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MINIREVIEW

Fetal Globin Gene Repressors as Drug Targets for Molecular Therapies To Treat the β-Globinopathies

Mikiko Suzuki,[a] Masayuki Yamamoto,[b,c] James Douglas Engel[d]

Center for Radioisotope Sciences[a] and Department of Medical Biochemistry,[b] Tohoku University Graduate School of Medicine, and Tohoku Medical Megabank, Tohoku University,[c] Sendai, Japan; Department of Cell and Developmental Biology, University of Michigan, Ann Arbor, Michigan, USA[d]

The human β-globin locus is comprised of embryonic, fetal, and adult globin genes that are expressed in a developmental stage-specific manner. Mutations in the globin locus give rise to the β-globinopathies, β-thalassemia and sickle cell disease, which begin to manifest symptoms around the time of birth. Although the fetal globin genes are autonomously silenced in adult-stage erythroid cells, mutations lying both within and outside the locus lead to natural variations in the level of fetal globin gene expression, and some of these significantly ameliorate the clinical symptoms of the β-globinopathies. Multiple reports have now identified several transcription factors that are involved in fetal globin gene repression in definitive (adult)-stage erythroid cells (the TR2/TR4 heterodimer, MYB, KLFs, BCL11A, and SOX6). To carry out their repression functions, chromatin-modifying enzymes (such as DNA methyltransferase, histone deacetylases, and lysine-specific histone demethylase 1) are additionally involved as a consequence of forming large macromolecular complexes with the DNA-binding subunits of these cellular machines. This review focuses on the molecular mechanisms underlying fetal globin gene silencing and possible near-future molecularly targeted therapies for treating the β-globinopathies.

The human β-globin locus is composed of embryonic (ε), fetal (^{G}γ and ^{A}γ), and adult (δ and β) globin genes (Fig. 1). The ε-globin gene is expressed in the embryonic yolk sac during the first few months of pregnancy, while sometime during the second month of gestation, after definitive erythroid cells first emerge, the ε-globin gene is silenced and fetal γ-globin expression is activated in the fetal liver. Beginning around the time of birth, fetal γ-globin expression is extinguished and the adult β-globin gene is activated. It is in this way that the β-globin genes are each expressed at distinct stages of development through a process termed "hemoglobin switching." The abundance of all five β-like globin transcripts is regulated by the locus control region (LCR) (Fig. 1), an aggregate enhancer located far 5′ to the locus. The LCR was originally identified as a group of five dispersed, developmentally stable DNase I-hypersensitive sites localized far 5′ to the embryonic ε-globin gene (1, 2). The LCR harbors both enhancer and insulator activities for the β-locus genes (3–5). The most widely accepted model for how the LCR transcriptionally activates individual genes is through chromatin looping, creating direct contacts between the proteins bound at the LCR and those bound at the individual globin gene promoters (6–8).

Proximity to the LCR significantly affects the developmental expression patterns of β-like globin genes. In the human locus, ε (embryonic), ^{G}γ and ^{A}γ (fetal), and δ and β (adult) globin genes are spatially arranged in a proximal-to-distal direction from the LCR and expressed developmentally in the same order. Transgenic mice harboring transgenes in which the LCR was used to direct transcription of the individual γ- or β-globin genes showed misregulated expression of those genes (9–11). However, transgenic mice harboring transgenes in which both the γ- and β-globin genes were linked to the LCR in their usual order exhibited behavior that reasonably approximated normal switching (12–14). Furthermore, by inverting the gene arrangement relative to the LCR *in vivo*, the order of expression was altered in a manner that was consistent with then-current models (15). These lines of evidence comprise one of the first "rules" of hemoglobin switching: competition for the LCR (enhancer) between the β-like globin genes is generally advantageous for the gene closest to the LCR. However, in addition to competition among the globin promoters, the second "rule" of switching emerged when it was discovered that the embryonic and fetal globin genes were autonomously silenced (became transcriptionally repressed) by local regulatory sequences that acted at the appropriate developmental stages (16, 17). Concordant with this observation, when transgenic mice harboring transgenes in which the LCR was directly linked to both the γ- and β-globin genes in their natural order were examined, γ-globin expression was repressed in adult-stage erythroid cells, while β-globin expression was induced (13). Thus, the second rule of hemoglobin switching posits that developmental stage-specific repression of the embryonic and fetal genes overrides LCR proximity in the determination of which gene in the locus is to be expressed. In summary, the developmental stage-specific expression of the β-like globin genes appears to be governed by two competing principles: the promoters compete for the LCR enhancer activity (6–8), while autonomous silencing of the embryonic and fetal globin genes takes precedence. Although there are countless cryptic nuances lying under the umbrella that covers this broad conclusion, in the final analysis these two overriding concepts must be considered in any further elaboration of mechanisms that explain the remaining aspects of hemoglobin switching, some of which are still shrouded in mystery.

β-GLOBINOPATHIES

Inherited mutations in the adult β-globin structural gene cause two major β-globinopathies: β-thalassemia (Cooley's anemia, or

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Address correspondence to James Douglas Engel, engel@umich.edu.

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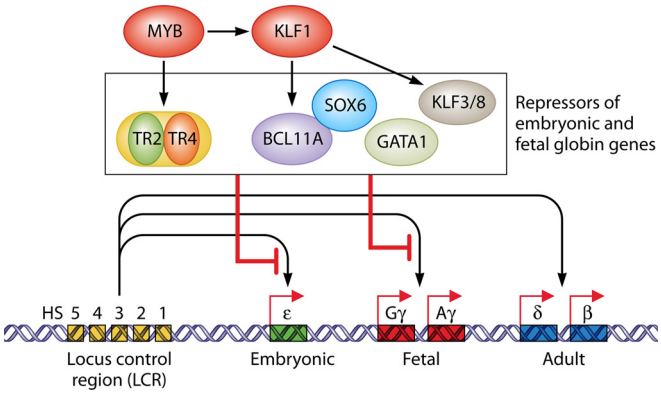

FIG 1 A current model for embryonic and fetal globin gene silencing. The human β-globin locus is composed of embryonic (ε), fetal (γ), and adult (β) globin genes and the LCR, itself composed of five DNase I-hypersensitive sites. The embryonic and fetal globin genes are autonomously repressed in adult-stage erythroid cells. TR2/TR4 represses embryonic and fetal globin genes directly by binding to DR elements in their promoters. MYB indirectly represses embryonic and fetal globin expression by activating TR2/TR4 and KLF1. KLF1 activates BCL11A, which represses embryonic and fetal globin genes in collaboration with SOX6 and KLF3/8. GATA1 may directly repress the fetal globin genes and additionally contribute to BCL11A-mediated repression.

CA) and sickle cell disease (SCD). β-Thalassemia results from reduced production of β-globin chains, which leads to an imbalanced accumulation of α- and β-globin proteins inside red blood cells (RBCs). SCD is caused by a mutation that results in a substitution of the sixth amino acid of adult β-globin from glutamic acid to valine (18). Hemoglobin tetramers bearing this mutation (HbS) polymerize inside RBCs and distort them into a characteristic distended appearance instead of their normal oblate ellipsoidal shape, which appears to be a biconcave disc. These rigid sickle RBCs are more prone to lysis as they traverse the circulatory system and have been shown to occlude blood flow in the microvasculature, causing pain and organ damage. While the symptoms are not nearly so serious in individuals who are heterozygous for the mutation (called sickle cell trait), patients bearing two sickle alleles commonly develop severe physiological problems.

Persistent expression of fetal globin gene expression in patients ameliorates both the symptoms and at least a subset of the pathophysiologies that occur as a consequence of these β-globinopathies. Musallam et al. observed a negative correlation between fetal globin levels and morbidity due to β-thalassemia intermedia (19). Induction of fetal hemoglobin (HbF) to above 8.6% was found to significantly relieve SCD mortality (20). Fetal γ-globin incorporation inhibits the extension of the long sickle polymers inside sickle cells (21) and thus ameliorates the symptoms and at least some of the pathologies due to SCD.

The only HbF-inducing agent that is currently approved by the U.S. Food and Drug Administration (FDA) for the treatment of SCD is hydroxyurea (HU). HU is an anticancer drug that induces cell cycle arrest by inhibiting ribonucleotide reductase activity for DNA synthesis. HU treatment was one of the very first compounds shown to increase HbF levels and thus improve some pathological effects of SCD (22). However, only about half of HU-treated patients generate sufficient levels of HbF to relieve the symptoms (23), and therefore novel therapeutic strategies must be developed in order to treat patients who are affected by SCD and β-thalassemia. The most direct strategy to induce HbF in adults would be to interfere with the gene-autonomous mechanisms that regulate fetal globin gene silencing. The rest of the review focuses on recent literature that explores the activities of the currently known transcriptional repressors (site-specific DNA-binding proteins) of the fetal globin genes in the temporal order of their original descriptions as participants in the γ-globin silencing process: TR2/TR4, MYB, BCL11A, SOX6, GATA1, and KLFs (Fig. 1).

THE DRED COMPLEX: A DIRECT γ-GLOBIN REPRESSOR

The embryonic and fetal globin genes have direct repeat (DR) elements in their promoters (24), while neither the mouse nor human adult β-globin genes bear such elements. DRs are consensus binding sites for nonsteroidal nuclear receptors. Significantly, a point mutation in either γ-globin promoter DR element leads to elevated, gene-autonomous γ-globin synthesis in adulthood (called HPFH, or hereditary persistence of fetal hemoglobin), indicating that mutation of this element is the underlying cause of aberrantly elevated fetal globin expression in the affected individuals (25, 26). Importantly, numerous point mutations in both the ^{G}γ- and ^{A}γ-globin promoter DR elements have since been identified that each lead to a fetal gene-specific HPFH phenotype. Phenocopying these natural variants, mutation of the DR elements in the ε- or γ-globin promoters of yeast artificial chromosome (YAC) or bacterial artificial chromosome transgenic mice bearing the entire human β-globin locus leads to persistent expression of the closest *cis*-linked gene into adulthood (27, 28).

Given that the DR *cis* element in the γ-globin promoters provided initial evidence for the possibility that the nuclear protein that bound to that site was a repressor, significant effort was expended to identify that presumptive nuclear receptor. The first suggestion for its identity as a transcription factor that was likely to be widely expressed came from gel shift analysis of biochemical extracts derived from erythroid cell lines (29, 30), but the identity of the protein(s) was unresolved. Somewhat later, some intriguing experiments identified this DR element-binding protein as a complex between the erythroid cell-specific transcription factor NF-E3 and a member of the COUP transcription factor family, possibly COUP-TFII (31). However, the bona fide identity of NF-E3 has never been firmly established, and although COUP-TFII is abundantly expressed in a human embryonic erythroid cell line (K562) (27, 31), and despite recent evidence suggesting the contrary (32), COUP-TFII is not expressed in definitive adult human erythroid cells (27; M. Sierant and J. D. Engel, unpublished observations).

We biochemically purified a DR element-binding protein complex that we named "direct repeat erythroid-definitive" (DRED) (Fig. 2), well before we had determined the actual identity of the DRED DNA-binding proteins and cofactors (27, 33); no other nuclear receptors were identified in this initial mass spectrometry-based experiment. The DRED complex appears to be composed of a heterodimer of the orphan nuclear receptors TR2 and TR4 (in current nomenclature NR2C1 and NR2C2, respectively) (33). TR2 and TR4 bind to the two DR elements in the ε-globin promoter with somewhat higher affinity than to the single DR element in each of the γ-globin promoters (33, 34). Mice bearing a targeted deletion in the *Tr2* gene reportedly display no overt phenotypes (35), while targeted mutation of *Tr4* leads to growth retardation and behavioral defects (36). Compound null mutations in both *Tr2* and *Tr4* leads to embryonic death prior to

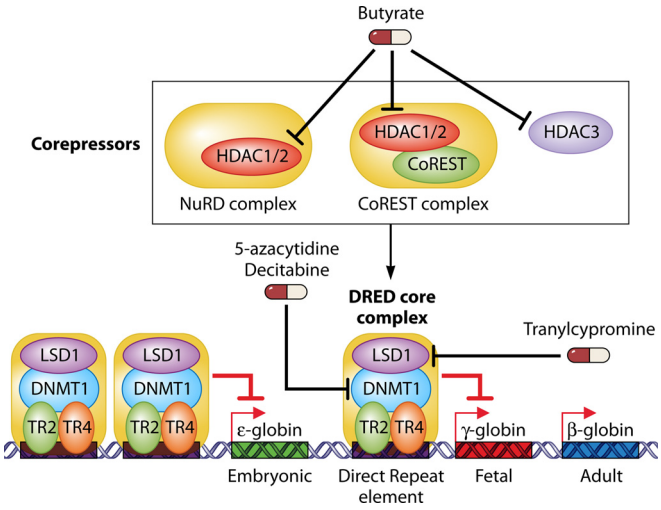

FIG 2 Embryonic and fetal globin gene repression by the DRED complex and molecular targets of inhibitory drugs. The DRED complex directly binds to the promoters of the embryonic and fetal globin genes to repress their expression in definitive adult bone marrow-derived erythroid progenitor cells. The DRED core tetrameric complex is composed of TR2/TR4, DNMT1, and LSD1. The core complex also associates with additional corepressors (NuRD and CoREST complexes that contain HDACs 1 and 2 and independently with HDAC3). Inhibitors of DNMT, LSD1, and HDACs, namely, 5-azacytidine/decitabine, tranylcypromine, and butyrate, respectively, induce fetal globin expression, which is at least partially dependent on DRED activity.

implantation, with incomplete penetrance (34, 37), and therefore TR2 and TR4 appear to have redundant but not identical activities. Silencing of the embryonic and fetal globin genes was delayed in definitive erythroid cells of *Tr2*- and *Tr4*-null mutant mice (34), consistent with a previous report that concluded that TR2 could repress transcription (38). Thus, the DRED complex directly represses embryonic and fetal globin gene expression in definitive erythroid cells.

In addition to TR2 and TR4, DNA methyltransferase 1 (DNMT1) and lysine-specific histone demethylase 1 (LSD1) participate as cofactors to form a tetrameric core DRED complex (39). DNMT1, previously characterized as the maintenance DNA methylase, is able to recognize and methylate CpG dinucleotides when they are found on the opposite strand of MeCpG, thus maintaining stable DNA methylation patterns in the genome after DNA replication (40). LSD1 removes the methyl groups from mono- and dimethyl-histone H3 lysine 4 (MeH3K4 and Me_2H3K4, respectively), which are widely regarded to be epigenetic signatures that mark transcriptionally active genes (41). This tetrameric core repression complex (TR2-TR4-DNMT1-LSD1) can additionally interact with histone deacetylase 1/2 (HDAC1/2), NuRD, CoREST, TRIM28 (also known as TIF1β), and HDAC3 to form an array of even larger multifunctional complexes (39) (Fig. 2).

Curiously (and incongruously), forced overexpression of TR2 and TR4 in erythroid cells leads to the induction of fetal globin expression, thereby reversing SCD phenotypes in mouse models (34, 42). Although the mechanism underlying this seemingly paradoxical phenomenon is unknown, one must consider two possibilities. One possibility is that overly abundant expression of TR2 and TR4 inhibits effective formation of the very large DRED complex because one or more of the enzyme components identified as DRED cofactors may be in limiting abundance, thereby "squelching" inactivation by the repressor (43). The other possibility is that TR2 and TR4, in addition to their repressive role, also function as activators of the fetal globin genes by association with cellular coactivators, such as PGC-1 (44). Several previous reports indeed contended that TR4 can function as a robust transcriptional activator (45–51). TR2 and TR4 are very broadly expressed, including in erythroid cells (52–54). In nonerythroid cells, TR4 has been shown to transcriptionally activate apolipoprotein E/C-I/C-II (46) and phosphoenolpyruvate carboxykinase in the liver (55), luteinizing hormone receptor in the ovary (56), and CD36 in macrophages (57). In erythroid cells, hundreds of genes are repressed (either directly or indirectly) by TR4, while even more genes are activated (58). Analysis of erythroid differentiation in comparative data sets from chromatin immunoprecipitation-sequencing (ChIP-seq) and RNA-seq experiments indicated that the DR motif is more prevalent in the proximal promoters of TR4 direct target genes that are involved in basic biological functions (e.g., mRNA processing, ribosomal assembly, RNA splicing, and primary metabolic processes [58, 59]). We also previously identified TRIM28 as an interacting protein of TR2/TR4 (39). TRIM28 is dispensable for fetal globin silencing but is required for erythroid differentiation, implying that TRIM28 may function as a coactivator of TR2/TR4 (60). However, it is equally plausible that TRIM28 functions as a corepressor, as it does in other contexts (61), to inhibit the transcription of a currently unidentified activator that is required for erythroid differentiation.

TR2 and TR4 are categorized as orphan nuclear receptors, since no ligand for either of them has been identified. However, structural analyses and biochemical investigations of the TR4 ligand-binding domain by Zhou et al. revealed that retinol and all-*trans* retinoic acid can act as very weak ligands for TR4 (62), although the concentrations at which they stimulated TR4 activity in transfected cells suggested that they may not be the genuine ligands *in vivo*. In addition, unsaturated fatty acids and their metabolites have also been reported to promote the transcriptional activity of TR4 (51, 57). In contrast, the amino acid sequence of the closely related TR2 protein suggests that it does not naturally associate with ligands, and thus may exert its activity either as an obligate TR4 partner or by homodimerization, thereby leading to competition for TR4 binding sites or for TR2/TR4 heterodimer binding.

In summary, even though TR2/TR4 was the first γ-globin repressor to be identified, the complexity of its interactions with multiple cofactors, the open question of whether or not either of this pair of orphan nuclear receptors is regulated by small-molecule ligands, and the fact that the TR2 and TR4 subunits are genetically compensatory all complicate potential strategies that can be envisioned by which one might inactivate the repressor and achieve the ultimate goal of increasing γ-globin synthesis. The hypothesis that TR4 could be a typical ligand-regulated nuclear receptor (51, 57, 62) seems promising, since identification of the genuine ligand could be used to subsequently identify antagonists that could prove to be clinically beneficial.

MYB: A CRITICAL HEMATOPOIETIC REGULATOR INVOLVED IN FETAL GLOBIN SILENCING

Thein et al. originally identified an intergenic region lying between the *HBS1L* and *MYB* genes on 6q23 as a region controlling fetal globin gene expression in adults based on quantitative trait locus (QTL) analyses (63, 64). Since the abundance of both the *HBS1L*

and *MYB* genes diminished in the patients with the *HBS1L-MYB* genotype, the gene responsible for that specific HPFH phenotype was unclear. The answer to this question was resolved via a serendipitous coincidence of similar conclusions from three independent studies.

The first insights regarding the nature of the effects of the *HBS1L-MYB* intergenic region emerged from a study of newborns bearing trisomy 13 who exhibited elevated HbF levels. Sankaran et al. identified two microRNAs, miR-15a and -16-1, on 13q14 as responsible for the elevated HbF levels in human trisomy 13 patients (65). At least one target of these two microRNAs was the *MYB* gene, and so inhibition of MYB mRNA function in human adult $CD34^+$ cell-derived erythroid cells resulted in induction of fetal hemoglobin. Therefore, it was concluded that *MYB* activity was responsible for this particular variant HPFH. In further support of this conclusion, Sankaran et al. recently reported analysis of a patient with a complete loss of function of the *HBS1L* gene (66). This patient exhibited a normal distribution of hemoglobin subtypes and no hematological abnormalities, but did bear other phenotypic deficiencies. Finally, it was demonstrated that disruption of the *Hbs1l-Myb* intergenic region by a random transgene insertion in the mouse led to greatly diminished Myb accumulation (*Myb* expression was significantly more affected than was *Hbs1l*) and was accompanied by a marked increase in the murine homologues of the human ε- and γ-globin genes (67). These three observations in concert validated the contention that *MYB*, rather than *HBS1L*, is responsible for the mutant intergenic region HPFH phenotype.

The *Myb* gene encodes a transcription factor that is critical for hematopoietic stem cells and progenitors. *Myb*-null mutant mice die at embryonic day 15 due to a failure in definitive erythropoiesis (68). Analyses of constitutive and conditional *Myb* mutant mice have revealed the contributions of this vital transcription factor to erythroid cell, T cell, and B cell development (69–71). While the *Myb* gene is highly expressed in hematopoietic stem cells and progenitors, its expression progressively diminishes as erythroid cells mature (72). Since the developmental stages at which the *MYB* and β-type globins are expressed differ markedly (67), the data can be interpreted to suggest that MYB probably induces fetal globin transcription through an indirect mechanism. In support of this contention, Bianchi et al. and we have found that MYB activates *KLF1* and *TR2/TR4* in human erythroid cells (67, 73). Since one of the several major functions of KLF1 is to activate the *BCL11A* gene, itself a robust fetal globin gene repressor (see below), current data support the hypothesis that MYB indirectly regulates γ-globin transcription because of diminished production of either or both of the repressors, BCL11A and TR2/TR4 (Fig. 1).

Importantly, the region on 6q23 that is responsible for elevated HbF levels is located not in the *MYB* structural gene, but in a putative gene regulatory region. Farrell et al. identified a 3-bp deletion within the *HBS1L-MYB* intergenic region that was first associated with high HbF levels (74). This tiny indel was located between a TAL1-binding site and a GATA-binding site, suggesting that the deletion may have affected the formation of a precisely structured higher-order TAL1/GATA factor complex, as first described almost 2 decades ago (75). More recently, Stadhouders et al. identified binding sites for the TAL1/GATA1 complexes in the mouse *Hbs1l-Myb* intergenic region and showed that the binding sites were in close three-dimensional proximity to the *Myb* gene promoter (76). Indeed, single nucleotide polymorphisms (SNPs) located at these binding sites were more recently shown to influence GATA1 and KLF1 binding and the interaction between those sites and the *MYB* gene promoter that led to a reduction in *MYB* gene expression (77). These reports suggested that the cell-type-specific loss of MYB function was achieved by inhibiting the ability of a TAL1/GATA1 complex to form an optimal association with a DNA site that regulates *MYB* transcription, thereby indirectly leading to an HPFH phenotype.

As described above, we originally identified an anemic mouse that had been created by random insertion of a transgene into the *Hbs1l-Myb* intergenic region (78). Such mice exhibited elevated levels of the mouse embryonic globins as well as anemic and thrombocytic phenotypes. In these mice, *Myb* gene expression was muted in megakaryocyte/erythrocyte progenitors (MEPs) but not in T cells, suggesting that the intergenic region contained a regulatory element, possibly an enhancer, that controlled *Myb* transcription in MEPs. Indeed, human HPFH individuals bearing mutations within the *HBS1L-MYB* region also exhibited modest (but quantitatively altered) hematopoietic deficiencies, including anemia and thrombocytosis (79). Evolution suggests that mutations or polymorphisms in gene regulatory regions can result in spatiotemporal defects of gene expression, which can in turn give rise to limited (but only rarely beneficial) phenotypic effects; such appears to be the case for the *HBS1L-MYB* intergenic mutation encountered in humans who additionally bear deleterious mutations in β-globin synthesis (80–83).

BCL11A: THE POSTER CHILD FOR GWAS

Genome-wide association studies (GWAS) represent a natural variation-based genetic strategy for interrogating the entire human genome for SNPs that are associated with specific phenotypes (84). GWAS performed on the genomes of sickle cell disease cohorts originally identified three chromosomal positions that influence HbF levels. While two of three represented loci that were already known to differentially modulate fetal and adult β-type globin levels (one within the β-globin locus as well as one in the *HBS1L-MYB* intergenic region), a third completely novel locus mapped at the position of the *BCL11A* gene, previously identified as a vital transcription factor for B lymphocyte development (63, 80, 85). In humans, a high level of HbF is associated with low *BCL11A* expression (86). *Bcl11a*-null mutant mice and erythroid cell-specific *Bcl11a* knockout mice both exhibited an HPFH phenotype (87, 88). These reports confirmed that the transcription factor BCL11A is a repressor of the fetal globin genes.

BCL11A forms molecular complexes with GATA1 and FOG1 and additionally with the NuRD chromatin remodeling complex (86). Furthermore, BCL11A physically interacts with transcription factor SOX6 (89). In a previous report, SOX6 was shown to bind to the promoter of the mouse embryonic εY-globin gene and silence its expression in definitive erythroid cells (90). Thus, BCL11A and SOX6 may collaboratively bind to a number of sites within the β-globin locus to repress fetal globin gene expression (89, 91).

Since *Bcl11a*-null mutant mice die in the perinatal period (85), as a therapeutic strategy a global reduction in BCL11A abundance could prove to be problematic in humans, especially since such a strategy could simultaneously affect unintended functions in other tissues. For example, *Bcl11a* germ line-null mutant and adult-stage-specific null mutant mice exhibit defects in lymphoid

development (85, 92). Additionally, BCL11A plays critical roles in neuronal morphogenesis and sensory circuit formation (93). However, Bauer et al. recently reported the identification of an erythroid cell-specific enhancer of the *BCL11A* gene, providing a possibly superior, and tissue-specific, alternative target for therapeutic development. As is also the case for the *MYB* intergenic enhancer, a TAL1/GATA1 complex activates the *BCL11A* erythroid enhancer (94).

GATA1 AND KLFs: ERYTHROID REGULATORY PROTEINS THAT INFLUENCE FETAL GLOBIN REPRESSION

KLF1, originally designated EKLF (95), has been known to be required for activation of the adult β-globin gene since shortly after its original discovery (96, 97). However, Borg et al. recently revealed a new function of KLF1 as a repressor of the fetal globin gene (98): from the analysis of a Maltese family with prevalent HPFH, the investigators identified a nonsense mutation in the *KLF1* gene. Further studies demonstrated that KLF1 represses fetal globin expression indirectly by activation of *BCL11A* (98, 99), and thus in haploinsufficient individuals a sensitivity to reduced KLF1 abundance in erythroid cells results in diminished production of the BCL11A repressor. In addition to KLF1, other KLF family factors have been shown to be involved in fetal globin silencing. KLF3-deficient (originally characterized as BKLF-deficient) mice exhibited derepression of the embryonic globin genes, and the effect was enhanced in *Klf3:Klf8* compound null mutant mice (100). Since the *KLF3*, *KLF8*, and *BCL11A* genes are all activated by KLF1, these factors all participate in fetal globin gene silencing, which is possibly mediated through KLF1.

SNP studies originally suggested that GATA transcription factors may also be directly involved in fetal globin gene silencing. Chen et al. identified an SNP located 567 bp 5′ to the fetal $^{G}\gamma$ globin gene (*HBG2*) in an Iranian-American family that exhibited elevated fetal globin levels (101). This polymorphism disrupted a GATA-binding motif in the $^{G}\gamma$ globin far upstream promoter that was also duplicated in the $^{A}\gamma$ globin (*HBG1*) gene (located 566 bp 5′ to the $^{A}\gamma$ start site) (102). Disruption of the −566 $^{A}\gamma$ GATA site in β-globin YAC mutant mice resulted in persistent expression of the $^{A}\gamma$ gene in adult mice (103). These studies suggest a contribution of a GATA transcription factor(s) to fetal globin silencing. As GATA1 also appears to play a significant role in BCL11A transcriptional activity (87), the mechanism of GATA1-related γ-globin repression could be functionally related to the BCL11A pathway.

CHROMATIN MARKS THAT DEFINE FETAL GLOBIN GENE EXPRESSION

As alluded to above, transcription factors that play roles as direct or indirect fetal globin gene repressors have been shown to interact with several enzymes that modify chromatin. While these enzymes are usually referred to as "epigenetic" modifying proteins, most have not been shown to heritably affect gene expression through a causal relationship. However, the marks that these enzymes impart to DNA and histones, and even to transcription factors themselves, are unquestionably affiliated with either activated or repressed transcriptional states, be they causal or simply a reflection of those states. Perhaps, intriguingly, DNMTs, HDACs, and the LSD1 enzymes are all implicated in fetal globin gene silencing by both TR2/TR4 and BCL11A (39, 86). DNA methylation within the locus has for many years been one of the hallmarks of globin gene inactivity through the strong correlation of methylation with repression (104–108). Histone acetylation of globin gene promoters also correlates with the expression levels of these genes (109), and HDAC inhibitors such as butyrate have been shown to induce HbF (110).

As mentioned above, methylation of cytosine residues in CpG dinucleotides by DNMTs correlates strongly with transcriptional repression (111). DNA methylation in the embryonic and fetal globin promoters inversely correlates with their expression. Small interfering RNA-mediated reduction of DNMT1 levels in baboon $CD34^{+}$ bone marrow-derived erythroid progenitors exhibited diminished methylation of the embryonic and fetal globin promoters and elevated gene transcription (112). Furthermore, mice that are genetically deficient for the methyl-CpG-binding protein gene *Mbd2* exhibit delayed (human YAC transgene-delivered) fetal globin gene silencing (113). The DNMT inhibitors 5-azacytidine and 5-aza-2′-deoxycytidine (decitabine) have been shown to lead to demethylation of the fetal globin gene promoters and to induce fetal globin gene expression (114–117); decitabine, in particular, is currently in clinical trials and is being tested as a possible therapeutic human γ-globin inducer.

In addition to DNMT and HDAC, LSD1 also binds (directly or indirectly) to both TR2/TR4 and BCL11A (39). LSD1 demethylates mono- and dimethyl-histone H3 lysine 4 (H3K4), an activating histone mark (41). We recently showed that inhibition of LSD1 via use of either RNA interference or a small-molecule LSD1 inhibitor (the FDA-approved antidepressant tranylcypromine [TC], trade name Parnate, is a monoamine oxidase inhibitor [MAOI]) robustly induces fetal globin expression in human adult erythroid cells that can be derived in culture from mobilized circulating $CD34^{+}$ progenitors (118, 119). Furthermore, the combinatorial application of TC and decitabine had synergistic effects on HbF induction in this cell culture model, achieving >50% HbF production in differentiated $CD34^{+}$ cells *ex vivo*, while the combination of TC plus HU was much less effective (118). Since it was later shown that higher concentrations of TC inhibited erythroid differentiation (120) and as there exist a number of potentially dangerous drug-metabolite interactions with this compound (121, 122), TC is unlikely to represent a popular medication for treating the β-globinopathies. Perhaps more-specific compounds that target LSD1 activity and that act at lower concentrations for longer periods of time in the bloodstream and have fewer side effects can be identified in the not-too-distant future.

CONCLUDING REMARKS

Development of novel therapies for treating the β-globinopathies by fetal globin gene induction has been contemplated and pursued for decades. Recent studies have identified a number of transcription factor molecules that are directly or indirectly involved in fetal globin gene silencing, and their associations with additional enzyme cofactors that impart distinguishing activating or repressing marks on DNA and histones near the transcription factor binding sites have provided many new potential interaction surfaces and enzymatic mechanisms for interruption of the activity of these proteins and for possible therapeutic development. Due to these achievements, we should be able to find new molecularly targeted drugs or new uses for existent medications to treat these disorders. In this regard, the greatest challenge may be to selectively impair the activity of broadly expressed factors that we might envision as potential drug targets (e.g., TR2, TR4, LSD1,

and DNMT1) specifically in erythroid cells or to inactivate molecules that are more highly tissue restricted (e.g., MYB, KLFs, GATA1, and BCL11A) without affecting the essentially universal requirement for MYB, GATA1, and KLF function in hematopoietic cells or the physiological requirement for BCL11A in other tissues. In this regard, the identification of enhancers that control the erythroid cell- or hematopoietic cell-specific transcription of *MYB* (67, 76) and *BCL11A* (94) could represent a significant conceptual advance in providing additional, more highly refined, interaction targets that could lead to disruption of regulatory pathways in a tissue-specific manner.

The progression of regenerative medicine utilizing human embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) in combination with genome editing portends cures for the β-globinopathies after correcting these lesions by essentially autologous transplantation of modified patient-specific iPSC or hematopoietic stem cells. Hanna et al. reported correction of sickle cell disease by transplantation with hematopoietic progenitors derived from genome-targeted iPSC generated from sickle cell model mice (123). In addition, recently developed techniques for genome editing (e.g., TALENs and CRISPR/Cas9) enable rapid and increasingly accurate manipulation of the genome (124, 125). Nevertheless, since those developments as molecular cures likely lie some time away, and since they will have the same restrictions for advanced medical care as any other strategy requiring bone marrow transplantation (and thus will be available to only a small fraction of the affected patient population), the development of safer and more effective drug treatments for the hemoglobinopathies is required. The goals in this field are and 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 that suffer from these devastating disorders.

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