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Understanding α-globin gene regulation and implications for the treatment of β-thalassemia

Sachith Mettananda,[1,2] Richard J. Gibbons,[1] and Douglas R. Higgs[1,3]

[1]Medical Research Council (MRC) Molecular Hematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom. [2]Department of Pediatrics, Faculty of Medicine, University of Kelaniya, Sri Lanka. [3]Oxford National Institute for Health Research Biomedical Research Centre, Blood Theme, Oxford University Hospital, Oxford, United Kingdom

Address for correspondence: Douglas R. Higgs, MRC Molecular Hematology Unit, Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK. doug.higgs@imm.ox.ac.uk

Over the past three decades, a vast amount of new information has been uncovered describing how the globin genes are regulated. This knowledge has provided significant insights into the general understanding of the regulation of human genes. It is now known that molecular defects within and around the α- and β-globin genes, as well as in the distant regulatory elements, can cause thalassemia. Unbalanced production of globin chains owing to defective synthesis of one, and the continued unopposed synthesis of another, is the central causative factor in the cellular pathology and pathophysiology of thalassemia. A large body of clinical, genetic, and experimental evidence suggests that altering globin chain imbalance by reducing the production of α-globin synthesis ameliorates the disease severity in patients with β-thalassemia. With the development of new genetic-based therapeutic tools that have a potential to decrease the expression of a selected gene in a tissue-specific manner, the possibility of decreasing expression of the α-globin gene to improve the clinical severity of β-thalassemia could become a reality.

Keywords: α-globin; β-thalassemia; gene regulation; epigenetic drug targeting; genome editing

Introduction

Hemoglobin, the highly specialized oxygen carrier molecule in human red blood cells (RBCs), is composed of two α- and two β-like globin chains and four heme groups, each having an iron (Fe^{2+}) ion as a cofactor. The α- and β-globin gene clusters are two of the most extensively studied regions of the human genome, and the genes in both loci demonstrate well-coordinated, tissue- and developmental stage–specific patterns of expression. Over the past three decades, a vast amount of new information has been uncovered on the precise regulation of these genes, which includes *cis*-acting regulatory elements (enhancers), *trans*-acting transcription factors (TFs), and epigenetic mechanisms mediated via chromatin modifications. Extensive knowledge on how these genes are regulated has provided significant insights into the general understanding of the regulation of human genes.[1]

Thalassemia is caused by impaired synthesis of either α- or β-globin chains. Reduced or absent production of β-globin chains, associated with nearly 300 genetic defects in and around the β-globin gene, result in β-thalassemia,[2] the most clinically significant form of thalassemia. Despite being one of the first molecular diseases identified and to have its pathophysiology characterized, the management of β-thalassemia is still far from optimal, limited to supportive therapy in the majority of patients.[3] It has been known for many decades that natural reductions of α-globin output, in the form of coinherited α-thalassemia, ameliorate the disease phenotype in patients with β-thalassemia.[4] However, except for a limited number of studies, the usefulness of this pathway for treatment of β-thalassemia has not been explored.

During the past few years, genetic therapies have made tremendous progress and generated considerable excitement in the field of translational

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medicine. Thanks to this, the scope of understanding of gene regulation has transformed from merely academic exercises to wider applications, including the ability to treat diseases. The range of potential genetic therapies now spreads far beyond standard gene therapy approaches to methods that fine-tune the expression of specific genes in a tissue-specific manner.[5,6]

In this review, we first summarize current knowledge on the regulation of the α-globin genes. We then present the clinical, genetic, and experimental evidence describing how silencing of the α-globin genes ameliorates the clinical phenotype of β-thalassemia. Finally, with the help of new genetic-based therapeutic tools, we propose various pathways that could be utilized to selectively silence the α-globin gene during erythropoiesis.

Regulation of α-globin

Distal enhancers of α-globin genes. The human α-globin gene cluster is located on the short arm of chromosome 16 (16p13.3) very close (∼150 kb) to the telomere. The *cis*-acting regulatory network of this gene cluster has been well characterized, and it is now known that expression of the α-globin genes is controlled by distant enhancers located 10–48 kb upstream of the genes (Fig. 1). Distal enhancer regulators of α-globin genes were first identified in the early 1990s, following a report of a family with α-thalassemia due to a large (62 kb) deletion upstream of the α-globin locus, which leaves the α-globin genes intact.[7,8] A more recent comparative sequence analysis of 22 species identified a conserved syntenic region of ∼135 kb containing the α-globin cluster and all of the *cis*-acting regulatory elements, including multispecies conserved sequences (MCSs) required for full tissue- and developmental stage–specific expression of α-globin.[9] Mapping the positions of DNAse1 hypersensitive sites to the MCSs showed that in humans there are four erythroid-specific distant enhancers situated 10 kb (MCS-R4), 33 kb (MCS-R3), 40 kb (MCS-R2), and 48 kb (MCS-R1) upstream of the gene locus within the conserved syntenic region. These elements enhance human α-globin expression.[9,10]

The four upstream enhancers vary in their capacity to enhance α-globin expression. A variety of experiments have suggested that MCS-R2 (previously known as HS-40) is the most critical regulatory element, capable of enhancing α-globin expression on its own.[8,11] Multispecies analysis revealed that MCS-R2 is the most highly conserved regulatory element and was detected in all mammals.[9] In a humanized mouse model, in which the mouse α-globin locus was replaced by a 117-kb segment of DNA containing the human α-globin gene cluster and regulatory elements, the deletion of a 1.1-kb segment covering MCS-R2 in the humanized chromosome resulted in a reduction of human α-globin expression to very low levels.[12] Similarly, all naturally occurring human deletions that cause α-thalassemia by removing the upstream regulatory elements include MCS-R2.[13] A rare patient homozygous for a naturally occurring deletion limited to the MCS-R2 region developed α-thalassemia with hemoglobin H disease.[14] These observations confirm that MCS-R2 is crucial for the expression of the human α-globin genes. Of the remaining enhancer-like elements, only MCS-R1 has been shown to have significant activity *in vivo*.

Epigenetic regulation of α-globin genes. The human α-globin gene cluster is located in a gene-dense, early replicating, and open-chromatin region of the genome surrounded by a number of housekeeping genes that are expressed in all cell types.[1] DNA at this locus is substantially unmethylated, and the promoters of all α-like globin genes are associated with unmethylated cytosine–guanine dinucleotide (CpG)–rich islands. Because the human α-globin locus is in an open chromatin environment, the genes are actively silenced through a transcriptional repressive protein complex known as polycomb repressive complex (PRC) 2 in nonerythroid cells, in which α-globin is not expressed.[15] The transcriptional repressor action of PRC2 is associated with a histone methyltransferase (EZH2) that increases trimethylation of histone H3 lysine 27 (H3K27me3), a chromatin signature associated with gene silencing.[16] The mechanism involved in the binding of the PRC2 complex to the target genomic sites is not fully understood but clearly depends on the unmethylated CpG island promoters.[17] In nonerythroid cells in humans, PRC2 is recruited to the α-globin gene promoter and the chromatin is modified by H3K27me3; this is associated with transcriptional silencing.[15]

In humanized mouse erythroid cells, when the α-globin gene is activated, PRC2 is displaced and the H3K27me3 chromatin mark is erased through both

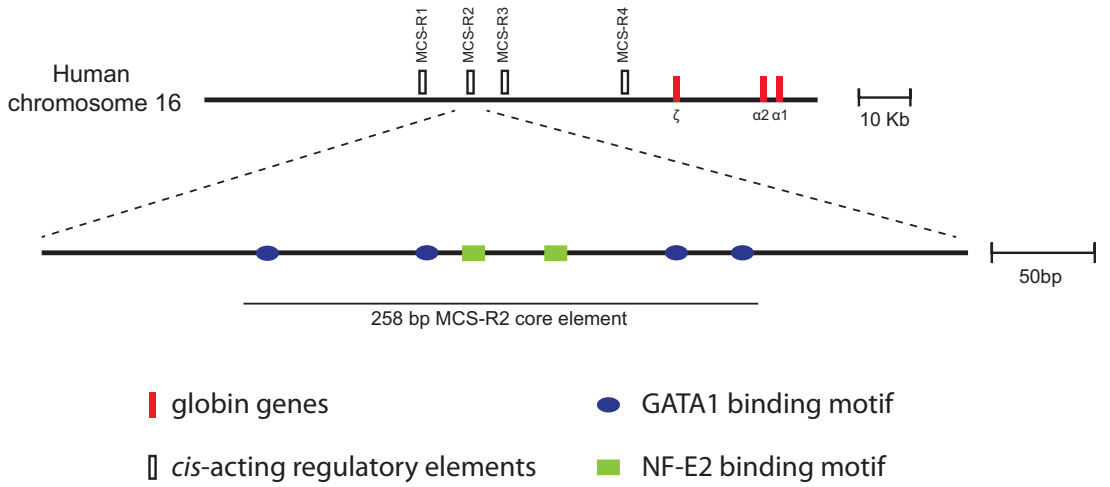

Figure 1. Schematic representation of the α-globin gene cluster (top) and the MCS-R2 enhancer element (bottom). The main globin genes in the α-globin gene cluster are ζ (embryonic) and α1 and α2 (adult). The four upstream regulatory elements of the α-locus situated 10–48 kb upstream to the genes are known as MCSR1–MCSR4. The most critical MCS-R2 enhancer has binding sites for erythroid-specific TFs GATA1 and NF-E2.

passive and active mechanisms. The active mechanism of demethylation of H3K27me3 is thought to be mediated via histone lysine demethylase (KDM) 6B (also known as Jumonji domain–containing protein 3 (JMJD3)).[18] In addition, as cells differentiate into erythroid cells, a dramatic increase in the levels of the H3K4me3 chromatin modification that signals transcriptional activation is also seen at the promoter of the α-globin genes.[19] This is believed to be mediated through the action of Set/MLL histone methyltransferases. The net effect of these chromatin modifications and recruitment of TFs is the activation of transcription of the α-globin genes to produce large amounts of globin mRNA and protein required to make hemoglobin.

TFs controlling expression of α-globin genes. During human erythroid differentiation, the key TFs, GATA-binding factor 1 (GATA1), Kruppel-like factor-1 (KLF1), stem cell leukemia (SCL), TF E2-alpha (E2A), Lim-only 2 (LMO2), and LIM domain–binding protein 1 (Ldb1) are recruited to the four upstream enhancers of the α-globin genes. In addition, another erythroid TF, nuclear factor-erythroid 2 (NF-E2), is bound to two of the enhancers: MCS-R2 and to a lesser extent MCS-R1.[20] Finally, general TFs together with RNA polymerase II (PolII) (collectively referred to as the preinitiation complex) are recruited to the MCS-R1 and MCS-R2 enhancers and then subsequently to the α-globin promoters, thereby initiating transcription. During this process, a physical interaction between enhancers and promoters is thought to occur through the mechanism of chromatin looping.[21,22]

Contrasting regulation of α- and β-globin gene expression. Despite being expressed in the same cell type, in a similar developmental stage–specific manner, the regulation of β-globin demonstrates significant differences from that of α-globin. The human β-globin gene is located on chromosome 11 in a relatively gene-sparse, late-replicating region of the genome that, in nonerythroid cells, is in a closed heterochromatic environment.[1,23] The promoter of β-globin is methylated and not associated with CpG islands. Therefore, in contrast to α-globin, in nonerythroid cells the promoter of β-globin is not bound by PRC2 and does not have the repressive chromatin mark H3K27me3.[15,24] Instead, silencing of the β-globin gene is mediated through DNA methylation and colocalization with nuclear heterochromatin.[1]

In erythroid cells, when the β-globin gene is activated, in a process similar to α-globin regulation, the distant enhancers (the β-locus control region) and the gene promoters are bound by erythroid-specific TFs, which include (but are not limited to) GATA1, NF-E2, KLF1, and SCL.[25,26] Similar to α-globin gene regulation, the interaction of β-globin gene enhancers and promoters is believed to be

maintained by chromosomal looping.[27] The promoter is marked by the H3K4me3 active chromatin modification. However, in contrast to the α-globin gene, the repressive chromatin modification H3K27me3 is not present at the β-locus, and histone demethylases have not been shown to play a role in the activation of β-globin.[15]

Reduction of α-globin as a therapeutic option for β-thalassemia

Role of free α-globin chains in the molecular pathology of β-thalassemia. Unbalanced production of α- and β-globin chains within erythroid cells is the main causative factor leading to hemolysis and ineffective erythropoiesis, which are the two most important contributing factors to anemia in thalassemia.[4,28] During every stage of normal development, production of α-like and β-like globin chains is closely balanced to prevent an accumulation of free globin chains that damage RBCs. The deleterious effects of small amounts of free α-globin chains are diminished by the erythroid-specific molecular chaperone α-hemoglobin stabilizing protein (AHSP). AHSP specifically binds to α-globin and stabilizes free α-chains by promoting protein folding and resistance to protease digestion, thereby protecting erythroid cells from the toxic effects of free α-globin.[29]

In patients with β-thalassemia, impaired production of β-globin chains results in an excess of free α-globin chains. The levels of free α-globin chains exceed the capacity of AHSP to buffer their effect. Once this capacity is exceeded, highly unstable free α-globin molecules undergo auto-oxidation, forming α-hemichromes (α-globin monomers that contain oxidized ferric iron) and reactive oxygen species (ROS) that trigger a cascade of events leading to hemolysis and ineffective erythropoiesis (Fig. 2).[30,31]

Coinheritance of α- and β-thalassemia. Since the initial descriptions by Fessas *et al.*[32] and Kan and Nathan,[33] coinheritance of α-thalassemia has been predicted to ameliorate the severity of β-thalassemia. A number of family, cohort, and case-control studies have reported that the coinheritance of a deletion of two α-globin genes (– –/α α or – α/– α) is associated with a milder clinical phenotype in most patients with β-thalassemia, while deletion of a single α-globin gene (– α/α α) is beneficial in patients other than those with the most severe reduction in β-globin synthesis (β^0/β^0 genotype).[34–40] Furthermore, a recent study showed that the presence of a mutated α-globin allele provides an additive effect to induction of γ-globin expression, the other major disease modifier of β-thalassemia.[41]

Coinheritance of α-thalassemia appears to have its most pronounced effect on patients with HbE β-thalassemia, the most common subtype of β-thalassemia worldwide. Importantly, coinheritance of even a single α-globin gene deletion (– α/α α) is able to produce beneficial effects in these patients. In large-scale prospective studies, all patients with coinherited α-thalassemia displayed a milder phenotype, older age at presentation, smaller splenic and hepatic sizes, normal physical and sexual maturation, and significantly less transfusion requirement.[39,42–46] Furthermore, in HbE β-thalassemia patient cohorts, the frequency of α-thalassemia is significantly lower than in the normal matched population. Similarly, it is extremely rare to find a patient with HbE β-thalassemia and two α-globin gene deletions (– –/α α or – α/– α) requiring transfusion treatment.[42,44] These observations suggest that individuals with HbE β-thalassemia, who coinherit only two, rather than the normal four, α-globin genes, have a very mild phenotype and rarely come to medical attention.

Effect of excess α-globin chains on β-thalassemia. Contrary to the beneficial effect demonstrated by coinheritance of α- and β-thalassemia, inheritance of excess α-globin genes in the presence of β-thalassemia worsens disease severity.[47–49] Patients with heterozygous β-thalassemia mutations, who would otherwise have a β-thalassemia minor phenotype, develop a thalassemia intermedia phenotype when they inherit more than four functional copies of the α-globin gene. This is because the inheritance of excess α-globin genes in these patients increases the amount of free α-globin within RBCs and worsens the α-like/β-like globin chain imbalance. Increase in free α-globin chains exacerbates the premature erythroid cell destruction and worsens the severity of anemia. This observation further emphasizes that the number of functional α-globin genes has clear and direct effects on the clinical severity of patients with β-thalassemia.

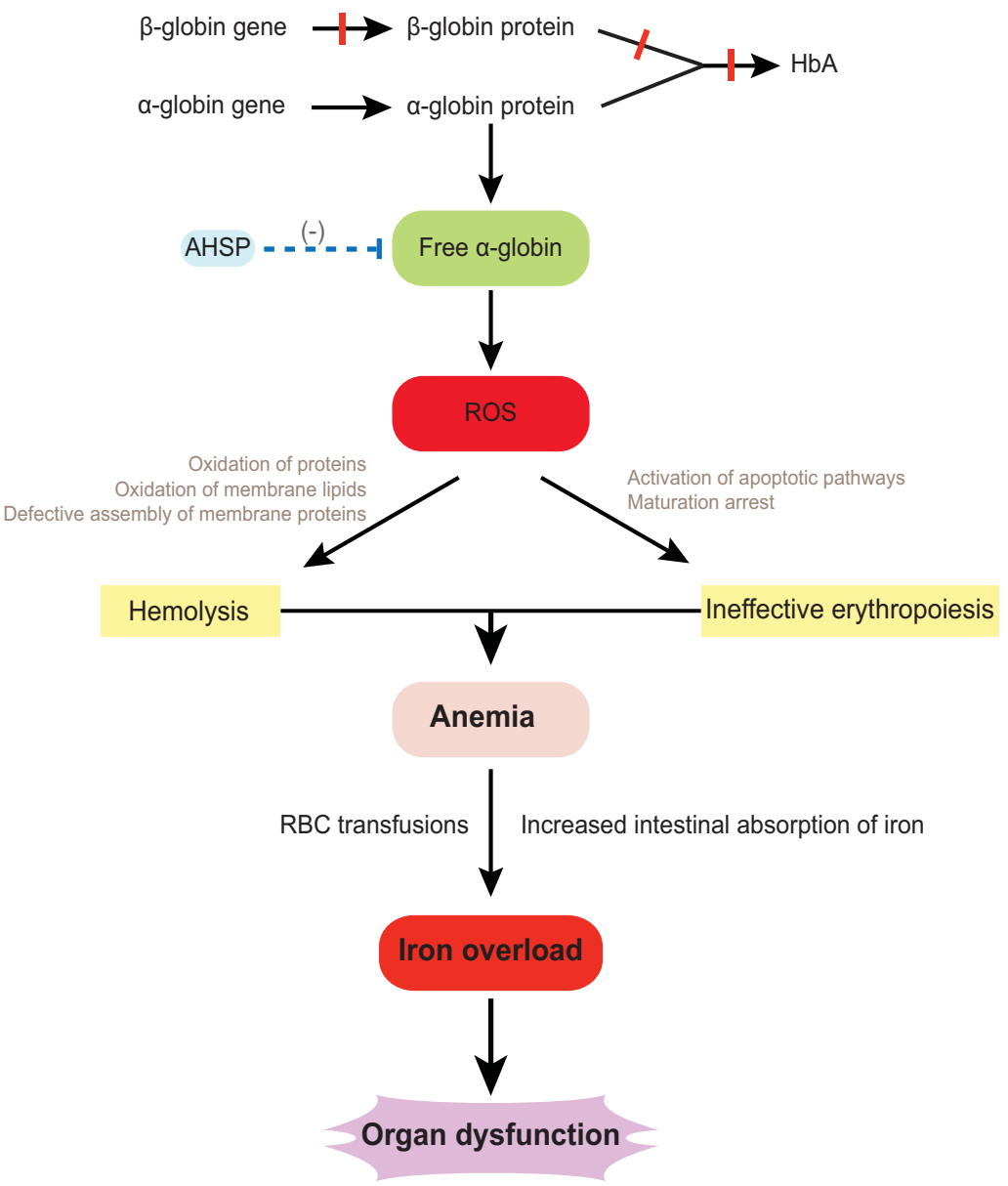

Figure 2. Pathophysiology of β-thalassemia. Absent or reduced β-globin production leads to an unbalanced excess of α-globin chains, which then trigger a cascade of events through the generation of ROS, resulting in hemolysis of mature RBCs and destruction of immature erythroid precursors in the bone marrow (ineffective erythropoiesis). The resultant anemia increases the intestinal iron absorption, which together with transfusion therapy leads to body iron overload and subsequent organ dysfunction.

Evidence from mouse models. Amelioration of β-thalassemia by reducing α-globin has also been recapitulated in the β-globin gene knockout (β-KO) mouse model. Voon *et al.* generated a double-heterozygous mouse knocked-out for both α- and β-globin genes (α + +/− −; β +/−) and compared the hematological parameters to those of the β-KO mice (α + +/+ +; β +/−).[50] Interestingly, all the observed parameters, including hemoglobin level, hematocrit, red cell distribution width, reticulocyte number, and the percentage of erythroid progenitors in the bone marrow and spleen were corrected to near-normal values in the double-heterozygous mouse. This observation further

emphasizes that a more balanced globin chain synthesis caused by coinheritance of α-thalassemia might make profound improvements to the β-thalassemia phenotype and provides an experimental proof that α-globin is a suitable target for developing therapies.

Desired level of reduction of α-globin. The clinical, genetic, and experimental evidence discussed in the preceding sections strongly support the idea that reducing α-globin expression in patients with β-thalassemia is a rational pathway to develop new therapies. Reducing α-globin output to levels comparable to a single or double α-globin gene deletion should be clinically beneficial to patients with β-thalassemia. Among patients with α-thalassemia, single and double α-globin gene deletions correspond to 75% and 50% α-globin levels, respectively, compared to normal controls. Therefore, these levels could be considered as rational therapeutic aims in β-thalassemia.[13,51] Although the data on coinheritance of β-thalassemia and three α-globin gene deletions are sparse, reductions down to 25% of the normal levels (as seen in patients with α-thalassemia due to three α-globin gene deletions) do not result in significant morbidity due to anemia and can be considered as a safe tolerable lower limit.

Selective silencing of α-globin expression: therapeutic pathways

The development of new drugs targeting specific epigenetic pathways and the advent of genome engineering using programmable, sequence-specific endonucleases have generated potentials to specifically regulate the expression of a single gene. Similarly, the current understanding of the regulation of α- and β-globin genes in erythroid cells has uncovered pathways to consider new strategies to selectively control α-globin expression to the appropriate degree to be useful for patients with β-thalassemia. Several pathways warrant careful consideration. Posttranscriptional gene silencing through RNA interference (RNAi) is one such plausible avenue that has been explored, to some extent, in the past. Pharmacological targeting of the contrasting epigenetic regulation of α- and β-globin genes and selective silencing of α-globin using genome engineering strategies are the other pathways that have potential for clinical application.

RNAi strategy. RNAi mediated through small double-stranded RNAs (small interfering RNA (siRNA) and short hairpin RNA (shRNA)) can produce efficient and specific posttranscriptional gene silencing.[52,53] Attempts to reduce α-globin expression using this technique have been reported in the past. Up to 50% knockdown of α-globin gene expression, using siRNA targeting the α-globin in β-thalassemia murine primary erythroid cells, demonstrated significant phenotypic improvement by reducing the levels of intracellular ROS.[54] Transgenic β-thalassemia mice that produced 20–35% less α-globin, generated by microinjecting shRNA targeting α-globin into single-cell embryos, demonstrated sustained phenotypic improvement in the RBCs with less poikilocytosis, fewer target cells, increased hemoglobin values and red cell counts, and low reticulocyte counts.[55] However, direct injection of siRNA plasmids targeting α-globin mRNA into the tail veins of the β-thalassemia mice did not produce improvements in red cell counts and hemoglobin levels.[56]

Pharmacological strategy through epigenetic drug targeting. Small molecules that alter the chromatin environment by selectively inhibiting epigenetic enzymes have become popular as new therapeutics in recent years.[6] Although most are indicated and trialled for various forms of cancers, their usefulness in the treatment of nonmalignant conditions are being investigated. In fact, the results from the very first clinical trial that tested the effect of a histone deacetylase inhibitor for sickle cell disease were recently published.[57] As outlined earlier, the chromatin environment and silencing mechanisms of the α- and β-globin clusters have very clear differences. This distinction may be very useful in the development of therapeutic strategies, and it is likely that drugs or small molecules that act by altering the chromatin environment will have differential effects on α- and β-globin expression.

Genome editing strategy. Genome editing using sequence-specific, programmable, artificially engineered nucleases has provided a realistic approach for the treatment of many human genetic diseases, including the hemoglobinopathies. These nucleases create double-strand breaks at specific chosen locations in the genome, which when repaired create mutations at the targeted sites and, depending on their position, result in either loss of gene

function or disruption of noncoding regulatory sequences. The zinc-finger nuclease (ZFN) was the first such technology to be developed. Although this held promise, many drawbacks, including cytotoxicity, low targeting efficiency, difficulties in engineering proteins for each target, and limitations in target sites (one per ∼100 bp), precluded its widespread use.[58] The second group, transcription activator–like effector nucleases (TALENs), were popular, as they dramatically increased the availability of target sites, allowing almost any sequence to be targeted. However, the construction of TALENs is challenging and time consuming.[59] Both ZFNs and TALENs use DNA-binding proteins to tether endonuclease catalytic domains to specific genomic loci. By contrast, in the recently developed RNA-guided CRISPR–Cas9 (clustered, regularly interspaced, short palindromic repeat–CRISPR associated system) system, the endonuclease Cas9 is guided by small RNAs through Watson–Crick base pairing with target DNA.[60] Therefore, it represents a system that is markedly easier to design, highly specific, efficient, and well suited for high-throughput and multiplexed gene editing for a variety of cell types and organisms.[61]

These genome editing approaches can be used to disrupt the expression of the α-globin gene. This can be done by directly targeting the genes or through targeting regulatory elements of the genes. If this strategy is successful, translation into clinical practice would broadly involve three steps. First, the hematopoietic stem cells (HSCs) will be harvested from patients with β-thalassemia after mobilization using granulocyte colony-stimulating factor. Then the harvested HSCs will be edited *ex vivo* using programmable nucleases and transplanted back to the same patient after myeloablation.

Conclusions

Advances in the understanding of the regulation of α- and β-globin genes have demonstrated clear and striking differences in the silencing mechanisms of these two gene clusters. Pathways are thus uncovered for selective silencing of α-globin expression to levels beneficial to patients with β-thalassemia. Novel genetic therapeutic tools now offer the potential to translate these theoretical mechanisms into practical realities. In this way, we are approaching the dawn of realizing the transformation of a long-standing clinical observation to an effective therapy in β-thalassemia.

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Conflicts of interest

The authors declare no conflicts of interest.

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