siRNA-mediated reduction of α-globin results in phenotypic improvements in β-thalassemic cells

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ABSTRACT

β-thalassemia is an inherited hemoglobinopathy caused by defective synthesis of the β-globin chain of hemoglobin, leading to imbalanced globin chain synthesis. Excess α-globin precipitates in erythroid progenitor cells resulting in cell death, ineffective erythropoiesis and severe anemia. Decreased α-globin synthesis leads to milder symptoms, exemplified in individuals who co-inherit α- and β-thalassemia. In this study, we investigated the feasibility of utilizing short-interfering RNA (siRNA) to mediate reductions in α-globin expression. A number of siRNA sequences targeting murine α-globin were tested in hemoglobinized murine erythroleukemic cells. One highly effective siRNA sequence (si-α4) was identified and reduced α-globin by approximately 65% at both the RNA and the protein level. Electroporation of si-α4 into murine thalassemic primary erythroid cultures restored α:β-globin ratios to balanced wild-type levels and resulted in detectable phenotypic correction. These results indicate that siRNA-mediated reduction of α-globin has potential therapeutic applications in the treatment of β-thalassemia.

Key words: siRNA-mediated reduction, α-globin, phenotypic, β-thalassemic cells.

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Introduction

Hemoglobin is a tetrameric protein comprised of two α-like and two β-like globin chains which are synthesized at a balanced rate during normal erythropoeisis.[1] β-thalassemia arises when α-globin is synthesized at levels exceeding the binding capacity of available β-globin chains, usually due to mutations affecting the β-globin locus which reduce β-globin expression.[1] The resultant excess α-globin, carrying a heme bound iron, is highly oxidized and binds to the membrane skeleton[2] where it induces generation of reactive oxygen species (ROS).[3] The increased ROS is capable of oxidizing adjacent membrane proteins leading to severe membrane abnormalities and unstable cell membranes, resulting in hemolysis and greatly exacerbating the anemic phenotype in β-thalassemia.[4]

The key role of globin chain imbalance in contributing to thalassemia severity is most clearly illustrated in individuals who co-inherit α-thalassemia with homozygous β-thalassemia. Reduction of α-globin synthesis in β-thalassemia restores globin balance and individuals demonstrate an improved phenotype.[5-8] In this study, siRNA sequences targeting murine α-globin were tested extensively to determine if significant reductions of α-globin could be affected in a manner which was conducive to therapy. One particularly effective sequence was identified and was found to reduce α-globin to therapeutic levels in primary erythroid progenitor cells derived from heterozygous β-KO mice.[9]

Design and Methods

siRNA sequences and shRNA cloning

All siRNA sequences targeting murine α-globin were ordered from Qiagen using the manufacturer's design algorithms. Direct and complementary oligonucleotides encoding short hairpin sequences were annealed and cloned into pSuperRed (Invitrogen, Carlsbad, CA, USA) under the control of a H1 promoter.

Cell culture and electroporation

Murine erythroleukemic (MEL) cells were induced to hemoglobinize by culturing in media containing 2% DMSO[10]

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(Sigma) and electroporated after six days of hemoglobinization. Electroporations were performed on the Gene Pulser (Bio-Rad, Hercules, CA, USA) at room temperature in 0.4 cm cuvettes (Bio-Rad) with 1×10^7 cells in 500 μL with the following conditions: 250 Volts, 1000 μF, ∞ resistance.

Quantitative real-time PCR

All samples were analyzed for α-globin, β-globin and β-actin expression with primers designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). Reactions were performed in triplicate on a 7300 Real-Time PCR System (Applied Biosystems). The formula $2^{-\Delta Ct}$ was applied to give fold-difference between α-globin relative to the reference gene. Changes in gene expression were determined by dividing fold-differences in experimental groups against fold-differences in mock electroporated cells.

Protein detection

Western blotting was performed according to standard protocols using antibodies for α-globin (SC-31333, Santa Cruz Biotechnology, Santa Cruz, CA), β-globin (SC-31116, Santa Cruz Biotechnology) and β-actin (SC-47778, Santa Cruz Biotechnology). Proteins were visualized with an Enhanced Chemiluminescent kit (Amersham) and protein levels were quantitated using Quantity One software (Bio-Rad). Levels of α-globin and β-globin were normalized to β-actin in each sample well. Expression of α-globin and β-globin was determined by dividing expression levels in experimental groups against mock electroporated MEL cells. Heme expression was determined by spectrophotometry at 414 nm using equal amounts of protein.

Primary erythroid culture system and reactive oxygen species detection

Cells were extracted from the bone marrow of wild-type (WT) and heterozygous β-KO mice and cultured using previously described conditions[11] with minor modifications. Following six days of expansion, cells were electroporated and resuspended at 4×10^6 cells/mL in differentiation medium. Levels of ROS were determined by incubating cells with 2',7'-dichlorofluorescin diacetate (DCFH) (Sigma) according to previously described conditions[12] with minor modifications.

Thalassemia mouse models

Double heterozygous (DH) α-KO/β-KO mice were obtained by crossing established heterozygous β-KO[9] mice and heterozygous α-KO[13] mice as previously described.[14] All animal experiments were conducted with the approval of the MCRI animal ethics committee.

Statistical analysis

All data are presented as mean average ± SD. Statistical significance was calculated using a two-tailed Student's t-test and $p<0.05$ was considered statistically significant.

For a detailed description of all methods, please refer to the Online Supplementary Appendix.

Results and Discussion

Comparisons of siRNA sequences targeting α-globin in murine erythroleukemic cells

In order to identify and evaluate effective α-globin-specific siRNA target sequences, four siRNA sequences targeting murine α-globin were electroporated into hemoglobinized MEL cells. Three sequences (siα1, siα3 si-α3 and si-α4) generated significant reductions in α-globin mRNA compared to mock electroporated MEL cells 24 hours post electroporation as detected by real-time PCR. The most effective siRNA, si-α4, reduced α-globin mRNA by 67%±10% relative to β-actin, while si-α1 and si-α3 generated modest, though significant, reductions of 44%±8% and 38%±8% respectively ($p<0.01$) (Figure 1A). Analysis of efficacy over time demonstrated that si-α4 generated reductions in α-globin mRNA which remained significant for 96 hours relative to β-globin expression (Figure 1B) with a slight recovery in α-globin expression at 96 hours (55%±9% reduction) ($p<0.005$).

Protein expression was detected at 48 hours post elec-

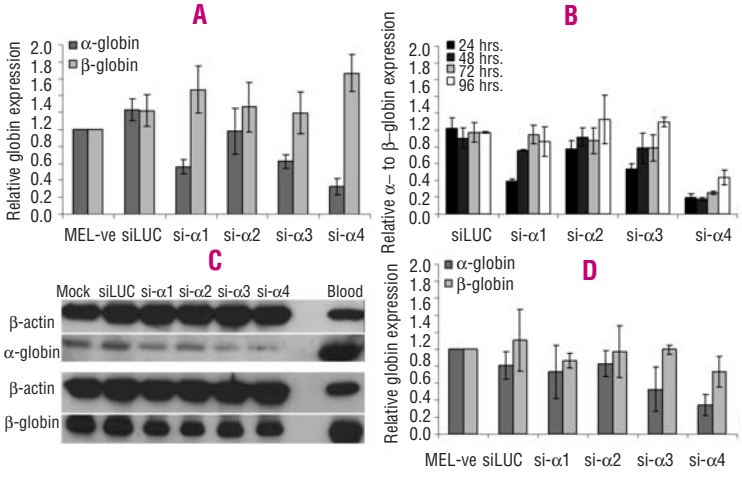

Figure 1. siRNA mediated reduction of α-globin in hemoglobinized MEL cells. (A) Relative globin RNA expression detected by real-time PCR 24 hours post electroporation with 10 μg of four siRNA sequences targeted to α-globin. Relative expression of α- and β-globin was calculated by normalizing to expression levels in mock electroporated MEL cells using β-actin expression as an RNA loading control. (B) Relative α-globin RNA detected by real-time PCR at various time points post electroporation with 10 μg siRNA. Values represent α-:β-globin ratios and were calculated by normalizing to expression levels in mock electroporated MEL cells. (C) Representative western blot. (D) Average α-globin and β-globin protein levels 48 hours post electroporation with 10 μg siRNA as determined by western blotting. Relative expression of α- or β-globin was calculated by normalizing to expression levels in mock electroporated MEL cells using β-actin as a loading control. An irrelevant siLuc siRNA was included in all experiments as an irrelevant control. All graphs represent the mean average of at least three independent experiments ± SD.

troporation by western blotting (Figure 1C). Slight reductions in α-globin expression were noted in MEL cells treated with si-α1 (25% ± 32%, p=0.14) and si-α3 (45% ± 26%, p<0.05) (Figure 1D) though these were not statistically significant. Confirming mRNA results obtained by real-time PCR, the most effective knockdown of α-globin was achieved using si-α4 which reduced α-globin protein levels significantly by 65%±12% (p<0.01). β-globin expression was monitored throughout all experiments and no statistically significant reductions were detected. An irrelevant siRNA targeting luciferase (siLuc)[15] was also included and had no significant effects on either α- or β-globin expression. The efficacy of the si-α4 sequence was further assessed in a time and dose-dependent manner. Dose effects became apparent at 48 hours when α-globin expression in cells treated with the three lowest doses (500 ng, 250 ng and 100 ng) increased from 20%±3%, 25%±9% and 30%±9% respectively at 24 hours to 40%±13%, 50%±8% and 80%±3% at 48 hours (Figure 2A). Having identified a promising sequence, the efficacy of the si-α4 sequence was further assessed as a plasmid encoding a short-hairpin sequence targeting the same region of α-globin as si-α4 (psh-α4). The psh-α4 plasmid was also shown to retain efficacy and reduced α-globin mRNA expression by 57% ± 17% relative to β-globin (p<0.01) (Figure 2B). Plasmids targeting the same regions as si-α1 (psh-α1) and si-α3 (psh-α3) were also compared.The psh-α3 construct reduced α-globin expression by 24%±7% (p<0.02) while psh-α1 had no significant effects. β-globin expression relative to β-actin was unaffected. A plasmid encoding an irrelevant shRNA sequence targeting enhanced green fluorescent protein (psh-EGFP)[16] had no effect on either α-globin or β-globin expression levels.

Both si-α4 and psh-α4 reduced α-globin to an extent which resulted in a visible decrease in hemoglobinization 48 hours post electroporation (Figure 2C). MEL cells electroporated with 10 μg and 100 ng of si-α4 both showed significant dose-dependent reductions in heme levels compared to mock electroporated cells with 73%±14% and 35%±4% reduction respectively (Figure 2D) (p<0.02). Cells treated with 10 μg of psh-α4 also generated significant reductions of 45%±13% in heme levels (p<0.05). MEL cells treated with 10 μg of siLuc RNA and psh-EGFP plasmid DNA irrelevant controls were unaffected. These experiments identified a particularly effective siRNA sequence (si-α4) and demonstrated that siRNA could effectively reduce α-globin in a relevant cell line expressing high levels of endogenous globins without affecting β-globin expression. A siLuc control had no effect on either α-globin or β-globin expression, indicating that the reductions were sequence specific and finally, time and dose effects confirmed that knockdowns were due to siRNA. However, it was crucial to ensure that the siRNA would remain equally effective in primary erythropoietic progenitor cells, which would need to be targeted for therapy.

siRNA mediated reduction of α-globin in erythroid progenitor cells

Analysis of globin ratios and ROS levels in RBCs of mice with various α-globin and β-globin genotypes indicated a clear relationship between excess α-globin chains and increased ROS production. Heterozygous β-KO mice exhibited higher levels of ROS compared to WT mice, while mice which were double heterozygous (DH) α-KO/β-KO with more balanced globin expression generated levels of ROS comparable to WT mice, a finding consistent with the markedly improved anemia previously reported for these DH α-KO/β-KO mice[14] (*data not shown*). Next, a primary erythroid cell culture system was utilized in order to model conditions *in vivo*.[11] Bone marrow derived cells were extracted from thalassemic heterozygous β-KO mice and cultured in conditions which favored the expansion of erythroid progenitor cells then treated with si-α4 prior to erythroid differentiation. Untreated WT and heterozygous β-KO cultured cells were analyzed for α:β-globin expression ratios as a control. Confirming results from bone marrow cells of these mice, α-globin expression in WT cells was half (50%±0.06) (p<0.01) that of heterozygous β-KO cells, which exhibited imbalanced, excess synthesis of α-globin relative to β-globin (Figure 3A (i)).

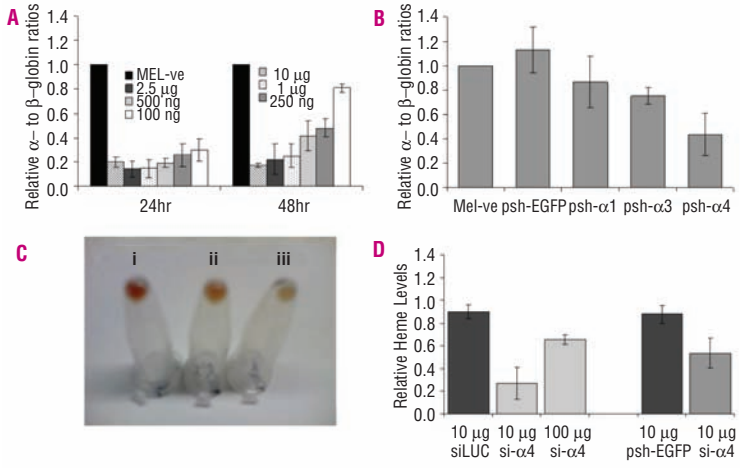

Figure 2. Efficacy of si-α4 at various doses and plasmid constructs encoding equivalent short-hairpin sequences. (A) Relative α-globin RNA detected by real-time PCR at 24 and 48 hours post electroporation with various doses of si-α4. Values represent α-globin: β-globin ratios and were calculated by normalizing to expression levels in mock electroporated MEL cells. (B) Relative α-globin RNA detected by real-time PCR at 48 hours post electroporation with 10 μg psh-α1, psh-α3 or psh-α4. Values represent α-globin : β-globin ratios and were calculated by normalizing to expression levels in mock electroporated MEL cells. 10 μg of pshEGFP plasmid DNA was included as an irrelevant control. (C) MEL cell pellets 48 hours post electroporation with (i) no DNA (ii) 10 μg psh-α4 and (iii) 10 μg si-α4 showing visible reductions in hemoglobinization. (D) Hemoglobin levels as detected by spectophotometry using equal amounts of protein 48 hours post electroporation with 10 μg si-α4 or 100 ng si-α4 and 10 μg psh-α4. 10 μg siLuc and 10 μg pshEGFP were used as respective irrelevant controls. Values were calculated by normalizing to mock electroporated MEL cells. All graphs represent the mean average of at least three independent experiments ± SD.

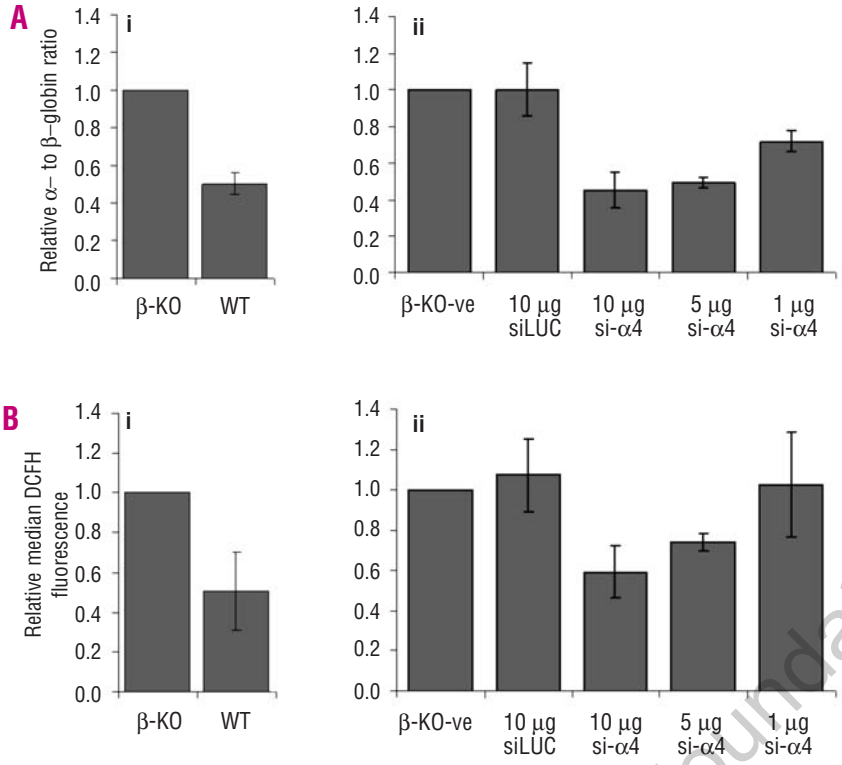

Figure 3. Restoration of globin balance in primary erythropoietic cells from heterozygous β-KO mice. (A) Relative α-globin: β-globin RNA ratios in cultured primary erythroid progenitor cells from (i) WT and heterozygous β-KO and (ii) heterozygous β-KO cells treated with 10 μg, 5 μg or 1 μg of si-α4 or an siLuc irrelevant control. (B) Relative levels of ROS in cultured primary erythroid progenitor cells from (i) WT and heterozygous β-KO and (ii) heterozygous β-KO cells treated with 10 μg, 5 μg or 1 μg of si-α4 or an siLuc irrelevant control. All values shown represent the mean average of at least three independent experiments ± SD.

This was reflected in levels of ROS production with cells from WT mice generating ROS at roughly half the levels (50%±19%, $p<0.005$) of identical cells cultured from β-KO mice (Figure 3B (i)).

Cultured primary erythroid progenitor cells obtained from heterozygous β-KO mice treated with 10 μg, 5 μg and 1 μg of si-α4 showed reduced α-globin expression of 55%±10%, 51%±3% and 28% ±6% ($p<0.02$) respectively relative to β-globin when compared to mock electroporated heterozygous β-KO cells. This was translated to a phenotypic improvement with cells treated with 10 μg and 5 μg of si-α4 exhibiting 41%±13% ($p<0.05$) and 26%±4% ($p<0.02$) reductions in ROS production respectively. This effectively demonstrates that siRNA can reduce α-globin expression to therapeutic levels in physiologically relevant primary erythroid progenitor cells and generate detectable phenotypic improvements.

Reduction of α-globin expression has long been known to improve anemia in β-thalassemia, illustrated in individuals who co-inherit α- and β-thalassemia.[1,5-8] However, until recently, the application of this knowledge in the form of therapy has been hampered by the lack of an efficient strategy to reduce gene expression. Although a recent study has demonstrated slight phenotypic improvements in transgenic mice expressing short-hairpin RNA targeting α-globin, the authors concede that this is not applicable in a therapeutic setting.[17] In this study, we report that siRNA can be used to reduce expression of a highly expressed gene such as α-globin at both the RNA and protein level. To the best of our knowledge, this is the first study demonstrating definitive reduction of endogenous globins mediated directly with siRNA without any DNA intermediates. A siRNA based strategy for reducing α-globin expression has some advantages over traditional models of gene therapy. Firstly, this approach targets one of the primary causes of the disease without necessitating permanent changes to the genome. Though this means the effects would be transient, there is a possibility of utilizing this system as a more flexible pharmaceutical treatment where dosage can be controlled and effects are completely reversible. Another advantage of siRNA over DNA based therapies, is that siRNA is active in the cytoplasm[18] and does not need to cross the nuclear membrane, one of the major barriers in gene therapy.[19,20]

Future investigations should include *in vivo* testing in a thalassemic mouse model as well as isolating effective siRNA sequences targeting human α-globin. Though gene replacement therapy would unquestionably be the ideal curative therapy for thalassemia, we believe that siRNA mediated reduction of α-globin could potentially provide a viable alternative or complementary strategy.

Authorship and Disclosures

HV designed and performed the experiments, analyzed the data and wrote the paper; HW managed the animal models and designed the research; JV designed the research. All authors were involved in drafting the article and revising it critically for important intellectual content. All authors have approved the final version to be published.

The authors reported no potential conflicts of interest.

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