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

Hsiao Phin Joanna Voon, Hady Wardan and Jim Vadolas

Cell and Gene Therapy Research Group, The Murdoch Children's Research Institute, The University of Melbourne, Royal Children's Hospital, Flemington Road, Parkville 3052, Melbourne, Australia

Citation: Voon HPJ, Wardan H and Vadolas J. siRNA-mediated reduction of α-globin results in phenotypic improvements in β-thalassemic cells. Haematologica 2008. doi: 10.3324/haematol.12555

Design and Methods

siRNA sequences and shRNA cloning

All siRNA sequences targeting murine α-globin were ordered from Qiagen using manufacturer's design algorithms. All siRNAs consisted of 19 nucleotides annealed sense and antisense RNA strands with two nucleotide 3' DNA overhangs. Sequences were as follows: si-α1 sense 5' r(GGAGCU-GAAGCCCUGGAAA)dTdT and si-α1 antisense 5' r(UUUCCAGGGCUUCAGCUCC)dAdT, si-α2 sense 5' r(AGGUCAAG GGUCACGGCAA)dTdT and si-α2 antisense 5' r(UUGCCGUGACCCUUGACCU)dGdG, si-α3 sense 5' r(CCGUGCUGACCUCCAAGUA)dTdT and si-α3 antisense 5' r(UACUUGGAGGUCAGCACGG)dTdG, si-α4 sense 5' r(CCUCUUGGUCUUUGA AUAA)dTdT and si-α4 antisense 5' r(UUAUUCAAAGACCAAGAGG)dTdA. Direct and complementary DNA oligonucleotides encoding a short hairpin based on the si-α4 RNA sequence were ordered from Oligoengine (Seattle, WA). Sense and antisense DNA sequences were as follows: sh-α1 sense 5' GATCCCCATG-GAGCTGAAGCCCTGGAAATTCAAGA-GATTTCCAGGGCTTCAGCTCCATTTTTA and sh-α1 antisense 5' AGCTTAAAAATGGAGCTGAAGCCCTG-GAAATCTCTTGA ATTTCCAGGGCTTCAGCTCCATGGG sh-α3 sense 5' GATCCCCCACCGTGCT GACCTCCAAG-TATTCAAGAGATACTTGGAGGTCAGCACGGTGTTTT-TA and sh-α3 antisense 5' AGCTTAAAAACACCGTGCT-GACCTCCAAGTATCTCTTGAATACTTGGAG-GTCAGCACGGTGGGG sh-α4 sense 5' GATCCCCTAC-CTCTTGGTCTTTGAATAATTCAAGAGATTATTCAAA-GACCAAGAGGTATTTTTA and sh-α4 antisense 5' AGCT-TAAAAATACCTCTTGGTCTTTGAATAATCTCTTGAA TTATTCAAAGACCAAGAGGTAGGG. The oligonucleotides were annealed and cloned into a pSuperRed vector (Invitrogen, Carlsbad, CA) under the control of an H1 promoter. The siLuc[27] and psh-EGFP[28] controls were designed according to previous published reports with sequences as follows: siLuc sense 5' r(CGUACGCGGAAUACUUCGA)dTdT and siLuc antisense 5' r(UCGAAGUAUUC CGCGUACG)dTdT. sh-EGFP sense 5' CCCGAACGGCATCAAGGTGAACTTCA AGAGAGTTCACCTTGATGCCGTTCTTT and sh-EGFP antisense 5' AAAGAAC GGCATCAAGGTGAACTCTCTT GAAGTTCACCTTGATGCCGTTCGGG.

MEL cell culture and hemoglobinization

Murine erythroleukemic (MEL) cells were maintained in continuous culture in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin according to standard protocols. Cells were induced to hemoglobinize by seeding at an initial concentration of 2×10^5 cells per mL in DMEM containing 2% DMSO (Sigma) and incubated at 37°C. After five days of hemoglobinization, cells were resuspended at 5×10^5 cells per mL in fresh DMEM with 2% DMSO[29] and electroporated approximately 16 hours later.

Electroporation of siRNA into cells

Immediately preceding electroporation, cells were washed three times with equal volumes of Opti-Mem reduced serum media (Invitrogen) and resuspended at a final concentration of 2×10^7 cells per mL in Opti-Mem. Electroporations were performed at room temperature in 0.4 cm cuvettes (Bio-Rad, Hercules, CA) with 500 μL cell suspension mixed with an appropriate amount of siRNA resuspended at a concentration of 1 μg/μL. Electroporations were performed on the Gene Pulser (Bio-Rad) using the following conditions: 250 Volts, 1000 μF, ∞ resistance. Cells were then cultured in 10 mL DMEM containing 10% FCS and 2% DMSO and incubated at 37°C.

Extraction of RNA and cDNA synthesis

Cells were collected at appropriate time points after electroporations and RNA was extracted using Tri-Reagent (Molecular Research Centre, Cincinnati, OH) according to the manufacturer's instructions. Quantity and quality of RNA was determined by spectrophotometry and cDNA was synthesized using SuperScript First-Strand kit (Invitrogen) according to the manufacturer's instructions.

Quantitative Real-Time PCR

All samples were analyzed using three different primer sets specific for α-globin (5' GCTCTGAGCGACCTGCATG 3' and 5' AGGTCACCAGCAGGCAGTC 3'), β-globin (5' GTGAGCTCCACTGTGACAAGCT 3' and 5' GCACAAT-CACGATCATATTGCC 3') and β-actin (5' CCCTAAGGC-CAACCGTGAA 3' and 5' CAGCCTGGATGGCTACGTA-CA 3'). All primers were designed using Primer Express software (Applied Biosystems, Foster City, CA).

Reactions were performed on a 7300 Real-Time PCR System (Applied Biosystems). 25 ng of template cDNA was combined with 2 pmol of the forward primer and 2 pmol of the reverse primer and 12.5 μL SYBR Green Master Mix (Applied Biosystems) to a final volume of 25 μL. Samples

were held at 50 °C for 2 mins. then 95 °C for 10 mins. followed by 40 cycles of 15 secs. at 95 °C and 1 min. at 60 °C with data collection occurring at 60 °C. A final dissociation stage was performed consisting of 15 secs. at 95 °C, 30 secs. at 60 °C and 15 secs. at 95 °C. All reactions were performed in triplicates. Triplicate Ct values for each sample and primer pair were averaged. Ct values of the gene of interest (α-globin) was subtracted from the reference gene (β-actin or β-globin) to calculate Δ Ct. The formula $2^{\Delta Ct}$ was applied to give fold-difference between α-globin relative to the reference gene. Reductions of α-globin expression were determined by dividing fold-differences in the experimental group against fold-differences in mock electroporated MEL cells.

Western blot

Protein was collected from cells by osmotic lysis followed by sonication. Protease inhibitor cocktail was added according to the manufacturer's recommendations (Complete Protease Inhibitor Cocktail Tablets, Roche Applied Science, Indianapolis, IN) and protein was quantitated using BioRad DC Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions. A total of 20 μg of protein was electrophoresed on a 12% SDS polyacrylamide gel for 90 mins. at 30 mA and electrotransferred onto a Hybond-P membrane (Amersham, Buckinghamshire, UK) at 45 mA for 1 hr. on a TE 77 PWR ECL semi-dry blotter (Amersham). Pre-stained molecular weight markers (Bio-Rad) were used to estimate position of protein bands and murine blood was used as a positive control. Membranes were blocked in 3% BSA at room temperature for 1 hr. Murine α-globin and β-globin expression were determined by staining separate gels containing identical experimental samples run in duplicates. Murine α-globin was detected with a goat polyclonal IgG anti-α-globin antibody (SC-31333, Santa Cruz Biotechnology, Santa Cruz, CA) and β-globin was detected with a goat polyclonal IgG anti-β-globin antibody (SC-31116, Santa Cruz Biotechnology) used at a 1 in 1,000 dilution overnight at 4°C. Membranes containing separated proteins were cut in half and stained separately for β-actin expression. β-actin was detected using a HRP-conjugated mouse monoclonal IgG β-actin antibody (SC-47778, Santa Cruz Biotechnology) used at a 1 in 250,000 dilution overnight at 4°C. HRP-conjugated donkey anti goat IgG antibodies were used at a 1 in 10,000 dilution for 1 hr. at room temperature as secondary antibodies. Visualization was performed using Enhanced Chemiluminescent kit (Amersham) according to the manufacturer's instructions. Radiographs were scanned and protein levels were quantitated using Quantity One software (Bio-Rad). Protein loading was controlled by normalizing α-globin and β-globin levels 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.

Spectrophotometry

Protein was collected from cells by osmotic lysis and centrifuged to remove debris. Protein concentration was determined using BioRad DC Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions and equal amounts of protein were analyzed by spectrophotometry at 414 nm to determine heme concentration. A heme standard curve was established using purified human hemoglobin (Sigma). A reduction in heme expression was determined by dividing heme expression levels in experimental groups against mock electroporated MEL cells.

Primary erythroid culture system

Cells were extracted from the bone marrow of wild-type (WT) and heterozygous β-KO mice and cultured using previously described conditions[30] with minor modifications. Briefly, cells were seeded into StemPro-34 medium (Invitrogen) containing 1 μM dexamethasone (Sigma), 20 ng/mL insulin like growth factor 1 (Promega, Madison, WI), 2 U/mL Aranesp (Amgen, Thousand Oaks, CA) and murine recombinant stem cell factor (180 ng/mL). Cell density was maintained at 4×10^6 cells/ml by daily media changes for five days.

On day 6 of expansion, cells were prepared and electroporated as described above. Following electroporation, cells were resuspended at 4×10^6 cells/mL in differentiation medium consisting of StemPro-34 medium (Invitrogen) containing 200 ng/ml biotin (Sigma), 8.3 ng/mL hypoxanthine (Sigma), 10 U/ml Aranesp (Amgen), 4×10^{-4} IU/mL insulin (Actrapid HM, Novo Nordisk, Bagsvaerd, Denmark), 1 mg/mL iron-saturated human transferrin (Sigma), 1 μM mifepristone (Invitrogen) and 100 μl/ml fetal calf serum (Thermo Electron, Melbourne, Australia).

Detection of reactive oxygen species

Levels of ROS were determined by incubating cells with 2',7'-dichlorofluorescin diacetate (DCFH) (Sigma) according to previously described conditions[31] with minor modifications. Briefly, cells were washed with PBS and resuspended at a concentration of 1×10^6 cells/mL. DCFH was dissolved in DMSO (Sigma) at a concentration of 10 mM and added to cell suspension to a final concentration of 10 μM. Cells were incubated with DCFH in a humidified incubator at 37°C and 5% CO_2 for 15 mins., washed and then resuspended in PBS. Cells were analyzed on an LSR II flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using FACS DIVA software (Becton Dickinson) and gated based on forward side scatter profiles.

Thalassemia mouse models

Double heterozygous α-KO/β-KO mice were obtained by crossing established heterozygous β-KO[32] mouse models and heterozygous α-KO[33] mouse models as previously described.[34] Progeny were genotyped using DNA obtained from tail biopsies using previously described multiplex PCR reactions to determine α-globin and β-globin genotypes. 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.