Chapter 6
Translational Research in Nucleic Acid Therapies for Muscular Dystrophies

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Abstract Nucleic acid therapies have gained significant traction in recent years as a promising new approach to treating various genetic diseases. Such therapies employ synthetic, small molecules called antisense oligonucleotides (AOs) which are capable of modulating the transfer of genetic information from nucleic acid to protein through various mechanisms, including the augmentation of pre-mRNA splicing and downregulation of expression. Thus, AOs can prevent the incorporation of genetic mutations causing disease into final protein transcripts as well as reduce levels of mutant transcripts, potentially ameliorating disease phenotype. This process, also known as antisense therapy, has recently been the subject of several preclinical and clinical trials aimed at treating muscular dystrophies. Thanks to recent advancements in antisense drug chemistries, numerous studies have demonstrated the safety, tolerability, and efficacy of AOs administered to patients with Duchenne muscular dystrophy, the most common form of muscular dystrophy. In the wake of promising clinical trial data, it may well be that the first federally approved marketable antisense drug for treating muscular dystrophy could be on the horizon.

Keywords Antisense oligonucleotides • Muscular dystrophy • Exon skipping

6.1 Introduction

A potential therapeutic strategy for the treatment of the muscular dystrophies which has gained significant momentum in the last several years is antisense therapy. Antisense therapy involves the use of short, synthetic, nucleic acid-like molecules known as antisense oligonucleotides (AOs). AOs are designed to bind in a

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sequence-specific manner to target regions on pre-mRNA, whereupon they can modulate splicing or inhibit protein synthesis through various mechanisms, such as translational arrest and RNase-mediated degradation of target RNA [1, 2]. By modulating the transfer of information from transcription to translation, antisense therapy could provide therapeutic treatment for a wide range of diseases, including muscular dystrophy, by preventing the incorporation of deleterious disease-causing mutations found in patients' genetic information from inclusion in the final protein product or by silencing the expression of a given mutant protein [3]. This chapter focuses on antisense oligonucleotides and their use as a therapeutic agent for the treatment of muscular dystrophies.

6.2 AO History and Early Challenges

The regulatory role of antisense RNA was first alluded to in studies involving transcription in Phage lambda [4] but was later defined through the study of *E. coli* plasmid ColE1, wherein plasmid replication is negatively controlled by antisense RNAs that modulate the formation of a replication-initiating primer [5–8]. Several other prokaryotic antisense systems were later described [5, 7, 9–11]. Antisense-mediated regulation of gene expression through the application of AOs was first reported by Zamecnik and Stephenson [12] who demonstrated the inhibition of Rous sarcoma virus (RSV) replication in chick embryo fibroblasts through the addition of a 13-mer oligonucleotide complementary to the 3′- and 5′-redundant terminal sequences of the RSV 35S RNA. Subsequent studies supporting the feasibility of antisense-mediated regulation of gene expression in eukaryotic systems were conducted using mouse β-actin, chicken thymidine kinase, and *Drosophila* hsp26, to name just a few [13, 14]. These early studies helped prepare the way for translational research utilizing AOs to target specific nucleic acid sequences to treat human diseases.

As the potential applications for AOs became apparent, so did the challenges and limitations of early antisense technology. These challenges have, in part, been responsible for the slow progress of antisense therapeutics into clinical application, but they have also propelled the breakthroughs that have brought nucleic acid therapies to where they are today; a few such early challenges will be discussed briefly as follows.

Initially, the method of AO delivery presented a major problem. The effectiveness of early AO chemistries was greatly hindered by their inefficiency at crossing the cell membrane, thus being unable to reach their intracellular targets at therapeutically beneficial quantities [15–18]. Off-target toxic effects caused by the immunostimulatory nature of traditional nucleic acids were another challenge faced by early AOs. Toll-like receptors, key players in the body's immune response, can be activated by the hybridization of DNA or RNA and can even recognize some AO chemistries [19]. Another challenge of AO-based therapies lies in the fact that depending on the nature of the disease or mutation, a very large proportion of

nucleic acid targets (possibly greater than 90 %) must be specifically silenced through direct base-pairing with an AO in order to prove therapeutically beneficial [20]. Determining biochemically effective concentrations of AOs can therefore prove quite challenging when compounded with other challenges such as toxicity. A final challenge to consider in nucleic acid therapy is the intracellular sequestration of AOs. Administered AOs can sometimes be taken up and sequestered in phagolysosomes, oligo-protein complexes, and the reticuloendothelial system [20]. To overcome all of these challenges, AOs have undergone extensive changes in their chemistries since their initial discovery and development. These chemical modifications have produced several classes of AOs that exhibit increased stability and greater effectiveness at reaching and hybridizing with their transcript targets.

6.3 Comparative AO Chemistries

To counter the many opposing barriers which have impeded the development of effective nucleic acid therapies, many unique chemical modifications have been developed. These "next-generation" antisense chemistries exhibit improved target specificity, biological stability, intracellular delivery, and reduced off-target toxic effects. Some of these AO modifications will be discussed presently.

A widely popular antisense chemistry is the phosphorodiamidate morpholino oligomer (PMO, morpholino) [21]. Unlike DNA or RNA, base pairs within the PMO chemistry are bound to morpholine rings rather than the traditional deoxyribose/ribose rings. Additionally, PMOs differ from classical nucleic acid structures in the design of their backbone which is comprised of phosphorodiamidate linkages as opposed to phosphodiester linkages [22]. PMO antisense oligos exhibit high nuclease stability as well as binding affinity for their mRNA targets. PMOs are also well tolerated and do not activate toll-like receptors, the interferon system, or the NF-κB-mediated inflammatory response [23]. The encouraging toxicokinetic profile of PMO-based antisense drugs has helped catapult them to the forefront of clinical research, and there have been several clinical trials employing them as a potential therapeutic agent for treating neuromuscular disorders. A phase II trial for Duchenne muscular dystrophy has already been concluded and has yielded very promising results [24].

Another AO chemistry which has also been the focus of several clinical investigations is the 2'O-methyl phosphorothioate-modified (2'OMePS) antisense oligo [25]. The increased stability and cellular uptake of this particular AO come from a 2′-ribose ring modification and the substitution of sulfur for oxygen groups along its backbone, creating a phosphorothioate linkage. As mentioned, this AO has demonstrated its safety and effectiveness through several preclinical and clinical investigations for a host of diseases and is also currently being studied as a potential therapeutic agent for the treatment of Duchenne muscular dystrophy [26].

While not all AO chemistries have advanced to the clinical trial phase, many have demonstrated great potential through extensive in vitro and in vivo

investigations. For example, vivo-morpholinos (vPMOs) are morpholino AOs conjugated to a cell-penetrating octa-guanidine moiety. These have been shown to effectively modulate splicing of the *FCMD* gene for the potential treatment of Fukuyama congenital muscular dystrophy, as well as facilitate multiple exon skipping of exons 45–55 and 6–8 in mice and dogs, respectively [27–29].

2'-methoxyethoxy (2'-MOE)-modified oligonucleotides contain 2'-O-alkyl-substitutions and function as antisense gapmers, supporting RNase-H-mediated cleavage of target RNAs [30–32]. Preclinical studies involving 2'-MOE AOs to treat spinal muscular atrophy (SMA) in the mouse model have shown the ability of this AO to ameliorate disease pathology in severely affected mice [33]. This antisense chemistry has been employed by Isis Pharmaceuticals in human clinical trials for SMA. Isis Pharmaceuticals' lead candidate drug, ISIS-SMN_{Rx}, is designed to target a splice enhancer region of the *survival of motor neuron 2* (*SMN2*) gene, facilitating inclusion of exon 7 and producing full-length SMN protein. Current phase II clinical trials involving children with infantile-onset (type I) as well as type II and type III SMA have demonstrated the safety and tolerability of ISIS-SMN_{Rx} (clinical trials ID: NCT01703988, NCT01839656).

6.4 Antisense Therapy and Muscular Dystrophy

As newer and more effective AO chemistries have been developed, their potential to treat a wide range of genetic diseases has been vigorously investigated. One promising group of genetic diseases for which antisense therapy holds great promise is the muscular dystrophies. Despite there being more than 30 different types of muscular dystrophy, with new sub-types being added regularly, only a relative few have found their way to the forefront of antisense therapy-based investigations.

6.4.1 Duchenne Muscular Dystrophy

Of all the muscular dystrophies, Duchenne muscular dystrophy is the most prevalent and, not surprisingly, it is also the foremost muscular dystrophy under investigation as a target for antisense therapy – specifically, exon skipping therapy (Fig. 6.1). Antisense-mediated exon skipping is a process that employs AOs to restore the open reading frame by either removing in-frame mutation-carrying exons or by removing exons flanking deletions [3, 34]. Numerous in vitro and in vivo studies have demonstrated the feasibility of exon skipping in the *DMD* gene [35–38]. The first successful *DMD* exon skipping in vitro was demonstrated in lymphoblastoid cells. In that study, researchers achieved successful skipping of exon 19 using a 2′O-methyl AO targeted to an exon recognition site and delivered via cationic lipid transfection [39]. The first demonstration of *DMD* exon skipping in vivo was shown in *mdx* mice using a 2′O-methyl AO designed to catalyze the

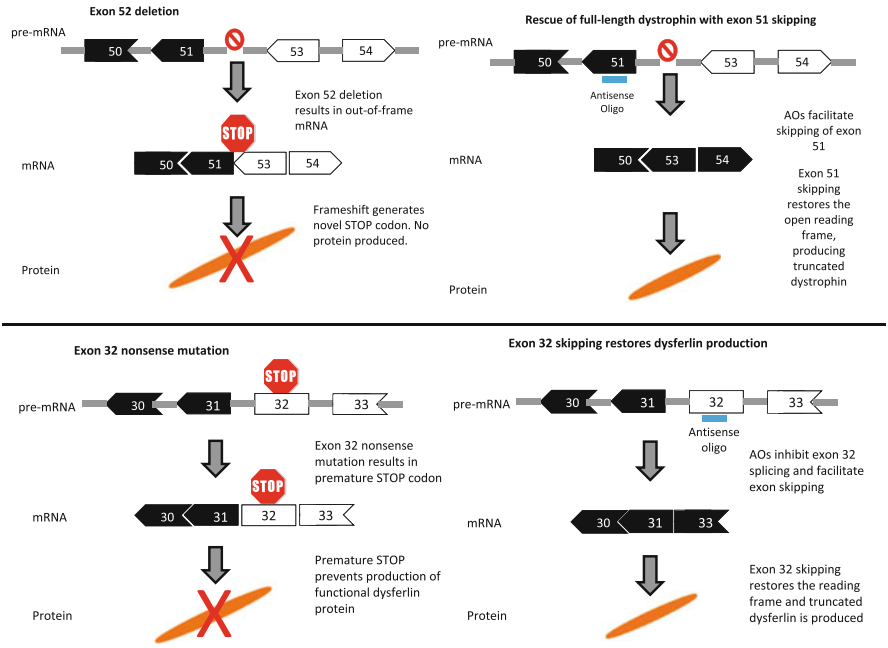

Fig. 6.1 Mechanism of exon skipping therapy in *DMD* and *DYSF*. The deletion of exon 52 in the *DMD* gene results in an out-of-frame transcript that does not produce functional protein (*upper left*). Antisense oligonucleotides are able to bind in a sequence-specific manner to regions of pre-mRNA and modulate splicing through interference with the spliceosome, resulting in the in-frame skipping of exon 51 and restoration of the reading frame (*upper right*). A nonsense mutation in exon 32 of the *DYSF* gene generates a novel stop codon, disrupting the reading frame and preventing the production of functional protein (*bottom left*). Antisense oligonucleotides can bind to a region of exon 32 and prevent incorporation of the exonic sequence into the final mRNA transcript, restoring the reading frame and producing a truncated protein that may retain some functionality (*bottom right*). Phasing or "framed-ness" of each exon is denoted by the shape of the ends of the exon – ends that fit together are in-frame

removal of exon 23 [40]. Lu et al. conducted the first systemic study of AO-mediated exon skipping in mice and were able to achieve bodywide dystrophin expression in all skeletal muscles using a 2′O-methyl AO chemistry [41]. Systemic administration of PMOs and local injection of 2′O-methyl AOs were able to restore dystrophin expression in the canine X-linked muscular dystrophy (*CXMD*) dog model; furthermore, this was the first successful demonstration of multiple exon skipping, wherein researchers used a cocktail of multiple AOs to achieve bodywide skipping of exons 6 and 8 [42, 43]. These discoveries have paved the way for AO clinical trials, and several have been conducted, with others ongoing or planned in the near future. The current status of nucleic acid therapies in clinical trials will be discussed in the section below.

One possible explanation for the remarkable success in antisense-based therapies for the treatment of DMD lies in understanding the pathology of the disease

and the changes which take place at the cellular level. Without dystrophin protein, the structural integrity of the sarcolemma is severely compromised, resulting in small perforations in the cell membrane [44]. These perforations result in a "leaky" cell membrane and could help facilitate easier transport of AOs into the muscle fiber [18]. This would result in the increased likelihood that AOs will be able to reach their intracellular targets. As discussed previously, cell delivery has always posed a major hurdle to nucleic acid therapies – but in the case of DMD, it may be that the very nature of the disease makes it more amenable to this particular kind of therapeutic approach.

6.4.2 *Fukuyama Congenital Muscular Dystrophy*

Fukuyama congenital muscular dystrophy (FCMD) is an autosomal recessive inherited form of muscular dystrophy more commonly associated with the Japanese population (about 1 in 10,000 births) [45, 46]. The *FCMD* gene encodes the protein fukutin, a putative glycosyltransferase [46–48]. Although its exact function is not fully understood, fukutin is a ubiquitously expressed protein (although expressed at much higher levels in skeletal muscle) thought to glycosylate cell-surface glycolipids or glycoproteins, including α-dystroglycan, a member of the dystrophin-associated glycoprotein complex (DGC) [47]. Patients with FCMD harbor a 3-kb retrotransposon insertion in the 3′-untranslated region (UTR) of the gene, which introduces a splicing error that causes premature truncation of exon 10 [28]. Researchers have successfully utilized a cocktail of multiple vPMOs targeting intronic and exonic splice enhancers to restore normal fukutin expression and protein levels both in a mouse model and in human patient cells (Fig. 6.2) [28]. Although initially promising, there have yet to be any further investigations into the antisense-mediated treatment of FCMD.

6.4.3 *Dysferlinopathy*

Mutations in the *dysferlin* (*DYSF*) gene are associated with three autosomal recessive inherited muscular dystrophies – limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy (MM), and distal myopathy with anterior tibial onset (DMAT) – and are collectively referred to as the dysferlinopathies [49–56]. The clinical symptoms of dysferlinopathy present across a wide pathological spectrum which narrows with disease progression until each subtype becomes practically indistinguishable. The protein product of *DYSF*, dysferlin, is a ubiquitously expressed transmembrane protein found at higher levels in skeletal and cardiac muscles where it plays an essential role in vesicle trafficking and plasma membrane resealing [54]. Providing "natural" proof of principle evidence that exon skipping could be amenable to treating dysferlinopathies, a female patient was

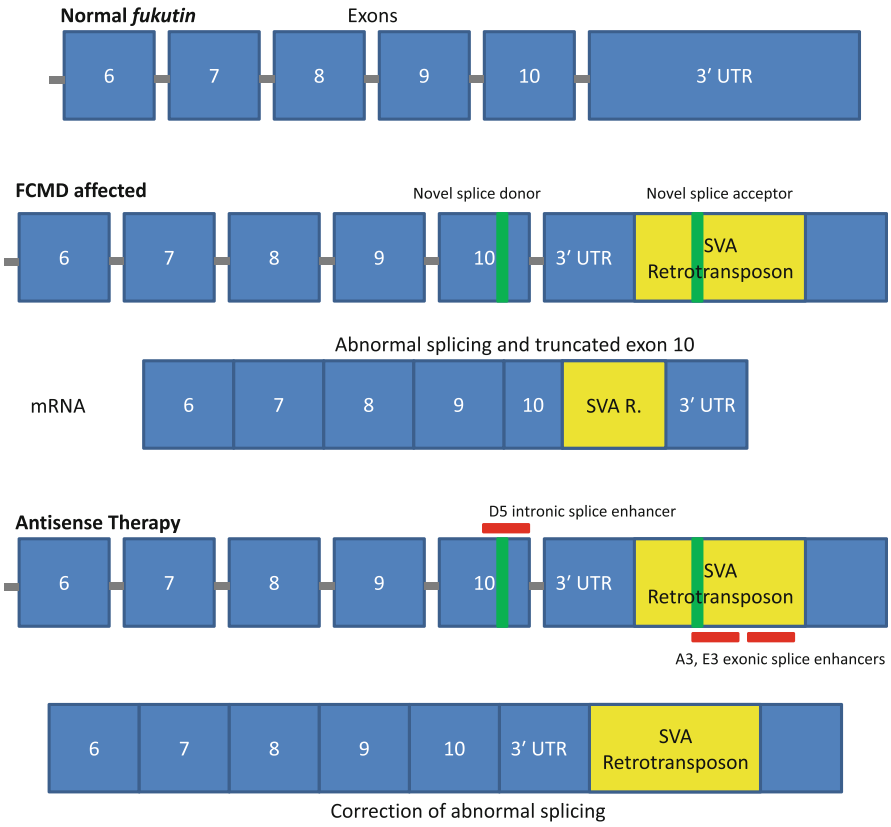

Fig. 6.2 Mechanism of antisense therapy in treating FCMD. The addition of a retrotransposal unit into the 3′ UTR of the *fukutin* gene generates novel splice acceptor and donor sites, resulting in aberrant splicing and truncation of exon 10. A cocktail of three antisense oligos targeting splice enhancer regions is able to correct splicing abnormalities and restore protein expression

previously reported as having a *DYSF* mutation causing the in-frame skipping of exon 32. Although the patient had two daughters with homozygous null mutations and severe LGMD2B, the patient herself presented with only mild symptoms, a phenotype attributed to the action of truncated DYSF protein caused by her mutation [57]. These observations drove subsequent investigations into the feasibility of *DYSF* exon skipping in vitro. One group utilized myoblasts generated from dysferlinopathy patients to test the effectiveness of 2′-O-methyl AOs targeting exonic splice enhancer/silencer sites and was able to achieve efficient exon skipping of exon 32 (Fig. 6.1) [58]. Another natural observation supporting the potential therapeutic action of truncated dysferlin comes from the report of a male dysferlinopathy patient who, despite proximodistal muscle weakness, was still ambulant unassisted at 41 years [59]. Intramuscular injections of a viral vector containing a *DYSF* construct corresponding to the patient's mutation were administered to *DYSF*-null mice and resulted in significant protein expression and restoration of

membrane resealing ability [59]. As yet, no in vivo investigations have been reported that assess the effectiveness of AOs in restoring membrane resealing ability and ameliorating dysferlinopathy phenotype, although several promising exonic targets have been identified and are under investigation for their amenability to antisense-mediated exon skipping based on in-frame translational shifts, confirmed redundancy, and their disassociation with any reported pathology [60].

6.4.4 Myotonic Dystrophy

Myotonic dystrophy type 1 (DM1) is a multisystemic neuromuscular disease characterized by progressive muscle wasting, myotonia, cardiac conduction deficits, mental retardation, insulin resistance, and cataracts, although the combination of these symptoms and their severity varies from patient to patient [61]. DM1 is caused by an expanded CTG tract in the 3′ UTR of the *dystrophia myotonica-protein kinase* (*DMPK*) gene, which is believed to result in RNA-gain-of-function toxicity [62]. A hallmark feature of DM pathogenesis is aberrant splicing (spliceopathy). DM spliceopathy is believed to occur due to protein binding with expanded repeat *DMPK* transcripts, and these spliceopathies are thought to be responsible for many DM symptoms, with more than 30 spliceopathic genes having been identified in DM patients [63–68]. Some spliceopathic genes which have been implicated in DM pathology, specifically in the progression of myotonia, include the *chloride channel 1* (*CLCN1*) and *muscleblind-like splicing regulator 1* (*MBNL1*) genes [69–71]. Antisense-mediated degradation of mutant *DMPK* transcripts and/or the correction of abnormal splicing in DM-related spliceopathic genes through nucleic acid-based therapy are, therefore, promising avenues for treating DM1 (Fig. 6.3). Using 2′-O-methyl AOs, a novel DM1 myoblast-myotube cell model, and a well-characterized DM mouse model (HSA^{LR}), Mulders et al. achieved efficient silencing of mutant *DMPK* transcripts in vitro and in vivo, a reduction in the amount of RNA nuclear foci, and normalization of spliceopathy in several affected genes [72]. In human cells, expanded CUG transcripts were significantly reduced following the addition of AOs, and in DM300-328-XXL mice harboring a 45-kb human genomic fragment containing human *DMPK* with an expanded CTG repeat, there were reduced levels of mutant transcripts following AO injection into skeletal muscles [73]. In another study, systemically administered 2'-MOE AOs were effective in reducing expanded CUG repeats and nuclear foci and achieved transcriptome normalization and normalization of splicing in four genes: sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (ATP2A1 or SERCA1), titin (TTN), LIM domain binding 3 (LDB3 or ZASP), and chloride channel 1 (CLCN1) with no signs of toxicity [74]. In the same study, persistent AO activity was detected up to 1 year following initial treatment and was accompanied by the sustained amelioration of several myopathic features including reduced numbers of centrally nucleated fibers and increased muscle fiber diameter [74].

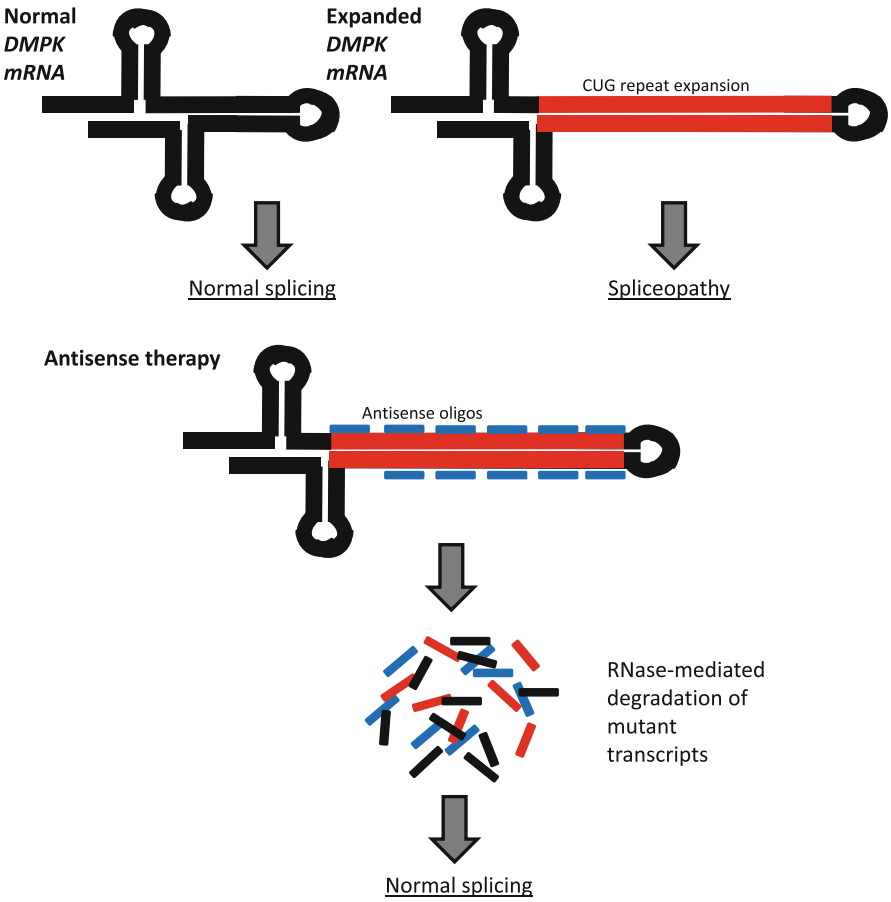

Fig. 6.3 Antisense-mediated degradation of toxic DMPK expanded repeat transcripts. DM1 is caused by a CUG repeat expansion in the *DMPK* gene, which results in spliceopathy in several other genes. Expression of mutant DMPK transcripts can be silenced through the use of antisense oligos which catalyze RNase-mediated degradation

Focusing on the correction of splicing in CLCN1, researchers employed PMOs to prevent incorporation of frame-shift-causing exon 7a and were able to suppress exon 7a inclusion, restore CLCN1 protein expression, rescue channel function, and reverse myotonia using two different DM mouse models [75]. Another group achieved amelioration of myotonia in mice who received PMOs delivered via bubble liposome-ultrasound, highlighting an effective method of AO administration and further demonstrating the feasibility of AO-based therapies for the treatment of DM [64].

6.5 Current Investigations and Clinical Trials

The road toward implementing AOs in the clinical arena has not been an easy one. Despite years of study, AOs as a licensed therapeutic agent have had very little success in reaching the market. To date, only two antisense drugs have ever been approved by the United States Food and Drug Administration: Vitravene (Isis Pharmaceuticals, Carlsbad, CA, USA), for the treatment of cytomegalovirus retinitis in HIV-positive AIDS patients, and Kynamro® (Isis Pharmaceuticals, Carlsbad, CA, USA), for the treatment of familial hypercholesterolemia [3]. Owing to recent and ongoing advancements in AO chemistries, several major hurdles facing nucleic acid-based therapies have been overcome. Some major hurdles remain, such as unknown long-term safety following AO administration, the limited efficacy of AOs in cardiac muscle, and limited applicability (only about 13 % of DMD patients are amenable to exon 51 skipping) [76, 77]. As research continues in this field, and it is already advancing at a rapid pace, it may not be long before an antisense drug for the treatment of muscular dystrophy comes to the clinic.

Based on the status of current clinical trials, the most promising candidate for an antisense drug is likely to be an AO designed for the treatment of Duchenne muscular dystrophy. To date, no other form of muscular dystrophy has received as much exposure in the preclinical and clinical trial world as that of DMD. These clinical trials have centered exclusively on single exon skipping, with exon 51 being one of the most promising targets [24, 26, 78–81], although clinical trials involving exons 44, 45, and 53 single exon skipping are also ongoing [2].

One of the leading developers of nucleic acid-based therapeutics is Sarepta Therapeutics. Their lead antisense drug, PMO-based Eteplirsen, is currently the focus of phase II/III clinical trials aimed at skipping exon 51 in the *dystrophin* gene for treating DMD. According to a recent report from a phase IIb trial, Eteplirsen significantly improved pulmonary function in patients treated through 120 weeks. This finding comes in the wake of preceding data from a 120-week study which found a significant improvement in walking ability in treated patients, as demonstrated by the 6-min walk test. No clinically significant negative effects due to Eteplirsen treatment have been reported. Another DMD exon 51-targeting drug, the 2′OMePS oligo Drisapersen, was developed jointly by GlaxoSmithKline (GSK) and Prosensa, and previous phase II clinical trial data demonstrated the safety, tolerability, and effectiveness of the drug at improving walking distance after 12 weeks of treatment. Unfortunately, Drisapersen did not demonstrate significant improvement in the 6-min walk test in a more recent phase III clinical trial. As a result, GSK is no longer involved in pursuing further clinical trials with Drisapersen, although Prosensa continues to be involved with the drug's development. There are plenty of lessons we can learn from this failure as recently pointed out [3]. For example, the doses of injections in this trial (up to 6 mg/kg) were much lower than the effective dose in *mdx* mice (2 mg/mouse; approximately 75–100 mg/kg) [41]. A very recent development in DMD clinical trials involves the joint efforts of Nippon Shinyaku Co., Ltd. and the National Center of Neurology and Psychiatry

(NCNP, Kodaira City, Japan), which have been developing a PMO-based antisense drug that facilitates exon 53 skipping in DMD patients. The first clinical trial involving their lead candidate drug NS-065/NCNP-01 is underway in 2014. This marks the first ever nucleic acid-based clinical trial in Japan and the first DMD exon 53-targeting clinical trial in the world.

While single exon skipping may be a focus of current clinical trials, future investigations involving multiple exon skipping could widen the door of applicability for AOs in treating muscular dystrophies, especially DMD. The majority of DMD patients (about 63 %) with deletion mutations harbor a mutation between the region of exons 45–55 in the *DMD* gene, a region known as the "mutation hot spot" [27, 37]. The development of a novel antisense approach to skipping exons 45–55 in the human *DMD* gene could overcome the enormous clinical heterogeneity observed in DMD by providing a single therapeutic tactic to treating a large proportion of patients. Furthermore, naturally occurring exons 45–55 deletions in patients are associated with an exceptionally mild, even asymptomatic phenotype [27, 35]. Bodywide exon skipping of exons 45–55 has been accomplished in a mouse model of DMD but has yet to be reported in human cells [27].

With successes in recent clinical trials, ongoing translational research using both in vitro and in vivo models, and continual advancements in AO chemistries, nucleic acid therapies for muscular dystrophies have become one of the most rapidly improving therapeutic strategies in medical research and may soon cease to be a technology exclusive to just the lab bench or to clinical trial cohorts.

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