

# Targeting mRNA for the treatment of facioscapulohumeral muscular dystrophy

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## Summary

Facioscapulohumeral muscular dystrophy (FSHD) is an inherited autosomal dominant disorder characterized clinically by progressive muscle degeneration. Currently, no curative treatment for this disorder exists. FSHD patients are managed through physiotherapy to improve function and quality of life. Over the last two decades, FSHD has been better understood as a disease genetically characterized by a pathogenic contraction of a subset of macrosatellite repeats on chromosome 4. Specifically, several studies support an FSHD pathogenesis model involving the aberrant expression of the *double homeobox protein 4 (DUX4)* gene. Hence, potential therapies revolving around inhibition of *DUX4* have been explored. One of the potential treatment options is the use of effective antisense oligonucleotides (AOs) to knockdown expression of the myopathic *DUX4* gene and its downstream molecules including *paired-like homeodomain transcription factor 1 (PITX1)*. Success in the suppression of *PITX1* expression has already been demonstrated systemically *in vivo* in recent studies. In this article, we will review the pathogenesis of FSHD and the latest research involving the use of antisense knockdown therapy.

**Keywords:** Antisense oligonucleotide therapy, *DUX4*, morpholino, gene therapy, *PITX1*, skeletal muscle

## 1. Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant gain-of-function genetic disorder involving asymmetric muscle weakness and atrophy particularly observed in the face, shoulder, upper arms, further extending into the trunk and legs (1). While there are a dozen forms of muscular dystrophy, FSHD is the third most common muscular dystrophy after Duchenne muscular dystrophy (DMD) and myotonic dystrophy, affecting approximately 1 in 8,000 - 20,000 individuals (2,3). However, since an individual can remain asymptomatic or exhibit mild symptoms, the

frequency of FSHD occurrence could be underestimated. While several candidate genes for FSHD have been identified and explored thus far, the role of *DUX4* as the causative gene in the pathogenesis of FSHD has predominated in the literature (4,5). Hence, the inhibition of *DUX4* expression and the suppression of its downstream molecules can potentially offer therapeutic benefit. The potential of antisense oligonucleotide (AO) therapy as a therapeutic treatment for neuromuscular diseases has recently been highlighted by several clinical trials involving DMD and spinal muscular atrophy (e.g. ClinicalTrials.gov identifier: NCT02193074). Recently, *in vitro* studies have demonstrated success in the suppression of *DUX4* mRNA expression by administering AOs into primary skeletal muscle cells of FSHD patients (5). Nonetheless, desired progress has been impeded by the lack of FSHD animal models and inefficient uptake of AOs into FSHD skeletal muscle fibers. This article will cover the pathogenesis of FSHD, the applicability of antisense oligonucleotide therapy in FSHD, as well as the limitations of antisense therapy in neuromuscular disorders.

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## 2. Pathogenesis of FSHD

FSHD is a genetic and epigenetic disorder primarily involving skeletal muscles. Unique to FSHD are asymmetric muscle weaknesses particularly seen in the face, shoulder and extremities (1). Typically, the onset of symptoms observed in FSHD patients occurs from 15-30 years of age. Depending on the genetic and epigenetic factors, disease manifestations will differ. Initially, FSHD phenotype involves facial muscle weakness resulting in difficulty with labial consonants, whistling and drinking through a straw (6). Upon progression of the disease, atrophy involving the upper arms, pelvic girdle, and lower limbs will occur. Hence, 10-20% of FSHD patients will progressively lose independent ambulation and become wheelchair bound by the age of 50 years (7). The most common presenting symptom is shoulder abduction weakness, seen in 82% of symptomatic patients. Shoulder weakness is the result of a more lateral positioning of the scapula than normal leading to a winged scapula appearance. Additionally, a clinical finding specific to FSHD is the Beevor's sign, which describes the ascending movement of the umbilicus when flexing the neck due to early truncal weakness. Extra-muscular manifestations of FSHD involve high-frequency hearing loss, and retinal telangiectasias in 75% and 60% of patients, respectively (8). Factors that contribute to the severity of phenotype include the age of symptom onset and also the extent of genetic changes.

### 2.1. Genetics of FSHD

Several candidate genes have reportedly been involved in the FSHD phenotype described previously: *DUX4*, *FSHD region gene 1 (FRG1)*, *FSHD region gene 2 (FRG2)*, and *adenine nucleotide translocase 1 (ANT1)* (9). Recent studies have primarily attributed pathogenesis of FSHD to the aberrant expression of a normally dormant gene, *DUX4* (10). *DUX4* is a double homeodomain transcription factor encoded within the D4Z4 tandem repeat. In a healthy individual, the subtelomeric region of chromosome 4q contains 11-100 copies of the 3.3 kb D4Z4 macrosatellite repeat, each with a copy of *DUX4* (11). However, *DUX4* is not expressed in normal functioning somatic tissues such as well-differentiated muscles fibers. While *DUX4* is expressed in early development, it is transcriptionally silenced during cellular differentiation of somatic tissues by CpG methylation of D4Z4 repeats (12). In early development, *DUX4* may play a role in activating a stem-cell-like transcriptional pathway (10). Expression of *DUX4* is maintained in the spermatogonia of the male testis (13,14). While the role of *DUX4* in the seminiferous tubule is not clearly defined, it may be involved in germ cell maintenance and development of stem cells (15). Snider *et al.* illustrated the expression

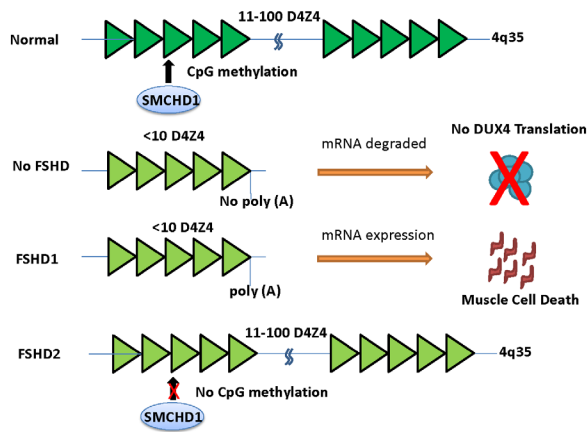
of full-length *DUX4* mRNA in induced pluripotent stem cells (iPSCs). However, the aberrant expression of *DUX4* is severely toxic to muscle tissues, resulting in oxidative stress and apoptosis (16-19). A recent study indicates that expression of *DUX4* in B cells was even capable of generating leukemia in mice *in vivo* (20). Additionally, *DUX4*-induced expression of antigenic proteins such as ERV may be involved in the inflammatory response seen in FSHD muscle histopathology, contributing to muscle atrophy (13).

### 2.2. Epigenetics of FSHD

In FSHD patients, several epigenetic changes take place to result in the pathogenic expression of *DUX4* in skeletal muscle cells. The first is the contraction of the D4Z4 array. Specifically, the deletion of D4Z4 repeat array in the subtelomeric region of chromosome 4 called 4q35 to less than 10 units results in reduced methylation and subsequently chromatin remodeling (21). This defect was first described as a reduction seen in EcoRI fragment of genomic DNA as compared to healthy individuals. While healthy individuals possess 11 to 150 D4Z4 repeats with EcoRI fragments being 40-300 kb in size, FSHD patients have between 1 to 10 repeats and EcoRI fragments at 10-38 kb in size (22). Following reduced DNA methylation due to contracted D4Z4 repeat, a more relaxed chromatin structure allows greater expression of genes located on that locus. The smaller the D4Z4 repeat size, the greater severity of the disease. Secondly, the presence of pLAM1 polyadenylated mRNA site at the distal D4Z4 unit is another condition for disease manifestation (23,24). Interestingly, the polyadenylation site is only intact on chromosome 4qA and not 4qB (25). As such, possible therapeutic strategy for FSHD may include inhibition of polyadenylation in chromosome 4qA leading to *DUX4* gene silencing. Ultimately, the deletion of D4Z4 array leads to a combination of DNA hypomethylation and polyadenylation allowing the aberrant expression of *DUX4*. Hence, *DUX4* are occasionally expressed in skeletal muscle nuclei (14). Detectable levels of *DUX4* up-regulation in myoblast was illustrated by Snider *et al.*, where 1 in 1,000 nuclei was positive for *DUX4* in proliferating primary FSHD myoblasts. Tassin *et al.* also confirmed low expression of *DUX4* in proliferating FSHD myoblasts *via* Western blot analysis. The study demonstrated increased *DUX4* protein expression within 1 in 200 nuclei after allowing FSHD primary myoblasts to differentiate for 4 days. Hence, *DUX4* transcription can be influenced by physiological stage of the cells and its surrounding environment (26).

### 2.3. Types of FSHD

Two types of FSHD exist: FSHD1 and FSHD2. The most common form, FSHD1, occurs in over 95% of



**Figure 1. Role of chromosome 4q35 D4Z4 repeat array in the pathogenesis of facioscapulohumeral muscular dystrophy (FSHD).** While contraction of D4Z4 repeats in FSHD1 results in the expression of *DUX4* and subsequent myopathy, expression of *DUX4* in FSHD2 is due to a mutation in the *SMCHD1* gene.

FSHD patients (21). Genetic analysis links FSHD 1 to the genetic contraction of macrosatellite D4Z4 repeat array on chromosome 4. FSHD2, however, has a normal number of D4Z4 repeats and instead involves a heterozygous mutation in the *SMCHD1* gene on chromosome 18p, a chromatin modifier (27) (Figure 1). Nonetheless, patients with FSHD1 and FSHD2 share similar clinical presentations. Over the last two decades, progress has been made in the better understanding of the pathogenesis of FSHD, potentially leading to therapeutic strategies for the treatment of FSHD.

### 3. Therapeutic Approaches to FSHD

No definitive curative treatment for FSHD has been established despite the recent progress made in understanding the genetic and pathophysiologic mechanism of the disease. Current standard clinical management options include physical therapy, aerobic exercise, respiratory function therapy, and orthopedic intervention (28,29). The current guideline for the management of individuals with FSHD is based on a principle of improving function and quality of life. Current drug therapy trials for the management of FSHD include myostatin inhibitor luspatercept and anti-inflammatory biologics (ATYR1940). The basis for anti-inflammatory biologics is to suppress inflammation commonly seen in muscle pathology of FSHD patients in order to slow phenotype progression. All the while, gene therapy has been explored to reduce pathogenic *DUX4* protein production in FSHD by controlling D4Z4 methylation, suppressing *DUX4* mRNA, and inhibiting *DUX4* pathway (5,17,30-32). Several inhibitory tools are available for use including small interfering RNA (siRNA), small hairpin RNA (shRNA), microRNA (miRNA) and antisense oligonucleotides (33,34).

#### 3.1. RNA interference

RNA interference-based approach has been explored by several studies as a prospective treatment for FSHD. siRNA are small double-stranded RNA molecules that act in the cytoplasm of cells to silence mRNA of targeted gene *via* a process of transcript degradation or translational inhibition (35). siRNA has been used to target the 3' untranslated region transcribed from pLAM (5). While shRNA (or artificial miRNA) shares a similar process of silencing target genes as siRNA, it acts on the nucleus of the cell instead. Hence, the advantage of shRNA lies in its ability to have long lasting effects at low doses. Wallace *et al.* have demonstrated *in vivo* success with an artificial miRNA by delivering miDUX4 through adeno-associated viral (AAV) vector into an AAV-based *DUX4* mouse model (32). The study illustrates a 90% reduction in *DUX4* protein and 64% reduction in *DUX4* mRNA. One of the limitations of RNA interference approach is its high dose cytotoxicity derived from its off-target effects (36,37). Additionally, the negative charge, size, and rigid structure of siRNA can complicate its passive diffusion across the target cell. Therefore, they require viral vectors for *in vivo* systemic delivery, which can cause significant side effects such as immune response.

#### 3.2. Antisense oligonucleotides

Antisense oligonucleotides (AOs) on the other hand are small single-stranded DNA-like molecules of 8 to 30 base pairs in length which can be chemically modified specifically to interfere with mRNA processing and stability (38). AOs can either act *via* exon skipping, splice modulation, or inhibition of gene expression. Importantly they do not require viral vectors for *in vivo* systemic delivery. The potential of AOs was initially demonstrated following discovery that transfection of short DNA sequence can inhibit gene expression (39). Currently, antisense therapy is used in preclinical studies and clinical trials of a variety of neuromuscular disorders including DMD, spinal muscular atrophy (SMA), and Fukuyama congenital muscular dystrophy (FCMD) (40-46). Currently, Sarepta therapeutics, Nippon-Shinyaku, and Prosensa are conducting clinical trials involving phosphorodiamidate morpholino oligomer (PMO) and 2'O methyl phosphorothioate oligonucleotide (2'OMePS). Beyond neuromuscular disease, antisense-mediated gene suppression therapy has taken ground in a spectrum of disease including cancer, thrombosis, and Ebola (47-50).

### 4. Antisense oligonucleotide therapy for FSHD

In light of recent success antisense therapy has in the study of neuromuscular disorders, its application to FSHD has been investigated in multiple studies (51).

Antisense therapy uses antisense oligonucleotides (AOs) which are short single-stranded DNA-like molecules to selectively hybridize pre-mRNA *via* base pairing (38). Since oligonucleotides have difficulty penetrating the lipid bilayer of cells and are also degradable by nucleases, several AO chemistries have been designed to continuously improve efficacy including PMO, octa-guanidine dendrimer conjugated PMOs (vPMO), and peptide-phosphorodiamidate morpholino oligomer (PPMO). Challengingly, the *DUX4* open reading frame (ORF) is located in the first exon and hence makes disruption of its reading frame by antisense-mediated exon skipping difficult (52). However, effective interference of *DUX4* mRNA using antisense oligonucleotide has been illustrated by Vanderplanck *et al. in vitro* (5). The study utilizes 2'OMePs to target splice sites of exon 2 and 3 and thereby disrupts the polyadenylation signal at the 3'UTR. Upon Western blot analysis, no *DUX4* protein can be detected following treatment with 600 nM of AOs. As well, the 2'OMePs administered was able to achieve 50% reduction in the intensity of *DUX4* gene upon RT-PCR analysis of *DUX4* gene fragments. However, the 2'OMePs chemistry developed by Prosensa targeting DMD has recently failed phase III clinical trial due to its toxicity and ineffectiveness (53,54).

#### 4.1. Phosphorodiamidate morpholino oligomer (PMO)

PMO is one of the most commonly used modified AO chemistry to offer sequence-specific inhibition of gene expression (55). PMO consists of short chains of 20-30 nucleic acid bases, a morpholino ring and a non-ionic phosphorodiamidate intersubunit linkage (56). Its structural chemistry provides high nuclease resistance, high affinity to target RNA, resistance to metabolic degradation, and reduced activation of toll-like receptors (57,58). The phosphorodiamidate backbone, in particular, helps the morpholino evade targeting by nucleases. The modified backbone also provides additional stability by helping the molecule evade immune responses. As well, compared to equivalent DNA-based antisense oligonucleotides, morpholino also has a higher binding affinity (56,57,59). Hence, morpholinos lead to less off-target effects. In addition, morpholinos have longer effective half-life due to its substitution for a six-membered morpholine ring. *In vivo* DMD studies have shown the efficacy of PMO by illustrating its ability to penetrate dysfunctional muscle fibers, increase dystrophin expression and ultimately improve muscle function (60-63). Marsollier *et al.* have shown the efficacy of transfecting PMO in immortalized FSHD cells to target *DUX4* mRNA polyadenylation signal in order to suppress the expression of *DUX4*. One of the challenges in building a therapeutic strategy around PMOs is its difficulty in crossing the lipid bilayer of cells and thereby resulting in reduced

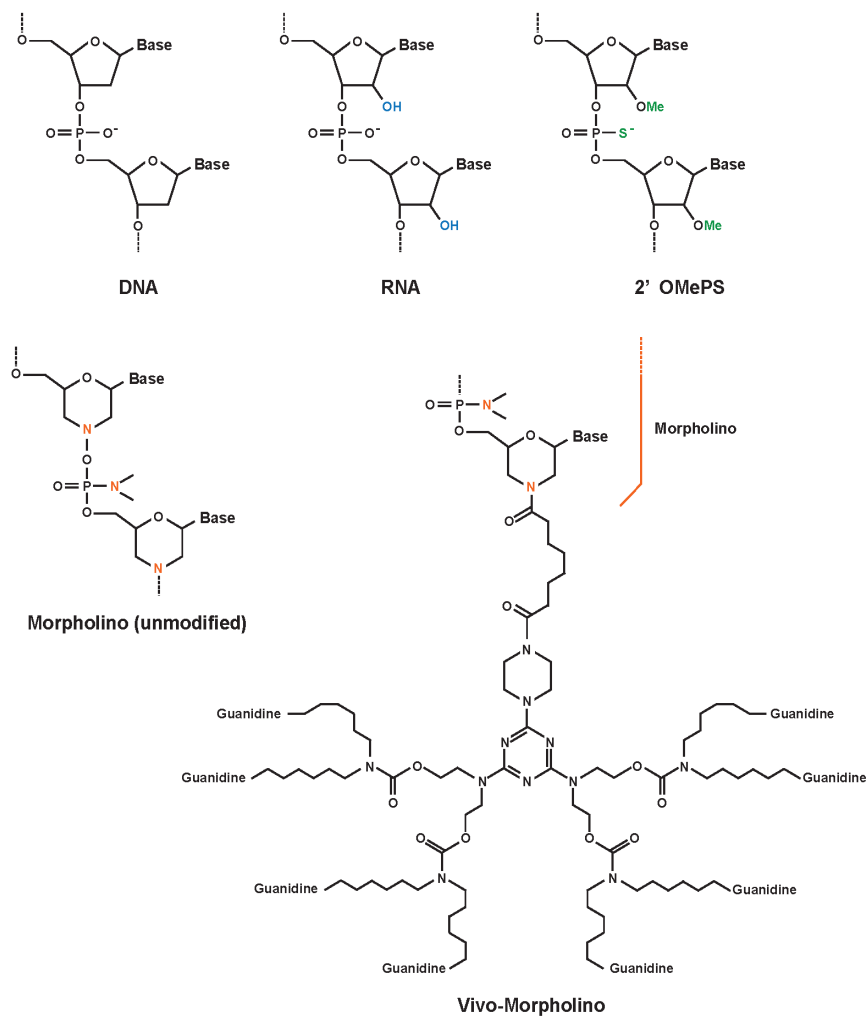
delivery to skeletal muscles (38). While the leaky muscle membrane of DMD assists in the uptake of AOs into a target cell, FSHD myofibers lack this leakiness (64-66). Hence, to achieve and maintain therapeutic efficacy, PMOs may need to be administered in large and repeated doses. However, a higher dose could result in harmful effects.

#### 4.2. Octa-guanidine dendrimer-conjugated vivo-morpholinos (vPMO)

In order to improve the cell-penetrating ability of antisense oligonucleotides, second-generation oligonucleotide such as octa-guanidine dendrimer-conjugated vivo-morpholinos (vPMO) have been used. Vivo-morpholinos essentially conjugates with a triazine core scaffold of eight guanidinium head groups to help penetrate the cell membrane and improve delivery of the morpholino (66-68) (Figure 2). The positive charge that accompanies vPMO assist in uptake and competes with splicing factors. *In vivo* studies with vPMO carried out by Yokota *et al.* have demonstrated greater efficacy in inducing exon 6-9 multiple exon skipping in dystrophic dogs compared to unconjugated PMO. In addition, no vPMO toxicity has been recorded upon systemic injection into mice up to 12 mg/kg (69). However, the positive charge does increase the risk of blood clot formation (70).

#### 4.3. Peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO)

Another candidate antisense oligonucleotide that has improved delivery efficacy while also minimizing toxicity is peptide-conjugated phosphorodiamidate morpholino oligomer (PPMO). Multiple peptide-conjugated oligonucleotide derivatives have been explored in recent studies, including arginine-rich peptide B-peptide, Pip-5c, Pip-6 and Pip6a (71-74). In particular, the newest modification, Pip-6a demonstrates improved stability and cardiac muscle penetration (71). Recent studies involving administration of PPMO into the *mdx* mouse model of DMD have shown promising results characterized by restored dystrophin at low doses, increased uptake and prolonged functionality (65,74-77). Specifically, an intramuscular injection of 2 µg of PPMO resulted in 85% dystrophin-positive fiber expression compared to only 14% observed in PMO treatment (65). Similarly, > 95% of exon-skipped RNA transcript was observed after IV injection of 20 mg/kg PPMO. Additionally, functional improvements were observed in various skeletal muscles after administration of PPMO (75). The improved effectiveness of PPMO compared to PMO is attributable to its active uptake process involving caveolae-mediated endocytosis (71). While access to the target cell has improved over the years, challenges



**Figure 2. Molecular structure of various antisense oligonucleotide chemistries derived over the years.** New generations of antisense chemistries such as 2'-O-methylated antisense oligonucleotides (2'OMePS) and octa-guanidine dendrimer conjugated PMOs (vPMO) are artificially modified to improve delivery into target cells.

remain in promoting the internal distribution of PMO to the nucleus for it to be active (65). Most of the PPMO are nonetheless distributed in the cytoplasm away from its site of action. As well, the toxicity of PPMO remains a challenge. PPMO however, benefits from its low therapeutic dose and sustained effects on the target cell. An ideal oligonucleotide therapy will be one that demonstrates long-term effects, sufficient efficacy at a low dose, and low toxicity.

### 5. AO-based therapy targeting *PITX1*

Since the aberrant expression of *DUX4*, a transcription factor, can lead to pathogenic deregulation of multiple genes in muscle, targeting of a downstream gene regulated by *DUX4* has also been explored recently. *PITX1*, a homeobox transcription factor, is a direct transcriptional target of *DUX4* (24). *PITX1* has previously been illustrated to be elevated in muscle fibers of FSHD patients and is understood to be

involved in the myopathy characterized in FSHD. Studies have found that the *PITX1* gene is 10-20 times up-regulated in the muscle fibers of FSHD patients. The role of *PITX1* in myopathy was shown *in vivo* via a tet-repressible muscle-specific *PITX1* transgenic mouse model (78,79). The *PITX1* transgenic mouse model with overexpression of *PITX1* in skeletal muscles demonstrates a similar disease phenotype to the muscular dystrophy seen in FSHD patients. Specifically, mice with over-expressed *PITX1* display reduced muscle fiber size and muscle strength. Hence, up-regulation of *PITX1* via *DUX4* overexpression contributes to the atrophy and wasting of skeletal muscles in FSHD patients. All in all, the downstream molecular changes due to ectopic *DUX4* expression are cytotoxic. Padley *et al.* have also illustrated the feasibility of suppressing *PITX1* using morpholinos *in vivo* (78). The study involves administration of 10 mg/kg of vPMOs into a tet-repressible muscle-specific *PITX1* overexpressing transgenic mouse model for 6

weeks. The vPMOs is used to inhibit the translation of *PITX1* by targeting the 25 base sequence at the translation start site of the *PITX1* mRNA transcript (78). Immunohistochemistry results illustrated 70% reduction in *PITX1* expression in triceps and 60% expression reduction in quadriceps. Muscle pathology results also illustrated a reduction in *PITX1* positive nuclei in muscle fibers as evidenced by 44% reduction in the number of angular shaped atrophic myofibers seen. Antisense targeting of the *PITX1* gene involved in myopathy is, therefore, an efficient therapeutic strategy for FSHD.

## 6. FSHD animal model

Despite advances in the design of oligonucleotide chemistry to promote increased uptake and efficacy, we still lack an adequate FSHD animal model to evaluate functional benefit and toxicity of antisense therapy. Namely, *DUX4* transgenic mouse model has not been able to capture the full disease phenotype of FSHD. For instance, the D4Z4-2.5 mice have normal histology of the limb, grip strength and creatine kinase (12,17). The challenge in generating a proper animal model for FSHD stems from the fact that D4Z4 macrosatellite encoding *DUX4* is unique to primates (80). Hence, introducing *DUX4* expression into natural laboratory models will be challenging. Currently, the best available system for *in vivo* study is the AAV-model developed by Wallace *et al.*, which demonstrates myopathy consistent with FSHD. The model is established by using adeno-associated viral vectors to deliver *DUX4* into mouse muscle fibers (34). Successful establishment of an FSHD animal model based on *DUX4* expression will assist in the understanding of the pathogenesis of disease and development of therapeutic approaches for FSHD.

## 7. Conclusion

Over the last two decades, progress has been made in our understanding of FSHD pathogenesis. As a gain-of-function disease characterized by the aberrant expression of *DUX4*, a knock-down approach involving antisense oligonucleotide has been explored. In particular, AOs have been especially useful by selectively inhibiting translation of target mRNA. Application of antisense oligonucleotide in the treatment of neuromuscular disorder has progressed in recent years, and its potential benefit has been observed from *in vitro* studies demonstrating successful suppression of *DUX4* expression. Additionally, promising benefits have been observed in the treatment of transgenic mouse model expressing *PITX1* with AOs. With the advancement of modified oligonucleotides providing enhanced delivery and increased efficacy, the movement towards gene therapy seems plausible.

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