Kautz L, et al. Blood. 2015;126:2031-7 6	Department of Medicine and Silarus Therapeutics, La Jolla, CA. David Geffen School of Medicine, University of California
Lentivirus carrying sequences of microRNAs that inhibit BCL11A	HbF induction	Erythroid progenitors from $\beta$ -TI patients	Lulli V, et al. PloS one 2013;8:e60436 56	Istituto Superiore di Sanità, Rome, Italy
		Erythroid progenitors from $\beta$ -TI patients	Guda S, et al. Mol Ther. 2015;23:1465-74 57	Boston Children's Hospital, Boston, Massachusetts, US
Antisense oligonucleotide BCL11A inactivator	HbF induction	Erythroleukemia series	Peralta R, et al. 55th ASH Annual Meeting. 2013 # 1022 58	Isis Pharmaceuticals
ZFN-driven (GG1-VP64) activation of the promoter of $\gamma$ -globin gene	HbF induction	Transgenic mice	Costa FC, et al. Anemia 2012;2012:507894 54	Department of Biochemistry and Molecular Biology, University of Kansas Medical Center
ZFN-driven BCL11A enhancer ablation	HbF induction	CD34+ cells from $\beta$ -TM patients	Urnov FD, et al. 57th ASH Annual Meeting; 2015 # 204 62	Sangamo BioSciences, Richmond, CA University of Washington, Seattle, WA Biogen, Cambridge, MA George Papanicolaou Hospital, Thessaloniki, Greece
CRISPR-Cas9-mediated BCL11A enhancer inactivation	HbF induction	Human adult-stage erythroid cell line	Bauer DE, et al. 57th ASH Annual Meeting; 2015 # 638 63	Boston Children's Hospital, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA
TALEN	$\beta$ -gene correction	Induced pluripotent stem cells (iPSCs) from thalassemic patients	Ma N, et al. The Journal of Biological Chemistry 2013;288:34671-34679 64	Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China

The use of resveratrol in a murine  $\beta$ -TI model resulted in amelioration of erythrocyte survival, increased hemoglobin levels and reduced reticulocyte count [26]. On the contrary, a recent study in double mutant Foxo3<sup>-/-</sup>/Th3<sup>+</sup> mice, showed that the loss of Foxo3 lead to improved early erythropoietic activity [27]. Clearly, further laboratory and clinical investigation is needed in this field.

**HRI-eIF2 $\alpha$ P stimulation.** eIF2 (eukaryotic initiation factor 2) is a factor required for the initiation of translation through the binding of tRNA to the ribosomes. In the erythroid precursors, eIF2 activity is regulated by a mechanism involving phosphorylation at its  $\alpha$ -subunit (eIF2 $\alpha$ P) by heme-regulated eIF2 $\alpha$  kinase (HRI). HRI acts as a result of stress or heme deficiency [28] and coordinates heme and globin synthesis in order to prevent excess globin translation in excess of heme. The expression of HRI increases during the late stage of erythroid differentiation and is important for the uneventful termination of erythropoiesis. Furthermore, eIF2 $\alpha$ P activates ATF4 (activating transcription factor 4) to diminish oxidative stress in nucleated erythroblasts. The dephosphorylation of eIF2 $\alpha$  and the regeneration of active eIF2 $\alpha$  is necessary for the recovery of protein synthesis of stress-induced gene expression [29,30].

HRI has been found increased in a mouse model of  $\beta$ -TI, indicating a protective role by inhibiting protein synthesis, including that of  $\alpha$ -globin. HRI deficiency has been shown to lead to embryonically lethal  $\beta$ -thalassemia [31]. Pharmacological enhancement of the HRI-eIF2 $\alpha$ P-ATF4 pathway could be beneficial in  $\beta$ -thalassemia. Salubrinal, a selective inhibitor of eIF2 $\alpha$ P dephosphorylation, has been tested in  $\beta$ -thalassemic erythroid precursors and has been found to augment the HRI signaling pathway and to reduce the production of hemichromes [30]. It has also been shown to increase HbF production with a concomitant decrease of HbA in differentiating human CD34<sup>+</sup> cells by a post-transcriptional mechanism [32]. These findings provide the basis for manipulating the HRI-eIF2 $\alpha$ P signaling pathway for the treatment of  $\beta$ -thalassemia.

**Prx2 activation.** Prx2 (peroxiredoxin-2) is an essential antioxidant protein that scavenges and inactivates ROS throughout the process of erythropoiesis ensuring its normal termination. Prx2 has been found upregulated during both murine and human  $\beta$ -thalassemic erythropoiesis [33]. The absence of Prx2 in mouse models worsens anemia and iron overload promoting a severe liver oxidative stress and down-regulation of hepcidin production by switching off Tfr2 (transferrin receptor 2) expression and Erk (extracellular-signal-regulated kinases) pathway. The use of a PEP1-Prx2 fusion protein reversed these phenomena with activation of Erk signaling pathway towards Tfr2 and the Sma and Mad (SMAD) system [34,35]. Modulation of Prx2 in  $\beta$ -thalassemia could provide a useful tool for the development of pharmacological agents targeting the amelioration of ineffective erythropoiesis and iron overload.

**HO-1 inhibition.** HO-1 (heme oxygenase-1) is an enzyme that catalyzes the degradation of heme in response to stress such as oxidative stress or hypoxia, both occurring in  $\beta$ -thalassemia [36]. The expression of HO-1 has been found especially augmented in EPO-dependent fetal liver erythropoietic cells from  $\beta$ -thalassemic mice fetuses, demonstrating the potential role of HO-1 in the mechanism of cellular damage of thalassemic erythroblasts. In the same mouse model, the administration of tin-protoporphyrin IX (SnPP), an HO-1 inhibitor, significantly improved ineffective erythropoiesis, decreased spleen size and liver iron and increased hemoglobin levels [37]. This approach of HO-1 induced iron damage of  $\beta$ -thalassemic erythroblasts is a very promising field for further research.

**Hsp70 chaperone machinery induction.** Hsp70 (heat shock protein 70) is a molecular chaperone needed for the normal termination of the late phase of erythropoiesis [38]. Hsp70 is most predominant in the human erythroblasts at late stages of maturation and is translocated to the nucleus, where it protects GATA-1 (globin transcription

factor-1), the principal transcriptional factor for erythropoiesis, from caspase-3 cleavage [39]. In  $\beta$ -TM erythroblasts, the presence of hemichromes leads to HSP70 sequestration in the cytoplasm leaving GATA-1 unprotected from caspase-3 cleavage, resulting in end-stage maturation arrest and apoptosis [40]. Exportins, such as XPO1, are factors that control the nucleocytoplasmic trafficking of proteins and RNAs. Inhibitors of XPO1 such as leptomycin B and KPT 251 have recently been tested in erythroid progenitors from  $\beta$ -TM patients. The most striking finding was the induction of HSP70 nuclear localization and the GATA-1 expression that resulted in improved erythroid terminal differentiation [41]. Understanding the underlying mechanisms that regulate HSP70 trafficking during erythroid differentiation may help to find new therapeutic agents to reduce ineffective erythropoiesis in  $\beta$ -thalassemia.

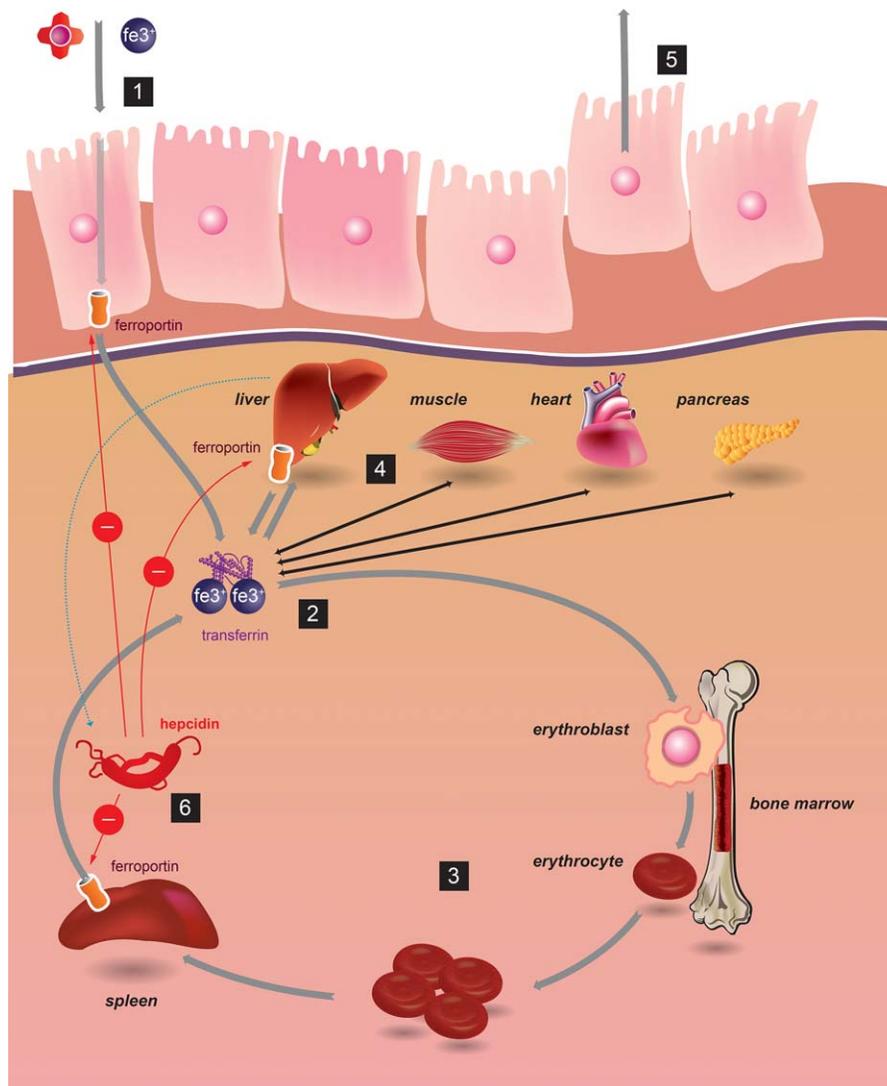
**Pyruvate kinase activation.** Pyruvate kinase (PK) is the enzyme that catalyzes the final step of glycolysis, the conversion of phosphoenolpyruvate to pyruvate. PK deficiency patients have signs of ineffective erythropoiesis [42]. The administration of the PK activator AG-348 has been shown to increase ATP in healthy subjects [43] and it currently in phase 2 studies in PK deficiency patients (NCT02476916). The same agent has been recently tested in  $\beta$ -TI mice and resulted in reduction of ROS production, amelioration of ineffective erythropoiesis as well as in decrease of liver iron overload and up-regulation of hepcidin [44].

## Iron metabolism manipulation

Iron homeostasis is a tightly regulated mainly by hepcidin, a protein produced in the liver and encoded by the HAMP (hepcidin antimicrobial peptide) gene. Brush border cells of the intestine, macrophages of the reticuloendothelial system and the hepatocytes are the target cells for hepcidin. Hepcidin degrades ferroportin, a transmembrane protein for iron transportation from intracellular to extracellular space. Consequently, it inhibits the iron absorption from the intestine and the release of iron stored in macrophages and hepatocytes [45] (Fig. 2). Hepcidin production is reduced in case of increased need for iron, such as in iron deficiency anemia, hypoxia and increased erythropoiesis. Conversely, hepcidin production in the liver is increased when iron storage is saturated to stop the iron absorption from the intestine and its release from the macrophages. Hepcidin is also increased during infections so as to avert microorganisms from using iron. In chronic inflammatory conditions, the stimulation of hepcidin production leads to iron deprivation and contributes to the anemia of chronic disease [46–48] (Fig. 3).

In  $\beta$ -thalassemia enhanced, yet inefficient activity of erythroblasts induces the release of factors (GDF15, TWSG1, HIF, and ERFE) that inhibit hepcidin [4–9]. As a consequence, iron absorption from intestine and release from macrophages are increased and lead to gradual iron overload in parenchymal organs. Thus, administration of hepcidin or factors inducing its expression could exert a positive impact on the improvement or prevention of iron overload. Hepcidin could also improve the ineffective erythropoiesis [49]. This hypothesis has been tested in murine models with  $\beta$ -TI. The inhibition of matriptase 2 (MT-2), a key regulator of hepcidin production, with silencing RNAs or antisense oligonucleotides resulted in increase of hepcidin and improvement of anemia and iron overload [50–53]. Comparable results derived with the use of minihepcidins, which are small peptides that mimic hepcidin activity and act as agonists [54]. Hepcidin production can also be increased with the use of exogenous transferrin through the down regulation of TFR1, leading to amelioration of iron overload and improvement of erythroid nucleation and terminal differentiation [55,56]. On the contrary, the absence of the other transferrin receptor, TFR2, on the erythroid lineage results in decreased hepcidin expression, possibly due to high ERFE production [57].

ERFE is a recently discovered erythroid suppressor of hepcidin produced in erythroblasts in response to EPO through the JAK2/



**Figure 2.** Iron recycling and homeostasis. 1. Only 10% of iron from food is absorbed (1-2 mg/day), mainly by duodenum and initial part of jejunum. The absorbed iron exits the intestinal epithelial cells through ferroportin, a transmembrane protein for iron transportation from intracellular to extracellular space 2. In blood circulation iron is bound to transferrin (3 mg). 3. Next, iron is used in the bone marrow for erythropoiesis (300 mg). The largest amount of iron is found in the circulating erythrocytes (>2 g) and it is recycled by the macrophages in the spleen (600 mg) which phagocyte the aged erythrocytes. Iron exits the spleen macrophages through ferroportin. 4. Excess iron is stored in the hepatocytes bound to ferritin (1 gr) and, in case of increased needs, it is released through ferroportin. Erythropoiesis is an exceptionally dynamic process leading on a daily basis to the production of  $2 \times 10^{11}$  new erythrocytes which show a life span of average 120 days. Daily needs of erythropoiesis for iron are about 20–25 mg. 5. However, daily absorption of food-derived iron (1–2 mg) is counterbalanced by its passive loss (1–2 mg, cellular apoptosis from skin or gastrointestinal tract, secretion through sweat or urine and blood loss during menstruation) and is not adequate for erythropoiesis. Thus, the tight regulation of iron recycling and homeostasis is absolutely necessary. 6. Hepcidin is the central regulator of iron homeostasis by inhibiting ferroportin and iron export from enterocytes, hepatocytes and tissue macrophages. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

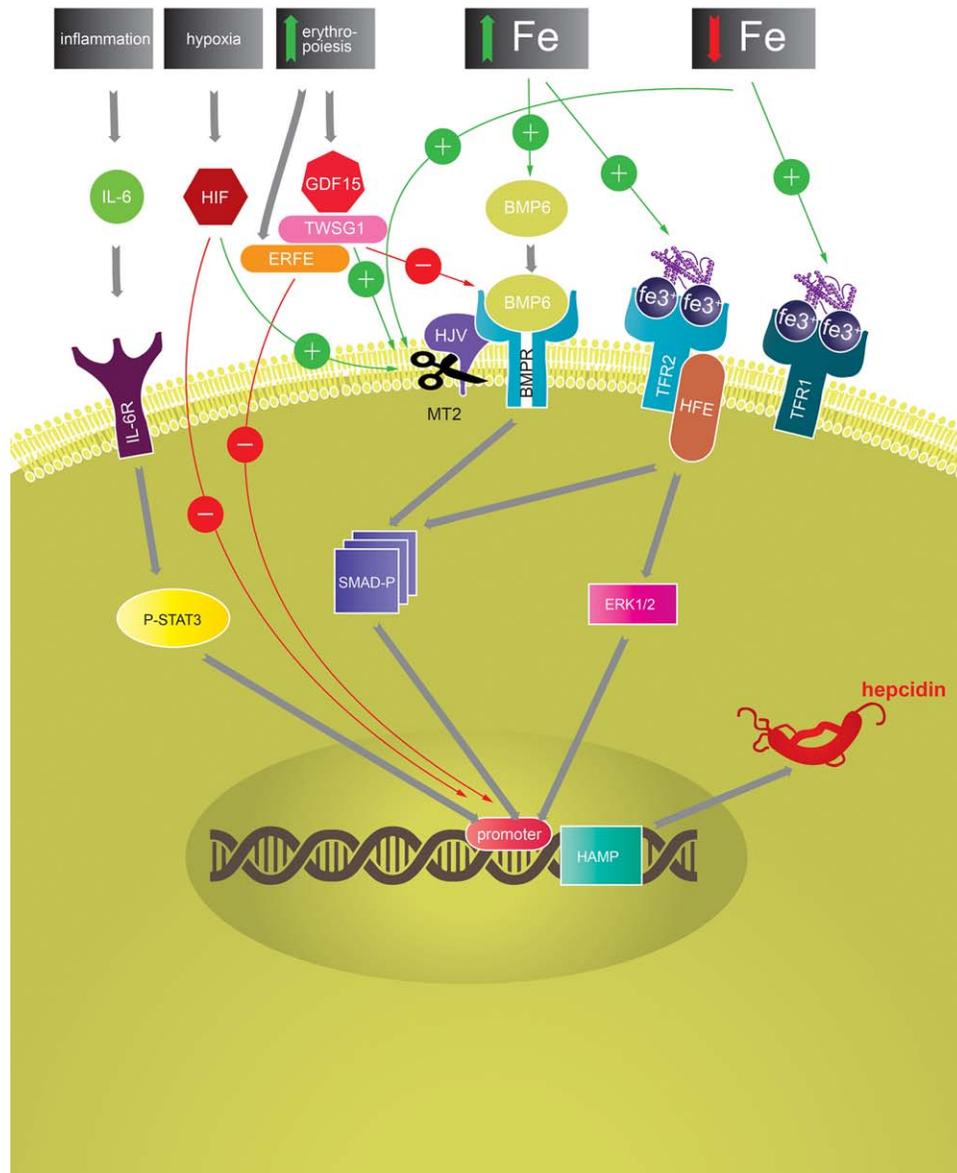
STAT signaling pathway [5]. ERFE has been found highly expressed in murine models with  $\beta$ -TI, whereas ERFE-deficiency resulted in increased expression of hepcidin, significant reduction in iron overload and slight amelioration of erythropoietic indices [6]. These findings indicate a role of ERFE inhibition as future target with therapeutic potential in diseases with iron overload and ineffective erythropoiesis, such as  $\beta$ -thalassemia.

### Gene therapy

Gene manipulation with the aim of therapy in  $\beta$ -thalassemia has been extensively investigated in several experimental systems. In vitro studies with lentivirus vectors in erythroid precursor cells from thalassemic patients and in thalassemic mouse models have focused on  $\beta$  or  $\gamma$ -globin addition, overexpression of endogenous  $\gamma$ -globin-activating transcription factors, silencing of  $\gamma$ -globin repressors, such as BCL11A (B-cell lymphoma/leukemia 11A) and genome editing of  $\beta$ -globin

mutations or  $\gamma$ -globin repressors. Several experimental methods are being studied in preclinical level with promising findings.

Overexpression of endogenous  $\gamma$ -globin has been induced in CD34+ cells from thalassemia patients with the use of lentiviral vectors expressing a zinc finger protein (GG1-VP64) which interacts with the promoter of  $\gamma$ -globin gene [58,59]. Silencing of  $\gamma$ -globin repressors, such as BCL11A, with lentiviruses carrying sequences of microRNAs has been shown to increase HbF production [60,61]. Furthermore, in vitro administration of antisense oligonucleotides inhibiting BCL11A has shown increase of HbF in an erythroleukemia series expressing BCL11A and KLF1 (Krüppel-like factor 1) [53,62]. KLF1 is the central regulator of the final differentiation of erythroid cells, the cell membrane and cytoskeleton formation, the iron metabolism and hemoglobin production. It also activates the expression of  $\beta$ -globin gene and plays a role in the transcriptional silencing of the  $\gamma$ -globin gene, possibly through BCL11A factor [63,64].



**Figure 3.** Regulation of hepcidin production by hepatocytes. A. Regulated by iron levels. In case of adequate iron levels, protein related to hemochromatosis (HFE, High iron Fe) is bound to transferrin receptor 2 (Tfr2) and hepcidin gene transcription is then induced through Erk (extracellular-signal-regulated kinases) and SMAD (Sma and Mad related proteins) pathways whereas protein BMP6 (bone morphogenetic protein 6) is increased contributing to the hepcidin production through SMAD cascade. In case of iron deficiency, however, hepcidin is inhibited due to HFE binding to transferrin receptor 1 (Tfr1) and Erk-SMAD pathway inhibition. Furthermore, BMP6 is reduced due to matriptase 2 (MT2) protein activation. MT2 destroys hemojuvelin (HJV) which is a co-receptor of BMP6 and consequently hinders its inductive activity on the transcription of hepcidin gene. B. Regulated by increased erythropoietic activity. Production of soluble factors (GDF-15: growth differentiation factor 15, and TWSG1: twisted gastrulation protein homolog 1 and ERFE: erythroferrone) by proliferating erythroblasts blocks transcription of hepcidin gene. GDF-15 and TWSG1 act by inhibiting BMP6 or inducing MT2 while ERFE employs a BMP6-SMAD independent mechanism. C. Regulated by oxygen levels. Hypoxia induces HIF (hypoxia induced factors) transcription factors which directly inhibit hepcidin gene promoter or act indirectly by activating MT2. D. Regulated by inflammation. Contribution of inflammation to hepcidin is attributed to cytokines, such as interleukin-6 (IL-6) and IL-1. Cytokines activate hepcidin gene promoter by activating the STAT (Signal Transducer and Activator of Transcription) pathway. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

The promoter of BCL11A is the target of genome editing, with the use of several nucleases such as engineered zinc fingered nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats linked to Cas9 nucleases (CRISPR-Cas9) [65]. It has been shown recently, that the ZFN-driven BCL11A enhancer ablation leads to increased

production of HbF in CD34+ cells from patients with  $\beta$ -thalassemia, which could be the source for autologous transplantation in these patients [66]. Similar effect has been achieved with the CRISPR-Cas9-mediated BCL11A enhancer inactivation in a human adult-stage erythroid cell line [67]. Apart from the  $\gamma$ -globin gene induction, genomic editing approaches have the ability to modify  $\beta$ -globin gene. TALEN-

**TABLE II.** Currently Planned, Ongoing, or Recently Completed Clinical Trials of Novel Therapeutics in  $\beta$ -Thalassemia

Drug	Mechanism	Route	Phase	ClinicalTrials.gov	Status	Institution/Developer
Ruxolitinib (INC424)	JAK inhibition	Oral	2	NCT02049450	Open	Novartis Pharmaceuticals
Sotatercept (ACE-011)	Ligand trap TGF- $\beta$ superfamily	Subcutaneous	2	NCT01749540	Active not recruiting participants	Acceleron Pharma, Celgene Corporation
Luspatercept (ACE-536)	Ligand trap TGF- $\beta$ superfamily	Subcutaneous	2	NCT01749540	Active not recruiting participants	Acceleron Pharma, Celgene Corporation
			2, extension study	NCT02268409	Active not recruiting participants	
HSCT	HSCT following reduced intensity conditioning	Matched sibling donor	3 1, 2	NCT02604433 NCT00920972 NCT01050855 NCT02435901	Open	US, Canada
		Family-related or cord-blood matched donor	2	NCT00408447	Open	Columbia University, US
HSCT	Matched unrelated HSCT		2	NCT01049854	Open	New York Medical College
HSCT	Nonmyeloablative haploidentical HSCT	Haploidentical transplants	1,2	NCT00977691	Open	National Heart, Lung, and Blood Institute (NHLBI)
HSCT	Nonmyeloablative peripheral blood mobilized HSCT	Allogeneic peripheral blood stem cell	1,2	NCT02105766	Open	National Heart, Lung, and Blood Institute (NHLBI)
HSCT	Unrelated umbilical cord blood following HLA-haploidentical HSCT	Umbilical cord stem cells	1	NCT02126046	Open	Nanfang Hospital of Southern Medical University
HSCT CordIn™	Umbilical cord blood-derived ex vivo expanded stem and progenitor cells	Ex vivo umbilical cord stem cells	1	NCT02504619	Open	Gamida Cell Ltd
LentiGlobin BB305 vector	$\beta$ -globin gene addition	Ex vivo autologous CD34+ stem cell transduction	1	NCT02151526	Open	bluebird bio, France
			1/2	NCT01745120	Open	bluebird bio, Northstar Study
TNS9.3.55 Lentiviral Vector	$\beta$ -globin gene addition	Ex vivo autologous CD34+ stem cell transduction	1	NCT01639690	Ongoing, but not recruiting participants	Memorial Sloan Kettering Cancer Center, US
GLOBE Lentiviral Vector	$\beta$ -globin gene addition	Ex vivo autologous CD34+ stem cell transduction	1	NCT02453477	Open	IRCCS San Raffaele, Italy

mediated gene correction has been used in induced pluripotent stem cells (iPSCs) from thalassemic patients [68].

### ■ Currently Planned, Ongoing, or Recently Completed Clinical Trials of Novel Therapeutics in B-Thalassemia (Table II)

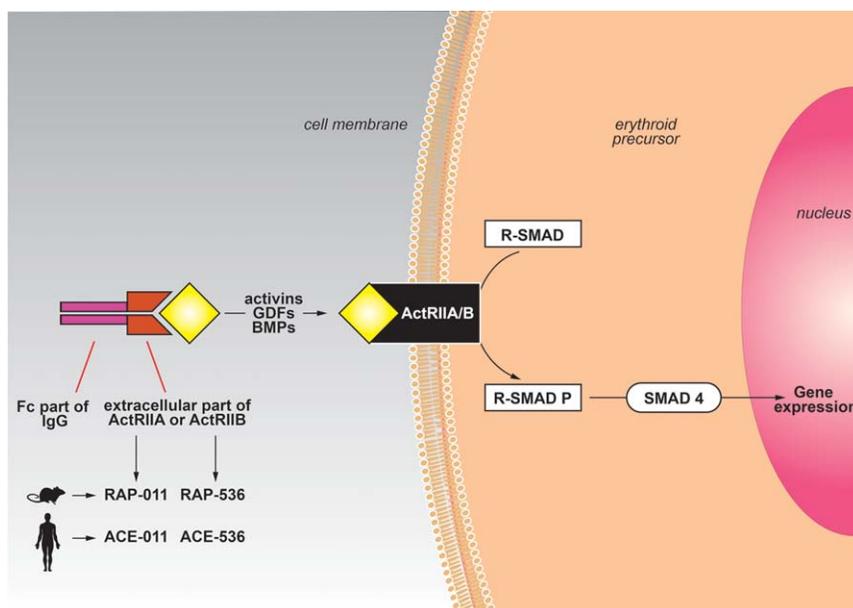
#### JAK2 inhibitors

EPO interacts with its receptor EPOR on the surface of the progenitor cells of the erythroid lineage inducing their proliferation, final differentiation and prevention from apoptosis. Erythrocyte production and maintenance in number is regulated by the response mechanism to hypoxia through HIF and the EPO-EPOR interaction.

Hypoxia can be the consequence of anemia and in this case iron absorption and release has to be increased for hemoglobin production. For this purpose, low blood concentration of oxygen induces the action of the HIF transcriptional factors. These factors consist of  $\alpha$  (HIF-1 $\alpha$ , HIF-2 $\alpha$ , HIF-3 $\alpha$ ) and  $\beta$  subunits (HIF-1 $\beta$ , HIF-2 $\beta$ , HIF-3 $\beta$ ) and are regulated by hydroxylation of an unstable  $\alpha$  subunit

which drives them in degradation under normal conditions. Prolyl-hydroxylases are involved in the hydroxylation process and their action depends on oxygen and iron levels. The activity of prolyl-hydroxylases in case of hypoxia or iron deficiency is inhibited resulting not only in the non-degradation of HIF factors but also in their stabilization, aggregation and transportation into the nucleus of the cell. There, they bind to certain gene promoters which are called hypoxia responsive elements (HRE) [69]. Among the genes which contain HRE sequences in their promoters is the gene of EPO and other genes which regulate iron homeostasis such as the hepcidin gene (HAMP), the DMT-1 (divalent metal transporter 1) gene, the SLC11A2 (solute carrier family 11, member 2) gene, the matriptase 2 gene (TMPRSS6, transmembrane protease, serine 6), and the transferrin (TF) gene [70]. The activation of EPO gene promoter induces the production of EPO in the kidneys. EPO acts through its receptor EPOR via multiple signaling pathways including the JAK2/STAT5, pI3K/AKT/mTOR and RAS-MAPK [3].

In  $\beta$ -thalassemia, ineffective erythropoiesis combined with peripheral hemolysis lead to anemia, hypoxia and increased, through JAK2/STAT5 pathway, EPO production. The central role of JAK2 kinase in this process has formulated the hypothesis that the administration of JAK2 inhibitors might have a positive impact on the prevention of the disease



**Figure 4.** TGF- $\beta$  signaling pathway and its ligand traps. TGF- $\beta$  signaling pathway: The transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily ligands such as activins, growth and differentiating factors (GDFs) and bone morphogenetic proteins (BMPs) bind to a type II receptor (ActRIIA, ActRIIB). This receptor phosphorylates receptor-regulated Sma and Mad related proteins (R-SMAD) which can now bind the coSMAD SMAD4. R-SMAD/coSMAD complexes accumulate in the nucleus where they act as transcription factors and participate in the regulation of target gene expression. TGF- $\beta$  ligand traps: Modified receptors of activin (activin receptor-II trap ligands) have been developed for its inhibition. These receptors derive from the fusion of the extracellular part of activin receptor (ActRIIA or ActRIIB) with the Fc part of IgG immunoglobulin. They exhibit reduced binding capacity to activins or other TGF- $\beta$  family members, such as GDF11, and function as selective trap ligand which inhibits the activation of the SMAD pathway. ACE-011 protein derives from the combination of extracellular part of the receptor ActRIIA with the Fc part of human IgG whereas the homolog RAP-011 requires murine IgG2. ACE-536 includes a modified extracellular domain of ActRIIB linked to the Fc region of human IgG1 whereas RAP-536 formation requires murine IgG2a. [Color figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

complications, especially splenomegaly, transfusion requirements and iron overload. Studies on mice models with  $\beta$ -TI (th3/+) have shown that short-term administration of JAK2 inhibitor (TG101209) reduced ineffective erythropoiesis and splenomegaly [71]. The JAK2 inhibitor ruxolitinib has recently been found to increase HbF production in cultured erythroid precursors from  $\beta$ -TI patients [72] and is currently under investigation in a phase 2 clinical trial in regularly transfused patients with thalassemia (NCT02049450). The primary endpoint of this study is the percent change of red blood cell transfusion requirement whereas the secondary outcome measures are the change of spleen volume, the change of the pre-transfusion hemoglobin level, the pharmacokinetics parameters and the number of participants with adverse events as a measure of safety and tolerability.

### The transforming growth factor- $\beta$ (TGF- $\beta$ ) superfamily ligand traps

The TGF- $\beta$  superfamily consists of four groups of proteins with similar structure and regulatory activity at the cellular level: TGF- $\beta$ , BMP (bone morphogenetic proteins), GDFs (growth and differentiating factors) and activins. Their receptors belong to the family of serine/threonine kinases and induce intracellular pathways recruiting the SMAD related protein group (Fig. 4). The TGF- $\beta$  superfamily plays a significant role in the regulation of basic biological processes such as development, differentiation and tissue homeostasis, including bone and hematopoietic tissue [73].

In vitro studies have shown that the effect of the TGF- $\beta$  superfamily members on the pluripotent stem cells and the erythrocyte progenitors (BFU-E, CFU-E) can be either promoting (TGF- $\beta$ , BMP4) [74] or inhibitory (activin, GDFs) [75,76]. The balanced action of these factors is essential for hematopoiesis. GDFs and activins inhibit the late phase of erythrocyte maturation and therefore are required for the normal termination of erythropoiesis. Furthermore, activins regulate bone formation by inhibiting osteoblastic function and

promoting osteoclastic action [77]. There are three types of activins, activin A, activin B, activin AB and they act through the type IIA and IIB receptors, ActRIIA and ActRIIB respectively. ActRIIB is also the receptor for GDF11 [78]. Inhibitors of GDFs or activins could have a positive impact on the late phase of erythropoiesis with therapeutic effect in conditions with anemia caused by ineffective erythropoiesis, such as  $\beta$ -thalassemia. Modified receptors of activin (activin receptor-II trap ligands) have been developed for its inhibition. These receptors derive from the fusion of the extracellular part of activin receptor (ActRIIA or ActRIIB) with the Fc part of IgG immunoglobulin. By this means, they exhibit increased binding capacity to activins or other TGF- $\beta$  family members, such as GDF11, and function as selective trap ligand that inhibit the activation of the SMAD pathway. ACE-011 derives from the fusion of the extracellular part of ActRIIA with the Fc part of human IgG whereas the homolog RAP-011 requires murine IgG2 [79,80]. ACE-536 includes a modified extracellular domain of ActRIIB linked to the Fc region of human IgG1, whereas RAP-536 requires murine IgG2a [81] (Fig. 4). RAP-011 and RAP-536 proteins have been administered in murine models with  $\beta$ -TI and exhibited positive impact on the reduction of ineffective erythropoiesis and subsequently beneficial results in the amelioration of anemia, splenomegaly and iron overload. In particular, the administration of these factors reduced the intracellular accumulation of hemichromes and the oxidation stress and improved the anemia by inducing the differentiation of the morphologically identifiable erythrocyte progenitors and by reducing the peripheral hemolysis. RAP-011 was shown to inhibit the expression of the growth factor GDF11 and the reduction of the apoptosis of the immature erythroblasts through Fas-Fas ligand pathway, whereas RAP-536 reduced the activation of the SMAD2/3 pathway [82–84].

Phase 1 clinical trials for ACE-011 (sotatercept) and ACE-536 (luspatercept) have already been performed on healthy volunteers and showed stable increase in hemoglobin levels [77,85,86]. Phase 2 clinical trials are now in progress on patients with thalassemia and other

diseases which chronic anemia and ineffective erythropoiesis, such as myelodysplastic syndromes (NCT01749514) and Diamond-Blackfan anemia (NCT01464164), and anemia due to chronic renal failure (NCT01146574). The results of sotatercept (NCT01571635) and luspatercept (NCT01749540) in adults with  $\beta$ -TI and  $\beta$ -TM show that there is a dose dependent improvement of anemia with a satisfactory drug safety profile [87–89].

The NCT01571635 sotatercept trial is a dose finding study determining safety and tolerability in adult patients with  $\beta$ -TM and  $\beta$ -TI who are either transfusion dependent (TD) or non-transfusion dependent (NTD). Secondary endpoints are the reduction of transfusion burden and the number of participants with adverse events. Recruitment has been completed and a total of 37 patients were separated in six groups and received every three weeks subcutaneously doses between 0.1 mg/kg and 1.5 mg/kg. Efficacy was assessed for NTD patients as a hemoglobin increase from baseline of 1 g/dl or more. For TD patients, it was assessed as the percentage of patients with a 20% or more decrease in transfusions. The maximum exposure to the drug was 18 months. Hemoglobin increases of at least 1 g/dl were noted in 67% of patients receiving 0.3 mg/kg and in 100% of patients receiving 0.75 mg/kg. Hemoglobin rise of at least 2 g/dl were noted in 33% of patients receiving 0.5 mg/kg and in 50% of patients receiving 0.75 mg/kg. Twenty percent or higher reductions in transfusion requirements were reported in 33% of patients receiving 0.3 mg/kg, in 50% of patients receiving 0.5 mg/kg, and in 67% of patients receiving 0.75 mg/kg of sotatercept. The drug was safe and well tolerated, and the maximum tolerated dose was not reached [87,88].

The NCT01749540 trial is an open label study evaluating the safety, tolerability and efficacy of luspatercept in adults with  $\beta$ -thalassemia. Primary endpoints are the proportion of patients who have an erythroid response defined as a hemoglobin increase of  $\geq 1.5$  g/dl from baseline for  $\geq 14$  days (in the absence of red blood cell transfusions) in NTD patients, or  $\geq 20\%$  reduction in transfusions compared to pre-treatment in TD patients. Secondary endpoints of the study are the number of patients with adverse events, the change in hemoglobin level in NTD patients, the changes in biomarkers of erythropoiesis, hemolysis, iron metabolism and bone metabolism, liver iron concentration (LIC) by MRI, pharmacokinetics of the drug and patient-reported quality of life questionnaires. Luspatercept was administered subcutaneously every three weeks for 3 months. Patients completing base study may have been eligible to enroll in an ongoing a 24-month extension study. Preliminary data are available for 59 patients (28 TD, 31 NTD). Twenty-one (75%) of 28 TD patients responded with  $>33\%$  reduction in transfusion burden whereas 16 (57%) of them achieved  $>50\%$  decrease over any 12-week period during the study. Reduction in transfusion burden correlated with a significant reduction in LIC. Regarding NTD patients, those who received higher doses (0.8–1.25 mg/kg) achieved greater mean increase in hemoglobin than the 0.2–0.6 mg/kg dose group. During the 24-month extension period, 11 of 17 patients (65%) had an increase in mean hemoglobin  $\geq 1.0$  g/dl over any 12-week period while almost half of them (8/17, 47%) had an increase of  $\geq 1.5$  g/dl. Five of 14 (36%) patients had a significant reduction in LIC at 16 weeks. Interestingly, the use of luspatercept led to rapid leg ulcer healing, evident within 6 weeks after the first dose in four patients. The drug was generally well tolerated in both TD and NTD patient groups. The most frequent mild-moderate adverse events ( $>10\%$  patients) were headache, bone pain, myalgia, and asthenia. The data from the questionnaires of the FACT-An anemia subscale and the SF-36 physical component summary showed an improvement in the quality of life with the increments correlated with the hemoglobin increases [90,91]. A phase 3 trial (NCT02604433, BELIEVE study) of luspatercept in adults with TD thalassemia is ongoing. Since there are currently no safe and effective alternatives to red blood cell transfusions, traps of activin ligands such as sotatercept and luspatercept may be proved to

be useful modalities and emerging data strongly support further evaluation of these agents in patients with  $\beta$ -thalassemia.

### Allogeneic hematopoietic stem cell transplantation (Allo-HSCT)

Allo-HSCT remains the only currently available option that has the potential to definitively cure the disease.  $\beta$ -TM patients with good risk features, according to the Pesaro criteria are expected to have a greater than 90% chance of a successful outcome [92,93]. Allo-HSCT in high risk patients is challenging due to high rates of graft rejection and transplant-related mortality [14]. A number of novel conditioning regimens are being evaluated in an effort to improve the transplant outcomes in high risk patients (NCT00920972, NCT01050855, NCT02435901). Favorable results were recently reported using modified or reduced intensity conditioning [94–96].

Bone marrow is the preferable source of stem cells, while the use of peripheral blood stem cells is being tried in order to reduce the risk of graft rejection in high risk patients (NCT02105766). Traditionally, fully matched HLA identical sibling donors are used, but matched unrelated donor HSCT can be considered in low risk patients with a fully matched donor (NCT01049854). There is a potential role for related cord blood transplants in low risk cases. Unrelated cord blood transplants and haploidentical transplants should preferably be done only in the setting of a clinical trial (NCT00408447, NCT02126046, NCT02504619, NCT00977691).

### Gene therapy

From the current gene therapy methodologies, only  $\beta$ -globin addition has been tried in thalassemic patients. Transfusion dependent  $\beta\epsilon/\beta 0$  patients has been transplanted with autologous CD34+ erythroid progenitor cells transduced ex vivo with lentiviral  $\beta$ -globin vectors. To date, there are a total of seven patients who have been treated successfully with encouraging results in terms of engraftment and transfusion independence, while long term follow up will clarify the possible insertional mutagenesis issues [97,98].

Phase 1 clinical trials have been initiated in order to assess these issues. Early phase, open label clinical trials of LentiGlobin BB305 will assess its efficacy and safety in patients with thalassemia major or sickle cell disease (NCT02151526, NCT01745120). Safety and tolerability of autologous CD34+ hematopoietic progenitor cells transduced with the lentiviral vector TNS9.3.55 (NCT01639690) or GLOBE lentiviral vector (NCT02453477) are also being assessed in ongoing trials. Preliminary data from the latter trial have been recently presented regarding three patients with thalassemia major. The first patient remains transfusion-free six months post gene therapy, the second 1.5 months and the third has completed mobilization of peripheral blood stem cells. All patients showed a satisfactory mobilization, high number of infused cells, with mild and reversible adverse events during and after the procedure [99]. Regarding the other experimental gene therapy methods, there are several ongoing preclinical studies with promising findings.

### Conclusion

Inefficient erythropoiesis is the main mechanism underlying the complications in  $\beta$ -thalassemia such as anemia, splenomegaly, bone malformations, and pulmonary hypertension. Recent research has revealed novel modulators of erythropoiesis which could be used as substantial treatment for ineffective erythropoiesis. Studies with JAK2 inhibitors have shown reduction of splenomegaly and ineffective erythropoiesis in thalassemic mice. A phase 2 trial is already examining the safety and effectiveness of the JAK2 inhibitor ruxolitinib in transfusion dependent patients. Administration of the TGF- $\beta$  family member inhibitors, sotatercept and luspatercept, have shown encouraging results in phase 2 trials, regarding the severity of the thalassemia phenotype with an acceptable safety profile and a phase 3 trial regarding the use of luspatercept has been recently initiated.

Agents that down-regulate the oxidative stress throughout the terminal erythroid maturation process have been investigated in  $\beta$ -TI murine models and showed hematological improvement. These agents aim systems such as Foxo3, HRI-eIF2aP, Prx2, HO-1, Hsp70, and PK and much is expected in the future. Studies on administration of hepcidin or factors that induce its expression (i.e., minihepcidins, inhibitors of MT-2, exogenous transferrin, ERFE inhibitors) are now at preclinical level and have shown promising results. Significant progress in the procedure of allo-HSCT has been achieved, mainly in the field of the optimization of the conditioning regimen. Gene therapy, the treatment of the future, has exhibited remarkable advances. Phase I trials are already recruiting patients with the aim to examine the

effectiveness and mostly the long term safety of transplantation of autologous CD34+ erythroid progenitor cells transduced ex vivo with lentiviral  $\beta$ -globin vectors. The introduction of epigenetic and genomic editing techniques that aim the silencing of  $\gamma$ -globin repressors, such as BCL11A, or the correction of  $\beta$ -globin gene have shown remarkable results in preclinical gene therapy studies.

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