Review

Oligonucleotide Therapies for Facioscapulohumeral Muscular Dystrophy: Current Preclinical Landscape

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Review

Oligonucleotide Therapies for Facioscapulohumeral Muscular Dystrophy: Current Preclinical Landscape

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Abstract: Facioscapulohumeral muscular dystrophy (FSHD) is an inherited myopathy, characterized by progressive and asymmetric muscle atrophy, primarily affecting muscles of the face, shoulder girdle, and upper arms before affecting muscles of the lower extremities with age and greater disease severity. FSHD is a disabling condition, and patients may also present with various extramuscular symptoms. FSHD is caused by the aberrant expression of double homeobox 4 (*DUX4*) in adult skeletal muscle, arising from compromised epigenetic repression of the D4Z4 array. *DUX4* encodes the DUX4 protein, a transcription factor that activates myotoxic gene programs to produce the FSHD pathology. Therefore, sequence-specific oligonucleotides aimed at reducing DUX4 levels in patients is a compelling therapeutic approach, and one that has received considerable research interest over the last decade. This review aims to describe the current preclinical landscape of oligonucleotide therapies for FSHD. This includes outlining the mechanism of action of each therapy and summarizing the preclinical results obtained regarding their efficacy in cellular and/or murine disease models. The scope of this review is limited to oligonucleotide-based therapies that inhibit the *DUX4* gene, mRNA, or protein in a way that does not involve gene editing.

Keywords: facioscapulohumeral muscular dystrophy (FSHD); DUX4; antisense oligonucleotides; RNAi; CRISPR; DNA aptamers; DNA decoys; targeted therapy; genetic therapy

1. Introduction

1.1. FSHD Overview

Facioscapulohumeral muscular dystrophy (FSHD, MIM: 158900 & 158901) is an autosomal dominant myopathy with a global incidence of approximately 1 in 8000–22000, making it one of the most common forms of muscular dystrophy worldwide [1–3]. FSHD is characterized by progressive muscle weakness and atrophy that develops in an asymmetric fashion, primarily affecting muscles of the face, shoulder girdle, and upper arms. Additional muscle groups can be affected with age, such as the ankle dorsiflexors and proximal leg muscles, resulting in obligate wheelchair use for approximately 20% of patients [1,4]. Some FSHD patients also experience extramuscular symptoms such as hearing loss, retinal vasculopathy, and/or cardiac conduction defects. FSHD is highly variable in terms of disease onset and severity [4]. There are no curative treatments available for FSHD, with current interventions limited to managing symptoms [5].

FSHD is a genetic condition with two distinct types. Patients can be classified as having either FSHD1 or FSHD2 depending on the genetic mechanism that results in the de-repression of the D4Z4 macrosatellite repeat array, located in the subtelomeric region 4q35 (Figure 1A). Healthy individuals have 11-100 3.3-kb D4Z4 repeat units. FSHD1, affecting 95% of patients, is caused by array contraction to ≤10 repeats [6]. FSHD2, affecting 5% of patients, is caused by mutation in genes involved in

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epigenetic methylation of the D4Z4 array (E.g., *SMCHD1*, *DNMT3B*, and *LRIF1*) [7–9]. Curiously, FSHD2 patients also tend to have fewer D4Z4 repeats than healthy individuals (12-16), demonstrating the complexity of this condition and how the distinction between FSHD1 and FSHD2 may not be as straightforward as initially thought. Regardless, the shared outcome in both types of FSHD is the loss of repressive methylation in the D4Z4 array. Therefore, clinical presentation is identical between FSHD1 and FSHD2 [4].

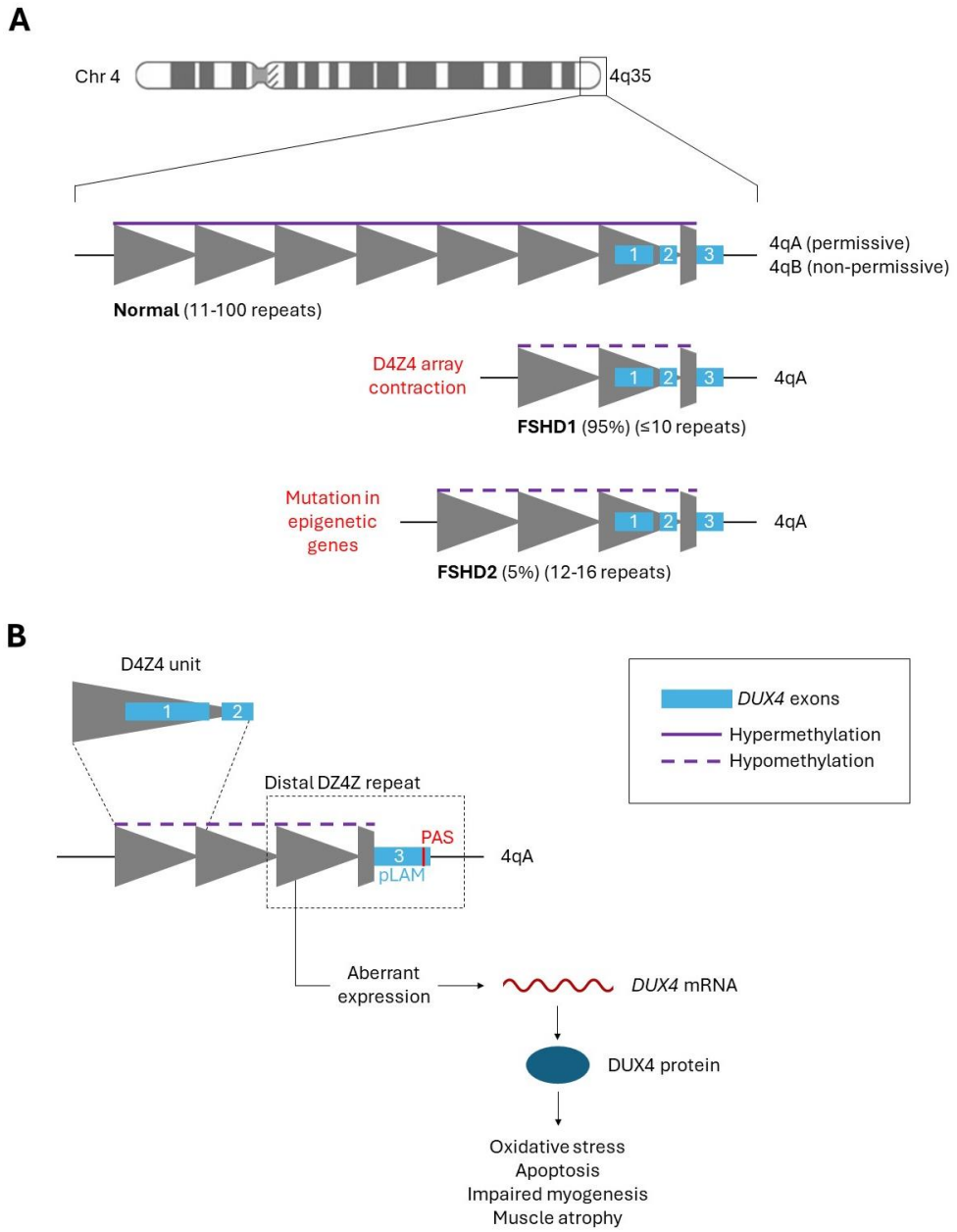

Figure 1. (A) Schematic representation of D4Z4 region in healthy individuals and FSHD patients. The D4Z4 macrosatellite tandem repeat array is found in the subtelomeric region 4q35. Each grey triangle indicates a 3.3 kb D4Z4 repeat unit, within each a *DUX4* retrogene is contained. The 1st and 2nd *DUX4* exons (blue boxes) occur in each full D4Z4 unit. A partial D4Z4 (grey trapezoid) occurs after the most distal full unit, followed by the 3rd *DUX4* exon. Healthy individuals have 11-1000 repeats and full epigenetic repression (purple line). FSHD patients have fewer repeats and compromised epigenetic repression (purple dotted line) arising from one of two genetic changes indicated in red text. **(B)** Schematic representation of aberrant *DUX4* expression from the most distal D4Z4 repeat within the FSHD-permissive 4qA haplotype. The 1st and 2nd *DUX4* exons are within each D4Z4 unit. The 3rd *DUX4* exon and PAS site are found directly downstream of the most distal D4Z4 unit. Compromised

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repression results in low-level DUX4 protein to exist within the skeletal muscle, perturbing downstream gene expression to cause the FSHD pathology (oxidative stress, apoptosis, impaired myogenesis, muscle atrophy, etc.).

1.2. DUX4 Is the Central Cause of FSHD

FSHD is caused by the aberrant expression of double homeobox 4 (DUX4) protein in adult skeletal muscle, arising from the loss of repressive methylation in the D4Z4 array. The *DUX4* gene encodes a transcription factor that is normally involved in zygotic genome activation during the 4-cell stage of early embryonic development [10,11]. Afterwards, *DUX4* is epigenetically silenced in all adult tissues apart from limited expression of unrelated *DUX4* isoforms in the testis and thymus [12,13]. Alternative splicing is known to occur for the *DUX4* transcript, however, only mis-expression of the full-length isoform in muscle tissue is relevant to FSHD. Any mention hereafter of *DUX4* mRNA is referring only to this full-length, pathogenic isoform.

Narrowing in on the genetic region from which FSHD arises, we return to the D4Z4 repeat array and the *DUX4* gene therein. Within each 3.3-kb D4Z4 repeat unit is a retrogene containing exons 1 and 2 of the *DUX4* open reading frame. A partial D4Z4 unit occurs after the most distal full unit, followed by the 3rd *DUX4* exon (Figure 1B) [14]. Aberrant *DUX4* expression occurs off this most distal D4Z4 unit in the FSHD-permissive 4qA haplotype. FSHD can only manifest in one of two major 4q allele variants: 4qA and 4qB. Unlike the non-permissive 4qB haplotype, the 4qA haplotype contains the pLAM region with a polyadenylation site (PAS), allowing transcription of a stable *DUX4* mRNA when epigenetic repression is compromised in the D4Z4 array [1,14].

Following transcription of the *DUX4* gene, stochastic, low-level DUX4 protein occurs in myofiber nuclei of the skeletal muscle. Inappropriate DUX4 protein in adult skeletal muscle is highly toxic, driving gene programs that result in oxidative stress, dysregulated transcript quality control, protein aggregation, inflammation, apoptosis, impaired myogenesis, and muscle atrophy (Figure 1B) [14–16]. DUX4 protein directly activates various genes including *TRIM43*, *ZSCAN4*, *MBD3L2*, *WFDC3*, *PRAMEF1*, *RFPL2*, and *KHDC1* [17,18]. Disruption of these signaling pathways by reactivated DUX4 produces the FSHD pathology that we see in patients, manifesting primarily in the muscle tissue. In this review, we discuss targeted therapies aimed at removing or inhibiting this mis-expressed DUX4 protein, summarize the preclinical results obtained so far, and discuss further considerations for these treatment approaches.

2. Oligonucleotide Therapies Targeting DUX4

FSHD is a condition arising solely from the aberrant reactivation of a dormant gene: *DUX4*. Therefore, therapies that directly target aberrant *DUX4* expression present a compelling treatment option. This review focuses on preclinical FSHD therapies that use a sequence-specific approach for targeting DUX4. This includes oligonucleotides with sequence complementarity to either the *DUX4* gene, *DUX4* mRNA, or DUX4 protein. This complementarity is used to inhibit *DUX4* somewhere along its gene>mRNA>protein expression axis, thereby preventing DUX4 transactivation and the resulting FSHD pathology (Figure 2). Notably, the scope of this review is limited to only non-gene editing approaches that target DUX4.

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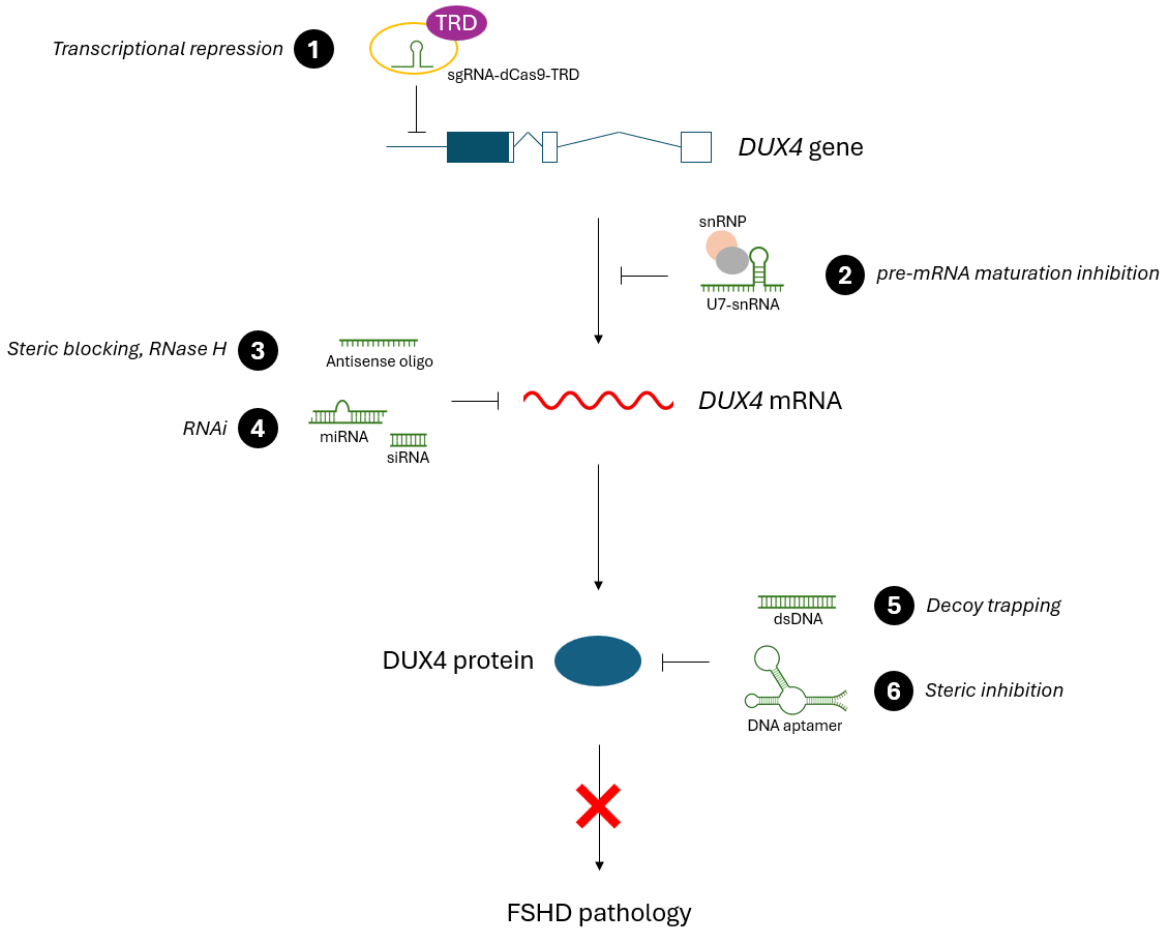

Figure 2. Overview of oligonucleotide therapies for FSHD and where they inhibit *DUX4* expression (gene, mRNA, or protein) to ameliorate FSHD symptoms. **(1)** sgRNA-dCas9-TRD targets the promoter or coding region of the *DUX4* gene, resulting in transcriptional repression. **(2)** U7-snRNA alters the specificity of a small nuclear ribonucleoprotein complex (snRNP) to inhibit *DUX4* pre-mRNA maturation. **(3)** Antisense oligonucleotides bind to *DUX4* mRNA, causing steric blocking (and downstream effects) or RNase H-mediated degradation, depending on the AO type. **(4)** Various RNA molecules (miRNA, siRNA, etc.) degrade *DUX4* mRNA through the RNA interference pathway. **(5)** Decoy dsDNA molecules have DUX4-binding motifs that trap DUX4 protein in a binding sink, inhibiting transactivation of downstream DUX4 targets. **(6)** DNA aptamers bind to DUX4 protein, inhibiting DUX4 activity through steric inhibition.

The targeted oligonucleotide therapies discussed in this review are divided into three categories: *Antisense Oligonucleotides (AOs)*, *RNA interference (RNAi)*, and *Other*. These therapies have shown promising preclinical results in cellular and murine models of FSHD, primarily in their ability to lower *DUX4* mRNA levels, reduce DUX4-target gene expression, and alleviate FSHD symptoms (Tables 1–3).

Table 1. Overview of preclinical studies investigating antisense oligonucleotides to treat FSHD.

Study	Type	Strategy	Chemical modification(s)	target site	Delivery mechanism	*in vivo* injection route	*in vitro* model	*in vivo* model	*in vitro* dose	*in vivo* dose (total #; dosing interval)	*DUX4* knockdown results	Other results
Vanderplanck et al. 2011 [31]	AO	Steric blocking	2'-OMe, PS	Ex2 SA, Ex3 SA	N/A	N/A	Primary FSHD myoblasts (differentiated post-treatment)	N/A	SA), 150 nM (Ex3 SA)	N/A	30% (Ex2 SA) and 50% (Ex3 SA) ↓*DUX4* mRNA (3 days post-treatment)	↓TP53 protein
Lim et al. 2015 [32]	Gapmer AO	RNase H degradation	2'-MOE gapmer, PS	Promoter	N/A	N/A	Primary FSHD myoblasts (differentiated post-treatment)	N/A	N/A	N/A	No ↓*DUX4* mRNA observed	None
Marsolier et al. 2016 [33]	PMO AO	Steric blocking	PMO	Ex3 PAS, Ex3 CS	N/A	N/A	Immortalized FSHD myotubes	N/A	50 nM	N/A	40% (Ex3 PAS) and 52% (Ex3 CS) ↓*DUX4* mRNA (2 days post-treatment)	↓*TRIM43*, *ZSCAN4*, *MBD3L2* expression
	PMO AO	Steric blocking	PMO	Ex3, Ex3 PAS	N/A	N/A	Primary FSHD myotubes	N/A	10 µmol/L	N/A	Not assessed	↓DUX4+ nuclei (Ex3 PAS only); ↓*TRIM43*, *ZSCAN4*, *MBD3L2*, *CCNA1* expression (Ex3 PAS only)
Chen et al. 2016 [34]				Ex3 PAS	N/A	Local (i.m., via electroporation)	N/A	FSHD muscle xenograft mice	N/A	20 µg (1x; N/A)	~100% ↓*DUX4* mRNA in FSHD muscle xenograft (14 days post-treatment)	↓*ZSCAN4*, *MBD3L5* expression
	AO	Steric blocking	2'-OMe, PS	Ex2 SA, Ex3 SA	N/A	N/A	Primary aFSHD and dFSHD myoblasts (differentiated post-treatment)	N/A	50 nM (Ex2 SA), 10 nM (Ex3 SA)	N/A	~90% (Ex2 SA, Ex3 SA) ↓*DUX4* mRNA (3 days post-treatment)	↓DUX4+ nuclei; ↓aFSHD myotubes; ↓*TRIM43* expression
Ansseau et al. 2017 [23]	Vivo-PMO AO		PMO	Ex3 SA	Vivo conjugate	Local (i.m.)	N/A	AVV-DUX4 mice	N/A	10 µg (1x; N/A)	30-fold ↓*DUX4* mRNA (10 days post-injection)	None
Derenne et al. 2020 [35]	Vivo-PMO AO	Steric blocking	PMO	Ex3 SA	Vivo conjugate	Local (i.m., via electroporation)	N/A	DUX4 IMEP mice		250 µg (1x; N/A)	Not assessed	2.5-fold ↓histological lesion compared to non-treated
Lim et al. 2020 [36]	Gapmer AO	RNase H degradation	LNA gapmer, PS	Ex1, Ex3	N/A	N/A	Immortalized FSHD myotubes	N/A	100 nM	N/A	~100% ↓*DUX4* mRNA (1 day post-treatment)	↓*TRIM43*, *ZSCAN4*, *MBD3L2* expression; partial transcriptomic restoration; ↑muscle cell fusion/size
				Ex3	N/A	Local (i.m.)	N/A	*FLExDUX4* mice	N/A	20 µg (3x; every 2 days)	~84% ↓*DUX4* mRNA in TA (1 day post-treatment)	None
Lim et al. 2021 [37]	Gapmer AO	RNase H degradation	2'-MOE gapmer, PS	Ex3	N/A	N/A	Immortalized FSHD myotubes	N/A	100 nM	N/A	~100% ↓*DUX4* mRNA (1 day post-treatment)	↓*TRIM43*, *ZSCAN4*, *MBD3L2* expression; partial transcriptomic restoration; ↑muscle cell fusion/size
				Ex3	N/A	Local (i.m.)	N/A	*FLExDUX4* mice	N/A	20 µg (3x, every 2 days)	~65% ↓*DUX4* mRNA in TA muscle (1 day post-treatment)	None
Bouwman et al. 2021 [24]	Gapmer AO	RNase H degradation	cEt gapmer, 5'-mC	Ex1	Palmitoyl conjugate	Systemic (s.c.)	N/A	*FLExDUX4* mice	N/A	50 mg/kg (13x; biweekly for first 4 weeks, weekly for next 5 weeks)	Mean 37% ↓*DUX4* mRNA in QUA, TRI, GAS, and TA (7 days post-treatment)	Mean 73% ↓DUX4+ nuclei; >60% ↓*Wfdc3*, *Agtr2*, *Serpinb6c* expression in QUA, TRI, GAS, and TA; ↓muscle pathology; ↓inflammation and fibrosis pathways
Falzarano et al. 2021 [38]	PMO AO	Steric blocking	PMO	Ex3 CS	Chitosan-shelled NBs	N/A	Immortalized FSHD myotubes	N/A	50nM	N/A	No ↓*DUX4* mRNA observed compared to naked PMO-CS3 control	Poor release of PMO-CS3 from chitosan-shelled NBs
	Vivo-PMO AO	Steric blocking	PMO	Ex3 PAS, Ex3 CS	Vivo conjugate	N/A	Immortalized FSHD myotubes	N/A	10 µM	N/A	>50% ↓*DUX4* mRNA	53-68% ↓*TRIM36*, 57-81% ↓*ZSCAN4*, 65-85% ↓*PRAMEF2* expression
Lu-Nguyen et al. 2021 [39]						Systemic (i.p.)	N/A	*FLExDUX4* mice	N/A	10 mg/kg (4x; weekly)	~50% ↓*DUX4* mRNA (7 days post-treatment)	~50% ↓*Trim36*, *Wfdc3* expression; 12% ↓muscle atrophy; 52% ↑*in situ* muscle strength; 17% ↓muscle fibrosis; ↑locomotor activity; 22% ↓fatigue level
Lu-Nguyen et al. 2022a [40]	Vivo-PMO AO	Steric blocking	PMO	Ex3 PAS, Ex3 CS	Vivo conjugate	Systemic (i.p.)	N/A	*FLExDUX4* mice	N/A	10 mg/kg (4x; weekly)	~50% ↓*DUX4* mRNA in DIA	↓DUX4+ nuclei; ~50% ↓*Trim36*, *Wfdc3* mRNA in diaphram; ↓muscle fibrosis; ↑muscle regeneration
Lu-Nguyen et al. 2022b [41]	Vivo-PMO AO	Steric blocking	PMO	Ex3 PAS, Ex3 CS	Vivo conjugate	Systemic (i.p.)	N/A	*FLExDUX4* mice	N/A	10 mg/kg (12x; weekly/biweekly)	60% (in DIA) and 40% (in TA) ↓*DUX4* mRNA (~3.5 days post-treatment)	18% ↑body-wide muscle mass; 32% ↑muscle strength; 29% ↓myofiber central nucleation; 37% ↓muscle fibrosis
Kakimoto et al. 2023 [42]	Gapmer AO	RNase H degradation	ALNA[Ms] gapmer, PS	Ex3	N/A	N/A	C2C12 cells; Primary FSHD myoblasts (differentiated post-treatment)	N/A	10-3000 nM	N/A	Significant ↓*DUX4* mRNA (measured by luciferase reporter)	↓*TRIM43*, *ZSCAN4*, *MBD3L2* mRNA; ↑myotube formation, myogenic fusion, myotube area (>300 nM)
						Systemic (s.c.)	N/A	*FLExDUX4* mice	N/A	15 or 30 mg/kg (3x; every 2 weeks)	~50% ↓*DUX4* mRNA in GAS (30 mg/kg only) (~17 days post-treatment)	↓*Wfdc3* mRNA (15, 30 mg/kg); ↓myofiber central nucleation, plasma CK level (15, 30 mg/kg)
						Systemic (s.c.)	N/A	*FLExDUX4* mice	N/A	10 mg/kg (5x; every 2 weeks)	~40% ↓*DUX4* mRNA in TA (~13 days post-treatment)	↑treadmill running speed, ↑muscle force

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Table 2. Overview of preclinical studies investigating RNAi-based oligonucleotides to treat FSHD.

Study	Type	Strategy	*DUX4* target site	Delivery mechanism	*in vivo* injection route	*in vitro* model	*in vivo* model	*in vitro* dose	*in vivo* dose (total #; dosing interval)	*DUX4* knockdown results	Other results
Vanderplanck et al. 2011 [31]	siRNA	RNAi	Ex2 SA, Ex3 SA	N/A	N/A	Primary FSHD myoblasts (differentiated post-treatment)	N/A	10 nM (Ex3 SA)	N/A	80% (Ex3 SA) ↓*DUX4* mRNA (3 days post-treatment)	↓DUX4, Atrogin1, and TP53 protein levels; ↓MuRF1+ nuclei; ↑muscle size
	amiRNA	RNAi	Ex1	AAV	N/A	HEK293 cells	N/A	Unknown	N/A	>50% ↓*DUX4* mRNA (measured by luciferase reporter)	↓DUX4 protein (miR-405, 1156)
Wallace et al. 2012 [48]					Local (i.m.)	N/A	AVV-DUX4 mice	N/A	3e10 particles (1x; N/A)	64% ↓*DUX4* mRNA (2-4 weeks post-treatment)	90% ↓DUX4 protein; improved histopathology; no caspase-3+ myofibers; ↑grip strength
Lim et al. 2015 [32]	siRNA	RNAi	Promoter, Ex1, In2, downstream elements	N/A	N/A	Primary FSHD myoblasts (differentiated post-treatment)	N/A	N/A	N/A	Up to ~50-90% ↓*DUX4* mRNA	↓DUX4+ nuclei; ↓*ZSCAN4* expression; *DUX4* knockdown is DICER/AGO-dependent
	amiRNA	RNAi	Ex1, Ex2, Ex3	AAV	N/A	HEK293 cells	N/A	Unknown	N/A	Up to >75% ↓*DUX4* mRNA (measured by luciferase reporter)	Up to >75% ↓DUX4 protein
Wallace et al. 2018 [49]			Ex1		Local (i.m.)	N/A	AVV-DUX4 mice	N/A	3e10 particles (1x; N/A)	Not assessed	miR-1155 was more toxic than miR-405 (measured by histological analysis)
	miRNA	RNAi	Ex1, Ex2, Ex3	N/A	N/A	HEK293 cells	N/A	N/A	N/A	36% (miR-675-5p plasmid) and 91% (lncRNA H19 plasmid, miR-675 precursor) ↓*DUX4* mRNA (measured by luciferase reporter)	↓DUX4-target gene expression; ↓cell death; ↓DNA damage
				N/A	N/A	Immortalized FSHD myotubes	N/A	N/A	N/A	Significant ↓*DUX4* mRNA	↓DUX4 protein; ↓DUX4+ nuclei; ↓caspase-3/7 activity
Saad et al. 2021 [50]				AAV	Local (i.m.)	N/A	AVV-DUX4 mice	N/A	5e10 particles (1x; N/A)	56% ↓*DUX4* mRNA in TA (14 days post-treatment)	↓DUX4 protein; 88% ↓*Trim36*, 57% ↓*Wfdc3* expression; 81% ↓myofiber central nucleation
Not fully released, see Arrowhead Pharmaceuticals, Inc. 2021 (press release) [51] and Jagannathan et al. 2021 (report) [52]	siRNA	RNAi	Unknown	Targeted RNAi Molecule (TRiM™)	N/A	Primary FSHD myotubes	N/A	10-100 nM	N/A	>75% ↓*DUX4* mRNA	↓DUX4-target gene expression
					Systemic (i.v.)	N/A	*FLExDUX4* mice	N/A	3 mg/kg (1x; N/A)	Significant ↓*DUX4* mRNA	↓DUX4 protein; ↓DUX4-target gene expression; ↓muscle fibrosis; ↑weight; ↑rotarod performance
Not fully released, see Mariot et al. 2022 (abstract) [53]	shRNA	RNAi	Unknown	AAV	Unknown	N/A	*FLExDUX4* mice	N/A	Unknown	High ↓*DUX4* mRNA	↓DUX4-target gene expression; ↓myofiber central nucleation; ↓inflammation
Not fully released, see Malecova et al. 2022 (abstract) [54] and Avidity Biosciences, Inc. 2024 (poster) [55]	siRNA	RNAi	Unknown	anti-mTfR1 mAb conjugate	N/A	Immortalized FSHD myotubes	N/A	Unknown	N/A	Not directly assessed	>75% ↓composite DUX4 and DUX4-target gene expression signature
					Systemic (i.v.)	N/A	*FLExDUX4* mice	N/A	8 mg/kg (1x; N/A)	Not directly assessed	>75% ↓composite DUX4 and DUX4-target gene expression signature in TA (3 weeks post-treatment); ↑treadmill running; ↑muscle force; ↑compound muscle action potential
Not fully released, see Dyne Therapeutics, Inc. 2024a (press release) [56] and 2024b (presentation) [57]	siRNA	RNAi	Unknown	anti-mTfR1 mAb conjugate	N/A	FSHD myotubes (unspecified)	N/A	Unknown	N/A	Not directly assessed	↓mean *TRIM36*, *ZSCAN4*, and *MBD3L2* expression
					Systemic (i.v.)	N/A	hTfR1/iFLEx D mice	N/A	Unkown (1x; N/A)	Not directly assessed	Long-term ↓DUX4-target gene expression in QUA, GAS, and TA; ↓hypotrophic myofibers; ↑treadmill running

Table 3. Overview of preclinical studies investigating other oligonucleotides to treat FSHD.

Study	Type	Strategy	Chemical modifica-tion(s)	DUX4 target site	Delivery mechanism	*in vivo* injection route	*in vitro* model	*in vivo* model	*in vitro* dose	*in vivo* dose (total #; dosing interval)	*DUX4* knockdown results	Other results
Himeda et al. 2016 [61]	CRISPR/dCas9-KRAB	CRISPR/dCas9-KRAB-mediated transcriptional repression of *DUX4* (by repressing *DUX4* directly)	N/A	Promoter, Ex1	N/A	N/A	Primary FSHD myocytes	N/A	N/A	N/A	~55% ↓*DUX4* mRNA (~2 days post-treatment)	↓*TRIM43*, *ZSCAN4*, *MBD3L2* expression; no off-target effect on expression of D4Z4-proximal genes (*FRG1* and *FRG2*)
Himeda et al. 2018 [62]	CRISPR/dCas9-KRAB	CRISPR/dCas9-KRAB-mediated transcriptional repression of *DUX4* (by repressing *DUX4* epigenetic activators)	N/A	N/A (targets ASH1L, BRD2, KDM4C, SMARCA5)	N/A	N/A	Primary FSHD myocytes	N/A	N/A	N/A	~50% ↓*DUX4* mRNA after activator repression (ASH1L, BRD2, KDM4C, SMARCA5, repressed separately) (3 days post-treatment)	↑H3K9me3 at the D4Z4 array
Klingler et al. 2020 [68]	DNA aptamer	Steric inhibition of DUX4 protein	N/A	DUX4 protein DNA-binding region (HD1, HD2)	N/A	N/A	N/A	N/A	N/A	N/A	Not assessed	SELEX-determined DNA aptamers have high affinity to recombinant DUX4 protein
Mariot et al. 2020 [69]	dsDNA	Decoy trapping (decoy binding sites to inhibit DUX4 transactivation)	2'-OMe, PS, HEG linker	N/A (dsDNAs contain DUX4 binding domain)	N/A	N/A	Primary FSHD myotubes	N/A	50 nM	N/A	Not assessed	39-91% ↓*TRIM43*, *ZSCAN4* expression
					AAV	Local (i.m.)	N/A	pCS2-mkgDUX4 electrotransfected mice	N/A	10 µg (1x; N/A)	Not assessed	34% ↓*Tm7sf4*, 51% ↓*DuxBl* expression
Himeda et al. 2021 [60]	CRISPR/dCas9-TRD (various domains)	CRISPR/dCas9-TRD-mediated transcriptional repression of *DUX4* (by repressing *DUX4* directly)	N/A	Promoter, Ex1	N/A	N/A	Primary FSHD myocytes	N/A	N/A	N/A	~30%-50% ↓*DUX4* mRNA (3 days post-treatment)	↓*TRIM43*, *MBD3L2* expression
					AAV	Local (i.m.)	N/A	*FLExDUX4* mice	N/A	5e5 particles	~30% ↓*DUX4* mRNA in TA (14 days post-treatment)	Modest ↓*Wfdc3* expression
Das and Chadwick 2021 [63]	CRISPR/dCas9-KRAB	CRISPR/dCas9-KRAB-mediated transcriptional repression of *DUX4* (by repressing *DUX4* directly)	N/A	Ex3	N/A	N/A	Immortalized FSHD myoblasts	N/A	N/A	N/A	Significant ↓*DUX4* mRNA (sgRNA CR-5A)	↓*TRIM43*, *ZSCAN4* expression (sgRNA CR-5A); partial ↑H3K9me3 at the D4Z4 array (sgRNA CR-5A)
Rashnonejad et al. 2021 [70]	U7-snRNA expression casette	Inhibition of pre-mRNA production or maturation (by altering the sn ribonucleoprotein complex to target *DUX4* pre-mRNA)	N/A	Start codon, Ex1 (SA, SD, SE), Ex3 PAS	N/A	N/A	HEK293 (co-transfected with CMV.DUX4-FL); Immortalized FSHD myotubes	N/A	N/A	N/A	60-95% ↓*DUX4* mRNA (HEK293+CMV.DUX4-FL and immortalized FSHD myotubes)	66-87% ↓DUX4 protein (HEK293+CMV.DUX4-FL); ↓*TRIM43*, *ZSCAN4*, *MBD3L2*, *PRAMEF12* expression (FSHD myotubes); ↓caspase-3/7 activity
Not fully released, see Rashnonejad et al. 2022 (abstract) [65]	CRISPR/Cas13	CRISPR/Cas13-mediated cleavage of *DUX4* mRNA	N/A	Unknown	AAV	Local (i.m.)	N/A	FSHD mice (unspecified)	N/A	Unknown	>50% ↓*DUX4* mRNA	Improved histopathological outcomes; immune response to treatment observed
Not fully released, see Saljoughian et al. 2022 (abstract) [66]	CRISPR/Cas13-ADAR	Cas13-ADAR-mediated *DUX4* mRNA editing (C>U nonsense mutation)	N/A	Unknown	N/A	N/A	Unknown	N/A	N/A	N/A	Not assessed	sgRNA optimization still ongoing
Sasaki-Honda et al. 2022 (preprint) [64]	CRISPR/dCas9-KRAB and dCas9-D3A/D3A3L	CRISPR/dCas9-(KRAB, D3A/D3A3L)-mediated transcriptional repression of DUX4	N/A	Unknown	N/A	N/A	KRAB(Neo) iPSCs (differentiated into muscle cells post-treatment)	N/A	N/A (1-3x tEP; every 7 days)	N/A	~50% ↓*DUX4* mRNA (2x and 3x tEP) (26 days post-treatment)	>50% ↓*TRIM43*, *ZSCAN4*, *MBD3L2* expression (2x and 3x tEP) (26 days post-treatment)

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Table abbreviations: 2′-OMe, 2′-O-methyl; PS, phosphorothioated; PMO, phosphorodiamidate morpholino oligomer; LNA, locked nucleic acid; 2′-MOE, 2′-O-methoxyethyl; cEt, constrained ethyl; 5'-mC, 5'-methylcytosines; ALNA[MS], 2′-N-methanesulfonyl-2′-amino-locked nucleic acid; HEG, hexaethylene glycol; Ex, exon; In, intron; SA, splice acceptor; SD, splice donor; SE, splice enhancer; PAS, polyadenylation signal; CS, cleavage site; NBs, nanobubbles; AAV, adeno-associated virus; mAb, monoclonal antibody; i.m., intramuscular injection; s.c., subcutaneous injection; i.p., intraperitoneal injection; i.v., intravenous injection; QUA, quadriceps; TRI, triceps; GAS, gastrocnemius; TA, tibialis anterior; DIA, diaphram; iPSC, induced pluripotent stem cells; aFSHD, atrophic FSHD; dFSHD, disorganized FSHD; tEP, transient electroporation; IMEP, i.m. injection and electroporation of naked plasmid DNA; SELEX, systematic evolution of ligands by exponential enrichment; TR-FRET, Time-resolved fluorescence energy transfer; AGO, argonaute; mRNA, messenger RNA; siRNA, small interfering RNA; miRNA, microRNA; amiRNA, artificial microRNA; shRNA, short hairpin RNA; snRNA, small nuclear RNA; sgRNA, single guide RNA; CRISPR, clustered regularly interspaced short palindromic repeat; Cas, CRISPR-associated protein; dCas9, dead (endonuclease deficient) Cas9; KRAB, Krüppel-associated box; TRD, transcriptional repression domain; ADAR, adenosine deaminase acting on RNA; N/A, not applicable.

2.1. Antisense Oligonucleotides (AOs)

Antisense oligonucleotides (AOs) are synthetic, single-stranded nucleic acids that target a complementary mRNA molecule, dictated by Watson-Crick base pairing, to initiate post-transcriptional gene silencing. AOs were first identified in 1978 by Zamecnik and Stephenson, who found that complementary oligonucleotides inhibited translation of Rous sarcoma virus mRNA [19]. AOs bind to a target mRNA in a sequence-dependent manner and prevent its translation, thereby reducing the amount of target protein [20].

AOs are known to utilize various chemistries, a fact that makes them distinct from other oligonucleotide therapies. Modern AO drugs have chemical modifications to improve pharmacological properties like tolerability, target affinity, nuclease resistance, and intracellular uptake [21,22]. Commonly used AO chemistries involve modifying the phosphate backbone (PS, phosphorothioate; PMO, phosphorodiamidate morpholino oligomer) or ribose sugar (2′OMe, 2′-*O*-methyl; 2′-MOE, 2′-*O*-methoxyethyl; LNA, locked nucleic acid) [21]. In addition, peptide and fatty acid conjugate modifications have been used to facilitate delivery of *DUX4*-targeting AOs [23,24]. AOs can also be synthesized as gapmers: a chimeric molecule comprised of a central DNA region and a flanking region of modified RNA [25].

The formation of an AO-mRNA duplex results in (1) RNase-H mediated degradation of the target mRNA, or (2) steric blocking of the target mRNA (Figure 3A) [20,22,26]. Only gapmer AOs recruit RNase H to cleave target mRNA. Gapmer AOs produce a DNA:RNA substrate when bound to mRNA, recognizable by RNase H [27,28]. Steric blocking AOs inhibit proper mRNA translation, splicing and/or stability in an RNase H-independent manner [20,22,26]. Additionally, these steric blocking AOs can initiate further downstream degradation pathways, such as nonsense-mediated decay and no-go decay [29,30].

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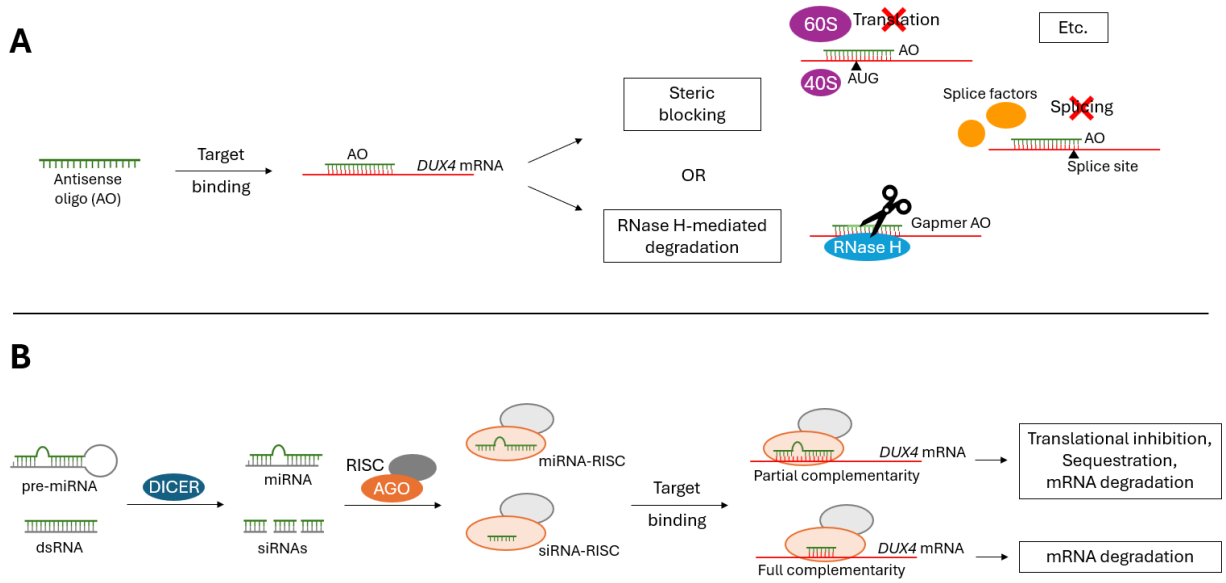

Figure 3. Mechanism of action for **(A)** AO and **(B)** RNAi therapies. **(A)** Antisense oligonucleotides (AOs) can degrade target mRNA transcript by recruiting RNase H, or by inducing steric blocking. AO-mediated steric blocking of a target mRNA transcript can inhibit proper translation, splicing and/or stability. Further downstream degradation pathways can be initiated on steric blocked mRNA transcripts. **(B)** RNAi-based therapies (siRNAs, miRNAs, etc.) degrade target mRNA transcripts using the RNA interference (RNAi) pathway. RNAi can be induced by miRNAs or siRNAs complementary to a mRNA transcript. DICER processes the precursor molecules to produce miRNA or siRNAs, which then get loaded into the Argonaute (AGO) protein of the RNA-induced silencing complex (RISC). Using the guide strand, RISC targets a complementary mRNA transcript and induces translational inhibition, sequestration, and/or mRNA degradation (miRNA-RISC) or simply mRNA degradation (siRNA-RISC).

As summarised in Table 1, numerous preclinical studies have demonstrated that AO therapies can effectively reduce the amount of *DUX4* mRNA and DUX4 target gene expression both *in vitro* and *in vivo* [23,24,31–42]. Other measures were also used to evaluate AO treatment efficacy, such as DUX4 protein levels, muscle fibre health, and murine functional performance. Most groups used primary or immortalized myoblasts/myocytes (often differentiated into myotubes) as an *in vitro* FSHD model, and *FLExDUX4* mice as an *in vivo* FSHD model [43–45]. Earlier studies used local (intramuscular) injection for *in vivo* AO treatment, while studies after 2021 evaluated systemic (intraperitoneal, subcutaneous) injection routes. Systemic injection, being more clinically viable, managed to yield a similarly efficient *DUX4* knockdown compared to local injection. Regarding the *DUX4* target site, the >10 studies produced since 2011 tend to target exons 2 and 3, often with an emphasis on the polyadenylations site (PAS), pre-mRNA cleavage sites, and/or splice sites therein (Figure 4).

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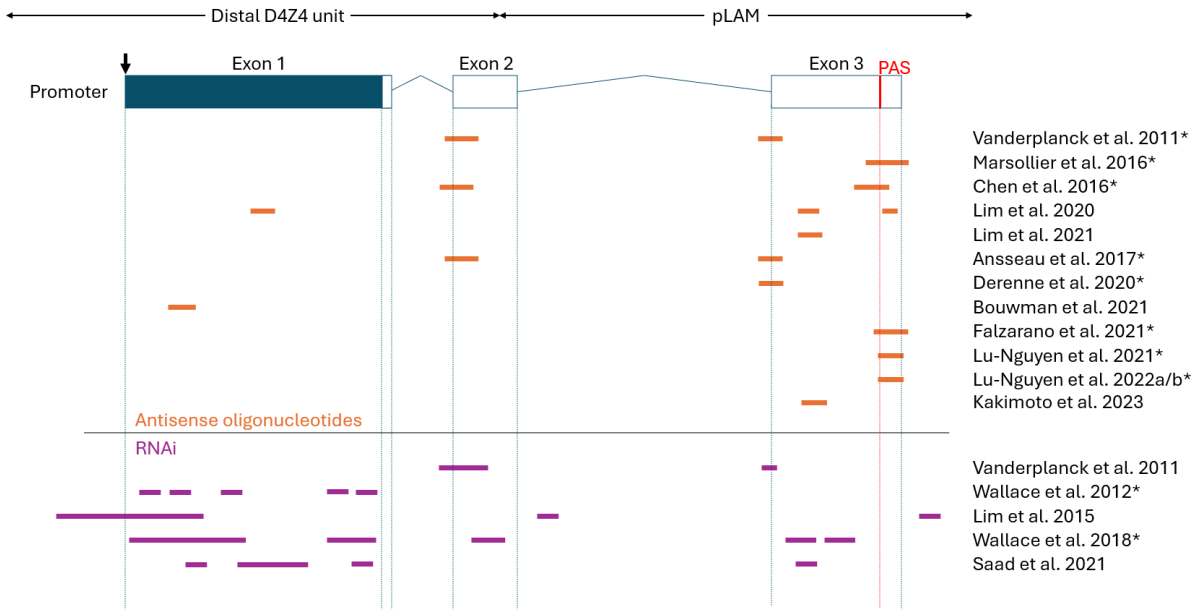

Figure 4. Overview of oligonucleotide target sites on *DUX4* mRNA. Orange (antisense oligonucleotides) and purple (RNAi) lines indicate oligo target sites. Partially overlapping target sites are simply represented as a continuous line. All attempted target sites are included, even oligos that showed poor indications in corresponding studies. Figure is not to scale and approximate oligo sites/sizes are shown. (Top) Schematic representation of *DUX4* gene (downwards arrow indicates start codon; blue, open reading frame; boxes, exons; lines, introns; red line, polyadenylation signal). The distal D4Z4 unit and adjacent pLAM region are indicated by double-sided arrows. Note that the following groups used some or all the same oligonucleotides (indicated by *): Vanderplanck et al. (2011), Ansseau et al. (2017) and Derenne et al. (2020); Marsollier et al. (2016) and Chen et al. (2016); Marsollier et al. (2016) and Falzarano et al. (2021); Lu-Nguyen et al. (2021), Lu-Nguyen et al. (2022a) and Lu-Nguyen et al. (2022b); Wallace et al. (2012) and Wallace et al. (2018).

2.2. RNA Interference (RNAi)

Like AOs, RNAi-based oligonucleotides act at the RNA level, binding to a target mRNA according to antisense sequence complementarity to initiate post-transcriptional gene silencing. Where these classifications differ is that RNAi-based oligonucleotides initiate the RNA interference (RNAi) pathway to knockdown target mRNAs. First defined by Fire and Mello in 1998, RNAi is a conserved, biological mechanism by which double-stranded RNA triggers the loss of homologous mRNA [46]. RNAi can be induced by miRNAs or siRNAs complementary to a mRNA transcript. DICER endonucleases cleave precursor molecules (pre-miRNA or dsRNA) to produce mature microRNA (miRNA) or small-interfering RNAs (siRNA), which then get loaded into the Argonaute (AGO) protein of the RNA-induced silencing complex (RISC). Using the guide strand, RISC targets a complementary mRNA transcript and induces translational inhibition, sequestration, and/or mRNA degradation (miRNA-RISC) or simply mRNA degradation (siRNA-RISC) (Figure 3B) [46,47].

The two types of RNAi-based oligonucleotide therapies for FSHD are miRNAs (natural or artificial) and siRNAs. Since 2011, several studies have shown that these RNAi-based oligos can knockdown *DUX4* mRNA and reduce DUX4 transactivation, in addition to improving other markers of FSHD symptom reversal (E.g., DUX4 protein levels, muscle fibre health, murine functional performance, etc.) (Table 2) [31,32,48–57]. Notably, the amount of published work is less for RNAi-based approaches compared to AOs. These studies commonly use primary or immortalized myoblasts/myocytes (often differentiated into myotubes) as an *in vitro* FSHD model, and AAV-DUX4 mice as an *in vivo* FSHD model [43,44,58]. *FLExDUX4* mice were also used as an *in vivo* model for testing RNAi therapies, but to a lesser extent [45]. Most studies used local (intramuscular) injection to evaluate preclinical efficacy *in vivo*, except for two groups with partially released findings using systemic (intravenous) injection [51,55]. All current preclinical studies opted for adeno-associated

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virus (AAV)-mediated delivery of siRNAs or miRNAs *in vivo*, except for the partially released findings from Avidity Biosciences, Inc. and Dyne Therapeutics, Inc., who each describe a proprietary anti-mTfR1 mAb conjugate for delivery [54–57]. Lastly, as summarized in Figure 4, these miRNAs and siRNAs target *DUX4* mRNA at all 3 of its exons, especially exon 1. Other sites like upstream D4Z4 regions, intronic regions, and pre-mRNA cleavage sites have also been tested.

2.3. Other

Other non-gene editing, preclinical oligonucleotide therapies have also been investigated as potential treatments for FSHD (Table 3). Unlike AOs and RNAi therapies which target *DUX4* mRNA, these oligos tend to target *DUX4* expression at the gene or protein level (Figure 2). These approaches present further compelling options for treating FSHD, in addition to the antisense approaches previously discussed.

2.3.1. CRISPR/dCas9 Transcriptional Repression

Multiple research groups have explored CRISPR/dCas9-mediated transcriptional repression of the *DUX4* gene as a targeted therapy for FSHD. This is a form of CRISPR inhibition (CRISPRi) which uses the sequence-specificity of the sgRNA-Cas9 complex to target the DUX4 promoter, but with a catalytically inactive, 'dead' Cas9 (dCas9) fused to a transcriptional repressor domain (TRD) [59]. This allows for specific re-silencing of the D4Z4 region, reducing *DUX4* expression and the resulting FSHD pathology. While various TRDs have been used, most studies opted for the Krüppel-associated box (KRAB) domain. Evaluation of this treatment approach has been largely done in primary FSHD myoblasts, myocytes, or myotubes, with the only *in vivo* testing performed by Himeda et al. (2021) using AAV-delivery and local (intramuscular) injection in *FLExDUX4* mice [60]. These studies have all reported reduction in *DUX4* mRNA following treatment, as well as reduction in DUX4 target gene expression and/or increased H3K9 tri-methylation at the D4Z4 array [60–64]. Notably, rather than directly repressing the *DUX4* gene, Himeda et al. (2018) used the CRISPR/dCas9-KRAB system to repress epigenetic activators of *DUX4* (*ASH1L, BRD2, KDM4C, SMARCA5*). Similar reduction of *DUX4* mRNA was observed for this approach [62].

Abstracts proposing other non-gene editing, CRISPR-based approaches have been recently published. Results are preliminary and not fully released, however. One group is developing CRISPR/Cas13-mediated cleavage of *DUX4* mRNA, reporting effective *DUX4* knockdown *in vivo* [65]. Another group suggests using CRISPR/Cas13-ADAR (adenosine deaminase acting on RNA)-mediated editing of *DUX4* mRNA to create a C>U nonsense mutation [66]. No definitive results have been published at this time.

2.3.2. DNA Aptamers

Aptamers are single-stranded oligonucleotides that can bind to a specific protein or protein family thanks to their secondary and tertiary folding structure. The unique 3D conformation of an aptamer allows target interaction like that of an antigen and antibody [67]. Therefore, aptamers can be used to specifically target a protein of interest, and in the case of FSHD, bind to and inhibit DUX4. Klingler et al. (2020) designed DNA aptamers with high affinity to DUX4 protein [68]. While not evaluated, these DNA aptamers could be used to treat FSHD by sterically inhibiting DUX4 protein in skeletal muscle.

2.3.3. dsDNA Decoy Trapping

Mariot et al. (2020) demonstrated a unique approach to prevent DUX4 transactivation known as decoy trapping [69]. Decoy trapping uses double-stranded DNA fragments whose sequence corresponds to DUX4 binding motifs, akin to the DNA regions that DUX4 normally binds to as a transcription factor. By saturating the cellular environment with dsDNA decoy binding sites, DUX4 protein is trapped in a binding sink and unable to activate its normal target genes. Mariot et al. (2020)

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found that dsDNA treatment was able to reduce expression or downstream DUX4 target genes *in vitro* and *in vivo* [69].

2.3.4. U7-snRNA pre-mRNA Inhibition

Rashnonejad et al. (2021) describe a strategy to inhibit *DUX4* mRNA expression using U7-small nuclear RNA (snRNA) antisense expression cassettes [70]. U7-snRNA is a part of the small nuclear ribonucleoprotein complex (snRNP), which is involved in 3' end processing of histone pre-mRNAs in the nucleus. This therapeutic approach uses modified U7-snRNA with antisense sequence specificity to *DUX4*, capable of inhibiting pre-mRNA production or maturation. Rashnonejad et al. (2021) showed that these U7-snRNA expression cassettes, delivered by AAV, effectively reduced *DUX4* mRNA, DUX4 protein, and DUX4 target gene expression in immortalized FSHD myotubes [70].

3. Further Considerations

Antisense therapies for FSHD, meaning AOs or RNAi drugs that target *DUX4* mRNA, are the furthest along in preclinical development compared to other oligonucleotide approaches. Many AO and RNAi therapies have shown promising indications both *in vitro* and *in vivo*, as discussed previously. Therefore, discussion of certain advantages and disadvantages of *DUX4*-targeting oligonucleotide therapies will focus on antisense approaches only.

3.1. Advantages of Antisense Approaches

Antisense therapies are ideal for monogenic diseases that can be attributed to a single root cause. In the case of FSHD, this is aberrant *DUX4* expression in adult skeletal muscle. DUX4 is an especially ideal therapeutic target for antisense therapies because it is practically absent in all adult tissues under normal, healthy circumstances, making concerns of unwanted *DUX4* knockdown elsewhere in the body largely insignificant [12,13]. This, however, is not something that should be entirely ignored when evaluating candidate therapies for clinical trials. Overall, antisense therapies are highly specific and potent molecules with a relatively simple mechanism of action, often taking advantage of conserved cellular processes [20].

Compared to gene-editing approaches that prevent *DUX4* expression, antisense therapies involve no changes to genomic DNA, acting only at the RNA level [20,26]. This makes them more acceptable from a regulatory standpoint, unburdened by the moral concern surrounding CRISPR/Cas9 editing of the human genome, even if for therapeutic purposes. For this reason, it may be fair to suggest that antisense therapies are a more clinically viable form of targeted, genetic therapy for FSHD.

3.2. Disadvantages of Antisense Approaches

Efficient delivery to muscle tissue is a considerable challenge for antisense therapies, often hindering the clinical utility of oligonucleotides that otherwise demonstrate good preclinical efficacy. AOs and RNAi oligonucleotides are relatively large nucleic acids that tend to be negatively charged and hydrophilic [21]. Molecules with such properties do not readily pass through the plasma membrane. Furthermore, upon systemic injection, these molecules must avoid nuclease degradation, mononuclear phagocyte system entrapment, protein entrapment, and high renal clearance [71–73]. If these can be overcome, there remains the issue of inefficient cellular uptake, as these oligonucleotides are also prone to endosomal entrapment within the cell [71–73]. All this means that only a small percentage of injected drug becomes bioavailable to provide therapeutic benefit to a patient. However, various strategies to improve delivery are currently being explored for AOs and RNAi oligos, including chemical modification, delivery conjugates, and carrier molecules [71].

Persistence of therapeutic effect is another notable disadvantage of antisense therapies. Given that antisense therapies act on the mRNA, a transient and replenishable molecule, regular lifelong administrations may be necessary to offer long-term reversal of FSHD symptoms for patients. This

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problem is not shared by other proposed genetic therapies that would permanently inactivate the toxic *DUX4* gene (E.g., CRISPR/Cas9 editing) such that multiple treatments are not needed.

3.3. Early-Stage Clinical Trials for Select FSHD Therapies

Two RNAi-based oligonucleotide therapies for FSHD are currently recruiting for Phase 1/2 clinical trials to evaluate the safety, tolerability, pharmacokinetics, pharmacodynamics, and efficacy in adult patients. First, ARO-DUX4, developed by Arrowhead Pharmaceuticals, is a *DUX4*-specific siRNA using an unspecified and proprietary delivery method (Phase 1/2 NCT06131983) [74,75]. Second, AOC 1020, developed by Avidity Biosciences, is a *DUX4*-specific siRNA using a proprietary anti-mTfR1 mAb delivery conjugate (Phase 1/2 FORTITUDE™ NCT0574792) [76,77]. Both therapies have previously demonstrated preclinical efficacy in cellular and murine models of FSHD [51,52,54,55].

4. Conclusions

Since *DUX4* was identified as the central cause of FSHD, numerous targeted oligonucleotide therapies have been proposed, many of which have shown promising results in preclinical stages. However, despite *DUX4* presenting itself as an ideal therapeutic target, there are still considerable challenges that may prevent these therapies from reaching clinical use and benefiting patients. Additionally, more progress towards fully characterizing FSHD is needed, as it remains an incredibly complicated condition with many unanswered questions. Further research into the molecular underpinnings of FSHD may offer additional therapeutic targets amenable to oligonucleotide therapies. Similarly, it is important to continue investigating the normal physiological role of DUX4 in the testis and thymus, as this is not fully understood and could impact decisions made when targeting *DUX4* in skeletal muscle, possibly in terms of off-target effects. Another consideration would be the potential synergistic effect of combining multiple therapies together, particularly those that target *DUX4* expression via different modes of action. This has not yet been attempted for FSHD. Overall, with a strong pipeline of candidate oligos from many different research groups, and two siRNA drugs entering early clinical trials, the future appears hopeful for a targeted treatment option for patients with FSHD.

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Conflicts of Interest: T.Y. is a co-founder and shareholder of OligomicsTx Inc., which aims to commercialize antisense technology. S.L.B. declares that this study was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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