Cell signaling pathways involved in drug-mediated fetal hemoglobin induction: Strategies to treat sickle cell disease

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Abstract

The developmental regulation of globin gene expression has shaped research efforts to establish therapeutic modalities for individuals affected with sickle cell disease and β -thalassemia. Fetal hemoglobin has been shown to block sickle hemoglobin S polymerization to improve symptoms of sickle cell disease; moreover, fetal hemoglobin functions to replace inadequate hemoglobin A synthesis in β -thalassemia thus serving as an effective therapeutic target. In the perinatal period, fetal hemoglobin is synthesized at high levels followed by a decline to adult levels by one year of age. It is known that naturally occurring mutations in the γ -globin gene promoters and distant cis-acting transcription factors produce persistent fetal hemoglobin synthesis after birth to ameliorate clinical symptoms. Major repressor proteins that silence γ -globin during development have been targeted for gene therapy in β -hemoglobinopathies patients. In parallel effort, several classes of pharmacological agents that induce fetal hemoglobin expression through molecular and cell signaling mechanisms have been identified. Herein, we reviewed the progress made in the discovery of signaling molecules targeted by pharmacologic agents that enhance γ -globin expression and have the potential for future drug development to treat the β -hemoglobinopathies.

Keywords: Fetal hemoglobin, sickle cell disease, β -hemoglobinopathies, NRF2, p38 MAPK, cell signaling

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Introduction

Sickle cell disease (SCD) and β -thalassemia are genetic disorders resulting in abnormal structure or decreased expression of the β -globin protein chain, respectively. SCD represents a significant global health problem, with more than 300,000 affected infants born each year, mainly in Africa. Fetal hemoglobin (HbF; $\alpha_2\gamma_2$) is expressed in persons with SCD at variable levels and is known to alleviate the clinical severity of the disease.¹ Although naturally occurring mutations in the β -locus or distant cis-acting factors contribute to HbF production during the adult developmental stage,² many studies have been conducted to design pharmacologic HbF inducers to offer treatment options to the majority of individuals not carrying these mutations. To date, hydroxyurea is the only FDA-approved drug for the treatment of SCD; currently, the recommendation is to offer this agent to all children with sickle cell anemia after nine months of age regardless of clinical severity. Hydroxyurea is a potent HbF inducer in adults^{3,4} and children;⁵ however, it does not possess the ideal combination of efficacy, safety and ease of use. Data generated in the Cooperative Study

of Sickle Cell Disease, verified that HbF levels above 8.6% significantly impacted mortality in SCD providing clinic proof of the protective effect of HbF.¹ Thus, understanding mechanisms involved in γ -globin gene regulation is critical to designing additional therapeutic agents and a cure.

Two major mechanisms for γ -globin silencing during development have been established including competitive interaction of the γ - and β -globin genes with the upstream β -locus control region during the fetal to adult switch^{6,7} and gene-autonomous γ -globin silencing.⁸ The latter mechanism provides the basis for direct gene-based strategies to induce HbF after birth to cure individuals with β -hemoglobinopathies. Several transcription factors play a major role in γ -globin silencing during development including TR2/TR4, MYB, BCL11A, GATA1 and KLF1 (Figure 1). The direct repeat erythroid-definitive (DRED) complex binds a direct repeat element overlapping the distal CCAAT box in the ϵ - and γ -globin promoters.⁹ Point mutations in the direct repeat at base -117 produces elevated γ -globin expression after birth.^{10,11} Work by Cui et al. demonstrated that binding of the DRED complex composed of TR2/TR4 and the cofactors DNA methyltransferase 1 (DNMT1) and lysine-specific

Figure 1 Major transcription factors involved in developmental γ -globin silencing. Shown is a schematic of the β -globin locus including the locus control region (LCR) comprised on hypersensitive sites 1–5 (white boxes). The major repressor proteins involved in γ -globin regulation demonstrated by developmental studies in transgenic mice and humans are shown. KLF1 bind directly in the β -globin gene promoter and interacts mainly with hypersensitive site 3 to facilitate competitive β -globin gene expression during adult development (green arrows). KLF1 also activates BCL11A to promote γ -globin silencing through BCL11A binding in hypersensitive site 3 and downstream of $A\gamma$ -globin (red lines). Displayed are different BCL11A protein-partners involved in γ -globin repression. The non-steroidal nuclear receptors TR2 and TR4 along with major components of the DRED complex, DNMT1 and LSD1 bind the direct repeat elements in the ϵ -globin and γ -globin promoters. LSD1: lysine-specific histone demethylase 1; DNMT1: DNA methyltransferase 1; BCL11A: B-cell CLL/lymphoma 11 A.

histone demethylase 1 (LSD1), directly represses γ -globin gene expression in definitive erythroid cells.¹² DNMT1 methylates CpG dinucleotides to maintain stable DNA methylation and long-term gene silencing.¹³ By contrast, LSD1 removes the methyl group from mono- and dimethyl-histone H3 lysine 4 to decrease epigenetic marks associated with transcriptionally active genes.¹⁴ Drugs that directly inhibit DNMT1 and LSD1 serve as a foundation for the development of novel HbF inducers.

Insights into γ -globin regulation have been garnered from human genetic studies. Through quantitative trait locus analysis, Thein et al.¹⁵ identified the intergenic region between the HBS1L and MYB genes associated with inherited HbF levels. Later studies in newborns with trisomy 13 and high HbF levels led to the discovery of two microRNAs, miR-15a and miR-16-1 mediating repression of MYB gene expression in adult CD34+ stem cell-derived erythroid progenitors.¹⁶ Disruption of MYB in mice produced increased ϵ -globin and γ -globin expression¹⁷ supporting a role of MYB in γ -gene silencing during development. Additional studies demonstrated the ability of MYB to activate KLF1 and TR2/TR4 in human erythroid cells¹⁸ as an indirect mechanism of γ -globin silencing. KLF1 is a master regulator of adult β -globin expression and many other aspects of normal erythropoiesis.¹⁹ In support of an inherited phenotype, Borg et al.²⁰ identified a Maltese family with elevated HbF levels associated with a nonsense mutation in the *KLF1* gene. Subsequently, other families have been identified with different *KLF1* mutations that produce a haplo-insufficiency phenotype and elevated HbF²¹; experimental data determined that KLF1 represses γ -globin indirectly through B-cell CLL/lymphoma 11 A (BCL11A) activation.²²

The discovery of BCL11A, a major repressor of γ -globin expression, has made a significant impact towards efforts to develop gene-based strategies to reactivate γ -globin

expression during adult-stage development. During hematopoietic cell differentiation, BCL11A is down-regulated and is possibly involved in lymphoma pathogenesis since translocations associated with B-cell malignancies also deregulate BCL11A expression.^{23,24} Related to inherited HbF levels, BCL11A was first identified by human quantitative trait locus studies,²⁵ and later confirmed to be a genetic modifier of γ -globin transcription by genome wide association studies in thalassemia²⁶ and sickle cell patients.²⁷ BCL11A interacts with GATA1, FOG1 and the NuRD repressor complex (Figure 1).²⁸ Disruption of the Mi2 β subunit of NuRD results in increased γ -globin gene expression in transgenic mice.²⁹ Recently, it was shown that partial knockdown of Mi2 β in primary erythroid cells results in significant γ -globin gene activation without affecting erythroid differentiation. Moreover, the effect of Mi2 β on γ -globin gene silencing in part was due to down regulation of BCL11A and KLF1.

Using molecular analysis of human primary erythroid cultures, interactions between BCL11A and SOX6 across the β -locus were demonstrated as another mechanism of γ -globin silencing.³⁰ However, significant concerns have been raised over the potential negative effects of knocking out *BCL11A* gene expression in hematopoietic stem cells and the risk for B-cell malignancies. Recent studies were conducted by Orkin and colleagues to characterize an enhancer in intron 1 of *BCL11A*, which can be target for erythroid-specific gene silencing.³¹ The guided knockout of this element in human stem cells using zinc finger nucleases and CRISPR-Cas approaches are underway for the development of gene therapy for β -hemoglobinopathies. Alternative strategies including drug-mediated modulation of major regulators of globin gene expression are also being explored. Macari et al.³² demonstrated inhibition of BCL11A and KLF1 along with activation of NRF2 signaling (discussed below) by the cholesterol reducing agent

simvastatin, as a mechanism of γ -globin activation in primary erythroid cells. The development of additional therapeutic agents is desirable.

Modulation of epigenetic marks to activate HbF expression

There exists a large group of chromatin modifying proteins, which regulate epigenetic marks through modification of DNA and histone proteins which allows the interaction of transcription factors to consensus motifs to fine-tune gene expression during development. DNA methylation of the embryonic and fetal globin genes inversely correlates with the level of gene expression. The therapeutic activity of the DNMT1 inhibitors 5-azacytidine and decitabine produces demethylation at the γ -globin promoters and HbF induction.^{33,34} Decitabine is currently in clinical trials for the treatment of SCD (ClinicalTrials.gov Identifier: NCT01685515). Another chromatin modifying protein LSD1 demethylates mono- and dimethyl histone H3 lysine 4, an active histone mark. The FDA-approved antidepressant tranylcypromine, which is an LSD1 inhibitor, induces HbF synthesis in adult erythroid progenitors; combination therapy with decitabine produced a synergistic effect.³⁵ Subsequent studies showed inhibition of erythroid maturation by tranylcypromine, which precludes further clinical development. However, another LSD1 inhibitor, RN-1, was recently shown to induce HbF in sickle cell mice with increased F-cells³⁶ and decreased numbers of sickle red cells in the peripheral blood, suggesting RN-1 may also reduce disease pathology.

Another epigenetic mark targeted to achieve high-level HbF induction is histone acetylation, which is most commonly associated with active gene transcription. Of the large number of histone deacetylase inhibitors tested in tissue culture systems and demonstrated to induce HbF, subsequent human trials verified the ability of arginine butyrate to induce HbF to clinically relevant levels.³⁷ Butyrate inhibits the function of several histone deacetylases producing hyperacetylation of mainly histones 3 and 4 and activation of the p38 mitogen activated protein kinase (MAPK) signaling pathway as a dual mechanism of γ -gene reactivation (discussed in detail below). However, further development of butyrate has been hindered by the requirement of intravenous administration. Major mediators of histone methylation (an active gene mark) are the protein arginine methyltransferases (PRMT). PRMT1 is associated with human γ -globin gene silencing through association with a repressor protein named friend of PRMT1.³⁸ Knockdown of this repressor protein mediates increased γ -globin gene expression in cultured human primary erythroid cells.

Discovery of novel HbF inducers

Tissue culture systems are an invaluable tool for the development of novel HbF inducers; however, they have limitations in their predictive value with regard to response in people with β -hemoglobinopathies. Tissue culture provides an *in vitro* model that can be easily manipulated

with different pharmacologic agents to determine the effects on globin gene expression; however, these observations may not accurately reflect developmental mechanisms involved in globin gene regulation. The most widely utilized cell lines namely K562 and mouse erythroleukemia cells serve as rapid screening tools, but the results obtained in these systems often are not reproduced in primary erythroid cultures or human trials.^{2,39,40} Our group characterized KU812 leukemia cells where significant γ -globin and low-level β -globin expression is observed at steady-state, as a system for chemical drug screens.⁴¹ Known HbF inducers and novel agents were identified in KU812 cells and later confirmed in primary erythroid cells.

Over the last two decades, numerous primary liquid culture systems have been established to generate hematopoietic progenitors from peripheral blood mononuclear and CD34+ stem cells.^{42,43} Primary erythroid cells provide a valuable model for identifying transcription factors and epigenetic targets for modulating γ -globin transcription. Yet, there is not a one-to-one correlation between drug-mediated HbF induction in primary cultures and clinical response in β -hemoglobinopathy patients. One caveat for studies using primary erythroid cells is that γ -globin gene expression should be measured after considerable erythroid differentiation has occurred, i.e. when γ -globin and β -globin expression levels approach those observed in adult erythroid cells. Drugs that interfere with erythroid differentiation might result in significant increases in γ -globin expression in this model but may have deleterious effects on erythropoiesis. Variation in erythroid maturation must be taken into consideration when assessing the therapeutic potential of different agents.

Over the last four decades, more than 70 pharmacologic compounds have been shown to induce HbF synthesis mainly *in vitro* in tissue culture systems with limited pre-clinical animal studies and clinical trials. While major transcription factors involved in developmentally regulated hemoglobin switching have been identified through human and murine genetic studies (described above), by contrast most drug therapies reactivate HbF through modulation of DNA-binding proteins and/or epigenetic modifications in adult erythroid cells. These agents include 5-azacytidine,⁴⁴ decitabine,^{45,46} hydroxyurea,⁴⁷ butyrate,^{48,49} short-chain fatty acid derivatives,^{50,51} simvastatin,³² pomalidomide,⁵² and fumaric acid esters⁵³ among others. HbF induction is accomplished by diverse mechanisms such as inhibition of DNA methyl transferases and histone deacetylases, DNA damage, and immune-modulation.^{2,54} Recently, expanded insights into γ -globin regulation have been gained through the investigation of cell signaling pathways activated by pharmacologic agents, which is the focus of the remainder of this review.

Cell signaling mechanisms involved in erythroid differentiation

Commitment to the erythroid lineage and terminal differentiation requires activation of multiple signaling pathways mediated by interaction between erythropoietin (EPO) and the erythropoietin receptor (EPO-R). The latter is activated

by EPO-induced dimerization^{55,56} and subsequent activation of signal transduction through Janus kinase 2 and signal transducer and activator 5A (STAT5A) and STAT5B⁵⁷; subsequent activation of MAPK and phosphatidylinositol 3-kinase also occurs.⁵⁸ MAPK signaling involves three pathways including SAPK/JNK, ERK1/2 and p38 MAPK.⁵⁹ ERK1/2 plays a role in proliferation of early erythroid precursors and terminal maturation,⁶⁰ while SAPK/JNK and p38 MAPK are involved in erythroid differentiation.^{61,62} During normal erythropoiesis in the adult bone marrow, hemoglobin synthesis is controlled by erythroid-specific and ubiquitous transcription factors²; for example, ATF-2 is phosphorylated and activated by p38 MAPK to alter target gene transcription.⁶³ The study of cell signaling molecules involved in γ -globin activation during erythropoiesis or in response to various pharmacologic agents has shed light on mechanisms of HbF induction thus providing therapeutic targets for the development of novel clinical treatments (Table 1). A full discussion of the complex network of proteins required to achieve normal erythropoiesis is beyond the scope of this review. However, these observations have provided a conceptual model for experimental approaches to ascertain the role of cell signaling effector molecules in γ -globin gene reactivation during adult development.

JAK/STAT signaling pathway

Erythropoietin was one of the first agents to be tested as an HbF inducer in baboons;⁶⁴ however, its efficacy was not demonstrated in human trials.⁶⁵ The STAT transcription factors are the most common downstream signaling molecules activated by EPO. There are six major STAT protein family members including STAT-1 through STAT-6, which exist as latent cytoplasmic proteins⁶⁶ that can be activated by pharmacologic agents. Studies by Boosalis et al. demonstrated that short-chain fatty acid derivatives induce prolonged expression of the growth-promoting genes MYB and MYC.⁶⁷ Furthermore, sodium butyrate and dimethyl butyrate mediate STAT-5 activation in the absence of histone hyperacetylation contributing to cellular proliferation and HbF induction by these agents.

Two STAT3 variants, STAT-3 α and STAT-3 β , have identical cognate DNA-binding motifs, but the latter acts as a dominant-negative isoform.⁶⁸ Work from our laboratory (Figure 2) demonstrated that STAT3 β silences γ -globin expression by means of a consensus-binding site at nucleotide +9 in the γ -globin 5'-untranslated region.⁶⁹ In subsequent studies, we observed *in vivo* binding of STAT3 and GATA-1 to this region by chromatin immunoprecipitation assay in K562 and mouse erythroleukemia cells.⁷⁰ DNA-

Table 1 Major cell signaling pathways involved in drug-mediated HbF induction

Drug	Class of drug	Cell signaling pathway	References
Erythropoietin	Growth factor	JAK/STAT, MAPK	Remy et al., ⁵⁶ Wojchowski et al., ⁵⁷ Seger and Krebs ⁵⁹
Thalidomide	Immune modulator	p38 MAPK, cAMP	Aerbajinai et al. ⁸⁸
Hydroxyurea	Ribonucleotide reductase inhibitor	p38 MAPK, cAMP, Cgmp	Park et al., ⁷⁵ Lou et al., ⁷⁷ Ikuta et al., ⁹³ Tang et al. ⁹⁶
Butyrate	HDAC inhibitor	p38, cGMP, cAMP	Witt et al., ⁷¹ Pace et al., ⁷⁸ Sangerman et al. ⁸¹
Trichostatin A	HDAC inhibitor	p38 MAPK	Pace et al. ⁷⁸
Scriptaid	HDAC inhibitor	p38 MAPK	Johnson et al. ⁷²
Apicidin	HDAC inhibitor	p38 MAPK	Witt et al. ⁷³
5-Azacytidine	DNMT inhibitor	cAMP	Keefer et al. ⁹⁸

cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; DNMT: DNA methyltransferase; HDAC: histone deacetylase; JAK/STAT: Janus kinase/signal transducers and activators of transcription; MAPK: mitogen-activated protein kinases; SCFAD: short-chain fatty acid derivative.

Figure 2 Transcription factors mediating γ -globin gene regulation via cell signaling pathways. Shown are the cells signaling pathways and downstream transcription factors demonstrated to bind the γ -globin gene promoter after drug treatments in erythroid cells.

ARE: antioxidant response element; ATF-2: activating transcription factor-2; CREB1: cAMP response element binding protein; Epo: erythropoietin; G γ -globin cAMP response element; Jak: Janus kinase; MAPK: mitogen activated protein kinase; NRF2: Nuclear factor (erythroid-derived 2)-like 2; ROS: reactive oxygen species; STAT3: signal transducer and activator of transcription 3

protein interaction studies confirmed that a STAT3/GATA-1 complex is assembled in this region. STAT3 β enforced expression in K562 cells reduced γ -globin expression which was reversed by GATA-1 over-expression. These data provide evidence that GATA-1 can reverse STAT3-mediated γ -globin silencing in erythroid cells.

γ -globin activation via p38 MAPK

A role for p38 MAPK cell signaling in HbF induction (Table 1) has been demonstrated for histone deacetylase inhibitors such as butyrate, trichostatin A, scriptaid, and apicidin^{71–73}; by contrast, ERK MAPK is a negative regulator of γ -globin expression.⁷¹ Studies from our laboratory showed that butyrate and trichostatin A stimulated p38 MAPK phosphorylation via a H₂O₂-dependent mechanism, supporting a role for reactive oxygen species in HbF induction.⁷⁴ Studies to define the mechanisms by which hydroxyurea induces HbF demonstrated its ability to active p38 MAPK signaling⁷⁵ and act as a nitric oxide (NO) donor.⁷⁶ We later demonstrated increased levels of nitrites and nitrates in K562 cells after hydroxyurea treatment, which was reversed by the NO synthase inhibitor NG-monomethyl-L-arginine⁷⁷ confirming the complexity of how hydroxyurea functions in erythroid progenitors.

Studies using K562 stable cell lines⁷⁸ and primary erythroid progenitors⁷⁹ have clarified molecular mechanisms for γ -globin gene regulation. Enforced p38 MAPK expression in K562 stable lines increased γ -globin transcription supporting transactivation by downstream DNA-binding proteins. Several transcription factors acting as downstream effectors of p38 MAPK such as ATF-1-4, CREB, and CREM^{80–82} are candidate regulators of γ -globin. We confirmed that butyrate and trichostatin A mediate CREB1 and ATF-2 phosphorylation and binding to an upstream cyclic AMP response element (CRE) located at -1222 in the G γ -globin promoter (G-CRE)⁸¹ (Figure 2). We then completed studies to determine if p38 MAPK signaling is required for steady-state γ -globin expression independent of drug induction. We observed γ -globin silencing after p38 or CREB1 siRNA knockdown.⁸³ Subsequent chromatin immunoprecipitation assays verified that CREB1 and its binding partner cAMP response element-binding protein (CBP) co-localize at the G-CRE region. These data support the role of p38 MAPK/CREB1 signaling in G γ -globin gene transcription under steady-state conditions.

CREB1 is known to have a wide array of binding partners including CBP, ATF-2, cJun,^{81,82} KLF4,⁸⁴ and MYB⁸⁵ among others. To determine whether a multiprotein complex bind to the G-CRE, we conducted studies using erythroid progenitors generated from normal adult bone marrow CD34+ stem cells. Through co-immunoprecipitation studies, we confirmed protein-protein interactions between cJun/ATF-2 and CREB1/ATF-2 but not between CREB1 and cJun. Promoter pull-down assay confirmed co-localization of cJun, CREB1, and ATF-2 on the G-CRE.⁸⁶ Subsequent studies were conducted using a chromatographically purified G-CRE/ATF2 protein complex and mass spectrometry analysis.⁸⁷ We identified the major binding partners of ATF-2 including CREB1, cJun, Brg1, and histone deacetylase 2

among others. Immunoprecipitation assays verified interaction of these proteins with ATF2 in human CD34+ cells undergoing erythroid differentiation, which was correlated with γ -globin expression. We are currently characterizing the changes in chromatin domains and epigenetic marks in the G-CRE region as it related to γ -globin expression during hemoglobin switching.

Another class of drugs that mediate immunomodulatory effects, namely thalidomide and pomalidomide were shown to mediate HbF induction; pomalidomide is a structural analog of thalidomide. Thalidomide induces γ -globin expression in a dose-dependent manner but had no effect on β -globin expression.⁸⁸ Furthermore, thalidomide treatment increased reactive oxygen species and p38 MAPK signaling along with histone H4 hyperacetylation suggesting different mechanisms of HbF induction by this agent. Using erythroid progenitors derived from human CD34+ stem cells isolated from normal and sickle cell donors, it was shown that pomalidomide is a potent HbF inducer but delays erythroid maturation.⁸⁹ Subsequent studies in the preclinical sickle cell transgenic mice confirmed HbF induction at levels similar to hydroxyurea.⁵² Whether pomalidomide activates p38 MAPK signaling, as a mechanism of HbF induction was not studied; however, it is an inhibitor of tumor necrosis factor- α , Interleukin-6 and Interleukin-1 β .⁹⁰

NO and cyclic guanosine monophosphate signaling pathway

NO is a second messenger signaling molecule generated from L-arginine by the action of NO synthase.⁹¹ In the vasculature, NO reacts with iron in the active site of the enzyme guanylyl cyclase to stimulate cyclic guanosine monophosphate (cGMP) signaling.⁹² Elevated soluble guanylyl cyclase levels have been observed in erythroid progenitors expressing a γ -globin program compared with those expressing β -globin.⁹³ Moreover, NO/cGMP signaling activates several transcription factors such as c-Fos and c-Jun,⁹² known to regulate γ -globin expression through binding at hypersensitive site 2 in the β -locus control region. In addition, NO increases Sp-1 binding to the CACCC box and may activate γ -globin by this mechanism.⁹⁴

Of the known HbF inducers, hydroxyurea, hemin, and butyrate have been shown to induce cGMP signaling to increase γ -globin expression in K562 cells and human erythroid progenitors.^{93,95} Hydroxyurea is metabolized to NO to activate cGMP signaling. Secretion-associated and RAS-related (SAR) is a small guanosine triphosphate-binding protein known to be involved in protein trafficking from the endoplasmic reticulum to the Golgi complex. Tang et al. demonstrated that SAR and γ -globin gene mRNAs are induced after hydroxyurea treatment.⁹⁶ Additional studies showed that stable expression of SAR in K562 cells increased γ -globin mRNA, while inhibiting ERK cell signaling consistent with a previously demonstrated negative role for ERK in γ -globin gene regulation.⁷¹

Later studies by the Ikuta lab focused on the ability of cGMP to stimulate cAMP-dependent pathways as a mechanism of γ -globin activation.⁹⁷ Ikuta's work suggested that

the cAMP-dependent pathway plays a negative role in γ -globin regulation in K562 cells. By contrast, another group demonstrated cAMP production is required for HbF induction by hydroxyurea, butyrate, and 5-azacytidine in human CD34+ erythroid progenitors, while perturbation of cGMP production had only minimal effects.⁹⁸ Therefore, the exact role of cAMP and cGMP in γ -globin regulation in erythroid cells requires additional studies.

In clinical trials, it was observed that hydroxyurea increases circulating NO levels in sickle cell patients;⁹⁹ therefore, we quantified the level of nitrate and nitrite generated by this agent in erythroid progenitors in the presence of three NO synthase inhibitors.⁷⁷ Hydroxyurea increased nitrates and nitrites levels and γ -globin transcription in this system. Furthermore, pretreatment with cGMP pathway inhibitors reversed γ -gene activation by hydroxyurea demonstrating direct activation of NO and cGMP cell signaling as a mechanism of HbF induction.

Oxidative stress and antioxidant response

When cells are exposed to high-level environmental oxidants such as ionizing and UV irradiation or elevated reactive oxygen species, they undergo oxidative stress.¹⁰⁰ To counter these extrinsic and intrinsic attacks, cells invoke defense mechanisms through basal and inducible regulation of phase II cytoprotective enzymes including heme oxygenase 1, superoxide dismutase, catalase, NADPH: quinone oxidoreductase 1 (NQO1), and glutathione S-transferase among others.¹⁰¹ These enzymes share a common consensus sequence TGACnnnGC encoded in the 5' upstream region of gene promoters known as the antioxidant response element (ARE).^{102–104} Later studies demonstrated the ubiquitously expressed transcription

factor NRF2 binds the ARE as a heterodimer with the small Maf proteins (MafF, MafG or MafK) in response to oxidative stress.¹⁰⁵ These data support NRF2 as a key regulator the cytoprotective enzymes through the ARE.¹⁰⁶ NRF2 was initially identified as a protein bound to tandem NFE2/AP1 repeats in the locus control region of the β -globin locus in K562 cells.¹⁰⁷ Subsequent knockout studies in mice determined that NRF2 was not essential for murine erythropoiesis.¹⁰⁸ However, recent publications have brought attention to NRF2 related to its role in γ -globin activation and HbF induction in response to several NRF2 chemical inducers.^{32,109}

NRF2/Keap1 repressor complex and NRF2 activation

The human NRF2 protein consists of 605 amino acid residues, which are organized in seven NRF2-ECH homology (Neh) domains (Figure 3a). The Neh2 domain interacts with the adapter repressor protein Kelch-like ECH-associated protein1 (Keap1)¹¹⁰ enriched with lysine residues that are targets for ubiquitination by ubiquitin ligase E3.^{111,112} The Neh 4 and 5 domains are bound by the transcription activators CBP and p300 when NRF2 interacts with different AREs to activate target gene transcription.¹¹³ Neh1 consists of a CNC-basic leucine zipper region, which dimerizes with small Maf proteins and serves as the NRF2 DNA-binding domain.¹¹⁴

The human Keap1 protein is composed of 625 amino acid residues (Figure 3b). It has three major domains, Broad complex, Tramtrack and Bric a brac (BTB), intervening region (IVR) and six Kelch/double glycine repeats (DGR) repeats. Keap1 forms homodimers and associates with the Cul3-dependent ubiquitin ligase E3 at the BTB domain.¹¹⁵

Figure 3 Schematic structure of NRF2 and Keap1 proteins. (a) Different domains in NRF2 including the Keap1 binding site (Neh2) and region of heterodimerization with small Maf proteins and DNA-binding domain (Neh1). Neh4 and Neh5 are domains for binding the co-activators CBP/p300. Neh6 domain is the binding domain to β TrCP, another ubiquitin ligase E3. (b) The regions in Keap1 required for binding to the E3 ligase Cul3/Rbx1 complex which ubiquitinates NRF2 and direct proteasome degradation. Also displayed is the NF2 binding domain including BTB, and DGR. The red circles represent residues reported to be phosphorylated by protein kinases. The triangles represent cysteine residues. The green triangles indicate reactive cysteine residues that function in oxidative stress sensing in the cell. Many antioxidant reagents target cysteine residue 151 which is labeled separately. NRF2: Nuclear factor (erythroid-derived 2)-like 2; IVR: intervening region; Keap1: Kelch-like ECH-associated protein1; DGR: double glycine repeats; Neh: NRF2-ECH homology

The DGR and the C-terminal regions associate with NRF2 under non-oxidative stress conditions.¹¹⁶ Keap1 protein has 27 cysteine residues, which mediates its role as a sensor for redox conditions in cells.

Mechanisms by which NRF2 is stabilized away from the Keap1-Cul3 inhibitory complex under oxidative condition have been studied extensively.^{101,117,118} In general, in the non-oxidative state, newly translated NRF2 is captured in the cytoplasm by Keap1 dimers through the DLG and ETGE motifs.¹¹⁹ The Keap1 dimer also interacts with the N-terminus of Cul3 to mediate NRF2 ubiquitination by a Cul3-dependent E3 ubiquitin ligase and rapid degradation through proteasome-dependent mechanisms.¹¹⁵ By contrast, when cellular oxidative insults or antioxidant drug treatment occur, reactive cysteine residues in Keap1 are oxidized.¹²⁰ Mutagenesis studies identified three cysteine residues in Keap1 (Green arrows in Figure 3b) that alter NRF2 function under oxidative stress conditions.^{121,122} This reaction results in a conformational change in the structure of Keap1, dissociation from Cul3 and stabilization of NRF2. Subsequently, NRF2 is phosphorylated and translocated to the nucleus where it forms heterodimer with small Maf proteins and bind to AREs of many genes encoding cytoprotective enzymes.¹²³

Recently, another ubiquitin E3 ligase, β -transducin repeats-containing protein (β TrCP) was shown to associate with NRF2 at the Neh6 domain after GSK3 β mediated NRF2 phosphorylation at two serine residues (Ser335 and Ser338).^{124,125} Inhibition of GSK3 β activity causes NRF2 activation by dephosphorylation and increased heme oxygenase 1 expression in Keap1^{-/-} mouse embryonic stem cells¹²⁶ suggesting the β TrCP-GSK3-NRF2 axis is another NRF2 regulation system independent of Keap1 regulation. Interestingly, transcriptome profiles demonstrated that Keap1 is highly expressed in CD34+ and CD71+ hematopoietic cells compared to other cell types whereas β TrCP is only highly expressed in CD71+ erythroid cells.¹²⁷ Other mechanisms of NRF2 regulation include activation by MAPK signaling in diverse cell types.¹¹⁸ Studies using inhibitors of p38 MAPK (SB203580), ERK (PD98059), and JNK (SP600125) signaling, showed attenuation of the ability of d-aminolevulinic acid/sodium ferrous citrate treatment to mediate NRF2 induction of heme oxygenase 1 expression in cardiomyocytes.¹²⁸ These studies demonstrate that MAPK signaling is involved in NRF2 activation and subsequent induction of cytoprotective enzymes.

HbF induction by NRF2 activators

The activators of NRF2 are categorized into nine chemical classes based upon differences in their capability to induce the NQO1 gene: (1) oxidizable diphenols and quione; (2) Michael reaction acceptor (olefins or acetylenes conjugated to electron-withdrawing groups); (3) isothiocyanates; (4) hydroperoxides; (5) trivalent arsenic derivatives; (6) divalent heavy-metal cations (lead and cadmium); (7) vicinal dithiols; (8) 1,2-dithiole-3-thiones; and (9) carotenoids and other conjugated polyenes.¹²⁹⁻¹³¹ These molecules are chemically active and able to covalently modify sulfhydryl groups by alkylation, oxidation or reduction. The presence

of ortho-hydroxyl group on the aromatic rings of the inducers enhances rapid reactivity with sulfhydryl compounds to raise inducer potency.¹³¹ By contrast, Keap1 serves as a sensor for the presence of NRF2 activators through its high content of cysteine residues. Many of these compounds have clinical utility for a wide range of diseases through NRF2 activation and induction of cytoprotective genes.¹³²⁻¹³⁵

Recently, NRF2 inducers have attracted attention in the globin field due to their ability to activate γ -globin transcription and HbF production in human erythroid cells (Table 2). Macari and Lowrey¹⁰⁹ reported that the known NRF2 inducers tert-butylhydroquinone (tBHQ), curcumin and 2-dithiole-3-thione, induce γ -globin and NQO1 transcription in K562 cells; moreover, increased NRF2 binding to the ARE region was observed in the promoters of these genes. In later studies, Lowrey and colleagues showed combining tBHQ and simvastatin (an FDA-approved drug used for cardiovascular disease), induced γ -globin and NQO1 transcription in an additive manner;³² simvastatin also induced HbF production in human primary erythroid progenitors (Table 2). To gain insights into molecular mechanisms, they observed that combined tBHQ and simvastatin treatment reduced the expression of two γ -globin repressors, KLF1 and BCL11A, suggesting a dual mechanism of γ -globin regulation. These agents could potentially be developed as γ -globin inducers to define a new class of therapeutic drugs for treating SCD and β -thalassemia.

Another group demonstrated a curcumin derivative, bisdemethoxy curcumin, induces γ -globin expression in primary erythroid progenitors.¹³⁶ Although it is unclear whether NRF2 signaling is involved in this induction, another report showed that this agent induces heme oxygenase 1 transcription through NRF2 nuclear localization in a mouse B-cell line.¹³⁷ Other drugs that target NRF2 have been investigated as HbF inducers; for example, sulforaphane is a potent NRF2 activator that is produced from its precursor, glucoraphanin.¹³⁸ Young broccoli sprouts and cauliflower are enriched with glucoraphanin, from which sulforaphane is released by myrosinase when the plant is physically damaged.¹³⁹ Recently, a clinical trial was initiated to test the ability of sulforaphane in broccoli sprout homogenates to induce HbF expression in human primary erythroid progenitors from healthy and sickle cell donors (ClinicalTrials.gov Identifier: NCT01715480). Triterpenoids are another group of NRF2 inducers¹⁴⁰ with potential use in sickle cell patients, since they experience inflammation and oxidative stress. These agents have anti-inflammatory and antioxidative stress properties, especially bardoxolone methyl and 2-cyano-3,12-dioxooleana-1,9-dien-28-imidazole. Additional research is required to demonstrate, if this class of drugs induces HbF in β -hemoglobinopathies.

Among the compounds discussed, tBHQ and sulforaphane preferably target cysteine residue 151 in the BTB domain of Keap1 (Figure 2), whereas the other agents do not target this cysteine.¹²² By contrast, it is known that NRF2 activation can occur by mechanisms independent of Keap1 inactivation. For example, in a recent report, the ability of the proteasome inhibitor MLN9708 to stabilize and

Table 2 Summary of drugs that induce HbF through the Keap1/NRF2 pathway

Drug	Structure	Effect	References
<i>tert</i> -Butylhydroquinone (tBHQ)		Induces NRF2 activation and binding to γ -globin promoter; Increase γ -globin expression and HbF level in K562 and primary erythroid progenitors	Macari and Lowrey ¹⁰⁹
Simvastatin		Increase γ -globin expression and HbF level in K562 and primary erythroid progenitors by NRF2 activation and inhibits β -globin transcription by suppression KLF1 and BCL11A	Macari et al. ³²
MLN9708 (proteasome inhibitor)		Inhibits proteasome mediated ubiquitination and degradation of NRF2; induces ROS generation and antioxidant response in B-CFU cells from SCD patient; induces NRF2 nuclear localization; Increases HbF level in K562 cells	Kunze et al. ¹⁴²
Dimethylfumarate (DMF)		Increase γ -globin expression and HbF level in primary erythroid progenitors; induces NRF2 to activate γ -globin promoter	Promsote et al. ⁵³

NRF2: NRF2: Nuclear factor (erythroid-derived 2)-like 2; SCD: sickle cell disease; HbF: fetal hemoglobin.

enhance NRF2 nuclear localization was demonstrated.¹⁴¹ Studies in erythroid progenitors from sickle cell patients showed HbF induction by MLN9708, which was reversed by siRNA-mediated NRF2 silencing. These data suggest other regulatory steps of the NRF2/Keap1/Cul3-Rbx1 pathway can be targeted to achieve HbF induction in erythroid cells.

HbF induction by dimethyl fumarate

The fumaric acid esters are known NRF2 activators in different cell types such as cerebral endothelial cells and astrocytes,¹⁴² and lymphocytes and monocytes.¹⁴³ These compounds have pleiotropic actions in a broad spectrum of tissues, and they have high tolerability and oral bioavailability. Pharmacokinetic studies indicate following oral intake, dimethyl fumarate (DMF) is rapidly hydrolyzed and converted into monomethyl fumarate (MMF), which mediates a covalent modification at cysteine residue 151 of Keap1 to inactivate the repressor protein followed by NRF2 stabilization and target gene activation.¹⁴⁴ Recent FDA approval of Tecfidera (DMF, Biogen Idec) for use in adults with multiple sclerosis makes this agent attractive for rapid extrapolation to clinical trials in SCD and β -

thalassemia. A phase 3 trial to test the safety and efficacy of Tecfidera in pediatrics was initiated in Europe (ClinicalTrials.gov Identifier: NCT02283853).

Recent studies from our laboratory confirmed the ability of DMF and MMF to induce γ -globin transcription and HbF expression to levels comparable to hydroxyurea in primary erythroid cells.⁵³ In our published works, DMF treatment for 48 h produced four-fold γ -globin induction and a concomitant 26-fold increase in HbF-positive cells by flow cytometry analysis with anti- γ -FITC antibody. Western blot confirmed an increase in HbF protein by DMF and its active metabolite MMF comparable to hydroxyurea. Later studies were conducted to address the question whether DMF induces HbF via NRF2 activation. To test this mechanism, we generated human primary erythroid cells in liquid culture from adult bone marrow CD34+ stem cells. As shown in Figure 4(a), after DMF treatment the level of NRF2 transcription increased 2.7-fold with a simultaneous increase in NRF2 protein by Western blot analysis (Figure 4b); by contrast, hydroxyurea did not increase NRF2 levels.

Subsequent studies were conducted in primary erythroid cells to test the effects of NRF2 gene silencing using

Figure 4 DMF induces NRF2 expression in human erythroid progenitors. Primary erythroid cells were generated in liquid culture established with adult bone marrow CD34+ stem cells as previously published.⁵³ On day 8, cells were treated with the various drugs for 48 h and then harvested for RNA isolation. NRF2 expression levels were measured by reverse transcription-quantitative PCR (a) and protein was isolated for western blot analysis (b). * $p < 0.05$ Primary erythroid cells were treated with siRNA targeted to NRF2 (siNRF2) using Dharmafect reagent (Thermo Fisher Scientific, Pittsburgh, PA) on day 8 in culture. After 48 h RNA was harvested to measure NRF2 expression by reverse transcription-quantitative PCR (c). In the same sample, the effect of siNRF2 treatment on the ability of DMF to induce γ -globin was investigated. Pretreatment with siNRF2 inhibited DMF-mediated γ -globin activation (d). ** $p < 0.01$. NRF2: Nuclear factor (erythroid-derived 2)-like 2; DMF: dimethylfumarate

siRNA treatment in the presence and absence of DMF; siNRF2 treatment alone produced 60% target gene silencing compared to scrambled siRNA controls (Figure 4(c)). Combination treatment with siNRF2 inhibited the ability of DMF to activate γ -globin transcription demonstrated by 50% γ -globin silencing, below steady-state levels (Figure 4(d)). These studies support a role of NRF2 signaling in DMF-mediated γ -globin activation; however, whether this agent will be useful for the treatment of β -hemoglobinopathies requires animal studies and clinical trials.

Based on these findings we propose the model shown in Figure 5 for γ -globin activation by DMF which is known to target cysteine residue 151 to deactivate the repressor protein Keap1.¹⁴⁴ As a result, NRF2 is stabilized and translocated to the nucleus to activate the ARE located in the γ -globin promoters. A similar mechanism for HbF induction has been established for simvastatin.³² We also have experimental data that DMF stimulates reactive oxygen species in K562 cells, which might activate p38 MAPK signaling as a second mechanism of γ -globin activation by this agent (B. P. unpublished). Additional studies are underway in our lab to test the ability of DMF to induce HbF *in vivo* using the preclinical β -YAC mouse model carrying the entire 248 kb β -locus.¹⁴⁵ This transgenic line has a γ -globin promoter inducible by 5-azacytidine, butyrate¹⁴⁶ and tranilcyproline, a lysine-specific demethylase inhibitor.¹⁴⁷ Our data support the possible re-purposing of Tecfidera (DMF) as a stand-alone therapy or combined with hydroxyurea for the treatment of SCD or β -thalassemia.

Future perspectives

Strategies to induce HbF expression include the use of pharmacologic agents that alter epigenetic marks or cell signaling pathways which allow DNA-binding proteins to transactivate gene expression. Alternate strategies include inhibition of major repressor proteins that silence γ -globin transcription during development. After decades of investigations only one drug, hydroxyurea, is FDA approved for the treatment of adults with SCD.⁴ However, hydroxyurea is not effective in all sickle cell patients and it is less effective in β -thalassemia.¹⁴⁸ These observations provide a rational for continued research to develop safe and useful alternative drug therapies. Changes in chromatin structure mediated by histone deacetylase and DNA methyltransferase inhibitors is a valuable approach to reactivate γ -globin but there remains challenges in translating tissue culture findings into clinical efficacy. For example, butyrate must be given intravenously⁴⁹ and decitabine requires stabilization with tetrahydrouridine to achieve oral bioavailability in sickle cell patients.¹⁴⁹

As cell signaling mechanisms of gene regulation have emerged, additional approaches have been pursued to reactivate γ -globin transcription. It is known that stage-specific transcription factors² activated by multiple cellular signaling pathways are required to achieve normal hemoglobin switching and erythroid differentiation. Interestingly, treatment with erythropoietin increases red cell production and HbF in culture¹⁵⁰ but was not effective in clinical trials in sickle cell patients.¹⁵¹ By contrast, a wide

Figure 5 Model of drug-mediated γ -globin activation via NRF2 stabilization. Under basal conditions, NRF2 is sequestered in the cytoplasm by Keap1 dimers, ubiquitinated by the Cul3-Rbx1 ubiquitin ligase E3 complex, and targeted for proteasome-dependent degradation (left side). Treatment with tBHQ, simvastatin and DMF result in chemical modification of the repressor protein Keap1 at cysteine residue 151 which changes the conformation of Keap1 and facilitates NRF2 stabilization and nuclear translocation (right side). Subsequent heterodimerization with small Maf proteins mediates binding of NRF2 to the γ -globin promoter ARE (antioxidant response element) located at -100 base pairs upstream of the cap site. NRF2: Nuclear factor (erythroid-derived 2)-like 2; Keap1: Kelch-like ECH-associated protein1; DMF: dimethylfumarate; tBHQ: *tert*-butylhydroquinone; ARE: antioxidant response element

variety of drugs stimulate p38 MAPK signaling including hydroxyurea,⁷⁵ butyrate,^{71,79,81} and thalidomide⁸⁸ thus distinguishing several agents with clinical potential. Despite progress made in identifying novel agents using tissue culture systems, there remains limitation on the number of preclinical sickle cell mouse and baboons models to test drug efficacy to for the translation of new agents in to clinical trials. Realizing the cost and length of time required to bring drugs from bench to bedside, the NIH partnered with pharmaceutical companies to support efforts to re-purpose FDA-approved drugs. This provides opportunity to test agents with different mechanisms of action to develop combination drug regimens for treating β -hemoglobinopathies similar to that used for cancer and other diseases.

In this review, we discussed several compounds which stimulate NRF2 signaling to reactivate γ -globin transcription, holding promise for development as HbF inducers. For example tBHQ, simvastatin and DMF activate NRF2 signaling to facilitate HbF synthesis in human primary erythroid cells. Other small molecules that target the Keap1/NRF2 axis have been identified such as supercurcumin, hemin¹⁵² and bardoxolone methyl that might be targeted for therapeutic development. Of interest to our group is the FDA-approved drug Tecfidera (DMF) which we established as an HbF inducer in human primary erythroid cells.⁵³ Recently, Belcher et al. demonstrated

that DMF stimulated expression of heme oxygenase 1, ferritin and other antioxidant proteins in NY1DD transgenic sickle mice.¹⁵³ Furthermore, blood cells isolated from DMF-treated sickle mice showed significantly decreased adhesion to heme- and TNF-activated human umbilical vein endothelial cells. They concluded that DMF may be an effective agent to prevent sickle pain episodes. Tecfidera holds promise for the treatment of SCD; however, additional preclinical data are needed to move this agent to clinical trials.

Gene therapy for the β -hemoglobinopathies involves several approaches either the addition of a normal γ - or β -globin gene using lentivirus-based vectors, correction of β -globin mutations or silencing of major γ -globin repressor proteins. The addition of a normal β -globin gene has been used to cure a few β -thalassemia patients;¹⁵⁴ work continues to extend this approach to sickle cell patients. To identify repressors of γ -globin expression, human genetic and developmental murine studies established TR2/TR4, BCL11A, and KLF1 as potential targets for gene therapy. Naturally occurring mutations in KLF1 provide nucleotides to target for mutagenesis to create a haplosufficiency state and high HbF levels in mature erythrocytes. An alternate approach was made possible with the discovery of the erythroid-specific enhancer in BCL11A,³¹ which address concerns about total knockout of this factor and risks for

carcinogenesis. Rapid advances in selective DNA mutation using CRISPR-Cas techniques provide the technology to achieve erythroid-specific BCL11A knockout to inhibit γ -globin silencing during development. Once these techniques are proven safe in human stem cells then gene therapy for β -hemoglobinopathies by this approach will be possible.

Other molecular molecules are being pursued as potential γ -globin inducers include miRNAs that target the 3'untranslated region of mRNA to mediate gene silencing. Recently, it was shown that miR-144 targets α -globin to prevent its precipitation in red blood cells,¹⁵⁵ and it is associated with severe anemia in sickle cell patients. Interestingly, miR-144 is a negative regulator of NRF2¹⁵⁶ a known γ -globin transactivator. Many miRNA molecules are targeted for clinical development, thus treatment with antagomirs to repress miR-144 might hold potential as a therapeutic option to induce HbF in β -hemoglobinopathy disorders.

Authors' contributions: BSP designed and supervised all experimental studies and writing and editing of the review article. LL conducted a literature search and contributed to writing the review article and previously published experimental data. LHM conducted studies to generate data related to mechanism of γ -globin induction by dimethyl fumarate and contributed to writing and editing the review article. BL conducted experiments to generate data related to dimethyl fumarate experiments and contributed to writing and editing the review article.

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