Antisense oligonucleotide–directed inhibition of nonsense-mediated mRNA decay

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Nonsense-mediated mRNA decay (NMD) is a cellular quality-control mechanism that is thought to exacerbate the phenotype of certain pathogenic nonsense mutations by preventing the expression of semi-functional proteins. NMD also limits the efficacy of read-through compound (RTC)-based therapies. Here, we report a gene-specific method of NMD inhibition using antisense oligonucleotides (ASOs) and combine this approach with an RTC to effectively restore the expression of full-length protein from a nonsense-mutant allele.

RNA-based therapeutic approaches show increasing promise, as exemplified by the recent progress in the development of ASOs and stop-codon RTCs as therapeutics for various genetic diseases. NMD is a quality-control process that degrades mRNAs with premature termination codons (PTCs) during translation. mRNAs targeted by small molecules that allow the ribosome to read through stop codons are often subject to NMD, reducing the therapeutic potential of such RTCs. NMD suppression has thus gained attention as a potential RNA-based therapy, but the approaches proposed so far have relied on general inhibition of the NMD machinery. Transcript-specific, as opposed to therapy, but the approaches proposed so far have relied on general inhibition of NMD has thus gained attention as a potential RNA-based approach with an RTC to effectively restore the expression of full-length protein from a nonsense-mutant allele.

ASOs can be designed to block the binding sites of RNA-binding proteins, resulting in effects such as splicing modulation and inhibition of translation initiation, among others. We tested whether ASOs can be used for targeted NMD inhibition by blocking EJC deposition on mRNA, downstream of a PTC. Although EJCs appear to be dispensable for most splicing events, they have roles in messenger ribonucleoprotein (mRNP) export from the nucleus and in translation. Our approach should not affect such processes, because we target only the EJCs downstream of a PTC on a given mRNA, leaving the remaining upstream EJCs unaffected.

We first tested a well-characterized NMD substrate, full-length, three-exon HBB (encoding β-globin) with a CAG-to-TAG nonsense mutation in exon 2 (HBB-T39) (ref. 14). We used Flp recombinase–mediated integration to stably express a single copy of this gene at a defined chromosomal location in U2OS T-REx cells. In this context, HBB-T39 mRNA expression was reduced to about 5% that of wild-type HBB (Supplementary Fig. 1). We designed a set of 2′-O-(2-methoxyethyl) (MOE) ribose– and phosphorothioate-modified 15-mer ASOs that together span the region of the HBB-T39 mRNA predicted to harbor an EJC and screened them individually for NMD-inhibiting activity (Fig. 1a,b). After transfection of the NMD reporter cells, many of the ASOs caused skipping of HBB exon 2; however, several ASOs resulted in minimal exon skipping and markedly increased the level of full-length spliced mRNA (Fig. 1c and Supplementary Fig. 2). We chose H-24 as the lead ASO and confirmed its dose-dependent activity, reflected in a threefold increase in HBB-T39 mRNA at the highest dose tested (Fig. 1d). Consistent with NMD inhibition, H-24 did not affect the levels of wild-type HBB mRNA (Supplementary Fig. 3).

To test the generality of our approach, we constructed a three-exon NMD minigene reporter based on the MECP2 gene encoding an S65X nonsense mutation (TCA to TGA) in the middle exon (exon 3 in the natural gene), similarly integrated at a defined location in U2OS T-REx cells. MECP2-S65X mRNA was efficiently degraded by NMD (Supplementary Fig. 4). We screened a corresponding set of 19 ASOs spanning the presumptive EJC binding site near the 3′ end of this exon and identified multiple ASOs that increased the amount of full-length spliced mRNA (Supplementary Fig. 5). One of several effective ASOs (M-33) for MECP2 resulted in a ~3-fold increase in mRNA (Fig. 1e). To verify the transcript-specific effect of NMD-inhibiting ASOs, we confirmed that the expression of several NMD-targeted endogenous mRNAs was unchanged after ASO treatment (Supplementary Fig. 6).

The above NMD substrates are simple three-exon genes or minigenes with a single predicted EJC downstream of a PTC. To test the broader applicability of our approach, we designed a pair of four-exon hybrid genes: one comprising HBB exons 1–3, with exon 3 and portions of the flanking introns from MECP2 inserted within HBB intron 2, and the other comprising MECP2 exons 2–4, with HBB exon 2 and portions of the flanking introns inserted within MECP2 intron 3 (Fig. 1f and Online Methods). Using our optimal ASOs for these two genes (H-24 for HBB

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and M-33 for MECP2), we compared the effects of single and combined ASOs in inhibiting NMD of these target substrates with two presumptive downstream EJCs. In both cases, the combined treatment was more effective than either ASO alone (Fig. 1g and Supplementary Fig. 7).

NMD is sensitive to the cellular state and environment and can be inhibited by stresses such as hypoxia and amino-acid deprivation. Furthermore, ASOs can have various off-target effects, although none has been linked to NMD efficiency. To test the presumptive mechanism of action, we performed in vitro splicing of HBB pre-mRNA, followed by glutathione-S-transferase (GST)-mediated pull-down of eIF4A3, the anchor component of the EJC, to confirm that an effective ASO blocks the interaction between the spliced mRNA and eIF4A3. Consistent with its predicted activity, H-24 ASO blocked the interaction between HBB mRNA and eIF4A3 in a dose-dependent manner (Fig. 2a).

To further establish the mechanism of action, we modified the HBB-T39 construct by adding 24 nucleotides at the 3' end of exon 2. Owing to the position-specific nature of EJC binding, this modification shifts the predicted EJC-deposition site downstream of the sequence targeted by the H-24 ASO1,18 (Fig. 2b). The mRNA expressed from this construct (HBB-T39+24) was not affected by H-24, indicating that the ASO target has been linked to NMD efficiency. To test the presumptive mechanism of action, we performed glutathione-S-transferase (GST)-mediated pull-down of eIF4A3, the anchor component of the EJC, to confirm that an effective ASO blocks the interaction between the spliced mRNA and eIF4A3. Consistent with its predicted activity, H-24 ASO blocked the interaction between HBB mRNA and eIF4A3 in a dose-dependent manner (Fig. 2a).

Additionally, to rule out the possibility that the increase in steady-state mRNA level reflects an increased rate of transcription, we performed an actinomycin D chase assay to measure the mRNA decay rate. As expected, cells treated with H-24 ASO expressed HBB-T39 mRNA with greater stability (Supplementary Fig. 8). HBB-T39 mRNA has been reported to undergo biphasic decay, probably owing to rapid decay of the NMD-sensitive mRNA molecules occurring along-with slower decay of a subset of mRNA molecules that evade NMD. Analysis of the half-lives in two phases revealed that the stability of HBB-T39 mRNA in ASO-treated cells was higher during the rapid-decay phase (estimated t1/2 = 6.8 min for untreated versus 16.5 min for ASO-treated), whereas the decay rate during the slow phase remained unchanged (estimated t1/2 > 12 h for both treatment groups).

To determine whether the target-mRNA increase results in an increase in the encoded protein after ASO treatment, we monitored the effect of ASO on protein synthesis via a GFP tag placed at the N terminus of HBB-T39. We observed a robust increase in the amount of truncated protein in response to ASO treatment (Supplementary Fig. 9). Notably, ASO treatment did not reduce the amount of full-length protein expressed from the wild-type GFP fusion construct (Supplementary Fig. 10). We conclude that protein synthesis is not inhibited by an exon-targeting MOE phosphorothioate ASO, presumably because it is displaced from the mRNA upon passage of a translating ribosome.

RTCs are diverse classes of molecules that cause the ribosome to misrecognize a PTC and incorporate an amino acid, terminating at
the natural stop codon downstream and thereby restoring synthesis of full-length protein from mRNA with a nonsense mutation\(^{20}\). Although this is a promising strategy currently being tested in clinical trials, NMD diminishes the efficacy of read-through therapy by degrading the mRNAs targeted by such compounds\(^{15}\).

To test the utility of our approach in improving the efficacy of RTCs, we designed a read-through reporter (HBB-T39(UGAC)), based on the GFP-tagged HBB-T39 construct described above, in which the TAG PTC and the following four nucleotides are replaced with TGACTAG. This stop codon and the downstream sequence weaken translation termination, allowing some spontaneous read-through and thereby increasing the sensitivity of detection of full-length HBB-GFP protein\(^{21}\). Translational read-through suppressed NMD to some extent, as was evident by the two- to three-fold increase in HBB-T39(UGAC) mRNA relative to HBB-T39 (Supplementary Fig. 1). Nevertheless, we observed strong increases in both the truncated protein and the read-through product upon further NMD suppression by H-24 ASO treatment (Fig. 2d and Supplementary Fig. 11).

G418 is an aminoglycoside with read-through activity\(^{4}\). Co-treatment of U2OS cells expressing HBB-T39(UGAC) with H-24 ASO and G418 increased the full-length protein level by 6-fold, compared to a 2-fold increase with G418 alone and a 2.5-fold increase with H-24 ASO alone. Thus, antisense inhibition of NMD can augment the efficacy of an RTC. The ASO-induced increase in expression of truncated protein could be deleterious in some cases, but this would depend on the properties of the particular mutant protein and on the efficacy of the RTC.

The antisense approach we present here, which we call gene-specific antisense inhibition of NMD (GAIN), allows gene-specific inhibition of NMD without relying on skipping of a PTC-containing exon. This gene-specific feature, along with the clinically demonstrated efficacy and safety profiles of various ASO chemistries such as MOE phosphorothioate\(^{2}\), makes this a promising approach to inhibit NMD.

METHODS

Methods and any associated references are available in the online version of the paper.
ONLINE METHODS

ASOs. All ASOs used were uniformly modified with MOE sugars, phosphorothioate backbone, and 5′-methyl cytosine. Synthesis, purification, and quantification were done as described23. All ASOs were dissolved in water and stored at −20°C. The sequences of all ASOs used in this study are provided in Supplementary Table 1. ASOs were named by taking the first letter of their target gene, followed by a number representing the distance of the ASO 5′ end from the exon-exon junction.

Preparation of NMD reporter cells. All the cell lines used in this study tested negative for mycoplasma contamination. The full HBB gene and MECP2 exons 2–4 were cloned into the pcDNA FRT/TO plasmid (Life Technologies), with GFP sequence added at the 5′ end of the first exon for monitoring protein expression. Only 200 bases at each end of MECP2 intron 3 (59,626 nt) were included in the construct. Chimeric constructs were made as follows: for construct 1, MECP2 exon 3, along with the last 173 nt of intron 2 and the first 145 nt of intron 3, was inserted into HBB intron 2 at nucleotide position +473. Likewise, for construct 2, HBB exon 2, along with the last 60 nt of intron 1 and the first 79 nt of intron 2, was inserted into MECP2 intron 3 at nucleotide position +335. Cloning was carried out by the sequence- and ligation-independent cloning strategy (SLIC)23, and PTCs were introduced by site-directed mutagenesis. HBB-T39+24 was made from HBB-T39 by replacing 6 nt at the 3′ end of HBB exon 2 with 30 nt from the 3′ end of HBB exon 1. The pcDNA FRT/TO (Flippase-recognition target/ Tet-on) constructs were co-transfected along with pOG44 helper vector into FRT-U2OS TREx cells with a single integration site (Life Technologies). Cells with successful FRT-mediated integration were obtained by hygromycin selection. Expression and correct splicing of the transgenes were confirmed by radioactive reverse transcription PCR (RT-PCR) following induction with 1 µg/ml tetracycline24.

Tissue culture and ASO transfection. U2OS cells were cultured in DMEM with 10% FBS. ASO transfections were carried out using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol for transfecting with 10% FBS. ASO transfections were carried out using Lipofectamine 2000 (Life Technologies). Tet-on constructs were cotransfected along with pOG44 helper vector into FRT-U2OS TREx cells with a single integration site (Life Technologies). Cells with successful FRT-mediated integration were obtained by hygromycin selection. Expression and correct splicing of the transgenes were confirmed by radioactive reverse transcription PCR (RT-PCR) following induction with 1 µg/ml tetracycline24.

RNA extraction and RT-PCR. Total RNA was extracted with TRIzol (Life Technologies) 48 h after transfection according to the manufacturer’s protocol. Oligo d(T)18-prime reversed transcriptase was carried out with ImpromII Reverse Transcriptase (Roche). Semi-quantitative RT-PCR was carried out in the presence of [32P]dCTP and primer pairs corresponding to the first and last exons of each construct. MECP2 cDNA was amplified using a forward primer specific to GFP sequence to avoid detecting the endogenous wild-type MECP2 mRNA. Primer sequences are provided in Supplementary Table 2. PCR products were detected with a Typhoon FLA7000 phosphorimager and quantified using MultiGauge v2.3 software (Fujifilm). Statistical significance was calculated with Student's t-test.

Protein extraction and western blotting. Cells were harvested 48 h after transfection and lysed in STE buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 0.1% SDS, 1% NP-40, and 2 mM EDTA + protease inhibitor cocktail (Roche)) by sonicating for 5 min at medium power using a Bioruptor (Diagenode), followed by 15-min incubation on ice. Protein concentration was measured by Bradford assay (Bio-Rad) with BSA as a standard. Rabbit anti-GFP (1:1,000; Sigma G1544) and mouse anti-α-tubulin (1:2,000; Sigma T9026) antibodies were used with IRDye 800CW secondary antibodies (LI-COR) for western blotting, and the blots were imaged and quantified using the Odyssey Infrared Imaging System (LI-COR).

In vitro transcription of HBB minigene. Pre-mRNA for in vitro splicing was transcribed using T7 RNA polymerase (Roche) in the presence of [α-32P]UTP using a PCR product as the template. The template contains portions of exons 2 and 3 and the intact intron 2 of HBB and was amplified from human genomic DNA using the primer pair T7HBB-Ex2-F and HBB-Ex3-R (Supplementary Table 2). The radiolabeled transcript was purified by denaturing PAGE, and its concentration measured by liquid scintillation counting.

In vitro splicing and GST pull-down. Expression and purification of GST-eIF4A3, HeLa nuclear extract preparation, and in vitro splicing were carried out essentially as described18,25,26. 50-µl splicing reactions were carried out for 90 min at 30°C with 4 µg GST alone or GST-eIF4A3 in the presence of varying concentrations of ASOs. A 5-µl aliquot was kept as input, and 400 µl of wash buffer (20 mM HEPES, pH 8.0, 150 mM KCl, 0.05% NP-40) was added to the remainder. mRNP was bound to 10 µl of glutathione-Sepharose beads (GE Healthcare) by incubating at 4°C for 2 h. The beads were washed three times with wash buffer and resuspended in 300 µl splice-stop solution (0.3 mM sodium acetate and 0.1% SDS). RNA was then recovered by phenol extraction and ethanol precipitation and analyzed by denaturing PAGE followed by detection and quantification on a Typhoon phosphorimager.

mRNA decay rate assay. U2OS cells stably integrated HBB-T39 constructs were pretreated with Lipofectamine 2000 (control) or 50 nM H-24 ASO. Expression of the transgene was induced 24 h after transfection by addition of 1 µg/ml tetracycline. Two hours after induction, the medium was replaced with medium containing 5 µg/ml actinomycin D and no tetracycline. Starting 30 min after actinomycin D addition (defined as 0 h), cells were harvested in TRIzol at different time points to assess the decay rate of HBB-T39 mRNA. The amount of HBB mRNA was normalized to ACTB mRNA. Half-lives were calculated using the two-component mixed exponential decay model \( y = a \times \exp(b \times t) + c \times \exp(d \times t) \); data from our experiment were fitted to this model using the Solver function of Microsoft Excel to solve for the decay constants b and d19,27.