Chapter

Transcriptional Repressors of Fetal Globin Genes as Novel Therapeutic Targets in Beta-Thalassemia

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Abstract

During development the human β-globin gene cluster undergoes two switching processes at the embryo-fetal and fetal-adult stages, respectively, involving changes in chromatin remodeling and in transcriptional regulatory networks. In particular, during the perinatal period, the switch from fetal-to-adult globin gene expression leads to fetal globin genes silencing and progressive decline of fetal hemoglobin (HbF). Impaired hemoglobin switching is associated with hereditary persistence of HbF (HPFH), a condition in which the fetal globin genes fail to be completely silenced in adult red blood cells. This condition, when co-inherited with hemoglobinopathies, has great therapeutic potential because elevated HbF levels can ameliorate β-thalassemia and sickle cell anemia. Therefore, there is a growing interest about the complex network of factors that regulate fetal globin genes expression. Here we discuss the activity of transcriptional repressors of fetal globin genes and their potential role as novel therapeutic targets in β-thalassemia.

Keywords: globin gene switching, HbF silencing, LCR, transcriptional repressors, genome editing

1. Introduction

The human β-globin locus consists of five functional genes, HBE, HBG2, HBG1, HBD, and HBB, positioned on chromosome 11 in the same order in which they are expressed during ontogenesis. This fascinating process that regulates changes in the globin gene expression, referred to as globin gene switching, is programmed in order to produce different hemoglobin molecules able to meet the changing oxygen demand of the developing organism [1]. According to this process, up to the eighth week of pregnancy, the embryonic globin gene (HBE) is predominantly expressed in the primitive erythroblasts, large nucleated erythroid cells originating in the yolk sac. Approximately between the sixth and eighth week of pregnancy, fetal liver assumes the hematopoietic role and produces small, enucleated erythroid cells. The transition of hematopoiesis from the yolk sac to the fetal liver coincides with the switch from the embryonic HBE gene to the fetal globin genes (HBG1 and HBG2). At the perinatal period, the bone marrow becomes the principal site of hematopoiesis...
and generates mature red blood cells. In conjunction with the transition from liver to bone marrow hematopoiesis, the second globin gene switch occurs from the fetal-to-adult globin genes (HBD and HBB) in which fetal hemoglobin (HbF) production gradually declines to be replaced by the adult type (HbA) [2].

In the last decades, large research efforts have been devoted to disclose the molecular mechanisms underlying this switching process, thus revealing how it is under the tight control of complex interactions involving dynamic changes in chromatin remodeling as well as in transcriptional regulatory networks. Nevertheless, it is rather common that residual amount of fetal globin genes continues to be expressed throughout adult life with the majority of adults having traces of HbF (approximately 1% of total hemoglobin). Impairment of this fetal-to-adult hemoglobin switching process, whose molecular basis has been associated with a heterogeneous group of inherited defects, leads to hereditary persistence of fetal hemoglobin (HPFH) in adult life. Based on clinical and genetic observations that HPFH has ameliorative effects in β-thalassemia and in sickle cell anemia (SCD), interest has been growing regarding the therapeutic potential of fetal globin gene reawakening for these disorders [3–5]. Therefore, much effort is currently underway in order to further clarify the molecular basis of hemoglobin switching and persistence of high HbF levels in adult life.

Fetal hemoglobin levels are regulated by complex mechanisms involving factors linked or not to the β-globin gene (HBB) locus. Several genetic determinants in cis to the HBB cluster, including large deletions in the HBB cluster or single nucleotide sequence variations in the regulatory regions of the fetal globin genes, are known to influence HbF levels [6, 7]. Also, HPFH quantitative trait loci (QTL) unlinked to the HBB cluster have been so far identified as being involved in the transcriptional control of fetal globin genes. In particular, in the last years, great attention has been focused on two genetic determinants, the HBS1L-MYB intergenic region located on chromosome 6q23 and the gene coding for the oncogenic transcriptional factor BCL11A on chromosome 2p16 that act as main QTLs in influencing HbF levels [8].

Below we discuss the contribution of some of the main cis- and trans-acting determinants so far identified in silencing fetal globin gene expression and the most recent therapeutic advances based on fetal globin gene reactivation in the treatment of hemoglobinopathies.

2. Regulatory elements and transcriptional factors involved in fetal globin gene silencing

2.1 The role of the locus control region (LCR) in chromatin remodeling and globin gene switching

The locus control region (LCR), located upstream of the HBE gene, represents the major regulatory element in the β-globin gene locus. It plays a key role in the switching process by controlling the transcription state of the locus as well as the transcription activity of each gene, thus conferring tissue- and stage-specific expression of the globin genes [9, 10].

The LCR contains five DNase I hypersensitive sites (HS 1–5), four of which (HS1–HS4) acting as erythroid cell-specific enhancers that are required for proper order of activation of the globin genes during ontogeny. However, differently from classical enhancer elements, LCR activity is both orientation- and distance-dependent and prevents position effects, therefore also indicating an insulator function for this regulatory region [9]. The last hypersensitive site (HS5) has a different role, acting as a structural and insulator element. Indeed, in erythroid cells it is closely
positioned to another hypersensitive site located at the opposite end of the \( HBB \) locus (3′HS1) to form an active chromatin hub that allows highly efficient globin gene transcription [10, 11].

It is now generally accepted that globin gene switching can occur through not mutually exclusive mechanisms involving direct competition between stage-specific gene promoters for LCR enhancer elements, gene expression activation, and/or silencing mechanisms promoted by specific activators and repressors as well as by epigenetic modifications. By chromatin conformation capture (3C) experiments, it has been shown that LCR exerts long-distance transcriptional enhancement through dynamic organization of the locus in stage-specific chromatin loops that allow the LCR enhancers to be positioned in close proximity of the genes that are to be activated [12]. This mechanism is also consistent with the evidence that the LCR can interact with only one promoter at a time. These interactions also strictly depend on the availability of specific trans-acting factors, non-DNA-binding factors linked to transcription factors and chromatin facilitators bound to regulatory regions of genes to be transcribed (Figure 1).

It has also been shown that epigenetic mechanisms at the \( HBB \) locus are involved in gene expression control. One of these mechanisms involves DNA and histone methylation processes that are generally associated with the repression state of globin genes in non-erythroid cells as well as with the erythroid stage-specific silencing of globin gene expression within the activated chromatin hub [13]. Recently, HS4 in LCR was found to regulate another important stage-specific epigenetic mechanism of gene expression control by contributing to drive the recruitment of different histone acetylation/deacetylation enzymatic activities at specific regulatory regions, with acetylation being a general hallmark of gene

![Figure 1](image)

*Figure 1.* Different molecular mechanisms proposed for LCR long-distance transcriptional enhancement through dynamic organization of the locus in stage-specific chromatin loops that allows the LCR enhances to be positioned in close proximity of the genes that are to be activated [9–12]. (A) The looping model is based on the concept that LCR acts as a holocomplex to stimulate gene expression by looping through the chromatin to activate the transcription machinery of a single gene at any given time. In this model, individual globin gene promoters are thought to compete for LCR activity. (B) The linking model requires the presence of a continuous protein chain from the LCR to the gene to be transcribed involving sequential stage-specific binding of transcriptional factors as well as of chromatin modifiers to define the chromatin region to be transcribed. (C) The tracking model is based on the idea that a signal such as an enhancer-bound protein complex comprising RNA polymerase II or histone modifiers is able to scan the DNA until a promoter is encountered to activate gene expression, with inactive genes looped out. (D) In the facilitated-tracking model, concepts of both the looping and tracking models are incorporated. In this model, enhancer-bound complexes track along DNA and, when the promoter of a to-be-transcribed gene is encountered, a stable DNA loop structure is formed.
activation and, on the contrary, deacetylation being associated with gene repression mechanisms. More recently, it has also been found that histone acetylation, besides activating high-level gene transcription, also contributes to the formation of specific chromatin looping leading to an open chromatin configuration at the HBB locus. Furthermore, it has also been proved that loss of histone acetylation increases methylation both in the LCR and in fetal globin genes, thus reinforcing the silencing mechanisms of these epigenetic modifications [11].

Chromatin looping, transcriptional protein complexes, and epigenetic modifications require a large array of protein factors whose list grows continuously, thus providing deeper insights into these mechanisms. The role of some of the most relevant of them will be herein discussed.

2.2 Cold-shock domain protein A (CSDA)

Cold-shock domain (CSD) proteins have been reported to be largely involved in a variety of gene expression regulation processes such as transcriptional activation and repression or posttranscriptional mechanisms including mRNA packaging, transport, localization, and stability. In these proteins the CSD domain does not bind to unique DNA sequence motifs, but it is able to recognize intramolecular triplex H-DNA structures, commonly generated by CT-rich sequences. The cold-shock domain protein A (CSDA), also known as DNA-binding protein A (dbpA), is a protein with a molecular weight of about 60 kDa [14]. The CSDA gene is located at position 12p13.1 and comprises 10 exons spanning a 24-kb genomic region. CSDA consists of three structural domains: an alanine/proline (AP)-rich N-terminal domain followed by a central cold-shock domain (CSD) and a C-terminal domain, containing four alternating clusters of basic and acid amino acids. The DNA-binding domain is encoded by exons 1–5. Within this region, the cold-shock domain, comprising about 70 amino acid residues, is encoded by exons 2–5 [14]. A RNP1 motif [GA] – [FY] – [GA] – [FY] – [IVA], conserved in this protein family [15], is present in the 3′ end of exon 2. The exon 6, encoding 69 amino acids, is alternatively spliced. The C-terminus (exons 6–9) C-terminal domain mediates protein-protein interactions [16]. Alternative splicing of exon 6 results in two main isoforms, namely, CSDA isoform a and isoform b, respectively, showing different C-terminal domains and thus potentially able to take part to specific protein complexes [14, 16].

Figure 2.

Binding of the transcriptional repressor CSDA to the −200 promoter region of HBG2 in adult erythropoiesis is impaired by HPFH mutations [18–22].
CSDA acts as a repressor of many cellular genes including the human granulocyte-macrophage colony-stimulating factor (GM-CSF), an important hematopoietic growth factor [17]. More recently, it has been demonstrated that CSDA acts as a repressor of fetal globin gene expression by binding a region −200 bp upstream of the HBG2 gene [18] (Figure 2). This region consists of alternating homopurine and homopyrimidine tracts generating an H-DNA structure [19]. Mutations (HBG2 -200 C → G, −202 C → T, −196 C → T e −195 G → A) that destabilize the intramolecular triplex structure have been found associated with HPFH [20]. Previous studies had suggested that disruption of the intramolecular triplex could abolish CSDA binding sites conditions in this HBG2 promoter region [19, 21, 22], thus leading to persistent expression of fetal hemoglobin in adult life [19]. Based on RNA interference (RNAi) and overexpression studies in human erythroleukemia K562 cells and in primary erythroid cells, CSDA was demonstrated to directly play a repression role in HBG expression [18]. In fact, down- and up-modulation of CSDA levels consistently corresponded to variations of HBG expression levels: CSDA knockdown induced by RNAi resulted in significantly increased expression of HBG genes, whereas its overexpression was associated with reduced HBG mRNA levels. Also, chromatin immunoprecipitation (ChiP) analysis in K562 cells showed that CSDA interacts with this promoter region, thus confirming that CSDA modulates HBG expression at the transcriptional level [18]. Subsequently, it has been proposed that NF-kB and histone deacetylase 2 (HDAC2) interact with CSDA to form a multiprotein complex which take part to the regulation of HBG expression by modulating local chromatin conformation [23], thus highlighting the relevance of the role played by CSDA in fetal globin gene expression and shedding novel light on the molecular mechanisms involved in globin gene switching (Figure 3).

2.3 B-cell lymphoma/leukemia 11A (BCL11A)

The B-cell lymphoma/leukemia 11A (BCL11A) is a multiple zinc-finger transcription factor encoded on chromosome 2p15, previously reported as being implicated in B-lymphocyte production and neurodevelopment [28]. More recently, genome-wide association studies (GWAS) identified single nucleotide polymorphisms (SNPs) in intron 2 of the BCL11A gene as strictly associated with HPFH conditions, thus revealing a hitherto unexplored but crucial role of BCL11A in HbF silencing [29]. Indeed, as was later demonstrated, such SNPs are able to disrupt erythroid-specific enhancer elements required for high-efficient expression of the BCL11A gene [30]. Similarly to CSDA, BCL11A knockdown experiments performed in K562 cells and in human erythroblasts led to increased expression of fetal globin genes, thus providing further evidence that this factor acts as a repressor of HbF expression.

In the last years, many efforts have been aimed at clarifying the repression mechanism exerted by BCL11A at the HBB locus. At this regard, an important contribution has been provided by the identification of the multiprotein complex
interacting with BCL11A that includes GATA-1, FOG-1, RUNX1, KLF1, and SOX6 [24, 31, 32]. In addition, further insights have been provided regarding the BCL11A repression mechanisms by demonstrating how these protein complexes drive the recruitment of a variety of epigenetic factors such as the nucleosome remodeling and deacetylase (NuRD) repressor complex, histone deacetylase (HDAC1 and HDAC2), lysine-specific demethylase (LSD1), and DNA methyltransferase (DNMT1) [25–27] (Figure 3). ChiP analysis also revealed binding sites for BCL11A at regulatory elements within the LCR, as well as at the promoter regions of both embryonic and fetal globin genes and in an intergenic region between the fetal and adult genes, thus indicating that these protein complexes are directly involved in fetal globin gene silencing as well as in long-range interactions that contribute to reshape chromatin loop domains in order to spatially separate the fetal and adult globin genes from the transcriptional machinery and, in the meantime, to promote long-range LCR interactions with the adult globin genes [33–35].

2.4 Krüppel-like factor 1 (KLF1)

The transcription factor KLF1 (Krüppel-like factor 1), formerly known as erythroid Krüppel-like factor (EKLF) for its restricted expression in erythroid cells and its similarity to the protein encoded by the Drosophila segmentation Krüppel gene), plays a multifunctional role in the regulation of a variety of cellular events leading to erythroid differentiation, including erythroid lineage commitment, heme synthesis, and globin gene switching [36–39].

The KLF1 gene (~3kb) is located on chromosome region 19p13.2 and consists of three exons encoding a 362 amino acid protein with an N-terminal region rich in proline residues and containing two short transactivation domains (TAD1 and TAD2) and a C-terminus with three highly conserved Cys2/His2 zinc-finger domains (ZF1, ZF2, and ZF3) that interact with the DNA sequence motif 5′CCMCRCCCN3′ located in CACCC boxes and GC-rich elements in the regulatory regions of its target genes [38].

In the HBB cluster, KLF1 exerts a dual role in fetal-to-adult globin gene switching by different mechanisms. On one hand, as also evidenced by studies on (+/−) KLF1 transgenic mice [12], KLF1 contributes to directly activate the HBB gene in adult life by inducing the formation of chromatin loops that relieve the HBB gene from competition with the HBG genes and favor interactions of the HS2 and HS3 sites in the LCR with the HBB promoter region [40]. On the other hand, KLF1 indirectly contributes to silencing fetal globin gene expression through the activation of BCL11A, a repressor of HBG globin genes transcription, as described above. To date, more than 65 loss-of-function mutations have been reported for the KLF1 gene, most of which are missense mutations found largely within the three zinc-finger domains [41]. Mutations in the KLF1 gene have been reported to interfere with its functions in the erythropoiesis process, thus leading to a wide range of hematological phenotypes, including high borderline HbA2 levels, mild microcytosis, and/or hypochromia but also, importantly, with persistence of fetal hemoglobin expression in adult life. It is thus not surprising that, although identification of carriers of KLF1 mutations may be quite challenging because their phenotypic traits do not present relevant clinical implications, the majority of KLF1 mutations have been detected in populations at risk for hemoglobinopathies according to the evidence that defective KLF1 activity can lead to impaired HbF silencing with ameliorating effects in SCD and in β-thalassemia. Therefore, KLF1 mutations have been considered as a natural model of impaired hemoglobin switching, and, accordingly, KLF1 has been proposed as a new potential therapeutic target in these diseases [39, 42].
2.5 Myb

Myb is a DNA-binding transcriptional regulator of approximately 75 kDa that plays a crucial role in hematopoiesis and erythropoiesis. The MYB gene is located on chromosome 6q23 and codifies for a protein with three functional domains: a highly conserved DNA-binding domain (DBD) near its N-terminus that recognizes and binds the consensus sequence PyAACG/TG, a central transactivation domain (TAD), and a C-terminal negative regulatory domain (NRD) containing a leucine zipper motif important for homodimeration and for protein interactions [43].

A large variety of proteins have been identified so far as Myb interactors: DNA-binding transcription factors that directly bind Myb, transcriptional coactivators that mediate interactions with the transcriptional machinery, and protein factors that are able to modify Myb activity. Each of these of protein factors can act either as activator or repressor of Myb function, depending both on cell type and protein microenvironment [43].

In the last decade, a GWAS approach led to the identification of a set of single nucleotide polymorphisms at the HBS1L-MYB intergenic region, subsequently identified as HMIP-2, displaying a strong association with persistence of fetal hemoglobin in adult life [44–46]. By demonstrating that these SNPs disrupt binding sites at key erythroid enhancers and cause reduced Myb expression levels, it was possible to provide an explanation for the association of the HMIP-2 region with HbF levels and, in the meantime, to identify Myb as a negative regulator of fetal globin genes.

Notably, further evidence of the repressive role of Myb on fetal globin gene expression has also been provided by clinical observations of higher HbF levels in patients with trisomy 13. It has been demonstrated that in this condition, the high HbF levels are related to the dysregulation of two microRNAs, miR-15a and miR-16-1, both localized on chromosome 13q14, which mediate Myb downregulation through the binding to its 3′-UTR region [47].

Nevertheless, in spite of all these evidences, the mechanism of repression of fetal globin genes exerted by Myb is not fully understood. It has been proposed that Myb could regulate HbF expression by two not mutually exclusive mechanisms: by activating the expression levels of fetal globin gene repressors as BCL11A and KLF1 or by controlling the kinetics of erythroid differentiation. In fact, low Myb levels have been found associated with reduced levels of these HBG repressors and also with accelerated erythroid differentiation leading to the release of early erythroid progenitor cells that are still synthesizing predominantly HbF [48]. Furthermore, it has also been found that Myb and BCL11A cooperate with DNMT1 to achieve developmental repression of embryonic and fetal globin genes in adult erythropoiesis [25], thus supporting the evidence that the network of factors involved in fetal globin gene silencing converges on common mechanisms and cooperates at different levels to sustain globin gene switching and to reinforce the process of HbF repression.

3. Novel therapeutic approaches for β-thalassemia

Currently, clinical management of β-thalassemia and SCD patients is largely dependent on regular blood transfusions associated with chelating agents for the treatment of systemic iron overload. Indeed, except for allogeneic transplantation strategies, whose favorable outcome is anyway restricted to the availability of a well-matched donor, or for the more recent haploidentical transplantation option that however carries substantial risks, no other curative option is currently available for hemoglobinopathies. Therefore, due to the relevant global health burden
of these diseases, there is still a great need for effective and definitive large-scale treatments.

In the last decades, given the ameliorative effects of elevated HbF levels that can lead to decreased hemoglobin polymerization in SCD as well as to reduced α-chain imbalance in β-thalassemia, a great deal of effort has been devoted to the development of new therapies aimed at reactivating fetal globin gene expression. Therefore, according to the evidences that hypo-methylated DNA and hyper-acetylated histones are well-known epigenetic marks of transcriptionally active regions, inhibitors of DNA methyltransferase (DNMT) and histone deacetylase (HDAC) have been developed as novel pharmacologic approaches to support transfusion therapy. Among the first drugs to be identified, 5-azacytidine and decitabine were found to induce increased HbF levels through DNA hypo-methylation process. Subsequently, the use of hydroxyurea (HU) was also investigated as a promising HbF inducer agent in adults with severe SCD. Although the mechanism through which HU induces HbF reactivation is still not completely understood, HU treatment was found to increase HbF levels and reduce HbS polymerization, thus improving clinical symptoms and quality of life of these patients. Another epigenetic approach was also exploited through the use of HDAC inhibitors, including butyrate and its derivatives that have been demonstrated to stimulate HbF production in β-thalassemia patients [49].

More recently, many hopes were directed toward the development of gene therapy procedures designed to introduce a normal copy of the β-globin gene by viral vectors that offer the advantages of long-term benefits and, even more importantly, being based on autologous transplant, do not require a matched donor. However, in spite of great expectations for a rapid development of this approach, for many years it proved a difficult goal, and only recently it has been successfully introduced in clinical practice. Notably, during the last years, additional promising therapeutic options have been provided by progress in gene-editing technologies, including the clustered, regularly interspaced, short palindromic repeat (CRISPR)-CRISPR-associated 9 (Cas9) (CRISPR-Cas9) tool, designed to introduce highly specific genome modifications for either gene disruption or correction [48, 50, 51]. At the same time, progress in understanding the molecular mechanisms underlying fetal globin gene silencing has contributed to identify several potential molecular targets, thus paving the way to novel therapeutic approaches for hemoglobinopathies that, besides correcting the defective β-globin gene, are now aimed at editing genomic segments that regulate fetal hemoglobin synthesis in hematopoietic stem cells (HSCs).

Interestingly, some of the most promising editing strategies for hemoglobinopathies that are being pursued include the reactivation of fetal globin genes that could be achieved either by silencing fetal globin gene repressors or, alternatively, by introducing known HPFH mutations in the HBB cluster. Since the beginning, targeting Myb or KLF1 appeared to be rather problematic given the pleiotropic roles played by these two factors in hematopoiesis and in erythropoiesis, respectively, whereas BCL11A emerged as one of the most appropriate candidate to be silenced, given its well-recognized role in HbF repression [48, 50, 51]. However, ubiquitous BCL11A knockdown rapidly turned out to be an unsuccessful strategy, given its role in other biological pathways including neuronal and B-lymphocyte development [28]. Consequently, alternative strategies are being underway focused on reducing its expression selectively in erythroid cells that could be achieved by disruption of its intronic erythroid-specific enhancer without affecting non-erythroid cells [51]. A similar approach could also be used to tune down Myb expression levels by editing SNPs at the HBS1L-MYB intergenic region that are known to disrupt the structure and the functionality of erythroid-specific enhancers causing reduced Myb expression levels. Anyway, other repressors such as CSDA or erythroid-specific
cofactors could represent potential suitable candidates to be considered for novel genome-editing strategies. Furthermore, genome-editing procedures leading to the introduction of KLF1 mutations that emerged as a natural model of impaired hemoglobin switching or HPFH mutations in the regulatory regions of fetal globin genes have been proposed either as alternative or supportive therapeutic strategies to induce clinically significant increases in HbF levels [48, 50, 51].

4. Conclusions

The research in this field should be immediately focused on the development of safe, effective drug therapies that can be accomplished through fetal globin gene induction and at the same time on the prospect of cures through bone marrow transplantation using the promise of genome-editing strategies that will bring a vastly improved quality and quantity of life to patients who suffer from these devastating disorders.

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Conflict of interest

The authors declare no conflict of interest.

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