OXFORD

Human Molecular Genetics, 2016, Vol. 25, No. R2 R99–R105

doi: 10.1093/hmg/ddw170
Advance Access Publication Date: 23 June 2016
Invited Review

INVITED REVIEW

Hemoglobin genetics: recent contributions of GWAS and gene editing

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Abstract

The β-hemoglobinopathies are inherited disorders resulting from altered coding potential or expression of the adult β-globin gene. Impaired expression of β-globin reduces adult hemoglobin ($\alpha_2\beta_2$) production, the hallmark of β-thalassemia. A single-base mutation at codon 6 leads to formation of HbS ($\alpha_2\beta^S{}_2$) and sickle cell disease. While the basis of these diseases is known, therapy remains largely supportive. Bone marrow transplantation is the only curative therapy. Patients with elevated levels of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) as adults exhibit reduced symptoms and enhanced survival. The β-globin gene locus is a paradigm of cell- and developmental stage-specific regulation. Although the principal erythroid cell transcription factors are known, mechanisms responsible for silencing of the γ-globin gene were obscure until application of genome-wide association studies (GWAS). Here, we review findings in the field. GWAS identified BCL11A as a candidate negative regulator of γ-globin expression. Subsequent studies have established BCL11A as a quantitative repressor. GWAS-related single-nucleotide polymorphisms lie within an essential erythroid enhancer of the BCL11A gene. Disruption of a discrete region within the enhancer reduces BCL11A expression and induces HbF expression, providing the basis for gene therapy using gene editing tools. A recently identified, second silencing factor, leukemia/lymphoma-related factor/Pokemon, shares features with BCL11A, including interaction with the nucleosome remodeling deacetylase repressive complex. These findings suggest involvement of a common pathway for HbF silencing. In addition, we discuss other factors that may be involved in γ-globin gene silencing and their potential manipulation for therapeutic benefit in treating the β-hemoglobinopathies.

Introduction

The β-hemoglobinopathies are classical Mendelian anemias caused by mutations in the adult β-globin gene. β-thalassemias result from mutations that decrease or ablate β-globin production. The majority of mutations are single-base substitutions or short deletions (1). Sickle cell disease (SCD) results from a structural alteration (glutamine-valine substitution at codon 6) in the β-globin protein. Natural history studies have documented considerable benefit of fetal γ-globin gene expression in lessening the clinical severity of both disorders (2). Understanding how the β-globin gene cluster is regulated, particularly how the γ-globin gene is silenced, is relevant to the design of new therapeutic strategies for treating the β-hemoglobinopathies. We briefly review recent work on the control of globin gene expression and implications for human genetics and therapy of these disorders.

Hemoglobin switching

Five β-like globin genes are located in the β-globin gene cluster on chromosome 11p in the order of their expression during

Received: April 28, 2016. **Revised:** May 20, 2016. **Accepted:** May 24, 2016

human ontogeny (3) (Fig. 1). In the first trimester, red cells formed in the yolk sac contain embryonic ϵ-globin (paired with the embryonic α-like ζ-gene expressed from chromosome 16p). Thereafter, expression of the fetal γ-globin, paired with adult α-globin, predominates during fetal erythropoiesis. Adult β-globin expression increases slowly during the second trimester and predominates around the time of birth as bone marrow hematopoiesis is established. In the normal adult, adult hemoglobin (HbA, $\alpha_2\beta_2$) comprises >95% of total hemoglobin and fetal hemoglobin (HbF, $\alpha_2\beta_2$) ~1–2%. The switch from HbF to HbA is a paradigm of developmental, stage-specific gene expression and critical to the pathophysiology of the β-hemoglobinopathies. During fetal life, the β-hemoglobinopathies exhibit no phenotype since red cell production and survival is dependent on γ-globin rather than β-globin. The level of HbF in adults is under genetic control, such that individuals with genetic variants display extended expression of HbF in the adult state (hereditary persistence of HbF, HPFH). Inheritance of a high HbF allele ameliorates the β-hemoglobinopathies. Despite the cloning and sequence analysis of the human β-globin cluster more than 35 years ago, an understanding of the basis of the hemoglobin switch has been elusive until the past several years.

Transcription of the β-globin gene cluster

High-level *in vivo* expression from the β-globin gene locus is under control of an upstream enhancer known as the locus control region (LCR) (4,5). The LCR, which is comprised of discrete elements that exhibit DNase I hypersensitivity in erythroid cell chromatin, is the prototype of 'stretch' or 'super' enhancers (6). Experiments in transgenic mice suggested that the LCR might loop to the individual downstream genes successively during ontogeny and thereby provide a mechanism for selective, mutually exclusive and developmentally appropriate activation of expression of the downstream globin genes. Blobel and coworkers employed forced looping directed by dimerization of the factor LIM domain-binding protein 1 (LDB1) to provide direct support for chromosomal looping (7). The essential erythroid transcription factors GATA1, friend of GATA1 (FOG1), Krüppel-like factor 1 (KLF1) and SCL/TAL1 cooperate to activate transcription (8,9). The looping process is dynamic, however, and transcription in an individual cell may alternate between globin genes. While the general features of gene expression from the β-globin cluster were established, the factors that control the switch from γ- to β-expression were entirely unknown.

GWAS contribution to hemoglobin switching

Genome-wide association studies (GWAS) provided the first evidence for genes that control HbF levels in humans. In 2007 and 2008, Thein and coworkers (10,11) and Cao and coworkers reported GWAS for the number of rare F-cells (those cells containing high concentration of HbF in adult blood) and the level of total HbF among individuals, respectively. These GWAS are notable for several features. First, only three regions of the genome reached statistical significance: the intergenic region between HBS1L and MYB on 6q, the β-globin locus on 11p and a new candidate the BCL11A gene on 2p (10–12). Both the β-globin gene cluster and the interval between the HBS1 and MYB genes had previously been linked to elevated HbF through naturally occurring deletions and linkage studies (13). Second, the three regions are associated with HbF levels in all populations studied to date. Third, taken together, the three loci account for at least 50% of the genetic variation in HbF level, an extraordinary contribution as compared with most GWAS-identified loci. Subsequent studies establish that the MYB gene, which encodes a transcription factor that affects erythroid cell proliferation and differentiation, is the relevant gene in the HBS1L-MYB region (14,15). Although BCL11A, a zinc-finger transcription factor, had been previously studied largely in the context of B-lymphocyte development, a potential role in globin gene expression was entirely unsuspected prior to the GWAS.

BCL11A as a regulator of the switch

Multiple lines of evidence establish BCL11A as a concentration-dependent negative regulator of γ-globin expression and therefore a silencer of HbF production (16,17). In erythroid precursors derived from primary human CD34+ cells, knockdown or

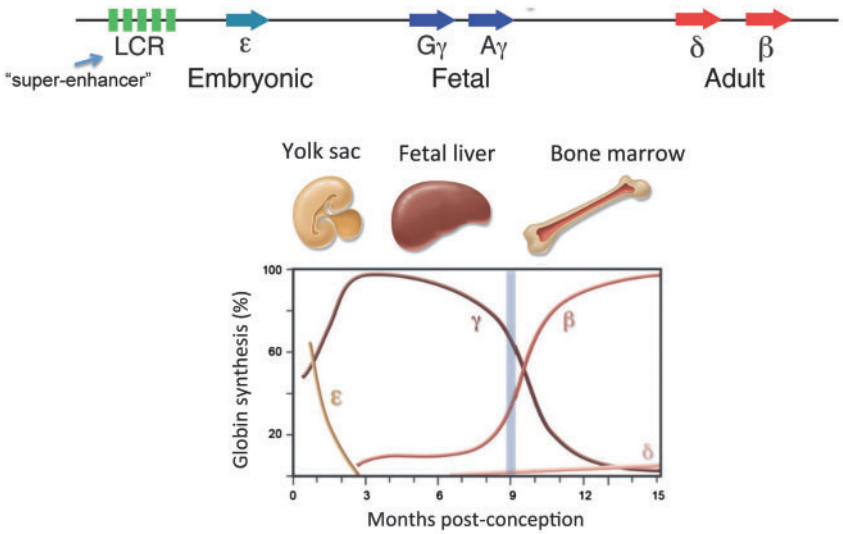

Figure 1. The spatial organization of the β-like globin genes of the human β-globin locus is illustrated above. The LCR, an erythroid super-enhancer, controls expression of the entire locus. The sites of red cell production and the relative levels of the β-like globins during pre- and post-natal development are depicted below. The first switch from ϵ-globin to the two γ-globins occurs between yolk sac and feta liver hematopoiesis. The second switch takes place gradually and accelerates after birth (indicated by a blue vertical line) and results in silencing of the γ-globins and dominance of β-globin.

knockout of BCL11A (by shRNA or clustered regularly interspaced short palindromic repeats [CRISPR]/Cas9 technology) results in elevated HbF. Mice lacking BCL11A show markedly delayed silencing of εy and βH1, mouse embryonic β-globins, at the fetal liver stage. In BCL11A knockout mouse embryos harboring a transgene for the entire human β-globin locus (β-YAC), silencing of γ-globin expression is greatly impaired. Given that many transcription factors have been implicated in globin gene regulation, one might question the extent to which any single factor contributes to the fetal-to-adult switch. The potency of BCL11A is best illustrated by experiments in genetically engineered SCD mice in which human genes replace mouse globins. In this setting conditional inactivation of BCL11A in the erythroid lineage alone is sufficient to rescue hematological parameters in the disease model due to high-level, pancellular HbF expression (18).

Recently, human genetics has provided a valuable insight into quantitative aspects of HbF silencing by BCL11A. Children have been described with rare microdeletions of 2p15-p16.1 associated with an autism spectrum disorder and developmental delay. These children retain one normal chromosome and harbor deletions on the other that involve BCL11A alone or in combination with contiguous genes. These individuals provide a unique opportunity to examine the consequences of haploinsufficiency for BCL11A in humans. In each instance, HbF levels are elevated and remarkably approach those in patients with classical HPFH (~20%) (19,20). Therefore, a modest reduction of BCL11A (to 50%) is associated with marked derepression of HbF. Moreover, other hematological parameters, including the numbers of B-lymphocytes, were normal. These clinical observations have important implications for directed reactivation of HbF for therapy either through genetic or biochemical means.

Given the critical role of BCL11A in HbF silencing, modulation of its activity or expression might constitute a new therapeutic strategy for β-hemoglobin disorders. The possibility of addressing both SCD and β-thalassemia with a single strategy, such as knockdown or knockout of the BCL11A gene by expressed shRNA or by gene editing, is attractive. Recent findings demonstrating a requirement for BCL11A in early hematopoietic progenitors and hematopoietic stem cells, however, suggest that a 'pan-hematopoietic' approach to BCL11A ablation would be unwise (21) (S. Luc *et al.*, manuscript in preparation and C. Brendel *et al.*, manuscript in preparation).

Genetic variation at the BCL11A locus

BCL11A is a hyperconserved gene. The trait-associated single-nucleotide polymorphisms (SNPs) identified by GWAS cluster within an ~12 kb region of intron 2 (22,23) (Fig. 2). The SNP-dense intronic region exhibits hallmarks of a regulatory element, including erythroid-specific DNase I hypersensitivity, erythroid transcription factor binding and histone modifications characteristic of enhancers. This region drives developmental, erythroid-specific expression from a minimal promoter in transient transgenic mice (22). The element is also essential for BCL11A expression in erythroid cells, as targeted deletion with TALENS or CRISPR/Cas9 in a murine erythroid cell line, immortalized human erythroid cells and erythroid precursors derived from primary human CD34+ cells reduces BCL11A expression >95%.

How does naturally occurring genetic variation at BCL11A impact expression? The enhancer region in human has three DNaseI hypersensitive sites (DHS), located at +55, +58 and +62 kilobases downstream of the transcriptional start site. An SNP in the +62 DHS is most correlated with high HbF level and is part of a haplotype with a SNP in the +55 DHS. The +62 DHS SNP (rs1427407) lies within a conserved half E-box/GATA-site, an element often found in erythroid and hematopoietic enhancers. Allele-specific chromatin immunoprecipitation revealed decreased GATA1 and TAL1 binding to the high HbF allele in erythroid cells derived from CD34+ cells. Moreover, transcript analysis showed decreased BCL11A mRNA transcripts from the variant allele. These findings suggest that rs1427407 is causal and leads to a modest reduction in BCL11A expression (~40%). In the SCD cohort from NIH GWAS analysis, the naturally occurring variant (rs1427407) is associated with an increase in total HbF from ~4 to 11% (22). Natural variation in erythroid BCL11A expression appears to be less than that seen in the haploinsufficient state.

Fine mapping of genetically implicated regulatory sequences

As in the instance of GWAS for HbF levels, genetic variation is observed most often in presumed regulatory elements rather than coding regions. Methods to interrogate enhancer function at high throughput and high resolution are needed to validate the regulatory potential of GWAS-identified regions. The

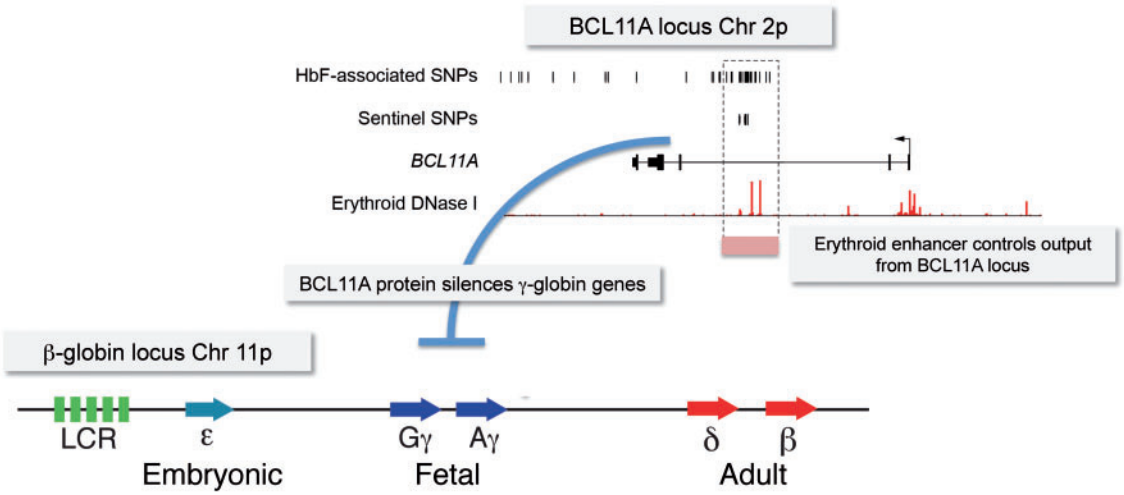

Figure 2. GWAS-identified HbF-associated SNPs within the BCL11A locus (chromosome 2) cluster within intron 2 of BCL11A that colocalize with erythroid-specific DNaseI hypersensitivity. These SNPs mark an area of cis regulatory potential that controls BCL11A expression. BCL11A protein acts in trans to regulate the β-globin gene locus on chromosome 11p.

erythroid specificity of the BCL11A enhancer is a feature that might be exploited in a strategy to reduce or ablate BCL11A expression only in the erythroid lineage as potential gene therapy for the hemoglobin disorders. While deletion of the entire 12kb enhancer region is feasible in cells in culture, efficiency is inversely related to deletion size and experience to date suggests that it would be too low for clinical application (24)

To interrogate enhancer function at high resolution, both to understand the organization of a complex enhancer and to search for discrete vulnerabilities, Canver *et al.* (23) developed a novel approach for an *in situ* saturation mutagenesis screen. This strategy allows for dissection of the regulatory element in native chromatin using a pooled library of guide RNAs (gRNAs) and Cas9, coupled with a functional readout of cells. Establishment of the method was facilitated by the availability of a novel human erythroblast cell line generated by oncogene immortalization of CD34+ cells. This cell line, HUDEP-2, resembles adult erythroid precursor cells and expresses HbA (25). Upon reduction of BCL11A with shRNA, HbF expression is enhanced, as in primary erythroid cells. Following introduction of Cas9 and a pooled collection of all possible gRNAs (based on the location of PAM sequences) to the DHS regions of the BCL11A enhancer, recovery of high HbF cells by FACS allows for identification of those gRNAs that lead to reduced BCL11A expression via disruption of the enhancer. The results were striking in several respects. First, most gRNAs did not alter HbF expression, indicating that cleavage followed by small insertions or deletions (indels) by nonhomologous end joining lead to no appreciable change in enhancer function. Second, a small set of gRNAs targeting a small region within the +58 DHS induced HbF expression, nearly to the extent evident by deletion of the entire 12 kb regulatory element or exon disruption. Detailed analysis revealed that indels neighboring a conserved GATA site correlated with loss of enhancer function. Third, slight reduction in enhancer function was observed with gRNAs targeting the +62 DHS, approximately in the position of the presumptive causal SNP detected in GWAS. In parallel studies using a knock-in reporter for embryonic β-like globin expression in murine erythroid cells, it was shown that the corresponding region of the mouse BCL11A locus exhibits discrete vulnerability in the +62 DHS rather than the +58 DHS region. Therefore, the BCL11A enhancer is subject to rapid evolution. The small segment of +58 DHS in the human gene that is critical for erythroid expression of BCL11A is a very attractive target for therapeutic gene editing (Fig. 3), as effects of its disruption are restricted to the erythroid cell lineage and thus spare hematopoietic stem cells and B-lymphocyte development.

Another major regulator: LRF/Pokemon

BCL11A is not the sole silencer of HbF. Its inactivation fails to completely reverse the hemoglobin switch at the adult stage. Quite recently, another potent silencer, called leukemia/lymphoma-related factor (LRF)/Pokemon/ZBTB7A, was identified through focused studies of its role in erythroid differentiation by Maeda and coworkers (26). In splenic erythroblasts of LRF conditional knockout mice, expression of mouse embryonic globin gene βH1 was dramatically increased, whereas that of the other embryonic gene εy was unchanged. This is in contrast to loss of BCL11A in which εy derepression is favored. Similarly, in LRF conditional mice harboring a human β-YAC transgene, human γ-globin expression was highly up-regulated compared to wild type. Although the mechanisms of LRF action need further study, initial findings indicate that LRF occupies the γ-globin genes and may maintain chromatin compaction. HUDEP-2 immortalized erythroblasts lacking either LRF or BCL11A express approximately 50–60% HbF. In double knockout cells, HbF is >95% of the total hemoglobin, suggesting that the two proteins comprise the majority of γ-globin silencing activity in adult erythroid cells. To date, neither GWAS nor searches for rare variants in patients with elevated HbF have associated genetic variation at LRF with HbF regulation.

Common pathway for HbF silencing

BCL11A and LRF proteins interact with components of the nucleosome remodeling deacetylase (NuRD) complex, an ATP-dependent chromatin remodeling complex comprised of multiple subunits, methyl cytosine domain 2/3 (MBD2/3), chromodomain-helicase DNA binding protein 3/4 (Mi2α/β), HDAC1/2, metastasis-associated protein 1/2, p66α/β and RbAp46/48 (27,28). However, BCL11A and LRF do not appear to be present in common complexes. Consideration evidence suggests that NuRD serves critical roles in globin gene repression and may represent a common pathway through which the two potent silencers of HbF expression act (Fig. 4). Prior work of Ginder and coworkers (29,30) have implicated NuRD in globin repression, both in chicken and mammalian erythroid cells, and indicated that physical interaction of p66 and MBD2 facilitates recruitment of CHD4/Mi2β to target globin genes. Interfering p66-MBD2 interactions leads to derepression of globin genes in several erythroid cell contexts. Independent support for a critical role of NuRD has been provided by shRNA knockdown of NuRD components in primary human erythroid cells derived from CD34+ cells (31). Of note, knockdown of CHD4/Mi2β leads to the greatest reactivation of HbF. Conditional knockout of CHD4/Mi2β in mice is consistent with these observations, though the extent of *in vivo* contribution cannot be assessed in the published data (32).

Other potential pathways explored largely through small molecule inhibition

In the hope of finding small molecules that might be developed into drugs for HbF reactivation in patients, investigators have

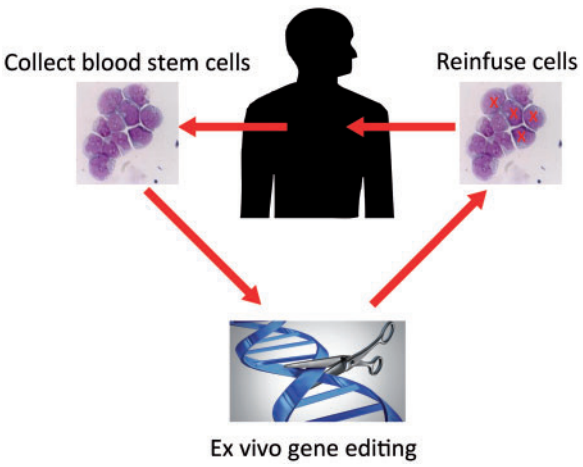

Figure 3. Schematic of gene therapy strategy for the β-hemoglobinopathies. Hematopoietic stem and progenitor cells are harvested from the patient and subjected to zinc finger or CRISPR/Cas9 mediated gene editing to interrupt the erythroid-specific enhancer of BCL11A. The cells, a mixture of unmodified and modified cells (marked with a red X) are then reinfused into the patient.

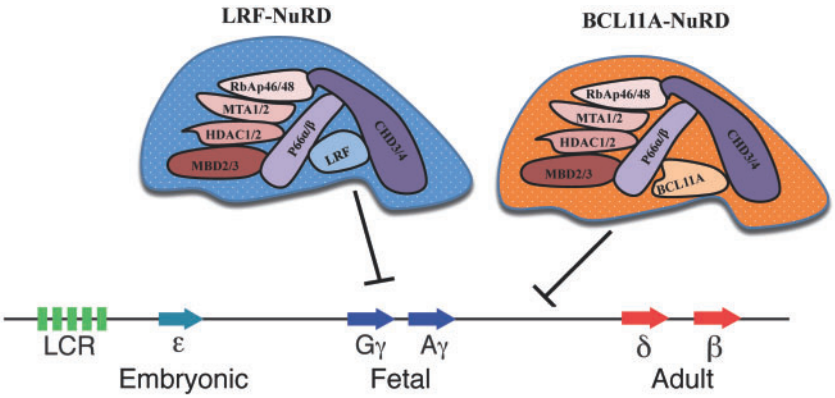

Figure 4. LRF and BCL11A each physically associate with the NuRD complex. However, the two proteins are not found within the same complexes. Therefore, it is hypothesized that they act through a common pathway mediated by NuRD. LRF is believed to directly act at the γ-globin genes, whereas BCL11A may affect silencing by interacting with sequences downstream of the γ-globin genes.

examined several pathways and potential targets, principally focused on epigenetic control mechanisms. For example, the histone demethylase lysine-specific demethylase-1 (LSD1) is present in proteomic analyses of BCL11A protein complexes and complexes associated with proposed repressive factors TR2/4 (31,33). Inhibition of LSD1 with small molecules is associated with increased HbF ratios in CD34+-derived erythroid cells. Engel and coworkers have suggested that LSD1 inhibitors may prove useful for treatment of patients. However, other findings indicate that the extent of derepression of γ-globin expression upon LSD1 knockout (or inhibition) is at least an order of magnitude lower than that achieved by BCL11A loss and is accompanied by impairment of erythroid differentiation (31).

Dimethylation of histone 3 lysine 9 (H3K9me2) is highly associated with gene repression. The enzymes EMHT1 (GLP) and EMHT2 (G9a), which are believed to form a heterodimeric complex, catalyze and maintain the repressive H3K9Me2 chromatin mark (34–40). UNC0638, a small molecule inhibitor of EHMT1 and EHMT2, strongly decreases global H3K9 dimethylation and causes delayed lineage specification of CD34+ cells (41,42). Two recent reports used UNC0638, shRNA knockdown or genome editing to reduce EHMT1/2 activity in erythroid cells (43,44). Miller and coworkers reported that LDB1 protein accumulates at the γ-globin gene promoters in the absence of EHMT2 and facilitates loop formation with the LCR. Ebert and coworkers focused on the altered chromatin state in the UNC0638 treated CD34+ cells. Global loss of H3K9me2 was accompanied by a specific increase in H3K9 acetylation, an activating chromatin mark, at both γ-globin gene bodies. Inhibitors of HDACs lead to some increased HbF in primary CD34+-derived erythroid cells (45–48). Combined EHMT1/2 and HDAC inhibition leads to an additive increase in γ-globin gene expression.

Reports of several years ago suggested that thalidomide-like agents, such as lenalidamide and pomalidomide, which are FDA-approved for myeloma and myelodysplastic disease, induce some HbF expression in erythroid cells. At the time of the initial studies, the mechanism of action of these drugs was unknown, though they were believed to be immunomodulatory. Elegant studies from the laboratories of Kaelin and Ebert, however, demonstrated that these drugs bind specifically to cereblon to direct ubiquitination to distinct protein targets, which differ depending on the drug-specific thalidomide-derivative (49,50). In myeloma cells pomalidomide directs specific degradation of IKZF1, which leads to cell death. Blanc and coworkers (51) recently reported that pomalidomide treatment increases HbF in CD34+-derived erythroid cells in association with downregulation of multiple factors, BCL11A, SOX6, GATA1, KLF1 and LSD1, at both protein and mRNA levels. They concluded that the drug may 'reprogram' erythroid progenitors to a more fetal-like state. The lack of specificity with respect to protein targets is equally consistent with an alternative hypothesis that the effects reflect general cellular stress. Further studies are needed to explore the potential utility of pomalidomide as a potential HbF inducer in patients.

Summary

Progress in understanding the precise control of globin genes has accelerated lately through convergence of human genetics and new methods for gene manipulation. The story of BCL11A illustrates general principles of genetic variation affecting regulatory sequences and also how exploration of a candidate locus identified by GWAS can build on preexisting biology and genetics. The validation of two major silencers of HbF expression, BCL11A and LRF, and a common pathway apparently shared by these factors, NuRD, provides opportunities to interrogate the mechanisms underlying gene-specific expression in the β-globin complex in detail. It is hoped that this path of research will ultimately provide a framework for discovery of compounds capable of reactivating HbF to substantial levels and thereby enhancing the lives of the many individuals afflicted with the β-globin disorders worldwide. In the meantime, the new findings may be exploited for improved genetic therapy. The critical region within the erythroid enhancer of BCL11A is an attractive target for gene editing, either by zinc fingers or CRISPR/Cas9. For clinical application, it is envisioned that the hematopoietic stem cells of patients will be subjected to gene editing *ex vivo* and then returned to the patients for reconstitution of their blood system (Fig. 3). Based on on-going preclinical work, it is anticipated that the first clinical trials might begin within the coming year.

Conflict of Interest statement. None declared.

Funding

Work from the Orkin laboratory was supported by the Howard Hughes Medical Institute and grants from the National Institutes of Health (HL032262 and HL032259). E.C.S. was supported by a Jane Coffin Childs Memorial Fund for Medical

Research Fellowship and a Postdoctoral Enrichment Program Award from the Burroughs Wellcome Fund.

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