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Authors
Barthélémy, Florian
Wein, Nicolas

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Review

Personalized gene and cell therapy for Duchenne Muscular Dystrophy

Florian Barthélémy[a,b], Nicolas Wein[c,d,*]

[a] Center for Duchenne Muscular Dystrophy, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA
[b] Department of Microbiology, Immunology, and Molecular Genetics, Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, USA
[c] The Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH, USA
[d] Departments of Pediatrics, The Ohio State University, Columbus, OH, USA

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Abstract

Dystrophinopathies are diseases caused by mutations in the Duchenne Muscular Dystrophy gene (*DMD*) encoding the dystrophin protein. Depending on the type of mutation, patients develop either the severe *DMD* or the milder Becker Muscular Dystrophy. Although substantial effort was made, the pathophysiology and variation in disease severity are still poorly understood. During the last two decades, relentless efforts were made to develop therapeutic strategies. Among these, gene therapy and cell replacement therapy appear very promising. These approaches are based on the replacement and/or repair of the mutated *DMD* gene or transcript at the molecular level, or at the cellular level via replacement of the damaged muscle cells. While highly successful in animal models, these therapies showed only modest efficacy in human clinical trials. More importantly, variable effects were observed in patients carrying the same mutation, suggesting that several factors (e.g., genetic modifiers, environmental factors) can affect treatment outcomes. In this review, we will describe recent advancements and new approaches of gene and cell therapies for dystrophinopathies that pave the way for a medicine "à la carte".
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Keywords: Dystrophin; Duchenne muscular dystrophy; Therapy; Muscle; Dystroglycan-protein complex.

1. Introduction

Duchenne muscular dystrophy (DMD) is one of the most common childhood neuromuscular diseases (1: 3500 male births [1]). It was named after the French neurologist Guillaume Duchenne, who was the first to describe the symptoms in 1861. The disease DMD is caused by mutations in the X-linked *DMD* gene that is one of the largest genes in the human genome (79 exons coding for a 14-kb transcript), producing the giant dystrophin protein (427 kDa) (Fig. 1). This subsarcolemmal muscle protein is associated with the dystrophin glycoprotein complex (DGC) [2]. The DGC, which links the intracellular compartment via the actin filaments to the extracellular matrix, is essential for the maintenance of the sarcolemma (Supplemental Figure). Loss of dystrophin protein causes a cascade of dysfunctions including the alteration of muscle tissue or function (increased degeneration/regeneration and replacement by fat or connective tissue) [3], deregulation of calcium homeostasis [4–6], appearance of oxidative stress [7], inflammation [8] and finally an increase in apoptosis of the mitochondrial-dependent energy metabolism [9,10]. Absence of dystrophin also leads to mislocalization of the DGC and neuronal nitric oxide synthase (nNOS) [11].

DMD is a progressive pathology with an age of onset around 3 years old. The first clinical symptom of DMD in boys is the use of hands and arms to raise their body from a squatting position (Gower's maneuver). Moreover, patients also have trouble running, jumping and display an unusual gait (stomach forward and shoulders back), or a pseudohypertrophy of calf muscles. Following the clinical examination, an elevated blood-test result for serum creatine kinase (CK), an enzyme that leaks out of damaged muscle, guides the diagnosis to a muscular dystrophy. To narrow down the diagnosis, a genetic testing is performed looking at particular regions of the DNA (if a known mutation is segregated in the family) or at the two most common mutational hotspots located between exon 2–20 and exons 45–55 [12]). Alternatively the

* Corresponding author.
E-mail address: Nicolas.Wein@nationwidechildrens.org (N. Wein).

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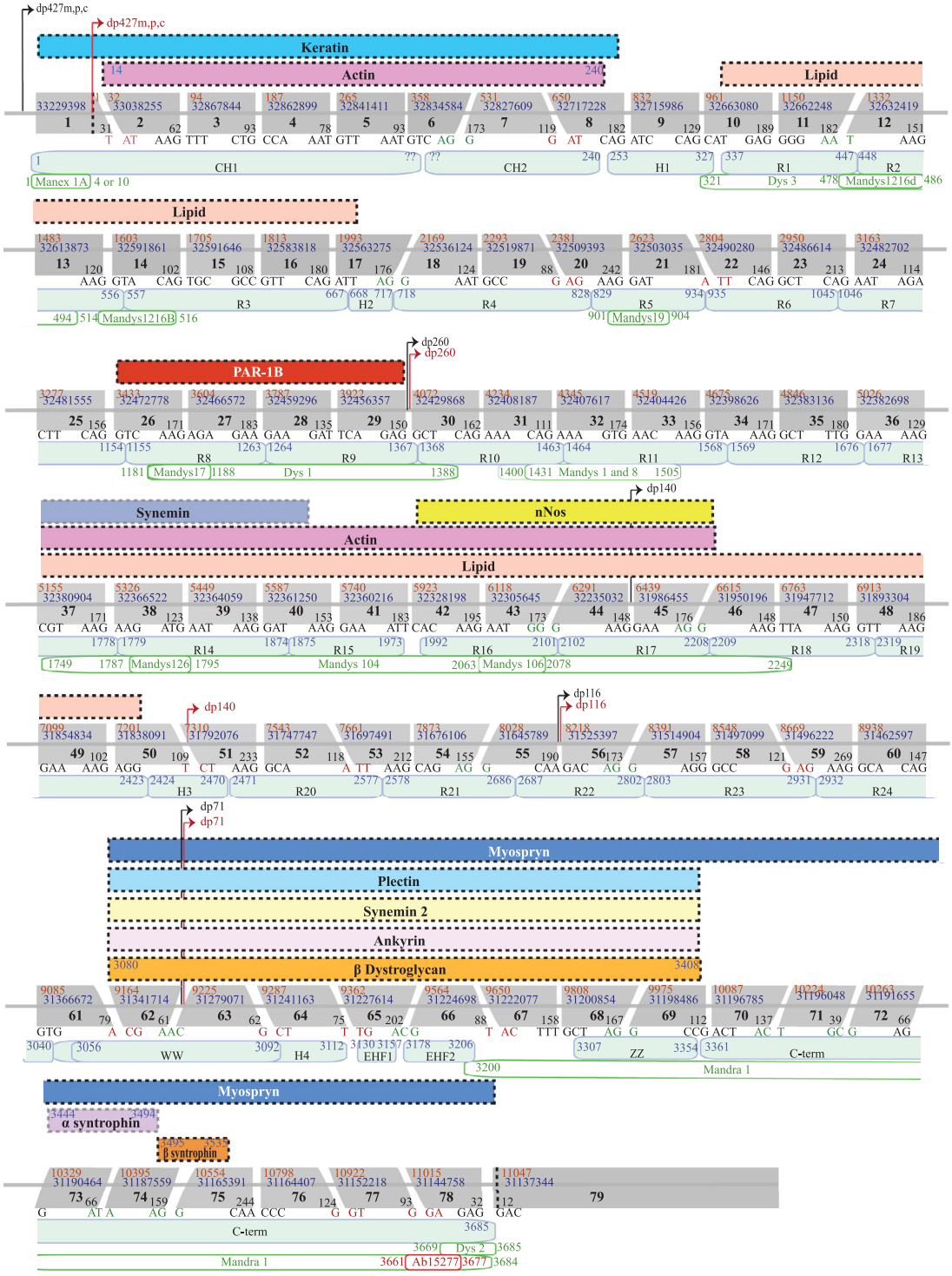

Fig. 1. Representation of the phasing of *DMD* exons. Black arrows indicate promoters of the different isoforms; red arrows indicate the corresponding start codon. All exons are in gray quadrilaterals. Orange numbers (upper left of each exon box) indicate the nucleotide number at the beginning of each exon (in correlation to dp427m mRNA). Blue numbers at the bottom right corner of each exon correspond to position at the genomic level (hg19). The sizes of each exon are indicated in the lower right corner. Below each exon, the position of the codon is indicated with a color code (black in +3, green in +2 and red in +1). Predicted Protein domains are represented as a light green ovoid form. Their respective amino acids position at the beginning and ending are indicated in blue numbers. The location of the most frequently used antibodies is indicated as outlined ovals: red for polyclonal or green for monoclonal antibodies. The dystrophin binding partner's location is also represented (dotted boxes).

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DMD gene is sequenced. This multistep diagnostic is necessary since *DMD* is one of the largest human genes. Among the different types of mutations identified to date, a majority is intragenic deletions and among these deletions, 65% are frameshift mutation but large duplications (12%) and small mutations (20%) are also found [13].

At the age of onset, muscle fibers are still able to regenerate, but this ability declines with age. At around 13 years of age, this decline results in the loss of ambulation as well as cardiac and/or respiratory failure that are consequently the most common causes of death in DMD [14]. Recent improvements in mechanical ventilation techniques helped to shift the life expectancy from typically between 20 and 30 years up to around 40 years in rare cases [15].

Interestingly, apart from DMD, mutations in the same gene can also cause a milder form of muscular dystrophy called Becker Muscular Dystrophy (BMD). Boys with BMD usually present with loss of ambulation and cardiac defects after age 15 [16], but they can also be asymptomatic far into adulthood [17]. Unlike DMD patients, BMD patients harbor mutations that preserve the reading frame of the *DMD* gene. These result in production of a truncated, but functional, protein.

To date, a curative treatment for DMD or BMD is not available [18] and the only options for patients are steroids that delay disease progression by reducing muscle inflammation and palliative treatments that improve patients' comfort (e.g., physiotherapy, hydrotherapy, physical stimuli) (for a detailed review see [19]). However, recent advances in fields related to cell therapy and gene therapy are promising. This review will summarize and discuss current personalized medicine (also known as precision medicine) strategies that aim to correct and/or compensate the genetic defect via gene therapy and/or cell therapy based on the specific biological context of each individual.

2. Gene therapy

2.1. Nature and evolution of viral vectors

Under investigation for nearly 30 years, gene therapy is an experimental technique that uses genes to replace a defective gene or to modify its content or expression to correct genetic abnormalities or prevent disease. Several of these approaches require the use of viral vectors. Adenoviruses were first considered the most plausible vectors for delivering modified copies of a gene due to their large encapsidation capacity and their high infection efficiency. However, studies showed that adenovirus based vectors are responsible for an elevated immunogenic reaction in non-human primates [20] and tragically were responsible for the death of a patient in a gene therapy clinical trial for the liver disease, partial ornithine transcarbamylase deficiency [21]. Despite the risks, adenoviruses are still the most commonly used vector for cancer gene therapy and vaccines [22].

For neuromuscular diseases, the two most prominent vectors are adeno-associated viruses (AAV) and lentiviruses. The choice to use either AAV or lentiviruses is based upon the specifics of each individual therapy as lentiviruses and AAV differ in many points (Table 1). Lentiviruses are single-stranded RNA viruses whereas AAVs are single-stranded DNA viruses. Both viruses can carry DNA within their envelope (also call capsid), a process is referred as packaging. While lentiviruses can replicate and package independently, AAV have a very limited capacity of replication and packaging when used alone. To maximize these processes, it requires the help of a second virus, usually adenovirus [23]. Lentiviruses integrate into the genome conferring infected cells the ability to transfer the transgene to daughter cells, which is a key advantage for long-term therapy. Lentiviruses also have a transgene-carrying capacity of up to 10 kb between the long terminal repeat (LTR).

A new generation of virus with self-inactivating LTRs and different insertion-site preferences is emerging with the promise of improved safety (for a review [24]). The size of the coding sequence of the *DMD* transcript (11 kb) makes its packaging in viral vectors challenging. However, in 2017, Counsell and colleagues demonstrated that it was possible to encapsidate successfully the full-length dystrophin into lentiviruses. This was possible because template switching occurs during lentiviral reverse transcription. This can lead to lentivirus DNA to recombine with the exogenic template producing chimeric proviruses. Using this approach, full length dystrophin was being expressed following lentiviral infection of human derived cells [25]. AAV naturally contains the *rep* and *cap* genes between two inverted terminal repeats. The creation of recombinant AAV (rAAV) vectors where the *rep* and *cap* genes are replaced by a sequence of interest allows for a maximum encapsidation capacity of ~4.9 kb [26]. AAVs have been engineered to show a robust transduction associated with a sizeable expression in hosts and a moderate immune response [27]. Inside the cell, recombinant AAV genomes remain episomal and only integrate into the genome with a low frequency of about 0.5% as demonstrated by Schnepp et al in muscle from adult male BALB/c mice [28]. Thus, while the episomal stability of AAV enables long-term transgene expression in non-dividing cells such as muscle fibers, this virus is less suited for dividing cells.

One major hurdle of gene therapy is to achieve proper targeting of disease-relevant tissues. This hurdle could be overcome using different AAV serotypes with high tropism for the desired target tissue [29]. In particular, AAVs 1, 5, 6, rh74, 8, 9 and 10 are serotypes that are often used to target human striated muscle when delivered through the blood [30]. In addition, using cell type specific promoters can ensure expression only in tissue of interest. In 2001, researchers generated self-complementary AAV (scAAV) viral vectors [31]. scAAV forms an intra-molecular double-stranded DNA template, preventing the need of synthesizing the second strand after entering the nucleus. Thanks to this alteration, transgene expression is more efficient compared to conventional AAVs. Despite this, the impact of scAAVs on the field of neuromuscular disorders remains limited due to their lower encapsidation ability (~2.3 kb instead of ~4.9 kb for AAV) [32].

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Table 1
Comparison of the gene therapy vectors.

Characteristics	Adenovirus	AAV	Lentivirus
Virus type	Double-stranded DNA	Single-stranded DNA	RNA
Envelope	No	No	Yes
Encapsulation capacity	~10–30 kb	~5 kb	~8 kb
High variety of tropism	No (if native)	Yes	Yes
Muscle tropism	Yes if modified	Yes (serotypes 1,5,6,rh74,8,9,10)	Yes
Ability to infect latent or quiescent cells	Yes	Yes	Yes
Ability to target satellite cells	No	not efficient	Yes
Genome integration	No	Yes (on chromosome 19 only) but rare event	Yes
Duration of expression	Transient	Transient	Persistent
High Production Scale* ($>10^9$ mcU/mL)	Yes	Yes	No
Immune response	High	Very Low	Low
Known pathological risks	Yes	No	Yes (oncogenesis and potential of replication-competent virus)
Ex vivo use	Yes	No	Yes

mcU/mL : microUnits per milliliter.

As mentioned above, one major limitation of using AAV is the lack of persistent expression in dividing cells. AAV-delivered transgenes show a decrease in expression each time cells divide, since unlike lentiviruses, AAV do not integrate into the genome. This decrease in expression might not be too detrimental if muscle is the target, since the myofibers are composed of post-mitotic cells. However, satellite cells, which are responsible for the re-population of damaged fibers, are not well targeted by current AAV vectors [33]. Therefore, every time the muscle is injured, satellite cells recruited to participate in the membrane repair process will not contain the transgene thus diluting/leading to loss of the transgene over time [33]. In an effort to resolve these issues, researchers are trying to create new AAV vectors with a high tropism for satellite cells. Current attempts involve using adjunction of specific receptors to the capsid of the AAV or manipulating the AAV genome to be able to be passed on to daughter cells for long-term correction [34].

Although AAV vectors are relatively safe, the use of viral vectors is always accompanied by concerns. For example, patients can be seropositive, meaning they produce antibodies against certain AAV serotypes. This could present a risk of reducing the vector effectiveness, as the immune system would recognize the treatment like a regular viral infection, leading to its neutralization [35,36]. Therefore, patients involved in clinical trials are often pre-screened for the presence of AAV antibodies. While rAAV vectors do not directly express any wild-type AAV proteins, the rAAV vector can still trigger immune responses [37], which could explain why AAV re-administration to muscle showed little to no success in mice when the exact same serotype was used [38,39]. However, unlike other viral vectors, most AAVs have not caused any severe side effects in clinical trials so far. Noteworthy, a recent publication described AAV related toxicity in non-human primates and piglets following high dose intravenous administration of a modified version of AAV9 [40]. While this finding is concerning, it is important to note that this has been shown in a pre-clinical stage and not necessarily using the standard clinical settings of AAV preparation/purification and titer quantification [41]. To date, the most severe side effect observed in more than 120 clinical trials using different AAV serotypes, was a transient tissue inflammation that did not cause any long-term damage, confirming the strong safety profile of AAVs [42]. Altogether, AAVs are very well tolerated and represent a powerful tool for gene therapy.

In the case of DMD, the size of the *DMD* mRNA prevents AAV from carrying the full transcript. To bypass this limitation, researchers used different AAV based approaches to improve DMD phenotypes including the use of mini and microgenes, or splicing alteration vectors. These strategies are explained in more details in the following section.

2.2. Alternative approaches

While most of gene therapy approaches are based viral vectors, alternatives exist based on non-viral delivery methods and engineered vectors. Non-viral vectors are often referred as naked DNA and particles or chemical based vectors. These approaches promise better uptake, lower immunogenicity and pathogenicity due to the low risk of insertional mutagenesis, low cost and ease of production. While, such nanoparticules have in the past been not efficient, recent advancement in the field rise expectations for this technology (for recent review see [43]).

2.3. Mini- and micro-dystrophin

As mentioned above, the dystrophin protein is one of the largest proteins in the body, making gene therapy approaches challenging due to packaging size limitations. Based on BMD patient phenotypes that can be very mild, it is evident that even large internal deletions in the dystrophin protein are tolerable, leading to minor defects in muscle performance [44]. This dispensability of subdomains has also been confirmed by the partial functional rescue observed in a mouse model of DMD (referred as *mdx* mouse model). AAV vectors containing internally truncated versions of the protein-mediated correction of the muscle defect in the *mdx* mouse [45–47].

The use of truncated or internally deleted versions of dystrophin, which allows the bypass of the AAV encapsida-

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tion limit, is of great interest. Truncated dystrophin genes are grouped into two main classes based on size: the mini-dystrophins and the micro-dystrophins. The mini-dystrophin genes, about 7 kb, encode a protein around half the size of the human native dystrophin protein and the inner part of the coding sequence (encoding the rod domains) is usually removed. It requires two AAVs to deliver the entire mini-dystrophin. Micro-dystrophins, about 4 kb, encode proteins of about one third the size of the human dystrophin protein. In addition to the removal of the inner protein part, the C-terminal part of dystrophin is also deleted, allowing encapsidation into a single AAV vector. While several versions of micro-dystrophin have been created (CS1, delCS1 and M3), all of them contain the cysteine rich domain (Fig. 2). First generation mini/micro-genes were delivered using adenoviruses at the end of the 90 s leading to restoration of the protein expression associated with very low phenotypic rescue [48]. Thanks to efforts in the AAV field and discovery of serotypes that better target muscle, researchers began focusing on a second generation of micro-dystrophin. This was key since the use of AAV allows good targeting of both skeletal muscle and heart in mouse models [49–51]. The current third-generation micro-dystrophins are constantly being improved either by swapping hinge and spectrin repeats, codon optimization (a modification that switches particular DNA sequence by changing its codons to match the most prevalent tRNAs resulting in a more efficient translation) and/or the addition of dystrophin parts that enable interaction with important protein partners (syntrophin, nNOS, etc…) [52–56]. Based on the promising results from experiments in golden retrievers with muscular dystrophy (GRMD dog model) [57,58], two constructs were moved forward and are currently being investigated in clinical trials: one mini-dystrophin (rAAV2.5-CMV-mini-dystrophin) is in phase I (NCT00428935) and one micro-dystrophin (rAAVrh74.MCK) (NCT02376816) is currently enrolling for a phase I/IIa (see Table 2 and Supplemental Table). SGT-001, another microdystrophin (Solid Biosciences, Cambridge, MA), is also being evaluated in a phase I clinical trial called IGNITE DMD (NCT03368742), based on evidence of phenotypic improvement without adversary effects in *mdx* mice (Solid Biosciences website). However the FDA due to serious adversary effect in one patient has put this on hold. Finally, PF-06,939,926/BMD-D001 (Pfizer, New York, NY),) is a mini-dystrophin currently tested in phase I (NCT03362502) (see Supplemental Table). Taken together, these approaches using mini or micro-dystrophin genes are promising since they could treat DMD/BMD patients independently of their mutations. However, the partial loss of functionality due to the omission of large protein subdomains remains a challenge and significantly reduces the efficacy of the treatment.

As a consequence, other approaches aim to produce nearly full-length dystrophin by targeting only the region that contains the mutation, in an attempt to bypass the genetic defect. During the last decade, several approaches have been developed to directly correct the genetic defects at the RNA level. Examples of such editing include the modification of transcripts through exon skipping during RNA maturation or stop codon read-through during translation. In the case of dystrophin, such approaches are designed to either restore the expression of a full-length transcript or of a nearly full-length *DMD* (for review see [59]).

3. Pre-messenger RNA (mRNA) surgery

3.1. Exon skipping

The goal of dystrophin exon skipping is to restore the reading frame of an out of frame mRNA in order to lead expression of a truncated but highly functional protein. This should transform a DMD phenotype into a milder BMD phenotype. The theory behind exon skipping therapies is based on the observation that some BMD patients with milder phenotypes present in-frame exonic deletions, encoding truncated, but highly functional, dystrophin proteins [60]. In comparison to mini or microdystrophin protein, this approach permits production of larger and thus more functional dystrophin.

Exon skipping strategies focus on interfering with the spliceosome during the maturation of the pre-mRNA into mRNA. Under physiological conditions, the spliceosome recognizes exons due to exonic definition motifs such as the branch point, acceptor site, and donor site. These exonic definitions can be hidden using small complementary DNA or RNA molecules called antisense oligonucleotides (AON), which are designed to bind to these motifs. This “hiding” of signaling sites then leads the spliceosome to skip one or more of the adjacent exons (Fig. 3A). These small nucleotide sequences can either be delivered under a promoter in form of gene therapy (discussed below), or by the application of “naked AONs” (discussed in section alternative approaches to increase dystrophin protein levels). Overall, the percentage of boys with DMD that could benefit from exon skipping therapies is about 80% [61,62]. Among these, 13% are eligible for exon 51 skipping; 8% eligible for exon 45 and 53 skipping; 6% for exon 44 skipping; 4% for exons 52, 50 and 43; 2% for exon 8 or 55; with the remaining 30% being eligible for other exons or multi-exon skipping.

Viral vectors such as AAVs are well suited to deliver a small, nuclear RNA (U1 or U7snRNA), which carries the antisense sequence [63–65]. U7 snRNA is normally involved in histone pre-mRNA 3′ end processing and U1 snRNA usually recognizes the 5′ splice site of an exon. By changing the RNA binding sequences of these small RNAs, they can be converted into versatile tools for splicing modulation [66]. The advantage of using U7 or U1 derivatives is that the antisense sequence is embedded into a small nuclear ribonucleoprotein (snRNP) complex, thereby protecting the antisense sequence from degradation and allowing accumulation in the nucleus where splicing occurs [67]. Moreover, when embedded into a gene therapy vector, these small RNAs can be permanently expressed inside the target cell after a single injection. The use of AAV-snRNA mediated exon skipping, such as U1 or U7, was shown to be particularly efficient in several animal models of DMD, including *mdx*

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Table 2
Overview of the therapeutic strategies for DMD currently tested in clinical trial. The table summarizes the results of therapeutic approaches currently in clinical trials. Ethical concerns and clinical trial numbers (NCT numbers) are also indicated. AAV: adeno-associated virus; CMV: cytomegalovirus; MCK: muscle creatine kinase; IV: intravenous; PMO: phosphorodiamidate morpholino oligomers.

Therapeutic approach	Ongoing clinical trial evaluation	Safety/Ethical concerns	NCT numbers
Mini-dystrophin (rAAV2.5-CMV-mini-Dystrophin;d3990 and rAAV9.muscle specific promoter-minidystrophin; PF-06,939,926)	-rAAV2.5-CMV-mini-Dystrophin is expected to start phase II very soon - Minidystrophin (PF-06,939,926) is currently enrolling for phase I	- potential acute or acquired immune response against mini or microdystrophin - potential liver toxicity when high dose of new engineered AAV9. however this was never shown using regular AAV9. several clinical trial using AAV9 have been proved to be safe.	NCT00428935 NCT03362502
Micro-Dystrophin (rAAV9-muscle specific promoter-microdystrophin	SGT-001 was put on hold by the FDA since the first patient dosed showed serious adverse reaction		NCT03368742
Micro-dystrophin (rAAVrh74.MCK micro-dystrophin and SGT-001)	rAAVrh74.MCK micro-dystrophin is currently enrolling for a systemic Gene Delivery Phase I/IIa		NCT02376816
Exon 51-skipping (eteplirsen/EXONDYS 51 - PMO)	In Phase III, 160 patients either treated with 30 mg/kg or untreated; study to run for 96 weeks (estimated date of completion in May 2019). Significant phenotypic improvement and protein expression were shown at 48 weeks.	No toxicity demonstrated but some local reaction. Repeated injection may be constraining for patients.	NCT00159250 NCT01396239 NCT00844597 NCT01540409 NCT03218995 NCT02420379 NCT02255552 NCT02286947
Exon 53 skipping (Golodirsen/SRP-4053 PMO)	Phase III is currently enrolling (previsional maximum of 126 patients) (ESSENCE trial). Expected end date by June 2021.		NCT02500381 NCT02310906
Exon 45 skipping (Casimersen/SRP-4045 PMO)			NCT02500381 NCT02530905
Gentamicin (readthrough)	Phase I studies showed : -No dystrophin expression and no clinical improvement after 2 weeks at 7.5 mg/kg of intravenous gentamicin in 2 DMD and 2 BMD. −3 out of 4 patients presenting a UGA stop codon showed dystrophin expression but no clinical amelioration with a regimen of two cycles, each for 6 days with a 7-week hiatus between dosing. -no side effect and decrease ck level after 2 weeks but rise up over the 6 month course of the study. No clear clinical efficacy demonstrated	Nephrotoxic and ototoxic at higher dose	NCT00005574 NCT00451074
Translarna/Ataluren (PTC124) (readthrough)	In phase III, modest effect in a subgroup with a baseline between 300–400 m in the 6MWT. New trial recruiting 250 subjects for a 72 week study followed by a 72 week open label period at 10, 20 mg/kg. Estimated study completion date end of 2021.	Potential off-target	NCT03179631 NCT02819557 NCT02090959 NCT03179631 NCT01826487 NCT01009294 NCT00847379 NCT01247207 NCT00759876 NCT00592553 NCT00264888
Ezutromid (Utrophin modulation)	A phase II trial is ongoing with 40 patients enrolled for a 48 week open label treatment. A significant reduction of muscle damage on muscle biopsies after 24 weeks of treatment has been reported.		NCT02858362 NCT02056808 NCT02383511
rAAVrh74.MCK.GALGT2	Currently under investigation with enrollment by invitation for a phase I trial.		NCT02704325
rAAV1.CMV.huFollistatin344	A phase II trial is planned to enroll soon	Muscle hypertrophy might be more susceptible to damage.	NCT01519349

(*continued on next page*)

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Table 2 (*continued*)

Therapeutic approach	Ongoing clinical trial evaluation	Safety/Ethical concerns	NCT numbers
BMS-986,089/RG6206 - Myostatin inhibitor	A phase II/III is ongoing with 159 randomized participants. Estimated completion date July 30, 2020.		NCT02515669 NCT03039686
DOMAGROZUMAB (PF-06,252,616) - Myostatin inhibitor	A phase II is ongoing with 3 dose levels (5 mg/kg, 20 mg/kg and 40 mg/kg) of IV infused for each of the 113 subject. Expected completion date 04/2018.		NCT02310763 NCT02907619 NCT01616277
Myoblasts	Myoblasts injections is currently under investigation in phase I. Estimated Primary Completion Date January 2019	Autologous or heterologous injection of precursor cells and risk of immune response	NCT02196467

mice, a mouse model carrying a duplication of *DMD* exon 2, as well as the GRMD dog [63,64,68–70](see Supplemental Table). A phase I clinical trial using this technology is currently planned for 2018 in the USA.

3.2. Trans-splicing

Another approach, trans-splicing, consists of promoting the exchange of nucleotide sequences from a mutated exon to a normal exon. This approach is of particular interest for patients not eligible for exon skipping (patients presenting mutations in the last third of the *DMD* gene that is essential to the dystrophin function). This method has only been tested in human cell lines and mouse models so far [71–73]. To mediate trans-splicing, a pre-trans-splicing molecule (PTM) is delivered by a viral vector (Fig. 3B). The PTM will induce a trans-splice event between endogenous pre-mRNA and the exogenous pre-mRNA, which results in a chimeric mRNA composed of a mutated exon and an exon free of mutations [74]. This approach is used to correct a splicing mutation occurring at the beginning of a donor or acceptor splice site because the cell will favor the non-mutated acceptor or donor sequence carried by the PTM [75]. The main limitations are the off-target splicing risks, restrains due to vector packaging limits, and generally lower efficiency of delivered exon incorporation compared to regular exons. This approach had variable efficiency in DMD skeletal muscle, but a good effect in the heart of *mdx* mice [71–73].

Trans-splicing can also be used to trigger recombination between viral genomes. For example, researchers have investigated the possibility of delivering either mini-dystrophin or the full length *DMD* cDNA by using 2 or 3 AAVs in combination each carrying a different area of the gene. These 2 or 3 genomes will then be trans-spliced to reconstitute the full-length dystrophin. In *mdx* mice, the use of dual AAV vectors for *DMD* resulted in improvement of histopathology, muscle strength, and protective effect over eccentric-contraction induced injuries [76]. The use of 3 AAVs leads to full-length dystrophin protein expression, although the distribution remained more patchy [77]. Even if this approach seems promising because it leads to the expression of a full-length transcript, the very low efficiency of this technique, especially when using three vectors, appeared to be technically challenging (see Supplemental Table). Extensive optimization will probably be required before being considered for a clinical trial. In addition, there is a higher risk for an acute immune response due to the massive amount of AAV required. While both exon skipping and trans-splicing approaches are promising, these approaches only target the mRNA and therefore do not correct the genetic defect per se.

4. Genome editing

Genome editing is the ultimate gene therapy approach aiming to correct the mutation at the genomic level on a permanent basis. The goal of genome editing, similar to exon skipping and trans-splicing, is to modify the genome of the cell of interest to remove the mutated exon(s) or exchange it with a mutation-free sequence. All these genome-editing tools work in a similar fashion. They are composed of a DNA-guiding molecule that locates and targets a nuclease to specific areas in the genome. Whenever the DNA guiding module finds a target, the nuclease cuts the DNA resulting in a DNA double-strand break (DSB) (Fig. 3C) triggering DNA repair mechanisms. Stimulation of these repair mechanisms using genome-editing tools could lead to either removal of the mutated exon(s) or its exchange with a mutation-free exon. Our focus in this review will mainly lie on the CRISPR/Cas9 system, but other genome editing tools exist and have also been used to treat *DMD* in cell lines and animal models. They have been engineered for efficient genome editing in various species, including zebrafish [78], mice [79], monkeys [80], and humans [81,82].

For example the zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), are two targeted nucleases. These two systems rely on DNA binding proteins that target the nuclease FokI to a particular region of the genome of interest. In 2015, ZFN were used to remove exon 51 from patient cells harboring a mutation in exon 51. These modified cells were then injected in *mdx* mice and dystrophin positive fibers were found following this treatment [83]. TALENs also successfully induced exon 51 or exon 44 removal in human DMD cells [84–86]. Unfortunately, despite their applications in pre-clinical research, ZFN and TALEN tools have numerous limitations, including the time consuming and experimental effort to design them and minimize the risk of off-target effects [87].

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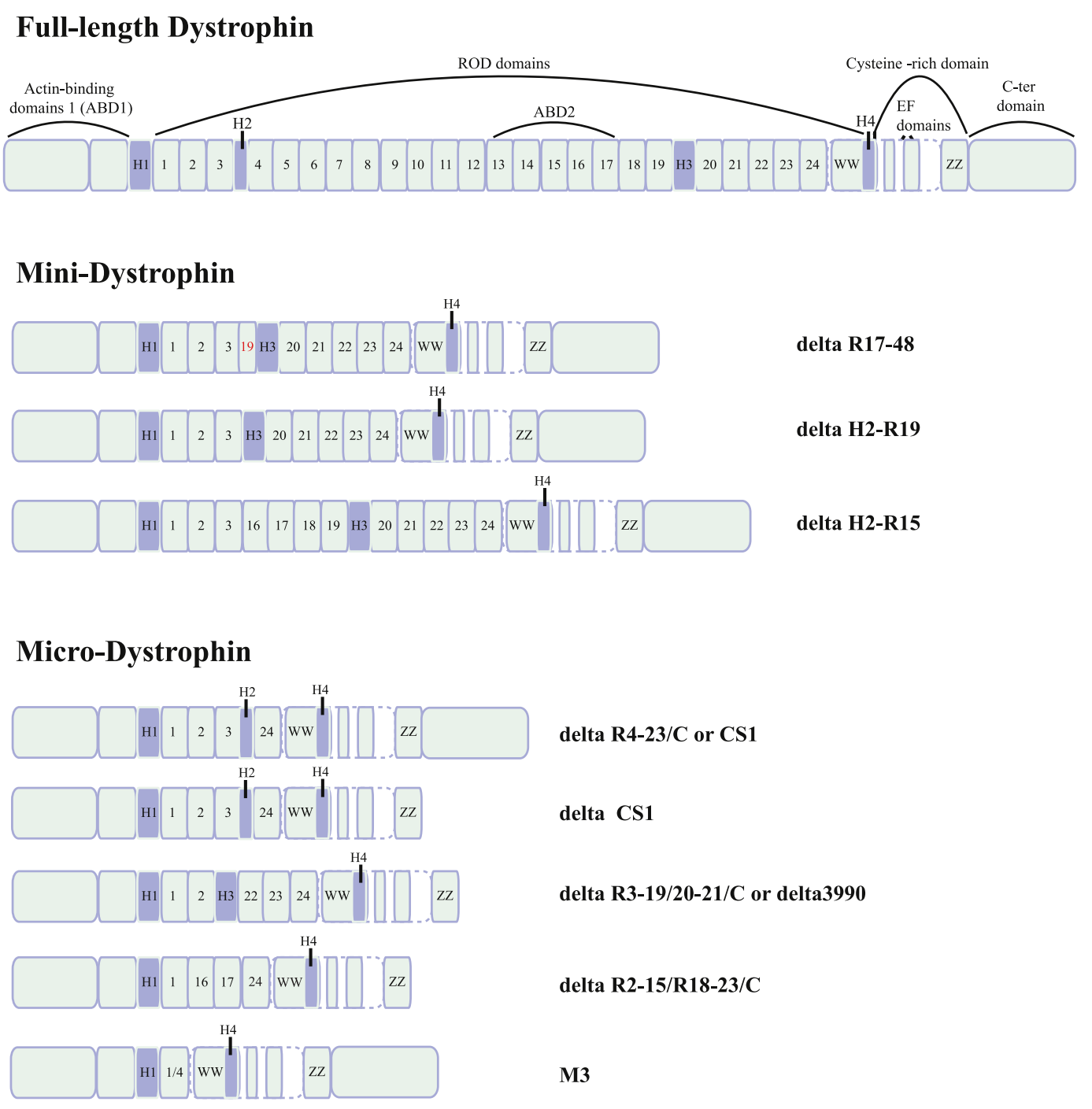

Fig. 2. The most promising Mini-/micro-dystrophins. Domains are identified as hinge domains (H); a domain presenting two tryptophan residues spaced 20–23 amino acids apart (WW), a cysteine-rich zinc-finger domain (ZZ), and a helix-loop-helix structural domain (EF); the different numbers indicate the different ROD domains. The rod domain 19 in the minidystrophin delta R17-48 is indicated in red as only the c-terminal part of the domain is present in the construct.

However in 2013, the first human application of a more powerful genome-editing tool was demonstrated: the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and its associated nuclease, CRISPR-associated system (Cas) [81,82]. *Streptococcus pyrogenes* Cas9 (SpCas9) nuclease, which has the ability to cut the DNA under specific conditions, has revolutionized the field of gene editing. The CRISPR/Cas9 system is brought to a specific genomic site by using a single guide RNA (sgRNA). If this 20-nucleotide sgRNA hybridizes DNA immediately preceding the canonical protospacer-associated motif (PAM) sequence 5′-NGG-3′, Cas9 cleaves both DNA strands resulting in a DSB [88] (Fig. 3C). In contrast to the site specific nucleases, ZFN and TALEN, the CRISPR/Cas9 technology is easy to develop since the experimental design is much more straight forward requiring only 20 nucleotides of se-

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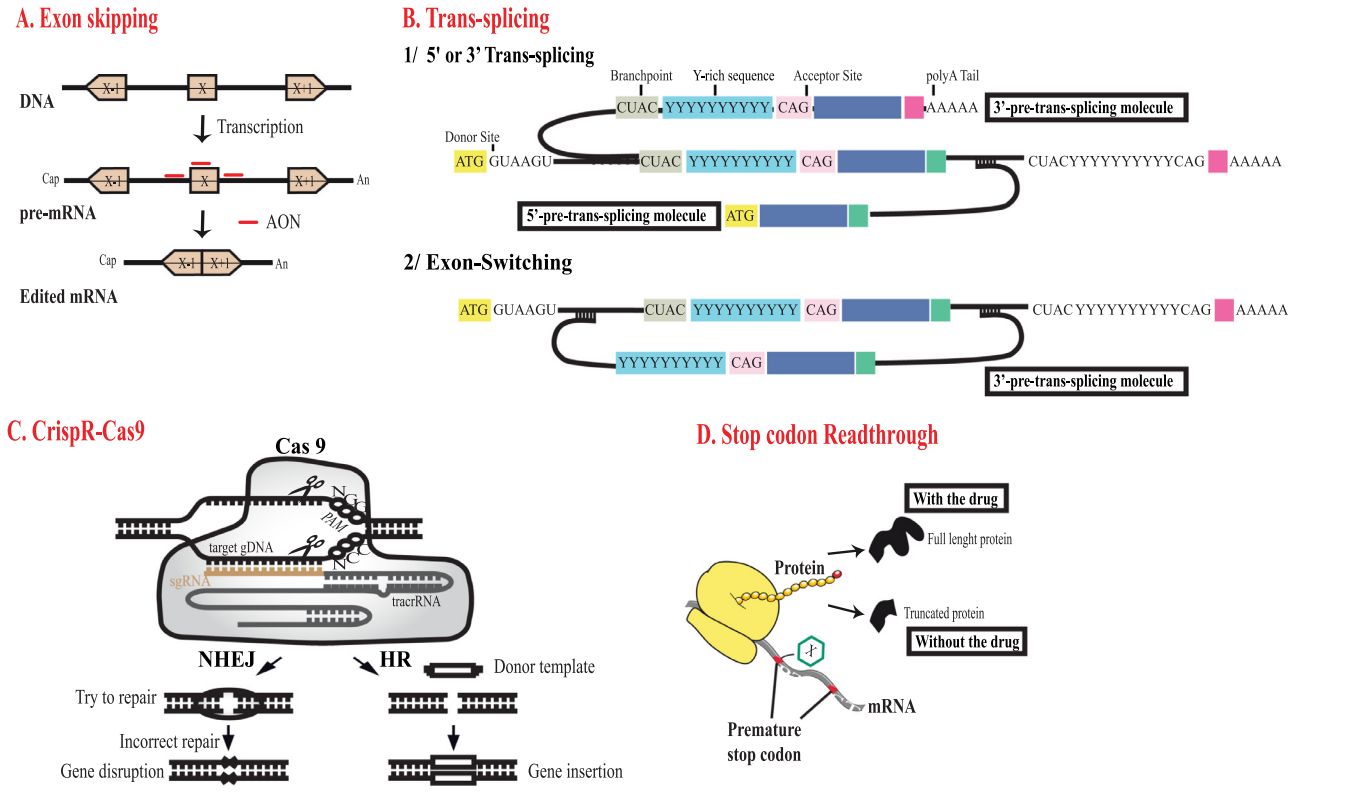

Fig. 3. Correction of the genetic abnormalities (A) Exon skipping strategies use an antisense molecule to hide one of the exon definition elements that are usually near the spliceosome (e.g., acceptor or donor site, branch point exonic splicing enhancer). This induces the spliceosome to not recognize the target exon resulting in the skipping exon. (B) Trans-splicing is generally used to correct the deleterious effect of a mutation on splicing. The entire sequence within the 5′(5′PTM) or 3′(3′PTM) of the chosen splicing site can be replaced. Thus, during the trans-splicing event, the corrected mRNA will be produced via different pre-mRNA, the native sequence, and the PTM. (C) Genome editing using CRISPR/Cas9. In this system, Cas9 nuclease is targeted to a specific genomic site by complexing with a single guide sgRNA, which hybridizes a 20-nucleotide DNA sequence immediately preceding a 5′-NGG-3′ (NGG) motif, introducing a double-strand break three nucleotides upstream of the NGG motif. These double strand breaks stimulate DNA repair mechanisms: homologous recombination (HR) and non-homologous DNA end joining (NHEJ). NHEJ is a prominent DNA repair pathway used by eukaryotic cells although it is error-prone (i.e., NHEJ can induce a small genomic deletion). (D) PTC readthrough this strategy apply only for mutation giving rise to a stop codon. Compounds such as Ataluren can bind the ribosome and the premature stop codon and replace the codon by another amino-acid resulting in expression of the full-length protein. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

quence complementary to the target region [89]. The mechanism of action of CRISPR/Cas nucleases is well characterized and they are essential for bacteria and archaebacteria adaptive immunity.

In 2013 a few studies raised concerns about the off-target mutation effects of the CRISPR/Cas9 system or immune responses to Cas proteins [90–92], however, more recent studies, using various genome wide sequencing techniques (reviewed here [93]), demonstrated that careful design of the sgRNA dramatically decreases off-target effects. Reduction of off-target raises the possibility of using this efficient and accurate tool for therapeutic intervention. Numerous papers have since demonstrated the in vitro and in vivo efficiency of this approach [84,94–103].

This genome-editing technology is especially promising due to its ability to mediate very large deletions. This is important for DMD/BMD patient, since creation of a genome editing tool that would remove exon 45 to 55 could benefit more than 60% of patients as this region of the gene is a well-defined hotspot for *DMD* mutations [95]. Young et al. successfully induced this 725 kb deletion of the *DMD* gene (from exon 44 to exon 55) in human induced pluripotent stem cells (iPSCs) derived from patients with DMD using CRISPR/Cas9 technology. Moreover, they successfully engrafted these corrected cells in the tibialis anterior of immunodeficient *mdx* mice between 5 and 7 weeks old (NSG-*mdx* mice) [95]. Lately another promising technology, CRISPR-gold, has been successfully used in *mdx* mice to promote dystrophin expression, ameliorate dystrophic phenotype and partially restore functionality [104]. This technology uses gold nanoparticles conjugated with DNA that are complexed with donor DNA, Cas9 RNP Cas9 ribonucleoprotein and the endosomal disruptive polymer poly(*N*-(*N*-(2-aminoethyl)−2-aminoethyl) aspartamide) (PAsp(DET)) (see Supplemental Table).

Recent efforts have also been made to identify new nucleases with more activities (sequence exchange instead of deletion), or a smaller size than the originally discovered spCas9 so the tool can be delivered via AAV [105]. Cpf1, a family of single-RNA-guided (crRNA) enzymes that recognize thymidine-rich PAM sequences and creates DSBs, is

among the most promising more specific enzymes discovered in 2016 [106,107]. Cpf1, which is expressed in several bacterial species, successfully ameliorated the pathophysiological hallmarks in *mdx* mice and also restored dystrophin expression in human-derived cardiomyocytes when targeting a *DMD* mutation [108]. While these genome-editing tools look promising, several concerns have been raised. There are safety questions about the constant expression of a nuclease if delivered via gene therapy vector and researchers are developing inducible systems to restrict expression. Finally, there are concerns with potential off-target cleavage of the nuclease and potential immune reactions due to the expression of the Cas9 bacterial proteins.

In summary, multiple promising gene therapy approaches for DMD are currently moving forward towards clinical trials (See Table 2 for a list of the current treatments in clinical trials and Supplemental Table for an overview of all the therapeutic strategies in the pipeline for DMD). Among these, exon skipping approaches targeting the *DMD* mRNA are the most frequent. More recently, CRISPR/CAS mediated genome editing strategies were developed that hold great promises to move towards clinic. Other gene therapies aiming to compensate for the consequences of the absence of dystrophin are also being explored (discussed in section compensatory approaches). While gene therapy bears great advantages such as long-term expression and high efficiency in muscle targeting, the drawbacks are risks associated with random integration of vectors into the genome, loss of gene expression over time or immune reactions to high vector doses. Therefore alternative approaches have been explored to mediate dystrophin expression (see hereafter alternative approaches to improve dystrophin protein levels).

Finally, one should note that ethical concerns have been raised regarding the application of this technology and the shifts (legislation, governments policy, emergence of new technologies etc) that may be encountered in a near future. Indeed, editing cells from the germ line will give raise to permanently modified embryos with unknown long-term consequences. The source of human germinal cells is also of concern in most of the countries around the world and is closely related to religious and cultural beliefs. The question of whether or not an early stage embryo is considered a human being that cannot be used for experiments is a large debate. Using iPSC or partially committed ES cells has been proposed to address such ethical question. However, ethical issues remain concerning the origin of the cell source. Autologous transplant is probably the easiest but depending on the age of the donor, the proliferation properties of the cells might be diminished.

5. Alternative approaches to improve dystrophin protein levels

5.1. Antisense oligonucleotide mediated exon skipping

Several chemical versions of AONs exist for exon-skipping including phosphorodiamidate morpholino oligomers (PMO), 2′-*O*-methyl-modified RNA, and tricycloDNA antisense. All of them have been used in vitro and in vivo in mouse and dog models and demonstrated efficient skipping that resulted in force and histology improvements (for recent review see [109] and Supplemental Table)). They also have shown promising results in phase II clinical trials [110–113].

Among them, Eteplirsen (Sarepta Therapeutics, Cambridge, MA), a PMO that targets the exon 51 of the *DMD* gene, demonstrated efficacy in production of low levels of dystrophin protein and some therapeutic potential in clinical trials (NCT01396239, NCT01540409, NCT02255552). In 2016, Eteplirsen obtained an accelerated approval from the FDA and is currently being reviewed for a European market authorization [114]. However, there were large disparities observed regarding the level of dystrophin protein produced between patients in ongoing clinical trials, suggesting that these drugs remain suboptimal [110,111]. Eteplirsen is still under evaluation for a subpopulation of patients in advanced stages of DMD (NCT02286947) and the evaluation of results is still pending for evaluation of this drug at the early stage of DMD (NCT02420379) or in young patients (NCT03218995)(see Table 2 and Supplemental Table).

Drisapersen, a 2′-O-Methyl-phosphorothioate AON, has been developed and tested by Prosensa to promote exon 51 skipping. The phase I and II clinical trials were promising with a dose-dependent increase in dystrophin expression and in the 6MWT. However, the later seemed to be less evident during the phase IIb extension phase [115]. Few advisory effects were also noted (mainly mild proteinuria, injection site reactions) although the phase III was engaged and later withdrawn based on the fact that the primary endpoint (improvement in the 6MWT) wasn't reached at the tested dose of 6 mg/kg/week [116]. This event leads to the denial of accelerated approval by the FDA. Finally to test safety, tolerability and pharmacokinetics of Drisapersen following a single subcutaneous injection of 3, 6 or 9 mg/kg in non-ambulant boys, a new study showed severe inflammation signs [117]. Taken together, both studies confirmed that the therapeutic window of this drug couldn't be achieved without safety concerns [118] (see Supplemental Table).

In an effort to maximize the benefits form antisense oligonucleotides, several approaches have been envisages (multi-exon skipping, enhanced delivery via peptide conjugation or nanoparticles delivery).

Multi-exon skipping approaches, which allow the skipping of several adjacent exons at once, are also currently being tested for DMD, enlarging the spectrum of eligible mutations [119]. The induction of multi-exon skipping is based on the use of a cocktail of antisense oligonucleotides to target several adjacent exons (two or more) within a given region. This approach is mostly considered for the main mutational hotspot (encompassing exon 45 to exon 55) [120]. However, the skipping of this region is extremely difficult to realize due to the large size of the introns [121].

One promising approach to increase the cellular uptake of the AON is based on their conjugation to arginine-rich CPPs

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(cell-penetrating peptides). This PMO-based AON are called PPMO. PPMOs were safely tested in *mdx* mice and allow a more persistent and efficient skipping in the tested tissues including heart and diaphragm [122,123]. Recently a systemic injection of PPMO for a combined exon 6 and 8 exon skipping in the canine X-linked muscular dystrophy model of DMD was conducted and showed an improvement of cardiac functions due to a dystrophin rescue in the heart [124]. A phase I clinical trial of SRP-5051-PPMO (Sarepta Therapeutics, Cambridge, MA) for exon 51 skipping is planned to start enrolling soon (see Supplemental Table).

The use of nanoparticles delivery to favor the uptake of nucleic acids was also tested since these particles can help with the targeting and/or penetration. In addition, they also confer a higher stability or protection against immune reaction. Among them, polymethyl methacrylate (PMMA) nanoparticles, that comprises two subgroups distinguished by their sizes (420 nM for T1 and 130 nm for ZM2), are very promising regarding their high transduction efficiency. Despite the broad biodistribution, T1 was shown to have a poor biodegradability and also to be able to form unwanted aggregates [125]. In the same manner, ZMN2 were also able to induce dystrophin expression in skeletal muscle and heart at lower doses than with 2′OMePS AON [126]. While promising, more studies are needed to understand the pharmacokinetics, biodisponibility and biodegradability of these new delivery systems (peptide-conjugated PMO and nanoparticles associated 2′OMePS) before envisaging applications in clinical settings.

Despite these single or multi-exon skipping approaches being attractive, AONs do not efficiently transduce all muscle, and repeated injections are required since these AONs are not constantly expressed. As a result, repeated injections required frequent hospital visits for the boys and their families.

5.2. Stop codon read-through

Around 15% of boys with DMD carry nonsense mutations that induce a premature termination codon (PTC) on the mRNA. The occurrence of a PTC can lead to ablation of protein translation and protein degradation, or release of a truncated non-functional protein [127]. Stop codon read-through approaches are based on the use of molecules that bind the ribosome and its partners leading to the transformation of the PTC into a different amino acid, thereby resulting in the continuation of the translation (Fig. 3D) [127].

Several drugs can induce stop codon read-through, and their efficiencies rely on the nature of the nonsense mutation and the surrounding nucleotide sequences [127]. These drugs can be divided into two main families: the aminoglycoside antibiotics (e.g., gentamycin) or chemical compounds without known analogues (e.g., ataluren or negamycin) [127]. Although gentamycin showed efficiency to induce stop codon read-through for several pathologies like cystic fibrosis [128] or DMD [129–133], nephrotoxic and ototoxic properties keep gentamycin from clinical consideration [134–136]. Negamycin, was demonstrated to be more efficient than gentamycin to induce the stop codon read-through in DMD cell lines visualized by a restoration of dystrophin expression in vitro and in vivo in *mdx* [137].

In 2007, PTC therapeutics (South Plainfield, NJ) screened 800,000 compounds for the ability to read-through premature nucleotide triplet "UGA" stop codons. Using high throughput screening, they discovered a molecule, ataluren, able to induce stop codon read-through and demonstrated its efficacy in vivo using the mdx mouse model [138]. Other laboratories failed to replicate this finding, questioning the specificity of Ataluren against stop codon and suggesting effects attributed to stop codon read-through in the original assays could instead be biased by a direct effect of ataluren on the firefly luciferase reporter used in these experiments [139,140]. Nevertheless, supported by evidence for functional improvement in mouse models of nonsense mutation-associated cystic fibrosis [141] and in a cell model of Hurler syndrome [142]. Ataluren entered clinical trials, and has received conditional approval in the European market for DMD. Although Ataluren can recognize any STOP codon, its efficiency decreased as follows: UGA > UAG > UAA [138]. Despite showing a good oral bio-distribution and a good tolerance, Ataluren has recently failed to reach its primary and secondary endpoints for cystic fibrosis in a phase III clinical trial for severe forms (NCT02139306). In addition, a phase 2b trial demonstrated, no significant improvement for DMD patients in the 6-minute walk test [112,143]. However, recent publication from the clinical phase III (NCT02090959, NCT01826487) results did show modest effects in a pre-specified subgroup of patients with a baseline between 300 m and 400 m for the six-minute walk test [144]. Based on these promising results, a new phase III trial is currently enrolling patients to measure the long-term outcomes in a 72 week phase followed by a 72 week open-labeled phase (NCT03179631). One hypothesis for this lack of efficiency is that this strategy relies on the need to produce an mRNA sufficiently stable to resist nonsense-mediated mRNA decay (NMD) [145], a process triggered by the presence of a premature STOP codon, which leads to mRNA degradation. Translarna™ (ataluren) is not able to abolish the NMD of newly synthetized mRNA or endogenous mRNA [138]. Translarna™/Ataluren has received a New Drug Application from the FDA in October 2017, but received a marketing authorization in Europe in 2014 (see Supplemental Table). A second-generation read-through compound, RTC13, also aiming to bypass nonsense mutations, has successfully restored functional dystrophin in muscles of *mdx* mice without any major drawbacks, paving the way for a phase I clinical trial [146] (see Supplemental Table).

6. Compensatory approaches

Some boys with DMD will not be eligible for genetic correction of their cells/DNA because of the rarity of their mutation or due to technical limitations. However, other approach can be imagined to compensate for the lack of dystrophin and/or deal with its consequence. For example, expression

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of a highly homologous protein could partially compensate for the absence of a protein. The major advantage of this approach is its benefit to all boys regardless of their mutation. In addition, this approach would avoid an immunological reaction since the protein is naturally expressed in hosts. Another advantage is that this technique doesn't necessarily require an extra copy of the transcript, since pharmacological drugs could stimulate the promoter of the targeted protein. Nevertheless, the non-toxicity of the given protein overexpression in the targeted tissue must first be verified [147].

6.1. Utrophin

Utrophin is a well-known homologue of dystrophin (80% amino acid homology with dystrophin in humans) despite its lack of an nNOS binding site. Utrophin, which is mainly expressed in adult muscle at the neuromuscular and myotendinous junctions, is also expressed at the sarcolemma of emerging and regenerating muscle fibers [148,149]. Evidence from several groups demonstrated that utrophin could substitute for dystrophin. For example overexpression of utrophin in *mdx* mice can correct the disease and was able to reform and relocalize the DGC to the sarcolemma, which is essential for muscle fiber integrity [150–152]. Similarly, it was found in DMD boys that the phenotype severity was milder in boys with higher utrophin expression [153]. Several extensive screenings and testing for molecules that increase utrophin expression were performed, however, only SMT C1100/Ezutromid (Summit Therapeutics, Abingdon, Oxfordshire, OX) showed success in a phase I clinical trial [154]. SMT C1100 was well tolerated at all doses, but with some variation in uptake, possibly due to the administration method [154]. This compound is now in phase II investigation and a significant reduction of muscle damage after 24 weeks of treatment has been reported (NCT02858362) (see Table 2 and Supplemental Table). In 2015, a second-generation drug, SMT022357, led to increased utrophin expression in skeletal, respiratory and cardiac muscles in mice [155] and a clinical trial is expected to be launched soon. Alternatively, upregulation of utrophin could potentially also be achieved via microRNA modulation. Among these, miR-206, let-7c, miR-150, miR-196b, miR-296-5p, and miR-133b can promote utrophin expression [156,157]. So far, the clinical relevance of miRNA regulation for DMD treatment has yet to be proven since very little is known about their other targets and thus, off-target effects are highly likely.

6.2. Members of the dystrophin glycoprotein complex

Overexpression of other DGC components is also being investigated as a potential therapeutic strategy for DMD. Biglycan, an extracellular matrix proteoglycan linked to the DGC, was used to upregulate the targeting of neuronal nitric oxide synthase (nNOS) and utrophin at the sarcolemma in *mdx* mice [158]. The use of a recombinant humanized non-glycanated biglycan is under investigation by pharmaceutical group Tivorsan (Providence, RI) who is about to launch a human phase I clinical trial [159] (see Supplemental Table). Laminin 1 (or laminin 111) is an isoform of laminin mainly expressed during the early embryonic stage [160]. It allows a better expression of utrophin and integrin at the sarcolemma via an increase of α7-integrin expression, which restores the muscle adhesion in congenital muscular dystrophies and DMD mouse models [161–163] (Supplemental Figure and Supplemental Table). The pharmaceutical group Prothelia, Inc. (Milford, MA) is developing a recombinant protein for therapeutic use in DMD with an expected start of enrollment in 2020 (see Supplemental Table).

6.3. Other muscle-related proteins

In DMD, several other genes/proteins, referred as modifiers (e.g., *SPP1* encoding osteopontin, *Galgt2*, etc.) can influence disease severity (Supplemental Figure) and are therefore evaluated for their therapeutic use since they could substitute certain functions for the missing dystrophin. For example, osteopontin also known as secreted phosphoprotein 1 (SPP1) does affect the disease progression. The polymorphism rs28357094 in the gene promoter of *SPP1* (G allele) is associated with a more rapid progression and less grip strength in human [164]. Ablation of this protein which is mainly involved in inflammatory processes and driving fibrosis [165] has been shown to promote muscle integrity via macrophage secretion of pro-myogenic growth factors like IGF1 in *mdx* mice [166].

Galgt2 ($\beta-1$, 4-N-acetylgalactosamine galactosyltransferase) is a glycosyltransferase mainly localized at the neuromuscular junction essential for its stability [167]. This protein also has a role in the maturation of the DGC proteins (notably α-dystroglycan). Galgt2 was shown to prevent injury caused by eccentric contractions in *mdx* mice [168]. This molecule is currently used in a phase I clinical trial using AAV delivery (NCT02704325) (see Table 2 and Supplemental Table).

Follistatin is a natural antagonist of several members of the TGF-beta family including myostatin, which when inhibited result in enlarged muscle. AAV delivery of follistatin (rAAV1.CMV.huFollistatin344) showed promising results in *mdx* mice and notably an increase of muscle force [169,170]. This approach is currently in phase I clinical trials for BMD (NCT01519349) but promising initial results (notably a reduction of fibrosis and centronucleation) were also obtained for DMD [171] (see Table 2 and Supplemental Table). Similarly, BMS-986,089 (Bristol-Myers Squibb/Roche, New York, NY), a myostatin inhibitor is currently in phase II/III clinical trial (NCT03039686) after promising preclinical data showing increase of muscle mass in *mdx* mice [172] and no severe adversary effect in phase I (see Table 2 and Supplemental Table). Domagrozumab/PF-06,252,616 (Pfizer, New York, NY), another myostatin inhibitor, is currently in phase II clinical trial with an expected completion date in April 2018. During preclinical trial, it has induced muscle anabolic activity in mdx and non-human primates and was safely tested in phase I with few adversary effects (see Table 2 and Supplemental Table).

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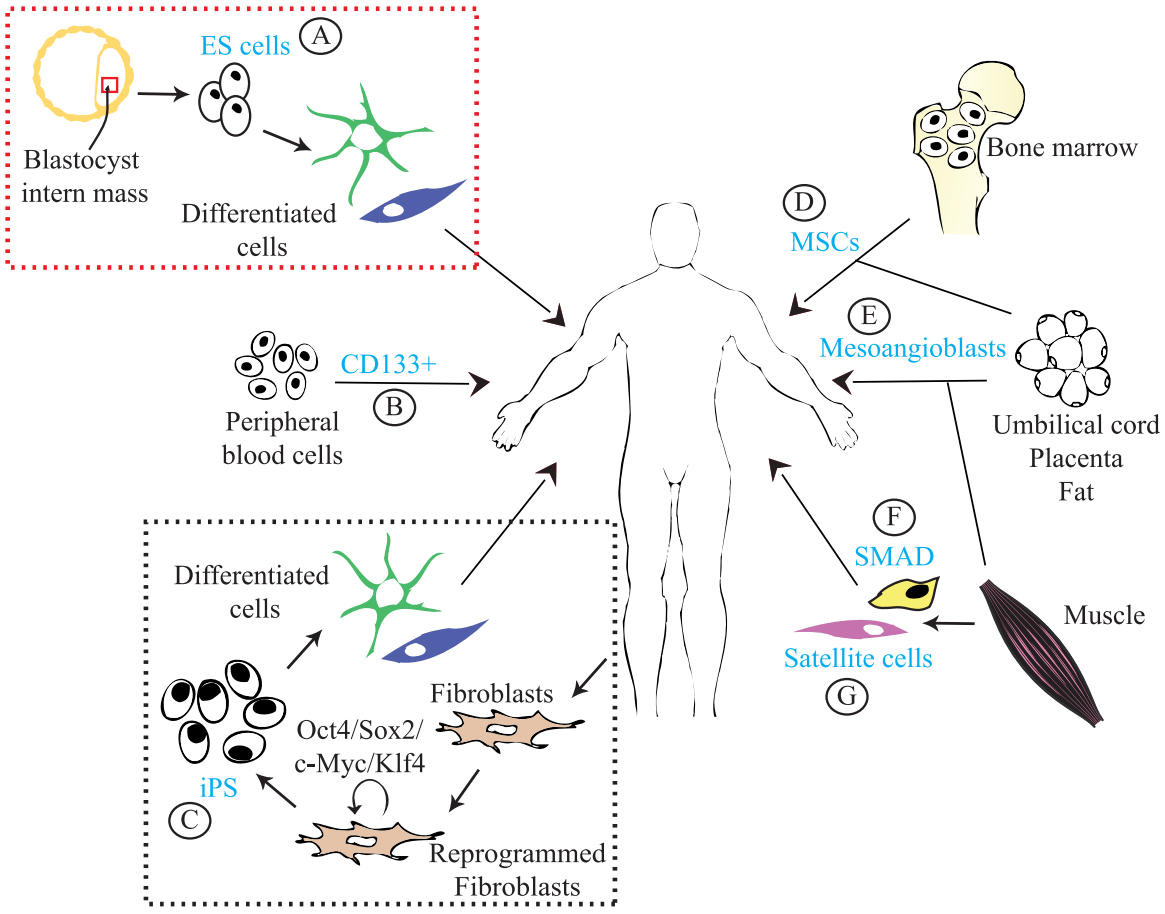

Fig. 4. Cell therapy strategies for Duchenne Muscular Dystrophies. Cell therapies under investigation for Dystrophinopathies. (A) Embryonic stem cells (ES cells) coming from the 5/6 days blastocyst, which have the ability to differentiate into all kind of cells; (B) CD133+ (coming from peripheral blood); (C) Induced pluripotent stem cells (iPS), which are cells such as somatic cells (e.g., fibroblasts) that will be de-differentiate into pluripotent cells by a cocktail of factors (e.g., oct4/sox2/c-Myc/Klf4). Several other approaches are based on the use of differentiated cells coming from healthy donors like; (D) MSCs (mesanchymal stem cells) coming from different kinds of tissue; (E) mesoangioblasts; (F) SMAD (skeletal muscle aldehyde dehydrogenase-positive cells); (G) muscle satellite cells.

7. Cell therapy

Cell therapy approaches aim to provide new cells to patients to compensate for their deteriorated cells in the targeted tissue. Cells can be from a donor or from the patient himself. Both approaches have advantages and disadvantages. While cells from the donor are mutation free; an immune reaction is a risk. Autologous cells have lower risk of causing an immune reaction; however, the cells would need to be repaired prior to reinjection. Currently investigated cell therapy approaches for humans can be classified into three groups according to the nature of techniques and/or cells used: (i) partially or fully differentiated cells, (ii) embryonic stem cells (ES cells), and (iii) induced pluripotent stem cells (iPS) (Fig. 4).

7.1. Partially or fully differentiated cells

This group includes muscle satellite cells; the SMAD (skeletal muscle aldehyde dehydrogenase-positive) cells; mesenchymal stem cells (MSC) and mesoangioblasts; and CD133+cells (from peripheral blood).

7.2. Muscle satellite cells and myoblasts

Muscle satellite stem cells are precursors to muscle cells. They represent a niche needed to allow muscle growth after-birth and injuries resealing [173]. These cells are located underneath the basal lamina on top of the sarcolemma [174]. They were first used for preclinical assays 30 years ago when Dr. Partridge's team injected satellite cells in *mdx* mouse muscle, and the deficient dystrophin fibers were able to re-express dystrophin after cell injection [175]. Although further studies were conducted on GRMD dogs, clinical trials in humans were not successful. Failure was mainly due to the short lifespan of the injected cells, their lack of dissemination within the tissue, or rejection by the host [176,177]. The main shortcoming of this approach is the inability of the cells to cross the endothelium, which prevents their systemic injection [178] (see Supplemental Table). However, a new clinical trial is ongoing for myoblast transplantation in DMD patients (NCT02196467), where they will transfer mature myoblasts instead of stem cells and hoping to get a sustained lifetime

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of transferred cells within the host (see Table 2 and Supplemental Table).

7.3. Skeletal muscle aldehyde dehydrogenase positive cells

Another type of muscle progenitors used for therapeutic trials are skeletal muscle cells positive for aldehyde dehydrogenase (SMAD cells), which are a small fraction (2–4%) of the mononucleated muscle cells [179]. These cells, once injected into muscles, can contribute to both the formation and regeneration of muscle [179]. The principal drawback of muscle progenitors is their very low abundance and thus the difficulty to obtain enough cells for transplantation (see Supplemental Table).

7.4. Mesenchymal stem cells

Alternatives to these muscle progenitors exist. The most studied ones for muscular dystrophy and DMD in particular, are cells found in peripheral tissues (e.g., blood, synovial membrane, umbilical cord). Mesenchymal stem cells (MSC) and Mesoangioblasts are the most promising, since they can be isolated from different tissues including muscle and adipose tissue. Both are of special interest for DMD as they can restore the expression of dystrophin in different models and improve the functionality in human cells [180–184], mice [185–192] and the GRMD dog model [193]. MSC have been tested in humans with no adverse effects and a transitory gain of function of 12 weeks [193] (see Supplemental Table).

7.5. CD133+

Rare cells like the CD133 + cells (0.06% in adult peripheral blood) could be valuable [194]. It was demonstrated that CD133 + cells were able to fuse in vitro with the C2C12 myoblast cell line and with severe combined immunodeficiency (scid)/*mdx* mouse fibers allowing dystrophin expression [195]. CD133 + cells have also been deemed safe to use in boys with DMD [196]. These multipotent cells, although promising, still require many steps of development to better understand their characteristics. In the meantime, researchers have developed tools for the differentiation of pluripotent cells such as embryonic stem cells into different kinds of cell lineages to facilitate muscle progenitor production (see Supplemental Table).

7.6. Embryonic stem cells

While there are only few studies using ES cells in a muscle context, the formation of multipotent mesenchymal precursors demonstrates their potential for commitment to the myogenic pathway [197]. Although promising, usage of these cells is still debated due to their high teratogenic potential [198] and their potential immunoreactivity in allogeneic transfer [199] (see Supplemental Table).

7.7. Induced pluripotent stem cells

Among these cells, it is necessary to distinguish between embryonic stem cells (ES) (i.e., cells at an undifferentiated stage early during embryogenesis) and the induced pluripotent stem cells (iPS) arising from somatic cells that can be reprogrammed to a prior commitment stage using reprogramming factors [200–202]. In contrast to ES cells, iPS cells acquire their pluripotency by genetic reprogramming using viral vectors, or DNA or RNA transfection, expressing a well-defined cocktail of transcription factors (for review [203]). Similar to embryonic stem cells, these cells can be differentiated into muscle progenitor cells. Currently, iPS cells are mostly used for molecular screenings, but the increasing numbers of new studies indicate their therapeutic potential [204], including patients suffering from DMD [95,205,206] (see Supplemental Table).

In summary, cell replacement holds great potential for treatment of neuromuscular disorders including DMD. Several clinical trials are currently ongoing. Challenges are the limited number of cells available, low migration ability of the cells, tumor formation due to mis-differentiation, and immune responses against donor cells. To overcome some of the limitations, *ex vivo* therapy - an approach that combines cell and gene therapy – could become an attractive solution.

8. From xenograft to ex-vivo therapy?

The *ex vivo* strategy begins with obtaining cells from the patient followed by reprogramming/correction of the defect in vitro with a virus carrying the therapeutic gene prior to re-administration to the patient (Fig. 5). This promising technique combining cell therapy (direct isolation of cells from patient or donor) and gene therapy (cell reprogramming) is under investigation for numerous diseases, especially for hematological disorders (for review see [207]). Exon skipping mediated therapy was recently performed on CD133+ cells from DMD patients and cells were injected into scid/*mdx* mice. Following injection of these corrected cells, significant dystrophin expression as well as improvement of muscle morphology and function was identified [208].

Similar approaches were performed using either autologous muscle precursor cells infected with lentiviruses encoding the micro-dystrophin and then injected in the muscle fibers of monkeys and mdx mice [209]; or freshly isolated satellite cells infected with micro-dystrophin and then injected in *mdx* mice [210]. Recently two other approaches have been evaluated. Filareto and colleagues introduced micro-utrophin into iPS cells from DMD patients. This technique showed promising results (e.g., overexpression of utrophin, DGC formation, better resistance to membrane rupture, better contractility of muscle fibers in *mdx* mice) [211]. In 2015 several labs corrected dystrophin mutations with CRISPR/Cas9 resulting in a partial restoration of the phenotype in *mdx* mice after cell transplantation [84,94,100,212].

Altogether, *ex vivo* therapy presents numerous advantages in comparison to conventional gene therapy techniques since

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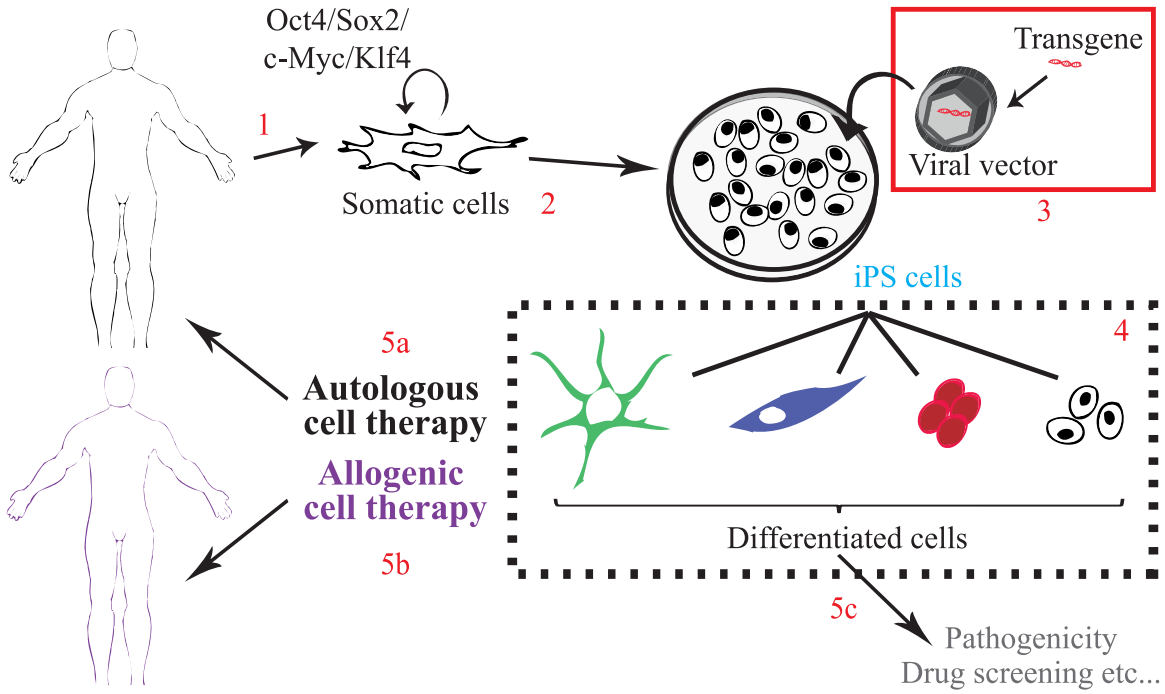

Fig. 5. Ex vivo strategy. 1) Collect patient's somatic cells (e.g., fibroblasts); 2) Convert into induced pluripotent stem (iPS) cells; 3) Correct the genetic defect using virus or gene editing approach; 4) Differentiate the iPS into progenitors; and finally, 5) Re-administer the modified cells to either 5a) the donor (autologous cell therapy) or 5b) another patient (allogeneic); or 5c) Use for drug screening, etc.

cells needing modification can be directly targeted, and a better control of the number of vector integrations into the genome as well as differentiation status of the cells prior to re-injection, results in lower tumorigenic risks and decreased toxicity. Nevertheless, some important limitations remain including the difficulty of reaching certain tissues or organs due to poor cell dispersion after the injection.

9. Financial and ethical aspects

With all these potential therapies, the scientific community and patient organizations are now more than ever confronted with the question regarding personalized medicine for genetic diseases. These treatments are often very expensive (hundreds of thousands of dollars per year –see Supplemental Table) and are therefore almost impossible to afford for most of the families. One frequent question patient organizations have is why is the cost of these therapies so high and how can it be justified to invest in a uncertain program that might prolong a patient's life, or might only address certain symptomes of the disease? Unfortunately, these treatments are intended for rare population(s) with therefore a low return on investment especially with the current costs of development, production and investments per patient over the years. One way to decrease the price of such therapy is to open their applications to multiple pathologies or widen their applications to different subpopulations of patients (status of ambulation, severity of the disease, age etc…). This could be achieved if we no longer consider one particular formulation as a treatment but if we consider that each individual piece that composed the drug could be re-used for another disease. For example, in the case of exon skipping, the development of the technology was used on a specific exon and disease but the experience gained and techniques used could in fact be helpful for other *DMD* exons and even other diseases. Therefore when considered investing in a program, one should see the long term consequence of that investment. In case of exon skipping, future programs will greatly benefit from prior examples and thus reduce the cost and time required for to generate proof of concept results that can support clinical trials.

In addition of cost, inclusion criteria for clinical trial are also often a debate. The efficacy of some treatments may differ depending on the clinical severity of the patient and on the disease progression rate. To ensure success of a clinical trial, inclusion criteria need to be carefully designed. Enrolling patients that cannot respond properly to the treatment reduces the success of a clinical trial and might slow down or even prohibit a promising new drug on the path to FDA approval. For instance, in order to improve muscles features, enough muscle mass should still be available. Thus younger patient populations are preferably targeted. In addition to the considerations that need to be made by the investigators, the families also face difficult decisions. It is hard for the patients' family to enroll their kids in clinical trials that bear a substantial risks and have an unknown outcome at an age where they still perform well and have a good quality of life. Older patients tend to be more willing to be enrolled in such exploratory clinical trials but this could be detrimental to the success of the clinical trial depending on the mechanisms the drug intends to target. There is no perfect answer to these

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questions and we should keep in mind that the influence of lobbyists and big pharmaceutical groups should not be underestimated in these discussions and decisions. In summary, clinicians and researchers should carefully listen to the need of patients and families when they design their clinical trial in order to address such debate.

10. Concluding remarks and future perspectives: combination therapy for a personalized approach?

While many potential therapies are being evaluated for DMD, the likelihood of one treatment to reverse/cure all pathological features for all boys with DMD is small. We expect various combinations of molecules or strategies will be necessary depending on the type of mutation and the disease severity. For example, despite data from recent exon skipping clinical trials being promising, the levels of protein produced following treatment, are suboptimal and vary between treated boys [111,213]. Therefore, even modest enhancement of efficacy or stabilization of the protein should enhance therapeutic efficacy. The use of small compounds to boost exon-skipping strategies is currently under investigation and shows some promising results. One of the molecules used for this is dantrolene, an already FDA approved drug that targets the Ryanodine receptor/Calmodulin pathway [214,215]. This compound enhances exon 51 skipping in patient cells when used in combination with phosphorodiamidate morpholino oligomers. Dantrolene also demonstrates a potential synergistic effect with AONs in the *mdx* mice that resulted in a phenotypic amelioration [216]. Likewise, the combination of micro-dystrophin (carried by AAV) and follistatin might be beneficial since follistatin increases muscle mass, thereby increasing the chance for a better uptake of the AAV as demonstrated in *mdx* mice [217]. Alternatively, the uptake of AAV-micro-dystrophin can also be increased when co-injected with insulin growth factor 1 [218] (Supplemental Figure).

To conclude, as the panel of available tools continues to increase and promising advancements in the DMD field are being achieved, both researchers and patients have good reason to be hopeful and optimistic. Moreover, multiple approaches described here are currently being explored for other rare muscular dystrophies like the Limb Girdle Muscular Dystrophies. Again, a one-size-fits-all treatment of all DMD cases does not seem likely. However, the increase in the number of patient registries and the ability to link natural histories greatly enhances opportunities to examine inter-individual differences and evaluate disease progression/regression. These opportunities will greatly facilitate the emerging field of personalized/combinatory therapies, which represent the future of effective DMD treatments. This will set the stage for other rare disease therapeutics.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.nmd.2018.06.009.

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