

# Epigenetic regulation of fetal globin gene expression in adult erythroid cells



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The developmental regulation of globin gene expression has served as an important model for understanding higher eukaryotic transcriptional control mechanisms. During human erythroid development, there is a sequential switch from expression of the embryonic  $\epsilon$ -globin gene to the fetal  $\gamma$ -globin gene in utero, and postpartum the  $\gamma$ -globin gene is silenced, as the  $\beta$ -globin gene becomes the predominantly expressed locus. Because the expression of normally silenced fetal  $\gamma$ -type globin genes and resultant production of fetal hemoglobin (HbF) in adult erythroid cells can ameliorate the pathophysiological consequences of both abnormal  $\beta$ -globin chains in sickle cell anemia and deficient  $\beta$ -globin chain production in  $\beta$ -thalassemia, understanding the complex mechanisms of this developmental switch has direct translational clinical relevance. Of particular interest for translational research are the factors that mediate silencing of the  $\gamma$ -globin gene in adult stage erythroid cells. In addition to the regulatory roles of transcription factors and their cognate DNA sequence motifs, there has been a growing appreciation of the role of epigenetic signals and their cognate factors in gene regulation, and in particular in gene silencing through chromatin. Much of the information about epigenetic silencing stems from studies of globin gene regulation. As discussed here, the term epigenetics refers to postsynthetic modifications of DNA and chromosomal histone proteins that affect gene expression and can be inherited through somatic cell replication. A full understanding of the molecular mechanisms of epigenetic silencing of HbF expression should facilitate the development of more effective treatment of  $\beta$ -globin chain hemoglobinopathies. (Translational Research 2015;165:115–125)

**Abbreviations:** Adox = adenosine-2',3'-dialdehyde; BCL11A = B-cell lymphoma/leukemia A; CID = chemical inducer of dimerization; CoREST = REST-co-repressor; DNMT = DNA methyltransferase; EKLf = erythroid Krüppel-like factor; FoP = friend of PRMT1 (protein arginine methyltransferase); HbA = adult hemoglobin; HbF = fetal hemoglobin; HDAC = histone deacetylase; KLF1 = Krüppel-like factor 1; LCR = locus control region; LSD1 = lysine-specific demethylase 1; MBD = methylcytosine-binding domain; Mi2 $\beta$ /CHD4 = chromodomain helicase DNA-binding protein 4; NCoR/SMRT = nuclear receptor co-repressor-1/silencing mediator for retinoid and thyroid receptors; NuRD = nucleosome remodeling and deacetylase; PCAF = P300-associated factor; PRMT = protein arginine methyltransferase; siRNA = small inhibitory RNA; shRNA = short hairpin RNA; TR2/TR4/DRED = nuclear receptor TR2/TR4 complex;  $\beta$ -YAC = beta-globin yeast artificial chromosome

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## INTRODUCTION

DNA methylation was the first well-described epigenetic signal and was long posited to have a role in gene regulation.<sup>1-3</sup> Vertebrate globin genes were among the first in which an inverse relationship between cytosine methylation and transcription was demonstrated.<sup>4-7</sup> Both histone and nonhistone chromosomal proteins after synthetic modifications have also been shown to have important roles in gene regulation, a concept formalized as the histone code.<sup>8-10</sup> These relationships have been described in detail in a review elsewhere.<sup>11</sup> The current discussion will focus primarily on the epigenetic mechanisms involved in developmental human  $\beta$ -type globin gene silencing (and hence fetal hemoglobin [HbF] silencing) and the preclinical and potential clinical translational avenues for overcoming this silencing in context of the treatment of inherited  $\beta$ -globin gene disorders.

In all vertebrates that have been studied, a switch from embryonic, or primitive, to definitive hemoglobin production occurs in erythroid cells during development. In humans and old world primates, as well as certain ruminants, an intermediate HbF predominates during mid to late gestational stages and persists at a low level postpartum in definitive erythroid cells after adult hemoglobin predominates (Table I). The details of this switch have been reviewed extensively.<sup>12,13</sup>

As with much of human biology, the ability to identify important regulatory mechanisms that are physiologically relevant is a major challenge requiring robust preclinical models for understanding  $\gamma$ -globin gene silencing in adults and successfully targeting those mechanisms therapeutically. Because of a high degree of evolutionary conservation of gene regulatory mechanisms in erythroid cells, transgenic mice bearing a yeast artificial chromosome (YAC) containing an intact human  $\beta$ -globin gene locus ( $\beta$ -globin YAC) have provided a valuable model system for studying developmental globin gene regulation. The transgenic mouse model also allows for testing the effects of modulating epigenetic processes in the context of whole animal physiology. At the same time, the  $\beta$ -globin YAC mouse model is limited by the fact that the mouse lacks a true analog of the human fetal erythroid compartment, such that the transgenic human  $\gamma$ -globin gene is regulated like the murine embryonic  $\beta$ -type globin genes, which are repressed several orders of magnitude more than the human  $\gamma$ -globin gene in adult humans<sup>14</sup> (Table I).

Cultured primary human erythroid cells derived from CD34+ progenitors induced to erythroid differentiation provide another powerful model for studying human

**Table I.** Developmental stage-specific human and mouse  $\beta$ -type globin gene and corresponding hemoglobin expression patterns

Species	Developmental stage	Predominant $\beta$ -type globin (hemoglobin)
Mouse	Primitive	$\beta^{H1}$ , $\epsilon^\gamma$ (Hb E) I-III
	Definitive	$\beta^{maj}$ (Hb $\beta^{maj}$ ) $\beta^{min}$ (Hb $\beta^{min}$ )
Human	Primitive	$\epsilon$ (Hb Gower-1, Hb Gower-2)
	Definitive	
	Fetal gestational and Postpartum	$\gamma$ (HbF), (Hb Portland-1) $\beta$ (HbA) $\delta$ (HbA2)

$\gamma$ -globin gene silencing.<sup>15,16</sup> The limitations of cultured primary erythroid cells include their limited life span, and the fact that achieving terminal erythroid differentiation while maintaining cell viability is often challenging.

The primate baboon model also has been quite useful given that the developmental  $\beta$ -type globin gene repertoire of the baboon is very similar to humans, including an HbF.<sup>17</sup> Other vertebrate models and cultured cell systems have provided important early insights into epigenetic control of globin gene silencing, but this discussion of preclinical translational studies is directed primarily at the aforementioned models.

Much of the focus of research on developmental  $\gamma$ -globin gene silencing has been on trans-acting transcription factors. The discovery of the quantitative trait locus B-cell lymphoma-leukemia A (*BCL11A*) on chromosome 2p16<sup>18,19</sup> identified this factor as an important regulator of HbF expression. Subsequent studies have shown that *BCL11A* binds to an intergenic region in the  $\beta$ -globin locus and has a dominant silencing effect on murine embryonic  $\beta$ -type  $\beta^{H1}$  and  $\epsilon^\gamma$ -globin, as well as human  $\epsilon$ - and  $\gamma$ -globin gene expression in  $\beta$ -YAC transgenic mice.<sup>12,20</sup>

Knockdown of *BCL11A* in cultured primary human adult erythroid cells also results in a significant upregulation of  $\gamma$ -globin gene expression, although the magnitude of this effect is much less than in the  $\beta$ -YAC mouse model.<sup>19</sup> The transcription factor *SOX6* also mediates embryonic  $\beta^{H1}$  and  $\epsilon^\gamma$ -globin gene silencing in the mouse, and it is known to interact with *BCL11A*.<sup>21,22</sup>

Krüppel-like factor 1 (*KLF1*), originally known as erythroid *KLF*, *EKLF* was initially shown to be critical for adult  $\beta$ -globin gene transcription,<sup>23</sup> and to increase the ability of the  $\beta$ -globin promoter to compete with the  $\gamma$ -globin promoter for the enhancer function of the erythroid-specific  $\beta$ -globin locus control region.<sup>24,25</sup> A more direct role of *KLF1* in  $\gamma$ -globin gene silencing occurs through its stimulation of *BCL11A* expression.<sup>26,27</sup>

The *MYB* gene has also been implicated in regulating HbF levels through both quantitative trait locus studies and functional assays.<sup>18,28-30</sup>

A number of other transcription factors have been implicated in embryonic-fetal  $\beta$ -type globin gene silencing. These include transcription factor that binds to the DNA sequence GATA (*GATA1*) in association with *FOG1* and the nucleosome remodeling and deacetylase (*NuRD*) complex,<sup>31-34</sup> nuclear factor erythroid 4 (*NFE4*),<sup>35</sup> the *TR2/TR4*/direct repeat erythroid definitive (*DRED*) complex,<sup>36,37</sup> *Ikaros* in association with the *SWI/SNF*-related protein complex coregulatory complex.<sup>38</sup>

As the transcription factors involved in fetal globin gene silencing have been recently reviewed, the remaining part of this review will focus primarily on epigenetic silencing mechanisms.<sup>39</sup>

### EPIGENETIC REGULATORS—WRITERS AND READERS

There are only a few examples in which an epigenetic modification of DNA or a chromosomal protein has a direct effect on structure or function.<sup>40</sup> An exception is histone acetylation, which does appear to directly alter chromatin structure.<sup>11,41</sup> In most cases, epigenetic marks serve as a recognition signal for a protein or protein complex, which ultimately carries out the specific associated regulatory function. A useful organizing concept for identifying potential targets for perturbing epigenetic fetal globin gene silencing is that of writers and readers. Writers are the enzymes that deposit or remove an epigenetic mark, whereas readers are the proteins or complexes that interpret those marks and carry out the associated regulatory function.

### DNA METHYLATION

DNA methyltransferase (*DNMT*) is a major category of epigenetic writers, as DNA methylation was the first well-established epigenetic regulatory signal. The most well characterized of these are the *de novo* methylases, *DNMT3A*, and *DNMT3B*, which symmetrically methylate cytosines in the dinucleotide Cytosine-phosphate-Guanine (*CpG*) on both strands of unmethylated DNA, and *DNMT1*, a so-called maintenance methylase, that adds a methyl group to the symmetric *CpG* on the unmethylated strand of DNA after DNA replication. From the time of the discovery that silent embryonic and fetal  $\beta$ -type globin genes are methylated and that the cytidine analog, 5-azacytidine, inhibits the processive methylation of hemimethylated DNA after replication, many studies have focused on *DNMT1* as a target for reversing globin gene silencing. Initial studies in animal models<sup>42</sup> were

followed by clinical interventions that demonstrated increased HbF expression in patients with both sickle cell anemia and  $\beta$ -thalassemia who were treated with 5-azacytidine.<sup>43-45</sup> The mechanism by which 5-azacytidine actually induces increased human fetal gamma globin gene expression has been debated, and mechanisms such as generalized cytotoxicity and induced erythroid cellular stress have been proposed.<sup>13,46-50</sup> Nonetheless in well-characterized primate and human  $\beta$ -globin gene locus-bearing transgenic mouse models, disruption of DNA methylation appears to be a major mechanism of relieving  $\gamma$ -globin gene silencing, although perhaps indirectly in part.<sup>51-56</sup> Despite the development of more specific inhibitors of *DNMT1*, such as decitabine, which, unlike 5-azacytidine, lacks effects on RNA metabolism, concerns about the safety of this class of agents have limited clinical application in  $\beta$ -hemoglobinopathies. However, a recent study of low dose decitabine in  $\beta$ -thalassemia patients reported an increase in HbF without detectable short-term cytotoxicity or genotoxicity.<sup>57</sup>

The readers of DNA methylation are a group of proteins that preferentially bind to DNA containing symmetrically methylated *CpG* dinucleotides. The largest family of these are the methylcytosine-binding domain (*MBD*) proteins, which include *MBD1*, *MBD2*, *MECP2*, and *MBD4*.<sup>58</sup> Of these, the role of *MBD2* in regulating embryonic/fetal  $\beta$ -type globin gene silencing in adult erythroid cells is the most well characterized. *MBD2* binds preferentially to DNA containing a high density of methylated *CpGs*. *MBD2* has been shown to bind directly to the avian embryonic  $\rho$ -globin gene, and knockdown of *MBD2* derepresses the gene in adult erythroid cells in culture.<sup>59</sup> Knockdown of *MBD2* has also been shown to induce a large increase in expression of the silent human  $\gamma$ -globin gene in human  $\beta$ -globin locus-bearing transgenic mice<sup>53</sup> and in human primary *CD34* precursor-derived adult erythroid cells.<sup>60</sup> Importantly, complete knockout of *MBD2* in transgenic mice does not result in any major deleterious phenotype,<sup>61</sup> suggesting that its disruption postnatally in somatic cells may have minimal toxicity.

*MBD2* mediates silencing by recruiting the *NuRD* complex to methylated DNA.<sup>62,63</sup> Structural studies of the *MBD2*-*NuRD* complex have identified a critical coiled-coil protein interaction between *MBD2* and *p66 $\alpha$ / $\beta$* , another *NuRD* complex component. Enforced expression of the *p66* coiled-coil protein results in release of the *Mi2 $\beta$*  chromatin remodeling ATPase from the *NuRD* complex, and derepression of the silenced embryonic and fetal  $\beta$ -type globin genes, presumably by decoupling *MBD2* from the *NuRD* chromatin remodeling function.<sup>60</sup>

A closely related member of the MBD family, MBD3, also associates with a NuRD complex, but does not bind to methylated vs nonmethylated DNA with high affinity.<sup>58,64</sup> Moreover, the presence of MBD2 and MBD3 in association with NuRD complex appears to be mutually exclusive.<sup>65</sup> MBD3-NuRD is associated with the  $\gamma$ -globin gene promoter primarily through association with the GATA1 transcription factor-associated protein, friend of GATA1 (FOG1),<sup>32,33</sup> or other complexes.<sup>66</sup> Disruption of expression of the Mi2 $\beta$  subunit of NuRD results in increased  $\gamma$ -globin gene expression in transgenic mice,<sup>34</sup> cultured mouse chemical inducer of dimerization (CID) hematopoietic cells bearing a human  $\beta$ -globin gene locus, and cultured primary human erythroid cells.<sup>67</sup>

Recently, it was shown that as little as a 50% knock-down of Mi2 $\beta$  in primary human erythroid cells results in a  $\sim$ 10-fold increase in  $\gamma$ -globin gene expression without affecting erythroid differentiation, compared with control CD34+ progenitor-derived erythroid cells treated with scramble short hairpin RNA.<sup>67</sup> The degree of differentiation in control cells in these studies leads to a level of 1%  $\gamma/\gamma+\beta$  RNA, which is comparable with normal adult reticulocyte levels. Interestingly, in these studies, the effect of Mi2 $\beta$  on  $\gamma$ -globin gene silencing did not appear to be chiefly because of an effect on MBD2-NuRD or MBD3-NuRD. Rather at least part of the effect was through downregulation of *BCL11A* and *KLF1* in Mi2 $\beta$  knockdown erythroid cells. The purported relationships of MBD2-NuRD, MBD3-NuRD, and Mi2 $\beta$  in  $\gamma$ -globin gene silencing in the context of other major epigenetic regulatory factors are depicted in Fig 1.<sup>67</sup> On the basis of the preponderance of evidence, it appears that MBD2 plays a greater role than MBD3 in silencing  $\gamma$ -globin gene expression, whereas Mi2 $\beta$  plays a greater role than either MBD2 or MBD3.

## HISTONE ACETYLATION

Increased histone acetylation has long been posited to be associated with decompressed chromatin and active gene expression.<sup>68,69</sup> The writers for histone acetylation are histone acetyltransferases including P300/CBP (CRE3 binding protein), PCAF, and TAF(11)250 (TBP associated factor),<sup>70</sup> as well as histone deacetylases (HDACs, which might be more properly thought of as “erasers”). The complexity of histone acetylation and its relationship to gene regulation have been intensively studied and will not be reviewed in detail here. The consensus is that acetylation of lysines at histone tails results in charge neutralization and loosening of the interaction of nucleosomes with their associated DNA.<sup>11</sup>

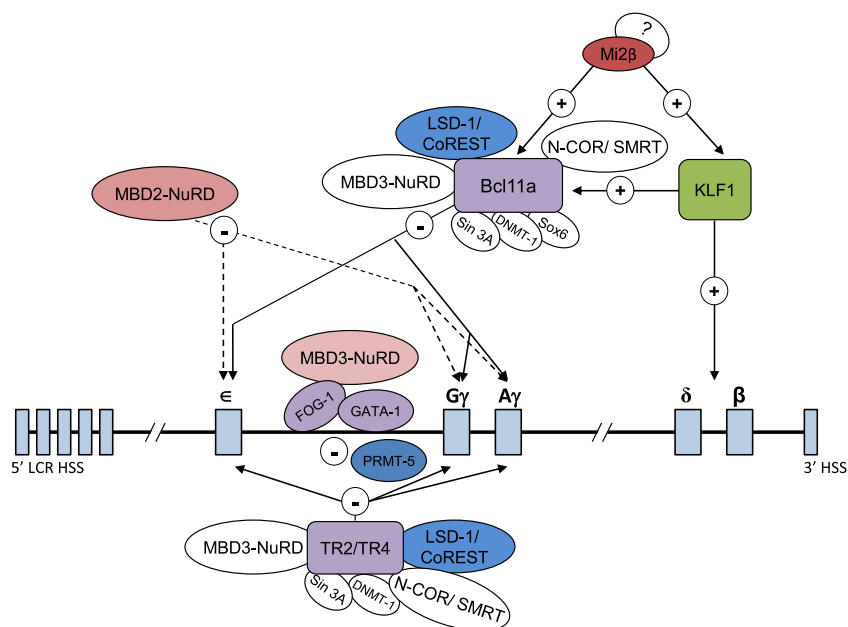
A number of inhibitors of HDACs have been identified or synthesized, the prototype being butyric acid.<sup>69</sup>

Butyric acid and derivatives were shown to induce the expression of silenced embryonic and fetal  $\beta$ -type globin genes in several animal models.<sup>71,72</sup> Although increased HbF expression was associated with increased histone acetylation in the vicinity of the  $\gamma$ -globin gene,<sup>54</sup> it is important to recognize that HDACs might potentially affect acetylation of transcription factors and other nonhistone proteins. Moreover, butyrate and other HDAC inhibitors have been shown to affect other signaling pathways including the Signal Transducer and Activator of Transcription 5, cyclic Adenosine Monophosphate, and Mitogen Activated Protein kinase systems.<sup>73-75</sup> Thus, the exact molecular mechanisms of  $\gamma$ -globin gene activation by HDAC inhibitors are not fully known. Nonetheless, treatment of patients with sickle cell anemia and  $\beta$ -thalassemia with sodium butyrate and butyric acid was shown to induce increased HbF expression.<sup>76,77</sup> The effect of naturally occurring butyrates is somewhat variable, possibly reflecting phenotypic differences in their metabolism or in the factors that are responsible for the mechanisms of action. Extensive efforts have been made to improve on the effectiveness of HDAC inhibitors, whereas decreasing unwanted adverse effects. Recent large-scale chemical genetic studies independently identified HDAC1 and HDAC2 inhibitors as inducers of  $\gamma$ -globin gene expression,<sup>78</sup> affirming the likely mechanism of action of butyric acid and its derivatives.

## HISTONE METHYLATION

Unlike histone acetylation, which is generally associated with both active chromatin configuration and gene expression, histone methylation can signal gene activation, gene silencing, or a bivalent state. For example, histone H3K4me3 methylation is usually associated with open chromatin and gene transcription, whereas histone H3K9 and H3K27me3 methylation are most frequently associated with gene silencing.<sup>8,79,80</sup> The presence of both H3K4me3 and H3K27me3 is associated with a poised bivalent state.<sup>81</sup>

The major writers of histone methylation are the SUV, Enhancer of zeste, Trithorax protein (SET) domain lysine-specific methylases and the protein arginine methyltransferases (PRMTs). A PRMT5-dependant multiprotein complex has been shown to contribute to human  $\gamma$ -globin gene silencing. Moreover, symmetric methylation of histone H4 arginine 3 (H4R3 Me2s) serves as a binding target for DNMT3A leading to methylation at the  $\gamma$ -globin gene promoter. The histone lysine methyltransferase Suv4-20h1 and components of the NuRD complex are also associated with these complexes.<sup>66,82</sup> Recently, the methyltransferase



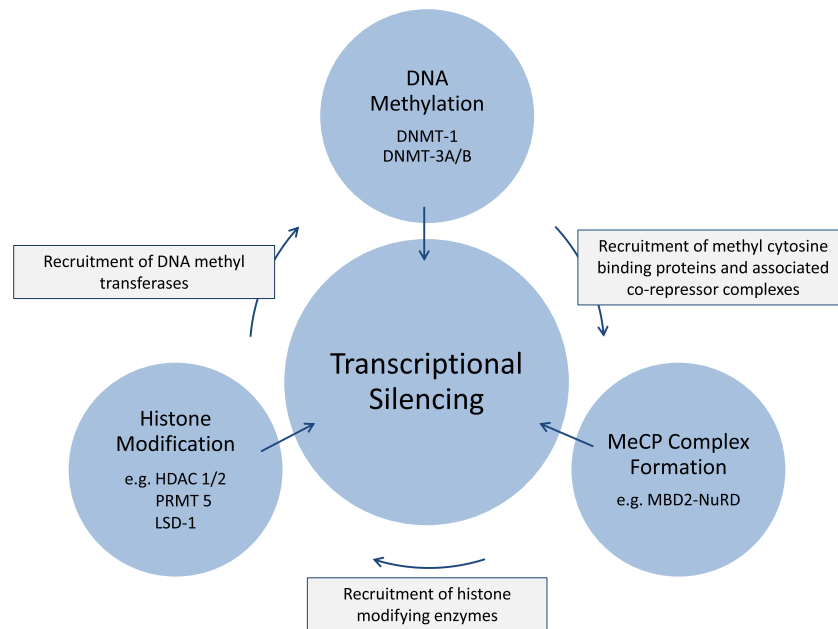
**Fig 1.** Mi2 $\beta$ -mediated epigenetic globin gene silencing through multiple mechanisms. Mi2 $\beta$  is a critical component of the MBD2/NuRD complex, which regulates developmental globin gene silencing independently of BCL11A and KLF1/EKLF. Mi2 $\beta$  also binds to the distal promoter region of the  $\gamma$ -globin gene as part of the MBD3/NuRD/GATA-1/FOG-1 silencing complex. Importantly, Mi2 $\beta$  binds to and activates expression of BCL11A and KLF1/EKLF, which in turn silence  $\gamma$ -globin gene expression. In each of these processes, Mi2 $\beta$  has been shown to directly promote  $\gamma$ -globin gene silencing. As depicted, Mi2 $\beta$  is also associated with the BCL11A complex and the TR2/TR4/DRED complex through its association with NuRD, but as indicated by the noncolored symbol, its role in the activity of these complexes has not been demonstrated directly. Likewise, other noncolored symbols designate epigenetic modulators that have not been shown directly to mediate silencing in association with factors designated by colored (shaded) symbols. BCL11A, B-cell lymphoma/leukemia A; CoREST, REST-co-repressor; DNMT, DNA methyltransferase; EKLF, erythroid Krüppel-like factor; HSS, DNase I hypersensitive site; KLF, Krüppel-like factor; LCR, locus control region; LSD, lysine-specific demethylase; MBD, methylcytosine-binding domain; N-COR, nuclear receptor co-repressor 1; NuRD, nucleosome remodeling and deacetylase; PRMT, protein arginine methyltransferase; SMRT, silencing mediator for retinoid and thyroid receptors. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

inhibitor, adenosine-2',3'-dialdehyde (Adox), was shown to induce  $\gamma$ -globin gene expression in human primary erythroid cells in culture, suggesting that PRMT5 enzymatic activity may be mechanistically linked to silencing. Given the possible off target effects of inhibitor studies, the possibility remains that the effects of Adox may be through another methyltransferase.<sup>83</sup>

Another member of the PRMT family, PRMT1, has been associated with human  $\gamma$ -globin gene silencing through association with a protein named friend of PRMT1 (FoP).<sup>84</sup> Knockdown of FoP protein resulted in increased  $\gamma$ -globin gene expression in cultured primary human erythroid cells. Interestingly, PRMT1 has also been shown to facilitate a number of histone acetylation events including acetylation of Lys9/Lys14 and subsequent transcription of the adult  $\beta$ -globin

gene.<sup>85</sup> This result suggests that the enzymatic activity of PRMT1 also may contribute to  $\gamma$ -globin gene silencing through increasing the  $\beta$ -globin gene's ability to compete for the  $\beta$ -globin locus control region enhancer activity.

Specific lysine demethylases are involved in  $\gamma$ -globin gene silencing in both murine and human adult erythroid cells. The lysine-specific demethylase 1 (LSD1) has been shown to associate with the transcription factor BCL11A through a complex containing the repressor element-1 silencing factor corepressor-1 (CoREST),<sup>86</sup> and to mediate part of BCL11A's strong  $\gamma$ -globin gene silencing activity. LSD1 also has been shown to associate with the TR2/TR4/DRED complex, along with several other corepressor complexes.<sup>87</sup> Inhibition of LSD1 by either RNA interference or the LSD1 enzymatic activity inhibitor, tranylcypromine, results



**Fig 2.** Interdependence of epigenetic gene silencing. Existing data suggest that DNA methylation and postsynthetic histone modification are dependent on one another and in turn reinforce each other. Either mark appears capable of establishing the other by recruiting epigenetic writer enzymes, thus resulting in a cycle that reinforces gene silencing. For example, as illustrated, DNA methylation recruits methylcytosine-binding proteins, which recruit corepressor complexes that contain histone-modifying enzymes that catalyze loss of activating marks (eg, histone acetylation and methylation) or addition of repressive marks (eg, histone methylation). In turn, repressive histone modifications such as H3K9 methylation can recruit DNMTs that deposit the repressive methylation mark at CpG dinucleotides. DNMT, DNA methyltransferase; HDAC, histone deacetylase; LSD, lysine-specific demethylase; MBD, methylcytosine-binding domain; NuRD, nucleosome remodeling and deacetylase; PRMT, protein arginine methyltransferase.

in increased  $\gamma$ -globin gene expression in  $\beta$ -globin locus-bearing transgenic mice and cultured primary human erythroid cells.<sup>86,88</sup> However, because LSD1 is required for normal erythroid maturation, it has been suggested that its inhibition potentially might adversely affect that process.<sup>86</sup>

#### THE INTERPLAY BETWEEN DNA METHYLATION AND HISTONE MODIFICATION IN REGULATING GENE EXPRESSION

Studies in vertebrate model systems have demonstrated a close and often reinforcing relationship between DNA methylation and repressive histone modifications in gene silencing.<sup>89,90</sup> In some instances, DNA methylation and associated methyl-binding domain proteins recruit corepressor complexes that contain SET domain proteins, which catalyze H3K9 methylation.<sup>91</sup> Other studies have demonstrated that repressive histone marks such as H3K9 methylation may recruit DNMTs.<sup>92</sup> Conversely, histone acetylation has been shown to prevent extinction of gene expression and subsequent DNA methylation.<sup>41,93</sup> The often self-reinforcing nature of these interactions is depicted in Fig 2.

#### MICRORNA AND RNA INTERFERENCE

Frequently microRNA (miRNA) and small inhibitory RNA are included in the category of epigenetic regulatory mechanisms. These small RNAs are capable of well-characterized post-transcriptional gene silencing, but also have been shown to direct epigenetic modifications in plants and animals.<sup>94</sup> Several miRNAs have been implicated in the regulation of  $\gamma$ -globin gene expression. LIN28B and the associated let-7 miRNA family are regulated during fetal to adult erythroid development. Enforced expression of LIN28B resulted in increased  $\gamma$ -globin gene expression in cultured adult primary human erythroid cells, whereas LIN28B knockdown decreased  $\gamma$ -globin gene expression in fetal cord blood-derived human erythroid cells which normally express high levels of HbF.<sup>95</sup> At least part of this effect was attributed to the effect of LIN28B on expression of *BCL11A*. Similarly, microRNA-486-3p was shown to bind to the *BCL11A* messenger RNA 3'-untranslated region and downregulate its expression concomitant with upregulation of  $\gamma$ -globin gene expression in cultured human erythroid cells.<sup>96</sup> The role of

epigenetic changes in the actions of either LIN28B or microRNA-486-3p remains unknown.

### INTERPLAY BETWEEN TRANSCRIPTION FACTORS AND EPIGENETIC REGULATORS

Any discussion of epigenetic regulation of globin gene expression must account for the interplay between transcription factors and coregulatory complexes with which they interact and which in turn often contain both “writers” (eg, histone acetylases and deacetylases), and “readers” (eg, methylcytosine-binding proteins) of epigenetic chromatin marks. Several transcription factors that are involved in embryonic fetal  $\beta$ -type globin gene silencing are known to associate with one or more corepressor complexes. Among these, BCL11A has emerged as a dominant regulator of developmental globin gene silencing in mice and is also implicated as a strong mediator of  $\gamma$ -globin gene silencing in cultured human primary erythroid cells.<sup>19</sup> BCL11A has been shown to associate with the MBD3-NuRD complex, as well as the LSD1/CoREST complex, Sin3A, NCoR/SMRT, and DNMT1.<sup>86</sup> Another transcription factor complex associated with embryonic globin gene silencing, the TR2/TR4/DRED orphan nuclear receptor complex, has been shown to associate with a number of epigenetic coregulatory proteins, including the MBD3-NuRD, LSD1/CoREST, Sin3A complexes, and DNMT1.<sup>87</sup> Thus, the effectors of these transcription factors may be in large part epigenetic.

Another connection between epigenetic regulators and transcription factors that are involved in  $\gamma$ -globin gene silencing is through epigenetic regulation of expression of the transcription factors themselves. It was recently shown that Mi2 $\beta$ /CHD4 (chromodomain helicase DNA-binding protein 4), independently of the NuRD complex, is required for high level expression of both *KLF1* and *BCL11A* in primary human adult erythroid cells and that Mi2 $\beta$ /CHD4 binds directly to BCL11A<sup>67</sup> (see Fig 1).

It is important to note that virtually all the epigenetic and transcriptional regulatory factors that are discussed here and depicted in Fig 1 have been shown to play a role in normal developmental globin gene switching. However, the relative effect of a given factor in the totality of  $\gamma$ -globin gene silencing appears to vary considerably in developmental globin silencing or “switching” vs maintenance of silencing in the adult erythroid compartment.

A summary of the coregulatory complexes that contain known epigenetic readers and writers and that are associated with transcription factors involved in developmental regulation of  $\gamma$ -globin gene expression is presented in Table II.

**Table II.** Association of  $\gamma$ -globin gene silencing transcription factors with epigenetic modulators

Transcription factors	Associated epigenetic modulators
BCL11A	MBD3-NuRD (HDAC1/2),* CoREST,* NCoR/SMRT,* LSD1,* Sin3A,* DNMT1,* Mi2 $\beta$ /CHD4 <sup>†</sup>
TR2/TR4	MBD3-NuRD (HDAC1/2)* LSD1,* Sin3A,* DNMT1*
GATA1/FOG1	MBD3-NuRD (HDAC1/2)*
NFE4	DNMT3A*
KLF1	BRG1/SWI/SNF,* Mi2 $\beta$ /CHD4 <sup>†</sup>
Ikaros	SWI/SNF*

*Abbreviations:* BCL11A, B-cell lymphoma/leukemia A; CoREST, REST-co-repressor; DNMT, DNA methyltransferase; HDAC, histone deacetylase; KLF1, Krüppel-like factor 1; LSD1, lysine-specific demethylase 1; MBD, methylcytosine-binding domain; Mi2 $\beta$ /CHD4, chromodomain helicase DNA-binding protein 4; NCoR/SMRT, nuclear receptor co-repressor-1/silencing mediator for retinoid and thyroid receptors; NuRD, nucleosome remodeling and deacetylase; PRMT, protein arginine methyltransferase.

\*The epigenetic modulator factor acts as effectors of transcription factor-mediated silencing.

<sup>†</sup>The epigenetic modulator factors may act to regulate expression of a specific transcription factor.

### TRANSLATIONAL CLINICAL TRIALS TARGETING EPIGENETIC HbF SILENCING

The first clinical trials aimed at reversing HbF silencing in patients with sickle cell anemia and  $\beta$ -thalassemia targeted DNA methylation with 5-azacytidine.<sup>43-45</sup> Although subsequent trials with cytotoxic agents raised questions as to the exact mechanism of 5-azacytidine-induced HbF expression,<sup>13</sup> a number of preclinical studies support a major role for DNA hypomethylation.<sup>51-53,55,56</sup> As noted previously, concerns about adverse effects of hypomethylating agents with known cytotoxicity have limited the widespread use of 5-azacytidine and decitabine in the clinic.

The use of HDAC inhibitors represents the other major example of clinical trials aimed at targeting epigenetic silencing of HbF expression in patients with  $\beta$ -globin gene disorders.<sup>76,77,97</sup> Recent trials with oral butyrate derivatives have shown activity in patients with  $\beta$ -thalassemia. One such agent sodium 2,2-dimethyl butyrate was shown to be tolerated in phase I/II trial.<sup>98</sup> Although butyrate and derivative compounds have demonstrated effectiveness in some patients, the effects are variable. The nature of this variability remains unknown and could involve differences in metabolism of various HDAC inhibitors or genetic heterogeneity in acetylated protein targets or downstream regulatory factors in different patients.

On the basis of the preclinical studies described previously, a number of epigenetic modulators are

either in early phase clinical trial testing or such trials are being planned. Among these are inhibitors of the histone lysine demethylase, LSD1,<sup>86,88</sup> the histone arginine methylase, PRMT5,<sup>83</sup> and selective HDAC1 and HDAC2 inhibitors.<sup>78</sup> The development of more selective HDAC inhibitors may increase their effectiveness, whereas decreasing the unwanted adverse effects of these agents.

In the face of a large number of epigenetic targets for inducing HbF expression in patients with  $\beta$ -thalassemia and sickle cell anemia, consideration of several factors should guide the further development of targeting strategies. The same considerations also apply to therapeutic targeting of transcription factors such as BCL11A and KLF1. The first is selection of the most informative preclinical model systems to identify promising agents. Human  $\beta$ -globin locus-bearing transgenic mice have provided a valuable model to identify important epigenetic and transcription factor silencers of embryonic/fetal  $\beta$ -globin gene expression. However, as noted previously, because mice only have embryonic and adult  $\beta$ -globin expression, this model may either underestimate or overestimate the effect that a given epigenetic or genetic fetal globin gene silencer will have in humans.

Cultured human primary erythroid cells derived from CD34+ progenitors also provide a valuable model for identifying epigenetic targets for inducing HbF expression. One important caveat for studies using these cells is that it is very important that the level of  $\gamma$ -globin gene expression be measured after extensive erythroid differentiation when the  $\gamma/\alpha+\beta$  expression level in control cells approaches that in normal adult erythroid cells. Agents that interfere with differentiation might result in a sufficient increase in  $\gamma$ -globin gene expression in this model to be clinically useful, but may have deleterious effects on erythropoiesis. Variation in the level of erythroid differentiation achieved in studies of agents that disrupt  $\gamma$ -globin gene silencing in this cell model system must be taken into consideration when assessing their relative therapeutic potential.

Another consideration is how specific the effect of a given type of epigenetic targeting might be. Clearly epigenetic regulatory factors have global effects on gene expression in all cell types, so that complete inhibition or ablation would likely be catastrophic in many instances. One exception might be the methyl-binding domain protein MBD2, whose complete absence is tolerated in knockout mice with only a minimal phenotype.<sup>63</sup> It is also generally believed that only genes that are in a poised state can be readily transcriptionally activated. Thus, if partial inhibition of multiple fetal globin gene silencing mechanisms can be achieved epigenetically, this might be highly effective with acceptable

**Table III.** Potential epigenetic therapeutic targets of  $\gamma$ -globin gene silencing

DNA methyltransferases
Histone deacetylases
Histone demethylases
Methyl-CpG-binding domain protein 2
Mi2 $\beta$ /CHD4 ATPase chromatin remodeler
Protein arginine methyltransferase 5
Friend of protein arginine methyltransferase 1

short- and long-term off target effects. Finally, the feasibility of targeting a given epigenetic regulator must be considered. Those that function through enzymatic activity such as DNA methylases, HDACs, histone demethylases, and histone methylases, and potentially the ATPase activity of Mi2 $\beta$ /CHD4, are more readily druggable. This is largely why clinical trials targeting these regulators already have been carried out or are underway. Like transcription factors, those epigenetic regulators such as MBD2-NuRD that function through protein-protein or protein-DNA interactions have been considered “undruggable” in the past. However, recent success in developing agents, such as covalently stapled peptides capable of disrupting protein-protein interactions in animal models,<sup>99-101</sup> and targeting specific proteins for degradation in the proteasome<sup>102,103</sup> suggest that this class of epigenetic regulators may be targeted successfully in the future (Table III).

## CONCLUSION

Epigenetic mechanisms play a key role in fetal globin gene silencing, both independently and in cooperation with specific transcription factor silencers such as BCL11A and KLF1. Among the first proof of principle targeted treatment trials in patients with  $\beta$ -hemoglobinopathies were those aimed at DNA methylation and histone acetylation, 2 key epigenetic marks of globin gene transcriptional activity. With further understanding of the specificity of epigenetic fetal globin gene silencing mechanisms, it is likely that targeting of this process will result in more successful treatment of patients with  $\beta$ -globin disorders through the induction of increased HbF levels.

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