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Correction of a Cystic Fibrosis Splicing Mutation by Antisense Oligonucleotides

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ABSTRACT: Cystic fibrosis (CF), the most common life-threatening genetic disease in Caucasians, is caused by ~2,000 different mutations in the CF transmembrane conductance regulator (*CFTR*) gene. A significant fraction of these (~13%) affect pre-mRNA splicing for which novel therapies have been somewhat neglected. We have previously described the effect of the *CFTR* splicing mutation c.2657+5G>A in IVS16, showing that it originates transcripts lacking exon 16 as well as wild-type transcripts. Here, we tested an RNA-based antisense oligonucleotide (AON) strategy to correct the aberrant splicing caused by this mutation. Two AONs (AON1/2) complementary to the pre-mRNA IVS16 mutant region were designed and their effect on splicing was assessed at the RNA and protein levels, on intracellular protein localization and function. To this end, we used the 2657+5G>A mutant *CFTR* minigene stably expressed in HEK293 Flp-In cells that express a single copy of the transgene. RNA data from AON1-treated mutant cells show that exon 16 inclusion was almost completely restored (to 95%), also resulting in increased levels of correctly localized CFTR protein at the plasma membrane (PM) and with increased function. A novel two-color *CFTR* splicing reporter minigene developed here allowed the quantitative monitoring of splicing by automated microscopy localization of CFTR at the PM. The AON strategy is thus a promising therapeutic approach for the specific correction of alternative splicing.
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KEY WORDS: cystic fibrosis; *CFTR*; splicing mutation; exon skipping; antisense oligonucleotide

Introduction

Cystic fibrosis (CF; MIM #219700) is the most common severe autosomal-recessive disease in Caucasians, with an estimated incidence of one in 2,500–4,000 newborns. Clinically, CF is a multiorgan disease but it predominantly affects the lungs, the dominant cause of morbidity and mortality. Other CF symptoms include pancreatic insufficiency, intestinal obstruction, elevated electrolyte levels in sweat (the basis of the most common diagnostic test), and male infertility [Amaral, 2015]. Classical CF is diagnosed early in infancy and suggested by one or more characteristic clinical features, a history of CF in a sibling or, more recently, a positive newborn screening result. Despite significant advances in symptomatic treatment and in our understanding of the pathophysiology of CF, life quality and expectancy are still limited for CF patients (~37 years in the USA) [Plant et al., 2013; Amaral, 2015].

CF is caused by mutations in the *Cystic Fibrosis Transmembrane Conductance Regulator* gene (*CFTR*; MIM #602421; GenBank NM_000492.3), which encodes a cAMP-regulated chloride (Cl^-)/bicarbonate (HCO_3^-) channel expressed at the apical membrane of epithelial cells [Choi et al., 2001]. To date, ~2,000 *CFTR* mutations have been identified, most of which are presumed to be disease causing, as they totally or partially disrupt CFTR channel function. Among these, a significant proportion (~13%) are reported to cause splicing alterations [Bell et al., 2015], a percentage which is similar to that in other monogenic diseases [Cooper et al., 2010; Fernandez Alanis et al., 2012]. However, given the potential pathogenic nature of many nonobvious splicing variants, the proportion of splicing defects can be higher than estimated. Indeed, recent characterization of "false" missense mutations and of variants located in noncanonical regulatory elements has highlighted the difficulties in correctly predicting the consequence of genomic variants on splicing [Sosnay et al., 2013; Sharma et al., 2014; Ramalho et al., 2015].

Pre-mRNA splicing is a finely regulated process that requires specific signals on RNA molecules: the classical 5′ and 3′ splice sites (5′ss and 3′ss), the polypyrimidine tract and the branch point sequence as well as less-conserved intronic or exonic splicing elements with enhancer or silencer functions (intronic splicing enhancers, intronic splicing silencers, exonic splicing enhancers and exonic splicing silencers: ISEs, ISSs, ESEs and ESSs, respectively). These in turn interact with splicing factors with a positive (SR proteins) or negative (hnRNPs) effect on the exon recognition [Wahl et al., 2009]. All these elements drive the spliceosome, the macromolecular ribonucleoprotein complex that promotes the splicing reactions, in the identification of the correct exon–intron boundaries [Wahl et al., 2009; Roca et al., 2013]. Indeed, factors associated to the splice sites and to the splicing regulatory elements stimulate a network of interactions across the exon that promotes splicing. Ultimately, the final product of splicing results from the balance among multiple positive and negative interactions over the exon toward a process called exon definition [Wahl et al., 2009; De Conti et al., 2013; Roca et al., 2013].

Additional Supporting Information may be found in the online version of this article.

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Disruption of exon recognition and alternative splicing (AS) are common causes of human disease. Intronic or exonic variants may cause disease by either disrupting canonical splice motifs or by creating cryptic splice sites, which lead to aberrant alternatively spliced mRNAs encoding nonfunctional proteins that totally replace or reduce normal protein expression [Spitali and Aartsma-Rus, 2012].

We have previously reported the molecular consequences of the *CFTR* c.2657+5G>A splicing mutation (legacy name: 2789+5G>A), located near the donor site in intron 16 (IVS16), and showed that it originates transcripts lacking exon 16 concomitantly with wild-type (wt) transcripts [Masvidal et al., 2014; Sharma, et al., 2014]. Herein, we tested whether an RNA-based antisense oligonucleotide (AON) approach would correct the skipping of exon 16. We used our previous mutant *CFTR* minigene model consisting of the full-length *CFTR* cDNA with intronic sequences, stably expressed in HEK293 Flp-In cells (hosting a single copy of the 2657+5G>A mutant minigene) [Masvidal et al., 2014] to assess splicing correction by two tailor-designed AONs. Data shown here demonstrate that through this strategy, we were able to modulate splicing and rescue normal full-length *CFTR* transcripts to 95% as well as restore normal levels of functional CFTR protein. Moreover, we also developed and validated a novel two-color fluorescent *CFTR* splicing reporter minigene, which allowed the quantitative monitoring of splicing by automated microscopy localization of CFTR at the plasma membrane (PM), thus being of high value in future gene or drug screens.

Materials and Methods

Minigene Construction

The *CFTR* minigenes were produced using a "sticky feet PCR" strategy to insert *CFTR* introns (IVS) IVS14, IVS15, and IVS16 consecutively into the pCDNA5/FRT/CFTR mammalian expression vector carrying the complete wt-CFTR cDNA as described previously [Masvidal et al., 2014]. Thus, "triple minigenes" consist of the full-length 4.5 kb *CFTR* cDNA plus relevant intronic regions (IVS14, IVS15, and IVS16), from herein referred as wt and 2657+5G>A mutant (Mut) CFTR splicing minigenes. Site-directed mutagenesis using KOD HOT start DNA polymerase (Novagen, Darmstadt, Germany) was used according to the manufacturer's protocol to insert the mutation 2657+5G>A. The mutation is numbered based on the cDNA sequence (GenBank Reference Sequence NM_000492) according to the Human Genome Variation Society guidelines (http://www.hgvs.org/mutnomen). Nucleotide 1 is the A of the ATG translation initiation codon. The *CFTR* cDNA full sequence that was inserted into pCDNA5/FRT/CFTR and used here is shown in Supp. Figure S1. Two-color *CFTR* splicing reporter minigenes were developed using the nonfluorescent wt and Mut minigene backbones: pcDNA5/FRT/mCherry-Flag-wt-IVS14/15/16-CFTR and the mutant minigene pcDNA5/FRT/mCherry-Flag-Mut-IVS14/15/16_2657+5G>A-CFTR, from herein referred as mCherry-Flag-wt-CFTR and mCherry-Flag-Mut-CFTR, respectively. The insertion of cDNA coding for mCherry upstream of *CFTR* minigenes was done using the same principle as for the IVS's. Flag-tag was inserted by mutagenesis using KOD HOT start DNA polymerase and used according to the manufacturer's protocol. Primer sequences are provided in Supp. Table S1.

Cell Culture and Stable Cell Line Production

Human embryonic kidney 293 Flp-In (HEK293 Flp-In) cells (Invitrogen, Carlsbad, CA) were cultured in Eagle's minimal essential medium supplement with 10% fetal bovine serum, selection antibiotic hygromycin B 100 μg/ml (Sigma-Aldrich, St. Louis, MO) kept at 37°C under 5% CO_2. HEK293 Flp-In stable cell lines were generated by cotransfection of *CFTR* splicing minigenes and pOG44 vector (Invitrogen) using Lipofectamine 2000 Reagent (Invitrogen), following the manufacturer's instructions. The Flp-In system contains only a single integrated Flp recombination target (FRT) and expresses no endogenous CFTR.

AONs Treatment and In Vitro Splicing Assays

AONs were synthesized by Integrated DNA Technologies, Inc. (IDT, Leuven, Belgium). The sequences of the AONs used were as follows: AON1, 5′ mU* mC* mU* mA* mC* mA* mC* mA* mA* mU* mA* mG* mG* mA* mC* mA* mU* mG* mG 3′; AON2, 5′ mC* mU* mA* mC* mA* mC* mA* mA* mU* mA* mG* mG* mA* mC* mA* mU* mG* mG* mA 3′; control AON (Ctrl), 5′ G* mC* mG* mU* mA* T* T* A* T* A* G* C* C* G* A* mU* mU* mA* mA* mC 3′. The letter m represents an O-methyl modification at the second position of a sugar residue and the asterisk represents a phosphorothioate modification of the backbone. HEK293 Flp-In cells stably expressing the wt- or Mut-CFTR splicing minigenes were transiently transfected with the AONs at a final concentration of 50 nM for 24 hr (RT-PCR assays) or 48 hr (other assays) using Lipofectamine 2000 Reagent (Invitrogen) following the manufacturer's instructions.

RT-PCR and Real-Time PCR Analyses

Total RNA was isolated 24 hr after AONs transfection using the NucleoSpin RNAII Kit (Macherey-Nagel, Düren, Germany) and 1 μg of cDNA was produced using the M-MuLV reverse transcriptase (NZYTech, Lisbon, Portugal). Expression of wt- and Mut-CFTR minigenes was assessed by semiquantitative RT-PCR (sqRT-PCR), with the primers: G15R-ex15 5′-CTACATGGAACACATACCTTC-3′ and G15L-ex17 5′-GGTGCTGGTGATAATCACTG-3′. The identity of each RT-PCR product was verified by sequencing. Quantification of spliced products was performed with ImageJ/Fiji software. Results were confirmed by four independent experiments. Expression of wt and Mut transcripts was also measured by quantitative real-time PCR (qRT-PCR) using the SsoFast EvaGreen system (Bio-Rad, Hercules, CA), with the primers Ex15-16-fw: 5′-CAGAGGTGGCTGCTTCTTTG-3′ (wt); Ex15-Ex17-fw: 5′-TTTTCTGGCAGAGCACTCCT-3′ (Mut); Ex17-rv 5′-ATAGCAAGCAAAGTGTCGGC-3′. ß-Actin was used to normalize expression of wt and Mut CFTR transcripts.

Western Blot Analysis

CFTR protein detection was performed by Western blot as previously [Masvidal et al., 2014]. Briefly, proteins were separated by SDS-PAGE 7% (w/v) gel and CFTR was detected using the anti-CFTR monoclonal antibody M3A7 (1:2,000 dilution) (EMD Millipore, Billerica, MA). As a loading control, we used calnexin detected by anti-calnexin antibody (1:3,000 dilution) (BD Biosciences, San Jose, CA) and the secondary antibody was horseradish peroxidase-labeled antimouse (1:3,000) (Bio-Rad). Image Lab™ Software (Bio-Rad) was

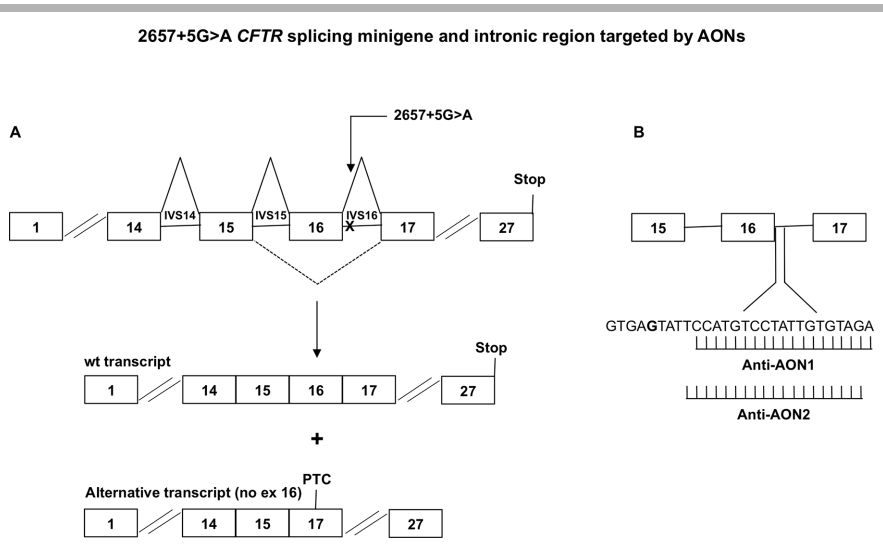

Figure 1. Effect of the 2657+5G>A *CFTR* splicing mutation and design of corrective AONs. **A**: Schematic representation of the 2657+5G>A *CFTR* splicing minigene, showing that the mutation 2657+5G>A produces wt *CFTR* transcripts and alternative transcripts lacking exon 16. PTC: premature stop codon. **B**: Diagram showing part of IVS 16 region targeted by AON1 and AON2. Mutation 2657+5G>A highlighted in bold.

used for CFTR protein quantification, which was then normalized to calnexin levels.

Automated Fluorescence Microscopy

HEK293 Flp-In cells stably expressing the two-color *CFTR* splicing reporter minigenes mCherry-Flag-wt-CFTR and mCherry-Flag-Mut-CFTR were grown in 96-well plates and transfected with either AON1 or control AON as above. After AONs transfection, extracellular Flag-tag was immunostained in nonpermeabilized cells, as previously done [Botelho et al., 2015]. Fluorescence microscopy was performed using a fully automated inverted Leica DMI6000B research microscope equipped with an EL6000 camera (Leica, Wetzlar, Germany) and a motorized stage. Fluorescence images were acquired in automatic mode using a 20x objective and a standard filter for mCherry (excitation 515–560, emission >590), Flag-tag (excitation 630–660, emission >670), and Hoechst (excitation 340–380, emission 450–490). Automatic image analysis was performed with open source software tools (CellProfiler, R), as previously performed [Botelho et al., 2015].

Iodide Efflux Assay

The CFTR-mediated iodide efflux assay used here was as previously described [Moniz et al., 2013]. Briefly, cells grown in 6-well plates were transfected with either AON1 or control AON (in duplicates). This is a well-established iodide efflux assay that consists in loading the cells with an iodide solution (as the channel is permeable to iodide), subsequent CFTR channel stimulation with forskolin (Fsk), and IBMX, followed by the measurement of iodide that remained in the cells. In this assay, increased channel activity corresponds to more iodide released from the cells and thus decreased iodide concentration remaining within the cells. Thus, after transfection with the AONs cells, were incubated with iodide-loading buffer for 30 min at 37°C, then thoroughly washed with iodide-free efflux buffer and equilibrated for 10 min in the same buffer. Cells were then incubated for 5 min either in the presence of iodide-free efflux buffer or in the presence of CFTR stimulators Fsk 10 μM and IBMX 50 μM (Sigma). Cells were then lysed and the iodide concentration in each sample was determined using an iodide-sensitive electrode (Orion 96–53; Thermo Scientific, Rockford, IL) with a pH/mV meter and normalized to the amount of protein.

Statistical Analyses

Data are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made using unpaired *t*-test (GraphPad Prism Software), and statistical significance was considered for $P \leq 0.05$ indicated by cardinal (♯) and asterisk (*) relative to wt and mutant (Mut) cells, respectively.

Results

Modulation of the 2657+5G>A Splicing Mutation by AONs

We have previously shown that the 2657+5G>A *CFTR* splicing mutation weakens the 5′ss of IVS16 originating transcripts lacking exon 16 concomitantly with wt transcripts (Fig. 1A) [Masvidal et al., 2014]. Here, we tested whether an AON strategy would correct such aberrant splicing, that is, skipping of exon 16. The 2657+5G>A mutation is located at the 5′ end of IVS16, that is, five nucleotides downstream from the intron, a region that is conserved among mammals and harbors the canonical base-pairing region for U1 snRNA, a key component of U1 snRNP required for correct splicing [Singh et al., 2006; Dal Mas et al., 2015]. Therefore, the AON (AON1) was designed a few nucleotides downstream from the U1 snRNA binding region (Fig. 1B; Supp. Fig. S2). Furthermore, we designed another AON (AON2) targeting the same IVS16 sequence as AON1 but one nucleotide upstream to assess whether interfering with the U1 snRNP binding impacts on exon recognition (Fig. 1B; Supp. Fig. S2).

The effect of AONs on *CFTR* splicing was determined by transfecting each AON into HEK293 Flp-In cells stably expressing the 2657+5G>A mutant (Mut) minigene, followed by the analysis of the splicing pattern by semiquantitative (sq) and quantitative (q)

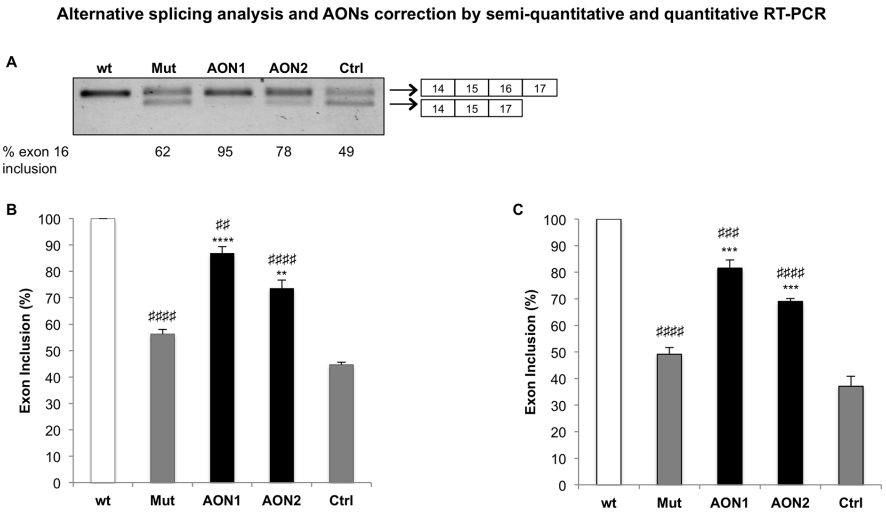

Figure 2. Correction of AS by AONs assessed by sqRT-PCR and qRT-PCR. AONs effect on splicing of the *CFTR* 2657+5G>A splicing minigene. **A**: RT-PCR analysis of wt and mutant (Mut) minigene transcripts expressed by HEK293 Flp-In cells transiently transfected with the AON1, AON2, or control AON (Ctrl). Representative result from agarose gel electrophoretic analysis where the upper band corresponds to correctly spliced products and lower band corresponds to alternatively spliced products, that is, exon 16 skipping. Numbers shown below correspond to the average percentage levels of exon 16 inclusion. The latter were calculated from the total value of exon 16 included and exon 16 skipped products. **B**: Graph showing quantification from four independent experiments. **C**: qRT-PCR analysis of wt and Mut-CFTR minigene transcripts expressed in HEK293 Flp-In cells transiently transfected with the AONs. Graph shows quantification from four independent experiments.

RT-PCR. Data from sqRT-PCR showed that AON1 was able to rescue correct splicing almost completely, by increasing exon 16 inclusion from 56 ± 2 % to 87 ± 3% in nontreated versus AON1-treated mutant cells, whereas AON2 only increased exon 16 inclusion to 74 ± 4% (Fig. 2A and B; Supp. Fig. S3). The AON1/2 effect was sequence specific since a nonspecific AON (Ctrl) did not rescue exon 16 inclusion. The presence of the entire exon 16 inclusion after AON1 treatment has been confirmed by sequencing the transcripts resulting from the above sqRT-PCR (Supp. Fig. S4). Evaluation of wt and Mut transcripts by qRT-PCR similarly showed that exon 16 inclusion increased from 49 ± 2% up to 82 ± 3% in nontreated versus AON1-treated mutant cells, whereas AON2 increased inclusion to 69 ± 1% (Fig. 2C). Moreover, we also assessed whether the 2562T>G *CFTR* variant in cis with 2657+5G>A (double mutant) could cause an effect on the rescue. However, similar to our previous study using the *CFTR* splicing minigenes with either 2562T>G alone or in combination with 2657+5G>A [Masvidal et al., 2014], we did not detect the alternatively spliced transcript that lacks exon 15 resulting from the 2562T>G SNP as was observed in the ex vivo analysis (Supp. Fig. S3). We also assessed the effect on our system of small molecules previously reported to rescue splicing in the context of other disorders [Anderson et al., 2003; Hims et al., 2007; Sumanasekera et al., 2008]. However, none of them had any visible effect (Supp. Fig. S5). Altogether, these results show that AON1 almost completely restored exon 16 inclusion into mature *CFTR* RNA of 2657+5G>A transcripts. As AON1 showed higher efficiency in correcting splicing, further experiments were performed only with AON1.

AON1 Increases CFTR Protein Levels

To assess whether AON1-mediated correction of the 2657+5G>A splicing mutation also leads to increased levels of full-length normal CFTR protein, we analyzed CFTR protein by Western blot in HEK293 Flp-In cells stably expressing the 2657+5G>A mutant minigene and treated with AON1. Indeed, data in Figure 3 show that AON1 treatment led to a significant ~twofold increase of full-length CFTR protein levels as compared with nontreated mutant cells. Nonspecific control AON (Ctrl) did not produce any detectable increase in the levels of full-length CFTR protein. This result shows that splicing modulation with AON1 at the mRNA level also restored levels of full-length CFTR protein.

AON1 Significantly Increases CFTR Levels at the PM

As normal CFTR localization is at the PM, next we investigated whether AON1 treatment caused an increase in CFTR trafficking to the cell surface. To this end, we developed a two-color *CFTR* splicing reporter that allows intracellular localization of CFTR by fluorescence microscopy. The mCherry red fluorescent protein was inserted at the N-terminus of both wt- and Mut-CFTR splicing minigenes to quantify the total amount of CFTR protein expressed by each individual cell. The Flag epitope tag inserted at the *CFTR* 4th extracellular loop, encoded by exon 17 (i.e., upstream of the 2657+5G>A mutation), allows quantification of CFTR localized at the cell surface [Botelho et al., 2015]. Mutant transcripts, however, will not result in the expression of the Flag-tag, since these are out of frame after exon 17 leading to a premature stop codon (PTC) at exon 17 and thus generating a truncated protein (Fig. 1A).

Results from fluorescence microscopy of HEK293 Flp-In stable cell lines expressing either wt- or Mut-CFTR reporter minigenes revealed that both cell lines show CFTR protein expression as observed by the mCherry fluorescence (Fig. 4A; upper and middle rows, 2nd column from the left). However, high levels of PM localized CFTR can be observed in the mCherry-Flag-wt-CFTR cells but not for the mCherry-Flag-Mut-CFTR ones (Fig. 4A; upper and middle rows, 3rd column from the left).

Yet, after AON1 treatment, mCherry-Flag-Mut-CFTR cells show significantly increased levels of PM-localized CFTR versus nontreated Mut-CFTR cells (see green fluorescence levels in Fig. 4A and B; middle and lower rows, 3rd column from the left, respectively).

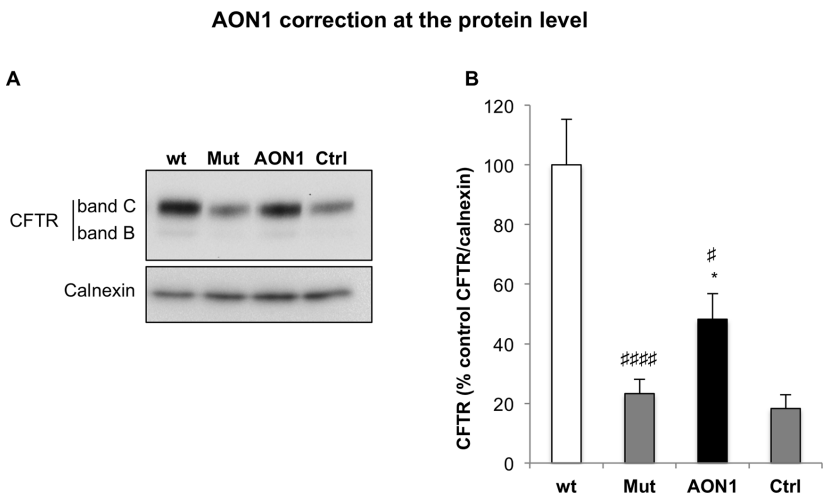

Figure 3. Correction of splicing by AON1 at the protein level. AON1 modulation increases levels of CFTR protein. **A**: Western blot analysis of wt- and Mut-CFTR minigenes expressed in HEK293 Flp-In cells transiently transfected with AON1 and control AON (Ctrl). Calnexin was used as a loading control. **B**: The amount of processed mature CFTR, that is, fully glycosylated form (band C) is shown as percentage of expression relative to nontreated wt cells; $n = 6$ independent experiments.

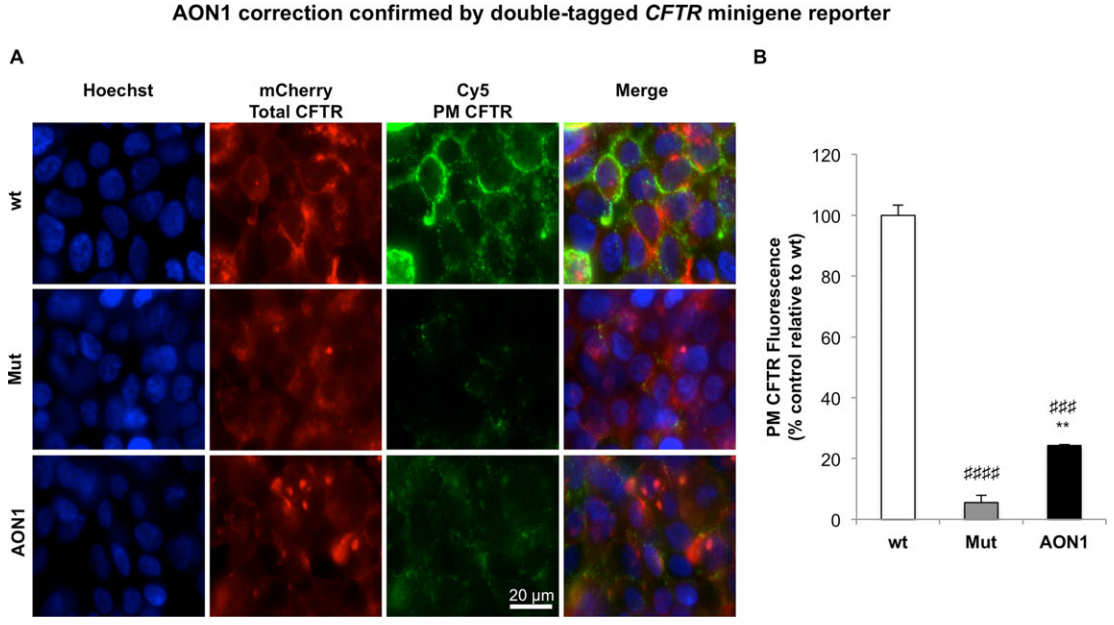

Figure 4. AON1 correction confirmed by double-tagged *CFTR* minigene reporter. Representative immunofluorescence images of HEK293 Flp-In cells stably expressing mCherry-Flag-wt-CFTR and mCherry-Flag-Mut-CFTR minigenes, after AON1 or AON control (Ctrl) treatment. **A**: Left column, Hoechst staining of the nuclei; middle columns, mCherry (red) and Cy5 (green) for total and PM CFTR, respectively. Cells expressing the 2657+5G>A intronic mutation generate an alternatively spliced mRNA that lacks IVS 16, causing disruption of the open reading frame and a PTC, thus resulting in a truncated protein, which is visible, although at low levels as observed by reduced mCherry fluorescence (middle row, 2nd panel from left). As in the experimental construct, the Flag tag was inserted at the CFTR 4th extracellular loop (by exon 17), that is, after the PTC, Flag staining is almost absent for the mCherry-Flag-Mut-CFTR (middle row, 3rd panel from left) as compared with wt (top row, 3rd panel from left). However, in AON1-treated cells, the Flag staining is recovered, indicating rescue of the full-length CFTR protein (bottom row, 3rd panel from left). Scale bar = 20 μm. **B**: Quantification of CFTR PM levels for Flag staining shown as percentage relative to wt cells; $n = 3$ independent experiments.

These results thus corroborate the mRNA and protein studies showing that AON1 corrects abnormal splicing and results in increased levels of CFTR protein correctly localized at the PM. Moreover, these data also demonstrate that our newly developed two-color *CFTR* splicing reporter minigene is suitable for quantitative monitoring of splicing correction through the appearance of CFTR at the PM.

AON1 Significantly Increases Levels of Functional CFTR

Finally, we tested the channel activity of CFTR protein in AON1-treated versus control-treated mutant cells as well as control-treated wt cells.

Data in Figure 5 show that AON1-treated cells had significantly higher levels of CFTR activity, as they exhibited a ~1.3-fold decrease

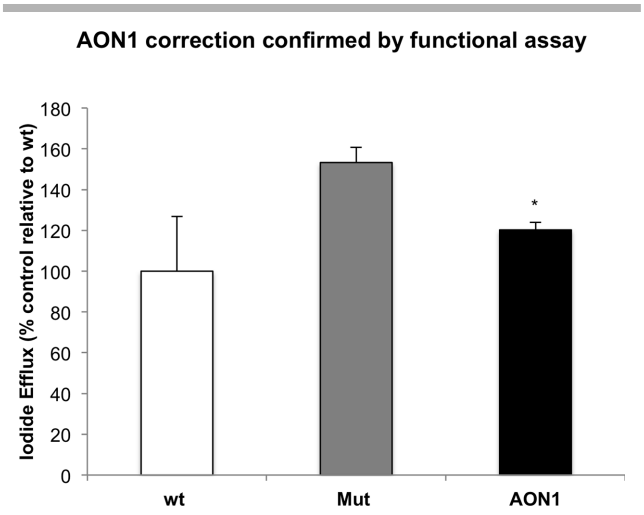

Figure 5. AON1 correction confirmed by functional assay. Iodide efflux of HEK293 Flp-In cells stably expressing 2657+5G>A mutant *CFTR* minigene after AON1 and AON control (Ctrl) treatment. Graph shown as percentage of control, that is, relative to wt, in cells stimulated by forskolin (10 μM) and IBMX (50 μM); $n = 3$ independent experiments. Iodide that remained in the cells was measured with an iodide electrode (see *Materials and Methods*).

in iodide concentration that remained in the cells in comparison to control-treated mutant cells (Fig. 5). This result further confirms all the above findings for the rescue of the splicing by AON1.

Discussion

Herein, we assessed whether RNA-based AONs can correct the splicing defect of the *CFTR* splicing mutation c.2657+5G>A (IVS16), that we previously reported to weaken the 5′ss of IVS16 originating exon 16 skipping, while drastically reducing the levels of wt-CFTR transcripts [Masvidal et al., 2014]. To this end, we used splicing minigenes consisting of the full-length *CFTR* cDNA and flanking intronic sequences, expressed in the HEK293 Flp-In cell system. To preclude the possible influence of the integration site of the trans-minigene on the activity of the splicing machinery, we used HEK293 Flp-In cells, as we described previously [Masvidal et al., 2014] and in which the Flp-In system leads to the insertion of a unique copy of either wt or mutant minigene at the specific FRT chromosomal site. Moreover, we are confident that this is a bona fide model for splicing because the splicing pattern resulting from our 2657+5G>A mutant minigene was comparable to that obtained in primary nasal cells obtained from patients carrying the c. 2657+5G>A mutation, as shown in our previous study [Masvidal et al., 2014]. We thus used this system to assess the corrective effects of two custom-designed AONs on the splicing mutation, which were analyzed at the mRNA and protein level. In addition, we used a two-color splicing reporter to quantitatively assess correction of splicing through the PM localization of full-length CFTR by automated fluorescence microscopy. In contrast to the very efficient rescue of normal splicing by AON1, we also tested the effects on splicing by several compounds previously reported to rescue splicing in the context of other disorders. However, none of the tested compounds evidenced any correction of the aberrant splicing. This may be due to these compounds acting in a sequence specific fashion.

The 2657+5G>A splicing mutation is located at the U1 snRNA-binding site, thus causing reduction of the complementarity with the U1 snRNA [Singh et al., 2006]. Accordingly, we have designed two AONs so as to target/mask the pre-mRNA region located a few nucleotides downstream of the mutation. Our data show that the antisense sequences (AON1 and AON2) significantly corrected exon exclusion and restored production of full-length CFTR mRNA. Splicing corrector AON1 showed more striking correction than AON2 (from ~49% to ~82% and ~69%, respectively), as it almost completely restored exon 16 inclusion. The more effective correction by AON1 could be due to two main reasons, namely, (i) AON2, being +1nt upstream, possibly interferes with binding site for U1 snRNA and thus also slightly impairs correct exon recognition; and/or (ii) AON1 masks a binding site for an inhibitory splicing regulator, that is, an ISS. It is thus possible that we have identified a putative ISS element downstream of the U1 snRNA binding site in intron 16 within the region targeted by AON1 (intronic position +10 to +28 relative to the donor splice junction). Notwithstanding, further studies are required to fully understand this mechanism, such as performing a series of deletions, insertions, and/or nucleotide substitutions within the region targeted by AON1. In addition, specificity and efficacy of correction could be assessed either by introducing mutations in AON1 and/or testing a panel of AONs hybridizing to different positions further downstream within IVS 16. By performing such studies, we would be able to: (i) confirm the silencer function of the putative ISS element identified in this study; (ii) prove that the corrective AON1 effect is solely due to masking of this element; (iii) delineate relevant intronic elements and investigate whether other AONs could promote greater exon inclusion. Consistently, a similar intronic cis element was identified in a nonconserved portion of the final intron (intron 7) of the extensively studied human *Survival Motor Neuron* (*SMN*) gene [Singh et al., 2006]. Such element was found to be an important component of a regulatory network that modulates AS of SMN exon 7, associated with spinal muscular atrophy (SMA) [Singh et al., 2006; Dal Mas et al., 2015]. Additional studies are necessary to fully address the mechanism and identify the factors involved in determining the AON1-mediated induction of exon inclusion.

Nevertheless, using our minigene system, we were able to show that AON1 modulation resulted in recovery of wt-CFTR protein synthesis, with consequent increase in levels of protein correctly localized at the PM exhibiting normal function as a Cl^- channel. Localization and quantification of the proportion of CFTR that reached the PM was assessed using a novel two-color splicing reporter system developed and validated here for automated fluorescence microscopy. Such a system is highly suitable for high-content gene screens to identify splicing regulators or in the discovery of drugs that restore splicing. Indeed, AS has emerged as a promising therapeutic target in a number of human disorders, but the discovery of compounds that target the splicing reaction has been hindered by the lack of suitable high-throughput screening assays. Although other splicing reporters were previously reported to assess AS [Stoilov et al., 2008; Gurskaya et al., 2012], our model has the advantage that besides quantitatively assessing correction of splicing, it also allows discrimination of the levels of the final product, that is, the resulting protein while enabling determination of its correct localization at the PM.

From the current study, we conclude that AONs deserve further consideration as a valuable therapeutic strategy for a significant proportion of CF-causing mutations (~13% of total) since they provide a very specific and efficient approach for correction of aberrant splicing, in this particular case exon skipping. Indeed, this approach has been successfully developed from preclinical cell and animal models into the clinical trial phase for Duchenne muscular dystrophy and SMA [Kole et al., 2012; Touznik et al., 2014]. Similarly, CF

patients carrying *CFTR* mutations affecting pre-mRNA splicing, that is, classes I and IV [Amaral, 2015] could benefit from AON therapies [Bell et al., 2015]. Previous studies have indicated that in CF 5% of normal *CFTR* mRNA is already enough to significantly attenuate severity of CF lung disease [Chu et al., 1992; Ramalho, et al., 2002]. In this particular case of patients with the c.2657+5G>A splicing mutation [Highsmith et al., 1997], they could significantly benefit from the AON therapy. Indeed, AON1 seems to provide sufficient correction for proper channel function in epithelial tissues [Bell et al., 2015]. Although previous studies have demonstrated effective in vitro approaches to correct mutation-specific *CFTR* splicing defects in CF those authors have used splicing factors [Nissim-Rafinia et al., 2004; Fernandez Alanis et al., 2012]. Here, the demonstration of splicing correction by AONs, which are resistant, easy to transfect molecules, highlights their therapeutic potential in CF. Yet, the clinical utility of these therapies for ameliorating CF lung disease requires suitable and safe in vivo delivery vectors [Ikpa et al., 2014]. Notwithstanding, a Phase I clinical trial supports the safety and tolerability of transferring a nonviral vector to treat CF, which consists of a lipid-mediated vector harboring a normal *CFTR* gene that is administered as a nebulizer [Yin et al., 2014].

AON1 could thus be used to induce in vivo similar correction to that demonstrated here in vitro for the c.2657+5G>A *CFTR* mutation, indicating that CF patients carrying such mutation could benefit from AON treatment. This study also highlights the therapeutic potential of splicing modulation by AONs for other genetic diseases caused by exon-skipping mutations.

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