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Gene and splicing therapies for neuromuscular diseases

Rachid Benchaouir[1], Valerie Robin[1], Aurelie Goyenvalle[1]

[1]Laboratory Biotherapies des Maladies Neuromusculaires, INSERM U1179, UFR des sciences de la sante Simone Veil, Universite de Versailles Saint Quentin, France

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1. ABSTRACT

Neuromuscular disorders (NMD) are heterogeneous group of genetic diseases characterized by muscle weakness and wasting. Duchenne Muscular dystrophy (DMD) and Spinal muscular atrophy (SMA) are two of the most common and severe forms in humans and although the molecular mechanisms of these diseases have been extensively investigated, there is currently no effective treatment. However, new gene-based therapies have recently emerged with particular noted advances in using conventional gene replacement strategies and RNA-based technology. Whilst proof of principle have been demonstrated in animal models, several clinical trials have recently been undertaken to investigate the feasibility of these strategies in patients. In particular, antisense mediated exon skipping has shown encouraging results and hold promise for the treatment of dystrophic muscle. In this review, we summarize the recent progress of therapeutic approaches to neuromuscular diseases, with an emphasis on gene therapy and splicing modulation for DMD and SMA, focusing on the advantages offered by these technologies but also their challenges.

2. INTRODUCTION

Neuromuscular disorders (NMD) refer to a group of diseases frequently inherited and characterized by progressive muscle weakness, loss of ambulation, chronic disability and early death worldwide. In Europe alone, it is estimated that 300,000 people suffer from NMDs (1). At present there is no effective therapy to stop the progressive loss of muscle and/or function and only palliative treatments exist, although several promising experimental strategies are currently under investigation. These include gene therapy aiming at reintroducing a functional recombinant version of the mutated gene using adeno-associated, lentiviral or adenoviral vectors, as well as RNA-based strategies using antisense oligonucleotides (AONs). Both strategies hold great promises with several clinical trials on going, in particular for Duchenne Muscular Dystrophy (DMD) and Spinal Muscular Atrophy (SMA). Challenges for NMDs treatment are particularly important because of the tissues involved: approximately 30-40 % of the human body consists of muscles, and delivery to the central nervous system is impeded by the blood brain barrier. The molecular tools developed for each disease are the same (being viral vectors or AONs) but they are tailored in different ways for the different NMDs.

In this review, we will focus on the main advances in gene therapy and RNA-based approaches for the treatment of NMDs. While the majority of them have focused on DMD or SMA, the basic principles and strategies summarized here are broadly applicable to many neuromuscular disorders. For both therapeutic strategies, we will present the different molecular tools currently used, the proof-of-concept results and clinical data already available, and finally discuss the limitations of each approach and promises they hold.

3. GENE THERAPY FOR NMDS

3.1. Generalities on gene transfer approaches

Gene therapy can be defined as a means to deliver DNA into cells in order to correct genetic disorder. Four major parameters have to be considered for this approach: (i) the nature of the therapeutic DNA construction, called the transgene, and its associated regulatory elements, (ii) the type of molecular tool able to deliver this transgene into the targeted cells, (iii) the mode of administration (*in vivo* or *ex vivo*), and (iv) the technological process giving an appropriate quantity to ensure high quality of the final therapeutic product.

3.1.1. Nature of the therapeutic DNA (or transgene)

The most common form of transgenes used for gene therapy approaches is a DNA coding sequence (cDNA) from a native gene, constructed and designed

to be transcribed and then translated into a protein to compensate the deleterious effect of the mutated form directly or indirectly responsible for the genetic disorder. The transgene cDNA can be designed to express a cytotoxic protein able to kill proliferative cancer cells for example (2). It can also be used to reinforce a metabolic state or to promote differentiation of a defined cell type towards another one as the capacity of the myogenic factor MyoD to push fibroblasts to differentiate into muscle cells (3). In all cases, the transgene expression can be controlled by different regulatory sequences as strong viral, tissue specific or constitutive promoters. Other kinds of DNA sequences, cloned under specific promoters and designed to have therapeutic role, are frequently embedded in the same type of vectors (see below) used to deliver classical transgenes. Among them, we can mention the micro-RNA (miRNA), small-interfering RNA (siRNA) or some antisense constructions. However, although this is a matter of debate, these constructions are generally not considered as "gene therapy" per se and some of these strategies will be largely discussed in the second part of this review. Gene therapy approaches for NMDs (especially DMD and SMA) will be essentially focused on strategies using cDNA sequences as therapeutic transgenes.

3.1.2. Type of vector for DNA delivery

Concerning the delivery of DNA into the targeted cells, two kinds of molecular tools can be used: viral or non-viral vectors. Several virus families are currently tested in gene therapy protocols: the most represented belong to Adenoviridae, Parvoviridae, Retroviridae, Herpesviridae, Baculoviridae and Poxviridae families. Viruses represent the most efficient gene transfer tool in living world due to their natural ability to infect organisms. They differ from each other by different characteristics such as nature and length of their genetic material (DNA or RNA), presence or absence of lipid envelope, and capacity to integrate or not their DNA into the cell genome (integration property especially dedicated to Retroviridae family). The morphological characteristics of viruses define another important property: their tissue tropism. This parameter has to be taken into account for the design of tissue specific gene therapy approach. The non-viral vectors, on the other hand, contain large group of compounds able to coat the DNA of interest to protect it against physical, chemical or enzymatic degradation into cells or extracellular fluids. Indeed, initial strategies using naked DNA alone for gene transfer have shown serious limitations in term of transfection efficiency and transgene expression, particularly after *in vivo* delivery (4). A broad range of synthetic compounds are known to vectorize DNA molecules (5). Among these synthetic vectors, lipoplexes (cationic lipids), polyplexes (cationic polymers as polyethylenimine), dendrimers and inorganic nanoparticles (calcium phosphates, silica, etc…) are the most widely used. Nevertheless, the harmlessness of these non-viral vectors is still investigated since many compounds present cytotoxicity and inflammatory responses after *in vivo* delivery. Moreover, their efficiency for the treatment of NMDs has not yet been rigorously evaluated so we will not detail their mechanism in this review.

3.1.3. Modes of vector administration

The mode of administration is the other parameter that has to be clearly defined to increase the chance of gene therapy success. Generally two modes are distinguished: *in vivo* and *ex vivo* delivery. The *in vivo* approach allows injection of vector directly into the organism to be treated. Parenteral injection is usually performed for gene therapy vectors: this can be done by intratissular (intramuscular, intra-cerebral, intra-cardiac, etc…), intravenous, intrathecal or subcutaneous injection. The route of administration is directly dependant on the targeted tissue, and will indirectly define the volume and quantity of vectors that will be necessary for treatment. For instance, the rescue of retinal defect by gene therapy, as for Leber's Congenital Amaurosis, with Adeno-Associated Virus (AAV) vectors (belonging to Parvoviridae family), requires injection of tiny volumes of product underneath the retina to be efficient (6-8), while treatment of DMD will necessitate huge amount of highly concentrate viral vectors to be relevant and compatible with the challenging whole body treatment (9). Therefore, processes of vector production will be drastically different depending on the targeted tissue. In *ex vivo* approaches, viral vectors are generally used to transduce specific group of cells *in vitro* before their *in vivo* transplantation. This combination of gene therapy and cell therapy is principally used with somatic cells or stem cells to regenerate genetically altered tissues. Retrovirus-derived vectors are generally chosen for cell transduction due to their capacity to integrate therapeutic transgene into the targeted genome, avoiding its lost during the cell proliferation stage.

3.1.4. Processes of vector production

Finally, the last important point concerns the vector biotechnology, and more precisely the protocols used for vector production and purification and its subsequent quality control before *in vivo* administration. For clinical perspectives, the entire process has to be easily implemented for industrial productions. It means that it has to be easily scalable and compatible with good manufacturing practices (GMP). Different upstream and downstream processes will be described in following sections for different type of vector.

Taken together, the gene transfer parameters described above have to be clearly defined according

to the specific applications and this is particularly true for NMDs such as DMD and SMA. Investigations in the field show that the nature of these genetic pathologies greatly influences and restrains the choices of therapeutic transgenes, vectors, route of administration and consequently the mode of vector production. After a brief description of these genetic pathologies, we will first give an overview of the most studied transgene constructions used, then present a panel of previously used viral-derived vectors and focus more particularly on AAV-derived vectors as they currently represent the most advanced viral vector towards clinical trials for DMD and SMA. We will finally conclude this part by presenting challenges and promises held by these approaches.

3.2. DMD and SMA: pathophysiology and candidate transgenes

3.2.1. DMD pathophysiology

DMD is a lethal X-linked progressive muscle-wasting disease caused by mutations, typically large deletions, in the *dmd* gene – the largest gene in the human genome (10) . Most mutations, including deletions (~65 %), duplications, point mutations or other small gene rearrangements disrupt the open reading frame, leading to aberrant translation and therefore to the absence of the essential muscle protein dystrophin. This protein is localized at the sarcolemma of the muscle fiber and forms a dystrophin glycoprotein complex (DGC) with dystroglycan, sarcoglycan, and syntrophin/dystrobrevin complexes. The DGC provides a mechanical and signaling link between the actin cytoskeleton and the extracellular matrix (11). The absence of dystrophin leads to recurrent muscle fiber damage during contraction and muscle fibers are eventually replaced by adipose and fibrotic tissue. Patients with DMD suffer from progressive loss of muscle function which generally leads to wheelchair dependency by the age of 13 and premature death, mostly before the age of 30 (12). Interestingly, the allelic disease Becker muscular dystrophy (BMD) which results in a much milder phenotype, is mainly caused by mutations maintaining the open reading frame and allowing the production of a partially deleted but functional dystrophin (13).

3.2.2. Candidate transgenes for DMD

The principle of gene therapy for DMD is to provide an alternative copy of functional dystrophin gene for patients. However, dystrophin cDNA is 14 kb long which is too large to be packaged in AAV vectors for example (having a carrying capacity of 4.5. to 5 kb – see section “AAV-derived vectors”). To circumvent this problem, researchers have proposed the possibility to use truncated but still functional dystrophin isoforms as alternative therapeutic approach, based on BMD genotypes (14-15). Different constructions of these mini- and micro-dystrophins were done and compared for their efficiency to recover high levels of dystrophin protein *in vivo* (Figure 1). For example, work from several labs has shown that two large regions of dystrophin – most of the rod domain (R), which normally contains 24 spectrin-like repeats, and part of the C-terminal domain – can be truncated with minimal functional impact (16-17). The best-studied spectrin-like repeat (ΔR4-R23) micro-dystrophin contains only the first 3 and the last spectrin-like repeats (18). Other examples of mini-dystrophins and micro-dystrophins genes that have been assessed for restoration of dystrophin are shown in Figure 1A.

3.2.3. SMA pathophysiology

SMA is an autosomal recessive disease with an incidence of 1 in 6000 births and a carrier frequency of about 1/40 (19-20). SMA is the second most common child disease after DMD and the second most common recessive disease after cystic fibrosis. SMA is clinically classified into four main types depending on the age of onset, the severity of the disease and the vital prognosis. SMAs are characterized by a degeneration of alpha-motor neurons in the anterior horn of the spinal cord resulting in muscular atrophy and weakness, due to mutations in the SMN gene. In man, the SMN locus is located in chromosome 5 (5q11.2.-13.3.) in a complex region which contains repetitive copies (21). The SMN locus presents a telomeric copy SMN1 which encodes the SMN protein, and centromeric copies SMN2 which produce about only 10 % of functional SMN protein. SMA occurs when SMN1 is mutated on its two alleles. However the severity of the disease is alleviated by the copy number of SMN2. For instance, severe forms (type I) carry two copies while the milder type III SMA patients carry three or four SMN2 copies.

3.2.4. Candidate transgenes for SMA

According to previous observations, gene therapy approach for SMA has emerged by replacing the mutated SMN1 gene by a non mutated copy. The goal is to increase the level of expression of the SMN protein in order to drastically attenuate the SMA physiopathology. During the past five years, several studies have demonstrated the efficacy of AAV-based vectors for the delivery of SMN1 cDNA in different SMA mice models. In particular, the serotype 9 has shown its capacity to cross the blood-brain barrier through the vascular system (22). For this reason, this serotype was particularly used in evaluation for SMA treatments. The SMN cDNA was also codon-optimized, showing better transcription efficacy (23). A schematic representation of SMN cDNA, mRNA and protein is shown in Figure 1B.

3.3. Viral-derived vectors for DMD and SMA gene therapy approaches

Amongst all the viral vectors used in gene therapy approaches for DMD or SMA, AAV vectors

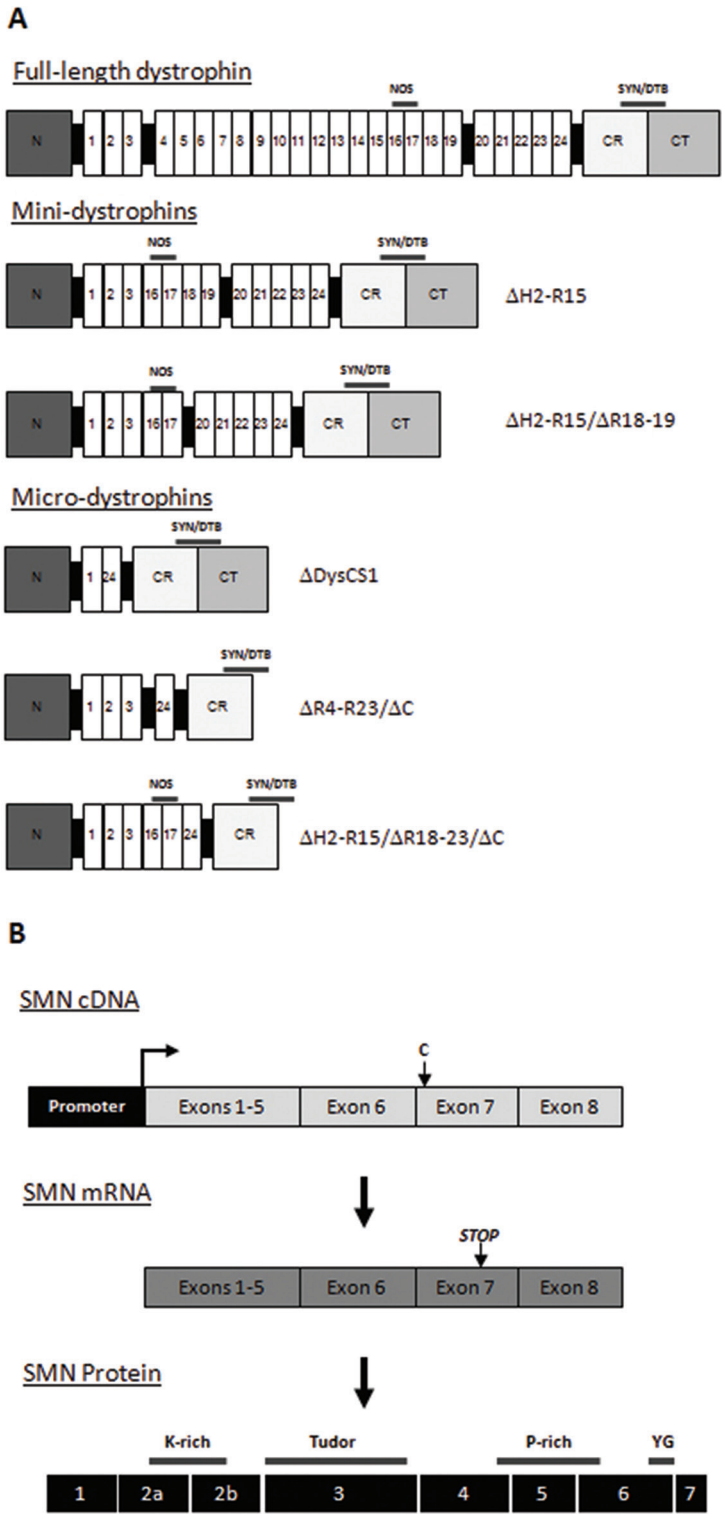

Figure 1. examples of transgenes used for DMD and SMA gene therapy approach. A. Some mini- and micro-dystrophin proteins used for dystrophin rehabilitation experiments, compared to full-length form. N: N-terminal actin binding site; CR: Cystein-rich dystroglycan binding site; CT: C-terminal syntrophin (SYN) / dystrobrevin (DTB)-binding sites; NOS: Nitric oxide synthase; Black boxes: hinge regions(H); White boxes: the 24 spectrin-like repeats (R) of the central rod domain. B. SMN transgene (upper part) showing the 8 exons and the localization of the cryptic splice site (C) at the beginning of the exon 7, the resulting SMN mRNA with the stop codon site, and the SMN protein with its specific domains. K-rich: lysine-rich domain (RNA binding domain); P-rich: proline-rich domain; YG: tyrosin – glycin domain involved in SMN self association. Note that several studies have used codon optimized isoform to improve the SMN transcription efficiency.

represent undoubtedly the most investigated and promising ones. Nevertheless, two other types of viral vectors have also been used: lentivirus- and adenovirus-derived vectors.

3.3.1. Lentivirus-derived vectors

3.3.1.1. Generalities

Lentiviruses are one of the Retroviridae genus that contains nine species amongst which the human immunodeficiency virus (HIV) is the most known. Their ~9 kb single stranded RNA genome (two copies per virus) is packaged in a capsid surrounded by a lipid envelop extracted from plasma membrane of infected cells during the virus budding. The viral surface glycoproteins recognize cell surface receptors and promote the fusion and the entry of virus into the cells. In the cytoplasmic compartment, the retrotranscriptase enzyme performs the transformation of the original single stranded RNA molecule in double stranded DNA form, and thus the integrase enzyme promotes the integration of the viral DNA into the cell genome. Classically, lentiviral vectors are produced using three-plasmid system composed of a packaging plasmid, coding for the regulatory enzymes (retrotranscriptase, integrase, protease) and structural protein; an envelope plasmid, coding for the surface glycoprotein ligand; and a cis-vector plasmid, coding for the transgene of interest and harboring the packaging signal sequence (24) (Figure 2). These plasmids are co-transfected in adherent human embryonic kidney 293 (HEK293) cells and supernatant is regularly harvested before its purification by diverse methods (ultracentrifugation, chromatography). Titers can reach 10^{10} transducing units per milliliter (TU/ml). Several generations of lentiviral vectors have been constructed to improve their safety and efficiency (25). These vectors have previously shown their capacity to transduce dividing and non-dividing cells such as neurons and skeletal muscles (26), and therefore appear attractive for DMD and SMA therapeutic strategies.

3.3.1.2. Lentivirus-derived vectors for DMD gene therapy

A recent study of *in vivo* gene transfer from Kimura *et al*. has shown that delivery of micro-dystrophin protein (ΔR4-R23/Δ71-78 isoform) by lentiviral vectors leads to successful transduction of both skeletal muscle and resident satellite cells into neonatal *mdx* mice (27). Although the level of transduction was not sufficient to completely halt ongoing myofiber turnover, dystrophin levels remained stable due to ongoing muscle regeneration supported by satellite cells, a proportion of which carried integrated copies of the micro-dystrophin vector. It is important to note that lentiviral vectors are generally pseudotyped with the stable vesicular stomatitis virus envelop glycoprotein (VSV-G), which displays a rather large tropism *in vitro* but performs low transduction efficiency in muscle tissues (28). This lack of muscle tropism has though been skirted by pseudotyping lentiviral vectors with other surface glycoproteins like those coming from Ebola or Mokola viruses (29), even if less stable than VSV-G. On the other hand, lentivirus-derived vectors have been successfully used in *ex vivo* strategies. Stem cell therapies have emerged to curb degenerative processes occurring in some genetic disorders (30). In 2007, we provided proof-of-concept that a category of adult stem cells extracted from DMD patients, harboring the CD133 cell surface marker, could be genetically corrected *in vitro* by lentiviral vectors, and participated in muscle regeneration after their *in vivo* transplantation (31). CD133 autologous transplantation was also tested for safety in a phase I clinical trial, and no systemic side effects were found after intramuscular delivery, which paved the way for future clinical trials (32). Other cell types, such as mesoangioblasts offer promising alternatives for stem cell mediated therapies for DMD (33). This review being focused on direct gene therapy, we will not develop the cell therapy strategies more fully, but one should keep in mind this exciting developing field and the promising combination of stem cell approaches and their *ex vivo* gene therapy correction mediated by lentiviral vectors for future clinical applications.

3.3.1.3. Lentivirus-derived vectors for SMA gene therapy

Compared to DMD, very few studies have investigated the use of lentiviral vectors for SMA. However, one of the most interesting studies was performed using an equine infectious anemia virus (EIAV) vector pseudotyped with a rabies virus surface glycoprotein (34). This vector had previously shown its capacity to transduce motor neurons by performing an efficient retrograde axonal transport (35). In the first study the authors have shown that intramuscular injections of this lentiviral vector expressing SMN in SMA mice restored SMN in motor neuron, reduced motor neuron death, and increased the life expectancy by an average of 20 to 38 % compared with untreated animals (34). Nevertheless, the efficiency of the latest proof-of-concept study was shown to be marginal and, according to the authors, is not currently sufficient to form a strong basis for clinical applications in humans (36).

3.3.1.4. Lentivirus-derived vectors: challenges

In vivo gene delivery of lentiviral vectors is hampered by some challenges, amongst which is the high risk of insertional mutagenesis due to the integration of their genome (37). Moreover, compared to other type of vectors (discussed below), lentivector production process do not currently allow the high yields and viral

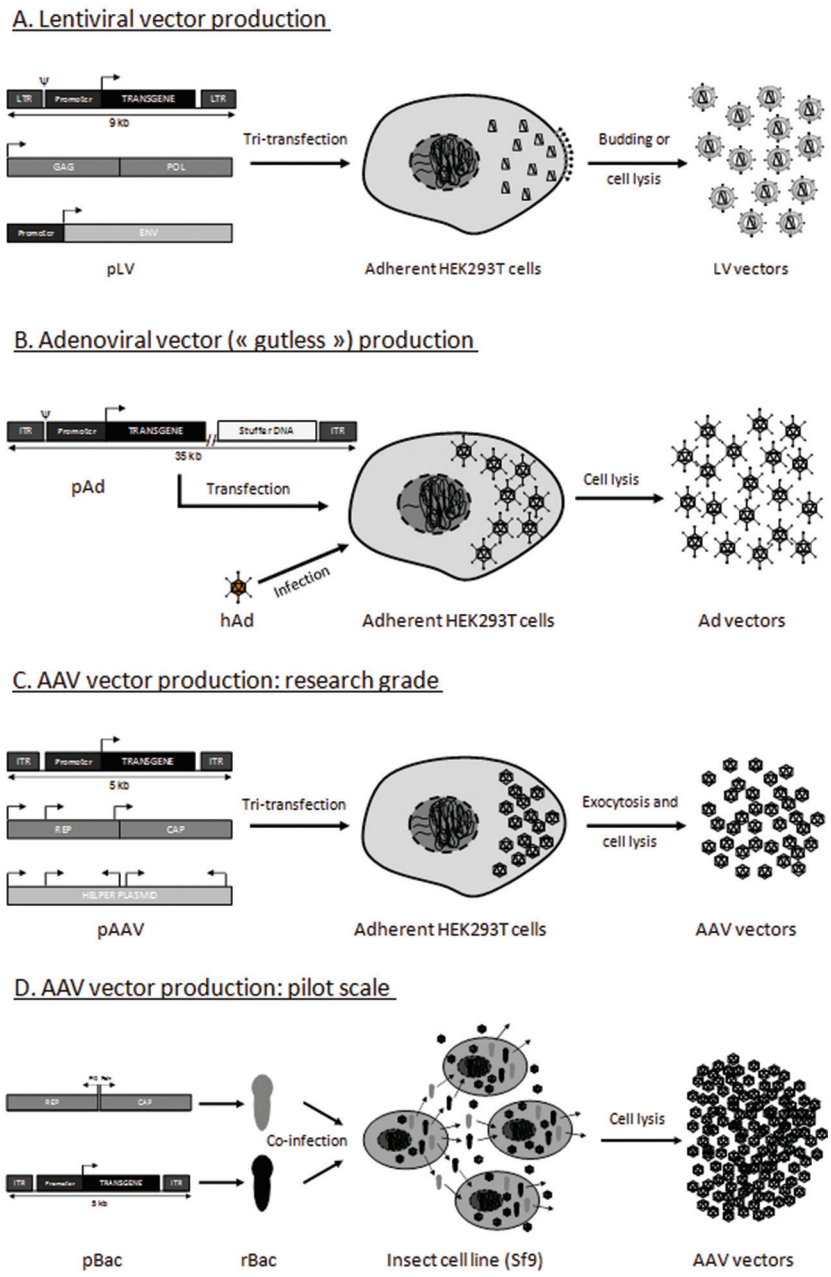

Figure 2. genetic constructions and mode of production of virus-derived vectors classically used in gene therapy approaches. A. Lentivirus-derived vectors (LV-vectors) are produced by tri-transfection of HEK293T cells with three lentiviral plasmid constructions (pLV). LTR: Long Terminal Repeats; Ψ: packaging signal; GAG: coding sequences for lentiviral structural proteins; POL: coding sequences for regulatory enzymes; ENV: coding sequence for surface envelop glycoprotein. B. The "gutless" adenoviral-derived vectors (Ad vectors) are produced by transfection of HEK293T cells with recombinant adenoviral genome (pAd) coding the transgene and completed with stuffer DNA, following by infection with helper adenovirus (hAd); ITR: Inverted Terminal Repeats; Ψ: packaging signal. C and D. AAV-derived vectors are produced following two different approaches. C. For research grade productions, HEK293T cells are tri-transfected with three AAV plasmids (pAAV); ITR: Inverted Teminal Repeats; REP: coding sequences for regulatory enzymes; CAP: coding sequences for structural proteins; the helper plasmid contains adenoviral genes necessary for the activation of AAV vector replication process. D. For pilot scale productions, AAV vectors are produced by the baculovirus / insect cell system. Two recombinant baculoviruses (rBac) are first produced from transfection of insect cell line (Sf9, from *Spodoptera frugiperda* species) by recombinant baculovirus genomes (pBac). Co-infection of insect cell line with these two rBac lead to (i) exponential infectious cycle through the whole insect cell culture (in suspension conditions), and (ii) concomitant AAV vector production. P10 and Polh: strong baculovirus promoters (polh: polyhedrin); REP and CAP: as described above.

titers compatible with whole body clinical applications. However, we have shown, through previous applications, that lentiviral vectors can use the benefit of pseudotyping to diversify their tissue tropism and to improve their cell transduction specificity. In this field, several developments are regularly done to improve stability of new surface glycoproteins by creating for instance chimeric proteins containing cytoplasmic part of the VSV-G and extracellular part of another surface glycoprotein (38). As discussed before, despite challenges for their use in direct gene therapy, lentiviral vectors remain the tool of choice for *ex vivo* approaches, especially for DMD therapeutic applications.

3.3.2. Adenovirus-derived vectors

3.3.2.1. Generalities

Another main type of viral vectors used in gene therapy derives from Adenoviridae family. Among the five current genera, the Mastadenovirus genus is composed of 7 human species (A to G), encompassing altogether 57 human adenovirus serotypes (hAd1 to 57). Unlike lentiviruses, adenoviruses are non-enveloped, and carry a double-stranded DNA genome of 30 to 40 kb, packaged in a 90 to 100 nanometers icosahedral shaped capsid. The capsid is formed from 252 capsomeres, splited in 240 hexons and 12 pentons. The virion has a unique "spike" or fiber (also called the fiber knob) associated with each penton base of the capsid that aids in cell attachment via the receptor on the surface of the host cell. The principal adenovirus receptor is the Coxsackie adenovirus receptor (CAR), which is expressed in various regions of the human body, including the heart, the brain and more generally, in epithelial and endothelial cell surfaces. After cell penetration by endocytosis, the adenovirus is transported to nuclear pore complex via microtubules, and the viral DNA is expelled into the nucleus where it starts its replication. Transcription of the early genes (E1 to E4) leads to expression of regulatory proteins, and the late genes (L1 to L5) allow expression of newly structural proteins. The first- and second-generation adenovirus vectors have been essentially based on human adenovirus type 5 (hAd5). These are depleted of some early genes, leading to replication-defective vectors. However, these adenovirus-derived vectors are able to transduce replicating and non-replicating host cells, which make them attractive candidates for the transduction of neurons or skeletal muscles. Like lentiviral vectors, the first-generation adenoviral vectors were produced in HEK293 cells which stably express E1 genes necessary for the recombinant viral genome replication. The deletion of the E1 genes from the wild-type adenovirus genome allows the cloning of transgene sequence limited to about 7 to 8 kb. High-capacity adenovirus vectors, also called helper-dependant or "gutless" adenoviral vectors were designed to increase the capacity for uptake of transgene DNA (39). In this vector type, all viral genes are removed, resulting in the possibility to integrate foreign DNA of approximately 35 kb (Figure 2). Several studies have indicated that toxicity and immunogenicity of this vector type is considerably reduced compared with first-generation vectors as briefly discussed in the "challenges" section.

3.3.2.2. Adenovirus-derived vectors for DMD gene therapy

First-generation adenovirus vectors have previously been used to successfully deliver a human mini-dystrophin cDNA to *mdx* mice via intramuscular injection (40). However, first and second-generation adenoviral vectors tend to have leaky expression of the remaining viral proteins *in vivo*, which elicit a host immune response. Delivery of full length dystrophin to adult and neonatal *mdx* mice using gutted adenoviral vectors results in long-term expression and amelioration of the physiological and pathological symptoms of muscle disease (41-42).

3.3.2.3. Adenovirus-derived vectors for SMA gene therapy

To date, very few direct gene therapy studies for SMA using adenoviral vectors were published. However, restoration of full-length SMN by adenoviral vectors was observed after delivery of RNA antisense oligonucleotides embedded in U7 snRNAs constructs (43). These U7 approaches will be developed in next part, but the results suggest that the adenoviral vector-mediated transgene application may be a suitable therapeutic approach to counteract SMA.

3.3.2.4. Adenovirus-derived vectors: challenges

The immunogenicity of the first- and second-generation adenoviral vectors precludes their use in systemic administration due to the danger of producing a life-threatening systemic immune response. Moreover, delivery of very high doses of adenoviral vectors can result in induction of innate and cellular immune responses that are potentially lethal and their production is still very cumbersome and difficult to standardize (44). Therefore, while adenoviral vectors are capable of delivering full length dystrophin, resulting in efficient and moderately long-term transduction, improvements are required for these vectors to be utilized safely in future gene therapy trials.

3.3.3. AAV-derived vectors

3.3.3.1. Generalities

AAV vectors currently appear as the best candidates for NMDs gene therapy strategies, due to several attractive characteristics. As discussed below, this non replicative vector is able to transduce muscle

and nervous tissues with high efficiency, expressing the transgene for long periods of time. Moreover, it is not integrative, avoiding problems of hazardous genomic insertions that can be the cause of tumorigenesis. These vectors induce also very low immunogenicity and inflammation following their *in vivo* injection. The next paragraphs provide more details about structure, tropisms and the processes to produce these AAV vectors at different scales. Several examples of therapeutic strategies for NMDs using these vectors will be described and limitations will be discussed together with different solutions proposed to improve their efficacy as a gene therapy vector in future clinical trials.

3.3.3.2. Morphological properties of AAV

Structurally, AAV are naked virions embedding a single stranded DNA genome inside an icosahedric shaped capsid formed of 60 capsomers. They are among the smallest animal viruses with a diameter comprises between 25 and 30 nm. The capsomers are composed of three capsid proteins, VP1, VP2 and VP3, represented in the whole particle by a ratio of 1:1:10 respectively. Without any envelop, tissue tropism of the virus is entirely driven by its capsid. In this context, the International Committee on Taxonomy of Viruses (ICTV) has recently re-evaluated the Parvoviridae family taxonomy by classifying AAV in different subspecies including the human and primates AAVs in *Adeno-Associated dependoparvovirus A*, which gathers 13 different serotypes. Among them, the well-known AAV2 represents the canonical agent of this group. 11 of these serotypes are used as gene transfer vectors. Note that the AAV5, largely used in gene transfer protocols, is currently classified in the subspecies B (including the bovine and caprine AAV), and adjusts the number of AAV-derived vectors to 12. Differences in capsid coding sequences, resulting in VP protein modifications, lead to differences in tissue tropism. For instance, the serotype 9 shows an interesting high affinity for cardiac tissue in rodents in addition to its brain and skeletal muscle tropisms (45-47). Investigations are currently underway to verify if such cardiac affinity is also applicable to other species such as the dog models (9), a prerequisite for human therapeutic applications. Multiplicity of AAV serotypes could be advantageously used to implement targeted gene transfer strategies. Table 1 gives the tissue tropisms of the most commonly used AAV vectors according to their described cell receptors and co-receptors. In addition to structural proteins, AAV genome encodes four regulatory proteins named Rep78, Rep68, Rep52 and Rep40 (according to their respective molecular weight). Rep78/68 are encoded from the same promoter (p5) while Rep52/40 are encoded by another one (p19). Rep 68 and Rep40 result from a unique splicing event present at both ends of the Rep78 and Rep52 mRNA sequences respectively. All of them encode for enzymes responsible for genome replication and packaging.

3.3.3.3. Life cycle and replication properties of AAV

The classification of AAV among the *dependoparvovirus* is due to their "dependence" towards "helper" virus to complete their viral cycle. Indeed, original studies on AAV2 show that after cell entry and in the absence of "helper" virus (issue from Adenoviridae, Papillomaviridae or Herpesviridae family), the AAV is unable to replicate its genome to form new particles, and this one inserts, via a Rep mediated mechanism, in a specific site located in human chromosome 19 (48-49). This integrative property is lost in AAV-derived vectors and these remain under episomal form into the nucleus of transduced cells. In contrast, co-infection of wild type AAV with helper virus promotes replication and formation of new AAV particles ready to sustain and amplify the infection cycle. One important characteristic about AAV viruses and derived vectors is the size of their genome. The AAV vector capsid cannot package more than 5 kb of single stranded DNA, which represents a significant limitation for gene therapy approaches and that will be discussed further. The 5' and 3' ends of the AAV genome present palindromic sequences folded in hairpin structures (50). These more or less 145 bases are named Inverted Terminal Repeats (ITRs) and serve as primers for the host cell's DNA polymerase, which converts the single-stranded virus genome into double-stranded DNA as part of the virus replicative cycle. They also play important roles in packaging the viral genome into preformed capsids (51). Due to these essential functions, the ITR structures cannot be deleted from a viral vector and need to be delivered in *cis*. These clarifications about viral cycle and genome structure will serve as a prerequisite to better understand the methods employed to produce AAV-derived vectors.

3.3.3.4. Biotechnological processes of AAV vectors

As explained above, gene therapy vectors have to be easily produced and, according to application, have to be scaled up. Upstream (production stage) and downstream (purification stage) processes for AAV vectors have considerably evolved during the last decade. Biotechnological methods have been implemented to propose different levels of production in accordance with type of projects: research, pilot or industrial. For research-grade vectors, used for *in vitro* experiments or to bring a proof of concept in small animal models, the classical method uses adherent human cell line as vector transient producer. HEK293T is once again the most widely used cell line for AAV production. Originally, co-transfection of adherent HEK293T cells with packaging plasmid (harboring the rep and cap

Table 1. Receptors, co-receptors and tissue tropisms of the mostly used AAV vectors

AAV serotype	Receptor	References	Co-receptor	References	Tissue tropisms	References
AAV1	N-linked sialic acid	217	ND		Skeletal muscle	218
					Brain	219
					Retina	220
					Pancreas	221
AAV2	HSPG	222	FGFR1	223	Vascular muscle cells	224
			HGFR	225	Skeletal muscle	226
			LamR	227	Brain	228
			CD9 tetraspanin	229	Kidney	230
AAV3	HSPG	231	FGFR1	232	Skeletal muscle	218
			HGFR	233		
			LamR	227		
AAV4	O-linked sialic acid	234	ND		Brain	235
					Retina	236
AAV5	N-linked sialic acid	234	PDGFR	237	Skeletal muscle	218
					Brain	236
					Lung	238
					Retina	220
AAV6	N-linked sialic acid HSPG	217	EGFR	239	Skeletal muscle	240
					Brain	241
					Heart	242
					Lung	243
AAV7	ND		ND		Skeletal muscle	244
					Retina	245
					Brain	246
AAV8	ND		LamR	227	Liver, Skeletal muscle	244
					Brain	246
					Retina	245
					Pancreas	247
					Heart, Skeletal muscle	248
AAV9	N-linked galactose	249	LamR	227	Liver, Heart	250
					Brain	22
					Skeletal muscle	251
					Lung, Kidney	45
					Pancreas, Heart	46

Abbreviations: EGFR: Epidermal growth factor receptor; FGFR1: Fibroblast growth factor receptor-1; HGFR: Hepatocyte growth factor receptor; HSPG: Heparan sulfate proteoglycan; LamR: Laminin receptor; PDGFR: Platelet-derived growth factor receptor. *ND: not determined*

genes) and a pAAV cis-plasmid (harboring transgene flanked by the two ITRs), was supplemented by infection of producer cells with an helper virus, generally belonging to human adenovirus serotype 2 or 5 (52). Because of the technical difficulties to separate AAV from helper virus, researchers have progressively identified the helper genes only necessary to promote replication of AAV genome. Finally helper plasmids were constructed and AAV vectors have started to be produced by triple transfection (53-55), ensuring better security and safety for experimenters and final products respectively (Figure 2). By using this classical system, productions can be carried out in plates, cell factories (CF) or roller bottles, allowing cultures from 150 cm^2 (15 cm plate) to more than 6000 cm^2 (CF10) of growth area, and ensuring the recovery of 10^{11} to $5.1.0^{13}$ total viral genomes (vg)

respectively. Technically, the producer cells are cultivated for 72 hours after transfection before AAV purification stage. Usually, vectors are collected from the cell pellet, avoiding the treatment of large quantities of media volumes. Nevertheless, recent advances have shown that supernatants contain a quantity of AAV particles similar to that contained in cells (56-58). The development of concentration methods, such as tangential flow filtration (TFF), has recently rushed purification methods from supernatants, drastically decreasing the time-consuming protocols using cell pellets. As introduced before, one of the main challenges for NMDs is the requirement of whole-body treatment as for DMD. It is generally admitted that a ratio of 10^{11} vg per gram of body weight is the minimal prerequisite to have an efficient *in vivo* tissue transduction (9). Suboptimal doses lead to rapid decrease of transgene expression and vector clearance (59). An adult mouse weighs about 30 grams, suggesting that a whole body treatment requires approximately $3.1.0^{12}$ vg. With a maximum of 10^{13} vg produced per run (using for example CF5 or Hyperflask® vessels), only 3 adult mice can theoretically be intravenously injected from one production. It is just sufficient to satisfy reproducibility and to be statistically relevant. However, the reduction of process duration allows rapid multiplication of productions with the possibility to treat 3 to 4 cell cultures in parallel (thus multiplying by 4 the number of mice to be treated). Despite the optimization of research-grade productions, “scaling” issues appear as soon as you consider treating a bigger animal model or even a human being. Even a young large animal model, such as Golden retriever muscular dystrophy puppy weighs around 1 to 2 kilograms, bringing the quantity of vectors around 10^{14} to $2.1.0^{14}$ vg. For a young DMD boy, weighing around 20 kilograms or more, we exceed 10^{15} vg. One can easily calculate that such quantities cannot be reached by triple transfections methods on adherent cells because of the total cell surface areas needed. An alternative process was proposed in 2002 by the group of Robert Kotin (60). They use the baculovirus / insect cell system to produce AAV, enabling the scalability of production. Indeed, this system has been used for decades by industrials to produce recombinant proteins. The robustness of the system lies in the capacity of insect cell line to grow in suspension, eliminating the obstacle of surface area. The second advantage is linked to the infection capacity of baculovirus vectors. Indeed, unlike transfection method, the baculovirus conserves its viral cycle and can thus perform an exponential viral infection from a very small quantity of starting material. The first described protocol used 3 distinct baculoviruses (60), each coding for genes essential for AAV production (rep, cap or ITR/transgene construction). Note that the baculovirus / insect cell system does not require the use of helper genes for AAV production and replication. Later developments reduced the number of recombinant baculoviruses to only 2 by combining the rep and the cap genes on a single vector (61), logically leading to an increased yield (Figure 2). Moreover cryopreservation instability of recombinant baculoviruses was circumvented by development of cell system allowing their conservation into baculovirus infected insect cells (62), enabling their use for large scale AAV productions (63). Therefore, production and purification of AAV vectors is currently amenable to industrialization, facilitating short term release of clinical applications.

3.3.3.5. AAV-derived vectors for DMD gene therapy

The previously described ΔR4-R23 mini-dystrophin transgene has been shown to almost fully prevent dystrophy in transgenic dystrophin deficient *mdx* mice (18). When combined with a C-terminal deletion, the ΔR4-R23/ΔCT micro-dystrophin was able to reverse most morphological abnormalities in young and old *mdx* mice and the more severe dystrophin:utrophin double-knockout mouse model following systemic delivery using rAAV vectors (64). Success of these preliminary results obtained with shortened dystrophin isoforms in mice had been confirmed in larger animal models.

Indeed, a number of preclinical approaches were done with dog models of DMD. While we focus here on AAV-mediated mini- and micro-dystrophin strategies, it should be noted that some adenovirus-derived vectors were also used to deliver these isoforms (65). In 2007, one study using the ΔR4-ΔR23/ΔCT canine micro-dystrophin isoform embedded in AAV vectors (serotypes 2 and 6) was intramuscularly injected in transiently immune-suppressed adult *cxmd* dogs (66). The results showed long-term and robust expression of the micro-dystrophin transgene in skeletal muscles and its expression restored localization of components of the dystrophin-associated protein complex at the muscle membrane. These encouraging results were corroborated by using an AAV2/8 serotype delivering a codon-optimized form of the previous canine micro-dystrophin (67). The same transgene construction was also injected in *cxmd* dogs through an AAV6 serotype (68). In this study, the authors used widespread intramuscular injections of the AAV6 vectors under a brief course of commonly used immune-suppressants. An impressive and robust micro-dystrophin expression was obtained for at least two years and was associated with reconstitution of the dystrophin-glycoprotein complex at the muscle membrane. Importantly, transgene expression was maintained for at least 18 months after discontinuing immune-suppression. Another interesting study, more focused on immune-suppression optimization, has used the canine R16-17/H3/ΔC micro-dystrophin cDNA embedded in an AAV6 serotype (69). After limb muscle injections in GRMD dogs, the authors have shown robust

and sustained micro-dystrophin expression for six months (end of the study). The same group recently reported equivalent results by using another canine micro-dystrophin construction (ΔR2-15/ΔR18-19/ΔR20-23/ΔC) embedded in an AAV9 serotype (70). Major components of the dystrophin-associated glycoprotein complex were restored and treated muscles showed less inflammation, fibrosis, and calcification. Importantly, the authors note that the therapy significantly preserved muscle force under the stress of repeated cycles of eccentric contraction. All these encouraging preclinical results established the proof-of concept for micro-dystrophin therapy in dystrophic muscles of large mammals and set the stage for clinical trial in human patients.

A phase I clinical trial for DMD (NCT00428935) used a human ΔR3-R21/ΔCT mini-dystrophin transgene (40 % of the 11 kb coding sequence of the human dystrophin cDNA) embedded in a chimeric AAV2.5. vector designed to be a serotype 2 capsid variant that contains 5 AAV1 amino acids (1 insertion and 4 substitutions) in the AAV2 VP1 background (71). The study, initiated in March 2006 and completed in April 2009, enrolled 6 DMD boys with frame shifting deletions that were injected with this vector in one of their biceps muscle. Unfortunately, no mini-dystrophin transgene expression was detected in any of the patients enrolled. Four out of six patients had detectable dystrophin-specific T cells, including CD4+ and CD8+ T cells in peripheral blood, with two patients having dystrophin specific T cells before vector treatment. The authors speculated that T cells targeting self or non-self dystrophin epitopes may have eliminated mini-dystrophin-expressing muscle fibers in the study. The same AAV2.5. vector was re-used in another phase I clinical trial that has enrolled six other DMD boys (72). In this randomized double-blind placebo controlled phase I clinical study, AAV2.5. vector was also injected into the bicep muscle in one arm, with saline control in the contralateral arm. Results show no cellular immune response against the vector. During the 2-year-long active phase of the trial monitoring, no serious or mild adverse events have been observed in any subject. However, among the six enrolled patients few or no mini-dystrophin positive fibers were detected, confirming the immunological rejection hypothesis of the previous trial. Despite the very low transgene recovery, these early stage trials shed light on dystrophin immunogenicity which should be carefully considered in designing and monitoring future experimental therapies for this disease. Immune challenges will be also discussed in a next paragraph.

3.3.3.6. AAV-derived vectors for SMA gene therapy

Among AAV-derived vectors, the serotype 9 has clearly demonstrated its efficiency to transduce motor neurons by bypassing the blood brain barrier, even after single systemic injection (22, 73). In particular, the development of the self complementary AAV (scAAV), packaging a double stranded genome, has shown an improving transduction efficiency *in vitro* and *in vivo* (74). In 2010, several studies showed successful rescue of SMA mice by gene therapy approaches. A first study by Foust *et al* reported that scAAV9-mediated SMN gene replacement in SMA mice models (SMNΔ7$^{+/+}$ and SMN$^{-/-}$) resulted in an unprecedented improvement in survival and motor function (75). The authors have conducted their study on early postnatal mice (P1 to P10) and have defined a therapeutic window by suggesting a developmental period in which scAAV9 therapy had maximal benefit. They also showed that scAAV9 crossed the blood brain barrier in a nonhuman primate and transduced motor neurons, supporting the possibility of translating this treatment option to human patients. In another study, the group of Brian Kaspar has described a compromised cardiac function in SMNΔ7 mice, resulting in at least partially early bradycardia (76). Delivery of scAAV9-SMN vector abolished the symptom of bradycardia and significantly decreased the severity of the heart defect. Another interesting report showed that delivery of scAAV9-SMN into the brain of severe neonates SMA mice models led to an impressive improvement of life span (157 days compared to 15 days in untreated controls) (77). Moreover, the injections resulted in robust improvement in skeletal muscle physiology, including increased myofiber size and improved neuromuscular junction architecture. Finally, treated mice also displayed substantial improvements on behavioral tests of muscle strength, coordination, and locomotion, indicating that the neuromuscular junction was functional. Valori *et al* reported similar results concerning the survival effect of exogenous SMN. Indeed, they showed that a single injection of scAAV9 expressing a codon-optimized version of the SMN cDNA into the facial vein 1 day after birth in SMNΔ7 mice resulted in a substantial extension of life span in these animals (23). Another study clearly showed an impressive life span extension when scAAV9 was systematically injected once in P1 SMA mice, increasing life expectancy from 27 to over 340 days (median survival of 199 days) in mice that normally survive about 13 days (78). More recent studies confirmed previous results on SMNΔ7 mouse model (79). In this original work, the authors have injected scAAV9-SMN vectors by intramuscular route and have shown a very efficient axonal retrograde transport, able to transduce motor neurons in entire spinal cord and the peripheral organs. Moreover, they showed that intramuscular injection of scAAV9-SMN vectors in SMA mice mediated high SMN expression levels in both the CNS and the periphery, and increased the median

lifespan from 12 days to 163 days. These encouraging results in mice models led to proof-of-concept studies in large animal models before future clinical assays. In this context, Bevan *et al* showed that systemic delivery of AAV9 in cynomolgus macaques, administered from birth to 3 years of age (to target brain and peripheral tissues), was efficient at crossing the blood brain barrier at all time points investigated (80). Moreover, systemic injection also efficiently targeted skeletal muscle and peripheral organs. Another large animal study was performed in pigs by evaluating an intrathecal (into the cerebrospinal fluid) scAAV9 vector administration (81). A total of 12 domestic farm pigs underwent a single-level lumbar laminectomy with intrathecal catheter placement for scAAV9 delivery. Three consecutive boluses via a temporary catheter resulted in diffuse transduction of motor neurons throughout the cervical, thoracic and lumbar spinal cords. Another recent intrathecal AAV administration was evaluated in non human primates (82), and the authors were able to achieve broad transduction throughout the brain and spinal cord parenchyma.

Altogether, these studies highlight the considerable therapeutic potential of scAAV9 for treating human SMA. Furthermore, the noninvasive mode of vector delivery and the high efficacy achieved provide a realistic and strong rationale for advancing vector-based SMN replacement approaches toward clinical trials in human patients. The group of Brian Kaspar and colleagues have recently been given approval by the FDA for an Investigational New Drug application to begin a phase I clinical trial of a systemic AAV9-delivered human SMN gene to SMA type I infants.

3.3.3.7. Challenges and promises of AAV vectors for NMDs

Many advantages have been described above concerning AAV vectors. Their capacity to transduce non-dividing cells, their long-term gene expression, their lack of pathogenicity (from wild-type infections) as their inability to replicate in absence of helper virus are strong arguments in favor of their use as gene therapy vector for the treatment of NMDs. Moreover, AAVs present numerous serotypes with different cell tropisms. Such heterogeneity enables researchers to choose the best candidate for specific therapeutic applications or tissue targeting. More specifically, some serotypes have demonstrated their ability to cross the blood-brain barrier after systemic delivery, such as the serotype 9. This rare characteristic represents a huge advantage for the treatment of disorders affecting whole body tissues such as DMD or SMA. We also described the capacity to produce these vectors at large scale using the baculovirus / insect cell system, allowing future industrial processes for clinical trial needs. Nevertheless, these positive properties are thwarted by some drawbacks that need to be overcome.

3.3.3.8. Improvement of packaging capacity

First, the limited packaging capacity of AAV particles is a serious challenge for transgenes exceeding 4.7. kb, especially taking into account several regulatory sequences (ITR, promoters, polyadenylation sites, intronic sequences,…) present in the final cassette (83-85). AAV genomes exceeding 5 kb show drastic diminution of the yields, preferentially due to proteasome-mediated degradation mechanism (86). To circumvent this problem, previous approaches have attempted to split the transgene cassette between two rAAV vectors containing splice donor and splice acceptor sites (87). This trans-splicing strategy has emerged from observations that AAV genomes produce intermolecular recombination between ITRs, leading to genome concatemerization (88). By this approach it was quickly possible to deliver therapeutic genes up to 9 kb in size, doubling the length of the AAV genome able to be expressed after cell transduction. By using appropriate AAV serotypes, the trans-splicing AAV vectors show beneficial results in different applications as for retina gene transfer (89-90), and mostly in DMD with efficient delivery of ΔH2-R19 mini-dystrophin gene in muscles (91). Moreover, systemic delivery of trans-splicing AAV9 vectors in *mdx* mouse model led to widespread transgene expression in the heart (92), mimicking the efficiency observed with classical AAV9 vectors. Recently, the group of George Dickson optimized the trans-splicing AAV strategy by proposing triple trans-spliced strategy able to reconstitute and express the 11.1. kb full length dystrophin *in vivo* (93). Nevertheless, faced with relatively low *in vivo* dystrophin expression the authors concluded that this new approach had to be optimized further to envisage future clinical application.

3.3.3.9. Improvement of transduction efficiency

The proteasome-mediated degradation of internalized AAV particles was also investigated to optimize their intracellular vector trafficking and to increase their transduction efficiency. It has been shown that phosphorylable amino acid residues (tyrosine, serine and threonine) located on the capsid surface play an important role in nuclear transport of AAV vectors. Indeed, previous work has described the significant increase of AAV2 transduction after substitution of surface-exposed tyrosine residues by phenylalanine (94-95). More recent investigations have identified a quadri-mutated variant, from mutations of tyrosine and threonine residues (Y444F + Y500F +Y730F + T491V), able to transduce target cells 24-fold more efficiently than wild-type AAV2 (96). Translated to other serotypes and combined with the trans-splicing approaches, these developments in capsid engineering should optimize the

AAV system for the selection of future clinically relevant vectors.

3.3.3.10. Immunity bypass

Another challenge for AAV gene delivery is the pre-existing host immunity to viral capsids (97). This immunity is mainly due to neutralizing antibodies (Nabs), for which the prevalence reaches approximately 70 % of the human population for AAV2 and to a lesser extent for the other AAV serotypes (98). This antibody response efficiently blocks gene transfer (99), and is also effective in several animal models such as mice, rats, pigs, dogs, sheep and rabbits (100). However, several solutions are under investigation (101). For human gene therapy, one solution to circumvent the seroprevalence could be the use of variant AAVs isolated from animals, mostly nonhuman primates. For instance, the AAVrh32.3.3, isolated from the spleen of a Rhesus macaque, having less than 70 % of amino acid homology with capsid sequence of human AAV1, 2, 7 and 8, exhibits a remarkably low seroprevalence among humans (102). Other nonhuman serotypes, belonging to dependovirus genus, and isolated from species as snakes (103), goat (104), bats (105), or even sea lions (106) could be an interesting alternative to circumvent antibody neutralization in human gene transfer assays. On the other hand, development of chimeric AAV variants could be an interesting way to both escape antibody neutralization and increase tissue specificity (107). These new chimeric AAVs are created by directed evolution (error-prone PCR, DNA shuffling) or by rational capsid engineering (transfer of specific capsid domains from one or several serotypes to another one). In this field, promising candidates were identified for NMDs applications. For example, the chimeric AAVM41, derived from AAV1, AAV6, AAV7 and AAV8 capsid components by DNA shuffling, shows an increased tropism for skeletal and cardiac muscle tissue (108). Moreover, this mosaic capsid surface AAV presents significant decrease in liver transduction *in vivo*, allowing better tissue specificity and antibody neutralization avoidance compared to "parental" serotypes. In the same way, the chimeric AAV2i8 vector was created by substitution of six amino acid residues of AAV2, involved in virus cell surface attachment, by the corresponding domain from AAV8 (109). This vector, compared to AAV2 and AAV8, clearly shows an improved tropism for skeletal and cardiac muscles, a liver de-targeting and drastic diminution of its neutralization by anti-AAV2 serum or human serum. According to these specific properties, AAV2i8 is a promising gene therapy vector for muscle disorders. Similar muscle tropism and liver de-targeting has been described with AAV9 variants obtained by specific mutations on the capsid sequence (110). As mentioned previously, a chimeric AAV2.5., produced using an AAV2 backbone in which four point mutations and one single residue insertion from AAV1 were substituted, was used in a phase I clinical study (72). All together, these new chimera approaches open therapeutic window for NMDs by using new vector generations with more specific muscle tropism and higher escape efficiency to antibody neutralization.

3.3.3.11. "Vaccination" bypass

As previously discussed, whole body treatment requires high vector doses to be efficient. In DMD, it has been demonstrated that suboptimal AAV doses lead to rapid loss of vectors with time, provoking drastic decrease of dystrophin in treated mice models (59) and even in dog model (111). These observations can be explained by the degeneration / regeneration process that is continuous in dystrophin-deficient muscles. Truncated forms of dystrophin (mini- and/or micro-dystrophins) used in gene therapy approaches may be more unstable than the full-length protein, leading to premature loss of their functionality. Furthermore, the efficiency of the transgene nuclear targeting can be problematic. For instance, in incomplete transduction of multinucleated skeletal muscle fiber, lack of dystrophin expression along short cell distance can be enough to destabilize the entire fiber by provoking holes along the sarcolemma, leading to influx of extracellular calcium and consequently cell death by apoptosis or necrosis (112). One optimal intravenous injection of AAV vectors has proven its efficiency to transduce large muscle territories (9), but the probability to miss some nuclei is not low, especially in some tissues that have more limited access as the brain. One way to circumvent these problems of stability and long term efficiency is to allow repeated injections of therapeutic vectors, leading to reinforcement of initial rescue and increase of newly targeted nuclei. Reinjection of viral vectors is thwarted by the immunological barrier due to the primo-injection (likened to a vaccination). One classical solution is brought through transient immune-modulation. Previous studies have shown that a brief course of immunosuppression with a combination of anti-thymocyte globulin, cyclosporine, and mycophenolate mofetil was sufficient to permit long-term and robust expression of a canine micro-dystrophin transgene in the skeletal muscle of the canine X-linked muscular dystrophy dog model (66, 69). In the same way, blocking the T-B crosstalk with anti-CD40 antibodies and CTLA4/Fc fusion protein was sufficient to totally abrogate the formation of anti-AAV antibodies and to allow for the correction of muscular dystrophy in multiple muscles of *mdx* mice model after AAV re-administration (113). However, immunosuppression strategies might not be applicable in all clinical settings, especially those concerning individuals with seriously compromised health conditions like DMD patients. Another interesting solution was proposed through several studies that show the possibility of re-administration of AAV by combining

different successive serotypes. For example, very low cross-neutralization was initially observed in mice between AAV1 and AAV2 (114), leading to synergetic effect of alternate *in vivo* administration of these two serotypes. In the same way, no cross-reactivity was detected in mice re-administrated with AAV6 after a primo-injection of AAV2 and vice versa (115). Another study, using an AAV2/5 hybrid vector shows no cross-immunization between this one and AAV2 (116). The absence of cross reactivity between AAV1, AAV2 and AAV5 was confirmed by other investigators (117). Re-administration strategies of different natural serotypes (for primates or non-primates species), between natural and hybrid vectors or between different engineered AAV could represent an interesting and powerful alternative for the reinforcement of initial level of tissue transduction and for a long term rescued phenotype stability.

4. SPLICING THERAPIES

The development of the antisense oligonucleotides (AON)-based approach started in the late 1970's when the oligonucleotides were used as tools to downregulate the expression of specific genes (118). In the past few years, attention has rapidly increased with the development of antisense molecules for manipulation of alternative splicing with the so-called splice switching oligonucleotides (SSO). In this context, oligonucleotides can be used to modulate the ratio of splicing variants or correct splicing defects, which opened far reaching implications in the treatment of a variety of diseases. The requirements for oligonucleotides that alter splicing are different from those for oligonucleotides used to achieve downregulation. In particular, they must not activate RNaseH, which would destroy the pre-mRNA before it could be spliced. They must also access their target pre-mRNAs within the nuclei of cells to efficiently compete with splicing factors. Several types of modified synthetic oligonucleotides fit these criteria. Amongst these, oligonucleotides with modifications to the 2' position, such as 2'-O-methyl (2'OMe), 2'-O-methoxyethyl (2'O-MOE) and 2'-O-aminopropyl, are RNaseH inactive and display higher nuclease resistance and affinity for target sequences than their 2'-deoxy counterparts. Similar characteristics are found in oligonucleotides with backbones based on morpholino (PMO), peptide nucleic acid (PNA), locked nucleic acid (LNA), phosphoroamidate and methyl-phosphonate derivatives (Figure 3). These advances in the development of antisense chemistries have led to numerous studies investigating the therapeutic potential of antisense technology (for review see (119)). AONs offer therapeutic options in several ways, including exon skipping, exon inclusion but also antisense mediated elimination of mRNA toxicity. The field has progressed very quickly over the last few years and promising results have been achieved, leading to several clinical evaluations. Notably, most of these advances have been made for NMDs such as DMD using exon skipping, SMA using exon re-inclusion or myotonic dystrophy (DM1) through elimination of mRNA toxicity. The remainder of this review focuses on the recent developments using these three NMDs as examples. In each case, we will first review the principle of the approach and the pre-clinical and clinical data from these initial trials and we will then discuss the promises and challenges of AON based therapy for NMDs, focusing on systemic delivery and novel molecules offering new perspectives.

4.1. Exon skipping approach for DMD

4.1.1. Background and rationale

As mentioned previously, most mutations causing DMD include deletions (~65 %), duplications, point mutations or other small gene rearrangements and disrupt the open reading frame, leading to aberrant translation and therefore to the absence of the essential muscle protein dystrophin. Interestingly, the allelic disease Becker muscular dystrophy (BMD) which results in a much milder phenotype, is mainly caused by mutations maintaining the open reading frame and allowing the production of a partially deleted but functional dystrophin (13). Antisense-mediated exon skipping strategies for DMD aim to remove the mutated exon alone or together with additional exons to restore the reading frame and consequently induce the expression of "BMD-like" shortened forms of dystrophin retaining crucial functions (Figure 4). Such exon skipping can be induced using antisense oligonucleotides (AONs) interfering with the normal splicing process by masking important splice sites (ss) such as specific 5' or 3'ss or bind to a splicing regulatory element (SRE), which can be intronic or exonic splicing enhancer (ISE or ESE) or intronic/exonic silencer (ISS or ESS).

4.1.2. Preclinical work

The principle of the exon skipping therapy for DMD has first been demonstrated by Pramono *et al* in 1996 in lymphoblastoid cells and by Dunckley *et al* in 1998 in cultured mouse cells *in vitro* (120-121). Since then, numerous *in vivo* studies have provided pre-clinical evidence for the therapeutic potential of an antisense strategy for DMD in several animal models. In particular, the mdx mouse model, which harbors a nonsense mutation in exon 23, has been used extensively to test efficacy of the AON approach using various oligonucleotides chemistries such as 2'OMe (122), phosphorodiamidate morpholino oligomers (PMO) (123-124), LNA or PNA (125-126). Systemic injections of 2'OMe AONs targeting the exon 23 in mdx mouse resulted in widespread dystrophin restoration in skeletal muscles but not in heart (127). More favourable pharmacokinetics and

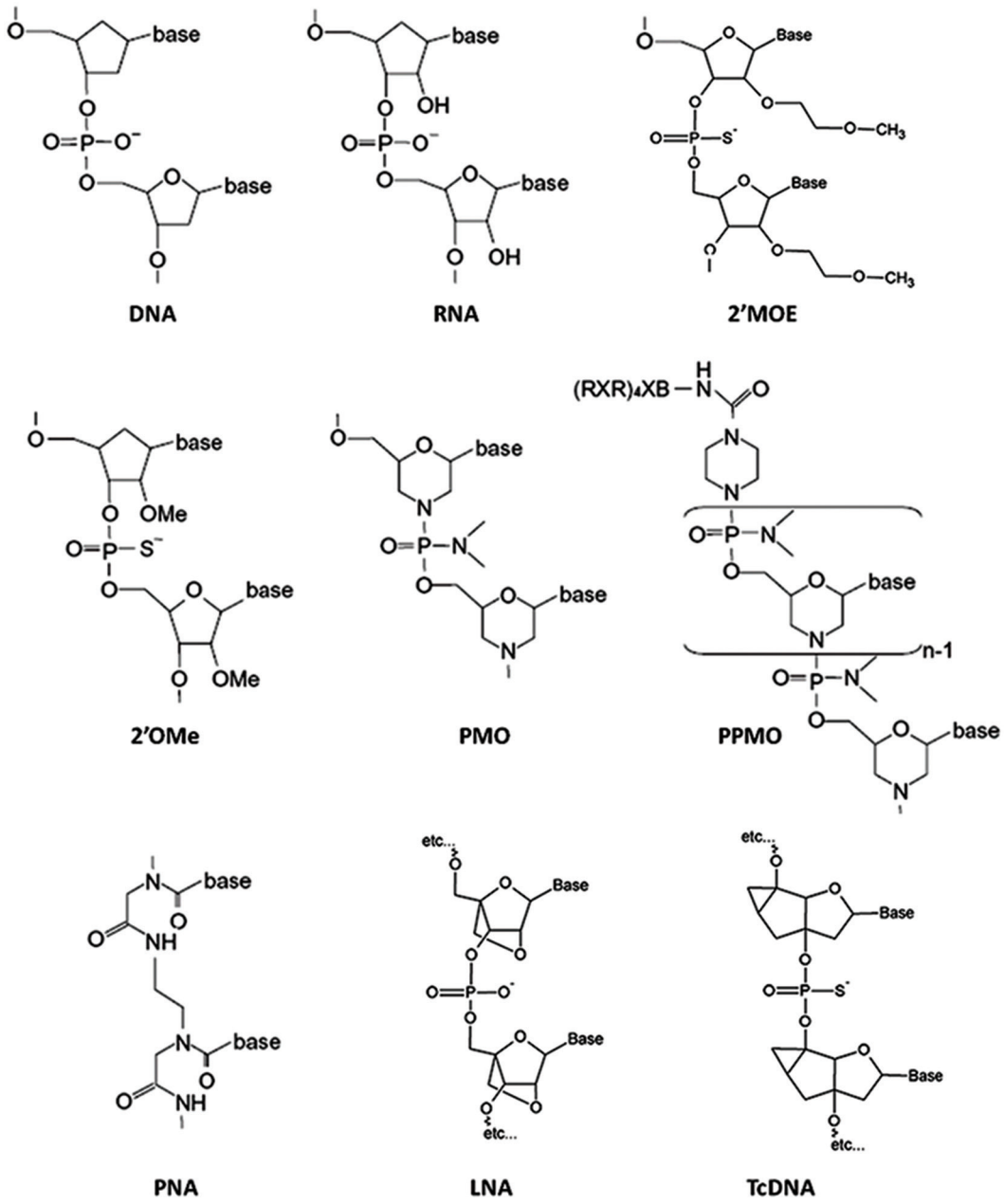

Figure 3. Various AON Chemistries. Artificially developed AON such as 2'OMethoxyethyl phosphorothiate (2'MOE), 2'O-Methyl-phosphorothiate (2'OMe), phosphorodiamidate morpholino oligomer (PMO), peptide conjugated PMO (PPMO), peptide nucleic acid (PNA), locked nucleid acid (LNA) and tricycloDNA (TcDNA) are shown for comparison with DNA and RNA.

pharmacodynamics were found following subcutaneous administration than intravenous or intraperitoneale, guiding the choice for future clinical applications (128). PMO AONs have also achieved widespread dystrophin restoration in mdx muscles following intravenous injections (129-130) and have been shown to significantly improve muscle pathology and locomotor activity after long term repeated administration (131). Evaluation of the therapeutic potential of PMO AONs has also been conducted in dystrophic dog, which requires skipping of both exon 6 and 8 for dystrophin restoration. Repeated administration of a cocktail of three PMO AONs led to significant levels of dystrophin restoration (132).

Following the encouraging results obtained on these animal models, antisense mediated exon skipping has rapidly moved towards clinical evaluation in DMD patients.

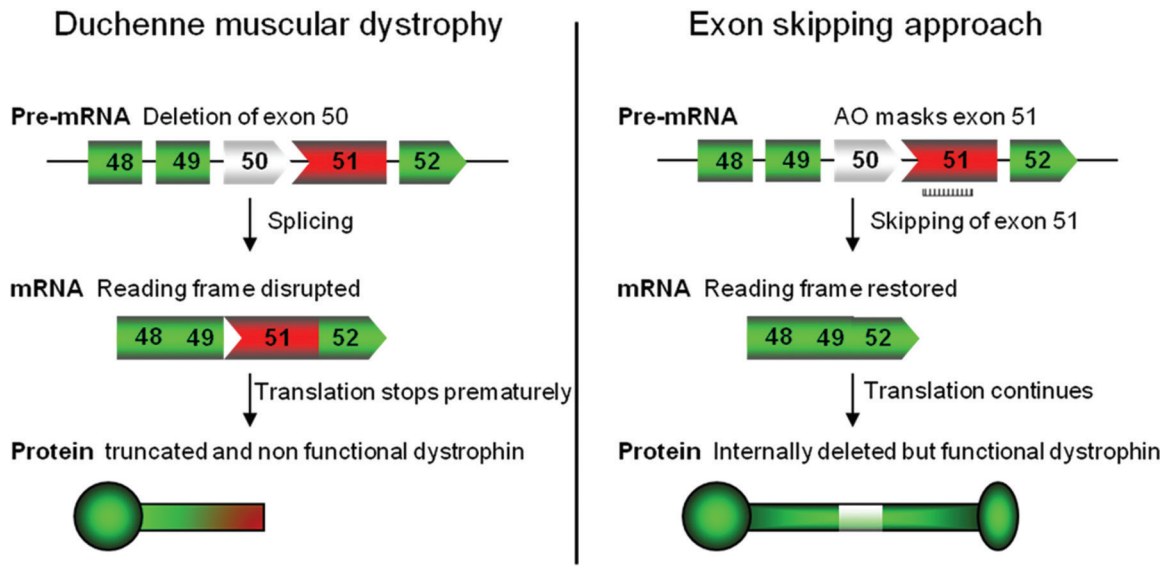

Figure 4. Antisense-mediated exon skipping rationale for DMD. Patients with Duchenne muscular dystrophy carry mutations disrupting the open-reading frame of the dystrophin pre-mRNA. In this example, exon 50 is deleted, creating an out-of-frame mRNA and leading to the synthesis of a truncated non-functional or unstable dystrophin (left panel). An antisense oligonucleotide (or modified small nuclear RNA) directed against exon 51 can induce effective skipping of exon 51 and restore the open reading frame, therefore generating an internally deleted but partly functional dystrophin (right panel).

4.1.3. Clinical trials of AON-mediated exon skipping in DMD

The clinical application of antisense mediated exon skipping for DMD raises the issue of the genetic heterogeneity of DMD patients meaning that this approach is mutation-specific and as such, an example of personalized medicine. According to the Leiden muscular dystrophy database, exon skipping is potentially applicable to ~83 % of all DMD patients if single and double exon skipping of deletions, small mutations and duplications can be achieved (133). Fortunately, the majority of deletions clusters into hotspot regions between exons 43 and 53, suggesting that skipping of the same group of exons is applicable to large groups of patients. The most notable example is exon 51 skipping, which is applicable to 13 % of all patients and has for that reason been targeted for first clinical trials.

2′OMe AONs were the first compounds to enter such trials led by the biopharmaceutical company Prosensa. Intramuscular injection of 0.8. mg PRO051 (also known as GSK2402968 or Drisapersen) into the tibialis anterior muscle targeting skipping of exon 51 resulted in 64–97 % of dystrophin positive fibres and 17–35 % of normal dystrophin protein levels (134). This led to an open-label, dose escalation systemic study, where five weekly injections of drisapersen at doses ranging from 0.5. mg/kg up to 6 mg/kg induced exon 51 skipping accompanied by low levels of dystrophin in 12 DMD boys (135). The follow up extension study for 12-weeks at 6 mg/kg reported stabilization of motor function in the boys. A subsequent Phase IIb placebo-controlled 6mg/kg/week study (ClinicalTrials.gov identifier: NCT01153932) of drisapersen again reported a significant benefit in 6MWT in treated DMD patients over the placebo group, which was maintained after 48 weeks of treatment, but with reduced significance (136). Unfortunately however, the concurrently ran phase III study (ClinicalTrials.gov identifier: NCT01254019), conducted on 186 DMD boys failed to meet the primary endpoint of a statistically significant improvement in the 6 minute-walk test (6MWT) (137). In January 2014, Prosensa and GlaxoSmithKline (GSK) announced that Prosensa had regained all rights from GSK to drisapersen and will retain rights to all other programs for the treatment of DMD. Although this transfer of rights represented the termination of the collaboration agreement between GSK and Prosensa executed in 2009, Prosensa announced in October 2014 that it has commenced the submission process for a New Drug Application (NDA) regulatory filing to the United States Food and Drug Administration (FDA) for drisapersen for treating DMD (http://ir.prosensa.eu/releasedetail.cfm?ReleaseID=875633).

PMO AONs have also entered clinical trials in DMD patients when intramuscular injections of a 30-mer PMO optimized to skip exon 51 (AVI-4658 also known today as Eteplirsen) was injected unilaterally into the

extensor digitorum brevis muscles of 7 patients, in a single-blind, dose-escalation protocol. Results from this trial demonstrated that PMO AONs were well tolerated by all patients and that dystrophin protein were expressed at up to 42 % of normal levels in dystrophin positive fibers of patients treated with the higher dose of 0.9. mg (138). Subsequently an intravenous PMO dose-escalation study was carried out on 19 DMD patients (139). This trial showed good tolerance of Eteplirsen from 0.5. mg/kg up to 20 mg/kg with no drug-related adverse events after weekly infusions for 12 weeks. Exon 51 skipping was detected at all doses and restored dystrophin protein was seen from 2 mg/kg. Degree of response was variable amongst patients, but the best DMD "responders" displayed 15, 21 and 55 % of dystrophin positive fibers. This was followed by a placebo-controlled phase IIb trial using higher doses of 30 and 50 mg/kg/week of Eteplirsen in ambulant boys. Following 48 weeks of treatment, results showed a number of dystrophin positive fibres up to 52 and 43 % in the 30 and 50 mg/kg cohorts respectively (140). Eteplirsen treated patients also showed a benefit of 67 m less decline in 6MWT over the placebo group and stabilization of the motor function in DMD boys participating in Eteplirsen extension study has now being observed over 2 years. However it should be noted that the patient numbers in this trial remain small (only 12 subjects) and no completely untreated control group is available for comparison beyond 24 weeks since placebo patients switched to 30 or 50 mg/kg Eteplirsen at week 25 (treatment was open label thereafter). Sarepta Therapeutics, the leading biopharmaceutical company has recently received guidance regarding an NDA submission from the FDA, which states that additional data are required as well as further discussion between the sponsor and the FDA to determine what would constitute a complete NDA. Sarepta is therefore planning a confirmatory phase III Study of Eteplirsen in DMD Patients (ClinicalTrials.gov Identifier: NCT02255552) as well as a safety study in advanced stage DMD patients (ClinicalTrials.gov Identifier: NCT02286947).

Apart from exon 51 skipping, both Prosensa and Sarepta are developing clinical applications targeting other exons, including exons 44, 45 and 53 and some of these trials are currently on going.

4.1.4. Vectorized approaches

Amongst the challenges faced by the AON-mediated exon skipping approach is the long-lasting effect of the therapy. Since the duration of the effects of AONs is limited *in vivo*, patients have to be re-injected weekly or monthly to maintain therapeutic levels of dystrophin. An alternative to this limitation is to deliver the antisense sequence using viral vectors and in particular AAV vectors which are extremely efficient for muscle gene transfer, as discussed in the first part of this review. Studies using U7 or U1 small nuclear RNA (snRNA) to carry the AONs have demonstrated efficient and sustained rescue of dystrophin in *mdx* mice (141-142). Linking the antisense sequence to a snRNA enhances its activity by allowing its proper subcellular localization and thereby facilitates the AON inclusion into the spliceosome (143-144). The AAV-U7 snRNA approach has also been evaluated in dystrophic dog following systemic injection and demonstrated efficient dystrophin and muscle function rescue (111). However, this study also highlighted a progressive loss of dystrophin positive fibers over a 5-year follow up period, which was also demonstrated in the severe double knock out (dys-/utr-) mouse model (59), suggesting that repeated administration may still be required. Nevertheless, this vectorized approach offers the possibility to efficiently deliver multiple snRNA cassettes to target multiple exons which could be extremely useful to overcome the personalized medicine issue for DMD treatment (145). In addition, this type of viral vector mediated exon skipping approach can be combined with cell-based therapies using lentiviral vectors to correct the reading frame of muscle stem cell before re-injection, as described previously in this review (31).

4.2. Exon inclusion for SMA

4.2.1. Background and rationale

As mentioned previously, SMA is caused by mutations in the SMN gene. The SMN locus presents a telomeric copy SMN1 which encodes the SMN protein, and centromeric copies SMN2 which produce very few SMN proteins. The SMN2 differs from SMN1 by only 5 nucleotides and only one is in the coding sequence, in exon 7, it is a C to T transition which does not affect the protein sequence. SMA occurs when SMN1 is mutated on its two alleles. However the severity of the disease is alleviated by the copy number of SMN2. As an example, severe forms (type I) carry 2 copies while type III SMA patients carry three or four SMN2 copies. This can be explained by the mechanism of splicing of exon 7 of SMN2 gene. The non-polymorphic transition located at position 6 of exon 7 in SMN2 (C6T) is responsible for alternative splicing; and when the nucleotide is a cytosine, exon 7 is correctly recognized and included as is the case for SMN1; when the nucleotide is thymine, as is the case for SMN2, exon 7 is excluded in 90 % of the transcripts (146-147). Based on this experimental observation some therapeutic approaches aiming at rescuing the splicing of SMN2 exon 7 have been developed.

4.2.2. Exon 7 definition

More fundamentally, two models have been proposed to explain the inhibitory effect of this transition C6T. According to the first model, the transition eliminates a cytosine-thymine activator exon element, an ESE (Exon

Splice Enhancer) crucial to the definition of exon 7 in the splicing reaction (148-149). ESE are small sequences of pre-messenger RNA recognized for protein permitting the recruitment of factors recognition of exon-intron junctions and the assembly of the spliceosome (150). In the case of exon 7 of the SMN1 gene, the ESE (+6, +12; CAGACAA) allows the recruitment of splicing protein SF2 / ASF, which could not be recruited in the SMN2 pre-mRNA. The other model goes further and assumes that the transition C6T causes the replacement of the ESE by an ESS (+6, +12; TAGACAA) (Exon Splice Silencer) that recruits splicing factors inhibitors such as hnRNP A1 protein (151). These two models are not mutually exclusive of each other and both apprehend what is happening in terms of phenomenological view: the strength of the acceptor site of exon 7 is greatly reduced contributing to the exclusion of exon 7 in 90 % of SMN2 mRNA (Figure 5).

Moreover, it was also proposed that the transition C6T near the 3' end side of exon 7 would cause an inhibitor splicing context in the 5' region of exon 7 of the SMN2 gene (152). We can observe the appearance of a stem-loop structure of RNA, called Terminal Stem-Loop 2 (TSL2) that sequesters the donor site of exon 7 (153). Finally, an ISS site (Intronic plice Silencer) has been identified in intron 7 separated by only nine bases from the 5' splice site of exon 7. ISS are small intron sequences that allow the recruitment of splicing inhibitors. The ISS, called ISS-N1 contributes to the low resolution of the splice donor site downstream of exon 7 (154). It is likely that this pattern reinforces the effect of the mutation on the TSL. Thus SMN2 gene produces approximately 90 % of transcripts missing exon 7 and only 10 % complete transcripts and thus functional protein. The spliced transcript lacking exon 7, named SMN2Δ7, encodes a protein missing 16 residues on the carboxy-terminal side which is very unstable and functionally defective (155).

4.2.3. Therapeutic strategies using antisense sequences

As part of the proposed therapies for SMA, the re-inclusion of exon 7 of the SMN2 gene is particularly promising. For this purpose, antisense sequences or vector AONs targeting different sites important for splicing have been used. Two main strategies have been investigated to re-include this exon, either by targeting the 3' splice site of exon 7 containing the ESS to strengthen the acceptor site, or by targeting the end of the exon and / or ISS present in intron 7 in order to make the donor site stronger.

4.2.3.1. Strategies with oligonucleotides

A SELEX study (Systematic Evolution of Ligands by Exponential enrichment) conducted *in vivo* on the exon 7 of SMN2 shows that both 5' and 3' regions seem to predispose to the exclusion (156). The SELEX method is a method of *in vitro* selection from combinatorial libraries of synthetic oligonucleotides. The selected oligonucleotides are capable of selectively bind a given ligand, with a high and specific affinity. Originally developed with RNA, this technique was then generalized to DNA oligonucleotides. This technique of *in vitro* selection has been adapted in this study to investigate the sequence of exon 7 *in vivo*.

One of these antisense strategies is based on the idea that the skipping of exon 7 is partly due to a competition between the 3' splice site of exon 7 and the one of exon 8. To change the balance in favor of the 3' site of exon 7, 2'OMe oligonucleotides targeting the 3' splice site and the branch point of exon 8 were tested. The oligonucleotides targeting the 3' splice site tested *in vitro* showed a 50 % inclusion of exon 7. However, this led to a retention of intron 7, but this does not normally affect the protein, due to the stop codon located in exon 7. Oligonucleotides targeting the BP have not shown re-inclusion of exon (157).

Another strategy is to use bifunctional AONs. Two groups simultaneously developed this method in 2003, which consists of adding a functional antisense group. Both teams have targeted C6U transition in exon 7 of SMN2, and used functional groups able to recruit splicing protein. In the first case, they have coupled a peptide containing a synthetic sequence of the RS domain of SR proteins to a PNA AON (158). In the second case, a repeated sequence mimicking an ESE recognized by the SF2 / ASF protein has been added to a 2'OMe AON (159). These two approaches showed re-insertion of exon 7 close to normal levels, but only a partial restoration of the level of SMN protein in fibroblasts of patients. Another group chose to reduce the strength of the acceptor site of exon 8 using 2'OMe coupled to a sequence capable of recruiting hnRNPA1 splicing inhibitory proteins (160). These molecules have been tested on fibroblasts from SMA patients and SMNΔ7 mouse models. After IV injection, they detected a rise in the level of protein in the liver and kidneys. The level of exon 7 inclusion was increased in the brain 24 hours after ICV injection. However, the longer term effect of the molecule was not studied on these mice. Another group targeted the ISS present in intron 6 (E1 intronic repressor sequence) coupled to a group capable of recruiting the splicing activator proteins (161). Such 2'OMe AONs induced a two fold increase in SMN protein, an increase in weight and a slight increase of the survival of mice SMNΔ7 after ICV injection. Another recent study used bifunctional 2'OMe AONs targeting ISS-N1 with a group capable of recruiting the SR protein. ICV injection of these AONs in SMA mouse models showed improved survival (20 days compared to 10 days for untreated mice), weight gain

and the presence of the SMN protein in the brain and spinal cord (162).

Some of the most promising studies using AONs for the inclusion of exon 7 are those of the Krainer group. Indeed, they used different 2'OMOE AONs covering the entire surface of exon 7 and its surroundings in order to find an optimal antisense sequence to induce exon inclusion (163). They chose a 17-mers antisense sequence targeting ISS-N1. This AON showed a rescue of the type III SMA mice phenotype (necrosis of the ears and tail) after ICV injection (164). They then expanded their study by injecting these 2'OMOE subcutaneous in a mouse model of severe SMA aged 1 and 3 days. They were able to show an increase in the survival of mice (108 days compared with 10 days for untreated mice) (165). Additional injections at 5 and 7 days post-natal increase survival of these mice to 137 days. This improvement on survival was shown to be dose dependent and increased to 248 days with two injections at the highest dose (160 mg/g). However, a similar treatment on mice aged 5 and 7 days showed no significant improvement. Subcutaneous injections in a severe SMA mouse model showed that peripheral systemic AON delivery was more effective than ICV injection at extending lifespan (165). These results suggest an important role for peripheral SMN function in the disease process. The role of the ubiquitous SMN protein is not totally known, but the snRNP assembly activity of the SMN/Gemin complex is clearly involved in a general cellular function required for survival of any cell or tissue type (166).

Studies have also been conducted using PMO AONs targeting ISS-N1 on SMNΔ7 mouse model. A single ICV injection at birth showed increased survival of these mice up to 100 days (compared to 15 days for untreated mice) (167). The best PMO sequence described targeting the ISS-N1 is a 25-mer (168). More recently, PMO targeting the E1 repressor sequence have also been used in two SMA mice model (severe and intermediate model), leading to significantly extended life span (54 days in severe model, and up to 700 % in intermediate model). The best results were obtained when the ICV injections were coupled with IP injection, confirming the peripheral role of SMN (169).

Currently, clinical evaluation is on going with 2'OMOE AONs targeting the ISS-N1. This molecule (ISIS-SMNrx) is still being tested in phase II/III clinical trial (ClinicalTrials.gov identifier NCT02193074). The first phase II trial of ISIS-SMNRx conducted in infants with type I SMA has provided encouraging results, with several improvements on standardized tests of muscle strength and function (ClinicalTrials.gov identifier NCT01839656). Similarly, the phase II trial of ISIS-SMNRx in children aged 2-15 years old with type II and type III SMA showed increased muscle function scores after multiple injections of ISIS-SMNRx into their spinal fluid, as well as evidence that the drug was biologically active in spinal fluid (ClinicalTrials.gov identifier NCT01703988).

4.2.3.2. Vectorized approaches for SMA

Several U7 SmOPT targeting the 3' end of the ss and the BP region of exon 8 of SMN2 were found to induce inclusion of exon 7, using the same antisense sequences as in Lim and Hertel (157). Inclusion of about 70 % has been observed *in vitro* with these U7snRNAs targeting the 3' splice site of exon 8 (170). The group of Daniel Schümperli has pioneered this strategy of vectorized antisense sequence with UsnRNA to induce the inclusion of exon 7. They first used modified U2snRNA complementary to the BP (171). However, they observed no improvement in SMN2 splicing. It was suggested that the U2 modification interferes with the U2 snRNPs assembly or that the limiting step in the definition of SMN2 exon 7 is not connected to the U2snRNP but prior recognition of the region by the factor BP splice SF1. Another strategy of this group was to change the U1snRNA to target the 5' ss of exon 7. This approach showed a strong inclusion of exon 7, but stable expression of U1snRNA was toxic to the cells (172). They then targeted ISS-N1 with U7smOPT, but this caused an even greater skipping of exon 7, presumably due to steric interference with the U7 snRNP near the 5 'ss (173). Overall, the most encouraging approach was to adapt the strategy of bi-functional AON into U7smOPT. A construct targeting the 3' region of exon 7 with an ESE tail showed 90 % of SMN2 mRNA with exon 7 of SMA patients cells. In SMA mouse model, they observed an increase in survival (124 days compared to 5-7 days for untreated mice), better muscle performance and a normal number of neurons at 1 month of age (174). Structural alterations of neuromuscular junctions present in the diaphragm of SMA mice were also corrected with this U7smOPT treatment (175).

4.3. Antisense strategy for Myotonic Dystrophy

4.3.1. Background on Myotonic dystrophies

Myotonic dystrophy is the most common adult-onset muscular dystrophy and the second most common cause of muscular dystrophy after Duchenne muscular dystrophy (DMD), affecting approximately 1 of 8000 (in Caucasians) to approximately 1:20,000 (Asian and African populations) people worldwide (176-177). Founder effects may increase the prevalence in specific regions, such as Quebec where the prevalence is approximately 1/500 (178). Myotonic dystrophy is an autosomal dominant inherited disease

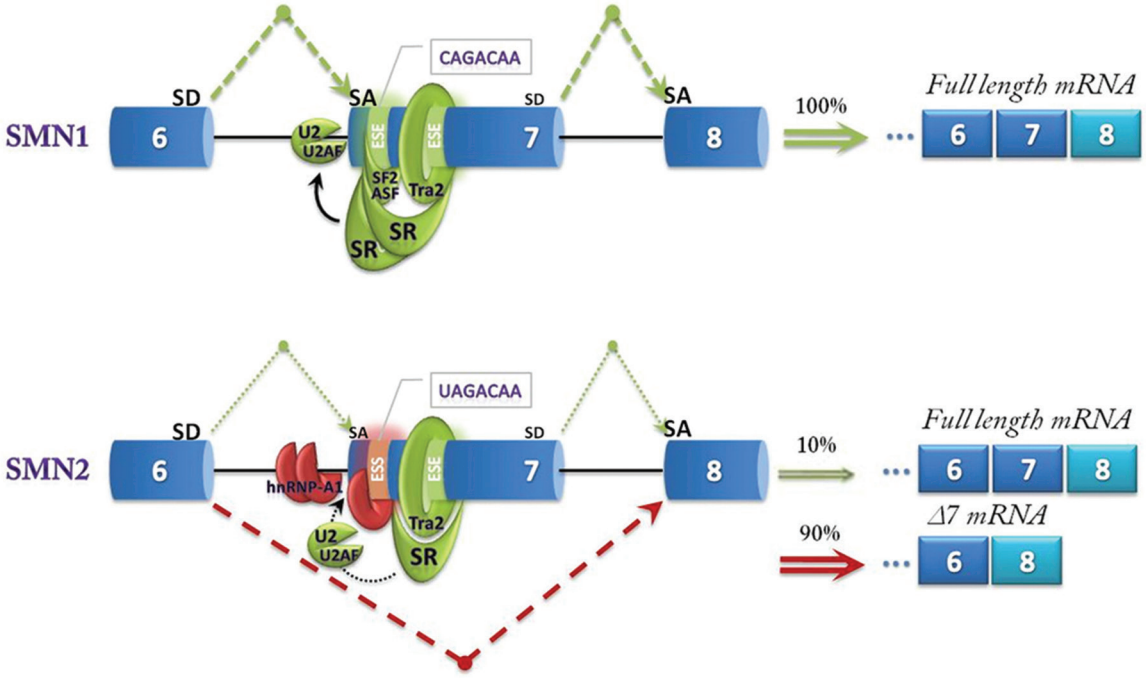

Figure 5. SMN1 and SMN2 mRNA splicing. Exon 7 of SMN1 gene is correctly included in 100 % of cases due to a strong acceptor site with the presence of an ESE, which allows the recruitment of essential protein splicing such as SF2 / ASF protein, SR proteins and the U2 factor. The exon 7 of the SMN2 gene is included in about 10 % of cases due to the transition C6U that converted the ESE to ESS which considerably weakens the acceptor site of exon 7. This ESS site does not allow the recruitment of splicing proteins but rather inhibitory proteins that will mask the site with specific protein interactions, such as hnRNP A1. Exon 7 is excluded in 90 % of cases by a conventional mechanism of alternative splicing, due to the strong acceptor splice site of exon 8.

affecting skeletal muscle, heart, brain, lens and endocrine organs. Two myotonic dystrophies can be observed: type I (DM1, Curschmann-Steinert) and type II (DM2).

DM1 is triggered by expanded CTG repeats in the 3' untranslated region of the Dystrophia Myotonica Protein Kinase (DMPK) gene (176, 179), and DM2 by expanded CCTG expansion in intron 1 of the ZNF9 gene (180). In patients with myotonic dystrophy type 1, CTG expansions range from 51 repeats to several thousands, whereas healthy individuals carry only 5 to 37 repeats (181). In patients with myotonic dystrophy type 2, the average of CCTG repeat length is about 5000 while healthy alleles contain fewer than 30 copies of the CCTG repeat (182). Results from several laboratories have shown that CUG or CCUG RNA repeats play a trans-dominant role in DM pathogenesis. By interfering with the function of RNA-binding proteins, these expansions may trigger downstream signaling pathways or other mechanisms that result in cellular toxicity (183-184).

At least two mechanisms account for the repeat RNA-induced trans-dominant toxic effects. First, the CUGexp RNA forms a double-stranded hairpin structure that directly binds to the muscleblind-like 1 (MBNL1) regulatory splicing fact with high affinity, resulting in MBNL sequestration and loss-of-function (185-186). The sequestration of MBNL1 leads to the misregulation of the alternative splicing of several transcripts (187). For example, the myotonia and weakness phenotypes of DM1 have been associated with the mis-splicing of the chloride channel (CLCN1), of the insulin receptor (IR), of the cardiac troponin T (TNNT3) and more recently the bridging integrator 1 (BIN1) transcripts (188-190). Secondly, CUGexp RNA activates protein kinase C (PKC) and upregulates CELF1 (CUGBP1 and ETR-3-like factor) protein levels by hyperphosphorylation, resulting in its gain-of-function (191-192) (Figure 5).

4.3.2. Therapeutic strategies using antisense sequences

4.3.2.1. Strategies with oligonucleotides

Inhibition of this RNA toxicity by targeting the CUG repeats represents a valuable therapeutic strategy for this disease (193) (Figure 5). Approaches that target directly the CUG repeat expansion by using small antisense (CAG) n sequences has been evaluated. The use of (CAG) 25 morpholinos in DM1 transgenic mice resulted in reduction of nuclear foci and redistribution of MBNL1 protein (194). The biochemical consequence of the MBLN1 release is the correction of alternative splicing for more than 14

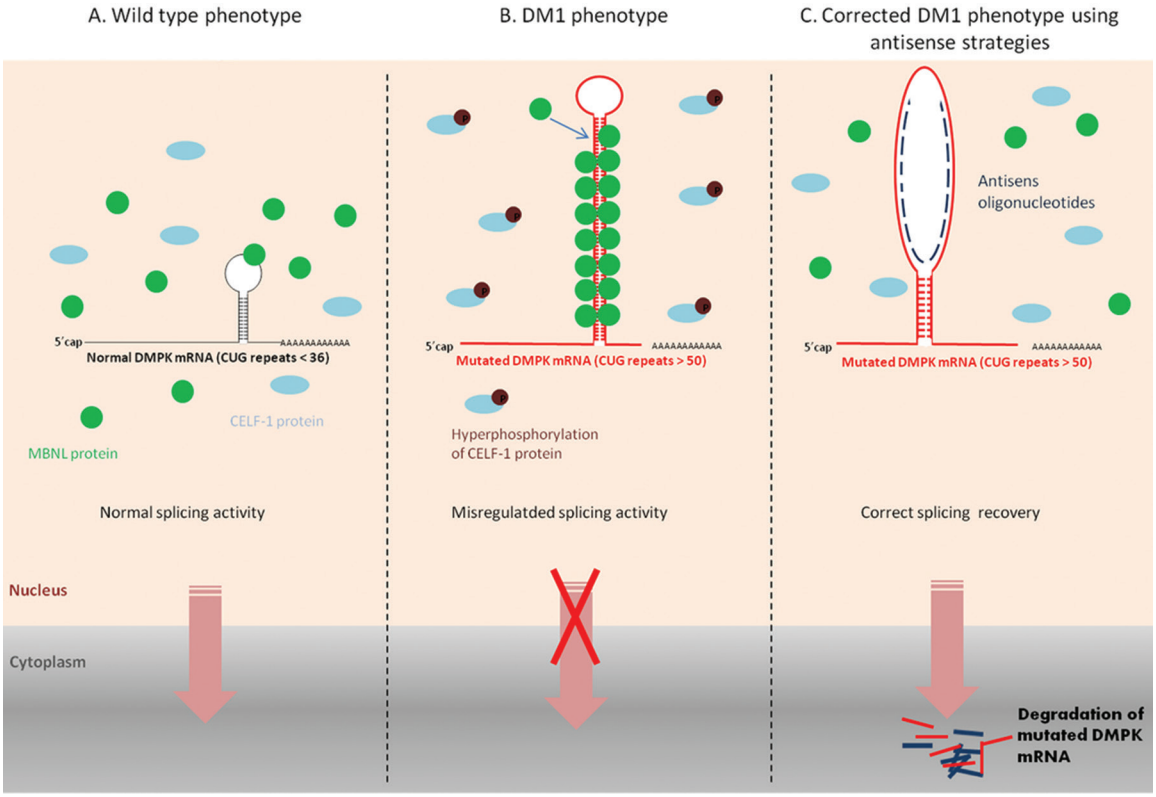

Figure 6. Therapeutic strategy using antisense oligonucleotides for DM1. A. In normal cells, the DMPK gene forming hairpin structures in its 3'non-coding region, is transcribed and exported in the cytoplasm to be translated. Alternative splicing of a specific group of transcripts is especially due to the activity of MBLN-1(in green) and CELF-1 RNA binding proteins (in blue). B. In DM1 cells, the expanded CUG repeats lead to the sequestration of MBLN-1, forming a ribonucleoprotein complex that aggregates into the nucleus. The nuclear export of the DMPK mRNA is inhibited and the CELF-1 protein is converted to its hyperphosphorylated form by mechanism implying PKC activation. Both, hyperphosphorylated CELF-1 and sequestered MBLN-1 cause misregulation of alternative splicing, in large part responsible for the DM1 phenotype. C. (CAG)n antisense molecules, able to strongly compete with MBLN-1 attachment by matching with expanded CUG repeats, release this protein into the nucleus. The mutated DMPK mRNA can be exported to the cytoplasm where it is degraded. Rehabilitation of normal concentrations of nuclear MBLN-1 regulates the CELF-1 activity, removes the nuclear foci, and corrects aberrant alternative splicing.

weeks after a single injection of the morpholino. Another therapeutic effect is the export of the CUG repeats into the cytoplasm and their subsequent degradation. Physiologically, (CAG) 25 corrects the expression, cellular localization and function of the muscle specific chloride channel (CIC-1), resulting in marked reduction of myotonia. In the same way, the use of (CAG) 7 2'OMe induces a selective degradation of the mutant transcripts in DM1 cells and clearly improves DM1 pathology in mice models by reducing nuclear foci and correcting alternative splicing of different transcripts (195). A recent study using PPMO AONs bearing a CAG repeat sequence show redistribution of MBNL1 protein in myonuclei and corrections of abnormal RNA splicing in mouse model of DM1 following systemic administration (196).

4.3.2.2. Strategies with RNAase H- active oligonucleotides

Another approach to eliminate RNA toxicity is to directly degrade the toxic RNA. Different studies have demonstrated the promising potency of RNase H-active ASOs targeting the CUG^{exp} RNA *in vitro* in patients cells and *in vivo* in DM mouse models. LNA- or MOE-flanked gapmers CAG sequences complementary to the CUG repeats can effectively reduce the levels of toxic RNA and disrupt RNA foci in a cell culture model of DM1 (197). These effects appeared highly specific to expanded CUG transcripts, as repeats of normal length were not significantly affected, suggesting the selectivity of the gapmer targeting the mutant allele. When administered to the skeletal muscle of a DM1 mouse model by direct injection, gapmers targeting the CUG repeats also showed efficacy in removing the CUG^{exp} RNA, but induced muscle damage. Another study using subcutaneous injection twice a week during 4 weeks of MOE gapmers in transgenic mice showed significant knockdown of hDMPK-CUG^{Exp} transcripts in hindlimb muscles, supporting the feasibility of silencing the pathogenic DMPK allele. Moreover, the effet of these AONs was sustained 1 year after treatment (198).

A phase I clinical trial is currently being conducted outside the United States to evaluate the safety of ISIS-DMPKRx in healthy volunteers. A Phase I/IIa study to evaluate the safety and dose-range finding of ISIS-DMPKRx in patients with DM1 is being planned for the end of the year 2014.

4.3.2.3. Strategies with vectorized antisense sequences

A modified human U7 small nuclear RNAs (hU7-snRNA-$(CAG)_{15}$) was also tested for its capacity to correct the DM1 defect (199). *In vitro* transduction of a lentiviral vector encoding this hU7-$(CAG)_{15}$ led to marked reduction of expanded DMPK mRNAs with little or no effect on the wild-type DMPK transcripts. Furthermore, this study shows that the length of the CAG antisense sequence is crucial since constructs with fewer than 15 CAG repeats can alter the expression of some transcripts bearing (CUG) n tracts. The precise mechanisms leading to the inhibition of RNA toxicity by using small antisense sequences targeting the expanded CUG repeats are not yet fully understood. However, breaking of the expanded CUG RNA/MBNL1 complexes and/or preventing the formation of new foci by using (CAG) n antisense sequences may allow degradation of the mutant transcripts by endogenous RNase H independent processes. Altogether, these antisense approaches indicate that the targeting of expanded repeats is a promising therapeutic strategy for DM1 and more extensively for many triplet expansion disorders.

4.4. Challenges and new advances for splicing therapies

4.4.1. Challenges

Although results from initial trials appear encouraging and raise expectations to a high level for the treatment of NMDs, there are several issues that pose challenge for the use of the current and first generation AONs as effective and affordable drugs for NMDs. As observed in the trial results for DMD, functional benefit is difficult to achieve mainly because of the poor cellular uptake and relative rapid clearance of 2'OMe or PMO AONs from circulation, which impose repeated administration to achieve some therapeutic efficacy. Experiments in animal models had already demonstrated that large doses ranging from 100 mg/kg/wk in mouse (129) to 200 mg/kg/wk in dogs (200) over several weeks were required for functional improvement. The fact that both animal models required large doses, despite their difference in body surface area, suggested that similar dose range might be required to achieve efficacy in humans. If so, the cost of an AON drug, type PMO for example, for life-long treatment will probably be prohibitive for many patients.

A second major hurdle with the systemic administration of AON is the high variability in exon skipping efficiency among muscle types. Again data collected from mice and dog studies have shown that some muscles respond better than others. Repeated intravenous injections of 100 mg/kg of PMOE23 to mdx mice resulted in higher restoration of dystrophin in quadriceps, abdominals, intercostals and gastrocnemii muscles compared to diaphragm, biceps, triceps and tibialis anterior for example (129). Even within the most responsive muscles, dystrophin was not uniformly expressed with detection of patches of dystrophin-positive fibers and patches of dystrophin-negative fibers and this was also observed in clinical trials.

An additional problem compromising the therapeutic potential of AON-mediated exon skipping for DMD at the moment is that both AON chemistry used in the clinical trials have shown very little efficacy in the cardiac muscle (127, 129). Studies have showed that even higher doses (nine doses of 100 mg/kg) induced only very low level (1-2 %) of dystrophin expression in heart with both 2'OMe and PMO (201). Only when used in huge amounts such as 300 mg/kg and 3000 mg/kg, PMOE23 could restore about 5 % and 30 % respectively of wild-type dystrophin level in the heart of mdx mice (202). Such extreme dose would be unsustainable for repeated administration and the long term treatment required for DMD. Clinically, cardiomyopathy is the second leading cause for the death in patients with DMD in countries where ventilator therapy has been introduced, accounting for 10-40 % of deaths in DMD populations (10), which implies a clear need for cardiac dystrophin correction.

Recent developments using cell penetrating peptides (CPP)-conjugated PMO (PPMO) and novel DNA analogs, tricyclo-DNA (tcDNA) AONs have addressed most of these delivery issues and could therefore represent an effective strategy to reduce dose level and dose frequency, as well as delivering the AON to non leaky fibers and the heart (202-207).

4.4.2. Promises of new molecules for DMD

Since the first study reported the enhanced PMO uptake mediated by a cell penetrating peptide, resulting in widespread restoration of dystrophin in mdx mice in 2007 (124), PPMO have gained attention for the systemic treatment of DMD. Many laboratories have demonstrated uniform and high level of dystrophin expression through the whole body using much lower

Table 2. Summary of the advantages and disadvantages between gene and splicing therapy approaches

Approaches	Advantages	Disadvantages
Gene therapy	. High transduction efficiency	. Reaction against viral particles : risk of anaphylactic shock
	. Long term effect	. Pre-existing host immunity : no or very limited re-injection
	. Several tissue tropisms: existing of several serotypes (see table 1)	. Weak tissue tropism specificity
	. No mutation-dependant approach	. Transgene under exogenous promoters (non natural)
	. Scalable processes	
Splicing therapy	. Natural promoter : conservation of natural gene expression levels	. Risk of toxicity at high doses
	. No immune response : possibility of repeated injections	. High production costs
	. Chemical modifications : improvement of bioassimilation	. Personalized medicine : mutation-dependant (DMD)

dose of PPMO compared to PMO (203, 208-209). PPMO are much more effective than PMO because of the ability of CPP to facilitate the internalization of PMO through an active process, unlike the passive diffusion process for PMO. PPMO are internalized by nearly all muscle cells, and therefore not restricted to leaky fibers as are PMOs (206). Moreover, evidence of significantly restored cardiac dystrophin has been demonstrated in PPMO-treated mice (202-205). A single intravenous injection of 30 mg/kg of PPMO indeed restored dystrophin to almost normal levels in the cardiac and skeletal muscles of mdx mice, which leads to increase in muscle strength and more importantly improvement of cardiac function (205). Further refinements and modifications of the arginine-rich peptide design have led to development of the Pip (PMO-internalizing peptide) series of CPPs. These peptides have two cationic arginine-rich domains either side of a hydrophobic central region and have been shown to improve both skeletal muscle and heart delivery of PMO in mdx mice (210-211).

Taken all together, these studies indicate that PPMO can be used at much lower dose than PMO and can achieve more widespread restoration of dystrophin throughout the whole body's muscle including the heart. These characteristics would qualify PPMO as an ideal candidate for the systemic treatment of DMD compared to unconjugated AON. However, the toxicity of current PPMO chemistry poses a challenge for determination of an effective and safe regimen in man. One PPMO targeting human exon 50 (AVI-5038) was tested in cynomolgus monkey (206) and found to cause mild tubular degeneration in the kidneys of monkeys injected weekly with 9 mg/kg for 4 weeks, although the same peptide conjugated to PMOE23 did not exhibit any toxic effect in the kidneys of mdx mice treated with higher doses (30 mg/kg biweekly for 3 months) (205). This indicates that monkeys are more sensitive to PPMO-related toxicity than mice. The nature of the toxicity is not well understood, but is likely to be due to the cationic nature of the peptide. A dose threshold for the toxicity seems to exist, which level depends on the amino acid composition of the peptide (206). These pre-clinical data provide valuable information and emphasise the difficulty in predicting exon skipping efficacy/toxicity across species and to plan efficient yet safe escalation in human patients.

More recently, a novel class of AONs made of tcDNA, which displays unique pharmacological properties and unprecedented uptake in many tissues after intravenous administration, demonstrated very encouraging results for the systemic treatment of DMD (207) and SMA (Robin *et al.*; manuscript in preparation). In DMD mouse models, we showed that systemic delivery of tcDNA-AONs allow high levels of dystrophin rescue in skeletal muscles as well as in heart, leading to physiological improvement of the cardio-respiratory functions. In this study, tcDNA were directly compared to 2'OMe and PMO AONs and induced 5-6 fold higher levels of skipping than 2'OMe and PMO at the higher dose (200 mg/kg/wk) and 3-4 fold higher levels with an equimolar dosing regimen. Remarkably, exon 23 skipping and dystrophin were evidenced in the central nervous system of animals exclusively following treatment with tcDNA, although levels were much lower than in skeletal muscles, suggesting the ability of tcDNA AONs to cross the blood brain barrier. Importantly, this restoration of dystrophin led a correction of neurobehavioural features linked to the absence of dystrophin in the brain. This makes the tcDNA-AON chemistry particularly attractive for future therapies in DMD patients as well as in other neuromuscular disorders or diseases eligible for splice-switching approaches requiring whole-body treatment. In particular, tcDNA have also been used in SMA mouse models and shown to induce efficient exon 7 inclusion in skeletal muscles, spinal cord and brain following systemic delivery (Robin *et al.*, manuscript in preparation). This could represent a promising advance since the currently most effective

delivery of AONs for SMA patients by intrathecal injection is invasive.

5. CONCLUSION

Within the last few years the prospect of successful gene or splicing therapy for neuromuscular disorders has moved a step closer, particularly for DMD and SMA but also for myotonic dystrophy. Many of these promising therapies have now entered clinical trials and encouraging results have been obtained in most cases. These trials have demonstrated how critical it was to design the endpoints and to collect data as accurately as possible, so that real guidance may be provided for later clinical trials targeting other exons or for gene replacement therapy. In the case of DMD for example, the efforts made by several large collaborative groups of physicians and scientists in the design and execution of these large clinical trials cannot be appreciated enough as we have learnt invaluable lessons about the natural history of the disease, the practicability and utility of clinical trial procedures and outcome measures. One of the most important lessons learnt is that, clinical endpoints such as 6MWT are important for assessing the disease progression, but total dominance of this principle could lead to misleading ideas. One should keep in mind that clinical benefit cannot be achieved without the production of a therapeutic amount of relevant protein product.

Despite the promising results of initial trials for these NMDs, many challenges remain such as optimal and targeted delivery for viral vectors as well as AONs, but also regarding the broad applicability of these therapies (as summarized in table 2). While gene therapy approaches could be applicable to all patients, the pathway for the development of essentially individualized novel therapeutic compounds for the treatment of the small numbers of patients with rarer mutations is less clear. The different deletions that cause DMD require skipping of different exons, and therefore design and optimization of many specific AONs. On the top of being an insuperable barrier in terms of money and time, it might be very problematic to find enough patients to even perform clinical trials. Some AONs may be applicable to a very restricted number of patients such as those targeting exons 71, 72, 75, 77 or 78 (representing 0.0.2 % of all mutations) (133). Close collaboration with regulatory agencies both in Europe and the USA will be required to hopefully lead to new ways of regulating such “personalized” therapies. Unlike DMD, a same antisense sequence could treat all SMA patients. The discovery of the ISS-N1 sequence in particular seems to be an effective target for the inclusion of exon 7 as we have previously mentioned.

Recent preclinical work in animal models using novel types of viral vectors, or new class of AONs such as PPMO or tcDNA suggest that solutions to the challenge of systemic and tissue targeted delivery are close at hand. While we focused this review on DMD, SMA and DM1, it should be noted that many other NMDs could benefit from the progresses described here for gene and splicing therapies. Several preclinical studies have actually demonstrated the therapeutic potential of gene therapy for limb girdle muscular dystrophies and clinical evaluation has shown promising results (as reviewed in (212)). More recently, AAV vectors have also been used successfully in a mouse model of DOK7 myasthenia (also called familial limb-girdle myasthenia) (213), underlying again the high therapeutic potential of gene therapy for NMDs. Similarly, splice-switching approaches have progressed into the *in vivo* proof of principle for many other NMDs as reviewed extensively by Van Roon-Mom and Aartsma-Rus (214) and Disterer and colleagues (215). For example, the recent study from Taniguchi-Ikeda *et al* showing correction of abnormal splicing pattern of the fukutin gene using AONs, opened new therapeutic avenue for Fukuyama muscular dystrophy (216).

With continued success, we will hopefully see a gradual implementation of new therapies over the coming years that will increasingly extend lifespan and improve the quality of life for patients with neuromuscular diseases.

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Abbreviations: EGFR: Epidermal growth factor receptor; FGFR1: Fibroblast growth factor receptor-1; HGFR: Hepatocyte growth factor receptor; HSPG: Heparan sulfate proteoglycan; LamR: Laminin receptor; PDGFR: Platelet-derived growth factor receptor. *ND: not determined.*

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Send correspondence to: Aurelie Goyenvalle, Chair of Excellence HandiMedEx, Laboratory Biotherapies des Maladies Neuromusculaires, UFR des sciences de la sante Simone Veil, Universite de Versailles Saint Quentin, 2 Avenue de la source de la bievre, 78180 Montigny le bretonneux, France, Tel: 33-1-70429432, Fax : 33-1-70429503, E-mail: aurelie.goyenvalle@uvsq.fr