

MIR29B mediates epigenetic mechanisms of HBG gene activation

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Summary

Sickle cell disease (SCD) affects over 2 million people worldwide with high morbidity and mortality in underdeveloped countries. Therapeutic interventions aimed at reactivating fetal haemoglobin (HbF) is an effective approach for improving survival and ameliorating the clinical severity of SCD. A class of agents that inhibit DNA methyltransferase (DNMT) activity show promise as HbF inducers because off-target effects are not observed at low concentrations. However, these compounds are rapidly degraded by cytidine deaminase when taken by oral administration, creating a critical barrier to clinical development for SCD. We previously demonstrated that microRNA29B (*MIR29B*) inhibits *de novo* DNMT synthesis, therefore, the goal of our study was to determine if *MIR29* mediates HbF induction. Overexpression of *MIR29B* in human KU812 cells and primary erythroid progenitors significantly increased the percentage of HbF positive cells, while decreasing the expression of DNMT3A and the *HBG* repressor MYB. Furthermore, *HBG* promoter methylation levels decreased significantly following *MIR29B* overexpression in human erythroid progenitors. We subsequently, observed higher *MIR29B* expression in SCD patients with higher HbF levels compared to those with low HbF. Our findings provide evidence for the ability of *MIR29B* to induce HbF and supports further investigation to expand treatment options for SCD.

Keywords: MIR29B, HBG, DNA methylation, fetal haemoglobin, MYB.

Sickle cell disease (SCD) is one of the most common genetic disorders affecting over 20 million people worldwide mainly in underdeveloped countries (Weatherall, 2013). Therapeutic intervention aimed at reactivating *HBG* (γ -globin) gene transcription and fetal haemoglobin (HbF) expression is an effective strategy for ameliorating the clinical symptoms of SCD and improving long-term survival (Platt *et al*, 1994; Poillon *et al*, 1993). Mechanistically, HbF blocks HbS polymerization and erythrocyte sickling through the formation of hybrid haemoglobin molecules in red blood cells. Based on these findings, hydroxycarbamide (HC) was developed as the only US Food and Drug Administration (FDA)-approved therapeutic intervention for HbF induction, however it causes bone marrow toxicity if not accurately dosed (Charache *et al*, 1995). Therefore, alternative approaches to induce HbF through epigenetic mechanisms of *HBG* β gene silencing targeting DNA hypermethylation of proximal promoter CpG rich regions was investigated in this study.

Therapeutic interventions to decrease *HBG* gene methylation and reactivate transcription have proven beneficial in clinical studies. For example, SCD patients treated intravenously with the DNA methyltransferase (DNMT) inhibitor decitabine (Dec) demonstrated robust induction of HbF and total haemoglobin levels (Molokie *et al*, 2017; Akpan *et al*, 2010; Lavelle *et al*, 2010; Desimone *et al*, 2002). However, when administered by mouth, Dec is rapidly inactivated by cytidine deaminase. In a recent Phase I clinical trial, where Dec was combined with tetrahydropyridine to inhibit its metabolism, DNMT1 protein levels and DNA methylation were decreased in peripheral blood mononuclear cells, while HbF levels increased in SCD patients (Molokie *et al*, 2017). Ideally, treatments for SCD will activate *HBG* expression without bone marrow toxicity and avoiding off target effects.

An attractive class of molecules under development for therapeutic intervention are microRNA (miRNA) mimics and antagonists. Due to their capacity to restore control of

aberrantly expressed genes, causing a wide array of human diseases, the development of miRNA therapeutics is highly investigated (Bianchi *et al*, 2009; Walker *et al*, 2011; Ward *et al*, 2016; Lulli *et al*, 2013; Starlard-Davenport *et al*, 2013). In previously published work, we demonstrated that *MIR29B*, inhibits *de novo* synthesis of the DNMT enzymes DNMT3A and DNMT3B, in breast cancer cells and restores control of aberrantly expressed tumour suppressor genes involved in cell cycle control, including *TP73*, *CDH1*, *RASSF1*, *CCNA1*, and *CDKN1C* (Starlard-Davenport *et al*, 2013). Recently, we identified *MIR144* as a repressor of *NFE2L2* and HbF expression in patients with SCD (Li *et al*, 2018) in a genome-wide microarray-based miRNA screen of RNA isolated from reticulocytes. Herein, we demonstrated that *MIR29B* mediates *HBG* activation and induces HbF in KU812 cells and human primary erythroid progenitors. To compliment *in vitro* studies, we observed higher *MIR29B* levels in the reticulocytes of SCD patients with high HbF compared to those with low HbF levels suggesting a functional role in *HBG* gene regulation.

Materials and methods

Subject recruitment and blood processing

Blood samples were obtained from individuals ($n = 12$) with homozygous sickle cell anaemia (HbSS) not on HC therapy followed in the Sickle Cell Program at Augusta University. Medical record review was completed to obtain complete blood counts with differential, reticulocyte count and HbF levels determined by high performance liquid chromatography. Blood samples were processed by ficoll histopaque separation of plasma, red blood cells, and buffy coat. Subsequently, the red blood cells were processed using the magnetic-activated cell sorting (MACS) column and *TFRC*⁺ MicroBeads (MACS, Miltenyl Biotec, Auburn, CA, USA) to isolate reticulocytes for total RNA extraction using TRIzol (ThermoFisher, Waltham, MA, USA). All research involving persons with SCD was approved by the Augusta University Institutional Review Board and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

MicroRNA microarray analysis

Reticulocytes isolated from the peripheral blood were used for miRNA analysis on the miRCURY LNA microRNA Array (Exiqon, Woburn, MA, USA). Raw data were quantile normalized using a model-based background correction algorithm as recently published by our group (Li *et al*, 2018). The miRNA genes differentially expressed between the high and low HbF groups were identified (Li *et al*, 2018) and raw data deposited in the National Center for Biotechnology Information Gene Expression Omnibus database, accession number GSE111356.

Erythroid differentiation of human CD34⁺ stem cells

Human primary erythroid cells were generated from adult CD34⁺ stem cells (STEMCELL Technologies, Vancouver, BC, USA) in a two-phase culture system as previously published (Li *et al*, 2014). During phase I, stem cells were grown in minimum essential medium- α (α MEM) containing AB serum, interleukin-3 (10 ng/ml), stem cell factor (10 ng/ml), and erythropoietin (2 iu/ml). On day 7, cells transitioned to Phase II media where they remained under erythropoietin (2 iu/ml) stimulation. Erythroid progenitors were transfected on day 8 with human mature *MIR29B* or control scramble (Scr) mimic (Applied Biosystems, Foster City, CA, USA) by nucleofection using the Amaxa[®] Human CD34⁺ Cell Nucleofector[®] Kit. For drug studies, cells were treated with 0.5 μ mol/l Dec alone or pretreatment with 100 nmol/l *MIR29B* on day 8 followed by drug treatment. After 48 h, cells were harvested for reverse transcription-quantitative PCR (RT-qPCR), Western blot, and flow cytometry analysis. Giemsa staining was used to monitor cell morphology and cell counts; viability was monitored using 0.4% trypan blue exclusion assay (Gibco, Carlsbad CA, USA).

KU812 cell culture and *MIR29B* transfections

Human KU812 cells were grown in Iscove's Modified Dulbecco's medium supplemented with 10% fetal bovine serum, penicillin (100 u/ml), and streptomycin (0.1 mg/ml) in 5% CO₂ at 37°C. Cell counts and viability were determined using 0.4% trypan blue exclusion assay. Cells were seeded at a density of 0.5×10^6 viable cells per 100 mm plate for different treatments. During log phase growth, cells were transfected with 25, 50, and 100 nmol/l of *pre-MIR29B* (Applied Biosystems) for 48 h in three independent replicates using Opti-MEM media (Gibco) and Lipofectamine[™] 2000 transfection reagent (Invitrogen Carlsbad, CA, USA) according to the manufacturer's instructions. The cells transfected with Scr oligonucleotide served as control. For drug inductions KU812 cells were treated with Dec (0.5 μ mol/l) or HC (75 μ mol/l) alone (Lou *et al*, 2009) or pretreated with 100 nmol/l *MIR29B* followed by drug treatments for 48 h, then harvested for subsequent analyses.

RT-qPCR analysis

Total RNA was extracted from KU812 cells using an AllPrep DNA/RNA/Protein Midi Kit (Qiagen, Valencia, CA, USA); TRIzol reagent was used to extract RNA from primary erythroid progenitors. Subsequently, cDNA was generated using the high capacity reverse transcription kit (Applied Biosystems) and qPCR was performed in a QuantStudio 3 Real-Time System using SYBR Green[™] Master Mix (Applied Biosystems). The level of *DNMT1*, *DNMT3A*, *DNMT3B*, *MECP2*, *HBG* and *HBB* gene transcripts were determined using gene specific primers (Table SI). To quantify *GYP A* (glycophorin A) and *TFRC* (transferrin receptor) gene

expression, we used the RT2-qPCR Primer system (Qiagen, Germantown, MD, USA). Relative quantification of gene expression was normalized to *GAPDH* as an internal control. Quantification of *MIR29B* was performed using the TaqMan miRNA assay (Applied Biosystems) according to the manufacturer's instructions and *SNORD48* was used as endogenous control. The $2^{-\Delta\Delta C_t}$ method was used for calculating the relative amount of target mRNA. All RT-qPCR reactions were performed in triplicate, repeated at least 3 times, and always included a no-template sample as a negative control. RT-qPCR results are presented as average fold change of target gene in cells relative to Scr control, which was normalized to one.

Western blot analysis

Total protein was isolated using the AllPrep DNA/RNA/Protein Midi Kit (Qiagen) according to manufacturer's instructions. For Western blot analysis, 20–40 µg of total or nuclear protein was loaded on a 12% acrylamide gel, transferred to a polyvinylidene difluoride membrane, and then blocked in 5% non-fat milk. Primary antibodies against DNMT3A (sc-373905), DNMT3B (sc-376043), MYB (05175 MI), HbF (sc-21756), and HbA (sc-21757) purchased from Santa Cruz Biotechnology (Dallas, TX, USA) were diluted in the range of 1:250 to 1:2000, incubated overnight and then followed by treatment with secondary antibody. The immunoblots were developed using SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) and analysed on a LAS-3000 gel imager (Fujifilm Medical Systems, Stamford, CT, USA). Blots were processed with Restore™ Plus Western blot stripping buffer and probed with β-actin (also termed ACTB, sc-53646) antibody as the internal control. The band intensity of different proteins was quantified by densitometry with MultiGauge Software (Fujifilm Medical Systems) and normalized to the band intensity of β-actin.

Flow cytometry analysis

To measure HbF positive cells (F-cells), the various experimental samples were harvested, washed in phosphate-buffered saline and then fixed with 4% paraformaldehyde and permeabilized with an ice-cold acetone/methanol (4:1) mixture before staining with fluorescein isothiocyanate (FITC) conjugated anti-HbF antibody (ab19365, Abcam) to quantify F-cells. Isotype control IgG antibody (MBS524511, MyBioSource, San Diego, CA, USA) was used to detect non-specific staining. In addition, the false positive attributed to Fc non-specific binding was subtracted by incubating the unfixed cells with Human TruStain FcX™ (422301, Biolegend, San Diego, CA, USA) prior to antibodies staining. Flow cytometry was conducted on a BD FACSCanto II or LSRII (BD Biosciences, San Jose, CA, USA) machine and percentage of F-cells determined by FlowJo Version 10.0.7 software (Tree Star, OR) as previously reported (Promsote *et al*, 2014; Zhu

et al, 2017). Briefly, a consistent flow of single events and live cells were sequentially gated using a Time gate, forward scatter (FSC)-H/FSC-A plot, FSC/sideways scatter (SSC)-plot and Live/Dead gates. The resulting population was classified into HbF negative and HbF positive populations.

Epimark site-specific quantitative analysis

Levels of 5-methylcytosine and unmodified cytosine at the –54 CCGG site 5' to the *HBG* gene cap site were quantitated by Epimark assays as previously described (Ruiz *et al*, 2015). DNA was purified from cell pellets using QIAGEN AllPrep DNA/RNA/Protein Minikits (Qiagen catalogue number 80234). DNA was analysed by qPCR using primers that target the *HBG* promoter and spanned the –54 CCGG site as published (Ruiz *et al*, 2015). The percentage of 5-methylcytosine and unmodified cytosine was determined using the comparative Ct method according to the manufacturer's instructions (EpiMark 5-hmC and 5-mC Analysis Kit).

Statistical analysis

The data are reported as the mean ± standard error of the mean for three to six replicates of independent experiments, each performed in triplicate. All data were analysed by a two-tailed Student's *t*-test and *P* < 0.05 was considered statistically significant. For the miRNA microarray analysis, two study groups consisting of SCD patients with HbF < 8.6% (low HbF; *n* = 6) and HbF > 8.6% (high HbF; *n* = 6) were analysed as previously reported (Li *et al*, 2018).

Results

Using *in silico* analysis, we discovered that *MIR29B* has a consensus sequence complementary to the 3'-untranslated region of *DNMT3A* and *DNMT3B* (Starlard-Davenport *et al*, 2013). Since *HBG* is silenced via DNA hypermethylation in adult erythroid cells (Lavelle *et al*, 2006; Ruiz *et al*, 2015) and *MIR29B* silences MYB (Martinez *et al*, 2011), a known *HBG* repressor protein, we determined whether *MIR29B* alters DNMT and MYB expression as a mechanism of HbF induction. Using KU812 cells, which express the *HBG* and *HBB* genes, we confirmed efficient transfection of *MIR29B* mimic into KU812 cells after 48 h by RT-qPCR analysis. In fact, KU812 cells treated with 25 nmol/l *MIR29B* showed a 200-fold (*P* < 0.05) increase in expression levels compared to Scr control (Fig 1A). Furthermore, a dose-dependent increase in *MIR29B* expression up to the 100 nmol/l concentration was observed. We next quantified *DNMT3A*, *DNMT3B*, and *MYB* mRNA and protein levels using RT-qPCR and Western blot, respectively. Transfection of KU812 cells with 100 nmol/l *MIR29B* significantly decreased *DNMT3A* and *DNMT3B* mRNA by ~50% and ~25% (*P* < 0.05) respectively (Fig 1B, C). Transfection of KU812 cells with 100 nmol/l *MIR29B* also significantly

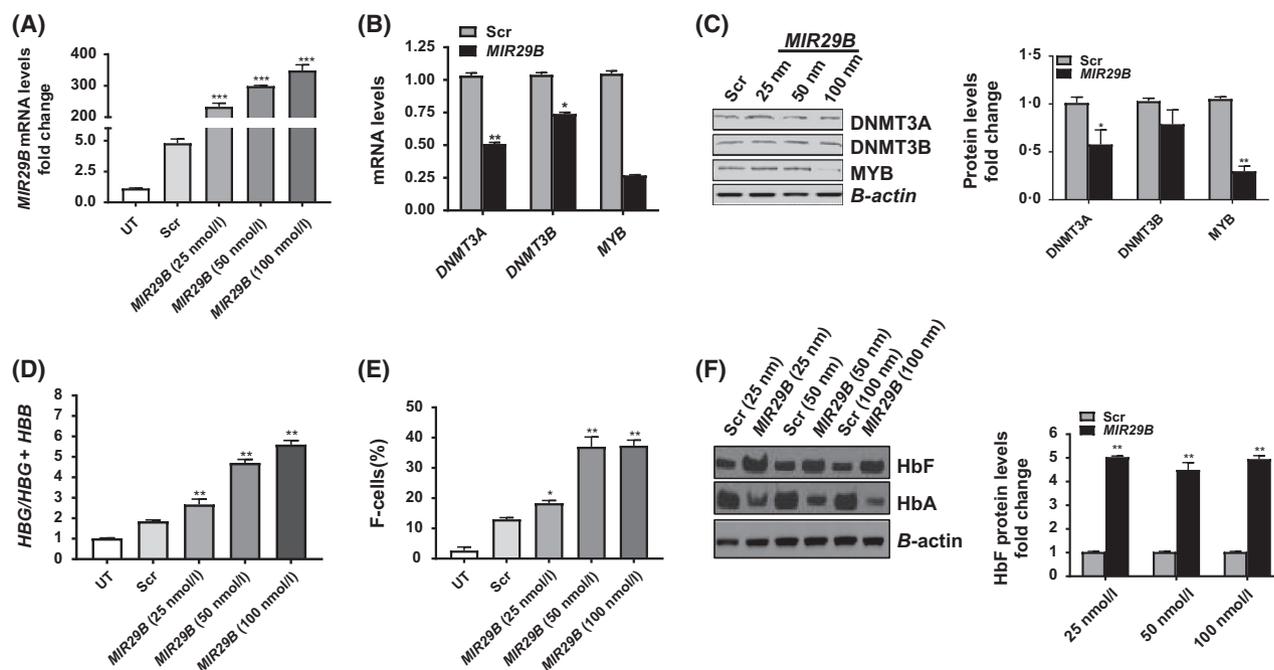


Fig 1. *MIR29B* regulates *HBG* gene expression in KU812 cells. (A) Fold change of *MIR29B* expression in KU812 cells following transfection for 48 h with miR-29b mimic or Scr control by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The expression of *MIR29B* level in Scr control was set to one after normalization to endogenous *SNORD48* levels. For all studies, the quantitative data are shown as the mean \pm standard error of the mean (SEM) of 3 to 5 independent experiments. * $P < 0.05$, is considered statistically significant; *** $P < 0.001$. (B) Fold change of *DNMT3A*, *DNMT3B*, and *MECP2* expression by RT-qPCR analysis after *MIR29B* 100 nmol/l (black bars) or Scr (grey bars) mimic transfection into KU812 cells. (C) Western blot for the proteins shown was completed in KU812 cells transfected with *MIR29B* (black bars) and Scr control (grey bars); β -actin was used as the internal control; *** $P < 0.01$. (D) The mRNA levels of *HBG* and *HBB* in KU812 cells was quantified by RT-qPCR and calculated as the ratio: *HBG*/*HBG* + *HBB* for the different conditions. (E) Flow cytometry analysis was completed to determine the % HbF positive cells (F-cells) after staining with fluorescein isothiocyanate labelled anti-HbF antibody. Shown in the graph is the quantitative data generated by FlowJo analysis. (F) Western blot of HbF and haemoglobin A (HbA) relative to β -actin protein levels in KU812 cells transfected with *MIR29B* mimic or Scr control at the concentrations shown. Scr, scramble; UT, untreated.

decreased DNMT3A protein expression; by contrast, the effect of *MIR29B* on DNMT3B levels was not significant. We quantified expression of *MECP2* (methyl CpG binding protein 2) which preferentially binds to DNA containing symmetrically methylated CpG dinucleotides and observed a 90% decrease in *MECP2* mRNA levels mediated by *MIR29B* mimic (Fig 1B). We subsequently determined the effects of *MIR29B* on its gene target the oncogene *MYB*, a known *HBG* gene repressor. *MYB* protein was decreased by 50% in *MIR29B* transfected cells compared to Scr control ($P < 0.01$) (Fig 1C). Thus, based on these findings we postulated that *MIR29B* activates *HBG* transcription, partly through DNMT and *MYB* silencing.

To ascertain whether *MIR29B* mediates *HBG* activation, we performed RT-qPCR analysis in KU812 cells. After 48 h of treatment, we observed a 2.3-fold ($P < 0.01$) dose-dependent increase in *HBG* mRNA mediated by *MIR29B* compared to Scr control (Fig 1D). By flow cytometry, we demonstrated the ability of *MIR29B* to increase F-cells from 15% (Scr control) to 40% (Fig 1E). To further support *HBG* activation, we observed a 2-fold increase in HbF protein levels in *MIR29B* transfected KU812 cells by Western blot, while adult

haemoglobin A (HbA) decreased by 50% ($P < 0.01$) (Fig 1F).

While these data in KU812 cells were supportive of the ability of *MIR29B* to regulate *HBG*, we next performed studies under physiological conditions, using primary erythroid progenitors generated from normal CD34⁺ stem cells. As previously reported by our group erythroid progenitors were generated in a two-phase culture system (Li *et al*, 2018). Transfection of erythroid progenitors with 50 and 100 nmol/l *MIR29B* produced a 7- to 8-fold increase in *MIR29B* expression (Fig 2A), while combining 100 nmol/l *MIR29B* with 0.5 μ mol/l Dec did not increase levels further. Under the same treatment conditions, we observed a significant decrease in *DNMT3A*, *DNMT3B* and *MECP2* mRNA levels (Fig 2B). Parallel these effects, *MIR29B* decreased DNMT3A, DNMT3B, and *MYB* protein levels by 50% in human primary erythroid progenitors (Fig 2C).

In our next set of experiments, we sought to determine whether combined treatment with *MIR29B* (silencing DNMT gene family expression) and Dec (incorporation into DNA to inhibit the action of DNMT), would produce an additive effect on *HBG* activation. Therefore, erythroid progenitors

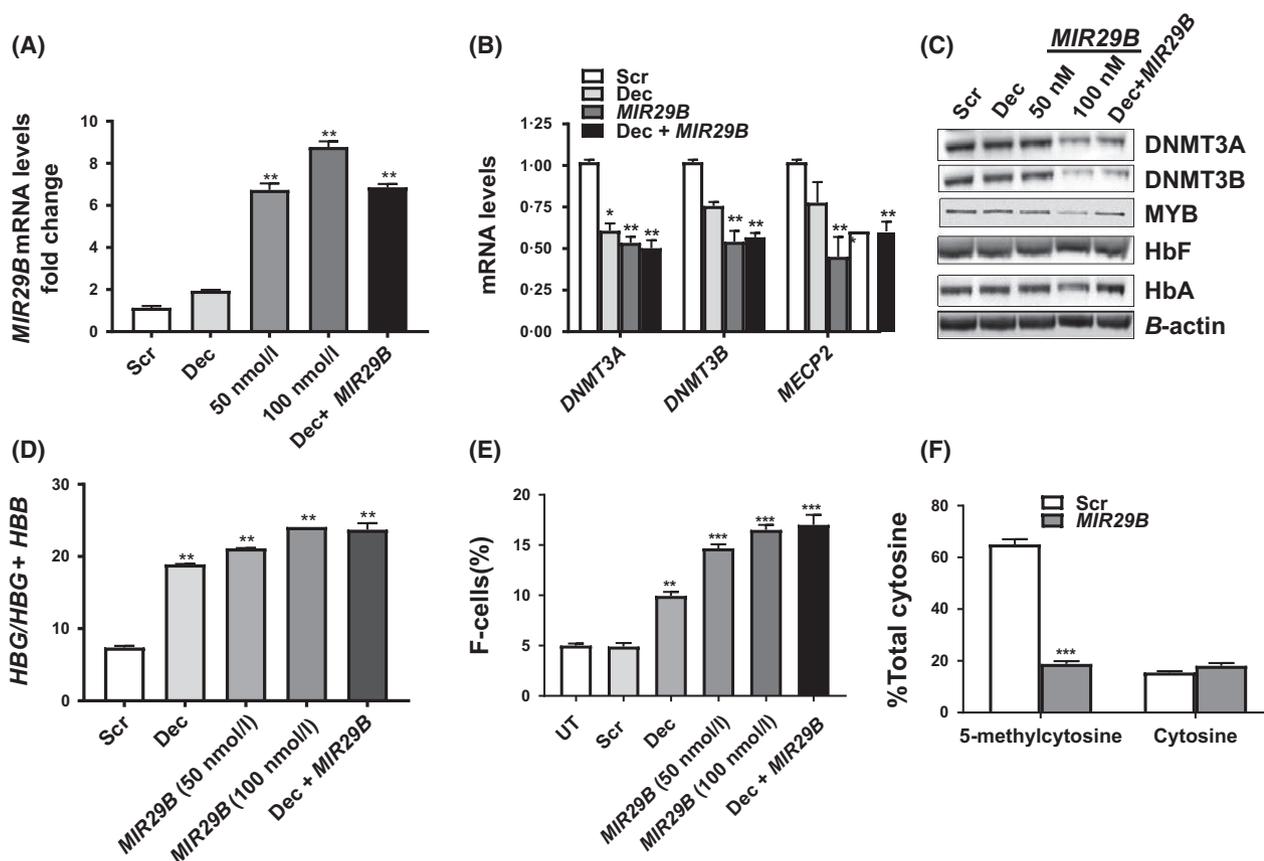


Fig 2. *MIR29B* regulates *HBG* gene expression through proximal promoter 5-hydroxymethylation in human primary erythroid progenitors. (A) Shown is the fold change of *MIR29B* expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in erythroid progenitors generated from CD34⁺ stem cells (see Material and Methods), treated with Scr (100 nmol/l), *MIR29B* (50 or 100 nmol/l) or 0.5 μ mol/l Dec alone or combined treatment with 0.5 μ mol/l Dec and *MIR29B* 100 nmol/l. The expression of *MIR29B* in Scr control was set to one after normalization to the *SNORD48* endogenous control. The data are shown as the mean \pm SEM. (B) Shown is the fold change of *DNMT3A*, *DNMT3B*, and *MECP2* mRNA levels in erythroid progenitors under the various conditions normalized to the internal control β -actin by RT-qPCR analysis. (C) Western blot analysis of the various proteins shown in erythroid progenitors for the different treatment conditions. (D) The mRNA levels of *HBG* and *HBB* in erythroid progenitors was calculated as the ratio: *HBG*/*HBG* + *HBB* for the different conditions by RT-qPCR. (E) Flow cytometry analysis was completed to determine F-cell levels in erythroid progenitors stained with fluorescein isothiocyanate-labelled anti-HbF antibody under the different treatment conditions. (F) Levels of 5-methylcytosine and unmodified cytosine (cytosine) at the *HBG* promoter -54 CCGG site for Scr control and *MIR29B* transfected erythroid progenitor cells. Scr, scramble; UT, untreated. * P < 0.05, ** P < 0.01, *** P < 0.005

were treated with 100 nmol/l *MIR29B* alone or combined with Dec (0.5 μ mol/l) as shown in Fig 2D, E. Similar to findings in KU812 cells, the *HBG*/*HBG* + *HBB* mRNA ratio was significantly increased 2.5-fold (P < 0.01) in erythroid progenitors treated with *MIR29B*; however, combination treatment with Dec did not increase *HBG* further (Fig 2D). Similarly, F-cell levels changed from 5.6% (Scr) to 9% and 16% with Dec and *MIR29B* (100 nmol/l) alone, respectively (Fig 2E, Figure S1). When both agents were combined, F-cells remained at levels observed for single agent treatments.

Lastly, we determined the effects of *MIR29B* on *HBG* promoter methylation levels in primary erythroid progenitors using the Epimark assay as previously described (Walker *et al*, 2011). Levels of 5-methylcytosine and unmodified cytosine at the CCGG MspI restriction site located at nucleotide -54 relative to the *HBG* gene cap site were determined. We

observed an 85% decrease in 5-methylcytosine levels in erythroid progenitors transfected with *MIR29B* alone compared to Scr control, whereas levels of unmodified cytosine remained unchanged (Fig 2F). These findings support the ability of *MIR29B* to mediate demethylation of the *HBG* promoter and HbF induction in normal human erythroid progenitors.

Given that Dec and *MIR29B* combined did not produce additive HbF induction, we performed studies with HC, which activated *HBG* expression by different mechanisms involving cytotoxicity and rapid erythroid regeneration, (Dover *et al*, 1986; Rodgers, 1992; Maier-Redelsperger *et al*, 1998) and activation of cyclic guanosine monophosphate signalling (Ikuta *et al*, 2011; Lou *et al*, 2009). Primary erythroid progenitors were treated with 75 μ mol/l HC or *MIR29B* (50 and 100 nmol/l) alone or in combination (Fig 3A, B).

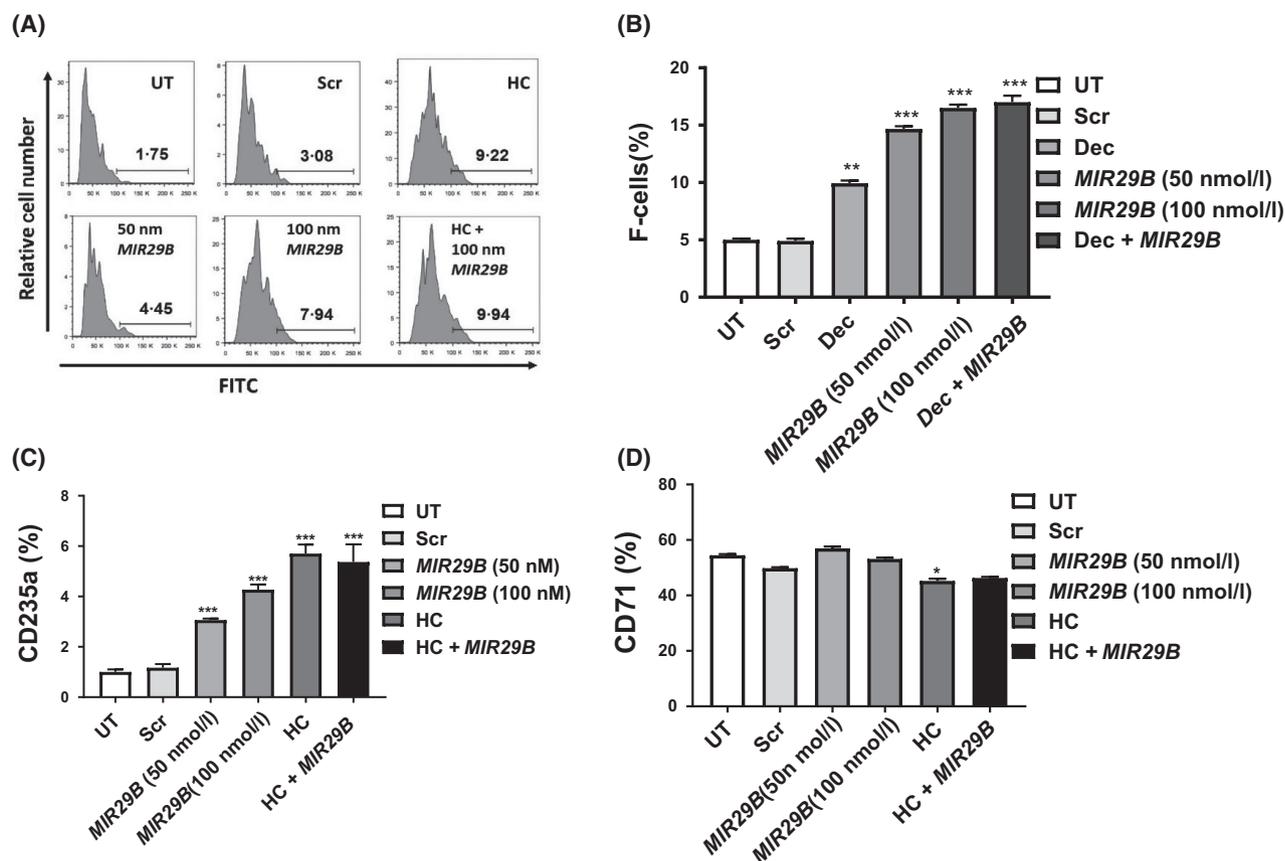


Fig 3. *MIR29B* increases expression of the late erythroid marker *GYP A* during erythropoiesis. (A) Shown is representative histograms generated by flow cytometry analysis of erythroid progenitors under untreated (UT), scrambled (Scr), hydroxycarbamide (HC) or *MIR29B* treatment conditions. (B) F-cell levels were determined by flow cytometry. Shown in the bar graph is the quantitative data generated by FlowJO analysis. (C and D) Shown in the graphs are data obtained by reverse transcription-quantitative polymerase chain reaction analysis of RNA isolated from erythroid progenitors under the various treatment conditions using gene-specific primers for erythroid differentiation markers *GYP A* (glycophorin A) and *TFRC* (transferrin receptor). FITC, fluorescein isothiocyanate; HC, hydroxycarbamide; Scr, scramble; UT, untreated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$

Treatment with *MIR29B* 100 nmol/l and HC alone increased F-cells to 8% and 9% respectively, compared to 2% for Scr control (Fig 3A, B). For combined studies with *MIR29B* (100 nmol/l), F-cell levels remained at 9%, which was similar to single agent treatments.

To determine if *MIR29B* has an effect on differentiation, we performed Giemsa stain for cell morphology to quantify erythroid cells at different stages of maturation by light microscopy. Both *MIR29B* (100 nmol/l) and HC (75 μ mol/l) produced an increase in early stage mononuclear cells by 1.9 and 2.5-fold; respectively, with a concomitant decrease in polychromatophilic and orthochromatophilic cells. Of note, *MIR29B* and HC also increased late stage reticulocytes and red blood cells by 4.3 and 5.3-fold respectively (Table I); combined treatment produced similar changes in erythroid maturation kinetics. To complement cell morphology, we also measured expression of the erythroid differentiation markers *GYP A* and *TFRC* (Fig 3C, D). The expression of *GYP A* significantly increased in erythroid progenitors overexpressing *MIR29B* or treated with HC alone and combination

treatment (Fig 3C); by contrast, only HC produced a 19% decrease ($P < 0.05$) in *TFRC* expression (Fig 3D). These data suggest that *MIR29B* expanded early erythroid progenitors and accelerated differentiation similar to HC.

While *in vitro* studies provide evidence for the ability of *MIR29B* to induce HbF, to gain evidence for its physiological relevance in SCD, we performed genome-wide miRNA expression profiling as recently reported (Li *et al*, 2018). After obtaining informed consent, blood samples were collected from individuals with SCD ($n = 12$) not on HC therapy, ranging from 4 to 20 years old. Male and female participants were equally distributed in the high HbF and low HbF groups. The clinical phenotype data collected included HbF levels, complete blood counts with differential and reticulocyte counts (Table II). There was no significant difference in haematological parameters between the two groups. The individuals with low HbF (average HbF: 3.4%) and high HbF (average HbF: 23.4%) used for miRNA microarray analysis which generated 180 differentially expressed miRNA genes (Li *et al*, 2018), are shown in

Table I. Summary of cell morphology from Giemsa stain.

	Mononuclear cells (%)	Basophilic erythroblast (%)	Polychromatophilic erythroblast (%)	Orthochromatophilic erythroblast (%)	Reticulocytes/red blood cells (%)
UT	8	32	11	47	3
Scr 50 nmol/l	9	41	13	28	5
MIR29B 50 nmol/l	7	44	4	33	2
Scr 100 nmol/l	15	42	6	33	3
MIR29B 100 nmol/l	16	52	2	16	13
HC 75 µmol/l	20	43	6	16	16
MIR29B 100 nmol/l HC 75 µmol/l	19	45	5	18	13

HC, hydroxycarbamide; Scr, scramble; UT, untreated.

Table II. Summary of clinical phenotype data for SCD patients used in miRNA microarray analysis.

Subject group	Hb (g/l)	Hct (%)	Platelets ($\times 10^9/l$)	WBC ($\times 10^9/l$)	Neut ($\times 10^9/l$)	Lymph ($\times 10^9/l$)	Retic count (%)
High HbF ($n = 6$)	86.3 \pm 4.9	26.11 \pm 1.42	404.67 \pm 60.17	11.7 \pm 2.00	5.2 \pm 0.72	4.917 \pm 1.25	8.467 \pm 1.15
Low HbF ($n = 6$)	78.0 \pm 13.0	24.13 \pm 1.56	521 \pm 54.35	12.95 \pm 0.94	6.35 \pm 0.73	4.750 \pm 0.59	9.783 \pm 1.42
<i>P</i> -values	0.1766	0.3698	0.1802	0.5943	0.2876	0.9067	0.4353

Hb, haemoglobin; Hct, haematocrit; Lymph, lymphocytes; Neut, neutrophils; Retic, reticulocytes; WBC, white blood cells.

$P < 0.05$ is considered significant.

Fig 4A. Of those identified, *MIR29B* was the only gene with known function as a DNMT inhibitor that was significantly up-regulated among SCD patients with high HbF compared to those with low HbF levels (Fig 4B). We extracted miRNA gene expression raw data for two *MIR29* isoforms; *MIR29B* levels were 2.5-fold higher ($P = 0.015$) among SCD patients with high HbF levels compared to the low HbF group (Fig 4B). However, as shown in Fig 4C, there was no significant difference in expression of miR-29c between the two groups ($P = 0.796$).

Discussion

Several research efforts have focused on identifying miRNA genes that regulate *HBG* expression during haemoglobin switching (Gasparello *et al*, 2017; Hojjati *et al*, 2017; Lai *et al*, 2017; Sun *et al*, 2017; Obeidi *et al*, 2016; Saki *et al*, 2016; Tayebi *et al*, 2017; Guda *et al*, 2015; De Vasconcellos *et al*, 2014; Bianchi *et al*, 2009). Bianchi *et al* (2009) identified the upregulation of *MIR210* by microarray analysis of erythroid precursors from a thalassaemia patient with high HbF levels; they also demonstrated that mithramycin targeted *MIR210* as a mechanism of *HBG* activation in K562 cells (Bianchi *et al*, 2009). In a subset of patients with SCD treated with HC, expression of *MIR26B* and *MIR151-3P* was associated with HbF levels at a maximum tolerated dose (Walker *et al*, 2011). Previous studies by our group demonstrated that *MIR34A* mediated HbF induction in K562 cells by repression of STAT3 expression, a known repressor of *HBG* (Ward *et al*, 2016). Previously, Lee *et al* (2013)

confirmed overexpression of LIN28B decreased *MIRLET7* miRNA family expression and increased HbF levels in primary erythroid cells. As further evidence of the therapeutic potential of miRNA, miR-486-3p induces HbF in adult erythroid progenitors by inhibiting *BCL11A* expression (Lulli *et al*, 2013). In this study, we demonstrated that *MIR29B* functions as an HbF inducer in human KU812 cells and normal human erythroid progenitors. To our knowledge, there are no other studies generating experimental evidence of a miRNA that targets the DNA methylation machinery as a mechanism of HbF induction.

DNA methylation is an epigenetic event that can be reversed by targeting key genes, such as *DNMTs* and *MECP2*, which are involved in mechanisms of gene silencing (Wu & Zhang, 2014). Elevated levels of 5-methylcytosine at the -54 methylation site in the *HBG* promoter during differentiation of erythroid progenitors is associated with *HBG* methylation and transcriptional silencing (Ruiz *et al*, 2015). In our current study, we observed significantly lower *HBG* promoter methylation following *MIR210* overexpression in normal human erythroid progenitors mediated by decreased 5-methylcytosine and *MECP2* expression. Previous studies also demonstrated that methyl-CpG binding domain proteins mediate embryonic *HBE1* and fetal *HBG* gene silencing in human adult erythroid cells and their gene silencing produced reactivation of *HBG* transcription (Gnanapragasam *et al*, 2011). Furthermore, strategies to inhibit the DNA methylation machinery have proven to be an effective therapeutic approach of HbF induction in SCD patients via *HBG* promoter hypomethylation (Molokie *et al*, 2017). We did

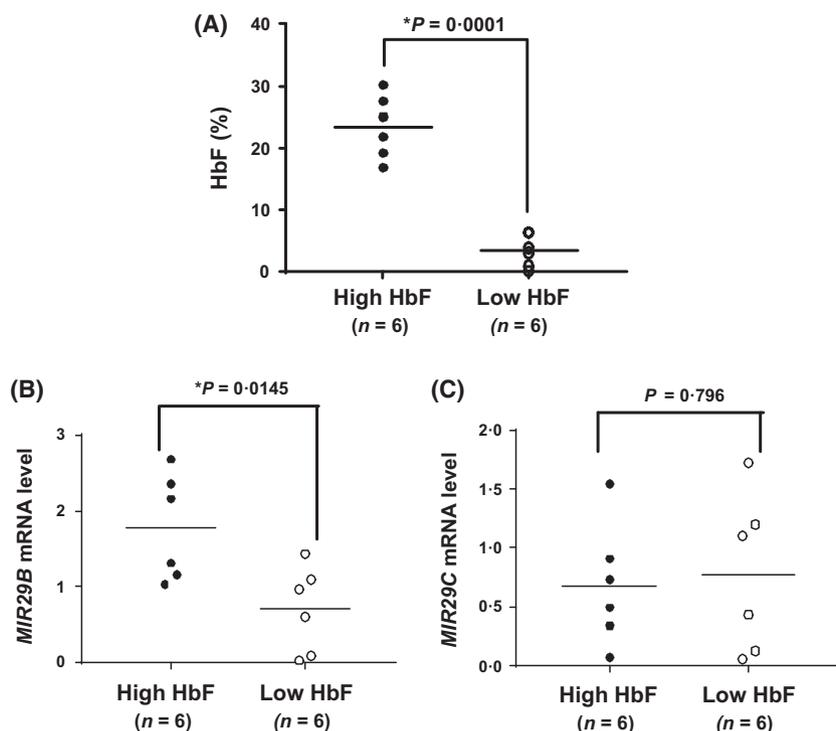


Fig 4. *MIR29B* correlates with higher peripheral red blood cell HbF levels in SCD patients. (A) Blood samples were obtained after informed consent from subjects with sickle cell disease (SCD) receiving medical care in the Sickle Cell Program at Augusta University. Shown is the distribution of HbF levels determined by clinical high-performance liquid chromatography for individuals with SCD in the high HbF group (black circles) and low HbF group (white circles); the vertical line represents the mean HbF level. (B) The raw data from miRNA microarray was analysis for *MIR29B* and (C) miR-29c, and expression levels correlated with the high HbF (black circles) and low HbF (white circles) SCD groups.

not observe additive HbF induction with combined HC and *MIR29B* treatment in erythroid progenitors generated from adult CD34⁺ stem cells. However, whether additive HbF induction would result for combination treatments under oxidative stress conditions, which exist in SCD, requires future investigations.

Experimental studies demonstrated that *MIR29* targets the tumour suppressor MYB (Martinez *et al*, 2011; Hu *et al*, 2014). In recent genome-wide association studies consisting of individuals diagnosed with SCD or β -thalassaemia, several loci, including the *HBS1L-MYB* intergenic interval, were associated with ~40% of the inherited HbF variance. The transcriptional activator MYB is essential for definitive haematopoiesis (Sheiness & Gardinier, 1984) and is highly expressed in immature haematopoietic cells and down-regulated during erythropoiesis (Bianchi *et al*, 2009). Overexpression of MYB inhibits *HBG* expression in K562 cells (Jiang *et al*, 2006); and its knockdown in primary human erythroid progenitors induces HbF expression (Sankaran *et al*, 2011). Moreover, MYB has been shown to regulate HbF expression in quantitative trait locus studies and functional assays (Jiang *et al*, 2006; Sankaran *et al*, 2011; Thein *et al*, 2007). Considering the essential role of MYB as a *HBG* repressor protein, we hypothesized that overexpression of *MIR29B* to silence MYB would mediate HbF induction. We observed downregulation of MYB by *MIR29B* and HbF induction in KU812 cells. To support a physiological role, Sankaran *et al* (2011) showed that overexpression of *MIR15A/MIR16-1* in patients with trisomy 13, down-regulated MYB protein as a mechanism of persistent HbF expression during adulthood. These

findings support the role of MYB as a negative regulator of *HBG* gene transcription *in vivo* directly targeted by *MIR15A/MIR16-1* in erythroid progenitors.

In summary, our study shows for the first time that *MIR29B*, a DNMT inhibitor, reactivates *HBG* gene transcription and HbF synthesis in human primary erythroid progenitors. Moreover, increased expression of *MIR29B* in reticulocytes isolated from individuals with SCD and high HbF levels provides additional evidence for the clinical relevance of this molecule. Future studies to evaluate ability of *MIR29B* to mediate epigenetic effects to activate *HBG* transcription and HbF expression in SCD progenitors or preclinical mouse models, are desirable to develop treatments for SCD and other β -haemoglobinopathies.

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Conflict of Interest

The authors declare no competing financial interests and no conflict of interests to disclose.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Summary of primer sequences.

Figure S1. Shown is representative histograms generated by flow cytometry analysis of erythroid progenitors under untreated (UT), scrambled (Scr), decitabine (Dec) or miR-29b treatment conditions.

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