Concise Review: Epigenetic Regulation of Hematopoiesis: Biological Insights and Therapeutic Applications

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ABSTRACT

Hematopoiesis is the process of blood cell formation starting from hematopoietic stem/progenitor cells (HSPCs). The understanding of regulatory networks involved in hematopoiesis and their impact on gene expression is crucial to decipher the molecular mechanisms that control hematopoietic development in physiological and pathological conditions, and to develop novel therapeutic strategies. An increasing number of epigenetic studies aim at defining, on a genome-wide scale, the cis-regulatory sequences (e.g., promoters and enhancers) used by human HSPCs and their lineage-restricted progeny at different stages of development. In parallel, human genetic studies allowed the discovery of genetic variants mapping to cis-regulatory elements and associated with hematological phenotypes and diseases. Here, we summarize recent epigenetic and genetic studies in hematopoietic cells that give insights into human hematopoiesis and provide a knowledge basis for the development of novel therapeutic approaches. As an example, we discuss the therapeutic approaches targeting cis-regulatory regions to reactivate fetal hemoglobin for the treatment of β-hemoglobinopathies. Epigenetic studies allowed the definition of cis-regulatory sequences used by human hematopoietic cells. Promoters and enhancers are targeted by transcription factors and are characterized by specific histone modifications. Genetic variants mapping to cis-regulatory elements are often associated with hematological phenotypes and diseases. In some cases, these variants can alter the binding of transcription factors, thus changing the expression of the target genes. Targeting cis-regulatory sequences represents a promising therapeutic approach for many hematological diseases. Stem Cells Translational Medicine 2017;6:2106–2114

SIGNIFICANCE STATEMENT

This review summarizes the epigenetic and genetic studies identifying genes and cis-regulatory regions involved in normal and pathological hematopoiesis. Novel potential therapeutic approaches targeting cis-regulatory sequences, which hold great promise for the treatment of many hematological diseases, are discussed. Readers will gain an overview of the epigenetic mechanisms regulating hematopoiesis and acquire knowledge about genome editing-based approaches for the treatment of β-hemoglobinopathies.

INTRODUCTION

Different cell types from the same organism or tissue are genetically identical but functionally heterogeneous because of the differential expression of genes. Epigenetic modifications are responsible for changes in gene activity that are not strictly dependent on the DNA sequence. Epigenetic markers can be used to identify cis-regulatory DNA elements (e.g., promoters and enhancers) that mediate developmental stage- and tissue-specific gene expression. High-throughput sequencing technologies have been extensively used to study the epigenetic regulation of gene expression programs in a wide range of hematopoietic cell types. The definition of regulatory regions controlling cell-specific gene expression programs is fundamental to understand the molecular mechanisms underlying hematopoiesis in health and disease.

HUMAN HEMATOPOIESIS

Human blood contains several different cell types with specific functions. Erythrocytes, also known as red blood cells (RBCs), transport oxygen from the lungs to the tissues and remove carbon dioxide. Leukocytes, also known as white blood cells (WBCs), are involved in inflammatory reaction and immune response. WBCs comprise granulocytes (neutrophils, basophils, eosinophils, and mast cells), lymphocytes (T cells, B cells, and natural killer [NK] cells), monocytes/macrophages, and...
dendritic cells. Platelets are cell fragments derived from megakaryocytes and play an essential role in the maintenance of hemostasis (Fig. 1).

Hematopoietic stem cells (HSCs) sustain the life-long production of all blood cells thanks to their ability to self-renew and differentiate toward multiple lineages. The hierarchical model of hematopoiesis places HSCs at the root of the tree composed of a series of multipotent, oligopotent, and unipotent progenitors that finally differentiate toward mature hematopoietic cells. Multipotent progenitor cells (MPPs), the immediate progeny of HSCs, have a full lineage potential but a limited self-renewal ability. MPPs give rise to oligopotent progenitors: the common myeloid progenitors (CMPs) and the multilymphoid progenitors (MLPs) [1]. CMPs differentiate into the granulocyte/macrophage progenitors (GMPs) and the megakaryocyte/erythroid progenitors (MEPs), which then give rise to unilineage progenitors and mature precursors. MLPs are able to generate both GMPs and the common lymphoid progenitors, which differentiate into B, T, and NK cell precursors (Fig. 1).

This current model of hematopoiesis (reviewed in [2]) is based on prospective isolation of hematopoietic cells carrying specific surface markers and evaluation of the lineage output using in vitro or in vivo assays. However, several studies at single-cell level challenged the classical hierarchy of human hematopoiesis. Combining new sorting strategies, optimized single-cell functional assays and single-cell gene expression profiling, Notta et al. showed that, in adult bone marrow, classically defined CMPs and MEPs are heterogeneous populations, composed mostly of unilineage committed cells [3]. Therefore, multipotent cells, such as HSCs and MPPs, give rise to committed unipotent progenitors, without an intermediate oligopotent CMP and MEP stage. Furthermore, the study of Velten et al. showed that HSCs and their immediate progeny, such as MPPs and MLPs, do not represent discrete cell types, but are included in a continuum of low-primed undifferentiated (CLOUD)-hematopoietic stem/progenitor cells (HSPCs) [4]. In the CLOUD, HSCs gradually acquire continuous transcriptional lineage priming into lympho/myeloid or megakaryocytic/erythroid major branches.

Despite these recent single-cell studies suggesting a revision of the hierarchical organization of human hematopoiesis, the current model is suitable and widely used to investigate the molecular mechanisms that drive HSPCS commitment and differentiation.

**EPIGENETIC CONTROL OF GENE EXPRESSION**

Gene expression is regulated by transcription factors and cofactors binding cis-regulatory DNA sequences, such as promoters and enhancers. Promoters are located at the 5’ end of the genes and consist of multiple DNA motifs for transcription factors that recruit the transcriptional machinery and define the transcription start site (TSS) [5]. Enhancers are clusters of transcription factors binding sites and can increase the transcription of the target promoters [5–7]. Enhancers function at various distances from their target genes and can be located upstream or downstream of
of human cell lines, primary cells, and tissues, thanks to different enhancers, respectively [9, 11]. Lysine 27 acetylation of histone H3 expression of the target genes (e.g., TF, transcription factor).

genes or within introns. Chromatin looping is thought to bring promoters and enhancers in close proximity to activate gene transcription. Enhancers are fundamental for the spatial- and temporal-specific expression of genes. These regulatory elements can be cell-type-specific: distinct enhancer elements can act on the same gene at different cellular stages or in different tissues, as well as in response to different stimuli. More recently, super-enhancers have been defined as clusters of cell-specific enhancers densely occupied by transcription factors and cofactors, and involved in the regulation of genes specifying cell identity [8].

Cis-regulatory elements present characteristic epigenetic features. They are typically devoid of nucleosomes to allow the binding of transcription factors to their DNA motifs [9, 10]. Nucleosomes surrounding these highly accessible DNA sequences are characterized by specific histone modifications. Histone H3 lysine 4 trimethylation (H3K4me3) and monomethylation (H3K4me1) are preferentially associated with promoters and enhancers, respectively [9, 11]. Lysine 27 acetylation of histone H3 (H3K27ac) marks highly active promoters and enhancers, and super-enhancers [8, 11, 12] (Fig. 2). Histone modifying enzymes that act as transcriptional coactivators, such as the histone acetyltransferase p300 and CREB binding protein (CBP), are localized in active regulatory elements, in particular enhancers [9, 13].

In the last 10 years, epigenetic features characteristic of transcriptional regulatory regions (DNA accessibility, histone marks, transcription factors, and cofactors binding) have allowed the mapping of cis-regulatory elements genome-wide in a multitude of human cell lines, primary cells, and tissues, thanks to different high-throughput technologies based on next-generation sequencing (NGS) [7, 9]. Chromatin accessibility can be directly assessed with different approaches: DNase-seq, formaldehyde-assisted isolation of regulatory elements (FAIRE-seq), and assay for transposase-activatable chromatin using sequencing (ATAC-seq) [9]. DNase-seq detects accessible DNA by DNasel enzymatic digestion of nucleosome-depleted sites, also known as DNasel hypersensitive (HS) sites, which represent cis-regulatory elements [14]. FAIRE-seq (formaldehyde assisted isolation of regulatory elements coupled with NGS) determines open chromatin regions by sequencing protein-free DNA, after removal of formaldehyde crosslinked protein-DNA complexes [15]. ATAC-seq is based on the ability of the hyperactive Tn5 transposase to fragment DNA and integrate sequencing adapters into open chromatin regions [16]. Chromatin immunoprecipitation (ChIP) combined with NGS (ChIP-seq) [17, 18] is also widely used to study chromatin modifications typical of cis-regulatory elements. ChIP-seq profile of H3K4me3 allows the identification of promoter regions [9, 17, 18], while H3K4me1 analysis defines enhancer regions [9]. H3K27ac can be used alone or integrated with H3K4 methylation profiles to identify highly active promoters and enhancers, and super-enhancers [8, 19, 20]. ChIP-seq analysis of binding sites of multiple transcription factors and/or coactivator proteins, such as p300 and CBP acetyltransferase, can also be used to identify enhancer regions [9, 21, 22].

Human hematopoiesis is one of the most established cell differentiation systems and is amenable to the study of gene transcription and chromatin structure. The transition through the hematopoietic hierarchy is regulated at transcriptional level. Master transcription factors control the activation of lineage-specific transcriptional programs through the binding of cis-regulatory elements. The identification of enhancers is a key step to understand how gene expression is finely regulated during hematopoiesis and how it is altered in pathological conditions [23]. Enhancers establish not only the transcription level, but also when and where a gene is expressed, thus determining cell identity. Most of the epigenetic studies characterizing the regulatory landscape of human hematopoietic cells rely on the prospective isolation of the different cell types defined using specific panels of surface markers (Fig. 1). In the last years, large international consortia, such as ENCODE (http://www.encodeproject.org), Roadmap Epigenomics (http://www.roadmapepigenomics.org), and Blueprint Epigenome (http://www.blueprint-epigenome.eu) collected epigenetic data of several human hematopoietic cell types, including rare blood cells [24–26]. These data represent an important public resource for basic biology and disease-oriented research. Several studies investigated the regulatory landscape of HSPCs in comparison with lineage-restricted progenitors or mature cells and described changes in enhancer dynamics during erythroid [27–31], myeloid [30–32], and lymphoid [28, 31, 32] commitment and differentiation. These studies showed that each individual cell type, within the hematopoietic hierarchy, displayed a set of cell-specific cis-regulatory regions associated to genes involved in cellular functions related to the given cell type. Interestingly, enhancer and super-enhancer landscape better define cell identity, compared with transcriptomic profile and promoter usage [12, 30, 31].

**Insights into Human Hematopoiesis from Genetic and Epigenetic Studies**

Human genetic studies allow the identification of DNA variants mapping to epigenetically defined regulatory regions and influencing gene expression. Genome-wide association studies (GWASs) allow the identification of sequence variations (single nucleotide polymorphisms [SNPs]) associated with human phenotypes and...
Figure 3. Genetic variants in the β-globin locus, HBS1L-MYB, and BCL11A loci influence fetal hemoglobin (HbF) levels. (A): Schematic representation of the β-globin locus on chromosome 11. Point mutations (HPFH 175 T > C and HPFH 198 T > C upstream of the γ-globin transcription start sites [TSSs]) and deletions (HPFH 13-bp in δγ-globin promoter and HPFH-S) are associated with HPFH. The 175 T > C and 198 T > C mutations create binding sites for TAL1 and KLFL1 transcriptional activators. A common SNP at position -158 bp of the γ-globin promoter is associated with moderately high levels of HbF. LCR and β-like globin genes (embryonic c, fetal δγ and αγ, and adult δ and β) are indicated. (B): The region between HBS1L and MYB genes on chromosome 6 contains three HMIP blocks 1, 2, and 3. Five SNPs associated with higher HbF levels map to the -84 and -71 kb MYB enhancers in HMIP-2. These variants reduce LDB1, GATA1, and KLFL1 occupancy, thus decreasing MYB expression and, as a consequence, increasing HbF expression. (C): Representation of the BCL11A gene on chromosome 2. Several SNPs, associated with high HbF levels, map to three erythroid-specific intronic enhancers located 55, 58, and 62 kb downstream of the BCL11A TSS. A SNP within the +62 kb enhancer impairs GATA1 and TAL1 binding, thus leading to a reduction of BCL11A expression and increase of HbF levels. Targeted disruption of a GATA1 binding site in the +58 kb enhancer is also associated with decreased BCL11A levels and high HbF expression. Abbreviations: HMIP, HBS1L-MYB intergenic polymorphism; HPFH, hereditary persistence of fetal hemoglobin; LCR, locus control region; SNP, single nucleotide polymorphism.

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Functional and mechanistic studies can be focused on the genes associated with the cis-regulatory regions and potentially involved in hematopoiesis. As an example, GWASs identified a SNP associated with low RBC number and high MCV in an enhancer region located 15 kb upstream of the cyclin D3 gene (CCND3) (Fig. 2) [39, 55]. Starting from this observation, Sankaran et al. unraveled the role of cyclin D3 in the regulation of erythrocyte number and size through gene disruption studies [41]. Other studies dissected the regulatory elements harboring genetic variants associated with particular phenotypes [46, 56]. A recent GWAS identified a SNP, associated with low basophil counts, 39 kb downstream of the gene encoding CCAAT/enhancer binding protein alpha (CEBPA), a master regulator implicated in basophil specification [56, 57]. This SNP overlaps with an enhancer targeted by the myeloid master regulators GATA2 and RUNX1 and identified specifically in CMPs, which give rise to several lineages including basophils. CRISP-RCas9-mediated mutagenesis of this region in HSPCs led to CEBPA downregulation and impairment of basophil production and maturation (Fig. 2) [56]. Interestingly, disease-causing variants affecting the binding sites of the erythroid master regulator GATA1 were identified in regulatory elements of the ALAS2 [44, 45], UROS [47], PKLR [48], and HBD [49] genes. Loss of GATA1 binding at promoter or enhancer regions was associated with decreased expression of the target gene (Fig. 2), which can lead to severe clinical phenotypes (e.g., X-linked sideroblastic anemia for ALAS2, congenital erythropoietic porphyria for UROS and pyruvate kinase deficiency for PKLR). Initial validation studies based on electrophoretic mobility shift and luciferase assays showed that these mutations impair GATA1 binding and the enhancer or promoter activity [45, 47, 49]. More recently, CRISPR-Cas9-mediated in situ disruption of the GATA1 motifs present in the ALAS2, UROS, and PKLR regulatory regions reproduced the gene downregulation observed in patients harboring mutation of these binding sites [46]. Interestingly, the disruption of the GATA1 motifs seems to affect the binding of the GATA1-dependent activation complex [46].

**cis-Regulatory Regions as Genetic Modifiers of Fetal Hemoglobin**

Hemoglobin (Hb) is a tetrameric protein containing two α-like and two β-like globins. The β-like globin genes (embryonic α, fetal αγ and δ, adult δ and β) are located on chromosome 11 within the β-globin gene cluster. Here, these genes are under the control of the β-globin locus control region (LCR), composed of DNsase I HSs. The LCR is able to recruit transcription factors and interact with the different β-like globin gene promoters to regulate their expression during development. A γ-to-β globin switching occurs shortly after birth. In adulthood, the expression of fetal hemoglobin (Hbf, α2γ2) is reduced to <1% of the total Hb output and the major Hb type is HbA (α2β2). β-hemoglobinopathies are the most common genetic disorders worldwide and are characterized by reduced or abnormal production of adult β-chains. In β-thalassemia, mutations affecting β-globin chain production cause the precipitation of unpaired α-globin chains within erythroid precursors, leading to their death and thereby causing ineffective production of RBCs. In sickle cell disease (SCD), a single amino acid substitution (E6V) in the β-globin chain causes the production of sickle hemoglobin (Hbs). Hbs has the propensity to polymerize and precipitate under deoxygenated conditions, resulting in RBC deformation and vaso-occlusions.

β-thalassemia and SCD display a remarkable variability in the clinical severity. However, reasons explaining this heterogeneity are not fully understood. Different studies have shown that individual variation in HbF expression may influence the clinical outcome of these pathologies, with high HbF levels correlated with less severe complications and longer life expectancy [58–60]. In β-thalassemia, γ-globin compensates the β-globin deficiency, whereas in SCD it exerts a potent anti-sickling effect thanks to a critical amino acid blocking the lateral contacts between β-like globin chains required for the formation of Hbs polymers.

A benign syndrome, referred to as “hereditary persistence of fetal hemoglobin” (HPFH), is characterized by increased Hbf levels (up to 90% of the total Hb) in the adult life without major impairment of RBC indices [61]. Molecular studies have identified two different types of HPFH, either caused by large deletions encompassing the β- and δ-genes (13–106 kb; e.g., HPFH5) or due to point mutations in the γ-globin promoters (Fig. 3A). Deletional HPFH are thought to either juxtapose the γ-globin promoters to enhancers normally located far away from the γ-globin genes or remove γ-globin inhibitory sequences [61, 62]. Non-deletional HPFH may alter the binding of transcription factors to critical regions of the γ-globin promoters. These mutations occur in three distinct regions of the highly similar γ-globin promoters: (a) approximately 200 bp upstream of the TSS of the γ-globin genes. The −198 bp T > C mutation in the α2γ-globin promoter has been recently shown to generate a de novo binding site for the erythroid transcriptional activator KLF1 [63], (b) at position −175 bp where the mutation T > C in both the γ-promoters creates a binding site for TAL1, a transcription factor activating the expression of many erythroid-specific genes [52], (c) between −117 and −102 bp within the CCAAT box and direct repeat (DR) elements targeted by potential Hbf repressors, such as NR2F2 (COP-THF), NR2C1 (TR2), and NR2C2 (TR4) [64]. A 13-bp HPFH deletion (−114 to −102 bp) disrupting the CCAAT box and the DR elements of the α2γ-globin gene has also been described [61, 65] (Fig. 3A). HPFH large deletions and point mutations are frequently associated with a pancellular Hbf distribution among the erythrocytes. Interestingly, pancellular Hbf expression in compound heterozygous with SCD and HPFH traits results in absent or milder SCD symptoms [61]. As an example, SCD-HPFH individuals have no features of SCD, including vaso-occlusive events and hemolytic anemia [66]. Beside HPFH mutations, other genetic factors can moderately raise Hbf levels above 1% of the total Hb and are associated with heterocellular Hbf distribution. Patients showing heterocellular Hbf expression still display anemia and vaso-occlusive complications, albeit less severe than in SCD [67]. Notably, this condition was associated with reduced pain crisis rate in SCD patients [68]. Genetic studies identified sequence variations at three genomic loci (β-globin locus, HBS1L-MYB, and BCL11A) that account for >30% of the variance in Hbf levels [69–71].

In the β-globin locus, a common SNP (C > T; commonly referred to as the “XmnI site”) at position −158 bp of the α2γ-globin promoter (chromosome 11p15) was linked with moderately high Hbf levels [71–73] (Fig. 3A). Other SNPs associated with Hbf levels were mapped in the β-globin locus [74, 75]. However, the functional cis-regulatory elements targeted by these SNPs have not been identified yet. Polymorphisms of the β-globin locus alone cannot explain the considerable variance in Hbf levels, as demonstrated by studies showing that high Hbf determinant segregates independently of the β-globin gene cluster [68, 76, 77],

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thus suggesting the involvement of other QTLs in the modulation of $\gamma$-globin expression.

Craig and colleagues demonstrated in a large kindred that another major determinant of HbF production is located on chromosome 6q23-q24 [78]. Further studies identified multiple genetic variants on chromosome 6q23 that are strongly associated with HbF levels [70]. These SNPs are distributed in three genomic regions (referred to as HBS1L-MYB intergenic polymorphism [HMP] blocks 1, 2, and 3) that span a 79-kb segment from the guanosine triphosphate (GTP)-binding elongation factor HBS1L gene to 45 kb upstream of the myeloblastosis oncogene MYB gene (Fig. 3B). HMP block 2 contains SNPs that are strongly associated with HbF and erythroid traits. Among these variants, five SNPs were mapped in two epigenetically defined erythroid-specific enhancers, located 84 and 71 kb upstream of the MYB TSS [79]. These variants impaired the binding of the looping factor LDB1 and GATA1, TAL1 and KLF1 erythroid master regulators (Fig. 3B). As a consequence, the long-range interaction between the erythroid-specific enhancers and MYB promoter were decreased, as well as MYB expression [79]. CRISPR-Cas9 mutagenesis confirmed the genetic association of the $\sim84$ and $\sim71$ kb enhancer regions with MYB expression levels [80]. MYB downregulation was associated with moderately increased HbF levels [79]. Lower MYB levels lead to a slow cell-cycle progression, which is associated with increased F-cell production. Alternately, increased HbF levels can be ascribed to the failed activation of genes encoding HBF repressors (e.g., BCL11A) by MYB [79] (Fig. 3B).

A GWAS performed in individuals with contrasting extreme F-cell distribution (below the 5th or above the 95th percentile) mapped an additional QTL in the gene encoding the zinc-finger transcription factor BCL11A on chromosome 2p15 [69]. Other GWASs validated the association between several SNPs in the BCL11A locus and higher HbF levels [68, 81–83]. These SNPs lie in an erythroid-specific intronic super-enhancer composed of three constituent enhancers located 55, 58, and 62 kb downstream of the BCL11A TSS (+55, +58, and +62 DNase HS) [84, 85] (Fig. 3C). A sequence variant in the $+62$ DNase HS affects the binding of GATA1 and TAL1 transcription factors, leading to a modest reduction of BCL11A expression and increased HbF levels in a SCD cohort [84] (Fig. 3C). These observations suggested a role of BCL11A in the repression of HbF expression in adult life. Further studies showed that shRNA-knockdown of BCL11A in primary erythroid cells increased $\gamma$-globin expression [86]. Notably, inactivation of BCL11A led to the correction of the murine SCD phenotype by inducing pancellular HbF expression [87].

**Therapeutic Strategies for $\beta$-Hemoglobinopathies Targeting cis-Regulatory Regions**

Current treatments for SCD and $\beta$-thalassemia involve symptomatic care and RBC transfusions, which, however, can lead to iron overload and organ damage. The only definitive cure for $\beta$-hemoglobinopathies is the allogeneic HSCT transplantation, which is available to a small proportion (30%) of the patients with an HLA-compatible donor [88]. Transplantation of autologous genetically corrected HSCs is considered an attractive therapeutic alternative for patients lacking a suitable donor. Gene therapy trials based on the use of lentiviral vectors expressing a $\beta$-globin transgene are currently ongoing [89–93]. Alternative promising approaches aim at reactivating therapeutic HbF expression [54]. Many of these strategies are based on targeted genome editing of cis-regulatory elements [85, 94–96].

Several groups attempted to mimic the beneficial HPFH mutations in the $\Delta\beta$- and/or $\gamma$-globin gene promoters. HPFH mutations in the $\gamma$-globin promoters may disrupt binding sites for $\gamma$-globin silencers [94] or generate new binding sites for $\gamma$-globin activators [52, 63] (Fig. 3A). Traxler et al. reproduced the 13-bp HPFH deletion in the $\gamma$-globin promoters using the CRISPR/Cas9 system, thereby inducing HbF levels sufficient to inhibit HbS polymerization in SCD HSPC-derived erythrocytes. This deletion is thought to reactivate fetal genes by removing the CCAAT box and the DR element, thus disrupting the binding sites for $\gamma$-globin transcriptional repressors [97]. The introduction of HPFH point mutations represents a potential strategy to reactivate HbF expression. In erythroid cell lines, the insertion of $\sim175T>C$ or the $\sim198T>C$ substitutions in the $\gamma$-globin promoters created de novo binding sites for transcriptional activators, and induced loop formation between the LCR and $\gamma$-globin promoters, thus increasing HbF expression (Fig. 3A) [52, 63]. This potential therapeutic approach has not yet been explored in human HSPCs. Another strategy aims at recreating deleitional HPFH, removing the $\beta$- and $\delta$-globin genes and the putative $\gamma$-globin inhibitory sequences. Ye et al. explored a CRISPR/Cas9 strategy in normal donor-derived HSPCs to excise a 12.9-kb region deleted in HPFH individuals (HPFH-5; Fig. 3A) [95, 98]. This approach resulted in a significant $\gamma$-globin induction and downregulation of the $\beta$-globin expression in HSPC-derived erythrocytes.

Downregulation of factors responsible for fetal $\gamma$-globin repression can be explored to achieve therapeutic HbF expression. Over the past decades, molecular studies unraveled the role of several nuclear factors in the regulation of the fetal-to-adult hemoglobin switching [99, 100]. However, interfering with the expression of HBF repressors, such as MYB, leukemia/lymphoma-related factor (LRF), KLF1, and BCL11A, can impair terminal erythropoiesis and the development of multiple hematopoietic lineages. MYB is essential for the hematopoietic system development [101] and its downregulation in human HSPCs determines a cell-cycle arrest and impairs erythroid differentiation [102]. The transcription factor LRF, encoded by the ZBTB7A gene, is necessary for HSC maintenance and B cell commitment [103]. In mice, loss of LRF leads to lethal anemia, due to increased apoptosis of erythroid precursors [104], and LRF knockdown increases HbF expression but delays human erythroid differentiation [105, 106]. KLF1 is a transcriptional regulator promoting erythroid lineage development at the expense of the megakaryocytic compartment [107, 108] and KLF1 knockout mice die in utero from severe anemia [109]. Although KLF1 haploinsufficiency can induce HbF expression [110], KLF1 is not an ideal target to develop a safe therapy for $\beta$-hemoglobinopathies, given its essential role in erythropoiesis. BCL11A is essential for proper development of B cells and BCL11A-deficient HSCs showed cell cycle and multi-lineage differentiation defects [111]. Therefore, erythroid-restricted BCL11A knockdown is mandatory. Interestingly, erythroid-specific expression of a shRNA targeting BCL11A induced HbF reactivation in normal donor and SCD erythrocytes circumventing HSC toxicity [112]. Recently, Chang et al. showed that complete BCL11A knockdown impair RBC enucleation [113]. However, in humans, BCL11A haploinsufficiency is associated with HbF persistence with normal hematological functions [114], suggesting that a fine modulation of BCL11A expression is required to develop a safe therapeutic strategy for $\beta$-hemoglobinopathies. An alternative strategy is based on the disruption of erythroid-specific BCL11A enhancers.
allowing a fine tuning of BCL11A expression. CRISPR/Cas9 dissection of BCL11A intrinsic enhancers identified a potential GATA1 binding site in the +58 DNase HS critical for erythroid expression of BCL11A. CRISPR/Cas9 and zinc finger nucleases-mediated disruption of this GATA1 motif led to reasonably decreased BCL11A expression and increased HbF levels in human erythrocytes. Importantly, targeting of the BCL11A erythroid-specific enhancer did not affect RBC enucleation.

For a potential clinical application of these novel approaches aimed at reactivating HbF expression, further studies are required to: (a) assess the frequency of genome edited bona fide human HSCs. To this aim, the delivery and the activity of the genome editing tools have to be optimized; (b) evaluate the potential toxicity due to the delivery and the on- and off-target activity of the genome editing tools in HSCs and their progeny; (c) perform a proper comparison of the different therapeutic strategies in terms of Hb content and functional correction of the patient phenotype.

CONCLUSION

In summary, the combination of genetic and epigenetic studies enabled the identification of genes and cis-regulatory regions involved in normal and pathological hematopoiesis. cis-regulatory elements represent in some cases potential therapeutic targets. The recent advent of genome editing technologies offers great promise for the development of targeted therapeutic approaches. Future studies will address the efficiency, efficacy, and safety of novel therapeutic strategies aimed at modulating gene expression by targeting cis-regulatory regions.

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AUTHOR CONTRIBUTIONS

C.A. and O.R.: conception and design, manuscript writing; A.M.: conception and design, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

REFERENCES

1. Doulatov S, Notta F, Eppert K et al. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. Nat Immunol 2010;11:585–593.
2. Doulatov S, Notta F, Laurenti E et al. Hematopoiesis: A human perspective. Cell Stem Cell 2012;10:120–136.
3. Notta F, Zandi S, Takayama N et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. Science 2016;351:aab2216.
4. Velten L, Haas SF, Raiff S et al. Human hematopoietic stem cell lineage commitment is a continuous process. Nat Cell Biol 2017;19:271–281.
5. Maston GA, Evans SK, Green MR. Transcriptional regulatory elements in the human genome. Annu Rev Genomics Hum Genet 2006;7:29–59.
6. Pennacchio LA, Bickmore W, Dean A et al. Enhancers: Five essential questions. Nat Rev Genet 2013;14:288–295.
7. Shlyueva D, Stampfli G, Stark A. Transcriptional enhancers: From properties to genome-wide predictions. Nat Rev Genet 2014;15:272–280.
8. Whyte WA, Orlando DA, Hnisz D et al. Master transcription factors and mediator establish super-enhancers at key cell identity genes. Cell 2013;153:307–319.
9. Heinitzman ND, Stuart RK, Hon G et al. Distinct and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. Nat Genet 2007;39:311–318.
10. Gross DS, Garrard WT. Nucleosome hyper-sensitive sites in chromatin. Annu Rev Biochem 1988;57:159–191.
11. Ernst J, Kheradpour P, Mikkelsen TS et al. Mapping and analysis of chromatin state dynamics in nine human cell types. Nature 2011;473:43–48.
12. Hnisz D, Abraham BJ, Lee TI et al. Super-enhancers in the control of cell identity and disease. Cell 2013;155:934–947.
13. Heinitzman ND, Hon CG, Hawkins RD et al. Histone modifications at human enhancers reflect global cell type-specific gene expression. Nature 2009;459:108–112.
14. Crawford GE, Holt IE, Whittle J et al. Genome-wide mapping of DNase hypersensitive sites using massively parallel signature sequencing (MPS). Genome Res 2006;16:123–131.
15. Giresi PG, Kim J, McDaniel RM et al. FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. Genome Res 2007;17:877–885.
16. Buenrostro JD, Giresi PG, Zaba LC et al. Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. Nat Methods 2013;10:1213–1218.
17. Barski A, Cuddapah S, Cui K et al. High-resolution profiling of histone methylations in the human genome. Cell 2007;129:823–837.
18. Mikkelsen TS, Ku M, Jaffe DB et al. Combinatorial patterns of histone acetylations and methylations in the human genome. Nature Genet 2008;40:897–903.
19. Wang Z, Zang C, Rosenfeld JA et al. Combinatorical patterns of histone acetylations and methylations in the human genome. Nature Genet 2008;40:897–903.
20. Creyghton MP, Cheng AW, Welstead CG et al. Histone H3K27ac separates active from poised enhancers and predicts developmental state. Proc Natl Acad Sci USA 2010;107:21931–21936.
21. Su MY, Steiner LA, Bogardus H et al. Identification of biologically relevant enhancers in human erythroid cells. J Biol Chem 2013;288:8433–8444.
22. Dogan N, Wu W, Morrissey CS et al. Occupancy by key transcription factors is a more accurate predictor of enhancer activity than histone modifications or chromatin accessibility. Epigenetics Chromatin 2015;8:16.
23. Cico A, Andrieu-Soler C, Soler E. Enhancers and their dynamics during hematopoietic differentiation and emerging strategies for therapeutic action. FEBS Lett 2016;590:4084–4104.
24. ENCODE Project Consortium. An integrated encyclopedia of DNA elements in the human genome. Nature 2012;489:57–74.
25. Roadmap Epigenomics Consortium, Kundaje A, Meuleman W et al. Integrative analysis of 111 reference human epigenomes. Nature 2015;518:37–130.
26. Astle WJ, Elding H, Jiang T et al. The allelic landscape of human blood cell trait variation and links to common complex disease. Cell 2016;167:1415–1429.
27. Xu J, Shoa Z, Glass K et al. Combinatorial assembly of developmental stage-specific enhancers controls gene expression programs during human erythropoiesis. Dev Cell 2012;23:796–811.
28. Abraham BJ, Cui K, Tang Q et al. Dynamic regulation of epigenomic landscapes during hematopoiesis. BMC Genomics 2013;14:193.
29. Huang J, Liu X, Li D et al. Dynamic control of enhancer repertoires drives lineage and stage-specific transcription during hematopoiesis. Dev Cell 2016;36:9–23.
30. Romano O, Peano C, Tagliazucchi GM et al. Transcriptional, epigenetic and retroviral signatures identify regulatory regions involved in hematopoietic lineage commitment. Sci Rep 2016;6:24724.
31. Corces MR, Buenrostro JD, Wu B et al. Lineage-specific and single-cell chromatin accessibility charts human hematopoiesis and
leukemia evolution. Nat Genet 2016;48:1193–1203.
37 Gonzalez AJ, Setty M, Leslie CS. Early enhancer establishment and regulatory locus complexity shape transcriptional programs in hematopoietic differentiation. Nat Genet 2015;47:1249–1259.
38 MacArthur J, Bowler E, Cerezo M et al. The new NHGRI-EBI Catalog of published genome-wide association studies (GWAS Catalog). Nucleic Acids Res 2017;45:D986–D991.
39 Li MJ, Li Z, Wang P et al. GWASdb v2: An update database for human genetic variants identified by genome-wide association studies. Nucleic Acids Res 2016;44:D869–D876.
40 Mauarona MT, Humbert R, Ryenes E et al. Systematic localization of common disease-association variation in regulatory DNA. Science 2012;337:1190–1195.
41 Paul DS, Albers CA, Rendon A et al. Maps of open chromatin highlight cell type-restricted patterns of regulatory sequence variation at hematological trait loci. Genome Res 2013;23:1150–1161.
42 van der Harst P, Zhang W, Mateo Leach I et al. Seventy-five genetic loci influencing the human red blood cell. Nature 2012;492:369–375.
43 Gieger C, Radakrishnan A, Cvejic A et al. New gene functions in megakaryopoiesis and platelet formation. Nature 2011;480:201–208.
44 Kamatani Y, Matsuda K, Okada Y et al. Genome-wide association study of hematological and biochemical traits in a Japanese population. Nat Genet 2010;42:210–215.
45 Nalls MA, Couper DJ, Tanaka T et al. Multiple loci are associated with white blood cell phenotypes. PLoS Genet 2011;7:e1002113.
46 Sankaran VG, Ludwig LS, Sicinska E et al. Cyclin D3 coordinates the cell cycle during differentiation to regulate erythocyte size and number. Genes Dev 2012;26:2075–2087.
47 Benyamin B, Ferreira MA, Willemsen G et al. Common variants in TMPRSS6 are associated with iron-refractory iron deficiency anemia (IRIDA). Nat Genet 2008;40:569–571.
48 Campagna DR, de Bie CI, Schmitz-Abe K et al. 5′-linked sideroblastic anemia due to ALAS2 intron 1 enhancer element GATA-binding site mutations. Am J Hematol 2014;89:315–319.
49 Kaneko K, Furuyama K, Fujiiwara T et al. Identification of a novel erythroid-specific enhancer for the ALAS2 gene and its loss-of-function mutation which is associated with congenital sideroblastic anemia. Haematologica 2014;99:252–261.
50 Wakabayashi A, Ulirsch JC, Ludwig LS et al. Insight into GATA1 transcriptional activity through interrogation of cis elements disrupted in human erythroid disorders. Proc Natl Acad Sci USA 2016;113:4434–4439.
51 Solis C, Alzencang GL, Astrin KH et al. Upregulation of the IL1B gene in human erythroid precursors results in overexpression of IL1B and IL1B-stimulated β-globin production by normal erythroid progenitors. Blood 2010;115:1015–1017.
52 Akinsheye I, Alusutana A, Soloviev I et al. Fetal hemoglobin in sickle cell anemia: Molecular characterization of the unusually high fetal hemoglobin phenotype in African Americans. Am J Hematol 2012;87:217–219.
53 Steinberg MH, Chui DH, Dover GI et al. Fetal hemoglobin in sickle cell anemia: A glass half full? Blood 2014;123:481–485.
54 Lettre G, Sankaran VG, Bezerra MA et al. DNA polymorphisms at the BCL11A, HBS1L-MYB, and beta-globin loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. Proc Natl Acad Sci USA 2008;105:11869–11874.
55 Menezel S, Gerner C, Gut I et al. A QTL influencing F cell production maps to a gene encoding a zinc-finger protein on chromosome 2p15. Nat Genet 2007;39:1197–1199.
56 Then I, Slenzel S, Peng X et al. Intergenic variants of HBS1L-MYB are responsible for a major quantitative trait locus on chromosome 6q23 influencing fetal hemoglobin levels in adults. Proc Natl Acad Sci USA 2007;104:11346–11351.
57 Then I, Waisscoat JS, Sampietro M et al. Association of thalassaemia intermedia with a beta-globin gene haplotype. Br J Haematol 1987;65:367–373.
58 Labie D, Buda-Delkhodjou O, Rouabhi F et al. The –158 site S′ to G gamma gene and G gamma expression. Blood 1985;66:1463–1465.
59 Labie D, Pagnier J, Lapoumeroulie C et al. Common haplotype dependency of high G gamma-globin gene expression and high Hb F levels in beta-thalassemia and sickle cell anemia patients. Proc Natl Acad Sci USA 1985;82:2111–2114.
60 Galanneau G, Palmer CD, Sankaran VG et al. Fine-mapping at three loci known to affect fetal hemoglobin levels explains additional genetic variation. Nat Genet 2010;42:1049–1051.
61 Akinsehiey I, Alusutana A, Soloviev I et al. Fetal hemoglobin in sickle cell anemia. Blood 2011;118:19–27.
62 Giampolo A, Mavilio F, Sposi NM et al. Heterocellular hereditary persistence of fetal hemoglobin (HPFH). Molecular mechanisms of abnormal gamma-gamma gene expression in association with beta-thalassemia and linkage relationships with the beta-globin gene cluster. Hum Genet 1984;66:151–156.
63 Martinez G, Novelletto A, Di Rienzo A et al. A case of hereditary persistence of fetal hemoglobin caused by a gene not linked to the beta-globin cluster. Hum Genet 1989;82:335–337.
64 Craig JE, Rochette J, Fischer CA et al. Dissecting the loci controlling fetal haemoglobin production on chromosomes 11p and 6q by the regressive approach. Nat Genet 1996;12:58–64.
65 Stadhouders R, Aktuna S, Thongjuea S et al. HBS1L-MYB intergenic variants modulate fetal hemoglobin via long-range MYB enhancers. J Clin Invest 2014;124:1699–1710.
66 Canver MC, Lessard S, Pinello L et al. Variant-aware saturating mutagenesis using multiple Cas9 nucleases identifies regulatory elements at trait-associated loci. Nat Genet 2017;49:625–634.
67 Uda M, Galanello R, Sanna S et al. Genome-wide association study shows BCL11A associated with persistent fetal hemoglobin and amelioration of the phenotype of
beta-thalassemia. Proc Natl Acad Sci USA 2008;105:1620–1625.
82 Sedgwick AE, Timofeev N, Sebastiani P et al. BCL11A is a major HbF quantitative trait locus in three different populations with beta-hemoglobinopathies. Blood Cells Mol Dis 2008;41:255–258.
83 Sebastiani P, Farrell JJ, Alsalun A et al. BCL11A enhancer haplotypes and fetal hemoglobin in sickle cell anemia. Blood Cells Mol Dis 2015;54:224–230.
84 Bauer DE, Kamran SC, Lessard S et al. An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level. Science 2013;342:253–257.
85 Canver MC, Smith EC, Sher F et al. BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis. Nature 2015;527:192–197.
86 Sankaran VG, Menne TF, Xu J et al. Human fetal hemoglobin expression is regulated by the developmental stage-specific repressor BCL11A. Science 2008;322:1839–1842.
87 Xu J, Bauer DE, Kerenyi MA et al. Corepressor-dependent silencing of fetal hemoglobin expression by BCL11A. Proc Natl Acad Sci USA 2013;110:6518–6523.
88 Lucarelli G, Andreani M, Angelucci E. The cure of thalassemia by bone marrow transplantation. Blood Rev 2002;16:81–85.
89 Cavazzana-Calvo M, Payen E, Negre O. A new hereditary persistence of fetal hemoglobin resulting from inactivation of the EKLF gene. Nature 1995;375:316–318.
90 Kier AB, McLain K, Mucenski ML. A functional c-myb gene is required for normal mouse fetal hepatic hematopoiesis. Cell 1991;65:677–689.
91 Bianchi E, Zini R, Salati S et al. c-myb supports erythropoiesis through the transcription of KLF1 and LMO2. Blood 2010;116:e99–e110.
92 Thompson AA, Kwiatkowski J, Rasko J et al. Lentiglobin gene therapy for transfusion-dependent β-Thalassemia: Update from the northstar HGB-204 phase 1/2 clinical study. Paper presented at: ASH 58th Annual Meeting; December 3–6, 2016; San Diego, CA.
93 Ferrari G. Gene therapy for hemoglobinopathies. Paper presented at: ASGCT Annual Meeting; May 4–7, 2016; Washington, DC.
94 Traxler E, Yao Y, Li C et al. Genome editing recreates hereditary persistence of fetal hemoglobin in human erythroblasts. Paper presented at: ASH 57th Annual Meeting; December 5–8, 2015; Orlando, FL.
95 Ye L, Wang J, Tan Y et al. Genome editing using CRISPR-Cas9 to create the HPFH genotype in HSPCs: An approach for treating sickle cell disease and beta-thalassemia. Proc Natl Acad Sci USA 2016;113:10661–10665.
96 Vierstra J, Reik A, Chang KH et al. Long-term engraftment and fetal globin reaction on genome editing of BCL11A in bone marrow-derived CD34+ hematopoietic stem and progenitor cells. Mol Ther Methods Clin Dev 2017;4:137–148.
97 Frontelo P, Manwani D, Galdass M et al. Novel role for EKLF in megakaryocyte lineage commitment. Blood 2007;110:3871–3880.
98 Siatecka M, Xue L, Bieker JJ. Sumoylation of EKLF promotes transcriptional repression and is involved in inhibition of megakaryopoiesis. Mol Cell Biol 2007;27:8547–8560.
99 Nuez B,Michalovich D, Bygrave A et al. Defective haematopoiesis in fetal liver resulting from inactivation of the EKLF gene. Nature 1995;375:316–318.
100 Borg J, Papadopoulos P, Georgitsi M et al. Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. Nat Genet 2010;42:801–805.
101 Tsang JC, Yu Y, Burke S et al. Single-cell transcriptomic reconstruction reveals cell cycle and multi-lineage differentiation defects in Bcl11a-deficient hematopoietic stem cells. Genome Biol 2015;16:178.
102 Brendel C, Guda S, Renella R et al. Lineage-specific BCL11A knockdown circumvents toxicities and reverses sickle phenotype. J Clin Invest 2016;126:3868–3878.
103 Chang KH, Smith SE, Sullivan T et al. Long-term engraftment and fetal globin reactivation upon genome editing of BCL11A in bone marrow-derived CD34+ hematopoietic stem and progenitor cells. Mol Ther Methods Clin Dev 2017;4:137–148.
104 Basak A, Hancarova M, Ullrich JC et al. BCL11A deletions result in fetal hemoglobin persistence and neurodevelopmental alterations. J Clin Invest 2015;125:2363–2368.