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Correction of splicing, chain imbalance, and adult hemoglobin synthesis in IVS-2-745 β-thalassemia specimens using 2'-o-methoxyethyl splice-switching oligos

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Table of Contents

Abstract 2

Introduction 4

Epidemiology and pathophysiology of β-globinopathies 4

Alternative splicing of the β-globin 8

Switch Splicing Oligos to restore canonic splicing 10

Chemical modification increase stability of SSOs 10

Methods 14

Human Ethics 14

Statistics 14

MEL Cell Culture 14

Human Erythroblast Cultures and Treatment 15

RNA, PCR, and Quantitative PCR (Q-PCR) 16

Tetrameric and single chain analysis by direct- and reverse-phase high performance liquid chromatography (HPLC) 17

Vector production and Titering 18

In vitro RBC sickling and morphological analysis 19

Copy Number Determination 19

Results 21

2'MOE-SSOs induce splice switching restoring adult hemoglobin (HbA) production in cells from β0/β-745 heterozygote patients 24

2'MOE SSOs show the most robust effects on a 745/745 homozygote sample 29

2'MOE SSOs prevents sickling in samples with a 745/sickle genotype 32

Discussion and Future Perspectives 34

Bibliography 37

Abstract

β-thalassemia is one of the most frequently-occurring disorders due to altered hemoglobin protein synthesis (Modell & Darlison, 2008). In this disease, the β-globin gene is mutated, causing severe anemia and ineffective erythropoiesis. Patients can present with a number of life-threatening co-morbidities, including iron overload, extramedullary erythropoiesis, hypogonadism and osteoporosis (Origa, 2016). Current standard of care for patients with severe anemia involves blood transfusion and iron chelation. Allogeneic bone marrow transplant is the only curative option, but is constrained by the availability of matching donors and the occurrence of graft-versus-host disease. (Breda et al., 2016)

As this is a monogenic disease, it makes it an attractive setting for genetic therapy at the DNA and RNA level. Currently, there are a few clinical trials and preclinical tests underway to evaluate the curative potential of lentiviral vectors to correct the mutated β-globin or add back a functional copy of β-globin (Jorge Mansilla-Soto, Isabelle Riviere, Farid Boulad, 2016, Negre et al., 2016)

One of the processes that affects β-globin synthesis in β-thalassemia is occurrence of aberrant splicing. IVS-2-745 is a splicing mutation that occurs in intron 2 of the β-globin gene. It creates an aberrantly spliced form that incorporates an extra exon and leads to a premature stop codon.

Here we report novel uniform 2'-O-methoxyethyl (2'-MOE) splice switching oligos (SSOs) to reverse this aberrant splicing. Lead 2'-MOE SSOs were generated to redirect splicing in the IVS-2-745 pre-mRNA. With these lead SSOs we have demonstrated aberrant to wild type splice switching. This switching led to an increase from 3-6% to up to 80% adult hemoglobin (HbA) in erythroid cells from thalassemic patients. Furthermore, we demonstrated a restoration of the balance

between β-like- and α-globin chains, and up to an 87% reduction in toxic α-heme aggregates. While next examining the potential benefit of 2'MOE SSOs in a sickle-thalassemic phenotypic setting, we found reduced HbS synthesis and sickle cell formation due to HbA induction, as a result of 2'MOE SSO treatment. In summary, 2'MOE-SSOs are a promising therapy for splicing forms of β-thalassemia. Their ability to functionally modulate the thalassemia and sickle cell anemia phenotype by correcting the underlying defect offers a pharmacological treatment that is both direct and specific.

Introduction

Epidemiology and pathophysiology of β-globinopathies

Inherited hemoglobin disorders are the most common monogenic diseases, representing a major health problem worldwide. Over 330.000 affected infants are born annually (83% sickle cell disorders, 17% thalassemias). Hemoglobin disorders account for about 3.4% of deaths in children less than 5 years of age. Globally, around 7% of pregnant women carry β or α thalassemia, or hemoglobin S, C, D Punjab or E, and over 1% of couples are at risk and currently approximately 68,000 children are born with various thalassemia syndromes each year (Modell & Darlison, 2008)

In developed countries healthcare for patients with hemoglobinopathies has achieved greater life expectancy, converting it into a long-term chronic disease. At the same time, in developing countries the lack of genetic counseling and prenatal diagnosis have contributed to the maintenance of a very high frequency of hemoglobinopathies in the population.

The extremely high frequency of hemoglobin disorders compared with other monogenic diseases reflects the selective pressure from *Plasmodium falciparum* with a high frequency of consanguineous marriages in many countries. In the United States thalassemia is becoming more prevalent due to immigration of people from affected regions, with an approximately 7.5% increase over the last five decades (Sayani & Kwiatkowski, 2015).

The β-globin (*HBB*) gene, which spans 1.6 Kb, contains three exons and both 5' and 3' untranslated regions (UTRs). It is regulated by an adjacent 5' promoter in which are located a TATA, CAAT, and duplicated CACCC boxes. A major regulatory region, known as locus control region (LCR), containing also a strong enhancer, maps 50 Kb

from the β-globin gene. This region contains four erythroid specific DNAse hypersensitive sites (HS-1 to HS-4), which are a hallmark of DNA-protein interaction. Each HS contains a combination of several DNA motifs interacting with transcription factors, among which the most important are GATA-1 (GATA indicates the relative recognition motif), nuclear factor erythroid 2, erythroid Kruppel-like factor, and friend of GATA 1. The importance of the LCR for the control of the β-like globin gene expression has been discovered by studying a series of naturally occurring deletions that totally or partly remove the HSs and result in the inactivation of the intact downstream β-globin gene (Cao & Galanello, 2010).

Consequences of mutations in the ß-globin could fall into two broad groups: structural variants that change the amino acid sequence and produce an unusual hemoglobin (such as HbS in Sickle Cell Anemia), and a group of autosomal recessive defects causing reduction or absent production of the ß-globin chain require for the structural integrity and functionality of the adult-hemoglobin tetramer (as in ß-globinopathies). People who inherit these mutations or their combinations may have a serious hemoglobin disorder (Dial, 1980).

Sickle cell anemia (SCA) is a disorder due to a single mutation in the β-globin gene, leading to the formation of hemoglobin S (HbS). HbS exhibits a marked decrease in solubility, and an increase in viscosity and polymer formation. Ischemic stroke, caused by large vessel arterial obstruction with superimposed thrombosis is one of SCA's common complications, along with painful episodes, and acute chest syndrome. Blood transfusions are administered to prevent thrombosis, but they are associated with significant risks of iron overload and other complications, and must be accompanied by iron chelation (Gardenghi, Grady, & Rivella, 2010; Musallam, Cappellini, Wood, & Taher, 2012).

β-thalassemia is caused by one or more of over 300 various mutations in the β-globin gene, which cause reduced (β^+) or absent (β^0) synthesis of the β globin chains of the HbA. Hematological and other features increasing severity of β-thalassemia are related to the extent of imbalance between α and β-like globin chains. The excess α-globin content in erythroid cells combine in insoluble hemichromes that damage cell membranes, while their heme component leads to the formation of toxic reactive oxygen species (ROS) and increased oxidative stress, resulting in ineffective erythropoiesis and apoptosis in the erythroid lineage (Camaschella & Cappellini, 1995; Rivella, 2015).

Based on the inherited mutations, patients might be affected by a milder form, indicated as β-thalassemia intermedia (TI) or the most severe form, β-thalassemia major (TM) (Musallam et al., 2012; Taher et al., 2013). TM requires regular transfusions to sustain life. Due to the negative progression of this disease, very often TI patients become transfusion dependent as well. Chronic blood transfusion requires co-treatment with iron chelators to prevent an increase in plasma iron levels and formation of non-transferrin bound iron (NTBI). Iron overload is observed also in untransfused patients because of ineffective erythropoiesis and for some patients it represents the first source of morbidity and mortality (Dong, Breda, & Rivella, 2014).

β-thalassemia patients develop hepato-splenomegaly, recurrent infections, and spontaneous fractures. Transfusion-associated infections and organ damage are side effects of long-term treatment and unsatisfactory iron chelation (Gardenghi et al., 2007; Musallam et al., 2012). Ineffective erythropoiesis triggers a cascade of compensatory mechanisms resulting in erythroid marrow expansion, extramedullary hematopoiesis, splenomegaly, and increased gastrointestinal iron absorption. Ineffective erythropoiesis triggers increased iron absorption by

reducing the expression of hepcidin, the hormone that controls dietary iron absorption (Ginzburg & Rivella, 2017; Parrow et al., 2014).

Although both transfusion and iron chelation treatments have remarkably improved over the years and, thus, improving the quality of life of patients with β-globinopathies, they do not provide a definitive cure, as they do not address the inherent genetic cause. To this end, hematopoietic stem cell (HSC) transplantation is the only presently available cure. Allogeneic bone marrow transplant (BMT) can be curative, but only a small proportion of patients have suitable donors. Furthermore, myeloablative HSC transplantation carries a 5%–10% mortality rate. Graft-vs-host disease and adverse immune reactions can limit the success of allogenic BMT as well (Angelucci, 2010).

Given these limitations, gene therapy using a patients' own HSCs represents an alternative and potential cure because it aims at the direct recovery of the hemoglobin protein function via the addition of a functional copy of the β- or γ-globin gene.

Gene therapy for β-globinopathies, particularly β-thalassemia and SCA, holds promise for the future as a definitive corrective treatment for these common and debilitating disorders. The development of this approach has been the object of research of the last few decades and has been proved successful in mouse model studies, *in vitro* human cell studies (Breda et al., 2012; Finotti et al., 2015). Correction of thalassemic mice was first shown in early 2000s (May et al., 2000) through delivery of the human β-transgene via a LV vector. Many different vectors with a wide-range of genetic elements have proven successful in preclinical studies and have resulted in the correction of animal models for globin diseases. Recent clinical trials show promising results in patients with TM (Jorge Mansilla-Soto,

Isabelle Riviere, Farid Boulad, 2016; Marina Cavazzana, MD, 2016; Miccio et al., 2008; Perumbeti & Malik, 2010; Roselli et al., 2010; Thompson Alexis A., MD, 2016)

Alternative splicing of the β-globin

Some β-globin mutations create new cryptic splice sites and, even though the original splice sites are intact, lead to incorrect splicing. Such mutations activate aberrant splice sites and change the splicing pathway even though the correct splice sites remain potentially functional (Sierakowska, Sambade, Agrawal, & Kole, 1996).

IVS-2-745 is a C→G mutation at position 745 within intron 2 that generates an aberrant splice site. This mutation creates an aberrant 5' splice site at nucleotides 745 of intron 2 and activates a common cryptic 3' splice site at nucleotide 579 within the same intron (Figure 1). Portions of the intronic sequence between the newly activated splice sites are recognized by the splicing machinery as exons and are retained in the spliced mRNA. The retained exon-like sequence of 165 nts generates a stop codon that prevents proper translation of the mRNA and causes a deficiency in β-globin leading to β-thalassemia (Gemignani, Sazani, Morcos, & Kole, 2002). Splicing of mutated β-globin pre-mRNA is shifted either completely or partially to aberrant pathway. The IVS-2-745 is a β+ allele, whose correct splice sites remain potentially functional and can produce some correctly spliced β-globin mRNA and consequently HbA.

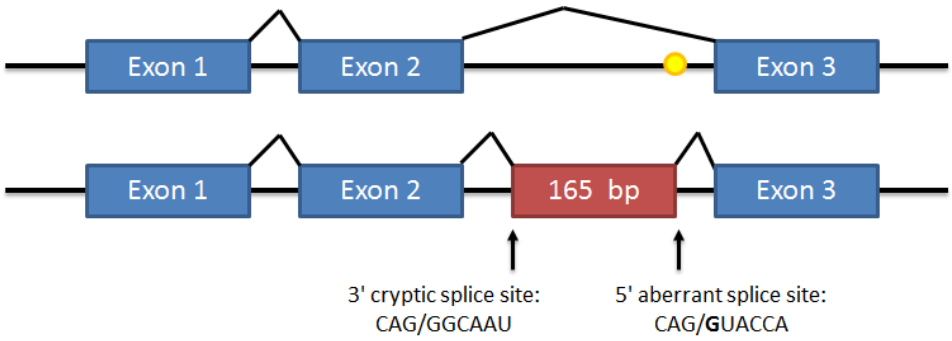

Figure 1. Splicing of wt human β-globin and aberrant splicing on mutant IVS-2-745. IVS-2-745 C→G mutation (in bold) generates an aberrant 5' splice site at nucleotides 745 (indicated by the yellow circle) of intron 2 and activates a cryptic 3' splice site at nucleotide 579. This causes the inclusion of an 165 bp- extra exonic sequence in the aberrant pre-mRNA

The IVS-2-745 allele is relatively common in the Mediterranean area, reaching as high as 15-20% of thalassemia mutations in regions of Spain, Jordan, Romania, and Serbia (Kountouris et al., 2014). A 745 mutation in a homozygous or a compound heterozygous state with other β-globin mutations results in a wide spectrum of phenotypes from non-transfusion dependent thalassemia (NTDT) to transfusion dependent thalassemia (TDT).

Any increase in synthesis of functional hemoglobin (whether adult or fetal) and decrease in transfusions would improve the patient's anemia and co-morbidities, with dramatic impact on the quality of life.

Switch Splicing Oligos to restore canonic splicing

Since defective β-globin genes as the IVS-2-745 preserve correct splice sites, an approach to use SSOs to hide the aberrant splice site and restore splicing to the original sites has been developed. A SSO has to achieve the following goals:

a) it must bind to the aberrant splice site and prevent it from being recognized by the splicing machinery

b) the duplex it creates must not be recognized by RNaseH, as to prevent degradation of the RNA.

Chemical modification increase stability of SSOs

Antisense oligonucleotides are 8 to 50 nucleotides in length that, *in toto* or in part, bind to RNA through Watson-Crick base pairing and upon binding to RNA, modulate the function of the targeted RNA. This definition includes a wide variety of oligonucleotide designs that modulate RNA through a diverse set of post binding mechanisms.

Because of the inherent instability of the phosphodiester linkage to nucleases, the oligonucleotide backbone presents an obvious first target for improvement with chemical modification (Figure 2). Phosphorothioate (PS)-containing oligonucleotides were one of the earliest and remain one of the most widely used backbone modifications for antisense drugs. The PS linkage greatly increases stability to nucleolytic degradation, and moreover they possess sufficient stability in plasma, tissues, and cells to avoid metabolism prior to reaching the target RNA.

After the PS-oligo, Peptide Nucleic Acids (PNAs) were generated, a radically different class of oligonucleotide. These contain a peptide replacement for the

sugar phosphate backbone, yet maintain both the ability to base pair with complementary RNA and DNA (Egholom M, Buchardt O, 1993) and a high resistance to degradation by nucleases and proteases (Nielsen, Egholm, Berg, & Buchardt, 2017). PNAs do not readily cross cell membranes, but their activity was observed in adipose tissue. Subsequently, modifications to the 2'-position of the sugar moiety have provided the most value in enhancing the drug-like properties of oligonucleotides as for 2'-O-methyl (2'-OMe).

Previous studies have demonstrated the feasibility to synthesize HbA in vitro with 2'-OMe Splice-Switching Oligonucleotides (SSOs) on HeLa and 3T3 cells (Sierakowska et al., 1996). The morpholino chemistry, which replaces the sugar moieties with morpholine rings, was used, in studies on thalassemic specimens (Lacerra et al., 2000). Morpholino oligos have poor prospects in clinical applications alone though, and need to be further chemically modified and conjugated with a cell-penetrating peptide for successful *in vivo* delivery (Svasti et al., 2009).

The 2'-MOE chemistry adds a methoxyethyl group to the 2' position of the ribose. It is currently the most advanced of the 2'-modified series and has entered clinical trials for multiple indications (Vickers, Wyatt, Burckin, Bennett, & Freier, 2001). 2'-MOE increases Tm by about +2°C per modification versus RNAs, relative to DNAs, and greatly increases resistance to nucleases. It also appears to reduce certain nonspecific protein binding, which can reduce toxicities. 2'-MOE oligonucleotides have unique structural features evident from structural studies that help explain the properties of 2'-MOE oligonucleotides (Teplova et al., 1999). 2'-MOE substitution at the 2'-position induces a C3'-*endo* (northern) conformation of the sugar and assumes a *gauche* orientation that traps water in a shell of hydration that includes the adjacent phosphate residue. This further increases rigidity of the C3'-*endo* sugar conformation, and the organization of the oligonucleotide into an

A-form geometry is thought to largely account for the increased affinity for the RNA. The increased nuclease resistance is most likely due to steric hindrance imparted by the 2'-MOE substituent combined with the shell of hydration created by the bound water. These attributes have translated from the test tube to human, resulting in numerous 2'-MOE modified antisense drugs entering the clinic (Bennett & Swayze, 2010).

For our study, we chose the uniform 2’-O-methoxyethyl. Uniformly distributed 2'-MOE SSOs do not mediate RNase H degradation when they bind their targets, which may be due to the steric hindrance conferred throughout the oligo by the methoxyethyl group. Safety studies of 2'-MOE oligos show they are well tolerated in multiple species from rodents to non-human primates; thus, they make an attractive setting for clinical applications. Over thirty compounds are currently being tested in clinical trials for various indications including cancer and cardiovascular, metabolic, and neurological diseases. Two examples are Mipomersen and Nusinersen: Mipomersen (marketed as Kynamoro) is a 2'-MOE antisense oligo that was approved by FDA for the treatment of familial hyper-cholesterolemia (Bennett & Swayze, 2010). Spinraza^{TM} (Nusinersen), a uniformly modified 2'-MOE SSO, has recently being approved by FDA after multiple phase 3 clinical trials to treat spinal muscular atrophy (SMA). Early studies have provided encouraging evidence that the 2'-MOE chemistry of Nusinersen treatment significantly improve motor neuron function in patients with SMA, through assessment of safety, tolerability and pharmacokinetics. (Chiriboga et al., 2016; Finkel et al., 2016).

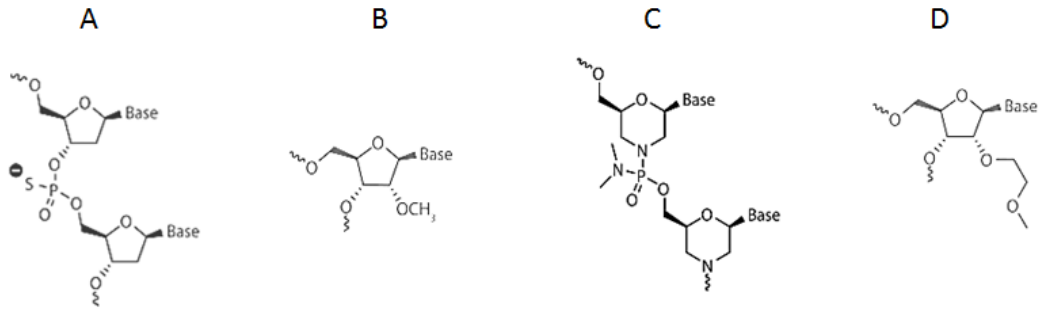

Figure 2. Examples of chemical modifications used in antisense oligonucleotides. (A) PS with the substitution of sulfur for oxygen in the phosphate ester. (B) 2'-OMe with the methoxy substitution on position 2' of the sugar. (C) Morpholinos have the sugar moieties replaced with morpholine rings. (D) 2'-MOE have a methoxyethyl group in the oxygen 2'.

Here, we demonstrate that uniform 2'-MOE SSOs are effective in treating erythroid cells from thalassemia patients without the requirement of additional chemical modification: they induce splice switching and HbA production. Furthermore, 2'MOE-SSOs alleviate other previously unstudied thalassemic cell parameters, such as a rebalancing of the stoichiometry of α to β chains, a reduction of toxic α aggregates, and the correction of erythrocyte deformities.

Methods

Human Ethics

Patients were recruited and samples obtained according to the Declaration of Helsinki, following approvals by the (A) Institutional Ethics Committee of the Fondazione Istituto di Ricovero e Cura a Carattere Scientifico Ca' Granda Ospedale Maggiore Policlinico, Milan, #391/2012 for P1, P2, and P4 (B) Cyprus National Bioethics Committee, National Grant ΕΕΒΚ/ΕΠ/2012/05, E.U. Grant ΕΕΒΚ/ΕΠ/2013/23 for P3 and P5 and (C) Children's Hospital of Philadelphia, Institutional Review Board (IRB) #15-012123 for S1. S2 was obtained during automated red cell exchange as part of routine clinical care; as S2 was unlinked and de-identified medical waste, the Montefiore Medical Center IRB deemed it IRB-exempt. All subjects gave their informed consent prior to their inclusion in the study.

Statistics

For three or more groups, we compared means with a one-way ANOVA test (for samples with normal distributions and equal variances by the Shapiro-Wilks normality test) or medians with a non-parametric Kruskal-Wallis test. For two groups, we compared means with a t-test (for samples with normal distributions by the Shapiro-Wilks normality test) or medians with a Mann-Whitney test. All tests were done using GraphPad Prism software, version 7.

MEL Cell Culture

MEL cells were grown under a 5% CO_2 atmosphere in RPMI 1640 medium (GIBCO, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (GIBCO,

ThermoFisher Scientific) and 100 U/ml penicillin 100 mg/ml streptomycin (GIBCO, ThermoFisher Scientific).

Erythroid differentiation was induced by adding *N*,*N*'-Hexamethylene bis-acetamide) (HMBA) (Sigma Aldrich) to the final concentration of 5 mM (Reuben, Wife, Breslow, Rifkind, & Marks, 1976). Cells were harvested after 7 days of HMBA-mediated induction for mRNA and hemoglobin content analyses.

Human Erythroblast Cultures and Treatment

Whole blood underwent CD34+ selection using immunomagnetic separation (Miltenyi Biotec Inc., Auburn, CA). CD34+ cells were kept undifferentiated in phase 1 media with bi- or tri-weekly media changes, centrifugation to remove both dead and spontaneously differentiating cells and by keeping cell density <0.5e6 cells/mL. After 8-10 days, cells were frozen in freeze media. Cells were thawed in thaw media, centrifuged 10' at 200g to remove dead cells, and resuspended in phase I. After 12 total days in phase I, cells were transferred into phase II media or a 2:1 mix of phase I and phase III media.

SSO treatment occurred on day 14 using 1-2e6 cells/condition; 5 µM dose was 4.5nmoles/1e6 cells. For syringe loading, cells were resuspended at 1e6/100µL, passed 10 times through a 25 gauge needle, and then kept at for 1 hr at 37ºC. Lipofectamine (ThermoFisher) was used according to the manufacturer's protocol, with 150µL Opti-MEM (ThermoFisher) and 6µL lipofectamine/1e6 cells. Following SSO treatment, cells were plated at 1e6 cells/mL in phase III media. Collection occurred on day 20 for all analyses. Toxicity was assessed by trypan blue staining and level of differentiation was assessed by benzidine staining (Breda et al., 2010).

CD34+ media: Phase 1: serum-free StemSpan with 10µL/mL CC-100 cytokine cocktail (both from Stemcell Technologies), 2 U/mL Erytrhopoietin (Amgen), 10−6 M dexamethasone (Sigma) and 100 U/ml penicillin 100 mg/ml streptomycin (GIBCO, ThermoFisher Scientific). Freeze Media: 50% characterized FBS (Hyclone), 10% DMSO (Sigma), and 40% Iscove's Modified DMEM (Cellgro, Manassas, VA,). Thaw Media: Iscove's Modified DMEM with 5% characterized FBS. Phase 2: Iscove's Modified DMEM with 3% AB serum (Atlanta Biologicals), 2% Human Plasma (Stemcell Tech), 10ug/mL Insulin (Sigma), 3U/mL Heparin, 200ug/mL Transferrin (Athens Research & Technology), 10ng/mL SCF (Peprotech), 3U/mL EPO (Amgen). For syringe loading, cells were resuspended at 1e6/100µL, passed 10 times through a 25 gauge needle, and then kept at for 1 hr at 37ºC. Following SSO treatment, cells were plated at 1e6 cells/mL in phase III media. Phase III media: same as Phase II, except for 1mg/mL transferrin.

RNA, PCR, and Quantitative PCR (Q-PCR)

Total RNA was isolated using Trizol (ThermoFisher). Retrotranscription of total mRNA was done using the SuperScript™ III First Strand Kit (ThermoFisher). PCR reactions were performed with the following primers:Fw: 5'-GGCAAGGTGAACGTGGATGAAGTT -3'; Rev: 5'-TAGGCAGAATCCAGATGCTCAAGG-3'. Q-PCR reactions were performed using the ABI 7900HT or Viia7 systems (Applied Biosystems), with either TaqMan (TaqMan PCR 2X Master Mix from ThermoFisher) or SYBR Green (Power SYBR from ThermoFisher or iTaqTM SYBR® Green Supermix from Bio-Rad) chemistry. Quantitative real-time PCR assays of globin, GAPDH, and glycophorin-A transcripts were carried out using gene-specific double fluorescently labeled probes. The following primer and probe sequences were used (forward, reverse and probe, when used, of each gene, respectively): WT correctly spliced β-

globin primers=Fw: 5′-CACCTTTGCCACACTGAGTGA-3′; Rev: 5′-GCCCAG GAGCCTGAAGTTCT-3′; 5′-FAM-CACTGTGACAAGCTGCACGTGGATCC-IOWA BLACK-3. The following TaqMan inventoried Gene Expression assays from Thermo Fisher were used: GAPDH: Hs02758991_g1; GYPA: Hs00266777_m1. Q-PCR results of WT β-globin were normalized by GAPDH to control for the total amount of cDNA and GYPA to control for the level of differentiation across samples. For PCR of human β-globin cDNA, the following primers were used: Fwd 5′-GTGCGAGAGCGTCAGTATTAAG-3′, Rev 5′-TCCCTGCTTGCCCATACTA-3′.

Tetrameric and single chain analysis by direct- and reverse-phase high performance liquid chromatography (HPLC)

Cell pellets were disrupted with Cytobuster (EMD Millipore) for single chain analysis and with water for tetramer analysis. For tetrameric analysis supernatant hemolysates were loaded into a System Gold 126 Solvent Module instrument (Beckman Coulter). Hemoglobines were separated on a weak cation-exchange PolyCAT A column (PolyLC), and detected at a wavelength of 415 nm. The Hb were bound to the column with mobile phase A (20 mmol/L Bis-Tris, 2 mmol/L KCN, pH 6.96) and eluted with mobile phase B (20 mmol/L Bis-Tris, 2 mmol/L KCN, 200mmol/L NaCl, pH 6.55). Single chain quantification was assessed by reverse-phase HPLC. Hemoglobin samples were injected on a Hitachi D-7000 HSM Series apparatus (Hitachi Instruments) using a Zorbax 5 µm 300SB-C8 300 Å, LC 150 x 2.1 mm column (Agilent Technologies) and a gradient from 20% to 60% acetonitrile in 0.1% trifluoroacetic acid in 25 minutes, with UV detection at 215 nm. Standards of HbA, HbF, HbS, and HbC were injected (Analytical Control Systems) and used to determine various hemoglobin peak types (Breda et al., 2010).

Vector production and titering

The human β-globin sequence was mutagenized with the IVS-2-745 β-globin sequence, creating a lentiviral vector expressing human IVS-2-745 β-globin. Viral stocks were generated by co-transfection of the IVS-2-745 β-globin plasmid together with the envelope plasmid (VSV-G), the packaging plasmid (pMDLg/p RRE), and the pRSV-REV plasmid into 293T cells (Lacerra et al., 2000). An aliquot (5e6) of 293T cells was seeded into cell culture dishes (10 cm) 24 hours prior to transfection in Iscove's modification of Eagle's medium (Cellgro, Manassas, VA, Manassas, VA) with 10% fetal bovine serum, 100 U/ml penicillin 100 mg/ml streptomycin (GIBCO, ThermoFisher Scientific), at 37°C under 5% CO_2. The culture medium was changed 2 hours prior to transfection. The precipitate was formed by adding the plasmids to 450 uL of 0.1× TE (0.1× TE is 10 mM Tris plus 1 mM EDTA) and 50 µL of 2 M $CaCl_2$, then adding 500 µL of 2× HEPES-buffered saline (281 mM NaCl, 100 mM HEPES, 1.5 mM Na_2HPO_4) drop wise after which the precipitate was vortexed and immediately added to the cultures. The medium (10 ml) was replaced after 16 hours. Viral supernatants were collected at 24 and 48 hours, cleared by low speed centrifugation, and filtered through cellulose acetate (0.2 µm). Following ultracentrifugation, serial dilutions of concentrated virus (5; 0.5 and 0.05 µL, respectively) were used to infect 1e5 NIH 3T3 cells (ATCC, Manassas, VA) in 1 mL of transfection buffer complemented with polybrene (Millipore, Billerica, MA) at a final concentration of 8 µg/mL. Genomic DNA was extracted after 3 days using phenol-chloroform-isoamylic alcohol. The multiplicity of infection (MOI) was calculated using the following formula: number of cells (1e5) X dilution factor (1 mL/µL viral preparation) X VCN (measured via real-time PCR, using oligos for WPRE element and Transferrin Receptor gene, see copy number determination) (Breda et al., 2016).

In vitro RBC sickling and morphological analysis

CD34+ cells were transduced with in expansion (phase I media). 2-3 days later, cells were put in phase II media and genomic DNA was extracted. Copy number was determined as below. SSO treatment with 50 µM scramble or 50 µM 91 oligo occurred 2-3 days after the start of phase II; cells were then placed for 6 days in phase III before harvest. We assessed the degree of cell sickling in our experimental samples using previously reported methodology, with slight modifications (Breda et al., 2016). Briefly, 0.5-1 million cells were suspended in isotonic Hemox buffer (TCS Scientific Corp, Southampton, PA), pH 7.4, supplemented with 10 mM glucose and 0.2% bovine serum albumin, in individual wells of a Costar polystyrene 96-well microplate (№ 9017; Corning, Corning, NY). The microplate was then transferred to a Thermomixer R shaker-incubator (Eppendorf), and maintained under hypoxia (Nitrogen gas), with continuous agitation at 500 rpm, at 37° C for 2 hours. At conclusion, aliquots of each sample were collected in 2% glutaraldehyde solution for immediate fixation without exposure to air. Subsequently, fixed cell suspensions were introduced into specialized glass microslides (Dawn Scientific, Inc., Newark, NJ) (Deng et al., 2014) for acquisition of bright field images (at 40x magnification) of single layer cells on an Olympus BX40 microscope fitted with an Infinity Lite B camera (Olympus) and the coupled Image Capture software.

Copy number determination

The number of integrations (VCN) was quantified by Q-PCR using Oligos for (Fw: 5'-GTGCGAGAGCGTCAGTATTAAG-3'; Rev: 5'-TCCCTGCTTGCCCATACTA-3') for a specific sequence present in the vector (GAG) and compared it to an endogenous control present in two copies within the genome (mouse Transferrin=Fw: 5'-TGTTGTAGTAGGAGCCCAGAGAGA-3'; Rev:5'-AGACCTGTTCCCACACTGGACTT-3';

human ID-1=Fw: 5’-AAGGTGAGCAAGGTGGAGATTC-3’; Rev: 5’-TTCCGAGTTCAGCTCCAACTG-3’).

Results

We screened a platform of SSOs developed by Ionis Pharmaceutical. These oligos are around 20 bp length, fully or partially complementary to the aberrant splice site of the IVS-2-745 β-globin gene.

As a prescreening tool, we used an artificial cell model, generated by introducing the human IVS-2-745 β-globin gene in Murine Erythroleukemia (MEL) cells (FRIEND, 1957) with a lentiviral vector, named AnkCT9W-745. This allowed us to detect both the human WT and 745 β-globin mRNA expression in MEL that otherwise only express murine β-globin genes. As a control, we also infected MEL cells with a lentivirus that carries the wt human β-globin gene, named AnkCT9W.

The direct-phase HPLC profile of non-treated MEL cells indicate the synthesis of two distinct adult hemoglobin tetramers, corresponding to mouse β-major and mouse β-minor hemoglobins (Figure 3A). Instead, MEL cells transduced with AnkCT9W presented an additional hemoglobin tetramer: a chimeric hemoglobin product of 2 mouse-α and 2 human-β chains (mα/hβ) (Figure 3B). This same vector was mutagenized as to create AnkCT9W-745, which express the human β-globin with the IVS-2-745 mutation. In MEL cell treated with AnkCT9W-745 the chimeric hemoglobin mα/hβ was almost undetectable (Figure 3C), due to the aberrant β-globin mRNA generated from this transgene.

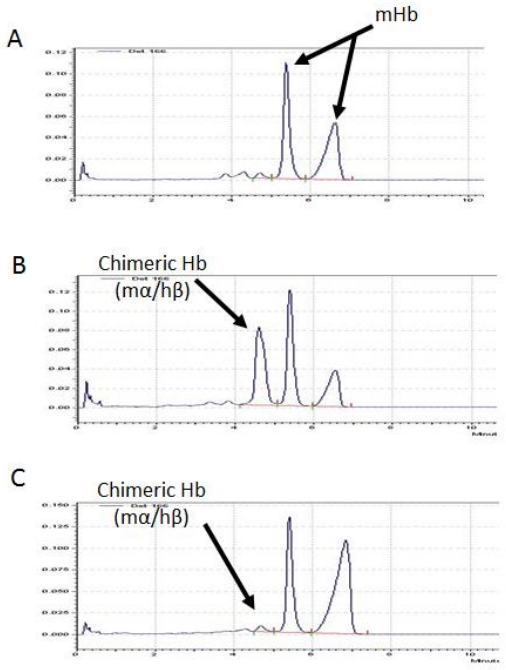

Figure 3. HPLC Analyses of murine Hbs tetramers of two α-chains associated with two β minor/major chains. Chromatographic profile of (A) untransduced MEL cells, (B) MEL cells infected with AnkCT9W β-globin lentiviral vector, and (C) MEL cells infected with AnkCT9W-745 β-globin lentiviral vector.

Pools of MEL cells carrying the IVS-2-745 mutation were treated with 11 different candidates SSOs and compared to untreated cells. Oligo treatment was performed on day 3 of differentiation as described from Reuben et al., 1976. SSOs were delivered by syringe loading (Clarke & McNeil, 1992) and at the end of differentiation, quantification of aberrant versus WT β-globin mRNA were assessed by Q-PCR.

We selected the 3 most effective SSOs among the panel of 11 different candidates. These were SSOs named 671991, 671992, 671993 (renamed 91, 92, and 93), which showed the highest reduction of human aberrant β-globin expression and highest levels of human WT β-globin expression (Figure 4). Concurrently, these oligos restored in MEL cells carrying the 745 mutation a production of chimeric

hemoglobin. The treatment with the SSO 91, 92 and 93 leads a production of mα:hβ of respectively 24%, 19% and 16% of the mα:hβ produced in the MEL transduced with AnkCT9W. A 2'-MOE antisense oligo not targeting any region was used as scramble to shown no correlation between treatment and hemoglobin expression. (Figure 5). Compared to untreated cells, MEL cells that received oligo treatment presented up to 3 fold more chimeric Hb. This indicated that the canonic splicing was restored by oligo treatment. Based on these results we identified oligo 91, 92 and 93 as top candidates to revert aberrant splicing in human cells derived from patients with the IVS-2-745 β-globin mutation.

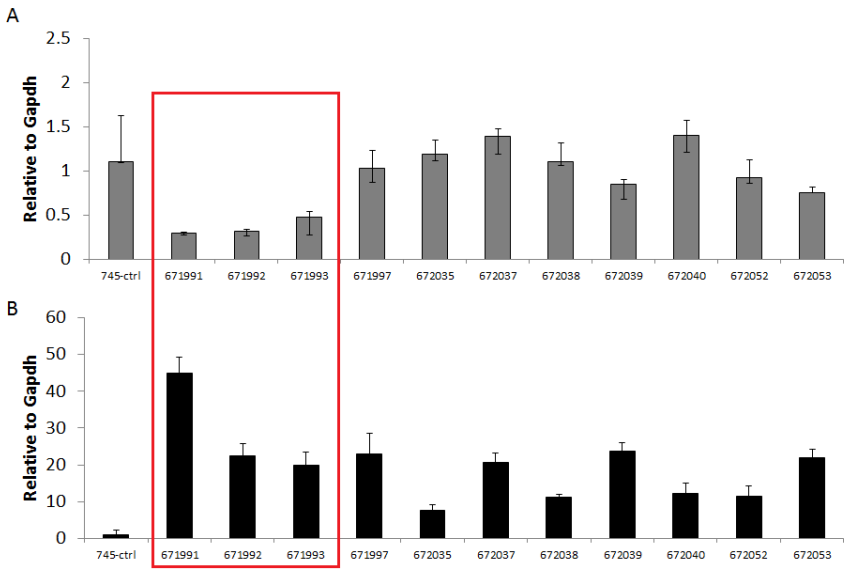

Figure 4. Expression of β-globins in MEL cells after SSOs treatment. (A) IVS-2-745 β-globin mRNA expression, (B) WT β-globin mRNA expression. Values of both globins are normalized by mouse Gapdh expression. Data acquired and analyzed by Q-PCR.

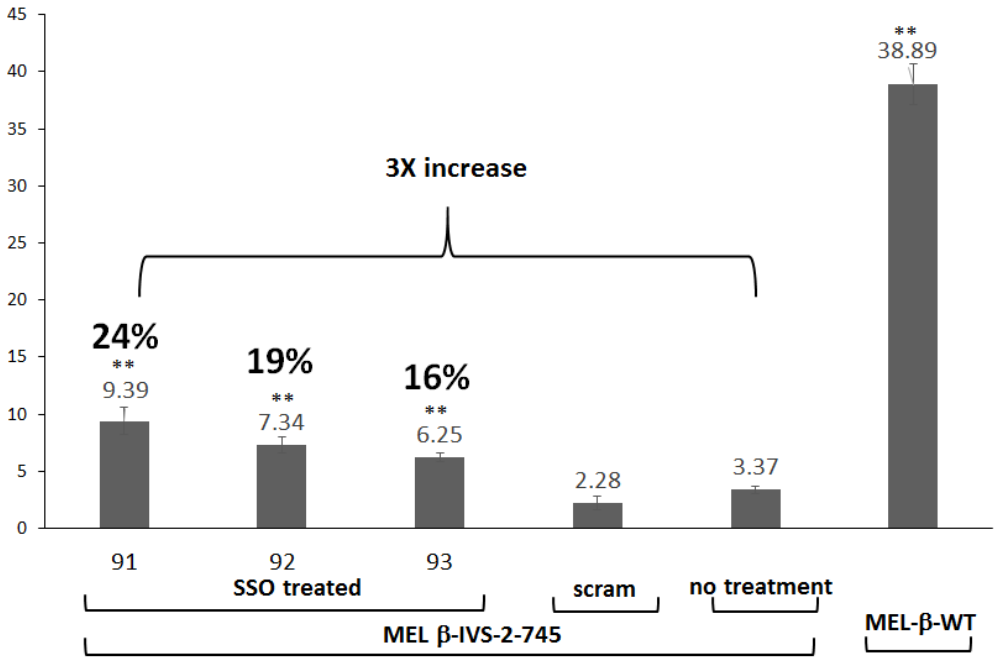

Figure 5. Chimeric hemoglobin (mα/hβ**) content in MEL cells treated with oligos 91, 92 and 93.** Compared to no treated MEL β-IVS-2-745, there is a 3 fold increase in HbA production. All statistics (ANOVA/Kruskal-Wallis) are compared to scramble control. NS=not significant *p<0.05, **p< 0.01 ***p< 0.001, ****p< 0.0001. Data acquired by HPLC.

2'MOE-SSOs induce splice switching restoring adult hemoglobin (HbA) production in cells from β0/β-745 heterozygote patients

We isolated via immunobeading hematopoietic stem cells (HSCs) derived from peripheral blood mononuclear cells from patients' blood. Consequently, HSCs were expanded and differentiated via a three-phase culture system, elaborated from a modified version of the two-phase liquid culture system (Breda et al., 2012). A complete description of the culture system is described in the Methods section, under the paragraph named "Human Erythroblast Cultures and Treatment". On day 3 of differentiation the SSO treatment occurred by syringe loading as described

by Clarke & McNeil, 1992. The differentiated erythroblasts were collected at completion of phase III.

Upon treatment with these oligos, we observed that differentiated IVS-2-745/β0 erythroblasts were able to undergo splice reversal at a dose as low as 5 µM. As expected, the scramble treated samples exhibited alternative splicing: both the longer 745 aberrant and the shorter WT mRNA forms were detected by electrophoresis (Figure 6). Overall, both oligo 92 and 93 were effective but the most significant effects were seen with oligo 91. The treatment with the 5µM dose (Figure 6A) showed a strong decrease of the 745 alternative splicing in specimen P1, most prominent with oligo 91. Based on this result, P2 specimen was treated with an additional 25 µM dose, which decreased aberrant mRNA form further, therefore indicating a dose-dependent response to the oligos (Figure 6B). Given the high cell viability seen up to a 25 µM dose, P3 and P4 specimens were treated with a 50 µM dose (Figure 6C and 6D). At this dose, we observed a maximization of splicing reversal and the 745 alternative mRNA is barely detectable, suggesting that the three oligos reach maximum effect at this concentration. The high WT mRNA signal in all the scramble treated specimens is most likely due to the higher stability of the wt compared to the aberrant mRNA form (Gemignani et al., 2002). Furthermore, we used primers that amplified both transcripts in the same RT-PCR reaction, which is a semi-quantitative method and does not necessarily reflect the absolute content of the two species in the samples. These results were obtained without affecting cell viability, as previously reported in other studies based on the use of 2'MOE (Disterer et al., 2014). We observed no significant difference on cell differentiation in most of the treatments (Figure 7).

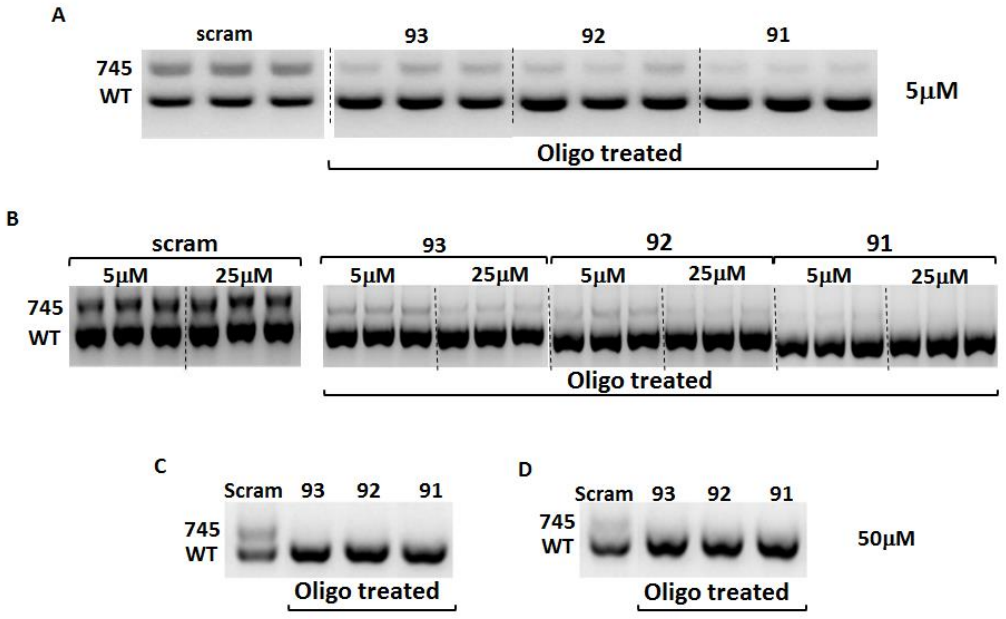

Figure 6. Electrophoresis of cDNA assayed by standard PCR for wt and 745 β-globin mRNA content. (A) P1 specimen treated with scramble and with the three oligos at a 5 µM dose (B) P1 specimen treated with scramble and with the three oligos at a 5 µM and 25 µM dose. (C) and (D) Specimen P3and P4 treated with scramble and with the three oligos 50 µM.

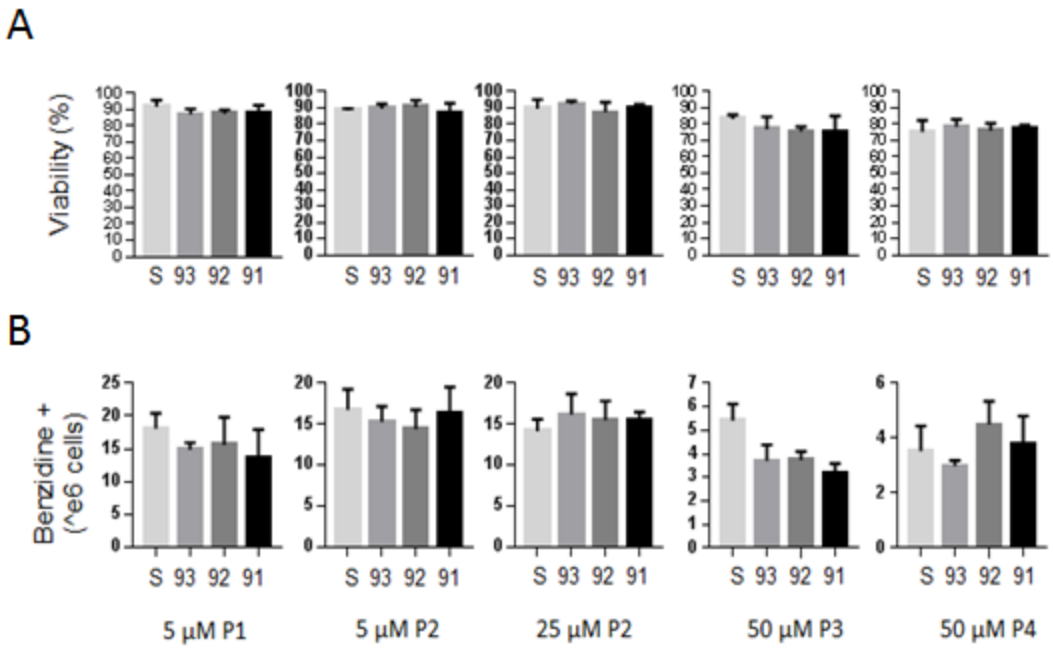

Figure 7. Cell viability and differentiation rate in patients' erythroblasts. (A) Percentage of live/dead cells counted by trypan blue essay (Strober, 2015). (B) Number of cells benzidine-positive

Despite the presence of detectable amount of WT mRNA, the amount of HbA measured by HPLC was very low in untreated specimens (Figures 7A, 8A). Upon oligo treatment HbA levels were increased and a significant reduction of free heme aggregates was observed. Once again, treatment with oligo 91 reached the most robust outcome. In fact, at the 5 µM dose of oligo 91, HbA increased from baseline levels of 2.60 ± 0.17% and 4.65 ± 0.46% to 29.21± 2.16% and 27.94 ± 1.09%, in specimens P1 and P2 respectively. The 50 µM dose induced an HbA increase in specimens P3 and P4 from baseline levels of 4.20 ± 0.75% and 2.97 ± 0.23% HbA to 59.14 ± 1.34% and 60.21 ± 2.61%, respectively. That sums up to a 20-fold increase in HbA in specimen P4. Q-PCR analyses show a similar trend (7B). The amount of correctly spliced WT β-globin mRNA increases up to 100-fold with a 50 µM dose.

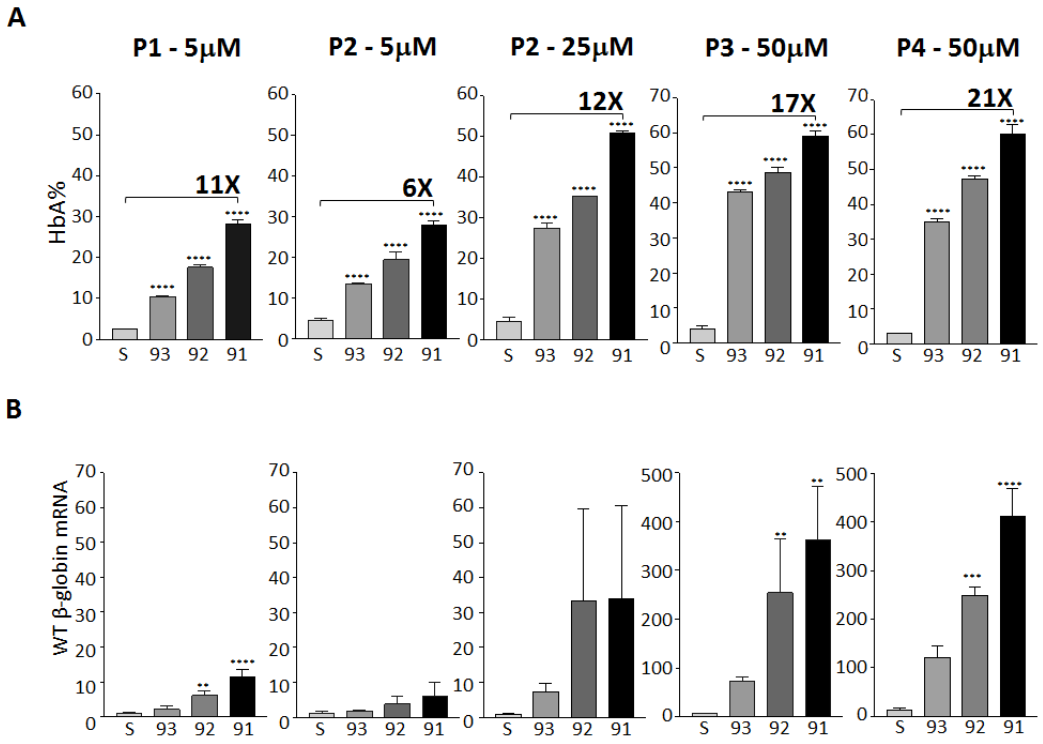

Figure 8. 2'MOE SSOs induce increase of HbA production and WT β-globin mRNA in IVS-2-745/β0 heterozygous sample. (A) % of HbA from cell lysates as detected by HPLC. (B) Q-PCR for only correctly spliced WT β-globin mRNA. Scale indicates relative expression as normalized to the housekeeping gene GAPDH and red-cell specific gene glycophorin A. n=3. S= scramble treated control at same dose. All statistics (ANOVA/Kruskal-Wallis) are compared to scramble control. **p< 0.01 ***p< 0.001, ****p< 0.0001.

The baseline aggregate level in heterozygotic cells was variable across samples, with average levels ranging from 22.00 ± 0.76% in P1 to 4.28 ± 1.12% in P4. In P1, a significant decrease in aggregates was evident across all oligo treatments, and was most significant in oligo 91 at a 54% reduction (Figure 9). In P2, a significant reduction is seen starting at a 25 µM dose. Although baseline levels of aggregates in P3 and P4 are lower, oligos 91 and 93 still elicit a significant aggregate reduction

at a 50 µM dose. Consistent with our previous observations, oligo 91 was the most effective, with as high as an 87% reduction in α-heme aggregates in P3.

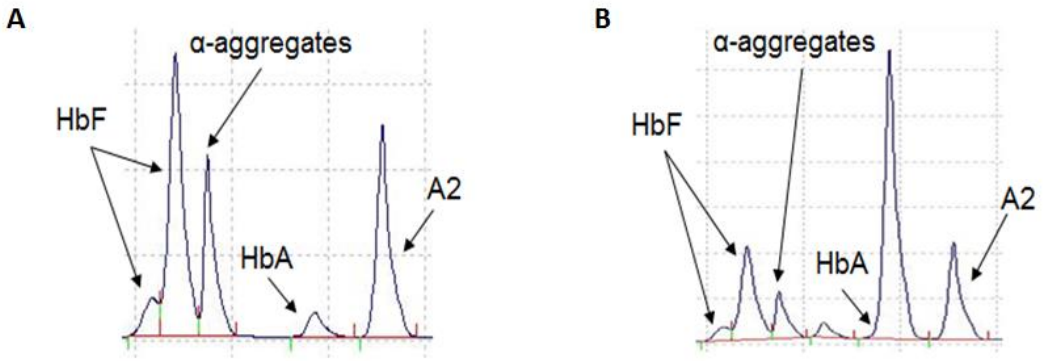

Figure 9. Representative HPLC profile of the separation of hemoglobins in of IVS-2-745/β0 heterozygous sample treated with oligo 91. (A) Presence of α-heme aggregates and low levels of HbA detected in untreated cells from P1. (B) After treatment with oligo 91 the production of adult hemoglobin was increased 11 folds and α heme aggregates were reduced two folds. Fetal and HbA2 levels were unaltered.

2'MOE SSOs show the most robust effects on a 745/745 homozygote sample

After demonstrating a dose-dependent response of oligo treatment, we next sought to determine if there was an "allele dose-dependency", i.e. whether treatment with SSOs had a higher impact on a homozygote sample, with two 745 target alleles, than on a heterozygote sample, with only one 745 target allele. In the specimen homozygous for the 745 mutation (P5) the aberrant and WT mRNA detected by PCR were about 50:50. A homozygote sample produces only 745 mutant pre-mRNA, and thus there could be more target 745 pre-mRNA for the oligos to splice switch than in the case of a single 745 allele. In the homozygous P5 sample baseline level of 6.75 ± 0.22% HbA was higher than in any heterozygous specimen, most likely due to the additive contribution of the endogenous WT

splicing of the two 745 β+ alleles. As hypothesized, the most striking effects of oligo treatment were seen in the 745 homozygote patient cells. The 745 mutant form was almost undetectable by electrophoresis in all treatments (Figure 10A); and there was a 300-700 fold increase in correctly spliced WT mRNA for the oligo 91 treated samples (Figure 10B). All three oligos had the highest effect in this homozygote sample. Oligo 91 produced 79.46 ± 0.94% HbA at a 50 μM dose. The 100 μM dose produced similar results with 77.92 ± 1.04% HbA, indicating the effect of this oligo reaches a protein plateau at a 50 μM dose. Oligo 92 and 93 produced 71.42 ± 1.22% and 58.48 ± 0.32% HbA at a 50 μM dose respectively (Figure 10C). The 100 μM oligo 91 dose in the homozygote led to a 60% reduction in α-heme aggregates. At this dose the effect on the protein production is plateaued. Quantitative PCR data show that at 25μM the effect of these oligos highly enhances and it is maximized at 50μM with a 20 fold increase in HbA production (Figure 10B).

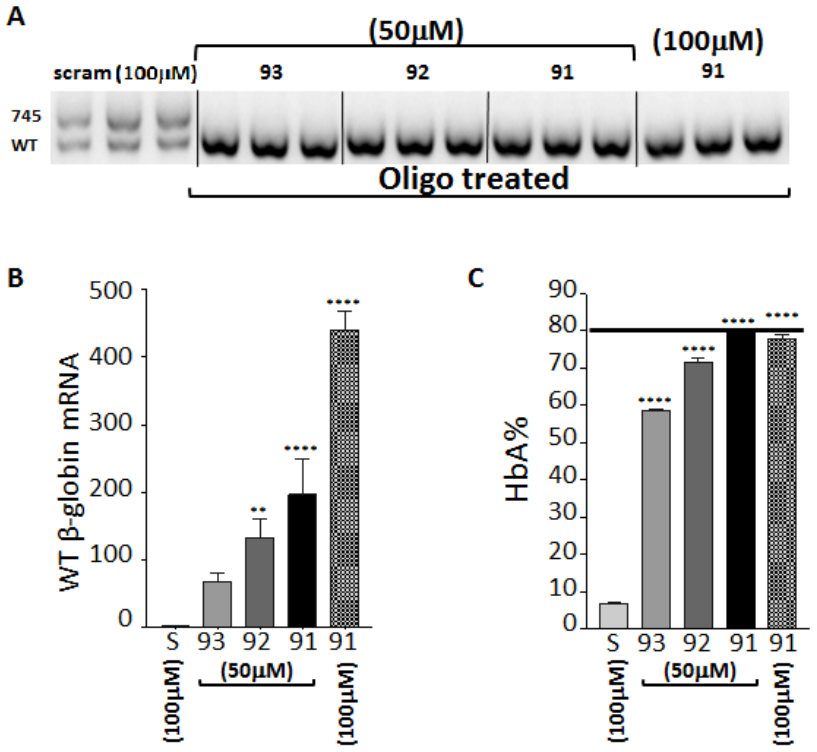

Figure 10. 2'MOE SSOs show the most significant results in homozygous IVS-2-745 patient cells. (A) Electrophoresis of cDNA assayed by standard PCR for wt and 745 β-globin mRNA content in untreated and treated specimens at the 50 and 100 μM dose. (B) Q-PCR analyses of correctly spliced WT β-globin mRNA. Scale indicates relative expression as normalized to the housekeeping gene GAPDH and red-cell specific gene glycophorin A. (C) % of HbA and. n=3 S= scramble treated control at same dose. All statistics (ANOVA/Kruskal-Wallis) are compared to scramble control. **p< 0.01 ***p< 0.001, ****p< 0.0001.

In order to examine the effects of 2'MOE SSO treatment on the α:β globin stoichimetry, cell lysates were separated by reverse-phase HPLC, as described in Methods under paragraph "Tetrameric and single chain analysis by direct- and reverse-phase high performance liquid chromatography (HPLC)". This allowed separation of the α chains from the β-like chains (β, γ, δ). Without treatment, the ratio of α:β-like chains ranged from 65:35 in P1 to 55:45 in P4/P5, all significantly different from a healthy control at 50:50 (Figure 11). Single-chain separation

showed though, that at as low as a 25 µM dose, oligo 91 was able to restore the 50:50 balance of α chains to β-like chains. Treated samples were no statistically different compared to samples isolated from a healthy control.

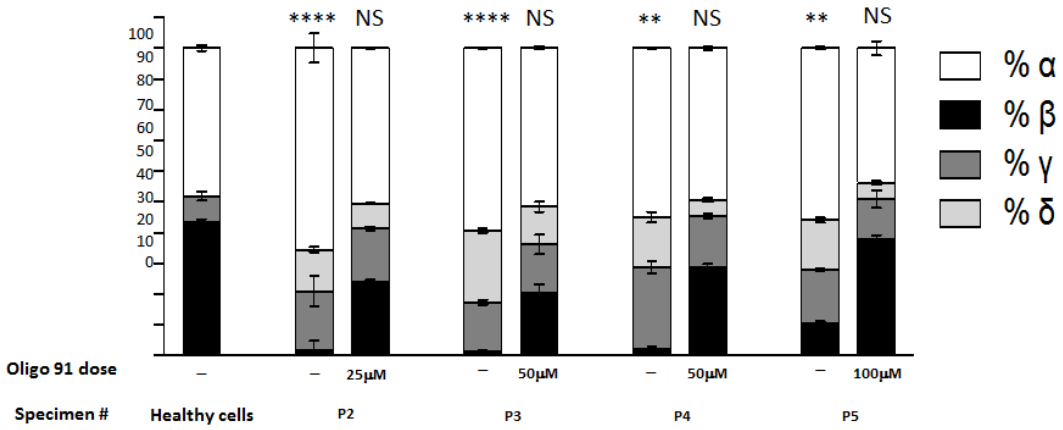

Figure 11. 2'MOE SSO 91 can rebalance the ratio of globins. (A) Single chain analysis of α chains to β-like chains (β, γ, δ) ratio. All statistics (ANOVA/Kruskal-Wallis) are compared to scramble control. n=3. NS=not significant, **p< 0.01, **** p< 0.0001

2'MOE SSOs prevents sickling in samples with a 745/sickle genotype

Lastly, we investigated the effect of these oligos on cells with the 745 mutation in heterozygosity with β-sickle allele, which could greatly benefit from an increased HbA synthesis.

As we could not obtain a patient sample with the 745/sickle genotype, we artificially created a model system, by transducing two homozygous sickle specimens (from patients S1 and S2) with AnkCT9W-745. With vector copy numbers of 2.02 and 1.62 for S1 and S2, respectively, this system replicated a heterozygotic state, as the 2 endogenous sickle alleles were matched by the two 745 alleles. Upon differentiation and exposure to hypoxia, S1 scramble-treated

cells showed prong-like polymers of sickle chains. Oligo 91 treatment of S1 resulted in an increase in HbA from 8.96 ± 1.88% to 59.82 ± 0.23%, and a decrease in sickle hemoglobin (HbS), from 75.34 ± 2.33% to 30.73 ± 0.32%. This increase in HbA at the expense of HbS led to 50% reduction in the sickling effect (Figure 12A). This was reflected in an amelioration in the cell morphology, due to lesser sickling (Figure 12B). These results show that 2'MOE SSOs could be indicated for use in compound heterozygous 745/sickle patients to reverse RBC deformities.

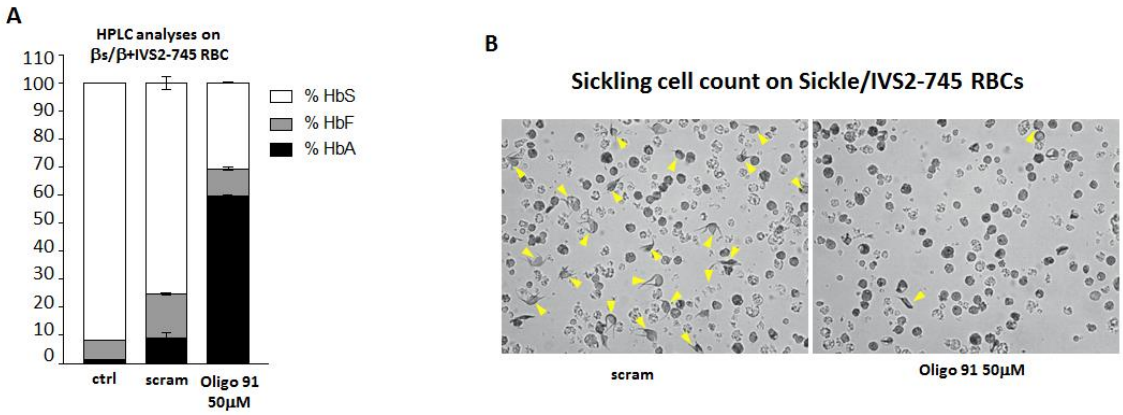

Figure 12. 2'MOE SSO 91 can rebalance the ratio of globins and produce enough functional HbA to prevent sickling. (A) Single chain analysis of the balance of α chains to β-like chains (β, γ, δ). n=3, scram=scramble treated control at same dose. NS=not significant, **p< 0.01, **** p< 0.0001. (B) In vitro sickling assay. 745/sickle cells were treated with scramble or oligo 91, then exposed to hypoxia. Barbed cells with long polymers of pointy sickle chains are indicated by yellow arrows

Discussion and Future Perspectives

2'MOE SSOs have strong therapeutic potential for splice mutants of β-thalassemia. They effectively act at the RNA level where the defect occurs, and lead to restoration of β-globin synthesis in the thalassemic cells. The novel production of β-globin chain leads to HbA production, a coinciding rebalancing of the α and β chains, and a reduction in α-heme aggregates. As demonstrated by the sickle assay, the oligos produce enough HbA to reduce formation of sickle cells.

When speculating on the clinical potential of 2'MOE SSOs *in vivo*, we can examine the data of two previously reported patients with a 745/sickle genotype (Gonzalez-Redondo et al., 1988). These patients expressed 4.8% HbA and their combined HbA/HbF/HbS levels average 8.6g /dL, therefore 0.41 g/dL is HbA from the correctly spliced single endogenous 745 allele. If, as shown in our study, 2'MOE SSOs were able to induce up to a 20-fold increase in HbA – this would translate to over an 8 g/dL increase. As 9 g/dL is transfusion-independent, these oligos could very feasibly help patients achieve transfusion independence. Transfusion independence is a key achievement factor, as transfusion is a contributing factor to iron overload and multiple organ damage (Musallam et al., 2012).

This is of course contingent on toxicity studies and drug delivery studies. As previously stated, 2'MOE show a favorable tolerability. We observed no significant difference in viability or differentiation for almost all of our treatments. If we were to look toward the future though, a side-by-side study would need to be done on multiple patient samples across the doses. For our study, the 5 µM and 25 µM dose were used on the same patient and produced no significant differences. However, the 50 µM and 20X dose were tested on different patient samples. As these samples were harvested from different patients, at separate time points, by

different collaborators, and shipped separately, we cannot make a true side-by-side comparison. We hope that in the future, easier access to patient databases and additional collaborations could allow such a large-scale study.

Another barrier to clinical translation is the ability to target the oligo to the bone marrow, as 2'MOE oligos accumulate the most in the kidney and liver. The spleen though, is the third highest accumulation area, with concentrations between 20-50% of that in the liver. The distribution within the spleen by cell type has not been well characterized, but data shows the most accumulation in the macrophages. 2'MOEs need physical or chemical manipulation to enter cultured cells. However *in vivo* and some primary cell cultures studies show natural cellular uptake pathways without this manipulation (Bennett & Swayze, 2010). Of course, *in vitro* systems do not always mimic *in vivo* systems, but as a future indication we could co-culture macrophages with our erythroid progenitors to determine if the macrophages are preventing oligo uptake. Per *in vivo*, the lack of an appropriate human 745 β-globin mouse model prevented us from fully executing these studies at this time, but other *in vivo* rodent studies show promise. Recent data suggests that the constrained ethyl (cEt) chemistry in antisense studies is successful at targeting the rat and mouse bone marrow (Peralta R, Yu XX, Katz M, Guo S, Hung G, 2015). The 2'MOE chemistry can potentially be combined with the cEt chemistry for better in vivo delivery. Nonetheless, a conjugated strategy that can direct SSO uptake by erythroid cells would be ideal. The oligo can potentially be conjugated to a protein that has an affinity for erythroid progenitors or the bone marrow to achieve specific targeting.

In summary, 2'MOE-SSOs are promising therapeutic tools for certain splicing forms of β-thalassemia. Their ability to correct the underlying splicing defect offers a pharmacological treatment that is both direct and specific. As such, this therapy

could help patients reduce their transfusion dependence or even reach transfusion independence.

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