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Engineered U7 snRNA mediates sustained splicing correction in erythroid cells from β-thalassemia/HbE patients

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ABSTRACT

Repair of a splicing defect of β-globin pre-mRNA harboring hemoglobin E (HbE) mutation was successfully accomplished in erythroid cells from patients with β-thalassemia/HbE disorder by a synthetic splice-switching oligonucleotide (SSO). However, its application is limited by short-term effectiveness and requirement of lifelong periodic administration of SSO, especially for chronic diseases like thalassemias. Here, we engineered lentiviral vectors that stably express U7 small nuclear RNA (U7 snRNA) carrying the splice-switching sequence of the SSO that restores correct splicing of β^E-globin pre-mRNA and achieves a long-term therapeutic effect. Using a two-step tiling approach, we systematically screened U7 snRNAs carrying splice-switching SSO sequences targeted to the cryptic 5′ splice site created by HbE mutation. We tested this approach and identified the most responsive element for mediating splicing correction in engineered U7 snRNAs in HeLa-β^E cell model cell line. Remarkably, the U7 snRNA lentiviral vector (U7 βE4+1) targeted to this region effectively restored the correctly-spliced β^E-globin mRNA for at least 5 months. Moreover, the effects of the U7 βE4+1 snRNA lentiviral vector were also evident as upregulation of the correctly-spliced β^E-globin mRNA in erythroid progenitor cells from β-thalassemia/HbE patients treated with the vector, which led to improvements of pathologies in erythroid progenitor cells from thalassemia patients. These results suggest that the splicing correction of β^E-globin pre-mRNA by the engineered U7 snRNA lentiviral vector provides a promising, long-term treatment for β-thalassemia/HbE.

1. Introduction

Hemoglobin E (HbE; $\alpha_2\beta_2^{26\text{Glu to Lys}}$) is a very common Hb variant found among Southeast Asian populations. It is caused by a G to A mutation at codon 26 (GAG to AAG) in exon 1 of human β-globin gene (*HBB*:c.79G > A), resulting in glutamic acid to lysine substitution and consequently to production of functional β^E-globin chains [1]. Furthermore, this mutation activates a cryptic 5′ splice site at codon 25 (Fig. 1A), resulting in aberrant splicing of β^E-globin pre-mRNAs, generating aberrantly-spliced β^E-globin mRNA that contains a premature termination codon at codon 55, leading to reduction of β^E-globin chains [2]. Homozygotes for HbE may be mildly anemic without any other clinical symptoms. However, compound heterozygote patients for β-thalassemia/HbE result in variable anemia, ranging from mild to severe, transfusion-dependent states [1]. In fact, the level of correctly-spliced β^E-globin mRNA is an important modifying factor that affects clinical severity of β-thalassemia/HbE patients [3]. This inspired us to apply the splice-switching technology [4] as a means to increase the level of correctly-spliced β^E-globin mRNA and HbE hemoglobin for therapeutic purpose.

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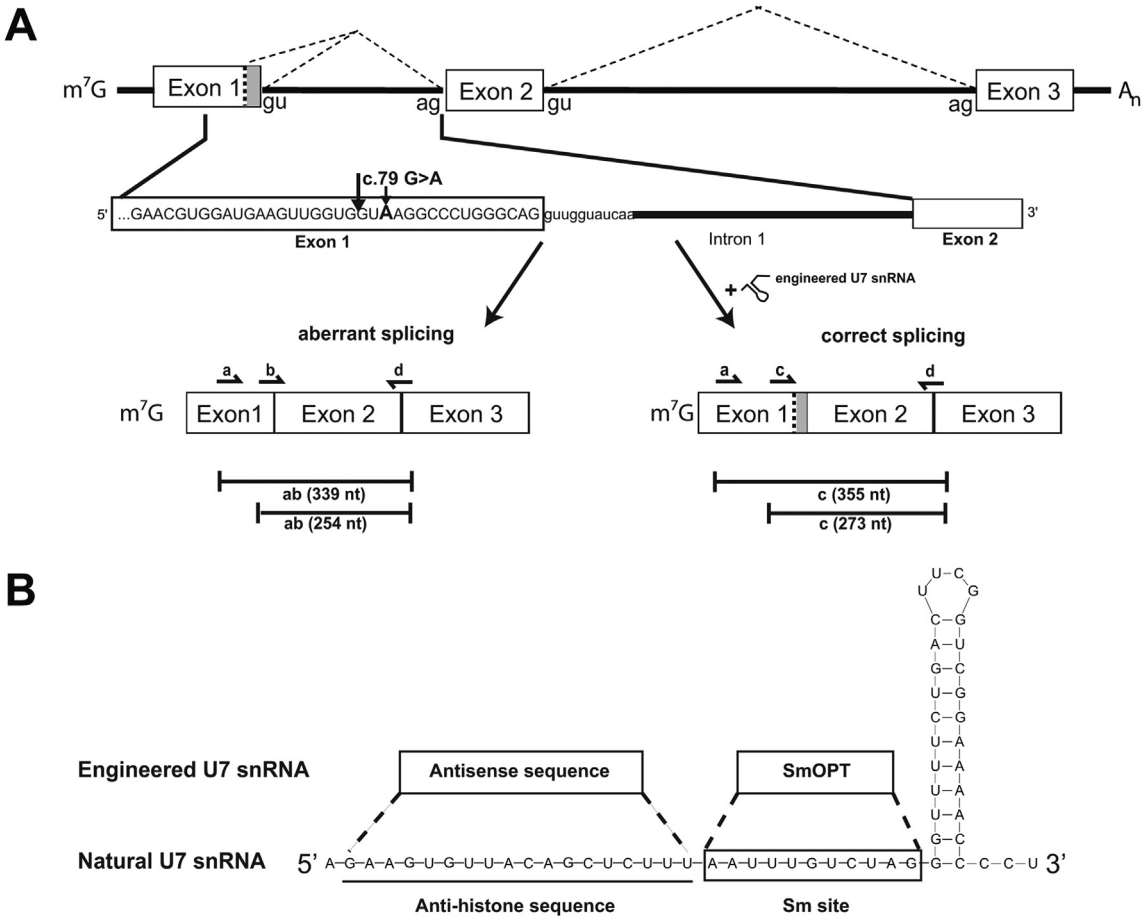

Fig. 1. Splicing of human β^E-globin pre-mRNA and its correction by engineered U7 snRNAs. (A) Correction of aberrant splicing of β^E-globin pre-mRNA by engineered U7 snRNAs. Boxes, exons; solid lines, introns; dashed lines, correct and aberrant splicing pathways. Exon and intron sequences are shown in upper- and lowercase, respectively. HbE mutation (bold letter) activates a cryptic 5′ splice site (down arrow) and causes aberrant splicing (left panel) leading to generation of the aberrantly-spliced β^E-globin mRNA lacking 16 nucleotides (grey area) at the 3′ end of exon 1. In the presence of the engineered U7 snRNA (right panel), the aberrant splicing is inhibited and the correctly-spliced β^E-globin mRNA is restored. Primers, half-headed arrows. (B) Structure of the engineered U7 snRNAs.

Splice-switching oligonucleotides (SSOs) have been successfully used to induce correct splicing or expression of therapeutic splice variants in several clinically-relevant targets including β-thalassemia [5]. Blocking aberrant splice sites created by mutations in intron 1 (IVS1-5, IVS1-6, IVS1-110), intron 2 (IVS2-654, IVS2-705, IVS2-745), and coding sequence (HbE) of human β-globin gene with SSOs has demonstrated therapeutic potential for β-thalassemia [5,6]. Specifically, it has been shown that treatment of β-thalassemia/HbE erythroid progenitor cells with phosphorodiamidate morpholino oligonucleotides (PMOs) targeted to the cryptic 5′ splice site of β^E-globin pre-mRNA effectively forced the splicing machinery to reselect the existing correct 5′ splice site, generating correctly-spliced β^E-globin mRNA and β^E-globin protein [6].

While SSO-induced modulation of pre-mRNA splicing is now employed as therapy for DMD and SMA, the approach requires frequent injections to promote sustained levels of therapeutic proteins. To address this problem, we set out to apply a form of gene therapy, exploiting a U7 small nuclear RNA (snRNA) as a vehicle to maintain stable levels of the appropriate splice-switching sequences that modulate pre-mRNA splicing in target cells. As shown previously [7] U7 snRNA, which normally is involved in processing of histone pre-mRNA 3′ end, can be engineered to affect and modulate splicing of a target pre-mRNA by replacing its natural anti-histone sequence with splice-switching sequences (Fig. 1B). Moreover, to enhance the efficacy of this process one can also replace the wild-type Sm-binding site of U7 snRNA with a consensus Sm-binding sequence derived from the major spliceosomal snRNAs (SmOPT). This augments its expression level and inactivates the histone pre-mRNA processing function of the U7 snRNA [8]. This system has been successfully used to achieve long-term effect in a variety of disorders including β-thalassemia [7,9], DMD [10] and SMA [11].

In this study, we generated a series of engineered U7 snRNA-based lentiviral vectors targeted to the cryptic 5′ splice site created by the HbE mutation and evaluated their long-term splicing correction efficiency of β^E-globin pre-mRNA in relevant cell models. We identified the optimal target region for blocking the aberrant splicing of β^E-globin pre-mRNAs and identified the most effective vectors, which promoted effective and long-term upregulation of correctly-spliced β^E-globin mRNA in erythroid progenitor cells from β-thalassemia/HbE patients and led to phenotypic improvements of β-thalassemic erythroid cells.

2. Materials and methods

2.1. Recombinant plasmid constructs

Human β^E-globin gene (HBB:c.79G > A) was amplified from DNA sample of a homozygous HbE subject using specific primers and

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