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Epigenetic activities in erythroid cell gene regulation ☆

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ABSTRACT

Interest in the role of epigenetic mechanisms in human biology has exponentially increased over the past several decades. The multitude of opposing and context-dependent chromatin-modifying enzymes/coregulator complexes is just beginning to be understood at a molecular level. This science has benefitted tremendously from studies of erythropoiesis, in which a series of β-globin genes are in sequence turned "on" and "off," serving as a fascinating model of coordinated gene expression. We, therefore, describe here epigenetic complexes about which we know most, using erythropoiesis as the context. The biochemical insights lay the foundation for proposing and developing novel treatments for diseases of red cells and of erythropoiesis, identifying for example epigenetic enzymes that can be drugged to manipulate β-globin locus regulation, to favor activation of unmutated fetal hemoglobin over mutated adult β-globin genes to treat sickle cell disease and β-thalassemias. Other potential translational applications are in redirecting hematopoietic commitment decisions, as treatment for bone marrow failure syndromes.

Introduction

Erythropoiesis is a highly regulated, complex process that progressively promotes commitment and differentiation of hematopoietic stem cells through intermediate multipotent progenitors into final terminal maturation steps to create an enucleated red blood cell [1,2]. The study of the regulation of this fascinating process continues to reveal many general principles of human biology and has provided a springboard to launch therapies for the large numbers of individuals affected by red cell disorders around the globe [3]. The classical branching hierarchy in hematopoiesis is currently being challenged by emerging single-cell transcriptome data that reveals the ever more apparent discovery that hematopoietic differentiation is a more continuous process than previously thought and may involve priming of progenitor cells into various unilineage progenitors [4-6]. The later processes of erythroid terminal differentiation, that are mainly controlled by the cytokine erythropoietin (epo), produced by the kidney under hypoxic stimulation, are currently better understood than earlier progenitor stages. Epo drives proliferation and terminal differentiation of erythroid committed progenitors (late BFU-E and early CFU-E) by stimulating intracellular signaling pathways (Jak2/Stat5; PI3K/AKT), activation of the red cell transcription factor GATA-1 and plays a direct role in reprogramming the epigenome through these intermediaries [7,8].

The hemoglobin tetramer is composed of 2 α-type and 2 β-type globin subunits and most mammalian species have evolved developmental switching of globin genes, most likely related to differing oxygen transport needs during embryogenesis [9,10]. Epigenetic mechanisms mediating hemoglobin switching have generated significant interest in red cell biology, with a particular focus on the β-globin locus, since preventing the switch from fetal (HbF) to adult hemoglobin (HbA) production has potential to treat the β-hemoglobinopathies (sickle cell disease/SCD, β-thalassemias) [11]. The human beta globin locus is arranged from 5' to 3' in a developmental fashion: the super-enhancer for this locus (called the LCR, for locus control region) is located 5' to the embryonic (*HBE*/ε), fetal (*HBG2*/ Gγ, *HBG1*/Aγ), and adult

Abbreviations: LCR, locus control region; HPFH, hereditary persistence of fetal hemoglobin; SCD, sickle cell disease; DRED, direct repeat erythroid-definitive; DR, direct repeat element; DNMT, DNA methyltransferase; 5-azaC, 5-azacytidine; HSC, hematopoietic stem cell; LSD1/KDM1a, Lysine-specific histone demethylase 1A; HSPC, (hematopoietic stem and progenitor cell); BAP1, BRCA1-associated protein-1; NuRD, nucleosome remodeling deacetylase; PRC2, polycomb repressive complex 2; HDAC, histone deacetylase; HAT, histone acetyl transferase; MBD, methylcytosine-binding domain; KO, knock-out; PRMT5, Protein arginine N-methyltransferase 5; EHMT1/2, euchromatic histone-lysine N-methyltransferase; CBP, CREB binding protein.

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Table
Key epigenetic cofactors in erythropoiesis.

Identity	Cofactor	Associated transcription factor(s)	Associated complex(es)	References
Corepressor	DNMT1	BCL11A, GATA1, GFI1b, TR2/TR4	DRED	31,34, 46, 49
	MBD2	BCL11A, KLF1	NuRD	51, 52
	HDAC1/HDAC2	GATA2, TR2/TR4	NuRD, CoREST, DRED	31, 58-60, 63
	LSD1	BCL11A, TR2/TR4	CoREST	34, 70-72
others	CHD4	BCL11A, KLF1, TR2/TR4	NuRD	76, 31
	EHMT1/EHMT2	NF-E2		77, 78
	PRMT5	LYAR	NuRD, SIN3	79,80
	EZH1/2, EED, SUZ12	BCL11A, GATA1	PRC2	33, 34, 82
Coactivator	P300	KLF1	SWI/SNF	87, 89
	CBP	KLF1	SWI/SNF	87, 89
	SIRT1	BCL11A, KLF1		90

(*HBD*/δ, *HBB*/β) globin genes. Mutations that lead to hereditary persistence of fetal hemoglobin (HPFH), which prevent the *HBG* to *HBB* switch, have revealed many key epigenetic factors that underpin this regulation, including the β-globin, *HBS1L-MYB,* and *BCL11A* loci [12]. The major β-hemoglobinopathies (β-thalassemia major and sickle cell disease) are highly prevalent global diseases: the β-thalassemias are caused by underproduction of normal adult β-globin, that can manifest as severe transfusion-dependent anemia while SCD is caused by production of mutant β-globin (sickle hemoglobin, HbS) that can polymerize in red blood cells when deoxygenated, impacting red cell health and rheology to cause multiorgan tissue hypoxia and damage. Natural genetic experiments, in which β-hemoglobinopathy patients co-inherent genetic traits that favor persistent expression of HbF beyond fetal stages of development, have demonstrated that HbF is the strongest known disease modifier of the β-hemoglobinopathies, associated with normal life expectancy in the best cases and an overall decrease in disease complications [13,14]. Thus, both β-thalassemias and SCD would significantly benefit from small molecule inhibitors to pharmacologically reverse the natural HbF "off switch," and a great deal of effort has accordingly been expended on understanding and targeting the regulation of HbF.

Epigenetics refers to the regulation of gene expression that is achieved independent of alterations in DNA sequence [15]. Most changes are reversible and are elicited through chemical modifications including DNA methylation/demethylation, histone tail modifications (eg, acetylation, deacetylation, phosphorylation, methylation, ubiquitination), chromatin remodeling and regulation by noncoding RNAs. Most examples of the best studied activities appear to be mediated by large complexes composed of transcription factors and their associated cofactors (coactivators and corepressors), which bind to cis elements in DNA such as promoters and enhancers [16-18]. Coactivators and corepressors are epigenetic enzymes and adaptor proteins that remodel chromatin to either promote gene transcription or repression (respectively) although some may have interchangeable functions in specific contexts. Epigenetic "writers" add histone modifications while "readers" recognize these modifications leading to chromatin binding and finally "erasers" can and do remove the activity of the writers. These cofactors are part of large macromolecular complexes, which are present ubiquitously in many tissues (eg, the NuRD and polycomb repression complexes) so their specificity in cells is likely dictated by the presence of transcription factors as well as the relative concentrations of coactivators and corepressors, which appear to be in dynamic flux on and off DNA [19]. Corepressors are, in general, in 100-fold excess when compared to coactivators, thereby globally limiting the number of genes expressed in a given cell [20]. As transcription factors (DNA binding proteins) are difficult to target therapeutically, many current strategies are aimed at blocking corepressor enzymatic functions. How these corepressors and coactivators function to epigenetically regulate the erythroid globin gene loci and induce fetal hemoglobin is the major focus of this review. The best defined cofactors in erythropoiesis with their associated transcription factors and complexes are summarized in Table and described in more detail below.

Coregulator complexes

The large multisubunit complexes that modify chromatin to allow the binding of transcription initiation complexes typically utilize ATP hydrolysis as the driving energy resource. Most epigenetic complexes have activities in nonerythroid cells as well as erythroid cells, and therefore their specificity must be determined by the sequence-specific DNA binding transcription factors (GATA1, ZBTB7a/LRF, TR2/4, BCL11a, or KLF1) and the environment related to other corepressor/coactivator complexes. The complex that has been exclusively defined in erythropoiesis is DRED (direct repeat erythroid definitive), which represses embryonic and fetal hemoglobin by binding to DR (direct repeat) elements in the *HBE1*, *HBG1/G2* promotors but are lacking in the human *HBB* gene [21,22]. Many HPFH mutations are located in this DR1 sequence and DR1 mutations in the ε-promoter of transgenic mice led to reactivation of embryonic globin in definitive erythroid cells [21,23-26]. The DRED complex DNA binding component is composed of TR2 and TR4, which are orphan nuclear receptors that bind directly to the DR1 elements in *HBE1*, *HBG1/G2* and *GATA1* genes with TR2/TR4 overexpression repressing transcription of *GATA1* during terminal erythroid differentiation [27]. Combined *Tr2*/*Tr4* or *Tr4* null mutant mice exhibit early embryonic lethality *in vivo* limiting their use to study erythropoiesis [28,29]. However, *Tr2* and *Tr4* conditional null mice exhibit delayed silencing of murine embryonic and fetal beta-globin genes in adult bone marrow [30]. The large DRED complex contains DNMT1 (DNA methyltransferase 1) and LSD1/KDM1a (lysine-specific demethylase 1), and small molecule inhibition of these corepressor enzymes reactivates fetal hemoglobin expression in definitive adult erythroid cells [31]. NCoR1 is another member of this complex that through mutational analysis appears to function as a scaffold for TR2/TR4 binding to the DNMT1 and LSD1 corepressors [32]. BAP1 (BRCA1-associated protein-1) is a more recently discovered deubiquitinase member of the DRED complex and knockdown of BAP1 led to increased ubiquitination of NCoR1 and reduced its localization to the beta globin locus [32]. TR2/TR4 has been shown to recruit other corepressor proteins such as CoREST as well as other complexes (eg, NuRD, the nucleosome remodeling and deacetylase complex), which are also important in silencing beta embryonic globin loci in definitive erythroid cells [31].

The polycomb repressive complex 2 (PRC2) is involved in transcriptional silencing by methylation of H3K27 in association with GATA1 and BCL11a [33,34]. SWI/SNF is another highly conserved, widely expressed complex with ATPase activity that is important

in erythropoiesis as mutation in the Brg1 catalytic subunit of SWI/SNF affects histone acetylation, DNA methylation, and transcription in the beta-globin locus thereby leading to anemia [35].

Corepressors

DNMT

DNA methylation is a major epigenetic modification that predominantly modifies Cytosine-phosphate-Guanine sites and generally leads to gene repression [36]. The DNA methyltransferases (DNMTs) are an important category of epigenetic writers, among which different members play the role of either adding new methyl groups to the C5 position of cytosine or maintaining the symmetric methylation of these sites during DNA replication [37]. DNMT1, the so-called maintenance methylase, is the most abundant DNMT in mammalian cells [38,39].

The increased methylation level of the γ-globin genes (*HBG2*, *HBG1*) as development progresses from fetal erythroid cells to adult bone marrow erythropoiesis led to studies of 5-azacytidine (5-azaC), an analogue of cytosine that cannot be methylated and that actually depletes the DNMT1 enzyme from cells, to induce γ-globin gene expression in the adult [40]. Increases of HbF synthesis after 5-azaC administration were observed separately in anemic baboons, a patient with β-thalassemia and a patient with sickle cell disease [40-42]. After that, the mechanism of 5-azaC in γ-globin gene reactivation remained controversial for some time although more recent work appears to have clarified this issue [43].

Dnmt1, *Dnmt3a,* and *Dnmt3b*-disrupted mice all evince embryonic or perinatal lethality, indicating their fundamental role in embryonic development [44,45]. Conditional knockout of the *DNMT1*gene in adult mice indicated that DNMT1 plays a key role in HSC (hematopoietic stem cell) homeostasis and hematopoietic lineage decisions by repressing the expression of key hematopoietic transcription factors, including GATA1 [46]. Lavelle et al showed that knockdown of *DNMT1* in baboon BM cells induces γ-globin expression and that decitabine (5-aza-2′-deoxycytidine) shows a similar effect in *in vivo* [47]. Decitabine has a better safety profile than 5-azaC and has already been in clinical use in AML (acute myeloid leukemia), CML (chronic myeloid leukemia), and MDS (myelodysplastic syndrome) [48]. However, decitabine alone has not been shown to be sufficient to induce HbF level for clinical efficacy. DNMT1 has been shown to associate with 2 critical transcription factors, TR2/TR4 and BCL11A, in repressing γ-globin expression [31,34,49]. We and others have also identified interaction of DNMT1 with TR2/TR4 in erythroid cells, indicating that DNMT1 could silence fetal globin genes in adult erythroid cells by interacting with multiple repressor complexes [31].

Methylcytosine-binding domain2

The methylcytosine-binding domain (MBD) proteins are a family of epigenetic DNA methylation readers capable of binding specifically to methylated DNA, thereby functioning as mediators of methylation signals [50]. Among them, MBD2 showed a role in regulating fetal globin gene silencing in adult erythroid cells [51]. MBD2 has been shown to be a repressor of γ-globin expression by examining MBD2$^{-/-}$ human β-globin locus–bearing transgenic mice [51]. Since treatment of MBD2-depleted mice with 5-azacytidine resulted in minimal induction of γ-globin expression, it was also suggested that DNA methylation might act primarily through MBD2 to maintain γ-globin suppression in adult erythroid cells [51]. Knockdown of MBD2 in CD34+ primary human erythroid cells and in HUDEP2 cells also resulted in significant increases of γ-globin gene expression [52]. Given that only mild phenotypes have been observed in MBD2-deficient mice, MBD2 may serve as a somewhat safe target to induce γ-globin as treatment for β-globin gene disorders [53,54]. MBD2 did not show binding to the γ-globin promoter region in ChIP assays [51]. It was shown to recruit the NuRD complex in γ-globin silencing, through recruitment of the chromatin remodeler CHD4 via a coiled-coil domain, and the histone deacetylase core complex via an intrinsically disordered region [55]. Disruption of the MBD2-NuRD complex by mutating either of these 2 interacting domains prevent γ-globin gene repression in HUDEP-2 cells [52]. Therefore these domains of MBD2 may be potentially viable therapeutic targets in reactivating γ-globin gene expression.

Histone deacetylases

The acetylation of core histones is one of the best understood histone modifications. Important positions for acetylation are Lys4, Lys9, and Lys14 on histone H3, and Lys5, Lys8, Lys12, and Lys16 on histone H4 [56]. Steady-state levels of acetylation of the core histones result from the balance between the opposing activities of histone acetyltransferases HATs and histone deacetylases (HDACs). In general, increased levels of histone acetylation (hyperacetylation) are associated with decompressed chromatin and increased transcriptional activity, whereas decreased levels of acetylation (hypoacetylation) generally have the opposite effects.

Butyrate, a prototype HDAC inhibitor, has been shown to stimulate fetal globin production in several animal models, human cultured cells, and β-globin disorder clinical trials [57-60]. Mechanistic studies of butyrate action observed increased histone acetylation and decreased DNA methylation of the γ-globin genes associated with elevated γ-globin expression after butyrate treatment [61,62]. However the target of butyrate and its derivatives is not fully understood. A small-molecule screen conducted by Bradner et al identified class-associated activity among HDAC inhibitors, pointing to a correlation between γ-globin induction and biochemical inhibition of HDAC1, HDAC2, and HDAC3, also known as class I HDACs [56,63]. RNA interference in primary human erythroid progenitor cells further identified HDAC1 and HDAC2, rather than HDAC3 as molecular targets mediating fetal hemoglobin induction [63]. A separate study by Mankidy et al, in contrast, showed that HDAC3 knockdown by siRNA induced γ-globin gene promoter activity in a modified luciferase assay and that butyrate-induced dissociation of HDAC3 but not HDAC1 or HDAC2 specifically from the γ-globin gene promoter [64,65]. This discrepancy might be due to the different assays employed in the 2 studies. The engagement of many HDAC isoforms and the lack of specific inhibitors for each member raise questions about their possible specificity or redundancy. Genetic depletion of HDAC1 or HDAC2 causes embryonic or perinatal lethality [62,66]. Inducible conditional knock-out (KO) of *Hdac1* and/or *Hdac2* in the hematopoietic system (using Mx1-Cre) showed that dual KO (but not *Hdac1* KO alone) reduced the number of erythrocytes and megakaryocytes, suggesting that HDAC1 and HDAC2 have overlapping functions in the development of the erythroid and megakaryocytic lineages [67]. The precise role for HDACs in γ-globin gene regulation remain to be elucidated. A better understanding may be achieved by establishing erythroid-specific deleted mice of each isoform.

HDAC 1/2 inhibitor treatment in primary erythroid progenitors resulted in a significant induction of GATA2 expression associated with the HBG upregulation, while knockdown of GATA2 attenuated increased γ-globin expression after HDAC 1/2 inhibition, suggesting that HDAC1/2 inhibition might induce *HBG* by activating GATA2 through histone acetylation [60]. In addition, increased histone acetylation of putative *Gata2* regulatory regions, including the +9.5 kb domain, was identified with HDAC 1/2 inhibition by ChIP-Seq [60]. HDAC 1/2 are subunits of both the NuRD and LSD1/CoREST complexes. The interaction between HDAC 1/2 and

TR2/TR4 has been shown by mass spectrometry and coimmunoprecipitation [31], suggesting that HDAC 1/2 can interact with the core NuRD complex and CoREST complex to form even larger complexes with TR2/TR4, thereby performing their function as corepressors of γ-globin transcription. It is noteworthy that HDAC3 was also shown to interact with this large complex [31].

Lysine-specific histone demethylase 1A

Lysine-specific histone demethylase 1A (LSD1) is the first histone demethylase "eraser" identified and functions to remove methyl groups from mono- and dimethyl histone H3 lysine 4, as well as H3K9 [68,69]. Knockdown or inhibition of LSD1 in human red blood cell precursors leads to induction of high levels of fetal hemoglobin [70]. Tranylcypromine, an irreversible active site prototype of many LSD1 inhibitors, results in significant enhancement of H3K4me2 accumulation at the γ-globin promoter [70]. *In vivo* studies in SCD mice and normal baboons showed that inhibition of LSD1 efficiently induced HbF synthesis and alleviated many pathological features associated with SCD [71,72]. LSD1 inhibitor administration alone still has not induced HbF to levels sufficient (>20%HbF) for therapeutic utility without also inducing significant negative side effects, therefore leading to the need for development of novel LSD1 inhibitors with improved efficacy and safety for clinical use. Of note, LSD1 conditional depletion in hematopoietic stem cells (using vavCre) or LSD1 knockdown in mice both resulted in anemia, as well as diminished cell numbers in some other lineages [73,74]. In addition, erythroid-specific loss of LSD1 (by EpoR-Cre) results in embryonic lethality [74]. Knockdown of LSD1 also impaired erythropoiesis from CD34+ HSPC (hematopoietic stem and progenitor cell)-derived erythroid progenitors, indicating that LSD1 is required for both HbF silencing and erythroid cell maturation, raising caution for possible therapeutic targeting of LSD1 [34].

LSD1 is a flavin-dependent monoamine oxidase and forms a stable complex with the CoREST corepressor complex, which has robust H3K4 demethylase activity. Coimmunoprecipitation assays indicated that TR2/TR4 recruit a number of corepressors including the LSD1/CoREST complex to execute its role in γ-globin silencing [31]. LSD1/CoREST has also been shown to interact with BCL11A in erythroid cells [34]. The combined loss of BCL11A and LSD1 more robustly induced γ-globin expression than does loss of either gene alone *in vivo*, suggesting a possible collaborative role for BCL11A and LSD1 in γ-globin gene silencing [34]. LSD1 was also shown to be associated with GFI1/GFI1B and to mediate transcriptional repression by Gfi proteins in hematopoietic differentiation, which might also be the case for fetal globin gene regulation [75].

Other corepressors

Another critical component of the NuRD complex is Mi2β (CHD4), which has been identified as a potent silencer of the γ-globin genes [76]. Knockdown of CHD4 induced γ-globin gene expression without disrupting erythroid differentiation in primary human CD34+ progenitors that was independent of MBD2-NuRD [76]. Mi2β was shown to interact with TR2/TR4 in adult erythroid cells, suggesting its role in the TR2/TR4 repressor complexes [31]. EHMT1/2 (euchromatic histone-lysine N-methyltransferase), which catalyzes mono- and dimethylation of H3K9, has been implicated in γ-globin gene silencing as shown by knockdown and administration of selective inhibitors in human primary HSPCs [77]. Inhibition of EHMT1/2 tends to reactivate γ-globin gene expression without impairing erythroid differentiation, suggesting that they may be ideal targets for therapeutic induction of γ-globin [77]. EHMT2 (G9a) is recruited by NF-E2 to the β-globin locus and establishes H3K9 and H3K27 dimethylation to repress E^y transcription in MEL (murine erythroleukemia) cells, indicating a role of EHMT2 in globin switching in a NF-E2-dependent manner [78]. Protein arginine N-methyltransferase 5 (PRMT5) is another identified potential epigenetic corepressor of γ-globin transcription. Expression of an enzyme activity-deficient mutant form of PRMT5 induced γ-globin gene expression, suggesting a role for PRMT5 in globin gene silencing [79]. A newly identified transcription factor nuclear protein Ly1 antibody reactive (LYAR), was shown to interact with PRMT5 to bind to the γ-globin gene, and mediate its function to silence γ-globin transcription [80]. However *Prmt5* conditionally mutated mice (using Mx1Cre) displayed a severe pancytopenia due to loss of functional HSPCs, possibly limiting its potential as a therapeutic target [81]. Components ofPRC2, which has histone methyltransferase activity for H3K27me3, was implicated in GATA-1-mediated gene regulation and exhibited fetal globin gene corepressor activity as well [33,82]. shRNA-mediated knockdown of core subunits of PRC2 (including EZH1/2, EED, and SUZ12) all resulted in diminished fetal globin gene expression in an RNAi screen in primary human erythroid cells [34]. Despite their roles in fetal globin repression, Ezh1, Ezh2, Eed, and Suz also have essential roles in HSC function as well as erythropoiesis, which indicates that they are likely not ideal drug targets to reactivate fetal globin genes [83-85].

Coactivators

Gene activation involves increasing chromatin accessibility (DNAse-I hypersensitive sites and assay for transposase-accessible chromatin) to allow the binding of transcription factors and coregulator complexes. Switching from corepressor to coactivator presence at some key sites has been shown to require an exchange complex [86]. Coactivators in erythropoiesis and hemoglobin switching have not been as extensively studied as corepressors. CBP (CREB binding protein) and p300 are HATs that function as transcriptional coactivators of adult beta globin [87,88]. These coactivators are part of the SWI/SNF complex and interact with KLF1 leading to acetylation of the transactivation domain enhancing KLF1 transcriptional activation of the beta globin promotor [89]. A recent publication has highlighted SIRT1 as a new coactivator that activates transcription of fetal hemoglobin [90]. SIRT1 is a deacetylase that displays both histone and nonhistone protein activities (transcription factors and coregulators) and can both activate and repress transcription. SIRT1 enhances LCR to *HBG* promoter looping and suppresses expression of factors that repress fetal hemoglobin and with further characterization could serve as a new therapeutic target for γ-globin induction.

Conclusion

From our current perspective, it unclear whether targeting of any of the single corepressors alone will be fully effective for full therapeutic induction of gamma globin, suggesting that pursuit of the most promising of these targets could eventually result in using multiple inhibitors targeting multiple pathways in combination, and that this could be the eventual safest and best option.

Declaration of Competing Interest

Sharon A. Singh has been a consultant for Emmaus Medical, Inc.; Doug Engel has in the past served as a consultant for Imago Biosciences, GSK, Amgen and Orphagen.

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