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Original Article

Antisense oligonucleotides targeting exon 11 are able to partially rescue the NF2-related schwannomatosis phenotype *in vitro*

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NF2-related schwannomatosis (NF2-related SWN) is an autosomal dominant condition caused by loss of function variants in the *NF2* gene, which codes for the protein Merlin and is characterized by the development of multiple tumors of the nervous system. The clinical presentation of the disease is variable and related to the type of the inherited germline variant. Here, we tested if phosphorodiamidate morpholino oligomers (PMOs) could be used to correct the splice signaling caused by variants at ±13 within the intron-exon boundary region and showed that the PMOs designed for these variants do not constitute a therapeutic approach. Furthermore, we evaluated the use of PMOs to decrease the severity of the effects of *NF2* truncating variants with the aim of generating milder hypomorphic isoforms *in vitro* through the induction of the in-frame deletion of the exon-carrying variant. We were able to specifically induce the skipping of exons 4, 8, and 11 maintaining the *NF2* gene reading frame at cDNA level. Only the skipping of exon 11 produced a hypomorphic Merlin (Merlin-e11), which is able to partially rescue the observed phenotype in primary fibroblast cultures from NF2-related SWN patients, being encouraging for the treatment of patients harboring truncating variants located in exon 11.

INTRODUCTION

NF2-related schwannomatosis[1] (NF2-related SWN) (Online Mendelian Inheritance in Man 101000) is an autosomal dominant (AD) disease caused by loss-of-function (LOF) variants in the *NF2* tumor suppressor gene (22q12.2).[2,3] The reported incidence is between 1 in 28,000 and 1 in 40,000[4] and the hallmark of the disease is the presence of bilateral vestibular schwannomas, which clinically present with hearing loss, tinnitus, and imbalance.[5] Common features of NF2-related SWN include cranial, spinal, peripheral nerve, and intradermal schwannomas; cranial and spinal meningiomas; and intrinsic central nervous system tumors, usually spinal ependymomas.[6,7] Despite their benign nature, tumors are associated with high morbidity because of their multiplicity and anatomic location, which cause pain and nerve dysfunction leading to a decreased quality of life and life expectancy.[5]

There are limited therapies available for these patients and radiotherapy and surgery for tumor resection are the classical treatments.[5] Currently, medical therapy is also an option since Bevacizumab, a monoclonal antibody targeting the vascular endothelial growth factor, has been demonstrated to be an effective treatment for vestibular schwannomas inducing the decrease in tumor growth and an improvement in hearing in adult NF2-related SWN patients.[7,8] However, bevacizumab does not seem to be equally effective in all patients, and it has no effect on other types of tumors arising from the disease, such as meningiomas.[9] Efforts have been made to develop other therapies for NF2-related tumors, such as losartan (an antihypertensive), brigatinib and lapatinib (tyrosine kinase inhibitors), or AR-42 (histone deacetylase inhibitor),[10–14] but there remains a need to identify clinically relevant therapeutic targets to treat NF2-related SWN patients.

The *NF2* gene consists of 17 exons[15,16] with a wide mutational spectrum. It is estimated that 85%–90% of the pathogenic variants in *NF2* are germline point mutations and 10%–15% are large deletions, distributed throughout the entire gene.[17,18] *NF2* encodes for Moesin-Ezrin-Radixin-Like protein (Merlin), a 595 amino acid scaffold protein involved in membrane protein organization, regulation

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of cell-cell adhesion, and with a regulatory role in the cytoskeleton architecture.[19,20] Merlin is comprised of three structural regions: an N-terminal FERM domain which is subdivided into three globular domains (F1, F2, and F3), an α-helical coiled-coil domain and a C-terminal hydrophilic tail.[21]

The genotype-phenotype correlation of NF2-related SWN has long been studied.[22–30] For NF2-related SWN, it is well reported that truncating variants and large deletions, which account for approximately 30% of cases, are associated with severe manifestations of the disease, whereas mis-sense or in-frame variants present milder forms of NF2-related SWN. Data reveal that individuals with the latter variants show a late disease onset and are diagnosed later compared with individuals with truncating mutations, as well as a lower risk of developing peripheral nerve tumors, spinal tumors, and meningiomas; thus, these genetic are variants associated with a milder disease burden.[22,24,28,31,32] In addition, approximately 25% of the point mutations affect the correct splicing of the *NF2* gene, and this mechanism is associated with a variable severity of the disease.[27,29,31] NF2-related SWN is also estimated to have a high presence of mosaicism, reported to affect at least 30% and up to 50% of *de novo* patients, which often challenges the molecular diagnosis of the disease.[33] NF2-related SWN mosaicism is associated with mild clinical presentations, although significant variability has been reported.[29,33] Thus, the well known association between the germline genetic variant type and disease severity may constitute an opportunity to develop personalized gene therapies based on the specific variant of the *NF2* gene.

Antisense gene therapy has been successfully used to regulate gene expression in some pathologies. Most of the target diseases are inherited in an autosomal recessive manner, such as spinal muscular atrophy (SMA),[34] homozygous familial hypercholesterolemia,[35] or Duchenne muscular dystrophy (DMD),[36] the latter two have been approved by the U.S. Food and Drug Administration (FDA) and are among others currently in the advanced stages of clinical trials.[37,38] Furthermore, there are also examples for AD diseases, such as retinitis pigmentosa (RP),[39] amyotrophic lateral sclerosis,[40] or Huntington's disease.[41] In all cases, antisense oligonucleotides (ASOs) have been used. These are single-stranded molecules that act at the RNA level by specifically correcting or modifying the expression of the target protein to a less deleterious form.[42,43] ASOs have been therapeutically used to reduce protein production whether via an RNase H cleavage or an mRNA-ASO complex to block mRNA-ribosome interaction. It is also possible to modulate the splice signaling and among the different types of ASOs, phosphorodiamidate morpholino oligomers (PMOs) have highly desirable molecular properties that make them suitable for this last approach. PMOs are DNA-analogue molecules that comprise a nucleic acid base, a methylenemorpholino ring and phosphodiester inter-subunit bonds are replaced by phosphorodiamidate linkages, all of which produce a structure that provides high-targeting specificity, stability, low toxicity, and resistance to nucleases, ensuring the maintenance of a good long-term activity within the cell.[44]

In this study, we propose an approach to test *in vitro* antisense therapy for NF2-related SWN. This consists of forcing the skipping of exons harboring a frameshift or nonsense variant while preserving the gene reading frame, being potentially applicable to 9 of the 15 *NF2* exons in which truncating variants have been reported. Thus, taking into consideration the NF2-related SWN genotype-phenotype association, frameshift or nonsense variants that generate a more severe phenotype could be altered at the RNA level and potentially generate less deleterious protein forms, resulting in a mitigation of phenotype severity. In addition, we tested the feasibility of modulating and correcting the aberrant splicing of the *NF2* gene originating from different point mutations located near canonical splice sites.

RESULTS

Antisense therapy strategies for *NF2*

The first approach of this study aimed to test if PMOs could be used to specifically force the skipping of in-frame exons carrying a truncating variant, thus maintaining the *NF2* gene reading frame, and test if the generated Merlin protein could act as an hypomorphic form, still preserving partial function and to some extent, able to rescue an *in vitro* phenotype. In this way, taking into account that nonsense or frameshift variants are associated with more severe phenotypes than in-frame variants, based on genotype-phenotype correlations, these truncating variants could be modified to less deleterious forms (Figure 1A), with the need to assess whether the effect of the exon skipping on both alleles was more beneficial than harboring a truncating variant in heterozygosis.

A second strategy was evaluated for *NF2* splicing variants. This experimental approach consisted of designing specific PMOs for a particular splicing variant to mask the aberrant splice signaling triggered by the pathogenic variant and promote the spliceosome to properly recognize the correct splice site without altering the wild-type (WT) allele. This could be used to target splicing variants that induce the skipping of an exon (Figure 1B) or for those that induce inclusion of intronic regions as coding sequences, as previously tested[45] (Figure 1C).

Evaluating the use of PMOs for truncating variants affecting the *NF2* gene

We first investigated *NF2* exons that could be appropriate candidates for generating an hypomorphic Merlin isoform after forcing its skipping, while preserving the gene reading frame. The evaluation of the *NF2* transcript (NM_00268.3) identified 9 out of 15 exons that could be skipped while maintaining the translational reading frame. In addition, for the remaining out-of-frame exons it would be possible to induce skipping of two consecutive exons (6 and 7, 12 and 13, and 14 and 15) (Figure S1). These 12 combinations were prioritized to be *in silico* analyzed. We also assessed exon length, since longer exons encode for a more significant proportion of the protein and, thus, could have a greater impact on its structure and stability and, as a consequence, functionality. Most *NF2* exons were in the range of normality without any exon overly long (>500 bp). We indicated

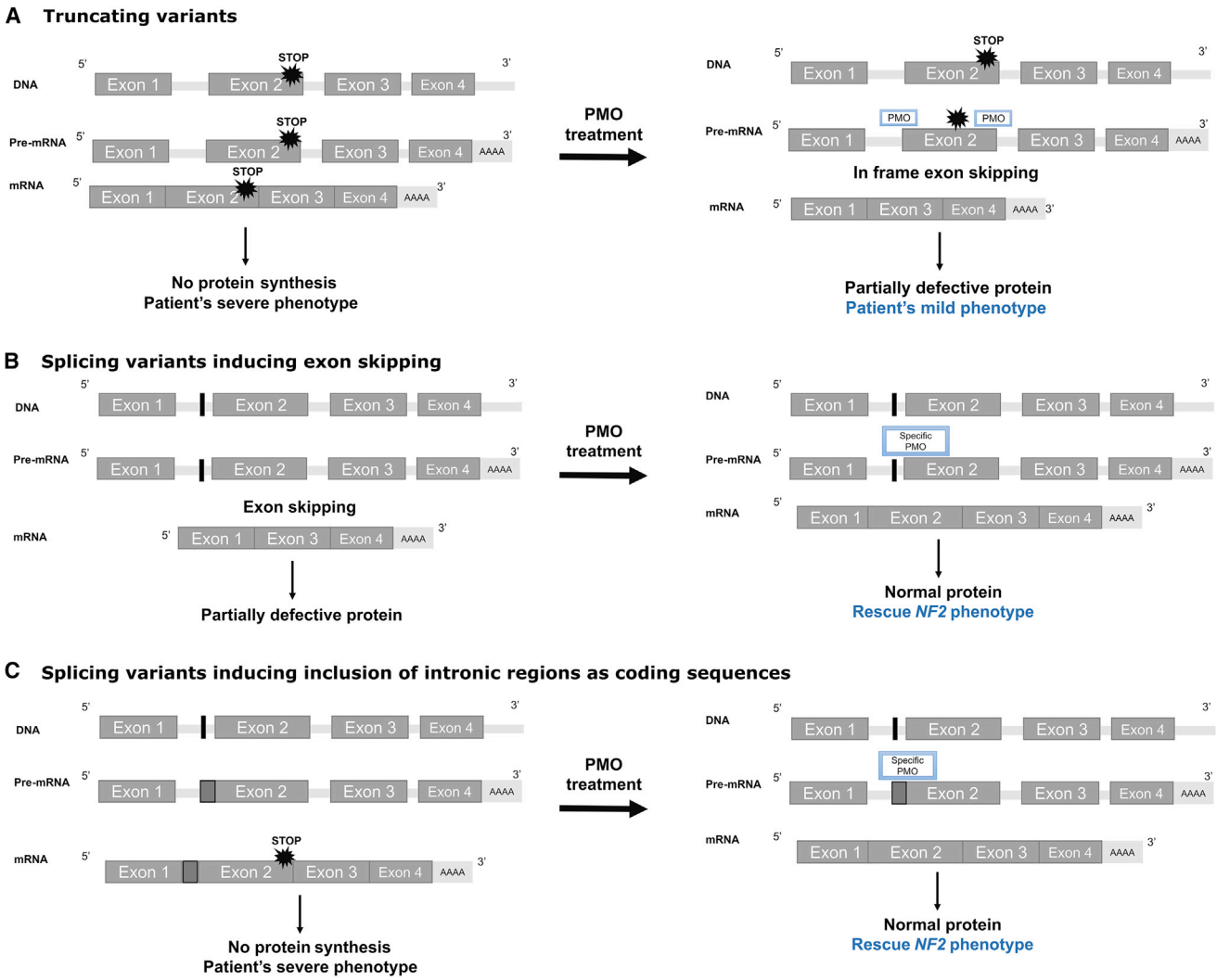

Figure 1. Experimental approaches to test antisense therapy for *NF2* variants

(A) Truncating variants. In blue, expected outcome after PMO treatment. PMOs are designed at the donor and acceptor sites of an in-frame exon to induce skipping of exons harboring truncating variants. The generated in-frame deletion is expected to ameliorate the phenotype *in vitro*. (B) Experimental approach to test antisense therapy for splicing *NF2* variants that induce exon skipping and result in a truncating protein and (C) that induce the inclusion of intronic regions as coding sequences, resulting in alterations in the gene reading frame that finally generate a truncating protein. In blue, expected outcome after PMO treatment. In both approaches, the PMO is expected to mask the aberrant splicing signal and rescue the NF2-related SWN phenotype *in vitro*.

all unique nonsense and frameshift variants described in the publicly available dataset Leiden Open Variation Database (LOVD) and also all truncating and splicing variants identified in our cohort. As previously described, no hot spots were detected and pathogenic variants were spread over the whole gene,[17,18] although several were described in the FERM domain[17,21] in the LOVD database. Next, we annotated Merlin protein post-translational modifications and the theoretical effect of skipping for each exon (or consecutive exons) in the resulting Merlin protein by *PredictProtein*.[46,47] Predicted features are shown in Figure S1. Seven candidate exons showed a high conservation score (>6.5), 3 were predicted to induce a high change of surface area, while 5 of 12 exons or combination of them showed several phosphorylation sites, which could be relevant for Merlin regulation. Considering all the different *in silico* indicators, exons 5 and 11 might be the most suitable candidates, since they showed less conservation score, did not present any phosphorylation site, and the predicted impact over the protein structure would be relatively low. Nonetheless, beside pairs of exons 12 and 13 and 14 and 15, the rest of the in-frame exons presented *in silico* metrics relatively similar among them; therefore, there was no strong evidence to discard any of the exons for further *in vitro* analysis. In the light of this, we tested the effect on Merlin after forcing exon skipping on all the available patient's primary fibroblasts with a truncating variant, corresponding with exons 4, 8, and 11.

Table 1. Exon-specific PMOs design to target *NF2* truncating variants

Exon	Target *NF2* variant	Target region	PMOs designed	PMO_Design 1	PMO_Design 2
4 (n = 2)	Any truncating at exon 4	Exon 4_5′	PMO pair	**PMO_5′ES4**: ACCTGAAAGGAGCAACAAGGGAGAC	
		Exon 4_3′		**PMO_3′ES4**: TTTCTTCTTTGAGCCTACCTTGGCC	
8 (n = 5)	Any truncating at exon 8	Exon 8_5′	PMO pair	**PMO_5′ES8**: TTTATTCTGTGGATCCAATAAGAAC	
		Exon 8_3′		**PMO_3′ES8**: TGCAGTACACACATGTCCTACCTCC	
11 (n = 2)	Any truncating at exon 11	Exon 11_5′	PMO pair	**PMO_5′ES11_v1**: AGGCGCTGCCGCTCCATCTGCGAGG	**PMO_5′ES11_v2**: CGCTCCATC TGCGAGGGGTGAAGAA
		Exon 11_3′		**PMO_3′ES11_v1**: CCAGCCCCTCAGAAATCACCAGTGC	**PMO_3′ES11_v2**: CCTCAGAAA TCACCAGTGCTTCGTT

n, number of patient-derived fibroblasts in which PMOs is tested.

A pair of PMOs were designed to be complementary to both 5′- and -3′′-intron-exon boundaries to induce skipping of exons 4, 8, or 11 of the *NF2* gene (exon-specific PMOs) and the induction of the exon skipping was first assessed in fibroblasts from healthy donors ($NF2^{(+/+)}$) (Table 1). PMOs were delivered through an endocytosis-mediated process with the use of the Endo-Porter. The effect of the treatment was considered in relation to the cells treated with Endo-Porter to determine exclusively the effect of the PMO treatment. Dose-response and time-course studies confirmed the efficiency of the PMO treatment at RNA level, showing full skipping of exons 4 and 8 at 20 μM at 24 h, 48 h, and 72 h (Figures 2A and S2). For exon 11, two different designs were tested (Table 1; PMO_ES11_v1 and PMO_ES11_v2): while the first (PMO_ES11_v1) failed to induce skipping (data not shown), for the second design (PMO_ES11_v2), the maximum skipping effect observed was at the highest tested dose (40 μM), achieving more than 50% of the exon-less form after 72 h of treatment (Figures 2A and S2).

Next, we analyzed whether the skipping of both the *NF2* alleles, the one harboring a truncating variant and the WT allele, was less deleterious than harboring a non-functional allele. First, we studied the capacity of restoring Merlin levels *in vitro* after the PMO treatment. Merlin western blot showed progressive loss of protein signal when targeting exons 4 (Merlin-e4) or 8 (Merlin-e8), studied at the 24 h, 48 h, and 72 h time points in two and five patient samples, respectively (Figure 2B). To verify that the induced skipping of exons 4 or 8 caused a decrease in Merlin levels rather than a lack of immunoreactivity, the exon-less *NF2* cDNAs were cloned to an N-terminal FLAG tag expression vector and transfected to HeLa cells. Flag-Merlin western Blot showed that there was little or no expression of Merlin-e4 or Merlin-e8, indicating that these Merlin forms could not be synthesized or were degraded shortly after synthesis (Figure S3).

Conversely, the skipping of exon 11 generated a shorter isoform of Merlin (Merlin-e11), and quantification of these results determine a slight increase in the total levels of Merlin (WT Merlin and Merlin-e11) (Figures 2B and S4). These results indicated that a potential hypomorphic Merlin-e11 could result from the skipping of this exon.

E11 PMO treatment of $NF2^{(+/-)}$ fibroblasts partially rescued the phenotype *in vitro*

The functionality of Merlin-e11 was studied in primary fibroblasts harboring a heterozygous truncating variant in exon 11: one from an adult patient (Patient_ES11_1) and another from a pediatric patient (Patient_ES11_2) (Table S1). Given the complexity of determining differences in the severity in the phenotype from studying the signaling pathways in which Merlin is involved,[29] physiological read-outs were assessed in patient-derived fibroblasts ($NF2^{(+/-)}$). Because of the role of Merlin in cytoskeletal organization,[21,48] Merlin-e11's functional activity was first tested through actin immunofluorescence. Phalloidin staining revealed an altered phenotype in both primary fibroblast samples, but it was slightly different in each patient tested: patient_ES11_1 showed prominent membrane ruffles, while patient_ES11_2 showed alterations in cytoskeletal abnormalities and a tendency to growth as aggregates, when compared with primary $NF2^{(+/+)}$ fibroblasts (Figure 3), and quite similar to the phenotype observed in other primary $NF2^{(+/-)}$ fibroblasts (Figure S5). After PMO treatment, an improvement in the organizational capacity of the cytoskeleton was observed, membrane ruffles were decreased in patient_ES11_1, and in patient_ES11_2 we observed an improvement in the cell-cell contact organization (Figure 3). Specifically, pediatric-derived $NF2^{(+/-)}$ fibroblasts recovered part of the cell-cell contact inhibition and recovered the capacity of growth as a monolayer after the induction of Merlin-e11.

In addition, proliferation rates were tested after 72 h of treatment by flow cytometry. Patient_ES11_1 showed a statistically significant depletion of proliferation when treated with PMOs (65.6%; $p < 0.05$) (Figure 4A), while patient_ES11_2 showed a less pronounced decrease (25.1%) (Figure 4B). Considering both samples, primary fibroblasts with a truncating variant in exon 11 and expressing Merlin-e11 by PMO treatment showed a 51% decrease in proliferation when compared with the vehicle (Endo-Porter) ($p < 0.01$) and 39.7% compared with treatment control (PMO with no effect over the *NF2* gene) ($p < 0.01$) on average (Figures 4C and S6). To determine absence of potential toxic effects, a viability test was performed, revealing that PMO treatment did not induce significant differences in cell viability (Figure S7).

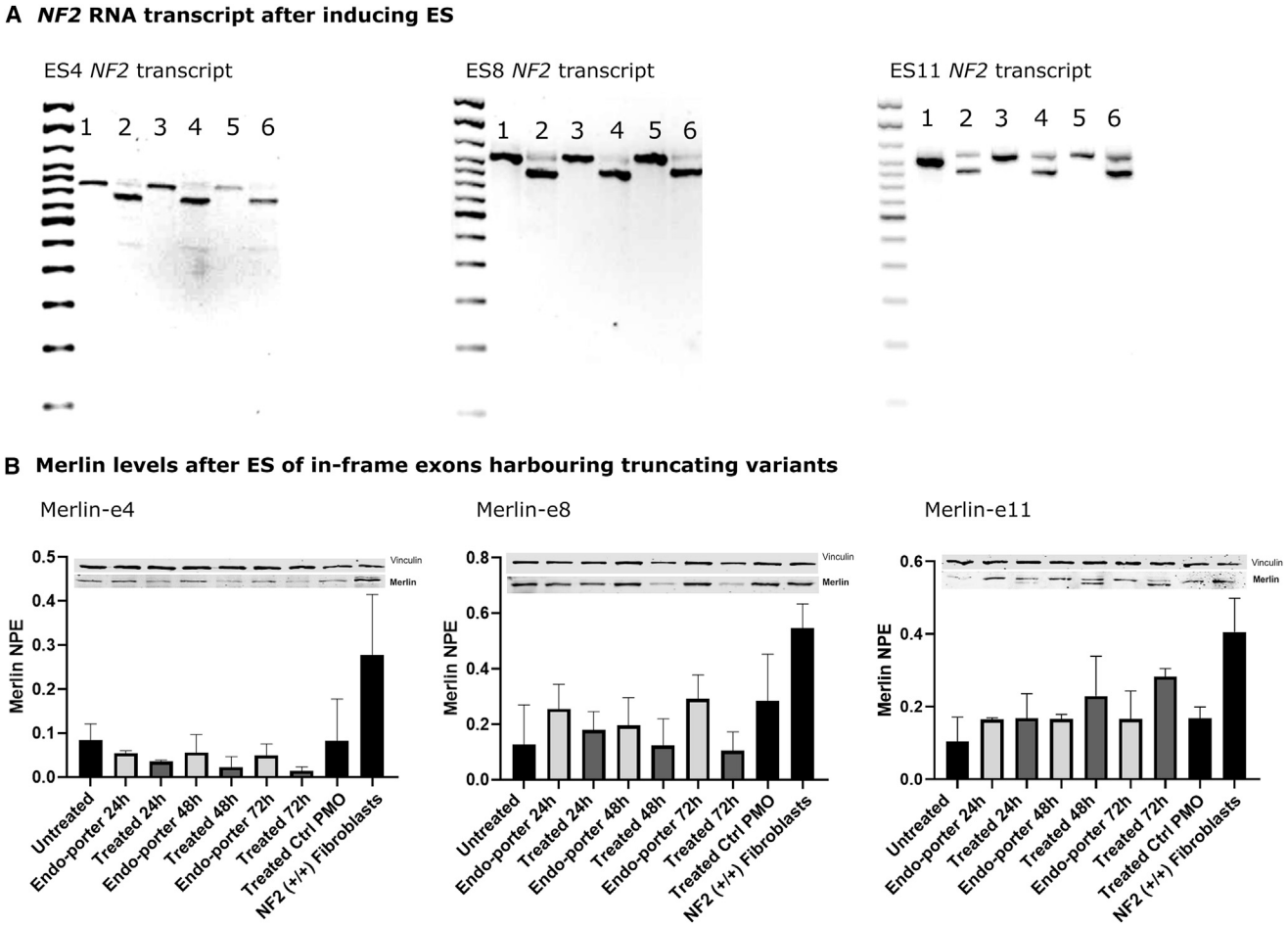

Figure 2. *NF2* RNA transcript and Merlin levels after inducing skipping of in-frame exons harboring truncating variants

(A) *NF2* RNA transcripts after inducing skipping of exons 4, 8, and 11 of the *NF2* gene showed that we were able to generate these exon-less forms at RNA level. The different lanes correspond to the following conditions: (1) untreated 24 h, (2) treated (20 μM for ES4 and ES8, 40 μM for ES11) 24 h, (3) untreated 48 h, (4) treated 48 h, (5) untreated 72 h, and (6) treated 72 h. (B) Merlin western blot after exon-specific PMOs treatment showed a decrease in Merlin total levels and no presence of exon-less, shorter forms of Merlin-e4 and Merlin-e8. Merlin-e11 was detected and a slight increase was determined in total Merlin levels. Graphs show total protein quantification from three independent experiments in fibroblasts from one patient. The mean ± standard deviation is represented in the bar. Increases or decreases in Merlin levels are considered in respect to the untreated sample. *NF2*$^{(+/+)}$ fibroblasts stand for control fibroblasts from healthy donors. When indicated, untreated stands for patient's fibroblasts (*NF2*$^{(+/-)}$) without PMO treatment, Endo-porter for patient's fibroblasts (*NF2*$^{(+/-)}$) treated with PMO vehicle, treated for patient's fibroblasts (*NF2*$^{(+/-)}$) treated with a pair of PMOs for each exon, and PMO Ctrl for patient's fibroblasts (*NF2*$^{(+/-)}$) treated with a control PMO that does not interfere with *NF2*. ES, exon skipping; NPE, normalized protein expression.

Antisense therapy for *NF2* splicing variants

This additional strategy intended to use PMOs, rather than to induce skipping, to mask specifically splicing variants and thus recover the correct splice signal. Two different PMOs were designed for each variant. The first design was a 25-mer variant-specific facilitated by *Gene Tools* according to their guidelines for intron and exon binding subunits and self-complementary moieties (data not shown). We made a second design using splice site predictors to study the variant surrounding sequence (acceptor splicing sites, exonic splicing enhancers or exonic splicing silencers) (based on *Human Splice Finder*) to avoid the induction of possible new splicing signals because of the effect of the PMO. In addition, the design avoided masking the canonical splicing GT-AG sites and branch points (YNYYRAY) when possible (Table 2, Figure 5A). We tested the PMOs' efficacy in primary fibroblasts from NF2-related SWN patients harboring heterozygous germline variants (*NF2*$^{(+/-)}$) located near the canonical splice site of exons 3, 8, and 15 (Table S1). A dose-response study was performed to test the effect on splicing of different concentrations of PMOs after 24 h of treatment, and the efficiency was assessed at cDNA level by reverse transcriptase polymerase chain reaction (RT-PCR). The effect of PMOs on the splicing of *NF2* was analyzed by electrophoresis and confirmed by Sanger sequencing, in which the maximum observed effect was at the highest tested dose (20 μM) (Figure 5B, dose response; Figure S8).

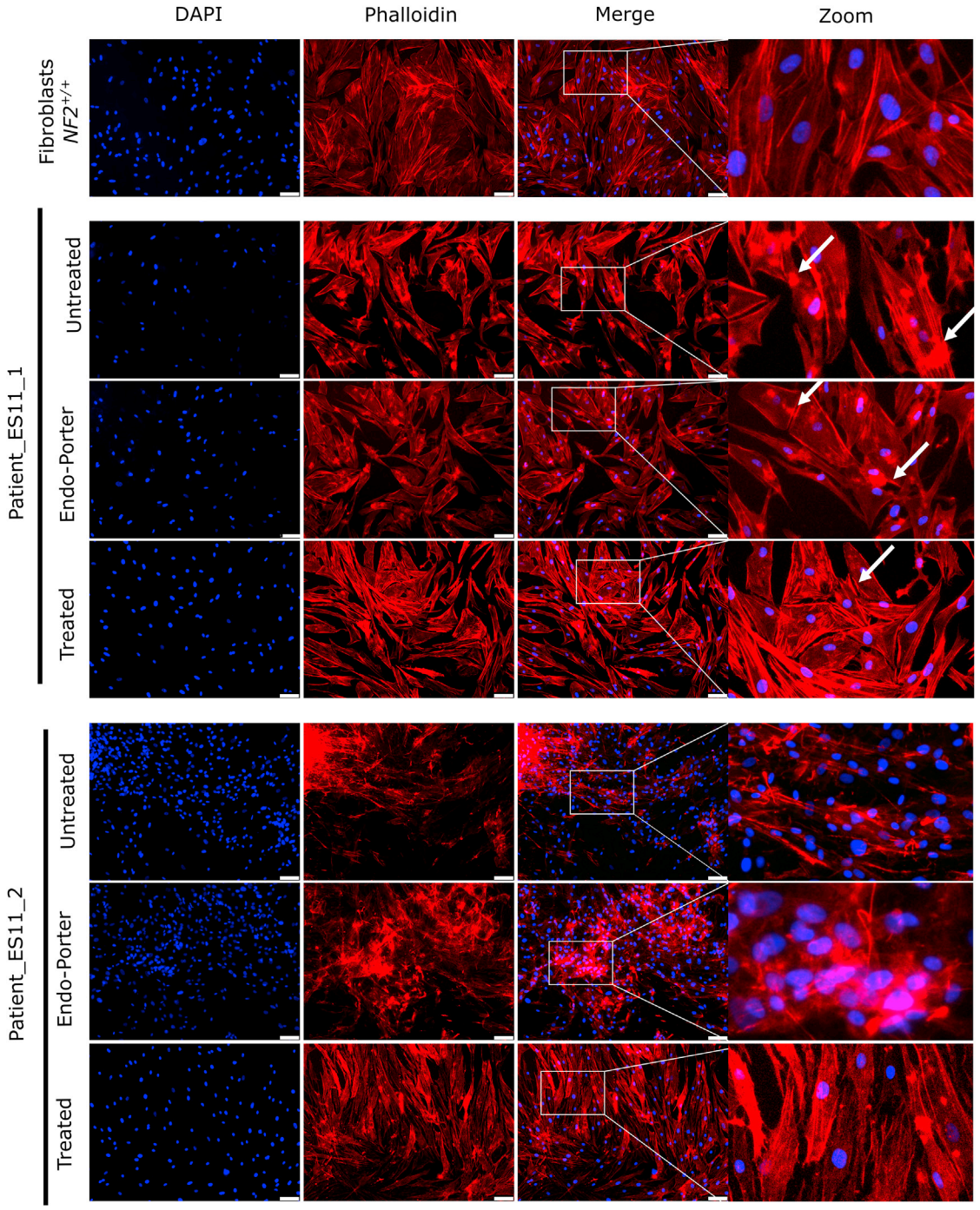

Figure 3. E11 PMO treatment of *NF2*$^{(+/-)}$ fibroblasts induced improvement in actin cytoskeleton organization

Actin immunocytochemistry analysis revealed cytoskeletal abnormalities in patient-derived fibroblasts. Patient_ES11_1 showed a decrease in membrane ruffle formation and Patient_ES11_2 responded to the treatment showing improved culture organization. 4′,6-Diamidino-2-phenylindole (DAPI) was used to stain cell nuclei and Phalloidin is shown in red. *NF2*$^{(+/+)}$ fibroblasts stand for control fibroblasts from healthy donors. When indicated, untreated stands for patient's fibroblasts (*NF2*$^{(+/-)}$) without PMO treatment, Endo-porter for patient's fibroblasts (*NF2*$^{(+/-)}$) treated with PMO's vehicle, and treated for patient's fibroblasts (*NF2*$^{(+/-)}$) treated with ES11 PMO. Scale bar, 75 μm.

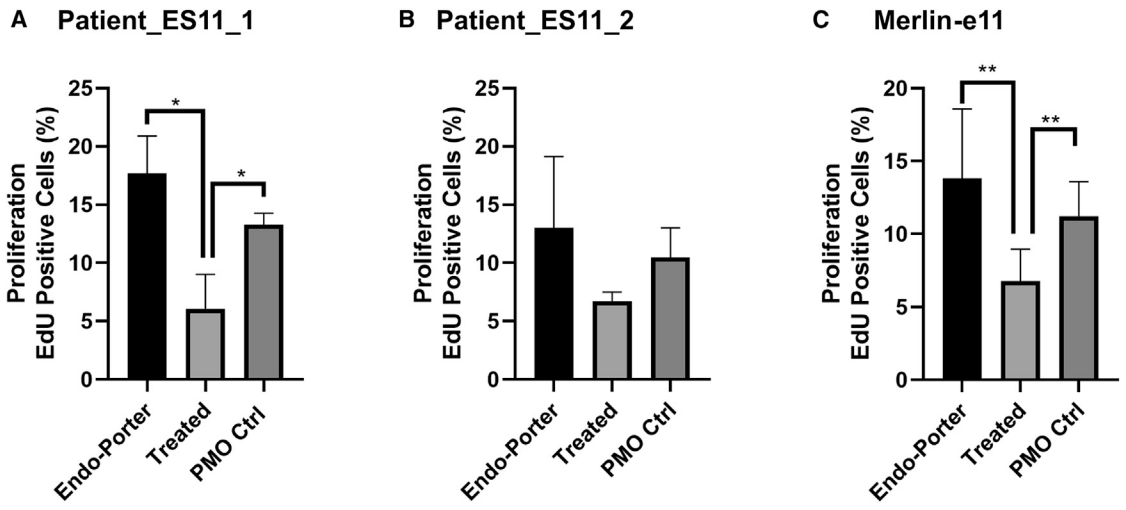

Figure 4. Expression of Merlin-e11 was able to reduce the proliferation capacity of patient fibroblasts

Quantification of EdU-positive cells (percentage over diamidino-2-phenylindole [DAPI]-positive nuclei). A significant decrease was detected in the proliferation rate of treated fibroblasts when compared with Endo-Porter for Patient_ES11_1 (A). Patient_ES11_2 (B) showed the same tendency. (C) Representation of the mean values of both patients. The mean ± standard deviation is represented in the bar, representing three independent experiments. *$p < 0.05$, **$p < 0.01$ denotes statistical significance using unpaired t test. See also Figure S5. In the figure, Endo-porter stands for patient's fibroblasts (*NF2*$^{(+/-)}$) treated with PMO's vehicle, treated for patient's fibroblasts (*NF2*$^{(+/-)}$) treated with specific PMO, and PMO Ctrl for patient's fibroblasts (*NF2*$^{(+/-)}$) treated with a control PMO that does not interfere with *NF2*.

A time course was carried out treating fibroblasts with 20 μM PMO during 24 h, 48 h, and 72 h. PMO treatment in Patient_Spl_1 and Patient_Spl_2 increased the skipping of exons 15 and 8, respectively, in contrast with the desired effect of inducing the inclusion of skipped exon without altering the transcription of the WT allele (Figure 5B: time course, B.I and B.II; Figure S8). Similarly, the treatment of PMOs of patients Patient_Spl_3 and Patient_Spl_4, both with nearby variants near the canonical splice site of exon 2, revealed that the variant-specific PMO caused the skipping of both exons 2 and 3 of the *NF2* gene instead of correcting the aberrant *NF2* splicing because of germline variants (Figure 5B: time course, B.III and B.IV; Figure S8). Their effect was confirmed by Sanger sequencing (Figure S9). *NF2*$^{(+/+)}$ fibroblasts from healthy donors were also treated with each designed variant-specific PMO, and they showed the same effect as *NF2*$^{(+/-)}$ fibroblasts derived from patients (Figures 5B and S8). The effect of the treatment on Merlin was assayed by western blot and no improvement in Merlin levels was detected at the studied time points (Figure S10).

DISCUSSION

NF2-related SWN is a multisystem genetic disorder for which the development of effective therapeutic options with no adverse consequences is needed. The clinical presentation of the disease is variable and related to the type of germline variant inherited in the *NF2* gene.[5] In this context, the development of personalized therapies based on the type of *NF2* variant constitutes an opportunity. In this study, we tested the use of PMOs as antisense therapy to decrease the severity of the effects of *NF2* germline truncating and splicing variants by changing them to milder hypomorphic forms *in vitro* by two different approaches.

In the in-frame exon skipping approach, we targeted nonsense and frameshift variants trying to benefit from the fact that these variants are associated with more severe phenotypes in comparison to in-frame germinal variants in *NF2*.[28,30,49] Thus, we proposed inducing skipping of the exon carrying the truncating variant with the hypothesis of generating potential homozygous hypomorphic forms of Merlin, to ameliorate the pathogenicity of the heterozygous truncating variant *in vitro*. We did not consider mosaic patients in this approach, since their phenotype could depend on the type of variant and the percentage of affected cells.[33]

First, we evaluated the theoretical effect of skipping any in-frame exon, and, although *in silico* studies did not show any outstanding candidate, the predicted effect on exons 5 and 11 was encouraging. Therefore, we decided to test all exons for which samples were available. The skipping of exons 4 or 8 harboring *NF2* LOF variants was not able to generate an exon-less Merlin, because Merlin-e4 and Merlin-e8 were degraded. Exons 4 and 8 of the *NF2* gene code for the subdomains B and C, respectively, of the FERM domain, where the majority of reported pathogenic variants occur.[17,21] The observed results could be due to the fact that the lack of an exon coding for the FERM domain would cause an unbearable aberrant folding of the protein and its synthesis would be prevented or it would be forcibly degraded, although these effects were not predicted *in silico*. In addition, the FERM domain is responsible for the intramolecular interaction with the C-terminal domain, which results in Merlin being in a closed and active conformation[50–52] to induce the most potential for anti-mitogenic signaling. Thus, if this folding fails, it is possible that the resulting protein would not be able to execute the tumor suppressor activity.

Table 2. *NF2* splicing LOF PMO design

Patient's code	c.	g.	p.	Variant effect	PMO_Design
Patient_Spl_Ctrl (PMO_Ctrl)	c-1447-240T>A	g.74408 T>A	p.Pro482Profs*39	Insertion of a cryptic exon of 167pb between exons 13 and 14	CATCCCTCAAATCTCT**T**ACCGTTCT[a]
Patient_Spl_1	c.1736A>G	g.83045A>G	p.Lys525Asnfs*19	ES of exon 15	**Spl1_PMO_v2**: C**C**TTTTAATGGTATTGTGCTTGCTG
Patient_Spl_2	c.810+1dupG	g.62785dupG	p.Phe271_295del	ES of exon 8	**Spl2_PMO_v2:** **C**CCTCCTTGTCACTGTACGAGATGT
Patient_Spl_3	c.241-9A>G	g.35526A>G	p.Val81Phefs*44	New acceptor site exon 3. Inclusion of 8 intronic nucleotides as coding sequence	**Spl3_PMO_v2**: GCAGAA**C**TGCAGAGCAAAAGACAAA
Patient_Spl_4	c.241-13T>A	g.35522T>A	p.Val81Phefs*44	New acceptor site exon 3. Inclusion of 10 intronic nucleotides as coding sequence	**Spl4_PMO_v2**: GCAGAATTGC**T**GAGCAAAAGACAAA

ES, exon skipping.
[a]Published in Castellanos et al. 2013.

When targeting exon 11 of the *NF2* gene, an exon-less Merlin was expressed (Merlin-e11). Merlin, as a member of the ERM protein family, is involved in the link between the actin cytoskeleton and adherent junctions with a relevant role in dynamic cytoskeleton remodeling, such as in membrane ruffling or in the formation of actin microspikes.[53–55] Consistent with other studies that described that Merlin loss leads to a dramatic increase of membrane ruffles and actin cytoskeletal disoganization,[45,56] we found that the $NF2^{(+/-)}$ primary fibroblasts tested in this study also showed actin cytoskeleton abnormalities. In addition, here we observed a decrease in membrane ruffle formation in response to the expression of Merlin-e11 that could be associated with a partial rescue of the phenotype and an improvement in the cytoskeleton organization after treatment, which could be due to a recovery of the Merlin function as a scaffold protein regulating the linkage between membrane proteins and the cortical cytoskeleton.[45,56,57] Consistent with the role as a tumor suppressor protein, Merlin-deficient cells lose contact inhibition,[50,54,58] and, although much remains to be discovered about the adhesive signaling pathways involved, in this study we showed that the induction of Merlin-e11 by PMO treatment contributed to the recovery of cell-cell contact inhibition of proliferation in fibroblasts $NF2^{(+/-)}$ derived from NF2-related SWN patients harboring truncating mutations in exon 11. The tested doses in this study are higher than the FDA-approved PMOs for other inherited diseases.[37,59,60] Thus, redesigns and modifications of the tested antisense molecule could be performed to improve its efficacy and decrease the required dose, in addition to further pharmacodynamics and pharmacokinetics studies to determine the appropriate range doses and the associated toxicity and effectiveness. Furthermore, a treatment effect should be tested in different cell types, especially in schwannoma and meningioma originating cells, as well as *in vivo* studies to determine if the treatment could prevent tumor formation or could reduce tumor burden, when administered systemically before considering the applicability of these molecules in patients.

Thus, this study constitutes a proof of concept of a potential medical approach for these variants, although there this remains much to be elucidated about the effect of the PMOs treatment and its therapeutically approach. Further data are required to assess whether this approach could delay the appearance of tumors, cause patients a reduced tumor burden or slower their growth.

A second strategy was directed at splicing variants with the aim of concealing the erroneous splice signal caused by the pathogenic variant and restoring levels of Merlin, in a similar way to the approach that our group used for a *NF2* deep intronic variant[45] or to induce exon retention by blocking an homozygous single nucleotide variant located six nucleotides within exon 7 of *SMN2*.[61] In this study, it has not been possible to design PMOs that permit specifically the correction of aberrant splicing signaling; furthermore, it was not possible to achieve enough specificity to target the mutated allele as the same effect was observed in either patient-derived fibroblasts or control fibroblasts. Based on our results, we hypothesize that, when the splicing variant is within ±13 nucleotides of the acceptor or donor splice site, the PMO could allosterically interfere with the branchpoint and the U2 small nuclear RNA incorporation into the spliceosome.[62] The spatiotemporal localization of the two alleles during splicing could prevent the PMO from specifically targeting the mutated allele and, thus, it would interfere in the cis-regulation of RNA elements of both alleles equally, resulting in an increased pathogenic effect.[63] Since the approach requires the PMO to be specific for the allele harboring the pathogenic variant, there is little room for variation in the PMO design for the variants tested in this study.

To the best of our knowledge, there are no studies in the literature that have been able to repair the LOF variant effect and induce exon retention through the use of ASOs for a splicing variants located in the canonical splice regions in genes causing AD diseases. The most described effect of these molecules targeting the intron/exon boundary is exon skipping. It has only been possible through CRISPR/Cas technology by the induction of single nucleotide substitutions.[64,65] Allele-specific approaches have been successfully tested for intronic variants through splice modulation ASOs that mask the erroneous signaling and, given their intronic location and distance to splicing regulatory regions, proper protein synthesis can be recovered.[45,66–69] Few works have applied exon-specific splicing enhancement by small chimeric

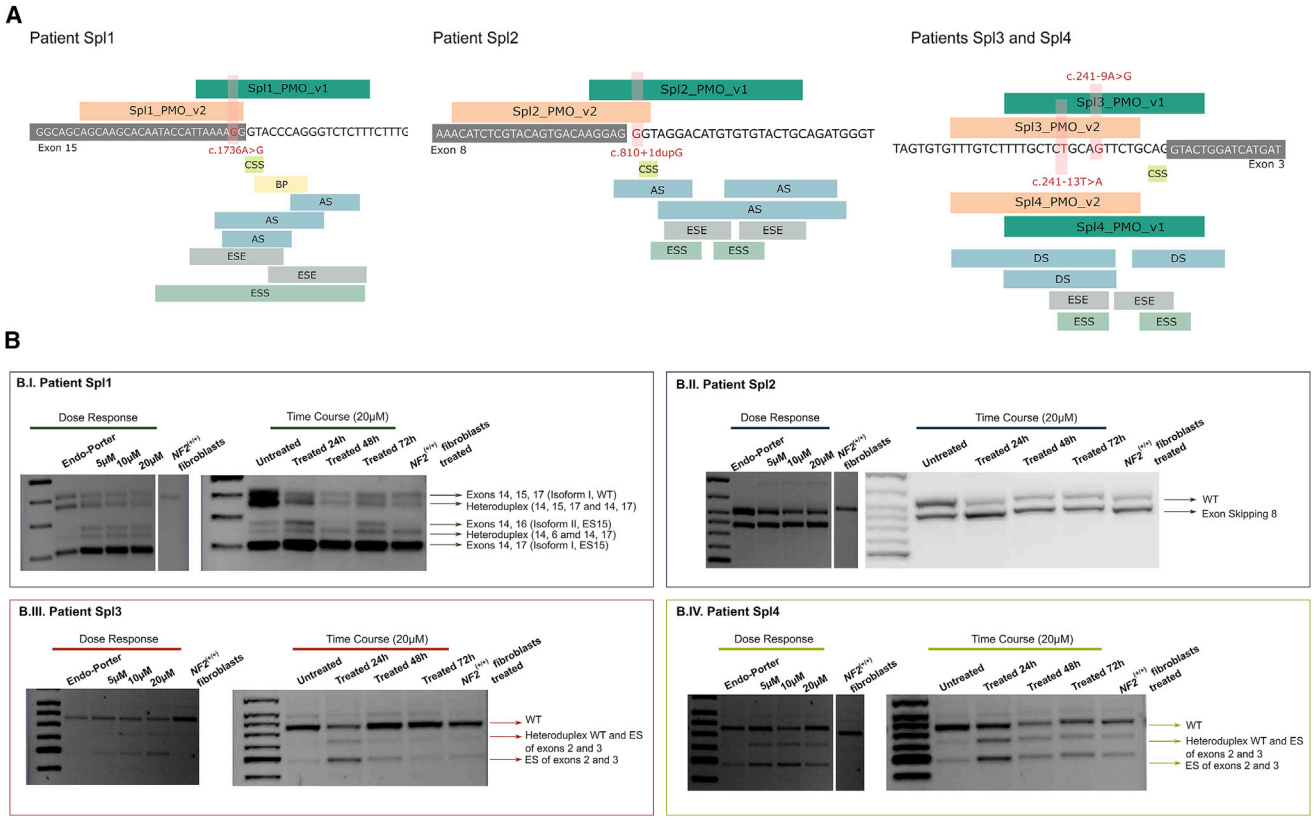

Figure 5. Design and testing PMOs for *NF2* splicing variants

(A) Design of the PMO specific for each variant. For the second design (v2), canonical splice sites (CSS), branch points (BR), acceptor and donor sites (AS and DS), exonic splicing enhancer sequences (ESE), and exonic splicing silencer (ESS) sequences indicated were taken into account and excluded, when possible, for the sequence design to avoid masking the correct splicing signals. The exonic part of the sequence is shown in a gray box and the unlabeled part constitute intronic regions. Tested genetic variants are highlighted in red. (B) The use of variant-specific PMO did not allow correction of the aberrant splicing signaling. The effect of the PMOs v2 is shown at cDNA level through dose response and a time course experiments for each patient (Spl_1, Spl_2, Spl_3, and Spl_4). *NF2* transcripts according to the expected molecular weight are indicated next to the bands in the agarose gel. *NF2*$^{(+/+)}$ fibroblasts stand for control fibroblasts from healthy donors and *NF2*$^{(+/+)}$ fibroblasts treated indicate that have been treated with the specific PMO of each patient at 20 μM. When indicated, untreated stands for patient's fibroblasts (*NF2*$^{(+/-)}$) without PMO treatment, Endo-porter for patient's fibroblasts (*NF2*$^{(+/-)}$) treated with PMO's vehicle, and treated for patient's fibroblasts (*NF2*$^{(+/-)}$) treated with specific PMO. ES, exon skipping.

effectors method to rescue disease-associated exon skipping and modulate alternative splicing.[34,70,71] Furthermore, for autosomal recessive diseases, such as SMA or DMD,[72,73] or for AD diseases with a dominant negative effect, such as frontotemporal dementia with parkinsonism-17 or RP, allele-specific ASOs have been used to induce degradation of the disease-causing allele via RNAse H activity and for this purpose, the native DNA backbone is not modified.[62,74–76] Some evidence suggests that Merlin could act in a negative dominant manner through its self-dimerization through FERM-FERM interactions,[77–79] but this seems to be dependent on patient's variant.[80] In the light of our findings, and if a Merlin-dominant effect is confirmed, the degradation of the *NF2* mutated allele by the use of RNAse H-dependent ASOs could be a possible antisense approach for nonsense, frameshift, and splicing pathogenic variants located in the FERM domain, in both in-frame and out-of-frame exons, and then bringing an improvement in the *in vitro* phenotype and opening the door for future gene therapy approaches.

In addition, for those truncating variants that do not exert a dominant-negative effect, the generation of hypomorphic Merlin variants could be beneficial for the patient, and, therefore, more variants and hypomorphic Merlin forms should be analyzed to understand the contribution of this effect over patient phenotype.

This work is a proof of concept of the use antisense therapy *in vitro* as a personalized therapy for NF2-related SWN patients harboring germline truncating variants at exon 11 of the *NF2* gene. Our approach has shown encouraging results when targeting exon 11, although further characterization assays need to be performed to better characterize the function of Merlin-e11 before considering it for further *in vivo* or pre-clinical assays.

In addition, further studies are needed to elucidate whether there are other mechanisms or designs to apply ASOs as a therapeutic approach for this disease, as in our experience it has not been possible

to recover Merlin levels for variants located at in-frame exons 4 or 8, or to correct splicing signals *in vitro* in the four variants located near canonical splice sites tested, although other splicing variants should be assessed to confirm these results.

MATERIAL AND METHODS

All procedures performed were in accordance with the ethical standards of the IGTP Institutional Review Board, which approved this study and with the 1964 Helsinki declaration and its later amendments.

Samples

Samples from 14 NF2-related SWN patients from the Spanish National Reference Center on phakomatoses were included in the study (Table S1) after providing written informed consent. Samples included in the study were those harboring a splicing variant or a truncating variant in an in-frame exon of *NF2*. In controls, fibroblasts $NF2^{(+/+)}$ stand as non-affected cells for experimental control (WT), with no mutation in the *NF2* gene, obtained from healthy donors.

Genetic *NF2* test

Genetic testing was performed using the customized I2HCP panel[81] in blood or tissue when available. To detect splicing variants, patients were studied both at the RNA and DNA levels. Variants were validated by Sanger sequencing and analyzed using CLC-workbench (Qiagen, Hilden, Germany) in all primary cultures established using (NM_000268.3; NG_009057.1; LRG_511) as reference sequences.

In silico NF2 analysis

NF2 exons were evaluated using NM_000268.3 and NG_009057.1 reference sequences. *NF2* pathogenic variants described in the LOVD in addition with our genetic variants database identified in our NF2-related SWN cohort.[29] All *in silico* analyses of exons (and combinations of them) that when skipped, an in-frame Merlin protein is theoretically maintained were based on the analysis performed by Leier et al. 2021.[82] Post-translation modifications (PTM) were retrieved from Phosfosite.[83] Only phosphorylation, acetylation, and ubiquitylation were considered as PTMs. Analyses of *in silico* protein modifications by the exon skipping were performed using the web-server version of PredictProtein (https://www.PredictProtein.org).[46,47]

Antisense specificity and efficacy

The 25-mer-specific PMOs for four patients with splicing variants, and a pair of PMOs to induce exon skipping of exons 4, 8, and 11 of the *NF2* gene were designed (NM_000268.3; NG_009057.1; LRG_511), synthesized and purified by Gene Tools (Philomath, OR). Endo-Porter (Gene Tools) was used as vehicle to deliver PMOs into cells at 6 μM (manufacturer-recommended dose), according to the manufacturer's instructions. Patient primary fibroblasts carrying *NF2* germline pathogenic variants were treated with the specific single or paired PMOs. A dose-response and a time course study were performed to set-up treatment conditions and the effect of PMOs was assessed by RT-PCR and Sanger sequencing. Levels of Merlin after PMO treatment were analyzed by western blot. For all experiments, the correct effect of the treatment at RNA level was confirmed before western blot analysis.

Analysis of Merlin-e11 phenotype recovery

An actin immunocytochemistry assay was performed to study the cytoskeletal organization after PMO treatment using Phalloidin (Molecular Probes [Eugene, OR], Invitrogen [Waltham, MA]). AClick-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Molecular Probes, Invitrogen) was used, according to the manufacturer's protocol, to determine cell proliferation. Cell viability was assessed using RealTime-Glo MT Cell Viability Assay (Promega) following the manufacturer's instructions.

NF2 cloning and expression

The *NF2* gene was cloned using the Gateway Gene Cloning system (Invitrogen) after treatment to induce the exon-skipping to an FLAG Tag Expression Vector. The expression vector was transfected into HeLa cells (Lipofectamine LTX with Plus Reagent, Invitrogen) according to the manufacturer's instructions. Flag expression was assessed by western blot (Monoclonal ANTI-FLAG M2 antibody, Sigma-Aldrich, St. Louis, MO).

Statistics

Statistical analysis was carried out using GraphPad Prism software v7. The Shapiro-Wilk test was used to test normality and for multiple group comparisons, a two-tailed unpaired t test was performed. Statistical significance is indicated by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. For more information, see the supplemental materials and methods.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its Supplemental materials.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.10.026.

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AUTHOR CONTRIBUTORS

E.C. conceived the study and wrote the manuscript that was revised, corrected, and improved by all the authors. N.C. performed most of the experimental work, analyzed the data, generated the figures for the paper, and contributed to writing the manuscript. I.R, S.B., and A.N. performed experimental work. M.T-M. performed bioinformatics analysis. A.P. and H.S. contributed with sample collection. E.S. and I.B. provided scientific input. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest.

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