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RNA repair restores hemoglobin expression in IVS2–654 thalassemic mice

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Repair of β-globin pre-mRNA rendered defective by a thalassemia-causing splicing mutation, IVS2–654, in intron 2 of the human β-globin gene was accomplished in vivo in a mouse model of IVS2–654 thalassemia. This was effected by a systemically delivered splice-switching oligonucleotide (SSO), a morpholino oligomer conjugated to an arginine-rich peptide. The SSO blocked the aberrant splice site in the targeted pre-mRNA and forced the splicing machinery to reselect existing correct splice sites. Repaired β-globin mRNA restored significant amounts of hemoglobin in the peripheral blood of the IVS2–654 mouse, improving the number and quality of erythroid cells.

oligonucleotides | RNA splicing | thalassemia | therapy | morpholino oligomers

One of the most common inherited diseases, β-thalassemia, is caused by defects in the β-globin gene that affect production of β-globin, a subunit of hemoglobin (1). Current therapy consists of frequent blood transfusions combined with iron chelation (2). The only cure, bone marrow transplantation, is limited by the scarcity of suitable histocompatible donors (3). Although more than 200 mutations cause the disease, some of the most common ones induce aberrant splicing of β-globin pre-mRNA, interfering with proper translation of β-globin protein. The IVS2–654 (C > T) mutation creates an aberrant 5′ splice site and activates a cryptic 3′ splice site within intron 2 of the β-globin pre-mRNA, leading to retention of the intron fragment in the spliced mRNA even though the correct splice sites remain undamaged and potentially functional (Fig. 1*A*) (4). The retained fragment prevents proper translation of β-globin, leading to hemoglobin deficiency and β-thalassemia. The IVS2–654 mutation is a common cause of thalassemia in Thailand and other countries of Southeast Asia (5). Work in this laboratory showed that splice-switching oligonucleotides (SSOs), which block aberrant splice sites in IVS2–654 and other pre-mRNAs (IVS1–5, IVS1–6, IVS1–110, IVS2–705, and IVS2–745) as well as in the coding sequence (HbE) of the β-globin gene, force the splicing machinery to reselect the existing correct splice sites, repairing the splicing pattern of β-globin pre-mRNA. This repair, which restores production of correctly spliced β-globin mRNA and protein, was accomplished in several in vitro systems and ex vivo in erythroid progenitor cells from thalassemic patients (6–12). In this study, we investigated the effectiveness in thalassemic splicing correction of a modified morpholino oligomer, SSO 654-P005, in a mouse model of IVS2–654 β-thalassemia.

Results and Discussion

To be effective in splicing repair, SSOs must hybridize tightly to the targeted splicing elements, such as aberrant splice sites, and prevent their recognition by the splicing factors. Furthermore, the resulting double-stranded structures must not be recognized by RNase H, which degrades RNA in RNA-DNA duplexes (13). These conditions are satisfied in cell culture and in vivo by modified oligomers with various backbones, including 2′-O-methoxyethyl phosphorothioate (MOE), locked nucleic acid (LNA), peptide nucleic acid conjugated with four lysines (PNA-4K), and phosphorodiamidate morpholino oligomers (14–16). However, MOE and LNA SSOs were ineffective at correcting aberrant splicing of IVS2–654 pre-mRNA when delivered ex vivo to cultured erythroid progenitor cells from IVS2–654 murine bone marrow cells (data not shown). PNA-4K and morpholino SSOs had only a modest effect at 45 μM on IVS2–654 pre-mRNA splicing in these cells (Fig. 1 *B* and *C*). Clearly, cell uptake of these oligonucleotides followed by translocation from the cytoplasm to the nucleus, the site of splicing, was limited. In this investigation, we found that a morpholino oligomer conjugated to a cell-penetrating peptide P005 (SSO 654-P005; in short is SSO P005) (17) was effective in a previously developed cell-based assay (18) and also in IVS2–654 pre-mRNA repair in cultured murine erythroid progenitor cells.

The shift in splicing from aberrant to correct was almost complete at 15 μM SSO 654-P005 (Fig. 1*D*). This SSO was then used for treatment of the previously generated thalassemic IVS2–654 mice (19). These mice are heterozygotes with 1 human IVS2–654 thalassemic β-globin gene "knocked-in" to replace 2 *cis*-β-globin genes that code for murine adult β-globin (β-major and β-minor). The average level of total hemoglobin in these mice is 11.7 ± 0.5 g/dL (n = 18). Despite a relatively high level of total hemoglobin, these mice exhibit thalassemic characteristics, including abnormal hematological indices, splenomegaly and iron deposits in advanced age. This phenotype is somewhat different from that of humans, who, as heterozygotes, are essentially symptom-free but similar to other thalassemic mouse models. IVS–654 mice produce reduced amounts of murine β-globin chains, leading to imbalance of α- to β-globin production, which may be damaging to erythroid cells (19 and references cited therein).

The IVS2–654 mice were treated with SSO 654-P005, delivered intravenously at 25 mg/kg with 4 once-daily injections followed by a 3-day break for 3 weeks (*Materials and Methods*). This treatment restored correctly spliced human β-globin mRNA in the peripheral blood up to ≈12% (Fig. 2*A*). The scrambled negative control SSO, P005 scr SSO, yielded no correction (Fig. 2*A*, lanes 6 and 7). Quantification of in vivo mRNA repair in the peripheral blood of SSO 654-P005-treated mice (n = 3–4) using RT-quantitative real-time PCR (qPCR)

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Conflict of interest statement: H.M.M., M.H.N., and R.K. are employed by AVI Biopharma, Inc.

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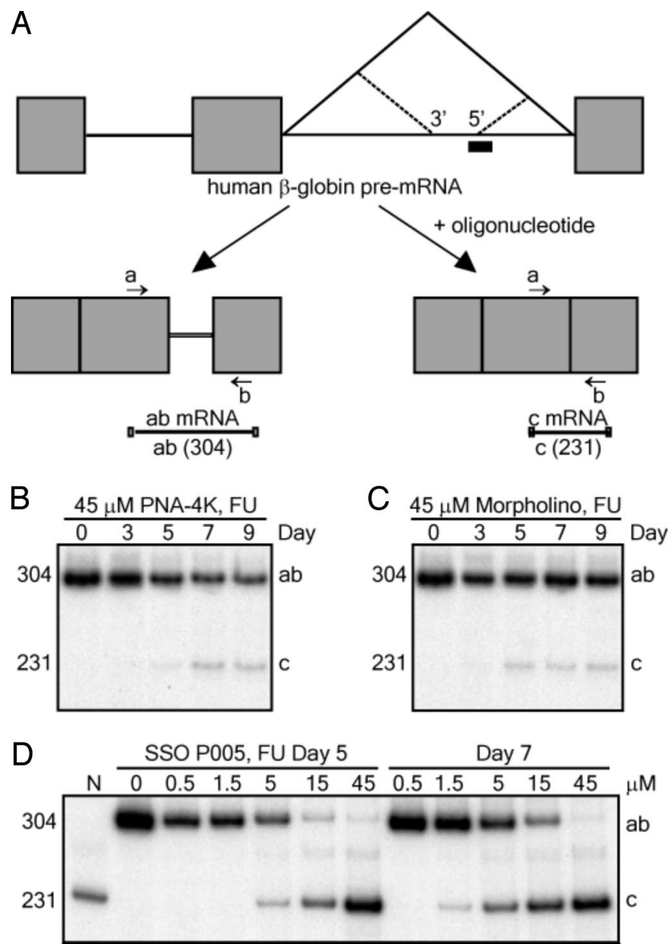

Fig. 1. (*A*) Splicing of IVS2–654 β-globin pre-mRNA and its repair by SSO. Boxes, exons; solid lines, introns and correct splicing pathway; dotted lines, aberrant splicing pathway. Thick bar, SSO; arrows, primers used in the RT-PCR assay. The length (in nucleotides) of RT-PCR products representing aberrantly (ab) and correctly (c) spliced mRNAs is shown. (*B*–*D*) Restoration of correctly spliced human β-globin mRNA in cultured erythroid progenitor cells. Cells were isolated from the bone marrow of IVS2–654 mouse and treated with PNA-4K (*B*), morpholino (*C*), and SSO P005 (*D*) SSOs. Treatment was by free uptake (FU) of SSO from the culture medium. SSO concentrations and the time of cell harvest are indicated. Total cellular RNA was analyzed by RT-PCR. N, RNA from normal human blood.

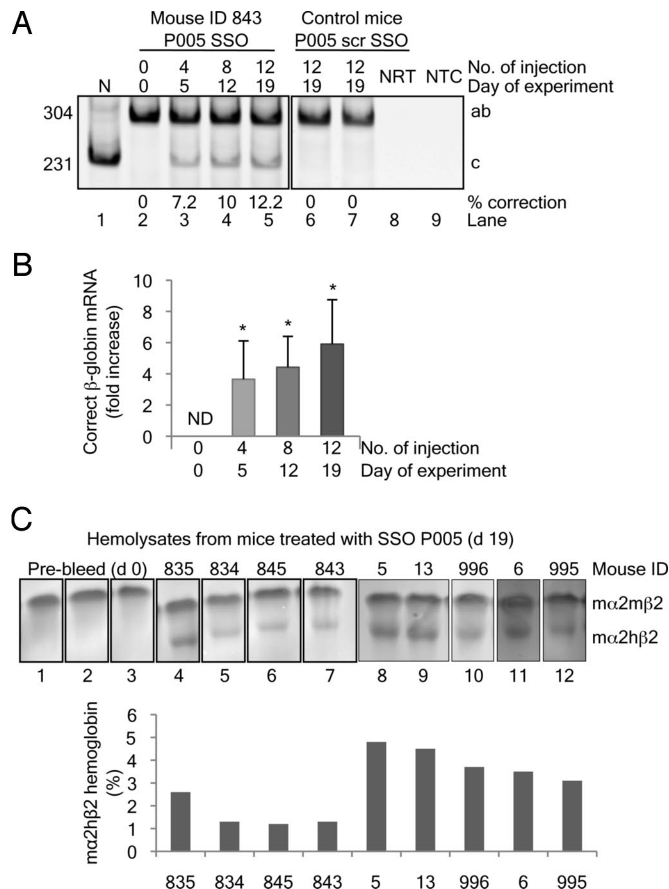

Fig. 2. Repair of human β-globin mRNA in vivo in IVS2–654 thalassemic mice by i.v. injections of SSO P005. (*A*) Conventional RT-PCR of total RNA from SSO P005-treated mice. RNA was isolated from peripheral blood. Lane 1, normal human blood (N); lane 2, prebleed; lanes 3–5, blood collected after 4, 8, and 12 SSO injections, respectively; lanes 6–7, blood collected from IVS2–654 mice treated with negative control P005 scr SSO; lane 8, no reverse transcription (NRT); lane 9, no template control (NTC). Days of bleeding and percentages of correctly (c) spliced mRNA compared with aberrantly (ab) spliced mRNA are indicated. No product was detectable without the RT step or in the absence of mRNA template. (*B*) Quantification of RNA repair by RT-qPCR. The mice were prebled and intravenously injected with 4 ($n = 4$), 8 ($n = 3$), and 12 ($n = 3$) doses of SSO P005. Total RNA from blood collected before [day (d) 0] and after every 4 SSO injections (d 5, 12, and 19) was analyzed. Data presented as mean ± SD. *P* values <0.05 were determined by one-way ANOVA. Asterisks denote statistically significant difference. The correctly spliced human β-globin mRNA was not detectable (ND) in prebled samples because the probe was designed to hybridize exclusively to the correct human β-globin mRNA (*Materials and Methods*). (*C*) Chimeric mouse-human hemoglobin ($m\alpha_2h\beta_2$) generated by SSO P005 treatment. IVS2–654 mice ($n = 9$) were dosed intravenously with SSO P005 for 3 weeks on a schedule: 4 once-daily injections and 3 days off. (*Top*) Hemoglobin levels were assayed by immunoblots with anti-human β-globin antibody of cellulose acetate electrophoregrams. Blood samples were taken before injections (lanes 1–3) and 1 day after the last injection (d 19) (lanes 4–12). (*Bottom*) Immunoblots were quantified using ImageQuant TL software.

showed that the correct human β-globin mRNA was restored up to 6-fold after treatment (Fig. 2*B*). The newly repaired β-globin mRNA was properly translated in circulating red blood cells (RBCs) and consistently yielded chimeric mouse-human hemoglobin ($m\alpha_2h\beta_2$), which reached ≈1–5% of the total in treated mice ($n = 9$; Fig. 2*C*).

Analysis of RBCs was carried out on mice 834, 835, 843, and 845. Despite a relatively low level of hemoglobin expression (Fig. 2*C*, lanes 4–7), improvement in RBC morphology was clearly detectable. Abnormal RBCs, including fragmented cells and those with abnormalities in shape and size (poikilocytosis and anisocytosis), were much less evident, indicating more effective erythropoiesis and less extravascular hemolysis. Concomitantly, the number of normal RBCs increased by ≈10% (Fig. 3 *A* and *B*). These results were consistent with a detectable and statistically significant reduction of the red cell distribution width (RDW), an indicator of red cell heterogeneity and increase in total hemoglobin level in the peripheral blood ($n = 5$; Fig. 4) of treated mice 5, 6, 13, 995, and 996 (Fig. 2*C*, lanes 8–12). In each mouse, the trend was reduced RDW and increased hemoglobin level, whereas in saline-treated mice, this trend was not observed. It is also noticeable that in mice with a lower initial hemoglobin level (≤11 g/dL), the increase was more pronounced (Fig. 4*B*).

The latter observation was confirmed in a single IVS2–654 mouse encountered in the colony with a low total hemoglobin level of 7.5 g/dL. We took advantage of this mouse and subjected it to an extended course of SSO 654-P005 treatment delivered by i.p. injection at 50 mg/kg on a schedule 4 days on and 3 days off for 6 weeks. In this particular experiment, the SSO treatment led

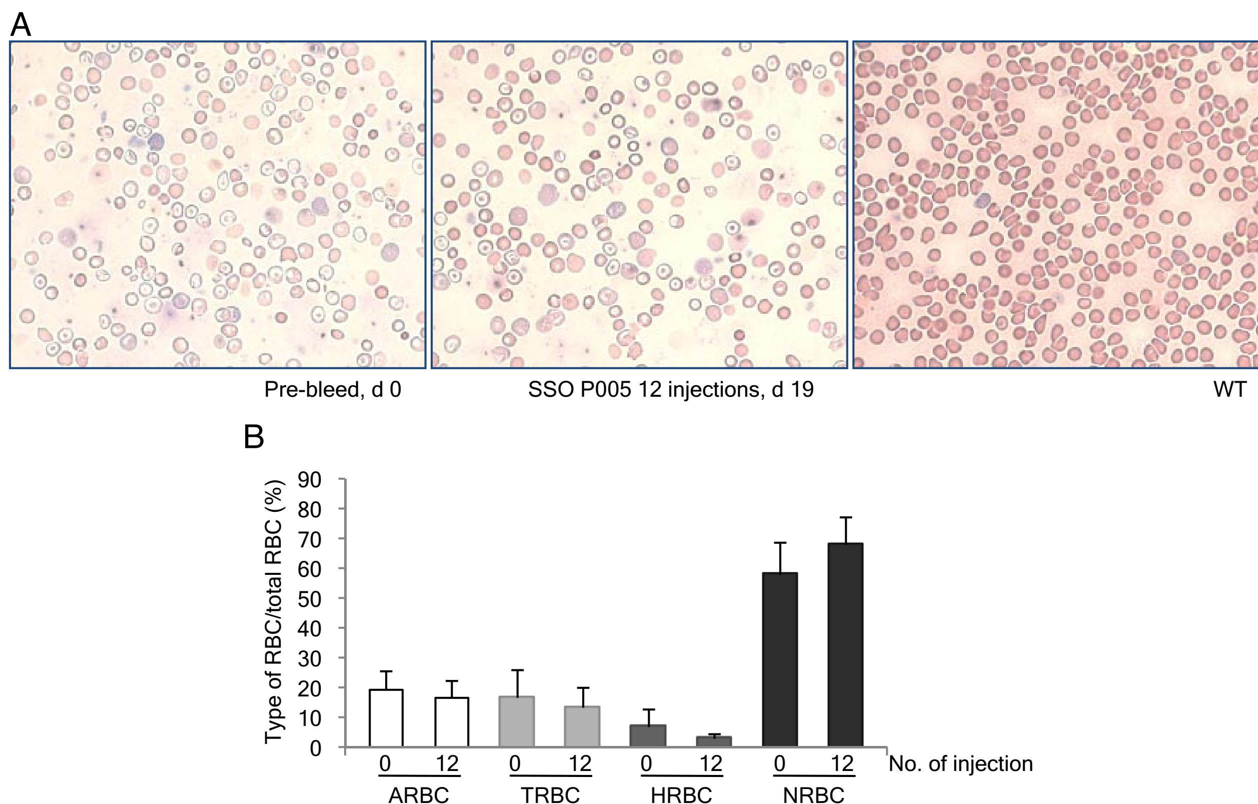

Fig. 3. Improvement of RBC morphology after SSO P005 treatment. (*A*) Wright-Giemsa-stained peripheral blood smears of IVS2–654 mouse were prepared before [day (d) 0] and after 12 i.v. injections of SSO P005 (d 19). WT, wild-type mouse. (*B*) Differential count of abnormal (ARBC), target (TRBC), hypochromic (HRBC), and normal (NRBC) RBCs. Blood smears before and after 12 i.v. injections of SSO P005 from IVS2–654 mice ($n = 4$) were stained with Wright-Giemsa. Three different field images were taken from each smear and used for the differential RBC count. Data are presented as mean ± SD. The difference between the numbers of NRBCs before and after SSO P005 treatment is 10% but not significant ($P = 0.06$; one-tailed Wilcoxon signed test).

to marked elevation of hemoglobin from 7.5 to 10.7 g/dL and of hematocrit levels from 19.9 to 29% (data not shown). Although a single mouse experiment has no statistical value, it further supports the notion that prolonged SSO treatment may be clinically valuable. It also suggests that at low hemoglobin levels, the response may be more robust than in mice with relatively high levels of hemoglobin. Thus, the nature of the model could have contributed to the modest response to treatment shown in Fig. 4.

This treatment was terminated at day 19 because we were unable to inject SSO 654-P005 into scarred tail veins reliably. Note that mouse RBCs have a lifespan of ≈60 days (20). Thus, during the period of injections, even if the restoration of splicing and hemoglobin production were 100% effective, only one-third of the existing RBCs, lacking additional hemoglobin, could have been replaced. Nevertheless, with prolonged SSO treatment, the fraction of healthy RBCs should continue to increase in the cell population in peripheral blood and further improve the overall hematological manifestations, including elevation of the hemoglobin and hematocrit levels.

We investigated the toxicity of SSO 654-P005 in IVS2–654 mice ($n = 4$) that received 16 SSO injections. Standard biochemical tests indicate no apparent liver or kidney toxicity (Fig. 5 *A–D*), nor was there any weight loss (Fig. 5*E*). To evaluate the primary inflammatory response that the treated mice might develop against the peptide-conjugated SSO 654-P005, we performed RT-qPCR to determine the change in transcriptional level of the IFN-γ and IL-12a cytokines after every 4 i.v. injections of the SSO at 25 mg/kg. The results shown in Fig. 5*F* indicate no significant change in the mRNA level of both cytokines. Furthermore, we found no significant change in IL-12 cytokines in SSO 654-P005-primed mice when they were challenged with the SSO 1 month after treatment, indicating that the SSO did not trigger the immune functioning cells to release their inflammatory cytokine (Table 1). More importantly, we did not detect SSO 654-P005-specific antibodies in the sera, indicating that the SSO did not induce a humoral immune response (data not shown). Finally, we investigated the cellular immune response of the lymphocytes from the treated mice to indicate whether they form memory cells that can recognize the SSO. To this end, we used the enzyme-linked immunosorbent spot assay (ELISPOT) to monitor the IFN-γ production and SSO 654-P005-specific antibody induction in the cultured lymphocytes that ex vivo encountered the SSO 654-P005. We found that the SSO challenge did not stimulate the lymphocytes to produce either IFN-γ or the antibody (data not shown).

We have found that repair of splicing-defective thalassemic pre-mRNA and concomitant production of adult hemoglobin (HbA) can be accomplished ex vivo in erythroid progenitor cells from thalassemic patients treated with SSOs with modified backbones (10, 11), including peptide-conjugate morpholino, SSO 654-P005 (data not shown). Thus, this treatment is compatible with and effective in the human cells that will constitute its target in the clinic.

We have shown by several measures that SSO 654-P005 improved the efficiency of erythropoiesis and concomitant morphology of erythrocytes in the peripheral blood of treated thalassemic mice. The SSO-induced mRNA repair, production of human β-globin, more efficient cell hemoglobinization, increased number of improved mature erythrocytes, and small

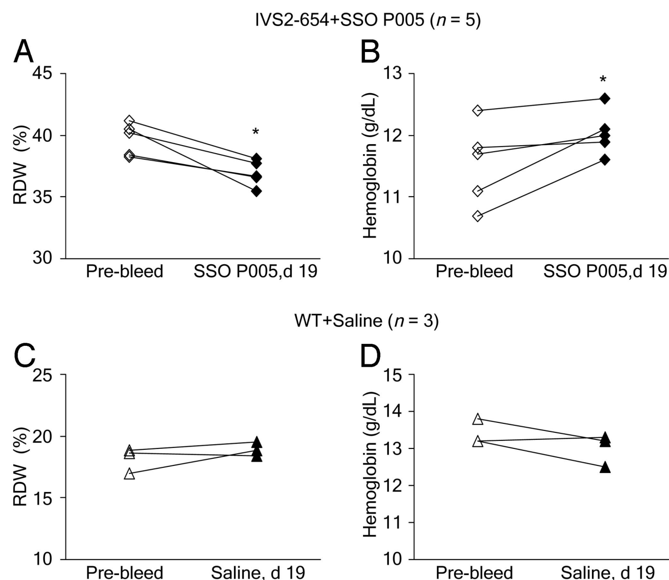

Fig. 4. Blood analysis for red cell heterogeneity (*A*) and hemoglobin level (*B*). Blood samples from IVS2–654 mice before (prebleed) and after treatment with 12 i.v. injections of SSO P005 ($n = 5$) were analyzed in Heska's animal blood counter. Asterisk indicates statistically significant (one-tailed Wilcoxon signed test) difference between prebled and SSO P005-treated mice. (*A* and *B*) $P = 0.03$. (*C* and *D*) RDW and hemoglobin levels of WT mice ($n = 3$) injected with saline (mean ± SD).

increase in the total level of hemoglobin were consistently evident after treatment, confirming that it had beneficial effects.

The fact that the effects were modest may reflect the nature of the model more than the performance of the oligonucleotide. The IVS2–654 mice are heterozygotes; therefore, at best, SSO treatment may only increase the hemoglobin levels by no more than one-half. Because the duration of the experiment was 19 days (i.e., one-third of the murine erythrocyte 60-day lifespan), the effect is reduced to ≈15%, assuming complete IVS2–654 repair in all targetable erythroid progenitor cells. SSO effectiveness less than 100%, which is to be expected, brings the results within statistical error, which, in our hands, was about 10% for hemoglobin and hematocrit assay. Furthermore, these calculations do not take into account that IVS2–654 gene may be expressed at a level that produced less mRNA, and hence β-globin, than the combined output of eliminated mouse β-major and β-minor genes. Another contributing factor to the poor response of the model appears to be the relatively high (11.7 g/dL) preexisting level of hemoglobin in untreated thalassemic mice. This is supported by the observation that in a mouse that had low hemoglobin (i.e., more pronounced anemia) and was treated with SSO 654-P005 for 6 weeks, the response was much more robust. Because the SSO is effective in human cells, the approach may be applicable to patients with IVS2–654/HbE thalassemia who do not produce functional HbA.

One notes that in transfused thalassemics, the hemoglobin level rises to about 20–30% and is clinically sufficient, whereas carriers with 50% hemoglobin are essentially asymptomatic. The results of this work already showed changes in blood morphology; the improvements need not be dramatic to achieve clinical significance. It is possible that continuous i.v. injections, impossible in mice for technical reasons, may be sufficient. The dose of SSO 654-P005 appears high at 25 mg/kg. However, if, as is frequently the case, dosing of morpholino oligonucleotides is scaled between mice and humans by mg/m^2, the human equivalent dose would be 2 mg/kg, which translates into 100 mg per dose for a 50-kg child, a likely patient.

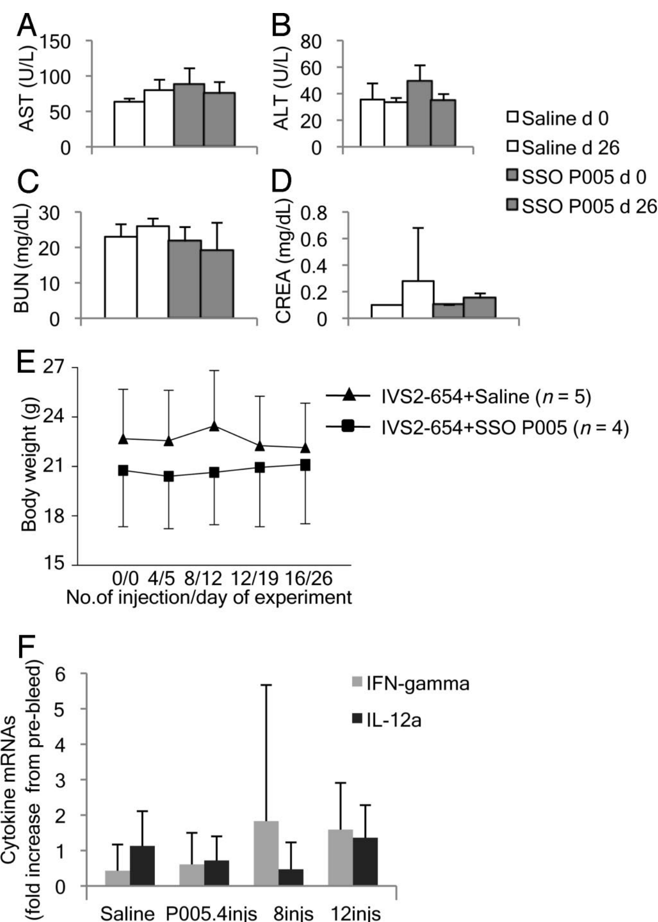

Fig. 5. Toxicity assay of SSO P005. Serum samples from IVS2–654 mice before [day (d) 0] and after treatment with 16 i.v. injections (d 26) of saline ($n = 5$) or SSO P005 ($n = 4$) were analyzed for (*A*) aspartate aminotransferase (AST, U/L), (*B*) alanine aminotransferase (ALT, U/L), (*C*) blood urea nitrogen (BUN, mg/dL), and (*D*) creatinine (CREA, mg/dL). (*E*) Body weights of the mice were recorded before (d 0) and after every 4 i.v. injections (d 5, 12, 19, and 26) of saline ($n = 5$) or SSO P005 ($n = 4$). (*F*) Quantification of cytokine mRNAs by RT-qPCR. The peripheral blood RNAs from saline mice ($n = 3$) and mice treated with 4 ($n = 5$), 8 ($n = 6$), and 12 ($n = 4$) injections of SSO P005 were analyzed for IFN-γ and IL-12a mRNAs. Data presented as mean ± SD.

Recent reports showed in vivo restoration of chloride channel function in myotonic dystrophy (21), factor VIII in a model of hemophilia (22), and dystrophin in the *mdx* mouse model of Duchenne muscular dystrophy (23–25) by oligonucleotide-induced modulation of splicing, an approach discovered in the Kole laboratory (6). These results, together with those reported here, indicate that the splicing-mediated pre-mRNA repair is not limited to a single disease. In fact, modulation of aberrant and alternative splicing by antisense oligonucleotides is likely to be useful in numerous indications, because 70% of human genes are alternatively spliced, with a number of them associated with cancer (26) and other diseases (27–29).

Materials and Methods

Oligonucleotides. The profiles of morpholino and PNA-4K oligonucleotides, including their sequences, were reported elsewhere (11, 14, 15). The 18-mer morpholino (5′-GCTATTACCTTAACCCAG-3′)-peptide-[(D)R(D)RRQRRKKRFFC] conjugate (P005) antisense to the aberrant 5′ splice site in the IVS2–654 β-globin pre-mRNA was synthesized at AVI BioPharma. The peptide contained 2 D-amino acid arginines at its N terminus as indicated in the sequence. The peptide was conjugated at the 5′ end of the morpholino oligomer through a noncleavable linker. The method for synthesis and purification of the conju-

Table 1. Measurement of mouse serum IL-12 p70 (pg/mL)

Samples	Preboosting	Day 3	Day 7	Day 28
Sal + Sal (7*)	0.67	28.49	ND	0.41
Sal + Sal (14*)	ND	ND	ND	0.21
Sal + Sal (998*)	ND	ND	ND	1.8
SSO + Sal (5†)	5.94	1.25	4.77	3.3
SSO + Sal (13†)	ND	ND	ND	ND
SSO + SSO (6‡)	ND	0.41	ND	ND
SSO + SSO (995‡)	ND	ND	ND	ND
SSO + SSO (996‡)	2.42	30.84	1.55	2.72

*Saline-treated mice; boosted with saline.
†SSO 654-P005-treated mice; boosted with saline.
‡SSO 654-P005-treated mice; boosted with SSO 654-P005 at 25 mg/kg.
ND, not detectable.

gate was described elsewhere (17). The SSO 654-P005 (lyophilized powder) was dissolved in sterilized water and was further diluted with saline to the desired concentration. The P005 scr SSO was used as negative control oligo.

Oligonucleotide Treatment

In Vitro. The oligonucleotides were tested ex vivo in IVS2–654 mouse erythroid progenitor cells, which were plated 18 h before SSO treatment using a free uptake technique as described (11). SSO 654-P005 concentrations were as shown in Fig. 1.

In Vivo. All animal protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of North Carolina. The 2-month-old IVS2–654 mice were weighed and prebled before i.v. (25 mg/kg) or i.p. (50 mg/kg) injection with the SSO at a volume not exceeding 200 μL. The injections were by means of 3 weekly courses, once daily for 4 consecutive days followed by a 3-day break. The i.p. injections were administered for 6 weeks on the same schedule.

RNA Isolation and Quantitative RT-PCR Analysis of Human β-Globin mRNA. Total RNA from cultured cells was isolated using TRI Reagent (Molecular Research Center). Blood RNAs were extracted using BD-Tri Reagent (Molecular Research Center). For end-point RT-PCR, 200 ng of the RNA sample was subjected to RT-PCR using the Thermostable r*Tth* Reverse Transcriptase RNA PCR Kit (Applied Biosystems) with 0.2 μCi of [α-^{32}P]dATP (Fig. 1 *B–D*) or with 0.05 nmol Cy5-AP3-dCTP (GE Healthcare) (Fig. 2*A*) per reaction. The RT step was carried out at 70 °C for 15 min, followed by PCR at 95 °C for 1 min and 65 °C for 1 min for 22 cycles. The primers used in RT-PCR assay were documented elsewhere (11). The RT-PCR products shown in Fig. 1 *B–D* were separated on 7.5% nondenaturing polyacrylamide gel and visualized by autoradiography. The RT-PCR products shown in Fig. 2*A* were analyzed on 10% nonpolyacrylamide 1.0-mm $\times$ 12-well 1 M Tris, 0.9 M Boric acid, 0.01 EDTA gel (Invitrogen), and the electrophoresis was conducted at 190 V for 1 h 40 min. The gels were scanned using a Typhoon 9400 scanner (GE Healthcare). ImageQuant TL image analysis software (GE Healthcare) was used to quantify spliced β-globin mRNA correctly. For the RT-qPCR assay, up to 1 μg of the RNA sample was treated with 1 U of RQ1 RNase-free DNase (Promega). Half of the RNA was used for first-strand cDNA synthesis utilizing a high-capacity cDNA RT kit (Applied Biosystems). One-tenth of the cDNA of each sample was used to perform qPCR using Taqman 2$\times$ universal PCR master mix (Applied Biosystems) together with 5′-GCAAGAAAGTGCTCG and 5′-CAGCACACAGACCAG as human β-globin mRNA-specific forward and reverse primers, respectively. The 5′FAM-AACT-TCAGGCTCCTGGGCAA-TAMRA was used as a probe to detect the correctly spliced human β-globin mRNA. The qPCR of each sample was done in duplicate on the ABI 7900HT fast real-time PCR system (Applied Biosystems). Data were normalized to β-actin that was simultaneously quantified, together with the correct β-globin mRNA in the same reaction, using 5′-GCACCACACCTTCTAC, 5′-GTCTCAAACATGATCTGG, and 5′HEX-ACCGAGGCCCCCCTGAAC-TAMRA as the forward and reverse primers and probe, respectively.

Detection of Chimeric Mouse-Human Hemoglobin. The chimeric hemoglobin ($m\alpha_2h\beta_2$) was detected using cellulose acetate electrophoresis followed by immunoblot analysis. The hemolysates were prepared by lysing the phosphate-buffer saline-washed RBCs obtained from peripheral blood of mice before (day 0) and after 12 i.v. injections of SSO 654-P005 (day 19) using hemolysate reagent (Helena Laboratories). The concentration of hemoglobin was determined by the cyanmethemoglobin method (Randox Laboratories). An equal amount of hemoglobin from each sample (OD = 0.17) was loaded onto Titan III-H cellulose acetate membrane, followed by electrophoresis at 350 V for 25 min. The hemoglobin proteins on cellulose acetate membrane were fixed using 0.5% Ponceau S, followed by 5% (vol/vol) acetic acid. The immunoassay was performed using mouse anti-human β-globin chain antibody with cross-reactivity against mouse β-globin (1 μg/μL; 1:1,000) (Accurate) and ECL Plex goat anti-mouse IgG-Cy 3 (1 μg/μL; 1:1,500) (GE Healthcare) as primary and secondary antibodies, respectively. The membranes were scanned using Typhoon 9400 with a setting for optimal Cy 3 detection [PMT 500 V, green (532) laser, and 580 BP 30 Cy3 TAMRA filter]. Quantification of chimeric hemoglobin was assessed by ImageQuant TL software.

Hematological Analysis. Peripheral blood samples were collected from the central tail artery, using heparinized microhematocrit capillary tubes, on day 0 (prebleed) and day 19 after 12 i.v. injections of SSO 654-P005. The complete blood cell count was performed using Heska's animal blood counter. Blood smears were stained with Wright-Giemsa for morphologic red cell analysis.

Toxicity Studies of SSO 654-P005. Peripheral blood samples were collected from the central tail artery immediately before (day 0) and after treatment with 16 i.v. injections of saline or SSO 654-P005 (day 26). The sera, obtained by clotting the blood at room temperature for 30 min, followed by centrifugation at 15,000 $\times$ g for 8 min, were subjected to liver and kidney function tests. Aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and blood creatinine were analyzed using an automatic chemical analyzer (VT250; Johnson & Johnson).

Immune and Inflammatory Response Assay. For the primary inflammatory response study, the IFN-γ and IL-12a mRNAs from peripheral blood of control mice (n = 3) and mice treated with 4 (n = 5), 8 (n = 6), and 12 (n = 4) i.v. injections of SSO 654-P005 at 25 mg/kg were quantified by RT-qPCR using an ABsolute SYBR green rox mix (ABgene), together with the SuperArray cytokine-specific primers (SuperArray Bioscience Corporation). Results were normalized to mouse β-actin expression level.

In the humoral immune response study, the 2-month-old IVS2–654 mice were given 12 i.v. injections of saline (n = 3) and 12 doses (n = 3) and 16 doses (n = 2) of SSO 654-P005. One month after the last treatment, mice were bled (preboosting) and the saline-treated mice and the 2 mice of 12-dose P005 group were given a single i.v. injection of saline, whereas 1 mouse of the 12-dose P005 group and the 2 mice of 16-dose P005 group were boosted i.v. with a single dose of SSO 654-P005 at 25 mg/kg. The sera were collected at days 3, 7, and 28 after boosting and analyzed for IL-12 levels using the Quantikine mouse IL-12 p70 immunoassay (R&D Systems). The remaining sera were diluted 1:50 in 5% (wt/vol) skimmed milk and tested for P005-specific antibody using standard ELISA.

For the cellular immune response study, IFN-γ and P005-specific antibody-secreting ELISPOT assays were carried out. One million spleen lymphocytes from mice treated 10 months earlier with 12 doses of saline (n = 4) or SSO 654-P005 at 25 mg/kg (n = 4) were cultured in the AIM-V medium (Invitrogen) containing 400 ng of the SSO for 22 h at 37 °C, 5% (vol/vol) CO_2. After incubating with biotinylated antibody and color development, the plates were scanned using a CTL plate reader (CTL Analyzers, LLC). The spots were counted using ImmunoSpot Software 1.7e (CTL Analyzers, LLC).

Statistical Analysis. The Wilcoxon signed test (one-tailed formulation) was used to compare data from 2 groups. Data from at least 3 groups were

compared using one-way ANOVA. Data with a P value <0.05 were considered statistically significant.

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