Original Research

Stable expression of miR-34a mediates fetal hemoglobin induction in K562 cells

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Abstract

Sickle cell anemia is a common genetic disorder caused by a point mutation in the sixth codon of the β-globin gene affecting people of African descent worldwide. A wide variety of clinical phenotypes ranging from mild to severe symptoms and complications occur due to hemoglobin S polymerization, red blood cell sickling, and vaso-occlusion. Research efforts are ongoing to develop strategies of fetal hemoglobin (HbF; $\alpha_2\gamma_2$) induction to inhibit sickle hemoglobin polymerization and improve clinical outcomes. Insights have been gained from investigating mutations in the β-globin locus or transcription factors involved in the mechanisms of hemoglobin switching. Recent efforts to expand molecular targets that modulate γ-globin expression involve microRNAs that work through posttranscriptional gene regulation. Therefore, the goal of our study was to identify novel microRNA genes involved in fetal hemoglobin expression. Using *in silico* analysis, we identified a miR-34a binding site in the γ-globin mRNA which was tested for functional relevance. Stable expression of the shMIMIC miR-34a lentivirus vector increased fetal hemoglobin levels in single cell K562 clones consistent with silencing of a γ-globin gene repressor. Furthermore, miR-34a promoted cell differentiation supported by increased expression of KLF1, glycophorin A, and the erythropoietin receptor. Western blot analysis of known negative regulators of γ-globin including YY1, histone deacetylase 1, and STAT3, which are regulated by miR-34a showed no change in YY1 and histone deacetylase 1 levels; however, total- and phosphorylated-STAT3 levels were decreased in single cell miR-34a K562 clones. These data support a mechanism of fetal hemoglobin activation by miR-34a involving STAT3 gene silencing.

Keywords: miR-34a, gamma-globin, fetal hemoglobin, STAT3

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Introduction

Sickle cell anemia is one of the most prevalent genetic disorders affecting over 25 million people of African descent worldwide. It is caused by a point mutation (A→T) in the sixth codon of the β-globin gene resulting in the replacement of valine for glutamic acid producing abnormal hemoglobin S synthesis. This substitution alters the chemical properties of hemoglobin S promoting polymer formation under deoxygenated conditions and generation of sickle red blood cells responsible for vaso-occlusion and the pathophysiology of sickle cell anemia. Other inherited single nucleotide polymorphisms (SNPs) in the β-globin gene when combined with the sickle mutation produce a group of hemoglobinopathies known as sickle cell disease.

The most effective genetic modifier of sickle cell disease is fetal hemoglobin (HbF; $\alpha_2\gamma_2$) which ameliorates clinical symptoms including anemia, severe painful episodes, and organ damage while improving long-term survival.[1] The therapeutic effects of HbF are achieved by its ability to block hemoglobin S polymerization through the formation of hybrid molecules in red blood cells.[2] During *in utero* development, HbF is the predominant hemoglobin produced; however, after birth HbF is replaced by adult hemoglobin A ($\alpha_2\beta_2$).[3] Therefore, understanding mechanisms involved in γ-globin gene silencing is critical to achieving therapeutic approaches to treat sickle cell disease and other β-hemoglobinopathies.

The control of γ-globin gene transcription has been studied extensively to discover molecular targets that can be adapted for therapeutic purposes.[4–11] Although under tight transcriptional control, the γ-globin genes are also modulated by translational events. For example, sickle cell patients treated with butyrate had rapid increases in HbF levels in reticulocytes supporting γ-globin regulation independent of transcriptional activation[12] implicating translational mechanisms of control. This fact was demonstrated

by rapid increases of HbF in sickle cell patients treated with arginine butyrate.[12] Subsequent in vitro studies established the ability of the chemical compound salubrinal to promote eukaryotic initiation factor 2α phosphorylation as a mechanism to enhance γ-globin mRNA translation and HbF induction.[13]

More recent studies have focused on posttranscriptional mechanisms of gene regulation through miRNA-mediated processes. MicroRNA (miRNA) molecules are small endogenous non-coding RNAs that regulate gene expression by interfering with mRNA translation or disrupting its stability to promote degradation. Azzouzi et al.[14] demonstrated that γ-globin mRNA is bound by an argonaute 2-containing miRNA-induced silencing complex in reticulocytes isolated from adults with an average 0.5% HbF when compared to umbilical cord blood reticulocytes with >90% HbF. miRNA screening studies identified miR-96 which targets the open reading frame (ORF) of γ-globin mRNA as a mechanism of gene silencing. More recently, Miller and coworkers[15] confirmed overexpression of LIN28B decreases let-7 miRNA levels and increased HbF levels in primary erythroid cells. Moreover, LIN28B expression in adult erythroblasts was accompanied by decreased BCL11A expression implicating this repressor in the mechanism of HbF induction by LIN28B.[15] These studies expand our understanding of the role of miRNA in γ-globin gene regulation; however, additional research is needed to ascertain the scope of miRNAs involvement in HbF synthesis to develop new therapies for the β-hemoglobinopathies.

The goal of our study was to discover novel miRNA molecules involved in γ-globin gene regulation. We conducted *in silico* analysis using the target prediction software Miranda[16] and TargetScan[17–19] and identified miRNA binding sites in the 3′untranslated region (3′UTR) of the *HBG1* (Aγ-globin) and *HBG2* (Gγ-globin) mRNA. Contrary to expected gene silencing, we observed increased luciferase activity after miR-34a mimic treatment in the Aγ-globin 3′UTR reporter plasmid. Moreover, subsequent stable expression of miR34a in K562 single cell clones induced γ-globin mRNA and HbF levels confirming a positive regulatory role. Subsequent studies to determine the effects of miR-34a on erythroid maturation established up-regulation of GATA1, KLF1, glycophorin A, and the erythropoietin receptor consistent with erythroid differentiation. Studies were conducted to identify genes regulated by miR-34a and known to be negative regulators of γ-globin. Stable miR-34a overexpression in K562 single cell clones reduced total- and phosphorylated-STAT3 levels supporting the ability of miR-34a to induce HbF expression through a mechanism involving STAT3 gene silencing.

Materials and methods

Cell culture

Human K562 and HEK293 cells were grown in Iscove's Modified Dulbecco's media supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (0.1 mg/mL) in 5% CO_2 at 37°C. Cell counts and viability were determined using 0.4% trypan blue exclusion assay (Lonza, Walkersville, MD).

HEK293 cell transient transfections

The luciferase reporter plasmids were purchased from Origene (Rockville, MD); each plasmid was constructed with a firefly luciferase gene upstream of the 3′UTR of *HBG1* (pMirTarget-HBG1) or *HBG2* (pMirTarget-HBG2). HEK293 cells were grown in six-well plates at a density of 2.0×10^5 cells per well in complete media. After 24 h, the growth medium was replaced with Opti-MEM (Invitrogen, Carlsbad CA). For each transfection, 0.5 μg of either pMirTarget-HBG1 or pMirTarget-HBG2 was combined with 100 nM or 200 nM of mature miRNA mimic including miR-374-3p, miR-4327, miR-922, miR-362-5p, miR-500b, and miR-34a or scrambled control using DharmaFECT DUO (Dharmacon, Lafayette, CO) according to the manufacturer's protocol. Each experimental condition was performed in triplicate, and cells were harvested after 48 h for total protein and luciferase assay. The plasmids constitutively express red fluorescent protein, which was used to determine transfection efficiency by flow cytometry on an LSRII machine (BD Biosciences, San Jose, CA) on the phycoerythrin channel at a wavelength of 565 nm. The data were captured using FACS Diva Software (BD Biosciences) and the percentage of cells positive for red fluorescent protein was determined by FlowJo Version 10.7× software (Ashland, OR).

Luciferase assay

Luciferase assays were conducted using the ONE-GLO™ Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, transfected HEK293 cells were washed with phosphate-buffered saline and lysed in Passive Lysis Buffer for 15 min. Protein lysates were combined with Luciferase Assay Reagent II and firefly activity was measured on a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA). Luciferase activity was normalized to red fluorescent protein expression.

K562 stable lines

miR-34a stable pools were established by transducing K562 cells at a multiplicity of infection of 20 with SMARTchoice™ shMIMIC miR-34a and scrambled (Dharmacon) lentivirus particles. To select for stable expression, puromycin (1.2 μg/ml) was added 48 h after transduction and maintained in culture up to 21 days. On day 9 and day 16, protein and total RNA were harvested for Real-Time PCR and Western blot analysis. Subsequently, we established single cell clones by serial dilution of miR-34a K562 pools in 96-well plates and functional studies were conducted after one month in culture.

Reverse transcription-quantitative PCR

Total RNA was extracted from cells using Trizol (Ambion, Carlsbad, CA) and analyzed by reverse transcription-quantitative PCR (RT-qPCR) as previously published by our laboratory.[20] Briefly, cDNA was generated using the Improm-II™ Reverse Transcription System (Promega, Madison, WI) with oligo $(dT)_{15}$ primers, then qPCR was performed in a CFX Connect Real-Time System using the

SSO Advanced™ Universal SYBR Green Super Mix (Bio-Rad, Hercules, CA) with gene specific primers. Expression levels of γ-globin and glyceraldehyde-3-phosphate (GAPDH) were quantified using standard curves generated with ΔTopo7 base plasmids carrying γ-globin (Topo7-γ-globin) or Topo7-GAPDH and γ-globin mRNA levels were normalized to GAPDH (γ-globin/GAPDH). Levels of CD71, CD235a (glycophorin A), erythropoietin receptor, GATA1, GATA2, and KLF1 mRNA were measured using the RT^2-qPCR Primer Assay system (Qiagen, Valencia, CA) and standard curves were generated using genomic DNA (0.5 to 500 ng).

miRNA quantification

To quantify mature miR-34a levels, the miScript II RT and SYBR® Green PCR kit were used (Qiagen) per the manufacturer's instructions. To generate miRNA cDNA, 500 ng of total RNA, HiSpec Buffer, Nucleic Mix, and miScript reverse transcriptase mix were incubated at 37°C for 60 min. qPCR analysis was conducted with diluted cDNA template, universal primer, and miRNA specific primer per reaction; miR-34a levels were quantified using the $2^{-\Delta\Delta Ct}$ method.[21]

Western blot

Total protein was isolated using RIPA buffer (ThermoScientific, Rockford, IL) supplemented with proteinase and phosphatase inhibitor cocktails. For nuclear protein extracts, cell pellets were suspended in Buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 0.05% NP-40) for 30 min. Subsequently, the nuclear pellet was mixed with Buffer B (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 25% glycerol) for 30 min and nuclear proteins were isolated. For Western blot analysis, 20–40 μg of total or nuclear protein was loaded on a 12% acrylamide gel, transferred to a PVDF membrane, and then blocked in 5% non-fat milk. Primary antibodies against HbF (sc-21756), HDAC1 (sc-8410), phosphorylated-STAT3 (sc-8059), and total-STAT3 (sc-483) purchased from Santa Cruz Biotechnology (Dallas, TX) and YY-1 purchased from Abcam (Cambridge, MA ab109228) 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 (ThermoScientific) on a Fujifilm LAS-3000 gel imager (Stamford, CT). Blots were processed with Restore™ Plus Western blot stripping buffer and probed with tubulin (sc-53646) or TATA binding protein (sc-204) antibody as internal controls. The band intensity of different proteins was quantitated by densitometry with MultiGauge Software (Fujifilm) and was normalized to the band intensity of the internal controls.

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. For miR34a stable expression studies each clone was analyzed individually, and then combined for comparison of average expression levels. All data were analyzed by a two-tailed student's *t*-test and $p < 0.05$ was considered statistically significant.

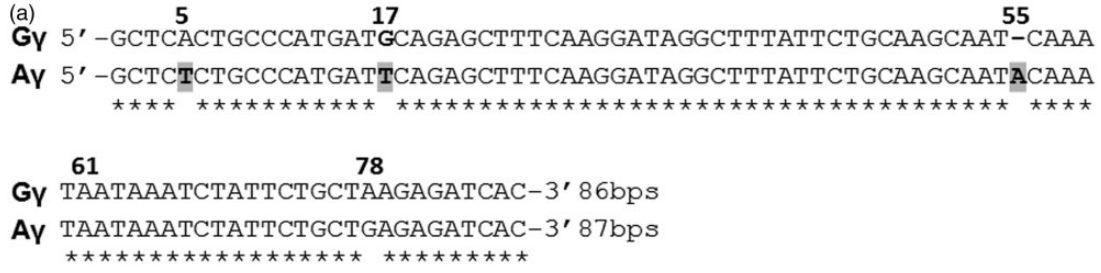
Results

The Gγ-globin and Aγ-globin 3′UTR contain novel SNPs

In humans, the γ-globin genes are encoded by the duplicated *HBG1* (Aγ-globin, NM_000559) and *HBG2* (Gγ-globin, NM_000184) genes that are identical in their protein coding sequences except at amino acid residue 136, where alanine and glycine are respectively encoded.[3] To determine differences between the 3′UTR, a ClustalW alignment was conducted using the validated reference sequences of *HBG1* and *HBG2* starting after the stop codon. The Gγ-globin (86 bps) and Aγ-globin (87 bps) 3′UTR differ in length by one nucleotide produced by a base deletion (Figure 1(a)). We next searched for SNPs in the 3′UTR using UTRdb, a database comprising annotated 5′UTRs and 3′UTRs.[22] Single base mismatches in the target mRNA seed sequence is capable of altering the strength of miRNA binding and regulation of its target mRNA.[23] The polyadenylation sequence (position 61) of both γ-globin genes is identical except at position 78. Furthermore, the Aγ-globin 3′UTR has three SNPs at positions 5, 17, and 55 numbered according to the first nucleotide after the stop codon (Figure 1(b)). These mutations may alter the affinity miRNA–mRNA interactions in the 3′UTR; therefore, we designed experiments using gene-specific luciferase reporters and miRNA mimics to determine the effect on luciferase activity.

Identification of miRNAs predicted to target the 3′UTR of the γ-globin mRNAs

The goal of our study was to identify miRNA molecules that exert posttranscriptional γ-globin gene regulation; therefore, computational approaches were used to identify potential miRNA genes that regulate γ-globin expression. Two open source software programs, Miranda[16] and TargetScan[17–19] were used to generate a list of miRNAs predicted to target the 3′UTR of the Aγ-globin and Gγ-globin mRNA. Both algorithms integrate experimentally confirmed properties of miRNA–mRNA interactions through the seed sequence of the miRNA and target site sequence within the 3′UTR. Thermodynamic free-energy between the miRNA–mRNA complex and comparative sequence analysis to predict miRNA targets across multiple species are used to achieve the prediction score. The majority of the miRNAs were predicted to bind both γ-globin gene 3′UTRs since they are 97% homologous (Figure 1(a)); however, miR-922 and miR-4291 were predicted to target Aγ-globin and miR-374-3p Gγ-globin alone. Table 1 summarizes the scores obtained from Miranda (miSVR) and context scores for TargetScan. We tested the top 10 miRNAs with highly negative scores indicative of favorable miRNA–mRNA interactions. To gain further evidence of functional relevance, we searched the literature for



(b)

SNPs in Aγ-Globin 3'UTR		
ID number	Base position	Base variation
rs1065686	5	-/A/T
rs11546324	17	G/T
rs3841756	55	-/A

Figure 1 Comparison of the nucleotide sequences of the γ-globin gene 3′UTRs. (a) A multiple sequence alignment of the base sequence in the 3′UTR of Gγ-globin and Aγ-globin generated using ClustalW software. The gray-shaded bases represent Aγ-globin 3′UTR single nucleotide polymorphisms (SNPs) reported in the UTRdb database. (b) The reference SNP cluster ID numbers and corresponding base positions and base variations observed in the Aγ-globin 3′UTR

Table 1 miRNA molecules predicted to bind γ-globin mRNA

miRNA	Gγ-globin Miranda	Aγ-globin Miranda	Gγ-globin TargetScan	Aγ-globin TargetScan
miR-374b-3p	−1.1370	no site	no site	no site
miR-4327	−0.9598	−1.0577	−0.4600	−0.4700
miR-922	no site	−1.1046	no site	−0.4600
miR-4291	no site	−1.0958	no site	−0.2800
miR-137[a]	−0.1022	−0.7796	no site	−0.2400
miR-362-5p[b]	−0.4882	−0.6814	−0.3700	−0.3700
miR-500b	−0.4230	−0.6358	−0.3700	−0.3700
miR-34a	−0.3962	ORF	ORF	no site
miR-449a	−0.3962	ORF	ORF	no site
miR-449b	−0.3962	ORF	ORF	no site

No site: no miRNA binding site detected by Miranda and/or TargetScan; ORF: open reading frame.
Note: Shown are the top 10 miRNAs predicted to target the Gγ-globin and/or Aγ-globin 3′UTRs. Miranda generates miSVR scores and TargetScan context scores. The miSVR algorithm utilizes machine learning to rank miRNAs by a down-regulation score, while the context score is an algorithm that utilizes context features to predict efficacy of miRNA–mRNA interactions.
[a]miR-137 was up-regulated in erythroid progenitors generated from umbilical cord blood $CD34^+$ stem cells[24] and is expressed in reticulocytes.[25]
[b]miR-362-5p is up-regulated by erythropoietin stimulation.[28]

evidence of miRNA involvement in globin gene regulation and erythroid differentiation.[24–28]

miR-34a mediates increased luciferase activity in the Aγ-globin gene 3′UTR

To identify novel γ-globin regulators, lead miRNAs predicted to bind the Gγ-globin and/or Aγ-globin 3′UTR were tested using the pMirTarget-HBG1 and pMirTarget-HBG2 reporter plasmids carrying the luciferase gene (Figure 2(a)). Luciferase activity was not changed significantly after transfection of miR-374-3p, miR-4327, miR-922, miR-362-5p, and miR-500b mimics at 100 nM and 200 nM concentrations (Data not shown). By contrast, miR-34a increased luciferase activity in the pMirTarget-HBG1 (Aγ-globin) reporter at the 100 nM (1.4-fold) and 200 nM (1.7-fold) concentrations compared to scrambled control (Figure 2(b)); however, the pMirTarget-HBG2 (Gγ-globin) reporter was not activated (Figure 2(c)). The observed increase in luciferase activity suggests miR-34a regulates Aγ-globin through an indirect mechanism involving silencing of a repressor protein which binds the 3′UTR; therefore, additional studies were completed to investigate this conclusion.

Stable overexpression of miR-34a increases HbF expression

To further investigate whether miR-34a plays a role in γ-globin gene regulation, we stably expressed shMIMIC miR-34a lentivirus particles in K562 cells using a puromycin selectable marker to establish three independent stable pools. At day 9 and day 16 in culture, we observed a >800-fold increase of miR-34a levels compared to scramble pools (Figure 3(a)) to demonstrate stable overexpression. We next analyzed the effects of miR34a on γ-globin expression at the mRNA and HbF levels by RT-qPCR and Western blot, respectively. On day 9, there was no difference in γ-globin mRNA in miR-34a pools; however, γ-globin mRNA levels were increased 2.0-fold by day 16 (Figure 3(b)). By contrast, HbF levels were increased 2.0-fold at both time points in response to stable overexpression of miR-34a (Figure 3(c) and (d)).

Under basal conditions, miR-34a mRNA is undetectable in wild-type K562 cells; therefore, we established single cell clones to determine the ability of miR-34a to induce HbF when expressed at lower levels and to avoid off target effects. From the miR-34a pools, cells were diluted to a

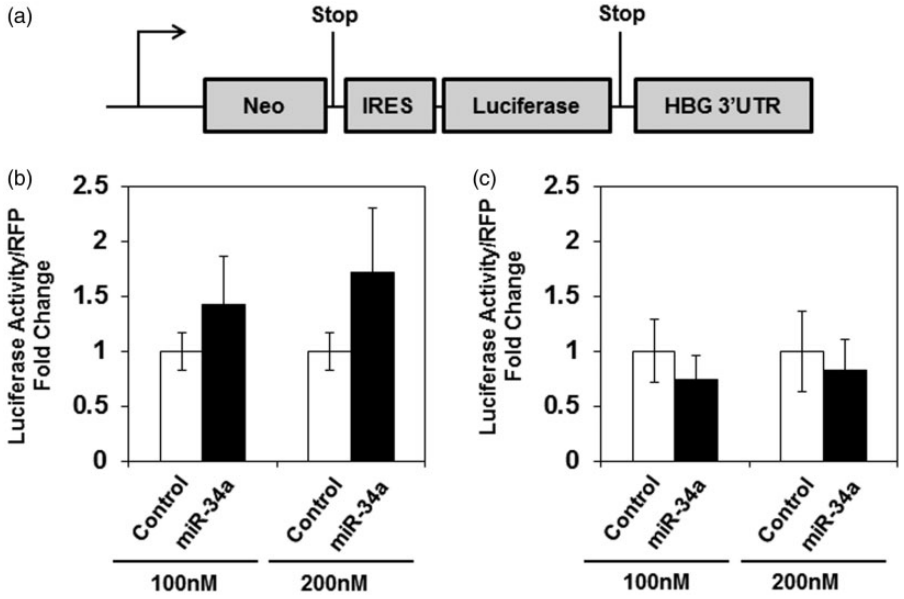


Figure 2 miR-34a alters Aγ-globin luciferase activity. (a) A schematic of the pMirTarget plasmids containing a firefly luciferase gene upstream of either the 3′UTR sequence of *HBG2* (Gγ-globin) or *HBG1* (Aγ-globin). (b) HEK293 cells were co-transfected with the pMirTarget-HBG1 plasmid and 100 nM or 200 nM of miR-34a or negative control mimic (see Materials and methods). The level of luciferase activity was normalized by red fluorescence protein (RFP) expression. Shown is the fold change in luciferase activity compared to control reported as the mean ± standard error of the mean. The data were pooled from three independent experiments each conducted in triplicate. (c) HEK293 cells were co-transfected with the pMirTarget-HBG2 plasmid and 100 nM or 200 nM of miR-34a or negative control mimic. The data analysis is the same as described in Panel B

single cell density in 96-well plates (see Materials and methods) and six independent clones overexpressing miR-34a and scrambled control clones were established. The levels of γ-globin mRNA were increased 2 to 6-fold (average 2.8-fold) in the six miR-34a clones compared to scrambled clones (Figure 4(a) and (b)).

We next quantified miR-34a overexpression in the individual clones using a kit designed to measure mature miRNA molecules (see Materials and methods). We observed miR-34a levels in the range of 1.2–3000 for the different K562 clones which was expected since they originated from a single transduced cell (Figure 4(c)). Mature miR-34a was not detected above background in the scrambled clones similar to wild-type K562 cells; however, there was an average 171-fold increase in miR-34a in stable clones (Figure 4(d)). For the individual clones, there appeared to be an association between miR-34a levels and γ-globin gene mRNA expression (Figure 4(a) and (c)). To corroborate the mRNA data, HbF levels were increased from 27 to 53-fold in the miR-34a clones compared to wild-type K562 cells (Figure 5(a) and (b)) with an average 13-fold increase in HbF (Figure 5(c)). Of note, there were two scrambled clones (S2 and S3) that exhibited non-specific increase in HbF expression (Figure 5(a)); to avoid off target effects, these clones were excluded from subsequent studies to define mechanisms of γ-globin regulation by miR-34a.

Stable overexpression of miR-34a promotes erythroid differentiation

Since miR-34a has been linked to phorbol-ester-induced megakaryocytic differentiation in K562 cells,[29] we next determined the effects of miR-34a on erythroid differentiation. Several proteins known to be altered during erythroid maturation including GATA1, GATA2, KLF1, CD71, CD235a (glycophorin A), and the erythropoietin receptor were analyzed in miR-34a (A1, A3, and A6) and scrambled (S1, S4, and S5) clones. GATA1 and KLF1 gene expression increased 1.4-fold and 2.4-fold respectively in miR-34a clones (Figure 6(a)). Furthermore, cell surface markers of erythroid differentiation including CD235a and the erythropoietin receptor increased 10-fold and 2.4-fold respectively in miR-34a clones (Figure 6(b)). By contrast, CD71 (transferrin receptor) was not changed significantly, suggesting miR-34a promotes erythroid differentiation while inducing HbF expression.

miR34a overexpression silences STAT3 gene expression

A wide variety of genes are targeted by miR-34a,[27,30–36] including the known γ-globin repressors YY1, HDAC1, and STAT3 among others. Therefore, we performed studies to determine whether miR34a indirectly regulates γ-globin expression through one of these negative regulators. Western blot analysis was performed with nuclear extract or total protein isolated from K562 clones to determine histone deacetylase 1 (HDAC1), YY1, and STAT3 levels. There were no differences in YY1 and HDAC1 protein levels in the three miR-34a clones (Figure 6(c)); in contrast, total- and phosphorylated-STAT3 levels were significantly reduced in the miR-34a clones (Figure 6(d)). Since miR-34a has been demonstrated to interact with STAT3 in pull-down experiments conducted with miRNA mimic,[36] our data

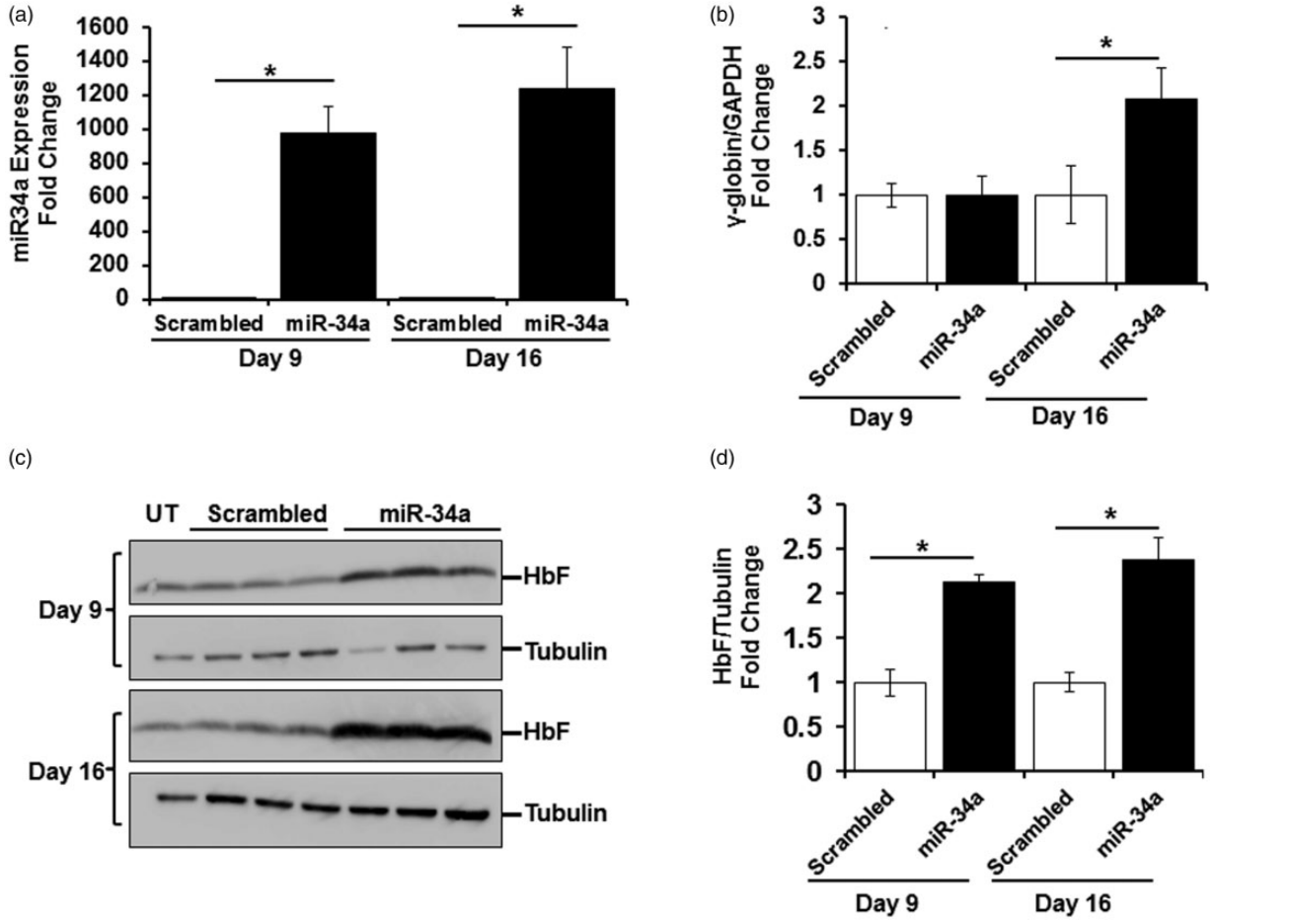


Figure 3 miR-34a overexpression mediates HbF induction in K562 stable pools. K562 cells were transduced with the SMARTchoice™ shMIMIC miR-34a or scrambled lentivirus particles. After puromycin selection, cells were harvested at day 9 and day 16 for analysis (see Materials and methods) (a) Mature miR-34a expression was measured by RT-qPCR using the Qiagen's miScript Primer Assay System. The ratio of miR-34a to RNU6 (housekeeping control) was plotted. The data were normalized to scramble control and reported as fold change of the mean ± standard error of the mean; $^*p < 0.05$. (b) The change in γ-globin gene expression was measured by RT-qPCR and shown is the fold change in γ-globin mRNA divided by GAPDH mRNA levels. The data were generated from two independent experiments with K562 cell pools performed in triplicate for each experiment. (c) Western blot analysis of HbF expression in K562 cell pools. Shown is a representative blot where tubulin was used as the loading control. (d) Data obtained by densitometry scan of western blot gels. The data are reported as fold change with respect to scrambled control normalized to one

support a possible indirect mechanism of γ-globin regulation by miR-34a through STAT3 gene silencing.

Discussion

miRNA genes represent a class of molecules under intense investigation for the treatment of a wide variety of clinical diseases. The discovery of novel effector molecules that can be targeted to alter HbF levels will expand strategies to develop therapeutic options for β-hemoglobinopathies. In vitro studies established that miR-96 directly targets the ORF of γ-globin to mediate gene silencing.[14] Furthermore, the LIN28B/let-7 interaction holds great promise for supporting the design of let-7 antagomirs and/or LIN28B expression systems to induce HbF in the erythroblasts of sickle cell patients.[15,37] A limited number of miRNA studies have been conducted in humans to elucidate mechanisms of HbF expression. Individuals with trisomy 13 have elevated HbF levels due to inhibition of the repressor protein MYB by miR-15a and miR-16-1.[38] A study in β-thalassemia patients demonstrated an association between elevated miR-486-3p and higher HbF levels linked to reduced expression of BCL11A, a developmentally regulated repressor of γ-globin.[39] Likewise, elevated miR-210 levels were observed in a patient with thalassemia and hereditary persistence of HbF; however, a direct causative relationship was not established.[40] The role of miRNAs in drug-mediated γ-globin activation has also been investigated. Walker et al.[41] verified that miR-26b was correlated with steady-state HbF levels in children with sickle cell disease, and miR-151-3p expression was associated with the maximal tolerated dose of hydroxyurea.

miRNA molecules have been implicated in other clinical phenotypes of sickle cell disease. Sangokoya et al.[42] established that miR-144 expression is correlated with the level of anemia in sickle cell patients and oxidative stress in sickle red blood cells. Reduced levels of the transcription factor NRF2 involved in the cellular stress response[42] was

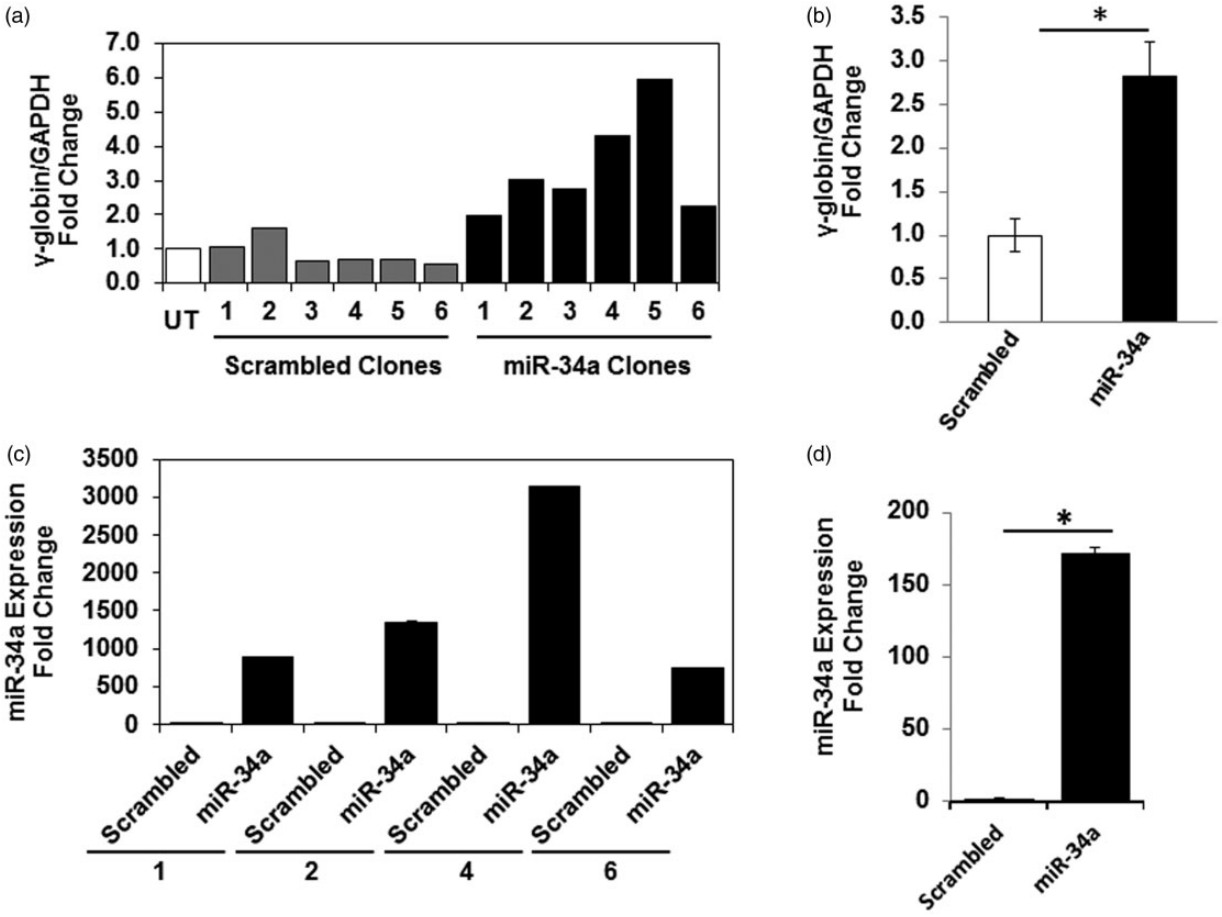


Figure 4 γ-Globin gene expression was increased in miR-34a K562 clones. K562 cells were transduced with the SMARTchoice™ shMIMIC miR-34a or scrambled lentivirus particles; after puromycin selection, the cells were subjected to serial dilutes to establish singe cell clones (see Materials and methods). (a) Fold change in γ-globin mRNA divided by GAPDH mRNA levels was measured by RT-qPCR. The data are reported with respect to untreated K562 cells which were normalized to one. Each data point represents the expression level of γ-globin at approximately one month in culture. (b) Average γ-globin mRNA levels for the six individual miR-34a and scrambled control K562 clones at one month in culture. (c) Mature miR-34a expression levels were measured by RT-qPCR and the ratio of miR-34a to RNU6 level (housekeeping control) was plotted. (d) Average mature miR-34a expression level for the six independent miR-34a and scrambled clones

implicated in the etiology of anemia. Recently, our group performed genome-wide miRNA expression profiling using reticulocytes isolated from sickle cell patients with extremes of HbF levels. We verified increased miR-144 and reduced NRF2 levels in the low HbF group[43] providing additional evidence for indirect mechanisms of HbF regulation by miRNA.

Currently, miRNAs are being investigated for the treatment of cancer, hepatitis C, myelodysplasia, and atherosclerosis among others diseases.[44–47] An excellent candidate for clinical development is miR-34a since it is a well-characterized tumor suppressor gene up-regulated by p53.[48] miR-34a is involved in the normal process of cell senescence, apoptosis, and differentiation[49] and signal transduction pathways.[50] Currently, the commercial grade miR-34a mimic MRX43 is in phase I clinical trials for the treatment of primary liver cancer.[50] As an ideal cancer therapeutic, miR-34a regulates a broad number of genes involved in proliferation, metastasis, and chemoresistance.[51] In our study, we demonstrated for the first time the ability of miR-34a to increase HbF levels in K562 cells. However, this cell line has limitations since it mainly expresses the ε- and γ-globin genes which restricted our ability to test the effects of miR-34a on adult β-globin gene transcription and often findings do not translate into clinical benefit. Consequently, studies using human primary erythroid progenitors and preclinical sickle transgenic mice are required to determine the potential of developing miR-34a as a useful HbF inducer. It may be possible to develop MRX34 for the treatment of sickle cell disease if this drug is proven to be safe and efficacious with low toxicity in human trials.

Our initial studies were focused on identifying miRNA that target the 3′UTR of γ-globin using *in silico* predicted binding sites which identified several candidate genes. Although target prediction algorithms are powerful tools, they are not always correlated with biological relevance. We predicted that luciferase reporter constructs carrying γ-globin 3′UTRs would be negatively regulated by target miRNA mimics; however, we observed enhanced luciferase activity by miR-34a. This finding suggests that despite the presence of a predicted seed binding region in the 3′UTR,

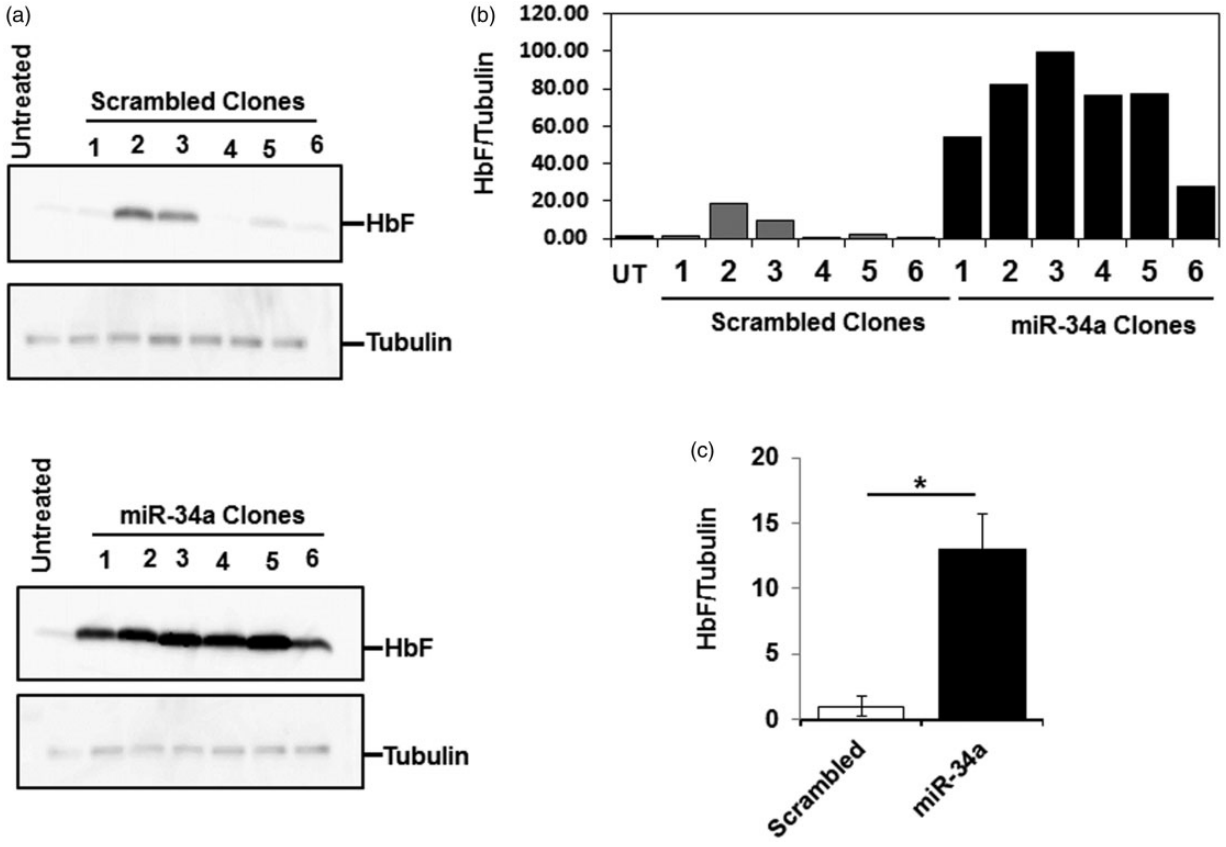


Figure 5 HbF expression was increased in miR-34a K562 clones. (a) Total protein was isolated from the scrambled control (top gel) or miR-34a (bottom gel) K562 cell clones and analyzed by Western blot for HbF expression. (b) Data obtained by densitometry scan of Western blot gels. The data are reported as fold change with respect to untreated K562 cells normalized to one. Each graph is representative of two independent characterizations of HbF expression levels. (c) Average densitometry readings from the six miR-34a clones compared to the six scrambled control clones

miR-34a probably does not bind directly and likely regulates γ-globin expression through silencing a negative regulator.

The main mechanism of gene regulation by miRNAs is repression of mammalian gene translation[52]; however, there have been reports of miRNAs that positively regulate gene expression. The two mechanisms proposed for the latter include activation through the 5′UTR and promoter binding. An example of the former was demonstrated by miR-122 and miR-10a, which induces the hepatitis viral genome through 5′UTR binding.[45,53] The second mechanism was demonstrated by miR-373 binding in the E-cadherin promoter to up-regulate gene expression.[54] However, we did not identify miR-34a binding sequences in the γ-globin 5′UTR or promoter region; therefore, it is unlikely that miR-34a increases HbF expression through either of these mechanisms.

To gain additional evidence for an indirect mechanism of HbF regulation by miR-34a, we analyzed proteins known to be negative regulators of γ-globin transcription and previously shown to be silenced by miR-34a including YY1, HDAC1, and STAT3.[32–36] YY1 binds the −1086 region in the upstream γ-globin promoter to silence gene expression[55]; by contrast, HDAC1 and HDAC2 are protein components of the nucleosome remodeling deacetylation complex[56] which plays a pivotal role in DNA methylation associated with globin silencing in β-YAC mice.[57] Histone deacetylase inhibitors such as sodium butyrate induce HbF[58] by inhibiting HDACs broadly producing histone hyperacetylation and a favorable open chromatin conformation to facilitate gene activation. The levels of YY1 and HDAC1 protein were not altered in the miR-34a K562 stable clones suggesting they are not involved in γ-globin regulation by miR-34a.

Subsequently, we investigated STAT3 which has been implicated in γ-globin gene regulation by experimental data.[59–61] Our laboratory confirmed that STAT3 and GATA1 compete for binding in the γ-globin 5′UTR, and that GATA1 overexpression reverses γ-globin silencing by STAT3.[60] Furthermore, as erythroid differentiation progresses, erythropoietin has the ability to activate STAT3 through serine 727 phosphorylation[62] as γ-globin gene silencing occurs. Consistent with these observations, miR-34a overexpression induced HbF and reduced total- and phosphorylated-STAT3 levels in K562 stable clones. TFSEARCH alignment of the γ-globin 3′UTRs did not identify STAT3 binding sites; however, a predicted miR-34a binding site was identified in the 3′UTR of STAT3 using TargetScan software. To further support a functional role, published data has established interactions between STAT3 and miR-34a using a pull-down system comprising a biotin-labeled miR-34a mimic transfected into K562 cells.

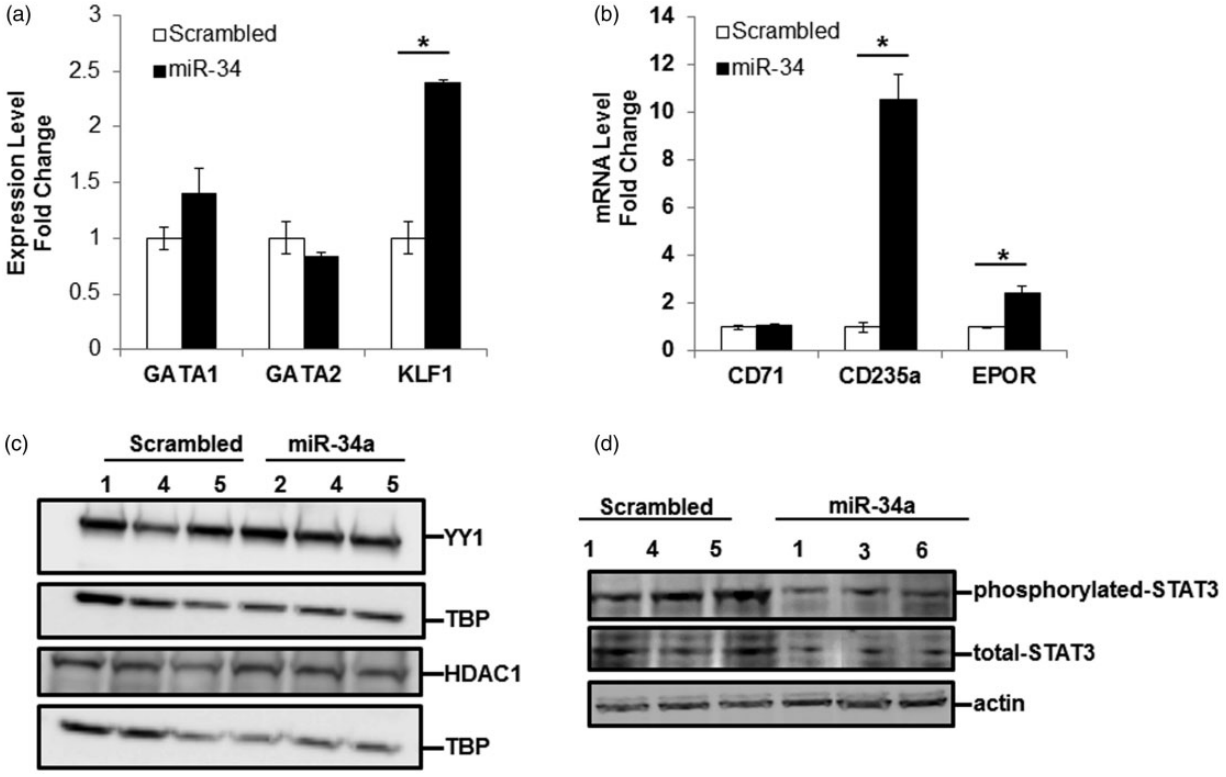


Figure 6 miR-34a overexpression facilitates erythroid differentiation and reduces STAT3 protein levels. (a) Total RNA was isolated from the individual clones and used for RT-qPCR analysis (see Materials and methods). The data are shown as the average for three scrambled clones (S1, S4, and S5) and three miR-34a clones (A1, A3, and A6). Target gene mRNA levels are normalized to GAPDH mRNA. (b) Data obtained by RT-qPCR using gene-specific primers for CD71 (transferrin receptor), CD235a (glycophorin A), and the erythropoietin receptor (EPOR). Data analysis is the same as described in Panel A. (c) Western blot analysis was performed with nuclear protein extracts to determine YY1 and HDAC1 levels; Tata binding protein (TBP) was used as the loading control. Shown is a representative gel for the S1, S4, and S5 scrambled and A2, A4, and A5 miR-34a clones. (d) Western blot was performed with total protein to determine STAT3 levels. Shown are representative gels from the miR-34a and scrambled clones

Therefore, our data support an indirect mechanism of γ-globin activation by miR-34a through STAT3 gene silencing.

Authors' contributions: All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; CMW conducted the majority of the experiments, the in silico analysis, literature search and contributed to writing and editing the manuscript. BL conducted Western blot studies of repressor proteins targeted by miR-34a and contributed to writing and editing the paper. BSP designed and supervised all experimental studies and contributed to writing and editing the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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