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Exon 32 Skipping of Dysferlin Rescues Membrane Repair in Patients' Cells

Florian Barthélémy[a,b], Cédric Blouin[c,d,1], Nicolas Wein[a,b,1], Vincent Mouly[e,f,g], Sébastien Courrier[a,b], Eugénie Dionnet[a,b], Virginie Kergourlay[a,b], Yves Mathieu[a,b], Luis Garcia[e,f,h], Gillian Butler-Browne[e,f,g], Christophe Lamaze[c,d], Nicolas Lévy[a,b,i], Martin Krahn[a,b,i] and Marc Bartoli[a,b,i,*]

[a]*Aix Marseille Université, UMR_S 910, Faculté de Médecine de la Timone, Marseille, France*
[b]*GMGF, INSERM UMR_ S 910, Marseille, France*
[c]*CNRS UMR 144, 26 rue d'Ulm, Paris Cedex 05, France*
[d]*Institut Curie, Centre de Recherche, Laboratoire Trafic, Signalisation et Ciblage Intracellulaires, 26 rue d'Ulm, Paris Cedex 05, France*
[e]*INSERM UMR_S 974, Institut de Myologie, Paris, France*
[f]*CNRS, UMR7215, Institut de Myologie, Paris, France*
[g]*Université Pierre et Marie Curie, UM76, Paris, France*
[h]*Université Versailles-Saint-Quentin, Versailles, France*
[i]*AP-HM, Hôpital d'Enfants de la Timone, Département de Génétique Médicale et de Biologie Cellulaire, Marseille, France*

Abstract. Dysferlinopathies are a family of disabling muscular dystrophies with LGMD2B and Miyoshi myopathy as the main phenotypes. They are associated with molecular defects in *DYSF*, which encodes dysferlin, a key player in sarcolemmal homeostasis. Previous investigations have suggested that exon skipping may be a promising therapy for a subset of patients with dysferlinopathies. Such an approach aims to rescue functional proteins when targeting modular proteins and specific tissues.

We sought to evaluate the dysferlin functional recovery following exon 32 skipping in the cells of affected patients. Exon skipping efficacy was characterized at several levels by use of *in vitro* myotube formation assays and quantitative membrane repair and recovery tests. Data obtained from these assessments confirmed that dysferlin function is rescued by quasi-dysferlin expression in treated patient cells, supporting the case for a therapeutic antisense-based trial in a subset of dysferlin-deficient patients.

Keywords: Therapy, membrane, neuromuscular diseases, dysferlinopathy, exon-skipping

INTRODUCTION

Mutations in *DYSF* (MIM# 603009, 2p13, GenBank NM_003494.2) [1, 2], the gene encoding dysferlin, cause a variety of muscular dystrophies collectively referred to as dysferlinopathies, the most significant of which are LGMD2B (LGMD2B; MIM# 253601) and Miyoshi Myopathy (MM; MIM# 254130) [1, 2].

At the onset of disease, usually in the second decade of life, proximal muscles in LGMD2B and distal muscles in MM are the principal muscle groups to be affected. In the early stages of disease, serum levels of creatine kinase are elevated and the histology appears clearly dystrophic with numerous inflammatory foci. In both phenotypes, most patients present

[1]These authors contributed equally to the work presented in this manuscript.

*Correspondence to: Marc Bartoli, "Myologie Translationnelle" Team, Faculté de Médecine de Marseille, Aix Marseille Université, UMR_S 910 "Génétique Médicale et Génomique Fonctionnelle", Marseille, France. Tel.: +33 491 324 906; Fax: +33 491 804 319; E-mail: marc.bartoli@univ-amu.fr.

with generalized muscle weakness after several years of disease progression, though in some cases, the early presentation is a proximo-distal muscle weakness [3]. Nevertheless, scapular winging is uncommon, and cardiac and respiratory complications do not typically develop amid the typical progression of these dysferlinopathies.

Dysferlin interacts with different muscle proteins involved in at least two pathways: membrane repair and myoblast/myotube membrane fusion. The process of membrane repair is now better understood since many actors have been identified at each of the different stages in the process. Among these participating factors, we have found specific types of lipids, sarcolemmal proteins like dysferlin as well as MG53 and annexins, and also muscle ubiquitous protein like calpain 3 [4–12]. The myoblast/myotube fusion, a key process for muscle formation during development and regeneration, is also poorly described but it appears to be associated with a substantial cytoskeletal rearrangement following the recruitment of a large protein network including dysferlin [10, 13–15]. The abnormalities observed in patients carrying function-modifying mutations in *DYSF* demonstrate the crucial role of dysferlin for muscle physiology and functionality.

As for some muscular dystrophies, several therapeutic strategies are currently being evaluated for dysferlinopathies, including gene therapy approaches [6, 16–19]. Based on the promising results of clinical trials for Duchenne Muscular Dystrophy (DMD) using Antisense Oligonucleotide (AON) approaches for exon 51 skipping of the dystrophin pre-mRNA [20–23] (Sarepta therapeutics and BioMarin, Inc., unpublished data), we recently evaluated the feasibility of a similar strategy for application in dysferlinopathies. In contrast to DMD where exon skipping strategies are intended to restore the reading frame (for patients presenting out-of-frame deletions), in dysferlinopathies the aim is to bypass the mutation in exon 32 without altering the reading frame and function. A prior report had described a mildly affected patient with a mutation causing in-frame skipping of exon 32 at one allele (caused by a lariat branch point mutation) together with a null-allele [24]. We considered these data to constitute a "natural" proof of principle than an exon 32-skipping approach could be therapeutic in dysferlinopathies. In a previous study, we were able to demonstrate its efficient skipping in patient cells carrying mutations in this specific exon, and thus demonstrated the feasibility of exon skipping targeting *DYSF* [25]. In the present work, we sought to characterize the functional recovery following exon 32 skipping in patient cells with the aim of translating our results to future clinical applications. The efficiency of the exon skipping was assessed by immunoblotting and immunochemistry. In addition, based on the role of dysferlin in myotubes, several functional tests were developed to quantify functional recovery. Our results demonstrate for the first time the rescue of dysferlin functions by a quasi-dysferlin generated by exon skipping in patient cells.

MATERIALS AND METHODS

Ethics statement

The relevant sample was provided, anonymously, by Myobank (Myology Institute) affiliated at EuroBioBank (www.eurobiobank.org). This affiliation certifies that the biopsy was obtained in accordance with the ethical standards laid down in the Declaration of Helsinki and the directive 2004/23/EC of the European Parliament.

Patients

Patient biopsies were obtained from a patient affected with Miyoshi myopathy: Patient 1 NM_0003494.3: c.[3477C>A]+[5979dupA]; p.[Tyr1159*] (stop codon in exon 32)+[Ala1993_Glu1994insArg; Ser1995*] (stop codon in exon 53); and a patient affected with LGMD2B: Patient 2: NM_0003498.3: c.[342+1G>A] (intron 4, splicing defect) +[3516_3517delTT]; p.[Ser1173*] (stop codon in exon 32). Mutational data are described using the nomenclature of the Human Genome Variation Society (www.hgvs.org/mutnomen).

Cell cultures

Myoblasts were expanded in Skeletal Muscle Cell Growth Medium (Promocell) adjusted at 20% FBS final, supplemented with 100 μg/ml of gentamycin (Sigma-Aldrich). At confluence, the medium was changed for a differentiation medium based on the Skeletal Muscle Cell Differentiation medium (Promocell) and supplemented with 100 μg/ml of gentamicin, 10 μg/ml of doxycycline (Sigma-Aldrich) and 100 μg/ml of apotransferrin (Sigma-Aldrich). When needed, cells have been differentiated into myotubes, maintained in cell culture supports coated using collagen I (BD Biosciences).

Identification of target sequences for dysferlin exon 32 skipping

Bioinformatics analyses, to find targetable sequences within exon 32 of the DYSF gene and its surrounding intronic sequences, were realized using www.umd.be/HSF/ and http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process5home [25–27].

AONs and transfection

The antisense oligonucleotides (AONs) used are as follows: AON B 5′-GCGUAGAUGGUAGCGGUUC CC-3′; AON D 5′-ACCUACCAGAAAAAGAGUCC UU-3′. AON were synthesized by Eurogentec and contain 2′-O-methyl RNA and full-length phosphorothioate backbones (Figure S1-A). Myoblast cultures were transfected using oligofectamine (Life Technologies) according to the manufacturer's instructions. Each AON was transfected one, two or three times at 800 nM every two days starting at day 0 of differentiation. Mock experiments were performed using the transfection reagent (oligofectamine) without AON. Control conditions were tested using a non-specific AON: 5′- GGAUCCCGCUCAGGAGUGCUG-3′. All experiments were conducted 48 hours after last transfection (day 6 of differentiation).

RNA extraction

RNAs were extracted from cultured cells using PureLink™ RNA Mini Kit (Life technologies) and RNA was purified using DNAse Free Kit (Life technologies). Five hundred ng of RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Saint Aubin, France) primed by random hexamers according to the manufacturer's instructions. PCR amplifications were performed with 1 µL of RT-product using primer pairs flanking the targeted exons (Forward: 5′-CCAGGAGCCAGAGATTCG-3′ Reverse: 5′- TGCTTTCAATGTCCTCCTTG-3′) for 35 cycles. Dysferlin RNA exon skipping was analyzed by classical RT-PCR for AON study. The products of PCR were separated by electrophoresis in a 1.5% agarose gel stained with 5% ethidium bromide.

Immunoblot

Detection of dysferlin was performed using the following protocol with primary anti-dysferlin antibodies (NCL-Hamlet 2, Cliniscience) diluted 1:300 or (NCL-Hamlet, Abcam) diluted 1:200 on untreated and treated patient cell protein extracts. Immunoblots were done using a whole protein extract sample prepared in lysis buffer (100 mM Tris buffer at pH 6.8, 4% SDS, 10% glycerol and 1% 2-mercaptoethanol). A 1% reducing agent (Life Technologies) was added and the samples were boiled for ten minutes. 20 µg of sample was loaded onto each lane of a 3–8% NuPage SDS–PAGE (Life Technologies). Proteins were then separated under electrophoresis at 80 volts at room temperature. Proteins were finally transferred onto nitrocellulose membranes (at 260 mA for 2 h at 4°C). Membranes were incubated with blocking buffer (1% non-fat milk powder in TBS-T) for one hour at room temperature. Primary antibodies were then diluted in blocking buffer and incubated with the membranes at room temperature for 1 h, with constant agitation. After washing in TBS-T, membranes were then incubated with the relevant secondary antibodies, which were diluted 1:10,000 in blocking buffer, for one hour at room temperature. The membranes were finally washed in TBS-T and developed using Pico Chemiluminescent Substrate (Pierce). GAPDH (Millipore) was detected using a dilution of 1:10,000.

Immunofluorescence

Cells were grown on Lab-TEK II™ (Fisher Scientific) according to the previously explained protocol. Throughout the procedure, cells were kept at room temperature. After treatment, cells were fixed with 4% paraformaldehyde for 10 minutes and then washed in PBS for 10 minutes. Cells were then incubated for 10 minutes with a permeabilization solution (200µL of PBS 1X +0.5% triton X-100 + protease inhibitors cocktail) (Roche). From there, cells were exposed to a blocking buffer (PBS+ 1%BSA + protease inhibitors cocktail) for 30 minutes. The primary antibody was applied in blocking buffer for 3 hours at room temperature, followed by one wash in PBS for 10 minutes and 1 hour of contact with the secondary antibody in blocking buffer. After a wash in PBS for 10 minutes, cells were fixed using a 4% paraformaldehyde solution in PBS for 10 minutes, then washed again. Finally, coverslips were mounted with Vectashield-Dapi 25 ng/mL, and kept at 4°C until pictures were taken. Dysferlin was detected using NCL-Hamlet at a dilution of 1:200. Caveolin-3 (BD Biosciences) was detected using a dilution of 1:1,000, and desmin (Fischer Scientific) using a dilution of 1:100. Scale bars are indicated on each picture.

Observation was performed using a Zeiss apotome microscope, and images were processed with AxioVision software and/or ImageJ software.

Membrane wounding assay

To induce damage, an area of the sarcolemma of the myotubes was irradiated at full power for 1 second with a two-photon laser-scanning microscope. The Multi-Photon apparatus consisted of a mode-locked Titanium-Sapphire laser system tuned to a 1035 nm excitation with 100 fs pulses at 76 MHz. The microscope was an inverted LEICA SP3. Images were captured for 3 minutes after the irradiation at 7-second intervals. Images were analyzed with ImageJ software.

Osmotic shock assay

Prior to osmotic shock, myotubes were incubated with 10 μM calcein-AM and 25 μg/ml DAPI (Life Technologies) for 20 minutes, followed by washing out the cells with PBS. Hypo-osmotic shock was performed by incubating cells with a differentiation medium diluted appropriately in deionized water [1:9 dilution (complete differentiation medium: H2O) approximating 30 mOsm hypo-osmotic shocks]]. Wide-field epifluorescence microscopy was performed using a 10X 0.3 NA objective and an EMCCD camera (Hamamatsu Photonics) in a Zeiss Axiovert 200 inverted microscope. Cell fluorescence was followed for 450 seconds immediately after medium change to hypo-osmotic medium. Acquisitions were performed with a time interval of 3 seconds. Images were analyzed with ImageJ software. Myotubes were selected as cells containing two or more nuclei as indicated by DAPI staining. Scale bars are indicated on each picture.

Statistical analysis

Individual means were compared using the parametric Student's *t*-test or with the non-parametric Mann-Whitney test. The powers of the tests were strictly superior at 75%. Differences were considered to be statistically significant if $p < 0.01$ and the size of the effect was >20%.

RESULTS

As reported previously, we were able to efficiently bypass exon 32-containing mutations in *DYSF* by use of AONs (Wein et al, 2010). We thus evaluated the efficacy of the AON-induced splicing event on muscle cells from a control individual. These cells were transfected, using oligofectamine, with two AONs (B and D) (Figure S1A). In mock conditions, the transfection reagent (oligofectamine) without AON was used. RT-PCRs were done using a forward primer at exon 31 and a reverse primer at exon 33. We were able to demonstrate exon 32 skipping by the presence of a 215 bp transcript corresponding to an mRNA deleted of the 78 nucleotides of exon 32. In this experiment both AONs were found to be efficient and were used in all subsequent experiments (Figure S1B). As dysferlin is supposed to be expressed during myotube formation, we chose to perform our AON treatment during the differentiation process. We performed single or multiple treatments (every two days) on cells from Patients 1 and 2, starting at cellular confluence until 6 days of differentiation, when myotubes are present. The presence of a 215 bp band indicated that exon 32 was successfully skipped by both AONs, and exon-exon boundaries were verified (Fig. 1A, Figure S1B-D).

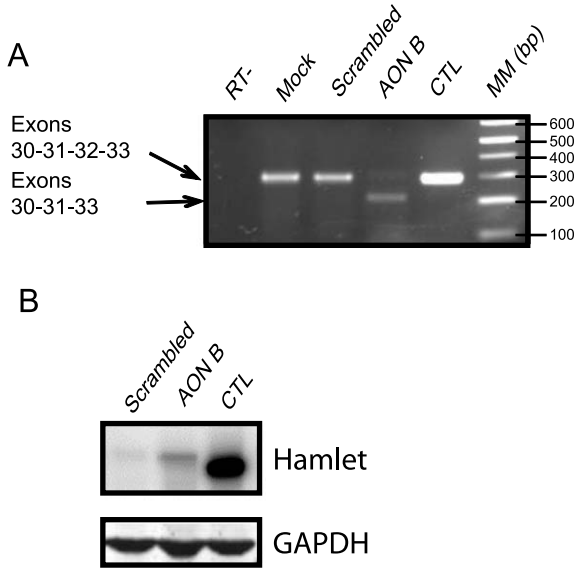


Fig. 1. AON B efficiently skipped dysferlin exon 32 in human patient cells. (A) Efficiency of exon skipping using AON B was assessed by RT-PCR on cells from patient 2. PCR was used to amplify the region between exons 31 and 33 (293 bp with or 215 bp without exon 32). RT::negative control of reverse transcription. Mock: negative control of transfection. Scrambled: nonspecific AON sequence. AON B: single transfection of AON B. CTL: positive control, cells were transfected with dysferlin full-length (native dysferlin). MM: molecular marker. (B) Western Blot experiment performed with proteins from (AON B or scrambled) treated patient cells or control cells. Hamlet was used to detect dysferlin and GAPDH was used for normalization.

We then determined that the quasi-dysferlin protein generated following the exon 32 skipping is stable in

treated patient cells. The predicted molecular weight of the quasi-dysferlin is close to the full-length isoform, 238 kDa and 241 kDa respectively. In protein extracts from cells treated with AONs B and D, we observed the expression of the quasi-dysferlin (Fig. 1B, Figure S2A). This protein is observed as early as 48 hours after the start of the treatment.

We evaluated the gain of function brought about by the expression of the quasi-dysferlin using different tests. Quasi-dysferlin is present and localized at the plasma membrane of myotubes only in treated patients' cells. Co-localization with caveolin 3 labeling confirmed the membrane targeting of quasi-dysferlin (Fig. 2).

We decided to verify if quasi-dysferlin could restore the myotubes' membrane repair capacities, which is the main function described for dysferlin [4].

We performed membrane wounding/repair assays both on muscle cells obtained from patient 1 as well as controls. To analyze this function we set up a laser wounding assay on *in vitro* culture. The extent of the lesion on myotubes was quantified in the presence of FM 1–43 dye and either with or without calcium as described previously [4, 6, 17]. In this assay, we observed that myotubes treated with AON successfully repaired membrane lesions in the presence of calcium, as seen by the low incorporation of FM1–43, whereas untreated patient myotubes exhibited a substantial level of incorporation (Fig. 3A). Quantitative analysis confirmed that both AON treatments restored the ability of patient cells to repair their membranes to a level which was similar to that observed in control myotubes (Fig. 3B).

In order to further investigate the function of the quasi-dysferlin, we carried out an osmotic shock assay adapted from a previous study [28]. Hypo-osmotic shock rapidly induces cell swelling which increases the membrane tension, and therefore applies a mechanical stress at the cell surface. Patient and control myotubes were subjected to a 30 mOsm hypo-osmotic shock for 7.5 min. To monitor the integrity of the plasma membrane, we followed the intracellular accumulation of calcein-AM, a cell-permeable non-fluorescent molecule that becomes fluorescent inside intact cells and leaks out of the cells if the plasma membrane is ruptured (Fig. 4A). We could restrict our analysis to fully differentiated myotubes by selecting cells with polynucleated syncytia as revealed by DAPI staining of the nucleus (data not shown). The level of fluorescence is maintained around its initial value in myotubes whose plasma membrane remains intact during osmotic shock. In contrast, cell bursting events lead, in all observed cases, to a ratio signal value that is below 0.5 and therefore represents the bursting

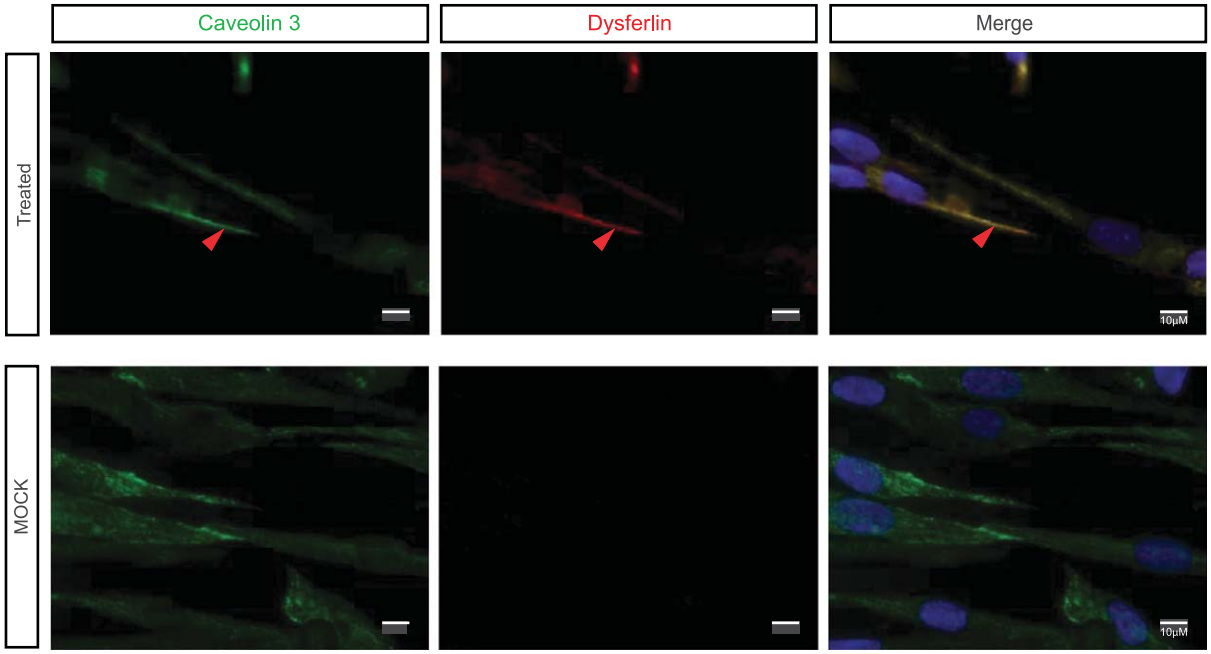


Fig. 2. Exon 32-skipped dysferlin is correctly localized. Caveolin 3 labeling: maturation of myotubes and production of dysferlin were evidenced by Hamlet 1 labeling (Bars = 10 µm, arrow pointed to colocalized signal). DAPI was used as a nucleus marker. All images were captured by an apotome microscope.

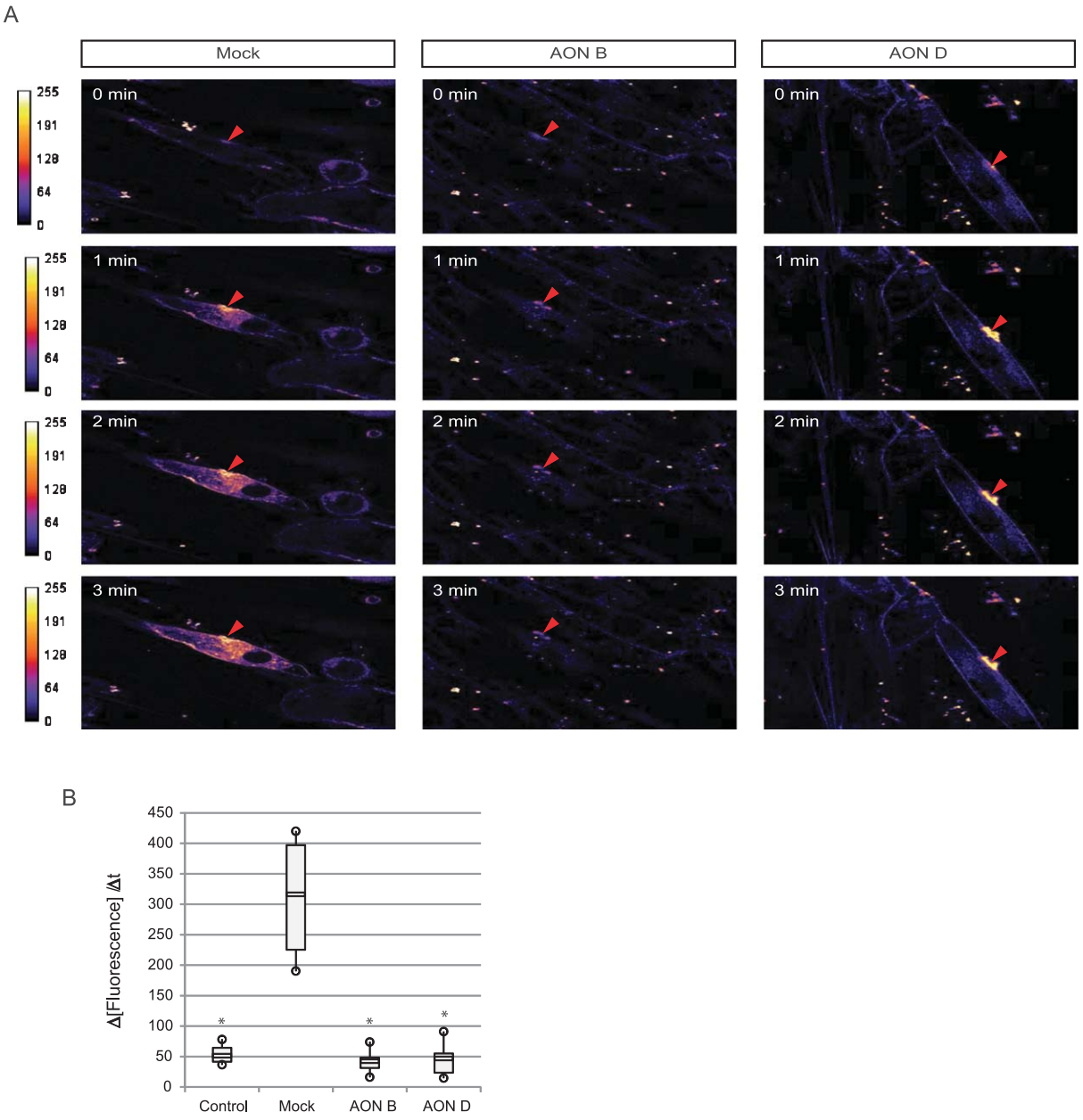


Fig. 3. Quasi-dysferlin increases the membrane repair capacity. (A) Membrane repair consequent to a bi-photon laser injury at the sarcolemma was visualized in presence of FM1-43 dye. Mock treated cells along with AON B and AON D treated cells from patient 1 were compared for three minutes at intervals of 7 seconds. Pictures at 0, 1, 2 and 3 minutes are shown. Arrows indicate the site of lesion. The scale (of false colors used) is represented on the left. (B) Box plots represent the rate of change of fluorescence (Δ[fluorescence]/Δt) in the myotubes from control ($n=9$), mock-treated patient cells ($n=9$), AON B treated patient cells ($n=10$) and AON D treated patient cells ($n=9$). Boxes extend from the 25th to the 75th percentile values. Minimum and maximum values are indicated by the dots at the ends of the vertical lines. Horizontal bars indicate the median value. $^{*}p<0.01$ compared to mock-treated patient cells.

threshold. Mock-transfected patient myotubes showed a high rate of bursting events (85.2%) compared to control cells (36.5%), in agreement with the known defect of membrane repair observed in these patients (Fig. 4B). One single AON transfection in patient cells was sufficient to increase membrane integrity since the measured level of bursting was close to that of control myotubes (41.9%) (Fig. 4B). These results show that restoration of dysferlin by AON treatment protects patient cells from mechanical stress. The total number

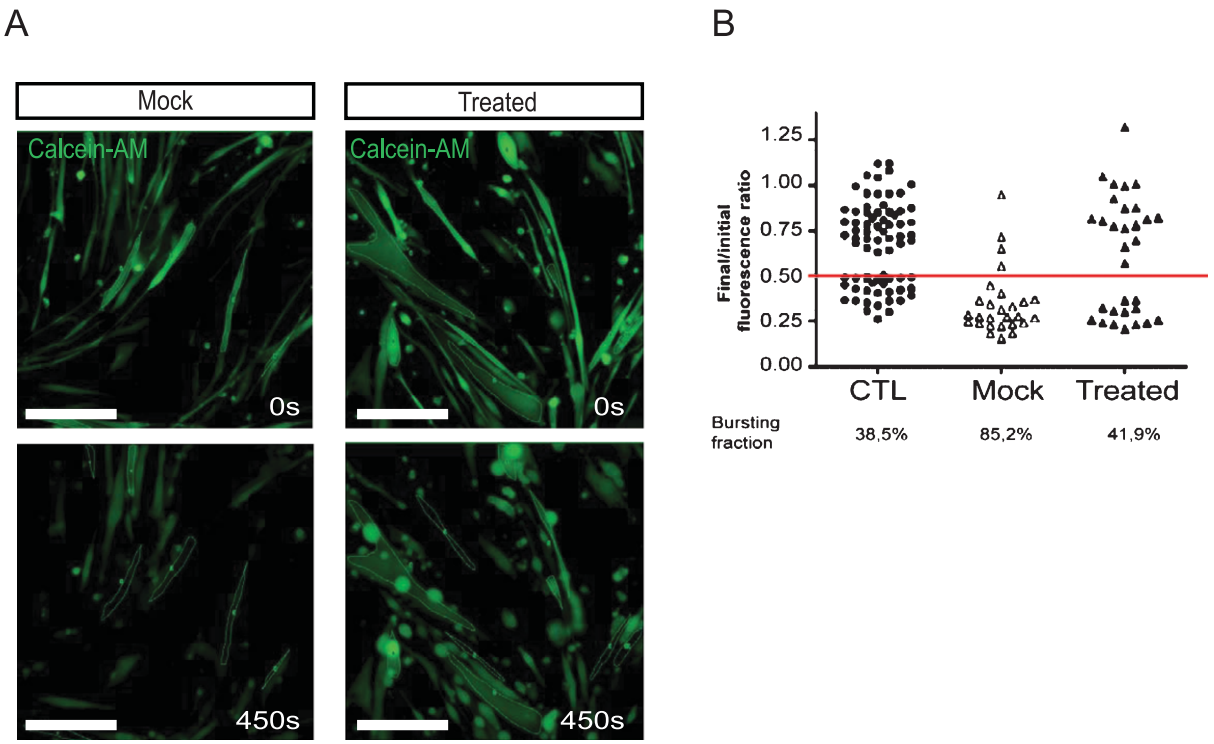


Fig. 4. AON-treated patients cells are resistant to hypo-osmotic shock. (A) Membrane rupture in response to hypo-osmotic shock was assessed using calcein-AM to distinguish between intact and burst cells. Low-magnification epifluorescence snapshots of calcein-AM in mock and treated cells at the beginning ($t = 0$ s, top) and at the end ($t = 450$ sec, bottom) of the hypo-osmotic shock (Bar = 100 μm). (B) Plot of final/initial fluorescence intensity ratio for control myotubes (WT, $n = 74$, dark dots), for patient 2 myotubes untreated (MOCK, $n = 27$, white triangles), treated with AON D ($n = 31$, dark triangles) during the hypo-osmotic shock. The bursting fraction corresponds to the cell population whose fluorescence ratio is below the selected bursting threshold. The bursting threshold has been set to 0.5 of normalized fluorescence intensity (horizontal red line).

of nuclei per cells in these experiments was also calculated and represented as a graph in Figure S2B.

DISCUSSION

This study demonstrates the possibility of restoring functionality in dysferlin-deficient human muscle cells by skipping of an exon bearing a deleterious mutation. The functional normalization was obtained using AONs targeting exon 32. Both AONs induced the splicing out of exon 32 and the expression of a deleted isoform of dysferlin in two different patient cell lines. To evaluate the functionality of this isoform, we employed a variety of assays that measure the myoblast/myotube fusion process as well as the membrane repair ability.

In the present study we have improved the skipping efficiency obtained in our previous work [25], by designing the new AON D targeting the splice donor site of exon 32. The skipping efficiency seems to be quite high, given that almost all the transcripts produced seem to be skipped. This efficiency seems greater than what has been obtained in DMD using a similar AON chemistry [29]. The difference observed here can be explained by several parameters as they were previously discussed [30].

This skipped mRNA was stable enough to produce a quasi-dysferlin deleted of 26-residues, preserving the frame and located at the beginning of the C2D domain. When considering our approach, it can be observed that a discrepancy exists between the high level of exon skipping and the relatively low level of protein expression. This could possibly be attributed to a decrease in the stability between the full-length dysferlin and the quasi-dysferlin deleted of exon 32; this specific issue should be addressed in future experiments. Nevertheless, this low level of quasi-dysferlin expression is sufficient to rescue the membrane repair capacity of treated patient cells, confirming that pivotal aspects of dysferlin function are rescued upon bypassing the mutation in exon 32. Cells in affected patients are

more susceptible to membrane mechanical constraints or at least membrane disorganization. We have shown here that they are more sensitive to a localized lesion or to an increase of membrane tension induced by osmotic pressure. One could hypothesize that this is due to the abnormal recruitment of membrane vesicles at the sarcolemma in the absence of dysferlin. Vesicle recruitment involves various proteins, among them caveolin 3. However we did not detect any difference in caveolin 3 localization after AON treatment. Since we have demonstrated that AON treated patient cells were resistant to both osmotic pressure and sarcolemma lesions, we can hypothesize that this is due to the proper recruitment of membrane vesicles at the sites of injury.

It has been proposed that dysferlin could induce membrane blebbing on cells consequent to a hypo-osmotic shock; these blebs probably result from the rapid membrane surface increase and variations of membrane tension [5, 9, 31]. Absence of dysferlin could impair this mechanism. Based on these observations, we set up a test to follow and quantify the resistance of myotubes to increased membrane tension induced by hypo-osmotic shock in defined time bands. To resist the membrane tension, cells augment the surface of their sarcolemma by the recruitment of additional membrane from reservoirs. These membrane reservoirs could be either vesicles or large invaginations of sarcolemma that could be reorganized by the protein complex involving dysferlin [5, 7, 9–11, 28, 32]. Altogether, this hypothesis can explain how dysferlin-positive myotubes accommodate acute mechanical stresses and are in accordance with previous published data from other groups [4, 8, 31, 33].

Our results establish a proof of concept for exon skipping as a therapeutic perspective for dysferlinopathies. Another group has also demonstrated that a mutant dysferlin pseudoexon could be skipped [34]. Such an approach has already been successfully applied in the case of DMD. After the encouraging results obtained in two independent phase II/III clinical trials for the skipping of dystrophin exon 51 [20–22, 35], BioMarin is moving forward with market authorization for applications involving the skipping of this exon using Drisapersen (Food and Drug Administration approval has been given and European Medicines Agency approval is still under review). Meanwhile, Sarepta is currently extending a phase II/III trial (NTC02255552) using Eteplirsen. In addition, phase II clinical trials are ongoing for exons 44, 45 and 53 using 2'-*O*-methyl chemistry and for exon 53 using PMO chemistry [36].

Dysferlin exon 32 encodes the N-terminal portion of the C2D domain, therefore deletion of exon 32 would most likely lead to loss of function of the entire domain without major consequences [24, 37]. The C2D domain is composed of the exons 31, 32, 33 and 34 whose deletion would maintain the open reading frame of dysferlin mRNA. In addition to dysferlin exon 32, the skipping of exon 34 has also been reported from control cells [38]. It would therefore be feasible to attempt multiple exon skipping so as to bypass the entire C2D domain of dysferlin using the previously validated AONs. These methods of multiple exon skipping have already been used with success in DMD treatment for the simultaneous skipping of at least 6 consecutive exons [39–51]. Exon skipping could also be used to remove pseudoexons. Recently, a mutation creating a pseudoexon between exons 44 and 45 of DYSF has been identified [34, 46]. Exon skipping using an AON targeting this pseudoexon restored a normal mRNA and increased dysferlin expression, opening the way for such an approach in dysferlinopathies [34].

Although some improvements may be necessary to bring about more routine use of AON-based therapies, rapid advances being made for DMD will certainly apply to other rare muscle disorders, and will allow a rapid translation of proof of principle into clinical applications in the future. At least a subset of 20 patients (with 6 different truncating mutations) should thus benefit from antisense-induced exon 32 skipping.

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CONFLICT OF INTEREST STATEMENT

MB, MK, LG and NL have a patent for dysferlin exon 32 skipping (#US20120208865).

SUPPLEMENTARY MATERIAL

The supplementary table and figure are available in the electronic version of this article: http://dx.doi.org/10.3233/JND-150109.

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