Targeting Genetic Modifiers of HBG Gene Expression in Sickle Cell Disease: The miRNA Option

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Abstract
Sickle cell disease (SCD) is one of the most common inherited hemoglobinopathy disorders that affects millions of people worldwide. Reactivation of HBG (HBG1, HBG2) gene expression and induction of fetal hemoglobin (HbF) is an important therapeutic strategy for ameliorating the clinical symptoms and severity of SCD. Hydroxyurea is the only US FDA-approved drug with proven efficacy to induce HbF in SCD patients, yet serious complications have been associated with its use. Over the last three decades, numerous additional pharmacological agents that reactivate HBG transcription in vitro have been investigated, but few have proceeded to FDA approval, with the exception of arginine butyrate and decitabine; however, neither drug met the requirements for routine clinical use due to difficulties with oral delivery and inability to achieve therapeutic levels. Thus, novel approaches that produce sufficient efficacy, specificity, and sustainable HbF induction with low adverse effects are desirable. More recently, microRNAs (miRNAs) have gained attention for their diagnostic and therapeutic potential to treat various diseases ranging from cancer to Alzheimer’s disease via targeting oncogenes and their gene products. Thus, it is plausible that miRNAs that target HBG regulatory genes may be useful for inducing HbF as a treatment for SCD. Our laboratory and others have documented the association of miRNAs with HBG activation or suppression via silencing transcriptional repressors and activators, respectively, of HBG expression. Herein, we review progress made in understanding molecular mechanisms of miRNA-mediated HBG regulation and discuss the extent to which molecular targets of HBG might be suitable prospects for development of SCD clinical therapy. Lastly, we discuss challenges with the application of miRNA delivery in vivo and provide potential strategies for overcoming barriers in the future.

Key Points
Experimental data support the association of microRNAs (miRNAs) with HBG activation or suppression via silencing transcriptional repressors and activators, respectively, of HBG expression.

The clinical utility of miRNAs involved in reactivation of HBG, potential off-target adverse effects, and specificity and sustainability for fetal hemoglobin (HbF) induction in vivo requires further investigation.

Strategies for overcoming barriers in miRNA delivery are needed for the development of HbF-inducing therapeutics for SCD.
1 Introduction

Sickle cell disease (SCD) is a group of common inherited hemoglobin disorders that affects millions of people worldwide, including more than 100,000 African Americans in the US [1]. Homozygous hemoglobin S (HbSS) and HbSβthalassemia or sickle cell anemia (SCA) are the most common and clinically severe phenotypes observed [1]. SCA is caused by a single amino acid base substitution (β6Glu > Val) in the β-globin gene (HBB), which produces abnormal sickle hemoglobin (HbS) [2, 3]. Disease severity and the clinical manifestations observed in SCD vary greatly in people with SCA, who experience more severe complications compared with other genotypes. These complications are often due to repetitive vaso-occlusive crises under hypoxic conditions that can lead to stroke, organ damage, and early death [4, 5].

Reactivation of HBG (HBG1, HBG2) gene expression and induction of fetal hemoglobin (HbF) is an important therapeutic strategy in SCD since an increase in the percentage of HbF inhibits polymerization of HbS molecules and ameliorates clinical severity. Historical data suggest that individuals with HbF levels >20% have fewer recurrent clinical events, such as crises or pulmonary complications and stroke [6]. A more recent clinical study by Estepp et al. supports the use of hydroxyurea (HU) in children with SCD, with the preferred dosing strategy targeting an HbF endpoint >20% to reduce clinical complications and hospitalizations [7]. Individuals with naturally occurring hereditary persistence of HbF variants have significantly higher HbF levels in the majority of their red blood cells (pancellular) producing benign clinical phenotypes. Thus, to achieve maximal clinical benefit, it is also critical that therapeutic agents produce a pancellular distribution of HbF.

To date, HU has been the only US FDA-approved drug with efficacy to induce HbF in SCA patients, yet serious complications have been associated with its use [8]. Over the last three decades, numerous additional pharmacological agents to reactivate HBG transcription have been investigated but few have proceeded to clinical trials, except arginine butyrate and decitabine; however, neither of these two drugs met requirements for routine clinical use due to difficulties with oral delivery and inability to achieve efficacious levels. Thus, therapeutic interventions that demonstrate sufficient efficacy, specificity, and sustainability for HbF induction with low adverse effects are desirable to mitigate the clinical complications observed in SCD patients.

Genetic mapping and genome-wide association studies discovered genetic loci in B-cell lymphoma/leukemia 11A (BCL11A), an Xmn1 variant upstream of hemoglobin subunit gamma 1 (HBG1), and the HBS1L-MYB intergenic region, which account for 20–50% of the common variation in HbF levels observed in SCA patients [9–13]. An investigation into the C > T genetic variant in Xmn1 (rs7482144), which is located at −158 upstream of the HBG1 gene, revealed a significant correlation of this variant with disease severity in adults with β-thalassemia [10]. In addition to the identification of genetic risk loci with HbF-modulating capabilities, a complex comprising of DNA-binding proteins, including GATA1, TAL1, E2A, LMO2, LRF/ZBTB7A, and LDB1 among others, have shown to be silence HBG transcription during hemoglobin switching by mediating DNA looping between the locus control region (LCR) and each globin promoter sequentially [14, 15]. Similarly, DNA hypomethylation and histone hyperacetylation of the HBG proximal promoter have been effective in inducing gene expression [16, 17] and have been associated with high levels of HbF [18]. Supporting studies reported that hemoglobin switching is controlled through the activity of epigenetic modulators such as BCL11A, KLF1, and HBS1L-MYB [19–22]. Collectively, these findings support molecular approaches to target genetic loci and/or their HBG-modifying transcripts and proteins to induce HbF to reduce the clinical complications of SCD.

Over the last decade, mounting evidence has highlighted the association of microRNAs (miRNAs) with HBG activation or suppression via transcriptional repressor proteins and transactivators, respectively, of HBG expression [23–25]. miRNAs are endogenous small RNAs that are naturally expressed at varying levels in mammalian tissues [26]. Since the emergence of the role of miRNAs in regulating distinct processes in mammalian cells, their applicability as a novel class of therapeutics for targeted gene expression in various human diseases, especially cancer, has been extensively explored and proven quite beneficial [27–30]. Moreover, due to their limited size, conservation among species, and capacity to disrupt expression of target genes, miRNAs are attractive therapeutic candidates for targeting gene expression in common mammalian diseases [31, 32].

Despite their high therapeutic potential, there are major concerns with the clinical utility of miRNAs due to off-target adverse effects. miRNAs function through imperfect base pairing to complementary regions of their target messenger RNA (mRNA) molecules and often produce downregulation of target gene expression, resulting in translational repression, mRNA cleavage, and deadenylation [33, 34]. However, the ability of a single miRNA to alter the expression of multiple genes and pathways, many of which may activate oncogenes or downregulate tumor suppressor genes [35], is an Achilles Heel when considering the development of miRNA-based interventions. Another concern is the lack of knowledge and uncertainty as to whether modulation of
miRNAs will produce sufficient efficacy, specificity, and sustainability for HbF induction in vivo. In this review, we propose to discuss the involvement of miRNAs in HBG gene regulation and critically assess the extent to which direct or indirect targeting of HBG expression by miRNAs might be a suitable prospect for therapeutic development for SCD. Lastly, we discuss challenges with the application of miRNA delivery in vivo and provide strategies for overcoming barriers in miRNA delivery.

2 Background on MicroRNAs

In 1993, miRNAs were first discovered as the product of the heterochronic lin-4 gene in C. elegans [36, 37]. They are small (~22 nucleotides), endogenous nonprotein-coding RNA molecules that interact with the miRNA-induced silencing complex (mRISC) to silence gene-specific mRNA in the cytoplasm [38]. miRNAs facilitate mRNA degradation and gene silencing by base-pairing with complementary sequences in the 3′-untranslated regions (3′UTR) of target mRNA [26, 39]. According to the miRBase database, release 22 (accessed January 2022), more than 38,000 mature miRNAs have been discovered [40]. Each miRNA produced potentially regulates the expression of hundreds of genes. In addition, a single transcript can be targeted by more than one miRNA to alter the expression of genes in the same pathway [40, 41]. Therefore, miRNAs have been widely investigated since discovery, evidenced by more than 126,900 published studies on miRNAs indexed in PubMed.

Since discovery, development of miRNA-based therapeutics for a wide variety of diseases, ranging from cancer to Alzheimer’s disease, has erupted. More recently, studies have examined an association between miRNAs in peripheral blood of several diseases, including neurodegenerative disorders, Alzheimer’s disease, and cancer [42–45]. Several miRNAs are abundant in erythroid cells and function as critical regulators of hematopoiesis, including miR-15a/16, miR-144, miR-150, miR-191, and miR-221/222 [46]. Supporting studies reported expression of genes targeted by differentially expressed miRNAs in fetal versus adult erythroid progenitor cells, including the let-7 miRNA family [47, 48]. These findings justify further investigations into the therapeutic potential of miRNAs involved in HBG gene regulation during adult erythropoiesis.

3 Targeting of HBG Transcriptional Regulators by miRNAs, Activation of HBG Expression, and Induction of Fetal Hemoglobin

3.1 EKLF/KLF1

The first major transcription factor identified to play a key role in adult HBB gene activation during hemoglobin switching was Erythroid Krüppel-like factor (EKLF/KLF1) [49]. Subsequent studies provided further evidence that KLF1 is a major player in controlling HBG-to-HBB gene switching by activating BCL11A [21], which indirectly represses HBG transcription [50–53]. More recently, Li et al found that miR-326 suppressed KLF1 expression directly by targeting its 3′UTR and activated HBG expression in K562 cells and CD34+ hematopoietic progenitor cells (Fig. 1, Table 1) [54]. By overexpressing miR-326 in K562 cells, KLF1 protein reduced and HBG expression increased approximately 50%. Moreover, miR-326 expression was positively correlated with HbF levels in β-thalassemia patients, but it is unknown whether miR-326 induces HbF in CD34+ hematopoietic progenitor cells. Obeidi et al. showed that miR-451 upregulation correlated with the induction of several transcription factors, including KLF1, and promoted erythroid differentiation in murine embryonic stem cells [55]. It is unknown whether miR-451 upregulation silences HbF expression during erythroid differentiation in murine embryonic stem cells. A study by Pule et al. found that ex vivo HU treatment of differentiated primary erythroid cells from seven unrelated individuals and of K562 cells significantly downregulated KLF1, which corresponded nicely to decreased expression of BCL11A and increased HBG gene expression [56]. Several miRNAs, specifically miR-15a, miR-16, miR-26b and miR-151-3p, were induced following HU treatment in both cell models [56]. Published studies by our group found that miR-34a promoted cell differentiation by increasing expression of KLF1, glycoporphin A, and the erythropoietin receptor while silencing STAT3 [57], a known repressor of HBG in K562 cells (Table 1) [58]. Additional investigations to understand the role of miRNAs in regulating HBG expression and inducing HbF through targeting KLF1 in vivo are needed.

3.2 BCL11A

The BCL11A gene encoding a C2H2-type zinc finger protein is a major transcriptional repressor of HBG expression during adult erythropoiesis [19, 59]. Inherited genetic variants in BCL11A associated with HbF levels in hemoglobinopathy patients [60] provided the catalyst for laboratory
studies confirming BCL11A as a major silencer of HBG gene expression. Table 1 highlights those miRNAs, including miR-451, which target BCL11A and influence HBG gene expression [25, 61, 62].

Other miRNAs besides miR-144 influence HbF levels via targeting BCL11A in vitro. For example, Gholampour et al. observed miR-30a expression correlated with higher HbF levels in erythroid precursors isolated from β-thalassemia intermedia patients [63]. Subsequent studies confirmed the role of miR-30a in silencing BCL11A in erythroid precursors and inducing HBG expression [63]. A similar study was conducted in the Democratic Republic of Congo in which 22 of 798 miRNAs were differentially expressed in peripheral blood of adult SCD patients receiving HU [64]. Interestingly, in that study several miRNAs, including miR-148b-3p, miR-32-5p, miR-340-5p, and miR-29c-3p were associated with silencing BCL11A (Fig. 1) [64]. Functional studies in K562 cells showed that healthy-donor exosomes and miR-138-5p regulated HBG expression by targeting BCL11A [43]. Lee et al. observed that suppression of let-7, which targets LIN28B, also reduced BCL11A expression and significantly induced HbF in adult human erythroblasts [65]. MiR-486-3p was also found to silence BCL11A and activate HBG in erythroid cells [66]. Future studies are needed to determine whether these miRNAs that target BCL11A will produce similar effects in preclinical sickle cell mouse models.

One of the earliest studies to implicate miRNAs in HbF induction by HU was published by Lopes et al. [67]. The authors observed a significant decrease in HIF1A expression, a marker of angiogenesis, and induction of miR-221, in human umbilical vein endothelial cell cultures following HU treatment in vitro [67]. Specifically, miR-210 and miR-486-3p were associated with increased HBG expression in
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HU responders [68]. A separate study by Sawant et al. further supported a role for miR-210 in erythroid differentiation and HbF induction in SCD patients receiving HU therapy [69].

### 3.3 c-MYB (MYB)

MYB is a well-characterized proto-oncogene [70] that plays an indirect role in silencing HBG transcription through targeting BCL11A and the repressor TR2/TR4 [52, 71]. MYB expression is high in immature hematopoietic cells but is downregulated during erythropoiesis [72]. Supporting studies showed that overexpression of MYB inhibited HBG expression in K562 cells [70], while silencing MYB induced HbF expression in primary human erythroid progenitors [73]. In human genetic studies, the association of MYB with HbF levels was demonstrated using quantitative trait loci studies and subsequent functional assays as previously described [10, 70, 73]. Later studies would provide evidence that MYB is a direct target of the tumor suppressor miR-15a/16 cluster (Table 1) [74]. Supporting data by Sankaran et al. showed that the higher expression of HbF observed in trisomy 13 subjects was due to increased expression of miR-15a and miR-16-1 [73]. In that study, miR-15a decreased MYB protein expression by inhibiting mRNA translation [73]. In contrast, Kouhkan et al. did not observe an effect of miR-16 on HBG expression in CD133+ stem cells [75]. Furthermore, miR-26b was shown to directly interact with the 3'UTR of MYB and induce HbF in both erythroid progenitors and K562 cells [56]. Our published findings also provide evidence that overexpression of miR-29b significantly decreased MYB protein expression by inhibiting mRNA translation [73].

![Adis]

Table 1 MiRNAs associated with the activation or suppression of HBG mRNA or HbF levels

| miRNA   | Phenotype              | Target mRNA | References |
|---------|------------------------|-------------|------------|
| miR-451 | Induce HbF levels      | KLF1        | [55]       |
| miR-15a/16-1 | Increase HBG expression | KLF1, MYB  | [56, 73, 74] |
| miR-26b | Increase HBG expression | KLF1, MYB  | [56, 94]   |
| miR-151-3p | Induce HbF levels     | KLF1        | [56, 94]   |
| miR-326  | Increase HBG expression | KLF1        | [54]       |
| miR-34a  | Increase HBG expression | STAT3       | [57, 58]   |
| miR-30a  | Increase HBG expression | BCL11A      | [63]       |
| miR-148b-3p | Increase HBG expression | BCL11A      | [64]       |
| miR-32-5p | Increase HBG expression | BCL11A      | [64]       |
| miR-340-5p | Increase HBG expression | BCL11A      | [64]       |
| miR-29c-3p | Increase HBG expression | BCL11A      | [64]       |
| miR-138-5p | Increase HBG expression | BCL11A      | [43]       |
| Let-7    | Induce HbF levels      | BCL11A      | [65]       |
| miR-486-3p | Increase HBG expression | BCL11A      | [66]       |
| miR-210  | Increase HbF expression | BCL11A      | [69]       |
| miR-105-5p | Increase HBG expression | MYB        | [64]       |
| miR-29b  | Increase HBG expression | MYB, DNMT3A/3B | [24]   |

**Activation**

**Suppression**

miR-96    | Suppress HBG expression | ORF of HBG | [76]       |
miR-223-3p | Suppress HBG expression | LMO2       | [77]       |
miR-150   | Suppress HBG expression | GATA1      | [78]       |
miR-23a/27a | Suppress HBG expression | KLF3, SP1  | [79]       |
miR-221/222 | Suppress HbF levels    | c-Kit      | [80]       |
miR-144   | Suppress HbF levels     | NRF2       | [25, 62, 81] |

KLF1 Krüppel-like factor 1, MYB MYB proto-oncogene, transcription factor, STAT3 signal transducer and activator of transcription 3, BCL11A B-cell lymphoma/leukemia 11A, DNMT3 DNA methyltransferase 3 alpha, DNMT3B DNA methyltransferase 3 beta, ORF of HBG open reading frame of hemoglobin subunit gamma 1/2, LMO2 LIM domain only 2, GATA1 GATA binding protein 1, KLF3 Krüppel-like factor 3, SP1 Specificity protein 1, c-KIT KIT proto-oncogene, receptor tyrosine kinase, NRF2 NF-E2-related factor 2, miR microRNA
peripheral blood of adult SCD patients receiving HU further showed that miR-105-5p was associated with silencing MYB [64]. Collectively, these studies highlight the ability of multiple miRNAs to fine-tune expression of MYB and influence HBG expression and HbF levels.

### 3.4 MiRNAs Involved in HBG Suppression

In contrast to miRNAs related to HbF induction, fewer have been associated with HBG gene suppression. To date, one miRNA has been shown to directly inhibit HBG mRNA, namely miR-96 [76]. Azzouzi et al. compared mRNA from reticulocytes of cord and adult blood and demonstrated miR-96 is highly expressed in adults [76]. When miR-96 was overexpressed in erythroid progenitors, HBG mRNA decreased 50% (Fig. 1). In luciferase-based studies, miR-96 directly inhibited HBG cDNA by targeting the open reading frame sequence [76]. In contrast, miR-223-3p suppressed HBG expression in β-thalassemia major exosomes by inhibiting the transcription factor LMO2 [77]. During erythropoiesis, miR-150 inhibits GATA1, which has been associated with HBG suppression in K562 cells [78]. Similarly, miR-23a and miR-27a enhance the expression of β-like globin genes by inhibiting KLF3 and SP1 binding to the β-like globin gene locus during erythroid differentiation [79]. A purified component of a natural Chinese medicine called emodin activates HBG expression in K562 cells through upregulation of c-KIT and downregulation of miR-221 and miR-222 [80].

miR-144 is one of the predominant miRNAs in peripheral blood erythrocytes that controls oxidative stress levels by suppressing NRF2 (nuclear factor erythroid 2-related factor) expression. A study by Sangokoya et al. showed that miR-144 was important in hemoglobin synthesis and SCD clinical severity [62]. Using prediction analysis of microarray to prioritize differentially expressed miRNAs, they further observed significantly higher expression levels of miR-144, miR-451, and miR-142-5p among erythrocytes isolated from adult SCD patients with severe anemia compared with normal erythrocytes [62]. In the same study, miR-144 directly targeted the 3′UTR of NRF2, a well-known transcription factor that modulates oxidative stress response, in K562 cells (Table 1) [62]. It is well documented that NRF2 directly activates HBG transcription while miR-144 targets NRF2 to silence HBG mRNA in normal CD34+ cells. Reversely, inhibiting miR-144 upregulates HBG in normal and sickle cell progenitors [25, 81]. Supporting studies from our group further showed that silencing of NRF2 by miR-144 significantly repressed HBG gene transcription twofold in normal erythroid progenitors while the miR-144 antagonist increased the percentage of F cells twofold in sickle erythroid progenitors [25].

### 3.5 HBG Regulation by DNA Methylation

DNA methylation is a critical epigenetic process that contributes to erythroid cell differentiation and hemoglobin switching mechanisms [82–84]. During developmentally regulated erythroid cell differentiation, the globin genes (5′-HBE, HBG1, HBG2, HBD, HBB-3′) in the HBB globin locus become sequentially demethylated and transcriptionally active. During adult erythropoiesis, the HBG gene promoter becomes hypermethylated and silenced by DNA methylation machinery, including DNMT3A, DNMT3B, DNMT1, and the MBD protein family [82, 85–89]. Thus, strategies to reverse HBG promoter hypermethylation and induce HbF have resulted in the development of pharmacologic DNMT inhibitors, particularly decitabine and 5-azacytidine, which reactivate HBG expression via hypomethylation of the CpG site within the HBG proximal promoter. Although decitabine and 5-azacytidine are potent HbF-inducing agents that impair de novo methylation activity, oral administration leads to rapid degradation by intestinal cytidine deaminases [90, 91] and there are concerns with possible adverse side effects [92], such as increased risk of hyperglycemia, neutropenia/leukopenia, and electrolyte imbalance. Thus, miRNAs, which are naturally found in the human body and that target genes involved in DNA methylation, resulting in an increase in HBG expression, may be an effective approach to address the clinical concerns raised by chemically induced DNA demethylation in general.

In our group, we took an alternative approach to discover miRNAs that target the DNA methylation machinery during hemoglobin switching. We initially conducted a genome-wide unbiased miRNA screen during switching in reticulocytes to identify miRNA expression patterns [25]. We collected blood samples from patients with confirmed HbSS genotype and contrasting HbF levels that ranged from 0.1% to 30.6%. SCD patients were separated into two groups based on their mean HbF levels: low HbF levels (average HbF_LOW = 3.37 ± 1.02) versus high-HbF (average HbF_HIGH = 23.48 ± 2.12). RNA was isolated from purified reticulocytes for microarray-based miRNA expression profiling. Following the miRNA screen, we identified an eightfold upregulation of miR-144 in the low-HbF group compared with those with high HbF levels [25]. We next discovered that miR-29b expression was associated with higher HbF levels [24], demonstrating the first evidence for a role of miR-29b as a DNA methyltransferase inhibitor to mediate HbF induction [24]. Subsequently, we observed that overexpression of miR-29b significantly increased the percentage of HbF-positive cells, while decreasing the expression of DNMT3A and MYB in human KU812 cells and primary erythroid progenitors (Fig. 1) [24].

These studies expand our previous findings showing that miR-29b has a complementary seed sequence with the
3'UTR of DNMT3A and DNMT3B, thereby inhibiting de novo DNMT synthesis in breast cancer cells [93]. Similar work by Walker et al. investigated the role of epigenetic mechanisms, specifically HBG promoter methylation levels and miRNA expression, on HbF induction in primary CD71+ erythrocytes of SCD patients before HU therapy and after reaching a maximum tolerated dose of HU therapy [94]. Their findings showed that expression of miR-26b and miR-151-3p known to target KLF1 were both associated with HbF levels at a maximum tolerated HU dose in SCD [94]. These findings highlight the importance for further investigation of the miR-29 family and other miRNAs in HBG regulation.

In support of these findings and the importance for targeting DNA methylation machinery, our group and others have shown that methyl CpG binding protein 2 (MeCP2) and/or other MBD proteins are involved in HBG regulation in adult erythroid progenitors [24, 95]. Considering epigenetic modifications, including acetylation and methylation, influence DNA looping of the LCR and access of transacting DNA-binding proteins at each globin promoter, it is plausible that targeting epigenetic pathways via miRNAs may enhance HBG gene transcription.

## 4 Overcoming Hurdles in miRNA Delivery

MiRNAs are attractive molecules for inhibiting repressors of HBG gene transcription and inducing HbF. In 2018, the FDA approved the first-of-its-kind targeted RNA interference (RNAi)-based therapy, ONPATTRO (patisiran) infusion, to treat peripheral nerve disease caused by hereditary transthyretin-mediated amyloidosis in adult patients [96]. Currently, 11 RNA-based therapeutics have been approved by the FDA and/or the European Medicines Agency [27]. These RNA-based therapies are delivered intravenously, intrathecally, or via subcutaneous administration to treat various diseases of the eye, muscle, or the central nervous system [27]. Recently, the FDA approved an emergency use authorization for the Moderna Vaccine (mRNA-1273) to prevent coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in individuals 12 years of age and older [97]. In addition to these RNA-based therapies, a substantial number of RNA therapeutics are in phase II or III clinical development, such as various anti-miRs and miRNA mimics [27], including a cholesterol-conjugated miR-29 mimic for skin keloid (NCT02603224, NCT03601052).

Despite the potential of RNA-based agents as therapeutic interventions, there are major concerns among the scientific community about the unwanted off-target effects mediated by miRNAs, or that straining the RNAi system may alter expression of multiple target genes [98–100]. However, miRNAs can potentially restore expression of proteins at physiological levels as previously documented [98–100], which is a major goal when considering the development of HbF-inducing therapeutics.

In addition to these concerns, critical hurdles involving delivery of miRNA-targeting agents remains a challenge for clinical translation [101]. Limitations to overcome delivery include, but are not limited to, poor in vivo stability and short half-life under physiological conditions, inappropriate biodistribution and degradation by ribonuclease enzymes, unfavorable immune response, and undesirable off-target effects, as previously described [101–103]. Thus, an appropriate carrier system and/or chemical modifications to overcome these limitations is critical to developing safe and effective miRNA-targeted therapeutics. Below, we briefly highlight promising platforms demonstrated to provide safe and targeted small-interfering RNA delivery in vivo.

## 5 Progress in the Development of miRNA Delivery Systems

### 5.1 Lipid-Based Vectors

Over the last 2 decades, the development of cationic liposome/lipid-based systems has seen a rapid expansion after initial description by Felgner et al. in 1987, who described the first successful in vitro transfection with a cationic lipid [104]. Since that time, a large number of commercially available cationic liposome/lipid-based systems, such as Lipofectamine and TransIT TKO, Oligofectamine, Lipofectin, DOTAP and RNAiFect, have been developed [105–109]. A major advantage for using liposomes is their flexible physicochemical and biophysical properties, which allows manipulation for different delivery considerations [110]. For instance, adding cholesterol modifiers and/or polymers, such as 1,2-Dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE) and polyethylene glycol (PEG), to liposomes has reduced inflammatory responses and enhanced the stability of the liposomes and cellular uptake, allowing for more effective delivery and hepatic clearance in vivo [111]. Introducing helper cationic polymers in the formulation has been further shown to increase the siRNA entrapment inside the liposome core [112]. Adding functional lipid groups, such as –COOH or –NH₂, at the distal terminal of polymers further assists with controlling pH and allows for easier linking with its target ligands [113]. Several cholesterol-conjugated small-interfering RNAs have advanced to clinical evaluation, such as RXI-109, SNALP®, and Atuplex, based on the ability to promote effective cellular uptake [114].
5.2 Nanoparticle-Based miRNA Delivery

To date, nanotechnology-based delivery systems are considered one of the most promising and exciting tools for precision medicine due to their ability to control the release of drugs and successful delivery in vivo [101, 115]. Nanoparticles are at the atomic or molecular level, with sizes ranging between 1 and 100 nm in diameter [116, 117]. Their small size is a major advantage, allowing them to move freely in the human body even when encapsulated or attached to therapeutic drugs that need to be delivered to target tissue sites [116, 117]. For instance, nanoparticle-based methods have been used to distinguish between healthy cells and cancer cells even when combined with chemotherapy and imaging modalities for cancer diagnosis [118–120]. Several small elements, such as gold, bismuth, and silver, have also been used as biomarkers for various molecular biology detection assays, and for magnetic, optical, and X-ray detection systems [121–123]. Other nanoparticle systems, such as the Rondel® platform, which uses the electrostatic force of attraction between negatively charged molecules and the positively charged linear polymer with alternate cyclodextrin molecules, has also been used to deliver small molecules [124].

5.3 Viral Vectors for miRNA Delivery

Viral-based delivery systems use retroviruses, lentiviruses, and adenoviruses or adeno-associated viruses (AVV) to deliver small molecules, including miRNAs, into somatic and germline cells [125, 126]. Viral vectors, especially AAV vectors, are more frequently utilized in delivering miRNAs due to the small size of miRNAs. This system enhances transfection efficiency and increased expression of miRNAs. A disadvantage of using viral vectors is their tendency to induce toxicity, inflammation, and immunogenicity caused by the inserted sequence [125, 127]. However, the development of the first-in-human lentiviral system targeting BCL11A (BCH-BB694 BCL11A shmiR) supported a phase I clinical trial for SCD patients (ClinicalTrials.gov identifier NCT03282656) [128, 129]. This open-label, nonrandomized, single-center, single-arm cohort study involved a single intravenous infusion of transduced CD34+ HSC cells containing a lentiviral vector encoding an shRNA targeting BCL11A mRNA embedded in an miRNA molecule that was evaluated in seven SCD patients [128]. Although adverse side effects (including pain, influenza, infection, priapism, and fever) were observed, individuals evaluated 6–24 months following infusion achieved robust and stable induction of HbF. Specifically, the median percentage of F cells among untransfused erythrocytes was 70.8%, which represented a substantial increase from baseline [128]. Of note, none of the patients evaluated had episodes of vaso-occlusive crises, stroke, or acute chest syndrome, which are common complications of SCD leading to emergency department visits and hospitalizations [130]. These findings warrant additional studies to determine the effect of vector-based targeting of other major HBG-modifying genes, such as BCL11A, MYB, KLF1, or other genes. However, there remains a considerable amount of concern with the clinical use of viral-based vectors due to their potential to increase risk of immunogenicity and insertional mutagenesis that needs to be addressed in future studies [131, 132].

6 Conclusions

For several years, miRNA-based targeted therapeutics have shown promise for the treatment of many diseases, by targeting multiple genes and pathways involved in oncogenesis and/or tumor suppression. Several genes, such as MYB, BCL11A, KLF1, and NRF2 are not only involved in oncogenesis or other biological processes but are also involved in HBG gene silencing. Supporting studies discussed in this review have shown the ability of various miRNAs to regulate HBG expression by activating or suppressing gene expression. Thus, identifying miRNAs associated with HBG activation or repression, such as MYB, BCL11A, KLF1, and NRF2, or other HBG repressor proteins, is critical for understanding the molecular mechanisms leading to HbF induction and reduced adverse effects in patients with SCD and other β-hemoglobinopathies.

Although there remains an unmet need to develop novel classes of drugs that are efficacious for HbF induction with limited adverse effects, utilizing miRNAs to target genes involved in HBG gene regulation shows promise. However, there are concerns with the implementation of miRNA-based interventions since they may alter the expression of genes involved in oncogenesis or tumor suppression. For instance, there are many miRNA signatures associated with cancer and progression but there is no consensus among multiple sera and tumor sample studies. As such, this is also a concern when considering how to ensure that multiple genes and their protein products are not altered when targeted by one or multiple miRNAs, resulting in unwarranted off-target adverse effects. Indeed, off-target and immunological effects remain an obstacle when considering the use of miRNAs as novel therapeutics. However, a major advantage for the use of miRNAs is that they can potentially restore expression of proteins, such as HbF, at physiological levels.

The challenge that remains with the utilization of miRNA molecules is the development of optimal drug delivery systems to prevent degradation in the blood. Recent developments in nanotechnology and drug delivery systems that target the tumor microenvironment may provide an alternative therapeutic approach with decreased toxicity. Thus,
to overcome this hurdle, it is necessary for investigators to include studies utilizing in vivo rodent animal models to evaluate the carcinogenic potential of miRNAs. There is a significant need to reduce the cost of chemical induction of miRNAs and miRNA-based applications for targeted delivery in SCD. Another concern is the lack of knowledge and uncertainty as to whether modulation of miRNAs, both chronically and systemically, will produce sufficient efficacy, specificity, and sustainability for HbF induction in vivo. Nonetheless, the remarkably successful clinical trial and development of BCH-BB694 gene therapy (Bluebird Bio, Cambridge, MA, USA) highlights the potential for developing miRNA as a therapeutic approach to treat SCD. However, a principal factor to consider, in addition to HbF levels, is the distribution of HbF in red blood cells (F cells) to achieve clinical efficacy. Indeed, the potential to develop miRNAs that directly activate HBG by indirect pathways, such as epigenetic mechanisms and/or silencing the BCL11A, MYB, KLF1 genes, provide new strategies to develop small molecules to increase HbF during adult development for the successful treatment of SCD. In conclusion, investigations to define the role of miRNAs in globin gene regulation and their ability to promote robust and stable HbF induction within preclinical SCD animal models to justify future clinical trials are needed.

Declarations

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Author contributions AS-D, QG, and BSP reviewed the literature, designed the review sections, and conducted a final review of the paper. AS-D designed the figures and tables and wrote several drafts of the paper.

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