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Review

Molecular basis of β thalassemia and potential therapeutic targets

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ABSTRACT

The remarkable phenotypic diversity of β thalassemia that range from severe anemia and transfusion-dependency, to a clinically asymptomatic state exemplifies how a spectrum of disease severity can be generated in single gene disorders.

While the genetic basis for β thalassemia, and how severity of the anemia could be modified at different levels of its pathophysiology have been well documented, therapy remains largely supportive with bone marrow transplant being the only cure. Identification of the genetic variants modifying fetal hemoglobin (HbF) production in combination with α globin genotype provide some prediction of disease severity for β thalassemia but generation of a personalized genetic risk score to inform prognosis and guide management requires a larger panel of genetic modifiers yet to be discovered.

Nonetheless, genetic studies have been successful in characterizing the key variants and pathways involved in HbF regulation, providing new therapeutic targets for HbF reactivation. BCL11A has been established as a quantitative repressor, and progress has been made in manipulating its expression using genomic and gene-editing approaches for therapeutic benefits. Recent discoveries and understanding in the mechanisms associated with ineffective and abnormal erythropoiesis have also provided additional therapeutic targets, a couple of which are currently being tested in clinical trials.

1. Introduction

The inherited disorders of hemoglobin (Hb) production are the most common human monogenic disorders, among which those affecting the adult β globin gene (*HBB*) – β thalassemia and sickle cell disease (SCD) – are the most clinically significant [1,2]. β thalassemia is caused by a spectrum of mutations that results in a quantitative reduction of β globin chains that are structurally normal [3], in contrast to SCD which is caused by an abnormal Hb variant (HbS, βGlu6Val) that results from a point mutation in the *HBB* gene [4,5]. This change predisposes HbS to polymerization when deoxygenated, a primary event indispensable in the molecular pathogenesis of SCD.

β thalassemia occurs widely in a broad belt including the Mediterranean, parts of North and sub-Saharan Africa, the Middle East, Indian subcontinent and Southeast Asia. It appears that heterozygotes for β thalassemia [6] are protected from the severe effects of falciparum malaria, and natural selection has increased and maintained their gene frequencies in these malarious tropical and sub-tropical regions. In these prevalent regions, gene frequencies for β thalassemia range between 2 and 30% [6]. However, owing to population movements in recent years, β thalassemia is no longer confined to these high-incidence regions, but have become an important public health problem in many countries, including North America and Europe [1]. Because carriers for α-thalassemia and HbS are similarly protected from the severe effects of falciparum malaria [7,8], regions where β-thalassemia is prevalent overlaps substantially with those of α-thalassemia and HbS. Hence, it is not unusual to encounter individuals who have co-inherited two or more Hb variants; for example, 30–35% of SCD individuals from West Africa have co-inherited α-thalssemia [9], and co-inheritance of α- with β-thalassemia is fairly common in the Mediterranean and South-East Asia [10,11].

2. The β globin gene (*HBB*) and normal expression

β globin is encoded by a structural gene found in a cluster with the other β-like genes on chromosome 11 (11p 15.15) [12]. The cluster contains five functional genes, ε (*HBE*), Gγ (*HBG2*), Aγ (*HBG1*), δ (*HBD*), and β (*HBB*), which are arranged along the chromosome in the order of their developmental expression to produce different Hb tetramers: embryonic (Hb Gower-1 ($\zeta_2\varepsilon_2$), Hb Gower-2 ($\alpha_2\varepsilon_2$), and Hb Portland ($\zeta_2\beta_2$)), fetal ($\alpha_2\gamma_2$), and adult (HbA, $\alpha_2\beta_2$ and HbA_2, $\alpha_2\delta_2$) [12] and Serjeant & Vichinsky 2017, this issue. Expression of the globin genes is dependent on local promoter sequences as well as the upstream β globin locus control region (β-LCR) which consists of five Dnase 1

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hypersensitive (HS) sites (designated HS1 to HS5) distributed between 6 and 20 kb 5′ of *HBE* gene [13–15]. There is one HS site at approximately 20 kb downstream of *HBB* gene. All these regulatory regions bind a number of key erythroid-specific transcription factors, notably GATA-1, GATA-2, NF-E2, KLF1 (also known as EKLF), and SCL as well as various co-factors (e.g. FOG, p300), and factors that are more ubiquitous in their tissue distribution, such as Sp1 [12,16,17] (See Philipsen and Hardison 2017, this issue).

The β-like globin genes are each expressed at distinct stages of development through a process referred to as hemoglobin switching (embryonic → fetal → adult). At six months after birth, fetal Hb (HbF, $\alpha_2\gamma_2$) which comprises < 5% of the total Hb, continues to fall reaching the adult level of < 1% at two years of age, when adult Hb becomes the major Hb, and mutations affecting the adult *HBB* gene, i.e. β thalassemia and SCD become manifested [18–20]. In contrast severe mutations which result in complete absence of α globin genes, become clinically apparent at the fetal stage.

The *HBB* locus is a paradigm for tissue- and developmental stage-specific regulation; expression of the individual globin genes relies on a timely and direct physical interactions between the globin promoters and the β-LCR, the interaction being mediated through binding of erythroid-specific and ubiquitous transcription factors [21,22]. A dual mechanism has been proposed for the developmental expression: 1) gene competition for the upstream β-LCR, conferring advantage for the gene closest to the LCR [23], and 2) autonomous silencing (transcriptional repression) of the preceding gene [24,25]. The ability to compete for the β-LCR and autonomous silencing depends on the change in the abundance and repertoire of various transcription factors that favour promoter-LCR interaction. While the ε and γ globin genes are autonomously silenced at the appropriate developmental stage, expression of the adult β globin gene depends on lack of competition from the upstream γ gene for the LCR sequences. Concordant with this mechanism, when the γ gene is upregulated by point mutations in their promoter causing a non-deletion hereditary persistence of fetal hemoglobin (HPFH), expression of the *cis* β gene is downregulated [26]. Further, mutations which affect the β globin promoter, which removes competition for the β-LCR, are associated with higher than expected increases in γ (HbF, $\alpha_2\gamma_2$) and δ (HbA_2, $\alpha_2\delta_2$) expression [27–29]. In recent years, an increased understanding of the repressors and co-repressors of the γ globin gene (e.g. BCL11A) and the switch from fetal to adult Hb expression has provided much insight on strategies of de-repressing expression of the fetal globin genes in adults for treating both β thalassemia and SCD.

3. Genetics of β thalassemia

> 300 β thalassemia alleles have now been described (http://www.ithanet.eu/db/ithagenes; http://globin.bx.psu.edu/hbvar) but only about forty account for 90% or more of the β thalassemias worldwide [30]. This is because in the areas where β thalassemia is prevalent, only a few mutations are common, possibly reflecting local selection due to malaria. Each of these populations, thus has its own spectrum of β thalassemia alleles.

Downregulation of the β globin gene can be caused by a whole spectrum of molecular lesions ranging from point changes to small deletions limited to *HBB*, to extensive deletions of the whole β globin cluster (Fig. 1) [3]. In contrast to α thalassemia, which is mainly caused by deletions (Harteveld and Farashi 2017, this issue), the vast majority of mutations causing β thalassemia are non-deletional.

Functionally, β thalassemia alleles are considered as β^0 when no β globin is produced, or β^+ in which some β globin is produced, but less than normal. A range of severity is encountered within the β^+ thalassemia group; the less severe forms are sometimes designated β^{++} to reflect the minimal deficit in β chain production. Some β^{++} alleles are so mild that they are 'silent', and carriers do not display any evident hematological phenotypes; their red cell indices and HbA_2 levels are within normal limits, the only abnormality being an imbalanced α:non-α chain synthesis [31]. These β^{++} thalassemia alleles have usually been uncovered in individuals with thalassemia intermedia who have inherited a silent β thalassemia allele in compound heterozygosity with a severe allele. In this case, one parent has typical β thalassemia trait, and the other is apparently normal. In contrast, carriers for β^0 and β^+ thalassemia alleles have clearly recognizable hematological phenotypes – mild or no anemia, microcytic hypochromic red cell indices, and elevated HbA2 with mildly increased HbF levels (Fig. 2).

3.1. Non-deletion β thalassemia

These non-deletional mutations, i.e. single base substitutions, small insertions or deletions of one to a few bases are located within the gene or its immediate flanking sequences. They downregulate the β globin gene via almost every known stage of gene expression, from transcription to RNA processing and translation of β globin mRNA. Approximately half of the non-deletional mutations completely inactivate the β gene with no β globin production resulting in β^0 thalassemia. (See Table 1 for categories of non-deletional mutations.)

3.1.1. Transcriptional mutations

Transcriptional mutants involve the conserved DNA sequences that form the β globin promoter (from 100 bp upstream to the site of the initiation of transcription, including the functionally important CACCC, CCAAT and ATAA boxes) or the stretch of 50 nucleotides in the 5'UTR. Generally, these transcriptional mutants result in a mild to minimal reduction of β globin output i.e. β^+ or β^{++} thalassemia alleles, and occasionally they are 'silent'. A silent β thalassemia allele which has been observed fairly frequently in the Mediterranean region is the − 101C → T mutation where it interacts with a variety of more severe β thalassemia mutations to produce milder forms of β thalassemia [32]. Other 'silent' mutations include those in the 5′ UTR; the extremely mild phenotype is exemplified in a homozygote for the + 1 A → C mutation who has the hematologic values of a thalassemia carrier, heterozygotes are 'silent' [33].

Within this group of transcriptional mutants, ethnic variation in phenotype has been observed. Black individuals homozygous for the − 29 A → G mutation have an extremely mild disease [34], while a Chinese individual homozygous for the same mutation had severe anemia and was transfusion-dependent [35]. The cause of this striking difference in phenotype is not known but likely to be related to the different chromosomal backgrounds on which the apparently identical mutations have arisen. One difference is the C-T polymorphism at position − 158 upstream of the $^{G}\gamma$ globin gene (*Xmn*1-$^{G}\gamma$ site) present in the β chromosome carrying the − 29 A → G mutation in Blacks but absent in that of the Chinese. The *Xmn*I-Gγ site, considered to be a quantitative trait locus for HbF, is associated with increased HbF production under conditions of erythropoietic stress (see later on 'Update on the genetic control on HbF).

3.1.2. Mutations affecting RNA processing

A wide variety of mutations interfere with processing of the primary mRNA transcript. Those that affect the invariant dinucleotide GT or AG sequences at exon-intron splice junctions prevent normal splicing altogether, causing β^0 thalassemia. Mutations involving the consensus sequences adjacent to the GT or AG dinucleotides allow normal splicing to varying degrees and produce a β thalassemia phenotype that ranges from mild to severe. For example, mutations at position 5 IVS1 G → C, T or A, considerably reduce splicing at the mutated donor site compared with the normal β allele [26]. On the other hand, the substitution of C for T in the adjacent nucleotide, intron 1 position 6, only mildly affects normal RNA splicing. Although the IVS1-6 T-C mutation is generally associated with milder β thalassemia, studies have shown differential severities for apparently identical mutations; again this is presumably related to the chromosomal background on which the mutations have

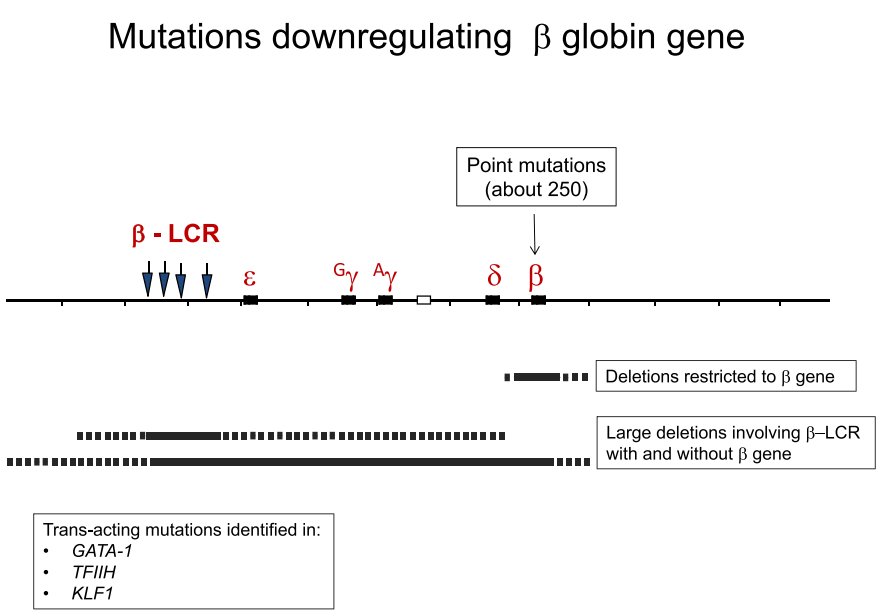

Fig. 1. Mutations causing β thalassemia.
A summary of the mechanisms downregulating β globin gene expression. The upper panel depicts the β globin gene (*HBB*) cluster with the upstream β locus control region (βLCR). The vast majority are point mutations affecting the structural *HBB* gene. Deletions downregulating *HBB* are rare and are either restricted to the gene or extensive, involving the βLCR with or without *HBB*. Dashed lines represent variation in the amounts of flanking DNA removed by the deletions.

arisen [36].

Both exons and introns also contain 'cryptic' splice sites which are sequences very similar to the consensus sequence for a splice site but are not normally used. Mutations can occur in these sites creating a sequence that resembles more closely the normal splice site. During RNA processing the newly created site is utilized preferentially, leading to aberrant splicing; incorrectly spliced mRNA is not functional because spliced intronic sequences usually generate a frameshift and a premature termination codon. Such mutations have been identified in both introns 1 and 2, and exon 1 of *HBB* gene. The associated phenotype may be either β^+ or β^0 thalassemia, depending on the proportion of normal and abnormal mRNA species generated. One such mutation is the (**G**AG → **A**AG) mutation in codon 26 in exon 1 [37] that results in the HbE variant. The single base substitution leads to a minor use of the alternative pathway; as the major β mRNA that codes for the variant is normally spliced, HbE has a mild β^+ thalassemia phenotype. Clinical phenotypes of compound HbE/β thalassemia heterozygotes resemble those with two β thalassemia alleles, ranging from severe anemia and transfusion-dependency to non-transfusion dependent states (i.e. thalassemia intermedia or non-transfusion dependent thalassemia (NTDT)), depending on the non-HbE β thalassemia allele and other genetic factors [38].

Other RNA processing mutants affect the polyadenylation signal (AATAA) and the 3′ UTR. These are generally mild β^+ thalassemia alleles [3].

3.1.3. Translational mutations

About half of the β thalassemia alleles completely inactivate the gene mostly by generating premature stop codons (PTCs), either by single base substitution to a nonsense codon, or through a frameshift mutation. As part of the surveillance mechanism that is active in quality control of the processed mRNA, mRNA harboring a PTC are destroyed and not transported to the cytoplasm in a phenomenon called (nonsense mediated RNA decay or NMD) to prevent the accumulation of mutant mRNAs coding for truncated peptides [39,40]. However, some in-phase PTCs that occur later in the β sequence, in 3′ half of exon 2 and in exon 3, escape NMD and are associated with substantial amounts of mutant β-mRNA leading to a synthesis of β chain variants that are highly unstable and non-functional with a dominant negative effect (see dominantly inherited β thalassemia) [41]. Other mutations of RNA translation involve the initiation (ATG) codon. Nine of these have been described; of these, all are single base substitutions apart from one mutation of 45 bp insertion and they result in β^0 thalassemia [26].

3.2. Deletions causing β thalassemia

β thalassemia is rarely caused by deletions. Eighteen deletions restricted to the *HBB* gene itself have been described, they range from 25 bp to ~6 kb of which two are minor intragenic deletions of 25 bp and 44 bp at the 3′ and of IVSI, and two (619 bp and 7.7 kb) remove the 3′ end of the gene but leave the 5′ end intact [3]. The 619 bp deletion at the 3′ end of the β gene is relatively common, but restricted to the Sind populations of India and Pakistan where it accounts for about one-third of the β thalassemia alleles [42]. The other deletion which removes 7.7 kb 3′ from the second intron of *HBB*, was described in compound heterozygosity with β^S gene in a woman with SCD from Cape Verde islands [43]. The other fourteen deletions differ widely in size (from 290 bp to > 67 kb) and remove in common a region in the β promoter (from position −125 to +78 relative to the mRNA CAP site) which includes the CACCC, CCAAT, and TATA elements. These deletions are extremely rare, but of particular clinical interest because they are associated with unusually high levels of HbA_2 and HbF in heterozygotes. These deletions result in β^0 thalassemia, yet the increase in HbF is adequate to compensate for the complete absence of HbA in homozygotes for these deletions [44–46]. It has been proposed that the mechanism underlying the elevated levels of HbA_2 and HbF is related to deletion of the β promoter removing competition for the upstream β-LCR and limiting transcription factors, resulting in an increased interaction of the LCR with the γ- and δ-genes in *cis*, thus enhancing their expression. This mechanism may also explain the unusually high HbA_2 levels that accompany the point mutations in the β promoter region [28].

3.3. Dominantly inherited β thalassemia

In contrast to the common β thalassemia alleles that are inherited typically as Mendelian recessives, some forms of β thalassemia are dominantly inherited, in that inheritance of a single copy of the β thalassemia allele results in clinical disease [47–49]. Carriers have moderate to severe anemia, splenomegaly and the hematological hallmarks of heterozygous β thalassemia – elevated HbA_2 and imbalanced globin chain synthesis [50]. More than thirty dominantly inherited β thalassemia alleles have now been described; they include a spectrum of molecular lesions – single base missense mutations and minor

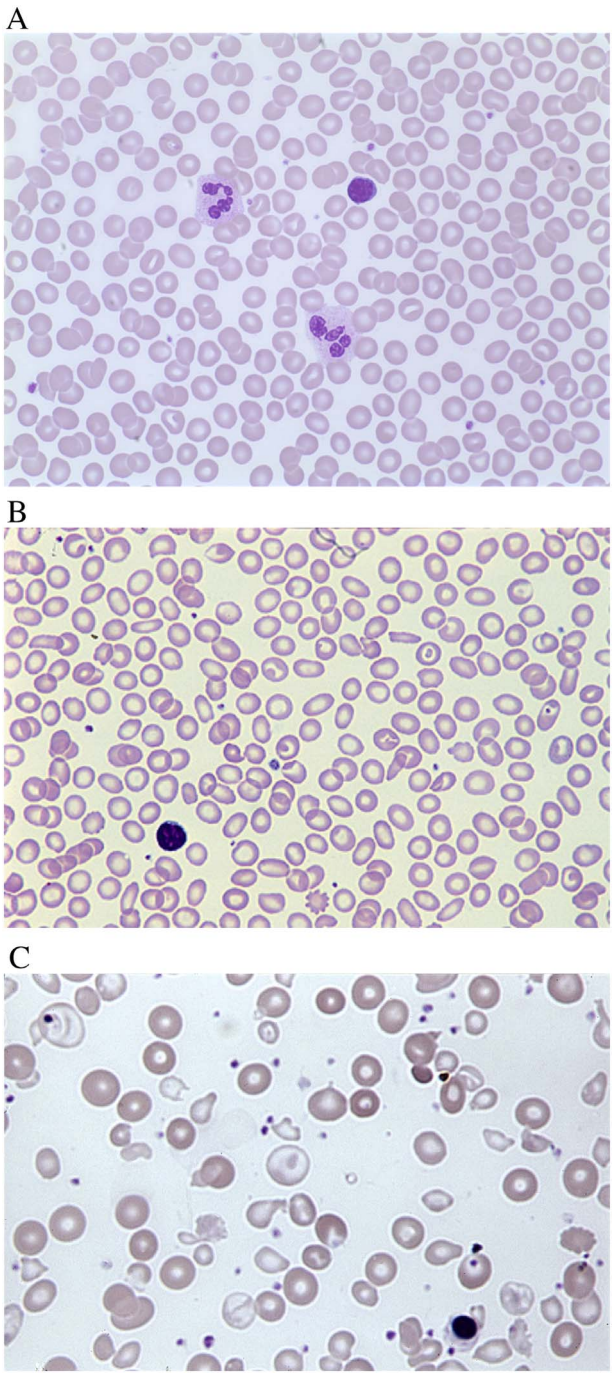

Fig. 2. Typical blood smears of normal, β thalassemia trait and β thalassemia major. A) Normal, B) β thalassemia trait showing hypochromic microcytic red blood cells, C) β thalassemia major showing gross aniso-poikilocytosis with poorly hemoglobinized and occasional nucleated red blood cells.

insertions/deletions that result in truncated or elongated β globin variants with abnormal carboxy-terminal ends [26,51]. The underlying denominator of these variants is the production of highly unstable and non-functional β globin variants that are not able to form viable tetramers with α globin. These precipitate in the erythroid precursors and together with the redundant α chains, overload the proteolytic mechanism causing premature death of these cells, and accentuating the ineffective erythropoiesis. Unlike the recessive forms of β thalassemia that are prevalent in the malarious regions, the dominantly inherited β thalassemia variants are rare, and found in dispersed geographical regions where the gene frequency for β thalassemia is very low. Furthermore, many of these variants are unique to the families described, and occur as *de-novo* mutations.

Table 1
Categories of the mutations causing beta thalassemia.
A. Deletions:
•Large deletions involving β LCR with or without β gene (cause εγδβ thalassemia).
•Deletions restricted to β gene.
B. *Trans*-acting mutations.
C. Point mutations.

I. Transcriptional mutations		Type
Promoter regulatory elements		
1)	CACCC Box	β^+ to β^{++} (silent)
2)	ATA Box	β^+
3)	5′ UTR (CAP +1 to +45)	β^+ to β^{++} (silent)
II. RNA processing		
Splice junction		
1) IVS1-position 1 and 2		β^0
2) IVS1–3′ end (minor deletion of 17 to 44 bp, and insertion of 22 bp)		β^0
3) IVS2-position 1 and 2		β^0
4) IVS2–3′ end		β^0
Consensus splice sites		
5) IVS1-position 5		β^+ or β^0 depending on nucleotide change
6) IVS1-position 6		β^+
7) IVS1-positions −3, 128, 129		β^+
8) IVS2–5		β^+
9) IVS2–3′ end		β^+ to β^{++} (silent)
Cryptic splice sites		
10) IVS1- positions 110, 116		β^+, β^0
11) IVS2-positions 654, 705, 745, 837		β^+, β^0
12) CD10 (GC**C** → GC**A**)		
13) CD19 (A**A**C → A**G**C) Hb Malay (Asn → Ser)		β^{++}
14) CD24 (GG**T** → GG**A**)		β^{++}
15) CD26 (**G**AG → **A**AG) (Glu → Lys, Hb E)		β^+
16) CD26 (G**A**G → G**C**G)(Glu → Ala, Hb Tripoli)		β^+
17) CD27 (GCC → TCC) (Ala → Ser, Knossos)$^{++}$		β^{++}
RNA Cleavage - poly A signal		
18) AA**T**AAA – single base substitutions, minor deletions,		β^+, β^{++}, β^{++} (silent)
Others – in 3′ UTR		
19) Term CD +6, C → G		β^{++} (silent)
20) Term CD +90, del 13 bp		β^{++} (silent)
21) Term CD +47 (C → G		β^{++}
III. RNA translation		
Initiation codon		
1) ATG – single base substitutions, 45 insertion		β^0
Nonsense codons-		
2) Numerous examples of single base substitions, all leading to premature termination codons		All β^0
Frameshift		
3) Numerous examples of minor insertions, deletions, shifting reading frame and leading to premature termination codons		All β^0

3.4. Unusual causes of β thalassemia

The unusual causes of β thalassemia that are extremely rare are mentioned here, not just for the sake of completeness but also to illustrate the numerous molecular mechanisms of downregulating the β globin gene. Transposable elements may occasionally disrupt human genes and result in their activation. The insertion of such an element, a retrotransposon of the LI family into intron 2 of the β globin gene has been reported to cause β^+ thalassemia [52].

Almost all variants downregulating *HBB* are physically linked to the gene and behave as alleles of the β globin locus (i.e. they are *cis*-acting). Rarely, mutations in other genes distinct from the β globin complex can downregulate β globin expression. Such *trans*-acting mutations have been described; one group affects the XPD protein which is part of the general transcription factor TF11H [53]. Eleven trichodystrophy (TTD) patients with mutations in *XPD* had hematological features of β thalassemia trait. In another case of a 4-generation family with X-linked

thrombocytopenia and β thalassemia, the causative mutation was shown to be in the amino finger of erythroid-specific GATA-1 affecting DNA binding [54].

Somatic deletion of the β globin gene contributed to thalassemia intermedia in three unrelated families of French and Italian origins [55,56]. The affected individuals with thalassemia intermedia were constitutionally heterozygous for β^0 thalassemia but subsequent investigations revealed a somatic deletion of chromosome 11p15, including the β globin gene complex, in *trans* to the mutation in a subpopulation of erythroid cells. This results in a somatic mosaic − 10 to 20% of the cells were heterozygous with one normal copy of the β globin gene, and the rest hemizigous, i.e. without any normal β globin gene. The sum total of β globin product is ~25% less than the asymptomatic β^0 carrier; these observations offer great promise for potential gene therapy as it shows that expression of a single β gene in a proportion of red blood cells appears to be sufficient to produce a non-transfusion dependent state. Late presentation of β thalassemia and transfusion dependency has been reported in a Chinese patient [57] and a Portuguese woman at the age of 15 years [58]. In both cases, the phenotype was caused by uniparental isodisomy of the paternal chromosome 11p15.5 that encompassed the β thalassemia allele.

4. Genetic modifiers of β thalassemia

Historically, the genetic modifiers in β thalassemia have been derived from an understanding of its pathophysiology, and subsequently validated by family and case control studies. Two important modifiers – co-inheritance of α thalassemia and variants associated with increased synthesis of HbF in adults have emerged from such clinical genetic studies. Elucidation of the modifying effects of HbF and α thalassemia has not been too difficult as these loci have a major clinical effect and the genetic variants are common, and thus would contribute substantially to disease burden. However, these two modifiers do not explain all the clinical heterogeneity. Recent advances in technology and reducing costs have prompted genome-wide association studies (GWASs) in an attempt to derive some of the genetic modifiers in such complex traits [59].

A major limitation in these genetic studies is the heterogeneity of the β-thalassemia alleles and phenotypes. In the Sardinian population where almost all the β thalassemia is β°, and the β thalassemia alleles are β°39, genetic association studies have been successful in unravelling some of the modifying factors [60,61]. GWASs involve an unbiased scan of the whole human genome and, by design, are more likely to reveal unsuspected interactions [62]. A case in point is the application of GWAS in the highly successful discovery of *BCL11A* (an oncogene that hitherto, was not known to have a role in erythropoiesis) as a quantitative trait locus (QTL) controlling HbF [60,63].

The central mechanism driving the pathophysiology of the β thalassemias relates to the deleterious effects of the excess α globin chain, leading to the formation of insoluble hemichromes which impair erythroid survival causing ineffective erythropoiesis [64]. Clinical studies have shown that disease severity correlates well with the degree of imbalance between α and normal globin chains and the size of the free α chain pool. Genetic modifiers can impact the phenotypic severity at the primary level by affecting the degree of globin chain imbalance, and at the secondary level by moderating complications of the disease related to the anemia or therapy e.g. iron chelation.

While the severity of beta thalassemia is primarily determined by the degree of beta chain deficiency, for any given beta thalassemia allele the severity of the disease can be alleviated by co-inheritance of α thalassemia or by co-inheritance of factors that increase γ globin chain production and HbF levels. In the latter case, γ globin chains combine with the excess α globin to form HbF; cells that contain a relatively higher percentage of HbF have selective survival as they are protected from the deleterious effect of excess α globin and premature death. Thus, all individuals with β thalassemia have variable increases in HbF due to survival of these F cells.

Certain β thalassemia mutations, notably those that involve small deletions or mutations of the promoter sequence of the *HBB* gene, are associated with much higher levels of HbF production than mutations affecting other regions of *HBB* (see deletions causing beta thalassemia) [3]. This might reflect the competition between the *HBG* and *HBB* promoters for interaction with the upstream β LCR and limited transcription factors. Heterozygotes for such types of β thalassemia mutations have unusually high HbA_2, and although the increases in HbF levels are variable, the increase in HbF production in homozygotes is adequate to compensate for the complete absence of HbA. HbF levels are normal or slightly elevated in β thalassemia heterozygotes. Higher HbF (and HbA_2) levels are found with mutations that involve promoter of the *HBB* gene, but variations in HbF levels also reflect the genetic background of the individual (e.g. -29 promoter mutation in Blacks and Chinese). In homozygous β thalassemia, the proportion of HbF ranges from 10% in those with the milder alleles to almost 100% in homozygotes or compound heterozygotes with β^0 thalassemia. In the most severe cases, the absolute amount of HbF is approximately 3 to 5 g/dL, produced as a result of extreme erythroid hyperplasia, selective survival of F cells, and some increase in *HBG* transcription. Non-transfusion dependent β^0 thalassemia intermedia with hemoglobin levels of 8 to 11 g/dL and 100% HbF has been observed. In some cases, the increase in HbF production reflects the type of β thalassemia allele, but in others

Table 2
Genetic modifiers of β thalassemia.
Adapted from Thein, S.L. Genetic association studies in β hemoglobinopathies. Hematology, ASH Education Program Book 2013; 354-61.

A. Primary at level of α: non-α globin chain imbalance	
Modifier	Mechanism
1. β globin genotype (one or two, and severity of β thalassemia alleles)	Directly affects output of β globin and chain imbalance
2. α globin genotype α thalassemia	Reduces chain imbalance and α globin excess
co-inheritance of extra α globin genes (ααα/, αααα/, or *HBA* cluster duplication	Increases α globin excess and chain imbalance
3. Innate ability to increase HbF (co-inheritance of HbF QTLs, eg, HbF-boosting variants in *BCL11A*, *HMIP*, *Xmn*1-*HBG2* and *KLF1*	Increased γ chains combine with excess α reducing chain imbalance
4. Potential modifiers include variants in ubiquitin proteolytic pathway	Promotes proteolysis of excess α globin
5. α hemoglobin stabilizing protein (*AHSP*)	Chaperones excess α globin (studies inconclusive)
B. Secondary at level of complications related to disease and therapy	
Complication	Modifier
1. Serum bilirubin and propensity to gallstones	UGT1A1 promoter $(TA)_n$ polymorphisms
2. Iron loading	*HFE* - H63D variants increase GI absorption of iron
3. Osteopenia and osteoporosis	Variants in *VDR*, *COL1A1*, *COL1A2*, TGFB*1*, modify bone mass
4. Cardiac disease	Apolipoprotein (*APOE*) ε4 – risk factor for left ventricular heart failure
5. Cardiac iron loading	Glutathione-S-transferase M1 – increased risk of cardiac iron in thalassemia major

co-inheritance of QTLs associated with increased *HBG* expression might explain their milder clinical severity [65]. The genetic modifiers of β thalassemia could be regarded as operating at the primary and secondary levels of the disease pathophysiology (Table 2).

4.1. Effect of the primary modifiers: HbF quantitative trait loci and α globin genotype

While selective survival of F cells provides an explanation for the increases in HbF in β thalassemia, the mechanism does not explain the wide variation in the amount produced. Much of this variability is genetically determined, in part from the co-inheritance of one or more of HbF-boosting alleles of the *Xmn*1-*HBG2*, *HMIP-2* and *BCL11A* HbF quantitative trait loci (QTLs) [60,61,66–68]. The *Xmn1-HBG2* (*rs782144*) QTL is a common sequence variation in all population groups, present at a frequency of approximately 0.35. Although increases in HbF and F cells associated with *Xmn1-HBG2* are minimal or undetectable in healthy adults, clinical studies have shown that under conditions of stress erythropoiesis, as in homozygous β thalassemia, the presence of *Xmn*1-*HBG2* leads to a much higher HbF response associated with a delayed transfusion need [65]. This could explain why the same mutation on different β chromosomal backgrounds, some with, and others without the *Xmn1-HBG2* variant, are associated with different clinical severity. High resolution genotyping studies suggest that *Xmn*1-*HBG2* may not be the causal element but in tight linkage disequilibrium to another, as yet undiscovered, variant(s) on chromosome 11p.

The *Xmn*1-*HBG2*, *BCL11A*, and *HMIP-2*, together with other loci, linked and unlinked to the *HBB* complex, constitute the loosely-defined entity of heterocellular HPFH that has been suggested to underlie the milder forms of β thalassemia. These HbF QTLs play an important role in fine-tuning γ globin production in healthy adults and in response to the stress erythropoiesis of sickle cell anemia and β thalassemia. The three QTLs are associated with HbF and severity of thalassemia in diverse population groups including Sardinian, French, Chinese, and Thai [60,67–69]. More than 95% of Sardinian β thalassemia patients are homozygous for the same codon 39 β^0 thalassemia mutation but have variable clinical severity. Co-inheritance of variants in *BCL11A* and *HMIP-2*, and α thalassemia accounts for 75% of the differences in disease severity [61]. In France, a combination of the β thalassemia genotype, Xmn1-*HBG2* and SNPs in *BCL11A* and *HMIP-2*, can predict up to 80% of disease severity [68]. In a cohort of 316 β^0 thalassemia patients, delayed or absent transfusion requirements correlated with status of the three HbF QTLs and the α globin genotype [70]. Using a combination of the HbF QTLs, the type of β thalassemia mutations, and the α globin genotype, a predictive score of severity has been proposed [71].

In many populations where β thalassemia is prevalent, α thalassemia also occurs at a high frequency and it is not uncommon to co-inherit both conditions [19]. Homozygotes or compound heterozygotes for β thalassemia who co-inherit α thalassemia will have less redundant α globin and tend to have less severe anemia. The degree of amelioration depends on the severity of the β thalassemia alleles and the number of functional α globin genes. At one extreme, patients with homozygous β thalassemia who have also co-inherited HbH (equivalent of only one functioning α globin gene) have NTDT [64].

In individuals with one β thalassemia allele (heterozygotes), co-inheritance of α thalassemia normalizes the hypochromia and microcytosis but the elevated HbA_2 remains unchanged. Increased α globin production through co-inheritance of extra α globin genes (triplicated – ααα/αα or ααα/ααα, quadruplicated – αααα/αα, or duplication of the whole α globin gene cluster – αα/αα/αα) with heterozygous β thalassemia tips the globin chain imbalance further, converting a typically clinically asymptomatic state to thalassemia intermedia [18,64,72,73]. Again, the severity of anemia depends on the number of extra α globin genes and the severity of the β thalassemia alleles.

At the primary level of chain imbalance, the proteolytic capacity of the erythroid precursors in catabolising the excess α globin has often been suggested, but this effect has been difficult to define. Alpha hemoglobin stabilizing protein, a molecular chaperone of α globin has also been suggested as another genetic modifier but its impact on disease severity has been inconclusive [74].

4.2. Secondary modifiers of complications of β thalassemia

These modifiers do not affect globin imbalance directly but might moderate the different complications of β thalassemia that are directly related to the anemia, or to therapy such as iron loading from intestinal absorption [75–77]. They include genetic variants which affect bilirubin metabolism, iron metabolism, bone disease and cardiac complications. Jaundice and a predisposition to gallstones, a common complication of β thalassemia, is associated with a polymorphic variant in the promoter of the *UGT1A1* gene. Individuals who are homozygous for 7 [*TA*]s, also referred to as Gilbert's syndrome, have higher levels of bilirubin and increased predisposition to gallstones, an observation that has been validated at all levels of β thalassemia. Several genes involved in iron homeostasis have now been characterized, including those encoding HFE (*HFE*), transferrin receptor 2 (T*fR2)*, ferroportin (*FPN*), hepcidin (*HAMP*) and hemojuvelin (*HJV*) [78]. The H63D variant, a common polymorphism in the *HFE* gene, appears to have a modulating effect on iron absorption. β thalassemia carriers who are homozygous for *HFE* H63D variant, have higher serum ferritin levels than carriers without the variant (see Table 1 for modifiers of β thalassemia).

The degree of iron loading, bilirubin levels and bone mass are quantitative traits with a genetic component; variants affect the genes that are involved in the regulation of these traits that contribute to the complications.

4.3. Update on the genetic control of fetal hemoglobin (HbF)

The production of HbF is not completely switched off at birth; all adults continue to produce residual amounts of HbF, with over 20-fold variation [79]. In 2000, we utilized twin studies to show that the common HbF variation in adults is predominantly genetically controlled with genetics explaining up to 89% of the inter-individual variation. We suggested that inheritance of HbF behaved as a quantitative genetic trait [80]. The β globin locus was the first chromosomal region that was shown to contain variants that affect HbF. Apart from the Mendelian forms of hereditary persistence of fetal hemoglobin (HPFH) that include deletions and point mutations in the γ globin gene promoters, epidemiological and clinical genetic studies [83] have long implicated *Xmn*1-*HBG2* (*rs782144*) as a common variant influencing HbF levels. SCD individuals in whom the β^S gene is on the Senegal or Arab-Indian β^S haplotype have the highest HbF levels and milder phenotypes, while SCD individuals with the β^S gene on a Bantu haplotype have the lowest HbF levels and the most severe clinical course [81,82]. The differences in clinical severity was ascribed to the difference in HbF levels implicating the *Xmn*1-*HBG2* site which is present on the Senegal and Arab-Indian β^S haplotype but not on the Bantu haplotype [83]. Recent high resolution genotyping, however, suggests that *rs782144* is not likely to be the variant itself, but in tight linkage disequilibrium with causal element(s) that remain to be discovered in the β globin cluster. Clinical studies in β thalassemia also showed that mutations on β thalassemia haplotypes that includes the Xmn1-*HBG2* site seemed to be associated with a milder disease.

Early studies in some families with β thalassemia and SCA suggested that high HbF determinants segregated independently of the *HBB*; these families were often discovered through the probands who had unexpectedly mild disease. The second QTL, the *HBS1L-MYB* intergenic region (*HM1P*) on chromosome 6q, was discovered through genetic linkage studies in one such kindred, and subsequently validated by genetic association studies [84]. These two QTLs were joined by *BCL11A* on chromosome 2p16 uncovered through GWAS. GWAS not

only 'rediscovered' the 2 known QTLs, but highlighted a novel gene whose relevance to HbF trait was previously unsuspected. Biological studies of these recently identified QTLs provide data supporting long-held views on mechanisms of hemoglobin switching – changes in *trans*-acting factor environment and perturbation of the kinetics of erythropoiesis [85–87].

Functional studies in primary human erythroid progenitor cells and transgenic mice demonstrated that *BCL11A* acts as a repressor of gamma-globin gene expression that is effected by SNPs in intron 2 of this gene [88]. Fine-mapping demonstrated that these HbF-associated variants, such as *rs1427407* in *BCL11A,* and *rs9399137* in *HMIP*, are localized to enhancers that are erythroid-specific [89,90]}. The variant *rs1427407* in *BCL11A* was further validated by systemic dissection using the CRISPR-Cas9 genome-editing approach [91].BCL11A does not interact with the γ globin promoter but occupies discrete regions in the *HBB* complex [92]. Functional studies in primary human erythroid progenitors and transgenic mice demonstrated that *BCL11A* represses γ globin and the silencing effect involves re-configuration of the *HBB* locus through interaction with GATA-1 and SOX6 that binds the proximal γ globin promoters [93,94].

With regard to *HMIP*, subsequent studies established that *MYB* is the relevant gene in the *HBS1L-MYB* region [95]. *MYB* encodes the MYB transcription factor that is essential for hematopoiesis and erythroid differentiation.

1) The causal variants reside in two clusters within the block, at − 84 and − 71 kb respectively, upstream of *MYB* [90,96]. The SNPs at these two regions disrupt binding of key erythroid enhancers affecting long-range interactions with *MYB* and *MYB* expression, providing a functional explanation for the genetic association of the 6q *HBS1L-MYB* intergenic region with HbF and F cell levels [90,97,98]. A 3-bp deletion is one functional element in the *MYB* enhancers accounting for increased HbF expression in individuals who have the sentinel SNP *rs9399137* that was found to be common in European and Asian populations, although less frequent in African-derived populations [99,100].
2) The *HBS1L-MYB* intergenic enhancers do not appear to affect expression of *HBS1L*, the other flanking gene [90]. A case report also excluded *HBS1L* as having a role in the regulation of HbF and erythropoiesis. In whole-exome sequencing of rare uncharacterized disorders, mutations in the *HBS1L* gene leading to a loss of function in the gene were identified in a female child [101]. The child had normal blood counts and normal HbF levels.
3) A delayed HbF to HbA switch, along with persistently elevated HbF levels, is one of the unique features in infants with trisomy 13 [102]. Compelling evidence has been provided that the increased HbF effect is mediated, at least in part, through down-modulation of *MYB* via targeting of its 3′ UTR by microRNAs 15a and 16-1 [103]. The gene encoding microRNAs 15a and 16-1 is localized on chromosome 13q14 that was unambiguously associated with the increased HbF trait in these infants.
4) *MYB* was also causally implicated by fine-mapping which identified rare missense *MYB* variants associated with HbF production [104].

It is proposed that MYB modulates HbF expression via two mechanisms: 1) indirectly through alteration of the kinetics of erythroid differentiation: low MYB levels accelerate erythroid differentiation leading to release of early erythroid progenitor cells that are still synthesizing predominantly HbF [12,105] and, 2) directly via activation of *KLF1* and other repressors (eg, nuclear receptors TR2/TR4) of gamma-globin genes [98,106,107].

Modulation of *MYB* expression also provides a functional explanation for the association of the *HMIP-2* SNPs with other erythroid traits such as red cell count, MCV, MCH, HbA_2 levels, and also with platelet and monocyte counts [108–112].

Variants in the *HBB*, *HMIP* and *BCL11A* loci account for 10% - 50% of the variation in HbF levels in adults, healthy or with sickle cell anemia or β thalassemia, depending on the population studied [61,63,68,113–119]. The remaining variation ('missing heritability') is likely to be accounted for by many loci with relatively small effects, and/or rare variants with significant quantitative effects on γ-globin gene expression that are typically missed by GWAS population studies. An example of the latter is the *KLF1* gene [120–122].

KLF1, discovered by Jim Bieker in 1993 [120] reemerged as a key transcription factor controlling HbF through genetic studies in a Maltese family with β thalassemia and HPFH that segregated independently of the *HBB* locus [123]. Linkage studies identified a locus on chromosome 19p13 which encompassed *KLF1* and expression profiling of erythroid progenitor cells confirmed *KLF1* as the γ-globin gene modifier in this family. Family members with HPFH were heterozygous for the nonsense K288X mutation in *KLF1* that disrupted the DNA-binding domain of KLF1, a key erythroid gene regulator. Numerous reports of different mutations in *KLF1* associated with increases in HbF soon followed [121,122]. The HbF increases occurred either as a primary phenotype or in association with red blood cell disorders such as congenital dyserythropoietic anemia [124,125], congenital non-spherocytic hemolytic anemia due to pyruvate kinase deficiency [126] and sickle cell anemia [121]. Several GWASs of HbF however, including ones in sickle cell anemia patients of African descent, failed to identify common *KLF1* variants [114,117]. On the contrary, targeted re-sequencing of *KLF1* identified variants that were over represented in Southern China where β thalassemia is prevalent compared to North China. *KLF1* variants were also over-represented in patients with milder β thalassemia when compared to thalassemia major [127].

KLF1 is a direct activator of *BCL11A* (see Chapter 1, read below) and is also essential for the activation of *HBB* expression [128,129]. Collectively, studies suggest that KLF1 is key in the switch from *HBG* to *HBB* expression; it not only activates *HBB* directly, providing a competitive edge, but also silences the γ globin genes indirectly via activation of *BCL11A*. KLF1 may play a role in the silencing of embryonic globin gene expression [126]. In the light of these findings, KLF1 has now emerged as a major erythroid transcription factor with pleiotropic roles underlying many of the previously uncharacterized anemias [130].

The emerging network of HbF regulation also includes LRF/Pokemon/ZBTB7A, SOX6, chromatin-modeling factor FOP and the NURD complex, the orphan nuclear receptors TR2/TR4 (part of DRED) and the protein arginine methyltransferase PRMT5, involving DNA methylation and HDACs 1 and 2 epigenetic modifiers [131–133]. Regulators of the key TFs, such as microRNA-15a and 16-1 in controlling MYB, could also have a potential role in regulating HbF levels [134]. LRF (leukemia/lymphoma-related factor) was identified through studies of its role in erythroid differentiation by Maeda and co-workers [131]. Inactivation of BCL11A or LRF in HUDEP-2 immortalized cells led to increase of 50–60% HbF. In double knockout cells, HbF comprised > 90% of total hemoglobin suggesting that the silencing pathway of LRF is independent of BCL11A [131]. To date, neither GWASs or targeted search for rare variants in individuals with elevated HbF have associated LRF genetic variation with HbF expression.

5. Potential therapies for β thalassemia

Hematopoietic stem cell (HSC) transplant from a fully matched-sibling donor (allogeneic transplantation) can cure β thalassemia effectively, approximately 2000 transplants have been done so far but this approach is limited by the availability of matched donors and is accompanied by potential immunological side effects (graft versus host disease (GVHD) or graft rejection) [135]. There is a need to explore novel therapeutic approaches – pharmacological and genetic – taking advantage of new insights on pathophysiology of the disease as well as development of genome technology. Gene therapy using patient-derived (ie. autologous) HSCs, avoids the risk of GVHD and is available for

all patients, but the HSCs have to be genetically modified ex-vivo [136,137]. Gene therapy approaches for modifying the HSCs include gene addition using lentiviral vectors (β-like globin transgenes, shRNA for erythroid-specific expression and lentiviral expression of a ZF-LDB1 fusion protein) targeting different aspects of the pathophysiology – increasing β globin or increasing γ globin. Genome editing involves correction of the β-thalassemic mutations via nuclease-induced HDR, disruption of modifier gene e.g. *BCL11A* to increase HbF expression, and creation of mutations in the *HBB* complex to simulate HPFH variants. Detailed coverage of the genome-editing approaches is beyond the scope of the review but the reader should refer to Cavazzana et al. [138] and Maeder and Gersbach [139].

5.1. Therapeutic induction of HbF

Advances in unravelling the molecular mechanisms controlling gamma-globin gene expression has led to new generations of agents that fall into 2 groups - those that affect chromatin regulators (such decitabine on DNA methylation and histone deacetylase inhibitors) and, others that affect DNA-binding transcription factors. Several trials of HbF-inducing agents are under investigation. Potentially three transcription factors – BCL11A, KLF1 and MYB – can be considered for manipulation of gamma-globin expression. BCL11A is a potent repressor of HbF but it has important roles in neuronal development and B-cell function. Potential clinical exploitation of reducing B*CL11A* expression by RNA interference, will require novel vectors that can restrict the effect to the erythroid lineage [140]. Dissection of the erythroid-specific enhancer down to a small region in the gene offers the possibility of disrupting this region and specifically targeting erythroid function using genome-editing technology such as zinc finger nucleases or CRISPR-Cas 9 [91,141]. Ubiquitous expression of MYB and its essential role in hematopoiesis, raises concerns on the ability to achieve adequate therapeutic window. KLF1 is erythroid-specific, and has pleiotropic effects on erythropoiesis. Experiments of nature have shown that loss of one copy of *KLF1* leads to HbF increases [122] and is clinically beneficial in β thalassemia [127]. But it will be very difficult to target KLF1 with small molecule inhibitors.

One could also “de-repress” gamma-globin expression by forcing interaction of the β-locus control region (β-LCR) with the γ-gene using a synthetic DNA-binding protein [142,143]. Genome-editing approaches involve gene therapy in which hematopoietic stem cells from the patient will be subjected to gene editing ex-vivo and then returned to the patient for reconstitution [132]. Safety profile of such a technology is still uncertain.

The last decade has also seen considerable advances in gene addition therapy using lentiviral vectors (antisickling β-globin or γ-increasing) in autologous hematopoietic stem cell transplantation (HSCT). The most advanced of these is the antisickling β-globin vector containing the HbA^{T87Q} mutation (Bluebird Bio), first tested in a patient with transfusion-dependent HbE/β-thalassemia [144,145]. This vector has also been used in patients with SCD, the first such treated case of SCD reported therapeutic > 50% antisickling β-hemoglobin, absence of crises, correction of disease hallmarks, and no evidence of insertional mutagenesis [146].

5.2. Targeting dyserythropoiesis

5.2.1. Activin receptor-II trap ligands

Erythropoiesis, the synthesis of new red blood cells, involves an intricate process of proliferation and differentiation from erythroid progenitors, and takes place primarily in the bone marrow. Bone marrow of patients with β-thalassemia shows immense erythroid activity but the erythropoiesis is ineffective due to premature death of the differentiating erythroid precursors. The hypoxic environment due to the ensuing anemia leads to increase in erythropoietin (EPO) production and other factors (such as members of the transforming growth factor-beta (TGF-β) and Activin receptor-II (ActR-II) trap ligands that form complexes stimulating further increase in erythropoiesis that continues to be ineffective. Binding of EPO to EPO receptor activates multiple transduction pathways, including Jak2/Stat5. Two compounds – luspatercept and sotatercept – that bind to trap ligands and GDF-11, have been developed in animal models and transferred to clinical use in humans [147]. Sotatercept and luspatercept prevent activins binding to ActR-II and the activation of Smad 4 dependent signaling pathway, improving erythroid maturation and red cell production. A phase 3 study multicentric, multinational study with luspartecept (BELIEVE, NCT02604433) is ongoing in β-thalassemia and HbE/β-thalassemia subjects. Preliminary results are promising, with a reduction in transfusion requirements.

5.2.2. Iron restriction

Iron availability affects erythropoiesis [148]. Hepcidin is the hormone that controls iron absorption, it targets ferroportin (FPN) an iron exporter. Patients with beta-thalassemia, in particular, the milder non-transfusion dependent forms, develop systemic iron overload from intestinal absorption due to inappropriately low hepcidin. Based on these observations, a target may be to increase hepcidin, which led to the development of hepicdin agonists, such as minihepcidin (MH) which are short peptide mimetics (AA) that are sufficient to target degradation of FPN [149]. An alternative method of increasing hepcidin in vivo is inhibition of the transmembrane serine protease TMPRSS6 which is essential in the activation of hepcidin [150]. Patients with iron-refractory iron deficiency anemia (IRIDA) have mutations in TMPRSS6 with elevated hepcidin levels and are refractory to oral iron, and partially to parenteral iron therapy [151]. Approaches reducing TMPSS6 include using antisense oligonucleotides (ASOs) and RNA interference (siRNA), both of which have been applied in murine models with beneficial effects [152,153].

Transferrin saturation is increased in all patients with beta-thalassemia, the unchaperoned iron underlies toxicity of the iron overload. This observation has prompted the approach of administering apotransferrin to beta-thalassemic mice, normalization of the labile plasma iron concentration normalized RBC survival, increased hemoglobin together with decreased reticulocyte, Epo levels and splenomegaly [154]. The data suggest the TF therapy could be beneficial in β thalassemic patients.

5.2.3. JAK2 inhibitors

β thalassemic mice have elevated Epo levels associated with increased levels of Jak2 phosphorylation leading to significant ineffective erythropoiesis and extramedullary hemopoiesis [155]. Jak2 inhibitors have been shown to be effective in reducing splenomegaly in β thalassemic mice. Several Jak2 inhibitors have now been developed with beneficial results in patients with myelofibrosis and Jak2-related polycythemia vera [156]. Jak2 inhibitors could be most beneficial for NTDT patients with splenomegaly.

5.3. Reducing α globin synthesis

The key pathophysiological mechanism leading to the ineffective erythropoiesis in β thalassemia is the continual production of α globin and accumulation of the free excess α globin in the erythroid precursor cells. Clinical studies over the last 30 years indicate that a natural reduction in α globin chain output through co-inheritance of α thalassemia ameliorates the disease phenotype in patients with β thalassemia [157]. The challenge here is tissue specific selective silencing of the α globin expression to an appropriate degree to be useful for patients with β thalassemia [158]. Plausible approaches include post-transcriptional silencing through RNA interference (RNAi) using small interfering RNAs, short hairpin RNA, epigenetic drug targeting to alter chromatin environment of the α globin genes, and genome-editing to disrupt expression of the α globin genes [159].

6. Concluding remarks

Early clinical and population studies provided evidence that an innate ability to produce fetal hemoglobin was clinically beneficial for patients with β thalassemia and sickle cell disease, prompting many studies and clinical trials of pharmacological agents for HbF reactivation in the 1980′s and 90′s. Of the agents, hydroxyurea with the lowest toxicity profile, remains the most widely used, and the only agent licensed for treatment of SCD, but its efficacy in β thalassemia is less proven. In the 1980′s allogeneic HSCT was introduced as a treatment option, to date ~2000 transplants have been carried out globally. Although the results have improved dramatically through improved conditioning, time of transplant and better support, allogeneic HSCT is limited by availability of fully-matched donors and potential immunological side effects. Gene therapy using patient-derived (i.e. autologous) HSCs, avoids the risk of graft versus host disease and is available for all patients, but the HSCs have to be genetically modified ex-vivo. Although tremendous progress has been made technically, with clinical trials underway, its safety profile is still being evaluated. Recent discoveries and understanding in the switch from fetal to adult hemoglobin has opened up new pharmacological and genetic targets for HbF reactivation. Similarly, improved understanding of the mechanisms associated with ineffective and abnormal erythropoiesis have also provided additional therapeutic targets, a couple of which are currently being tested in clinical trials.

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