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

CHIARA ANTONIANI,[a,b*] ORIANA ROMANO,[a,c*] ANNARITA MICCIO[a,b]

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[a]Laboratory of Chromatin and Gene Regulation During Development, INSERM UMR1163, Imagine Institute, Paris, France; [b]Paris Descartes, Sorbonne Paris Cité University, Imagine Institute, Paris, France; [c]Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy

*Co-first authors.

Correspondence: Annarita Miccio, Ph.D., Imagine Institute, 24 Boulevard du Montparnasse, Paris, 75015, France. Telephone: +33 1 42 75 43 34; e-mail: annarita.miccio@institutimagine.org

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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;00:000–000*

SIGNIFICANCE STATEMENT

This review summarizes the epigenetic and genetic studies identifying genes and *cis*-regulatory regions involved in normal and pathological hematopoiesis. We discuss novel potential therapeutic approaches targeting *cis*-regulatory sequences, which hold great promise for the treatment many hematological diseases. The 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

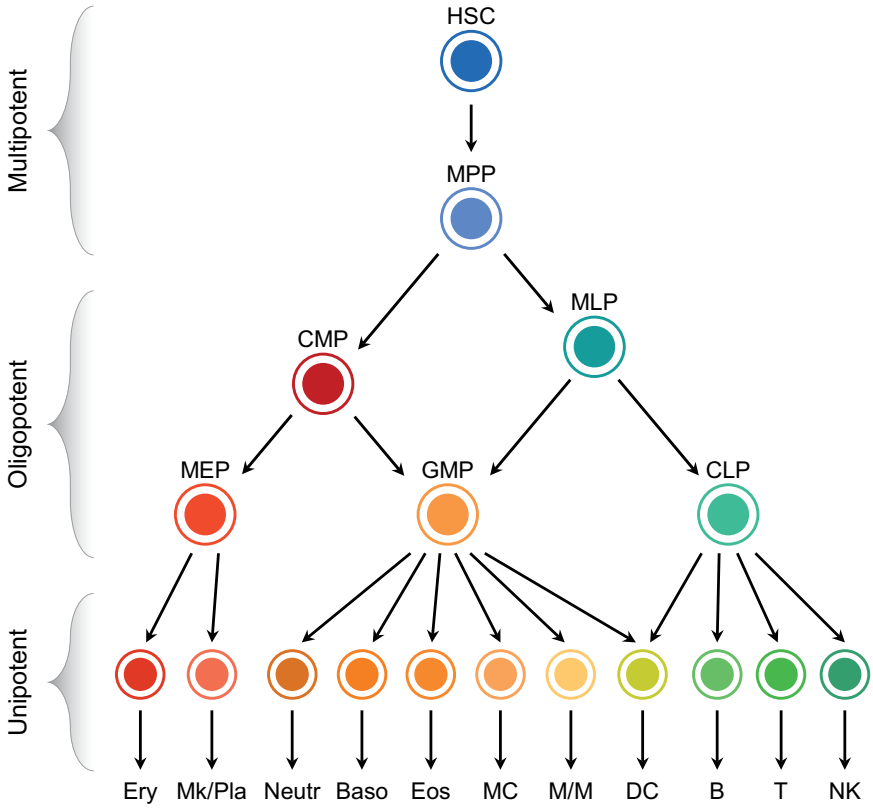

Figure 1. The current model of human hematopoiesis. Schematic representation of the hematopoietic hierarchical tree, composed of multipotent, oligopotent, and unipotent cell types. Abbreviations: B, B cells; Baso, basophils; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cells; Eos, eosinophils; Ery, erythrocytes; GMP, granulocyte/macrophage progenitor; HSC, hematopoietic stem cell; MC, mast cells; MEP, megakaryocyte/erythroid progenitor; Mk/Pla, megakaryocytes/platelets; MLP, multilymphoid progenitor; M/M, monocytes/macrophages; MPP, multipotent progenitor cell; Neutr, neutrophils; NK, natural killer cells; T, T cells.

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 transcriptomic 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

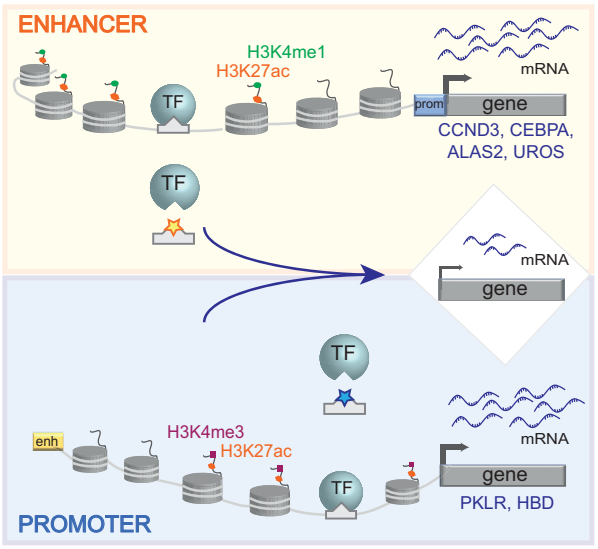

Figure 2. Genetic variants in *cis*-regulatory elements affect target gene expression. Genetic variants mapping to enhancers or promoters (indicated by yellow and blue stars, respectively) can prevent the binding of transcription factors and result in decreased expression of the target genes (e.g., *CCND3*, *CEBPA*, *ALAS2*, *UROS*, *PKLR*, and *HBD*). Active enhancers are characterized by H3K4me1 and H3K27ac histone marks, whereas H3K27ac and H3K4me3 mark active promoters. Abbreviations: prom, promoter; enh, enhancer; 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-accessible chromatin using sequencing (ATAC-seq) [9]. DNase-seq detects accessible DNA by DNaseI enzymatic digestion of nucleosome-depleted sites, also known as DNaseI 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

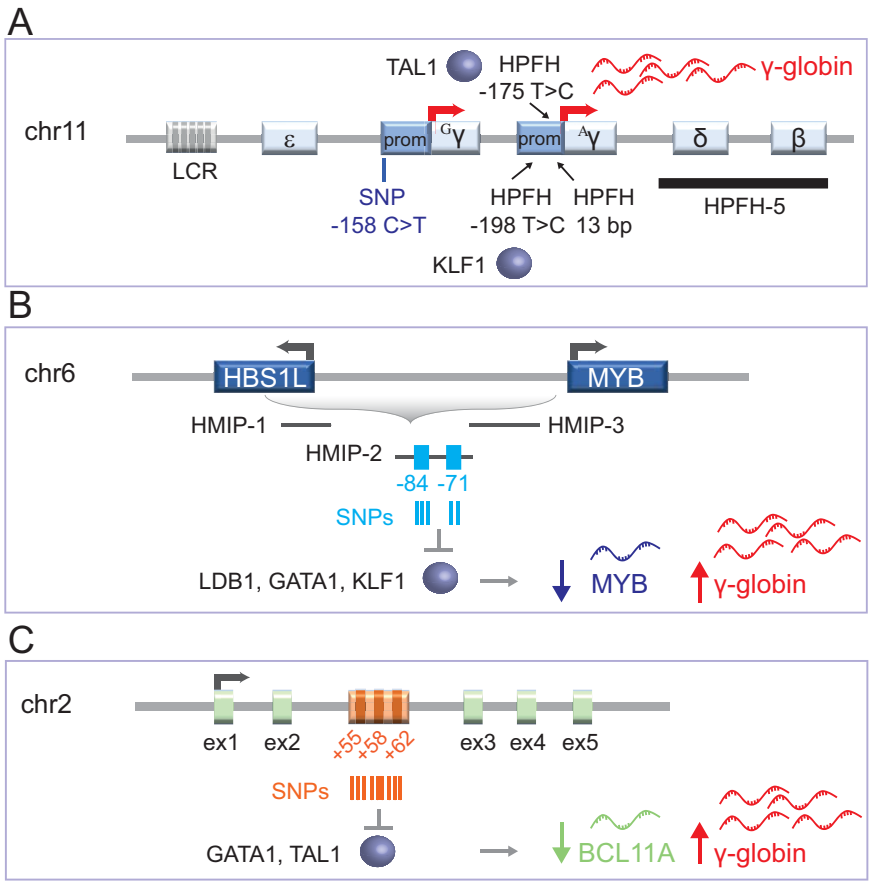

Figure 3. Genetic variants in the β-globin locus, *HBS1L-MYB*, and *BCL11A* loci influence fetal hemoglobin (HbF) levels. **(A):** Schematic representation of the β-globin locus on chromosome 11. Point mutations (HPFH −175 T > C and HPFH −198 T > C upstream of the γ-globin transcription start sites [TSSs]) and deletions (HPFH 13-bp in ^{A}γ-globin promoter and HPFH-5) are associated with HPFH. The −175 T > C and −198 T > C mutations create binding sites for TAL1 and KLF1 transcriptional activators. A common SNP at position −158 bp of the ^{G}γ-globin promoter is associated with moderately high levels of HbF. LCR and β-like globin genes (embryonic ε, fetal ^{A}γ and ^{G}γ, 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 KLF1 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.

diseases, which are collected in large databases, such as the GWAS Catalog [33] and GWASdb [34]. An increasing number of GWASs identified genetic variants linked to a variety of traits, ranging from height and hair color to metabolic and hematological phenotypes. SNPs can be also associated to disease susceptibility and represent genetic modifiers of diseases. Interestingly, numerous trait- and disease-associated sequence variations occur in *cis*-regulatory elements, such as enhancers [24–26, 35, 36]. Genes potentially targeted by regulatory elements associated with these SNPs are then identified as: (a) the nearest genes, (b) genes whose expression is correlated with the specific sequence variation (expression quantitative trait loci SNPs), (c) genes potentially implicated in the phenotype.

Several GWASs identified SNPs associated with hematological phenotypes [37–40]. Large studies described 68 genetic loci linked with platelet number and size [38], and 10 SNPs associated with WBCs number and subtypes [40]. Seventy-five genetic loci have been associated with RBC traits, such as RBC number and size (mean corpuscular volume [MCV]), hematocrit, and hemoglobin concentration [37]. In this study, a large fraction of SNPs co-mapped with epigenetically defined erythroid enhancers [37]. SNPs associated with *cis*-regulatory elements have in general small effects on gene transcription and disease phenotypes, explaining less than 10% of phenotypic variance, but can give us insights into human hematopoiesis in vivo. Indeed, they allow the discovery of new candidate genes involved in hematopoiesis [37, 38, 40, 41] and hematopoietic disorders [42, 43]. Interestingly, mutations in regulatory elements have been shown to be responsible for several genetic and nongenetic hematological diseases [44–51].

Genetic variants mapping to *cis*-regulatory elements can be experimentally validated to unravel the molecular mechanisms underlying the observed phenotypes. They can affect enhancer and promoter activity, for example, by either disrupting or creating de novo binding sites for transcription factors, thus deregulating the expression of the target genes [46, 52–54] (Figs. 2, 3).

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. CRISPR-Cas9-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 ε, fetal ^{A}γ and ^{G}γ, 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 DNase 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, $\alpha 2\gamma 2$) is reduced to $<$1% of the total Hb output and the major Hb type is HbA ($\alpha 2\beta 2$). 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 inter-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 ^{A}γ-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 (COUP-TFII), 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 ^{A}γ-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 ^{G}γ-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],

thus suggesting the involvement of other QTLs in the modulation of γ-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 [HMIP] 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). HMIP 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 −84 and −71 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. Alternatively, 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 γ-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 β-Hemoglobinopathies Targeting *Cis*-Regulatory Regions

Current treatments for SCD and β-thalassemia involve symptomatic care and RBC transfusions, which, however, can lead to iron overload and organ damage. The only definitive cure for β-hemoglobinopathies is the allogeneic HSC transplantation, which is available to a small proportion (30%) of the patients with an human leukocyte antigen (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 β-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 ^{A}γ- and/or ^{G}γ-globin gene promoters. HPFH mutations in the γ-globin promoters may disrupt binding sites for γ-globin silencers [94] or generate new binding sites for γ-globin activators [52, 63] (Fig. 3A). Traxler et al. reproduced the 13-bp HPFH deletion in the γ-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 γ-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 −175T > C or the −198T > C substitutions in the γ-globin promoters created de novo binding sites for transcriptional activators, and induced loop formation between the LCR and γ-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 deletional HPFH, removing the β- and δ-globin genes and the putative γ-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 γ-globin induction and downregulation of the β-globin expression in HSPC-derived erythrocytes.

Downregulation of factors responsible for fetal γ-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 β-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 β-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* intronic enhancers identified a potential GATA1 binding site in the +58 DNase HS critical for erythroid expression of BCL11A (Fig. 3C) [85]. CRISPR/Cas9 and zinc finger nuclease-mediated disruption of this GATA1 motif led to reasonably decreased BCL11A expression and increased HbF levels in human erythrocytes [85, 96]. Importantly, targeting of the *BCL11A* erythroid-specific enhancer did not affect RBC enucleation [113].

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.

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