Efficient recovery of dysferlin deficiency by dual adeno-associated vector-mediated gene transfer

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Deficiency of the dysferlin protein presents as two major clinical phenotypes: limb–girdle muscular dystrophy type 2B and Miyoshi myopathy. Dysferlin is known to participate in membrane repair, providing a potential hypothesis to the underlying pathophysiology of these diseases. The size of the dysferlin cDNA prevents its direct incorporation into an adeno-associated virus (AAV) vector for therapeutic gene transfer into muscle. To bypass this limitation, we split the dysferlin cDNA at the exon 28/29 junction and cloned it into two independent AAV vectors carrying the appropriate splicing sequences. Intramuscular injection of the corresponding vectors into a dysferlin-deficient mouse model led to the expression of full-length dysferlin for at least 1 year. Importantly, systemic injection in the tail vein of the two vectors led to a widespread although weak expression of the full-length protein. Injections were associated with an improvement of the histological aspect of the muscle, a reduction in the number of necrotic fibers, restoration of membrane repair capacity and a global improvement in locomotor activity. Altogether, these data support the use of such a strategy for the treatment of dysferlin deficiency.

INTRODUCTION

Dysferlinopathies are autosomal recessive muscle disorders caused by mutations in the DYSF gene, which encodes for the Dysferlin protein. The two most frequent phenotypes are limb-girdle muscular dystrophy type 2B (LGMD2B; OMIM253601), which presents with muscle weakness predominantly in the proximal pelvic muscles, and Miyoshi myopathy (MM; OMIM254130), which affects mostly the muscles of the distal lower limbs (1,2). Dysferlin deficiency was also associated with additional phenotypes such as distal myopathy with onset in tibialis anterior (TA), a proximodistal presentation, a congenital mode of onset or even hyperCKemia (3–6). Although all possible types of mutations have been identified in DYSF, including missense, nonsense, frameshift, small deletions or insertions, splicing mutations and large deletions, no correlation with the clinical presentations has been uncovered (1,2,5,7).

The DYSF gene is mainly expressed in skeletal and cardiac muscle and monocytes/macrophages (8) (http://genome.ucsc.edu/cgi-bin/hgGateway). DYSF is composed of 55 exons spanning 150 kb of genomic DNA and is transcribed as a 6.2 kb mRNA (1,2). It codes for a 237 kDa protein composed of a very long N-terminal cytosolic region containing seven C2 domains, two specific central ferlin domains and a transmembrane domain at the C-terminus. Dysferlin shares close homology with the Caenorhabditis elegans FER-1 protein, a mediator of calcium-mediated membrane fusion during spermatogenesis (9) and belongs to the mammalian ferlin family that also includes Myoferlin, Otoferlin, FER1-L4, FER1-L5 and FER1-L6 (10).

Several lines of evidence suggested a key role for dysferlin in sarcolemma repair through membrane fusion and vesicle trafficking. This hypothesis was initially postulated on the basis of structural and sequence similarities between dysferlin and FER-1. Interestingly, other members of the ferlin family have been demonstrated to participate in similar processes (11,12). The involvement of dysferlin in membrane repair was confirmed by the observation that dysferlin-deficient myofibers show reduced membrane resealing capacity after

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laser-induced damage (13). Confirmation that a defect in membrane repair is a contributing factor to the disease pathology was provided by the observation of numerous structural membrane defects in muscles of LGMD2B/MM patients (14,15). In addition, other data have suggested that an exaggerated immune response might also contribute to the pathophysiology of the disease and explain the marked inflammation observed in the muscles of LGMD2B/MM patients (16). Indeed, increased susceptibility to complement attack secondarily to the downregulation of the complement inhibitor, decay-accelerating factor/CD55 (17), exaggerated macrophage and dendritic cell activation (18) and release of exocytotic vesicle contents of a compensatory vesicular trafficking pathway (19) may all contribute to a deleterious inflammatory environment within the muscle.

Although finding a treatment for dysferlinopathy is a very active area of investigation, there is currently no treatment for this disease. With respect to a gene therapy approach, the size of the dysferlin cDNA exceeds the encapsidation capacity of adeno-associated virus (AAV), the safest and most efficient vector for muscle that has been identified to date. To bypass this limitation, we took advantage of the concatemerization ability of AAV (20,21). This strategy uses two independent AAV vectors, one carrying the 5’ part of the cDNA together with an intronic sequence that bears a donor splice site, and the other carrying an intronic sequence with an acceptor splice site followed by the 3’ part of the cDNA. Injection of both dysferlin vectors into the muscle of dysferlin-deficient mice led to the expression of full-length dysferlin, restoration of the membrane repair capacity of myofibers and increased locomotor activity of the mice, thus demonstrating that this approach may be relevant for gene therapy in LGMD2B/MM patients.

RESULTS

Backcross onto the C57BL/6 background maintains the deleterious character of the A/J mutation at a level similar to that seen on the native background

The A/J strain, a naturally occurring mouse model of dysferlin deficiency, was used. This mouse model carries a retrotransposon insertion in intron 4 that leads to out-of frame aberrant splicing and absence of the dysferlin protein (22). To obtain a dysferlin-deficient model that could be compared with a strain-matched control, A/J mice were backcrossed onto the C57BL/6 background. This model, B6.A/J-Dysf<sup>promd</sup> (Dysf<sup>promd</sup>), was characterized at the N4 generation at 1, 2 and 4 months of age (Supplementary Material, Fig. S1). Muscle sections were stained with hematoxylin phloxine saffron (HPS) and the number of centronucleated fibers per square millimeter were counted. Homozygous mice presented with their first dystrophic features at 2 months of age as indicated by the presence of centronucleated fibers and areas of inflammation. The morphological alterations worsened by 4 months of age as indicated by an increase in the presence of centronucleated fibers (Fig. 1A and B). The majority of muscles were impaired, with psoas being the most affected, followed by quadriiceps femoris and TA and then gastrocnemius. Comparison of Dysf<sup>promd</sup> with A/J mice of the same ages showed that backcrossing onto C57BL/6 maintains the deleterious character of the A/J mutation at a comparable level with that seen on the natural background (Fig. 1B). In addition, as shown by mutation analysis using allele-specific PCR (Fig. 1C), this new strain is now free from the deficiency in the C5 component of the complement that renders the A/J mice extremely sensitive to infections (23).

Dual AAV vectors express a full-length dysferlin in vivo after intramuscular injection

The human dysferlin coding sequence was split between exons 28 and 29 and cloned into two AAV vectors. Sequences corresponding to the human 28th intron were incorporated at the corresponding ends of the cassettes after amplification using primers carrying the appropriate restriction sites to obtain pAAV-Dysf<sup>5’-E28I28</sup> and pAAV-I28E29-Dysf<sup>3’</sup> (Supplementary Material, Fig. S2). Pseudotyped rAAV2/1 and rAAV2/9 vectors were produced for transfer experiments into 4-month-old dysferlin-deficient mice. Initial experiments were carried out in the A/J mice since they were performed before the generation of the Dysf<sup>promd</sup> mice and were performed using only the rAAV2/1 pseudotyped vectors. Subsequent experiments were performed on the Dysf<sup>promd</sup> mice using both rAAV2/1 and rAAV2/9. The left TA received a mix of both AAV vectors (1.5 × 10<sup>11</sup> vg of each for rAAV2/9; 4.5 × 10<sup>11</sup> vg of each for rAAV2/1). Muscles were sampled 1 month after injection for rAAV2/1 and rAAV2/9 in Dysf<sup>promd</sup> and at 1, 2, 6 and 12 months for rAAV2/1 in A/J.

To obtain molecular evidence of viral genome recombination, a Southern blot was performed on DNA extracted from rAAV2/1-injected and non-injected TA of A/J mice (1 month after injection). DNA was digested with AarI and AfeI to generate fragments, with sizes allowing distinction of concatemer orientation. A band at 1878 bp corresponding to the heterodimerization of the two AAV in the expected tail-to-head orientation was detected in the DNA of injected muscle but not in DNA of non-injected muscle (Supplementary Material, Fig. S3). After this validation, dysferlin mRNA expression analysis was performed using a Taqman quantitative assay that recognizes the junction between the two parts of the human cDNA and compared with the level of the dysferlin transcript in normal human muscle. Specificity of the primers to amplify only the human gene was checked on murine cDNA from muscle (data not shown). Production of the full-length dysferlin messenger in injected muscles was detected in all tested conditions with quantity corresponding to 3.2–18.4% of the dysferlin mRNA level found in normal human muscle (Fig. 2A).

To analyze the overall distribution of the mRNA in injected muscle sections, in situ hybridization was performed on samples from the rAAV2/9 experiments in Dysf<sup>promd</sup> using a specific 35S-antisense probe that detects the human exon 28–29 junction. A corresponding 35S-sense probe was used as a control. Specific labeling was indicated by a cluster of dots within or in the vicinity of a nucleus (Fig. 2B). About 5–10% of nuclei showed expression of the full-length message on sections of muscles injected with the dual AAV vectors (Supplementary Material, Fig. S4). Although scattered
dots were observed on non-injected sections and on sections with sense probe, they never occurred as clusters (Supplementary Material, Fig. S4) and therefore were not considered true positive.

Confirmation of dysferlin protein expression was obtained by western blotting (Fig. 2C). The full-length 237 kDa dysferlin human protein was detected in extracts on blots from injected muscles from all experiments, but not from non-injected muscles. Figure 2C shows western blots from the time-course experiment using rAAV2/1 (4.5 x 10^10 vg each vector) in A/J mice at 1, 2 and 12 months. The expected 237 kDa band was detected in all treated animals at every time point with only a decrease in signal at 12 months. It is worth to note that no obvious increase in inflammation was present at this time point in injected muscles. Quantification of the signal on blot indicated that the level obtained at 1 month corresponds to 54% of the dysferlin level in human control; at 2 months 59%, and at 12 months 30% (n = 3 for each time point). We also verified by western blot that no expression of partial forms occurred from single vector in muscles injected with one vector only (Supplementary Material, Fig. S5).

In parallel, to assess the distribution of dysferlin within the muscles, cross-sections from injected and non-injected Dysfprmd muscles were immunostained using a monoclonal antibody against dysferlin. Although no labeling was observed in Dysfprmd-untreated sections (Fig. 2D, left upper panel), sampled muscles from the rAAV-treated legs showed widespread positive staining (Fig. 2D, right upper panel). Dysferlin expression in the injected muscles was equally distributed between the cytoplasm and the sarcolemma (Fig. 2D, lower panel), whereas in wild-type (WT) mice and human, dysferlin is largely sarcolemmal (Supplementary Material, Fig. S6). Confocal double-staining immunofluorescence microscopy with antibodies recognizing dysferlin and the ryanodine receptor 1 (RyR1), a protein of the sarcoplasmic reticulum abutting the T-tubule membrane, was performed. Quantification of colocalization using the Colocalization Finder plug-in in ImageJ yielded a Pearson’s correlation coefficient of 0.68 ± 0.015, illustrating that the intracytoplasmic staining of dysferlin corresponds to the T-tubules (Fig. 2E). A western blot analysis of subcellular fractions was also performed showing that the transgenic dysferlin were detected fully in the membrane fraction (Supplementary Material, Fig. S6g). Taken together, these results indicate that injection of both AAV vectors can lead to the production of full-length human dysferlin protein.

Expression of the transgene after intramuscular injection is associated with improved histology

We performed a new set of experiments on 2-month-old Dysfprmd mice to analyze the histological aspect of injected muscles. The left TA received a mix of both rAAV2/9 vectors (1.5 x 10^11 vg each) and the muscles were sampled 1 month after injection. At the histological level, an improvement of the dystrophic features was seen. Upon analysis of non-treated Dysfprmd sections, a high number of fibers with
Figure 2. Expression of the transgene after intramuscular injection. Left TA muscle of 3-month-old Dysfprmtd mice was injected with either 4.5 × 10^10 vg of each rAAV2/1 or 1.5 × 10^11 vg of each rAAV2/9. Right TA muscle was injected with PBS as a control. Muscles were sampled and dysferlin expression was measured 1, 2, 6 or 12 months after AAV injection, (n = 3 for each condition). (A) qRT–PCR analysis of injected muscles. Dysferlin mRNA was detected 1, 6 and 12 months (mo) after injection by Taqman qRT–PCR analysis using a specific probe designed to overlap the human exon 28–29 junction. Dysferlin mRNA levels are normalized by P0 (acidic ribophosphoprotein) and expressed as ratio (in percentage) to human mRNA control. The units are presented on a logarithmic scale. Yellow bars correspond to rAAV2/1 in A/J mice, green to rAAV2/1 in Dysfprmtd mice and blue to rAAV2/9 in Dysfprmtd mice. NI, non-injected. Stars represent significant difference between injected and non-injected mice (P-value < 0.05) as calculated with the Mann–Whitney test. (B) In situ hybridization. Expression was determined by in situ hybridization on samples from rAAV2/9 experiments on Dysfprmtd, using an antisense-specific 35S-probe targeting the junction. Cross-sections of TA muscle from non-injected (left panel) and injected (right panel) mice were labeled with these probes and counterstained with hematoxylin and eosin. This figure shows representative images out of three different experiments. A positive labeling is indicated by small black spots with a higher concentration close to nucleus. Scale bar, 50 μm. (C) Western blot analysis of injected muscles. Muscle lysates at 1, 2 and 12 months from injected (+) and non-injected (−) animals were stained with mouse monoclonal antibody directed against dysferlin (NCL-Hamlet) and normalized by an antibody against actin. The full-length dysferlin (237 kDa) was detected. The bottom panel shows dysferlin expression in extracts from human and WT mouse muscle, showing similar level of expression (the sequence recognized by the antibody is identical between mouse and human). A histogram shows the level obtained at the different time points compared with human level. (D) Immunofluorescent analysis of dysferlin in muscle sections. Determination of dysferlin localization on injected muscles 6 months after injection was carried out by immunohistochemical labeling. Images represent TA cross-sections labeled with NCL-Hamlet antibody. In non-injected muscles (upper left image), no labeling was detected, whereas a clear positive signal was observed in injected muscles (objective ×20, upper right image). Higher magnification (objective ×63, lower right image) of the same view showed a signal both in the cytoplasm and at the periphery of the fibers. Scale bar, 50 μm. (E) Colocalization of transgenic dysferlin and T-tubules. Immunohistochemistry experiments show colocalization (merge, yellow) of dysferlin (red) with RyR1 (green) (objective ×100). Dysferlin is also observed at sarcolemma. Scale bar, 25 μm.
centrally located nuclei and many areas of inflammation were observed. Analysis of the contralateral treated leg revealed that only scant areas of inflammation remained (Fig. 3A). Since we injected adult mice and analyzed the muscles early after injection (1 month), centronucleation can still be observed. Treated \textit{Dysf} \textsuperscript{frmd} mice were also subjected to an eccentric contraction exercise protocol 8 h after an intraperitoneal injection of Evans blue dye (EBD). Non-injected muscles showed scattered positive fibers indicating disruption of the muscle membrane. Whereas in injected muscles, the proportion of positive fibers diminished by 90\% (Fig. 3B).

**Injection of vectors improved membrane repair capacity in treated muscle**

To evaluate the membrane repair capacity of injected muscles, \textit{flexor digitorum brevis} (FDB), a tiny muscle from the sole of the foot, was injected with both AAV vectors ($0.75 \times 10^{11}$ vg each). Beforehand, we performed an experiment with a YFP reporter vector (rAAV2.1-CMV-YFP) in \textit{Dysf} \textsuperscript{frmd} mice to monitor the level of fiber transduction. One month after injection, almost all the fibers were positive (Supplementary Material, Fig. S7). After 1 month of expression, muscles were sampled and fibers were isolated by enzymatic digestion. Laser-wounding experiments were performed in the presence of FM1-43 dye with fibers from WT, injected and non-injected \textit{Dysf} \textsuperscript{frmd} mice. In all cases, a patch of fluorescence was observed to form within 7 s at the lesion site just after injury (see arrows, Fig. 4A). In WT fibers in the absence of calcium and in non-injected \textit{Dysf} \textsuperscript{frmd} fibers, FM1-43 dye penetrated continuously into the damaged fiber as seen in representative images of Figure 4A. In contrast, WT fibers were able to repair the lesion in the presence of calcium and halt the rapid penetration of FM1-43 dye. As expected, the presence of dysferlin in the treated fibers gave rise to a pattern that closely parallels that of WT mice in the presence of calcium, showing rapid stabilization of fluorescence entry, which is indicative of an efficient repair process (Fig. 4A). For all fibers, we plotted the level of fluorescence entering the cell versus time (Fig. 4B). A significant difference ($P < 0.05$) was observed between WT fibers in the presence or absence of calcium, as well as between injected and non-injected fibers (Fig. 4C). It should be noted that after enzymatic digestion of the muscles for the preparation of samples for the membrane repair assay, a high level of dead fibers (detected by wide-field epifluorescence microscopy as fibers spontaneously taking a high level of the FM1.43 dye) was observed.

**Systemic injection of dual vector improved muscle histology and function**

The dual vectors were administered intravenously in the tail vein ($2 \times 10^{12}$ vg each vector) in 1-month-old \textit{Dysf} \textsuperscript{frmd} animals, and several limb muscles (\textit{TA}, \textit{quadriceps} and
as well as the diaphragm and heart were sampled 1 month after injection. The human dysferlin messenger was detected by quantitative RT–PCR (qRT–PCR) in all these samples (Fig. 5A). We also detected by western blot the expected 237 kDa band of the dysferlin protein in a number of muscles with a level corresponding to 1 to 4% of the quantity of dysferlin in a WT murine TA muscle (Fig. 5B). Since the injection was performed before the appearance of the pathological signs, it allowed us the analysis of the beneficial effect on the number of fibers with centrally located nuclei. Indeed, the expression was associated with a decrease of centronucleated fibers in treated animals.
confirming the improvement of histological features in transduced muscles. To study the effect of AAV injection on muscle function, locomotor activity of C57BL/6, Dysf\textsuperscript{promd}-untreated and Dysf\textsuperscript{promd}-treated mice of about 4 months of age was quantified in an open space assay during night hours. Dysf\textsuperscript{promd}-treated mice had been injected via the tail vein with $5 \times 10^{12}$ vg of rAAV2/9 5' and 3' vectors 1 month prior to locomotor assay. The absolute
maximum speed was almost equivalent for non-injected and injected Dysferlin mice (Fig. 5D). The WT mice had a mean period of activity of 36%, Dysferlin of 23% and the injected mice of 30% (Fig. 5D). Dysferlin-treated mice showed a 33% significant increase of period of activity compared with non-injected mice. In addition, the total covered distance was 395, 141 and 213 m for the WT, Dysferlin-untreated and -treated mice, respectively (Fig. 5D). Thus, the increase in distance was of 51% between untreated and treated Dysferlin mice.

**DISCUSSION**

Since the size of the dysferlin cDNA exceeds the packaging capacity of the AAV vector, correcting the gene defect by transfer of the full-length cDNA using a single-AAV vector is not feasible for LGMD2B as has been for other LGMD2s (24–27). In this report, we demonstrate efficient gene transfer by using a dual AAV vector approach which takes advantage of AAV’s ability to concatamerize. It is also based on the introduction of splicing sequences into the AAV cassettes to obtain the formation of full-length dysferlin. Overall, the treated dysferlin-deficient mice showed good improvement of the disease pathology, indicating that this strategy represents a viable therapeutic option for dysferlinopathies.

A similar strategy has been previously used to transfer, with various efficiency, a truncated factor VIII or mini-dystrophin into the liver and skeletal muscle, respectively (28–31). Since very few studies have used such a strategy, it is difficult to determine the parameters that will result in the best efficiency of gene transfer and expression. Nevertheless, there is room for improvements in several areas. First, the levels of full-length messenger reconstitution could be increased by directing the orientation of AAV dimerization through the use of different types of inverted terminal repeats (ITRs) at the two extremities of the AAV genome (20) or by homologous recombination between sequences introduced into the cassettes (32,33). Second, another critical parameter appears to be the nature of the sequences introduced into the cassettes to obtain splicing (21). Previous reports on dual AAV transfer for factor VIII and dystrophin showed that the splicing-directing sequences play an important role in the production of the transgene pre-mRNA. This could possibly occur through the reduction of steric hindrance exerted by the ITR-containing junction on RNA polymerase (21). In the dystrophin studies, two splitting regions (introns 60 and 63) were tested and they exhibited different efficiencies. Indeed, intron 63 showed a weaker ability to concatamerize compared with intron 60 and that this could be corrected by its replacement by a chimeric intron (28). In our case, because of its central position, sequence shortness and high splicing score, we selected the endogenous intron 28 of dysferlin and split it equally between the two cassettes. Our results indicate that this combination is functional. Experiments are ongoing in our laboratory to test other splicing-directing sequences and a combination of type 2 and 5 ITRs and to see whether these can further increase the dimerization efficiency. To go further along this line, the question of how much protein is required to prevent the pathology is an important one. In the case of dystrophin, it was shown that transgenic mice expressing <5% of the protein have a reduced myopathy and a level of 30% is sufficient to prevent any pathological manifestations in human skeletal muscle (34,35). We observed that 1–4% expression of a functional dysferlin in a number of muscles, as seen after systemic injection, is sufficient for the mice to display a reduction of symptoms. This observation is encouraging but it remains to be seen how much protein is needed for a full recovery.

Other gene therapy strategies have also emerged to transfer large cDNAs. In particular, it was demonstrated recently that a type-5 AAV serotype can accommodate up to 8.9 kb in one capsid. However, this was accompanied by a nearly two log decrease in titer (36). In addition, serotype 5 is a very poor muscle transducer compared with serotype 1 or 9 (37). However, it might be possible that more adequate AAV vectors will be obtained, considering the ongoing effort to generate new AAV serotype, using serotype chimeraism or DNA shuffling (38,39). Generating a truncated, although functional version of the protein, is another means to correct protein deficiencies caused by defects in large genes and has been effectively used with the dystrophin gene (40). The repeated C2 domain structure of dysferlin and the fact that proteins with only two C2 domains like synaptotagmin are competent in membrane fusion argue in favor of a possible modularity for dysferlin (41,42). However, each particular C2 domain of dysferlin may have a specific interaction network that could individualize it and render it indispensable for dysferlin function (42,43). Discovery of a patient with a mild form of LGMD2B associated with partial deletion of the gene would serve as proof-of-principle for a mini-dysferlin transgene. However, to date, only missense mutations or a one-exon skipping mutation, but not large deletion compatible with AAV construction, has been associated with mild forms of LGMD2B (5,43,44).

With respect to the location within the muscle fiber, dysferlin was mostly sarcolemmal both in WT mice and in human, whereas transgenic dysferlin was equally distributed between the cytoplasmic and sarcolemmal membrane fractions. Immunofluorescence colocalization analysis showed that the intracytoplasmic localization of dysferlin coincided with RyR1, a protein bound to the T-tubule. This result is in agreement with previous observations of association of dysferlin with the T-tubule network (45,46). It has been shown that dysferlin is predominantly expressed in this system during myotube maturation and muscle regeneration and that dysferlin is able to translocate rapidly from the T-tubule location to a site of injury at the sarcolemma (43). It is possible that the difference in ratio between the two membranous compartments compared with endogenous dysferlin comes from the fact that the transgenic dysferlin is a newly synthesized protein that has remained in the initial compartment. Interestingly, we observed that the proportion of dysferlin present at internal membranes seems to decrease with time since the injection, in accordance with a maturation-related process (Supplementary Material, Fig. S6e and f).

Northern blot analysis has shown that dysferlin is expressed not only in the skeletal muscle, but also in other tissues such as the placenta, lung and liver (42). Since clinical studies have not demonstrated any pathogenicity associated with the
absence of dysferlin in these organs, it may not be necessary to target them. However, it has been hypothesized that monocytes, which also express dysferlin, might contribute to the disease evolution (47). In fact, patient monocytes showed abnormally high phagocytic function compared with control monocytes (18). RNA interference on a macrophage cell line further validated that this inflammatory presentation is directly related to dysferlin deficiency and is not a secondary effect. Although AAV is known to infect monocytes, (48), the use of a muscle-specific promoter in our experiments prevented theoretically correction of the dysferlin deficiency in the monocyte population, raising the possibility that phagocytosis may still be inappropriately aggressive in our treated mice. The decrease in expression observed 12 months after injection may involve the immune system, in particular through this increased phagocytosis.

In conclusion, we have demonstrated that it is possible to transfer the full-length dysferlin cDNA into muscle, using a strategy that takes advantage of AAV concatemerization and splicing, and that expression of this full-length dysferlin protein has the ability to correct many aspects of the disease phenotype. These data provide a strong argument for the therapeutic use of this strategy to treat dysferlinopathies. In addition, the data presented here, together with the result obtained with the same strategy in DMD, support the use of such a strategy for other diseases.

MATERIALS AND METHODS

Construction and production of recombinant AAV

The 5′ part of the full-length human dysferlin coding sequence (GenBank number NM 003494) from exon 1 to exon 28 and the 3′ part of dysferlin from exon 29 to exon 55 were PCR-amplified. Both fragments were cloned independently into an AAV vector carrying type-2 ITR. The 5′ part was placed under the control of a C5-12 promoter (49), and the 3′ part was followed by a poly-adenylation signal from SV40. A PCR-mediated approach was used to insert the donor splice site sequence or acceptor splice site sequence of the 28th intron of the human dysferlin gene to generate the plasmids pAAV2-Dysf.E28I28 and pAAV2-Dysf.I28E29. Adenovirus-free rAAV2/1 and rAAV2/9 viral preparations were generated using a three-plasmid transfection protocol as described previously (50). See Supplementary Material for detailed information.

Animal models, vector administration and functional evaluation

All procedures on animals were performed according to the European guidelines for the humane care and use of experimental animals. The experiments were approved by the French Ministry of Research and Education (Agreement decision B91 228-101). A/J and C57BL/6 mice were purchased from Charles River Laboratories (Les Oncins, France). B6;A/J-Dysfprm (Dysfprm) were generated through successive backcrosses on C57BL/6 background. Briefly, A/J female mice were crossed with C57BL/6 male mice from the Jackson Laboratory. Genotyping for the Dysfprm mutation was performed on tail DNA as described (22). Heterozygous N1 female mice were then crossed with C57BL/6 male to generate N2 animals. This procedure has been performed successively until N4. Determination of the presence of the Hc0 allele was performed by an allele-specific PCR assay using the forward primers C5_WT-F 5′-TTTACAACAACTGGACTGCTATA-3′ for the WT allele and 5′-TTTTACAAACAAGTGGACTGACT-3′ for the Hc0 allele and the reverse primer 5′-GGCATCTTGTAAATGCCAAATG-3′. Genomic tail DNA was amplified by PCR with an annealing temperature of 60°C using the Herculase polymerase (Stratagene, La Jolla, CA, USA).

All our protocols were performed using male animals. Either intramuscular or intravenous injections were performed. For the in vivo experiments aimed at assessing AAV-mediated dysferlin expression, mice were injected into the left TA muscle with a mix of the rAAV viral preparations, Dysf.E28I28 and Dysf.I28E29, in equal proportion (4.5 × 1010 vg each for rAAV2/1 or 1.5 × 1011 vg each for rAAV2/9) or with one of the viral preparation alone (9 × 1010 vg for rAAV2/1 or 6 × 1011 vg for rAAV2/9). For the in vivo experiments aimed at assessing AAV-mediated functional rescue of membrane repair, FDB muscles were injected with 15 μl of a mixture containing the two AAV vectors (4.5 × 1010 vg in total for rAAV2/1 and 1.5 × 1011 vg in total for rAAV2/9). A control experiment was performed by injecting 15 μl (9 × 109 vg/ml) of an rAAV-CMV-YFP in the FDB of Dysfprm mice. For intravenous injection, 2 × 1012 vg each of the two rAAV2/9 were injected into the tail vein in a 250 or 800 μl volume for 1- and 4-month-old mice, respectively. In some experiments, mice were injected intraperitoneally with EBD (0.1 mg/10 g of body weight) the day before sacrifice. Locomotor activity was assessed in the LE 8811 IR motor activity monitor (BIOSEB, Chaville, France). Test chambers were shielded from external noise and light, but each test field was illuminated with a white fluorescent light and was fully ventilated.

RT–PCR, in situ hybridization and western blot

RT–qPCR analyses were performed as described previously (50). The primer pairs and Taqman probe used for spliced dysferlin-specific detection were as follows. Exon28F 5′-CTCAACCGGCTGTGAT-3′, Exon29R 5′-GTCGGGTGTGTTGTAGTACATCTTCTCA-3′ and Exons 28/29 5′-CAAGGCTTGAGGAG-3′. The ubiquitous acidic ribosomal phosphoprotein (P0) was used to normalize the data across samples. To perform in situ hybridization, an antisense oligonucleotide directed against the junctional part of the dysferlin message (5′-ATGCTATACTCCACCGCTTGACAGGCCGGG-3′) was designed, labeled with [35S]-dATP. See Supplementary Material for detailed procedures.

Western blotting was performed using standard procedures with the anti-dysferlin NCL-Hamlet antibody that recognizes both the human and murine dysferlin (Novocastra, dilution 1:500). Blots were scanned and quantified using the ‘Gel plotting macros’ (http://rsb.info.nih.gov/jij/docs/analyze.html#gels) in ImageJ. To determine the cellular compartment where transgenic dysferlin is expressed, proteins were extracted with the ProteoExtract O Subcellular Proteome Extraction Kit.
(Calbiochem, Darmstadt, Germany) from injected TA muscle sample. A western blot was then proceeded with 90 μg of protein.

_Biceps brachii_ muscle biopsies obtained after informed consent from individuals free from muscle disease were used as normal control for dysferlin expression.

**Histology and immunohistology**

Cryosections (8 or 10 μm thickness) were prepared from frozen muscles and boiled in PBS for 10 min and then cooled for 5 min at RT. Sections were incubated in blocking solution (3% BSA in TBS) and immunodetection was performed overnight using the monoclonal antibody dysferlin-Hamlet (NCL-Hamlet, Novocastra, diluted 1:20). After three washes in PBS for 5 min each, sections were incubated with a biotinylated goat anti-mouse IgG1 antibody (Southern Biotechnology) for 45 min. After three washes in PBS, sections were incubated with a streptavidin antibody conjugated with Alexa-594 for 20 min. For colocalization studies, sections were additionally first-labeled with a monoclonal RyR1 antibody (ab2868, Abcam, diluted 1:500) revealed with goat anti-mouse Alexa-488. Sections were examined under a Leica confocal microscope. Colocalization of dysferlin with RyR1 was quantified (on seven random regions-of-interest) with Pearson's correlation coefficient using ImageJ with the RyR1 was quantified (on seven random regions-of-interest) with Pearson's correlation coefficient using ImageJ with the Colocalization Finder plug-in after correction of background with FFT bandpass filter plug-in.

**Membrane injury and membrane repair monitoring**

The assay was performed on isolated single-fibers from FDB muscles of 3–4-month-old Dysf<sup>erm</sup> and C57BL/6 mice in the presence or absence of calcine and in the presence of FM 1–43 dye (final concentration 4 μM; Molecular Probes). To induce damage, an area of the sarcolemma on the membrane of the muscle fiber was irradiated at full power for 1 s with a two-photon confocal laser-scanning microscope. The multi-photon microscope consisted of a Radiance 2100 MP scan head (Bio-Rad) equipped with a mode-locked titanium–sapphire laser system (Coherent Verdi-Mira) pumped at 10 W and tuned to an 800 nm excitation with 100 fs pulses at 76 MHz. The microscope was an inverted Nikon TE300 (Nikon Instech Co.) with Nikon objectives (dry CFI Plan APO, ×20 NA0.75). Images were captured for 3 min after the irradiation at 7 s intervals. Images were represented in an inverted 16-color look-up table of ImageJ. For every image taken, the fluorescence intensity at the site of the damage was measured by ImageJ on an area of about 0.01 mm<sup>2</sup> and plotted on a graph with time on the x-axis and the amount of fluorescence on the y-axis. The rate of fluorescence influx for all fibers in a same group was plotted on histogram.

**Statistics**

Data are presented as means ± SEM. Individual means between two groups were compared using the non-parametric Mann–Whitney test. The non-parametric Kruskal–Wallis one-way analysis of variance was used for testing equality of population medians among groups. Differences were considered to be statistically significant at *P < 0.05 and at **P < 0.01.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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