Review

Current Therapeutic Approaches in FSHD

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Abstract. Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common muscular dystrophies. Over the last decade, a consensus was reached regarding the underlying cause of FSHD allowing—for the first time—a targeted approach to treatment. FSHD is the result of a toxic gain-of-function from de-repression of the *DUX4* gene, a gene not normally expressed in skeletal muscle. With a clear therapeutic target, there is increasing interest in drug development for FSHD, an interest buoyed by the recent therapeutic successes in other neuromuscular diseases. Herein, we review the underlying disease mechanism, potential therapeutic approaches as well as the state of trial readiness in the planning and execution of future clinical trials in FSHD.

Keywords: All neuromuscular disease, muscle disease, facioscapulohumeral dystrophy (FSHD), outcome measures

INTRODUCTION

Facioscapulohumeral dystrophy (FSHD) is the third most common muscular dystrophy after Duchenne muscular dystrophy and myotonic dystrophy, with a prevalence of ∼12–15 per 100,000 [1, 2]. Age of onset is variable with presentations at birth to late in life. On average, males tend to present earlier in their late teen years to mid-twenties whereas females present in their late twenties to early thirties. Classically, the disease presents with facial and proximal arm weakness with winged scapula followed by weakness of foot dorsiflexion and hip girdle muscles. Additionally, truncal muscles including the paraspinals and abdominal muscle are variably affected. Asymmetric involvement is frequent and often very prominent [3]. Bulbar, cardiac, and extraocular muscles are spared. Neuromuscular restrictive lung disease occurs in about 15% of individuals with a minority needing non-invasive ventilatory support [4]. Symptomatic hearing loss

and retinal vascular disease (Coats disease) are infrequent occurring exclusively in infantile-onset disease [4]. Nevertheless, FSHD can result in significant morbidity with 20% of the patients becoming wheelchair dependent after the age of 50 [5]. As the wide spectrum of age at disease onset suggests, the rate of disease progression is variable but generally slow [6, 7].

MOLECULAR PATHOPHYSIOLOGY OF FSHD

Genetics of FSHD

Over the past decade, consensus was reached regarding the primary cause of FSHD, the inappropriate expression of the *DUX4* gene on chromosome 4q35 in skeletal muscle (see Figure 1). There are multiple tandem copies of the *DUX4* gene, each contained in a 3.3 kb repeat unit, known as a D4Z4 macrosatellite repeat [8]. Unlike microsatellite repeats that consist of a few base pairs, macrosatellite repeats are several kilobases in size. Tandem repeated DNA composes a significant portion (>50%) of the human genome and this type of copy number variation accounts for much of human phenotypic variation.

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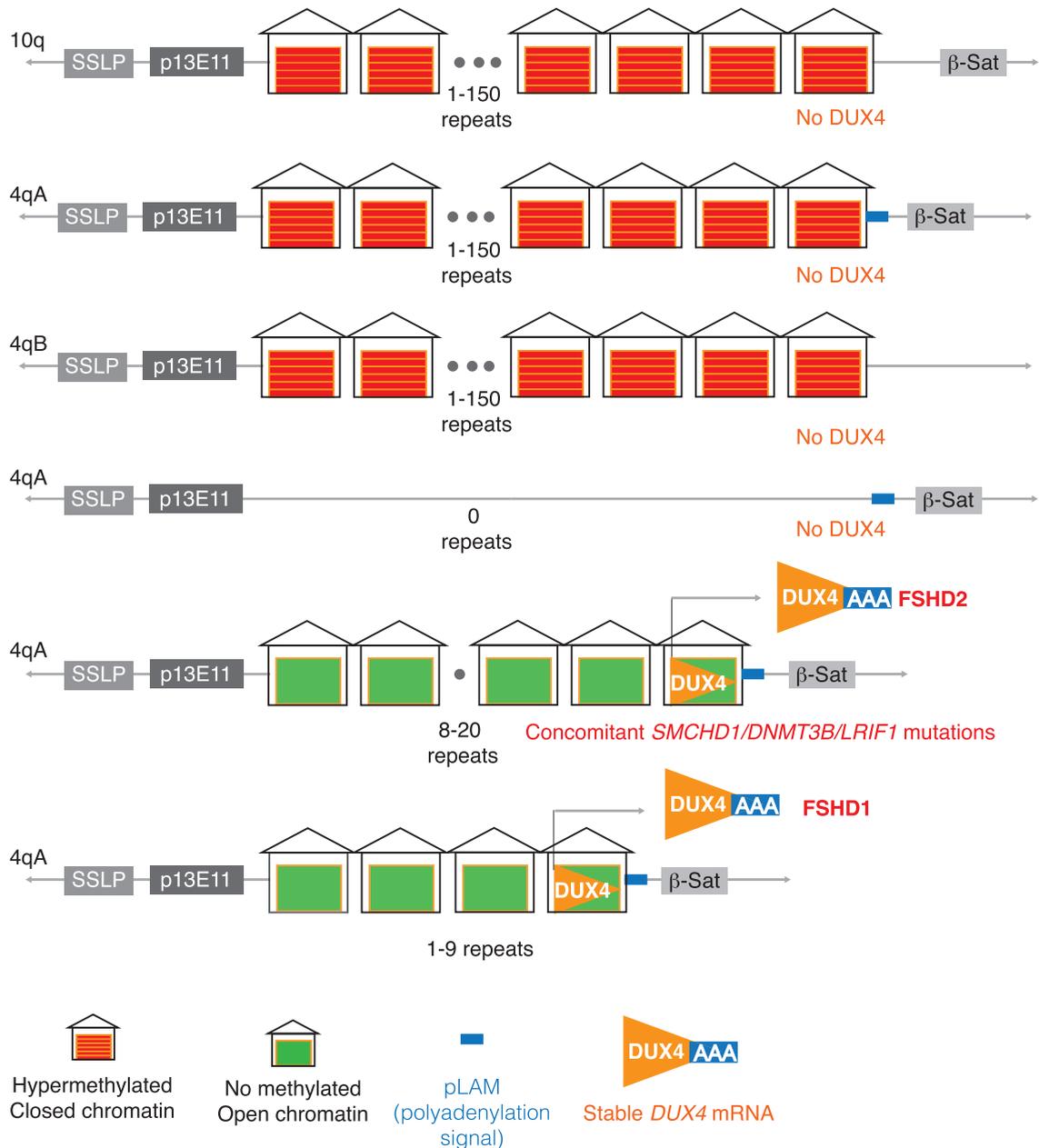
Fig. 1. DUX4 genetics. The production of DUX4 in human muscles requires the breakdown of the multiple genetic safeguards evolved to suppress its expression in somatic cells: 1) The presence of more than 10 tandem repeat units on 4q that allow for heterochromatin condensation [91]; 2) GC-rich sequence (73%) in the repeat that allow for *methylation* [92]; 3) a *polyadenylation signal* that cannot be used in somatic/muscle cells in ∼50% of the European population [93]; 4) *histone modification H3K9me3* to cause a repressive chromatin state. The utilization of the 4qA *polyadenylation signal* seems to be specific in somatic cells and may be aided by muscle-specific enhancers [94] in the proximal end of 4q that may aid in the transcription of DUX4 and the stabilization of the mRNA. The polyadenylation signal is critical for pre-mRNA processing and allows for DUX4 pre-mRNA cleavage and extension of polyadenylation to the mRNA [95]. The pathomechanism of derepression of DUX4 as the cause of FSHD was discovered because of careful study of the genetic structure of the 4q locus and the many naturally occurring cross-over events with the 10q subtelomere and the conclusions are: 1) A single repeat containing DUX4 is required; as an individual with complete loss of 4q subtelomeric region did not have FSHD [96]; 2) The region proximal to DUX4 on 4q and absent on 10q, including the upstream region with *FRG1*, *SLC25A4 (ANT1)* and *DUX4c* genes, is not required because a translocation of the most distal end of 4q to 10q resulted in FSHD; 3) 10q contraction (as found in ∼10% of the normal population) does not result in FSHD [97–99]—most likely because while 10q has similarity to the permissive 4qA alleles with the presence of 6.2-kb β-satellite sequence, it lacks the *polyadenylation signal*— similar to the non-permissive 4qB alleles.

The D4Z4 repeats are located at the telomeric end of chromosomes 4q and 10q with copy numbers from 11 to greater than 150 repeats [8]. FSHD results from a change in the non-permissive, highly methylated chromatin structure of the D4Z4 repeats to a more permissive euchromatic structure, allowing the expression of the *DUX4* gene from the most distal D4Z4 repeat. There are two sequence variants distal to the last repeat termed A and B. Only the A variant on 4q35 contains has a polyadenylation signal, allowing the production of a stable *DUX4* mRNA.

In FSHD type 1 (FSHD1), which constitutes about 95% of patients with FSHD, contraction of the D4Z4 repeat number to between 1–10 repeats results in chromatin relaxation. When a contraction occurs on a 4q35 with an A variant, stable *DUX4* mRNA and protein is produced leading to a toxic gain-of-function. In FSHD1, the shorter the number of residual repeats is broadly associated with younger disease onset, overall severity, and increase penetrance. Individuals with 1–3 repeats tend to have earlier onset and more severe muscle weakness and non-muscular manifestations such as symptomatic hearing loss, retinal vascular disease and more likely to develop restrictive lung disease [9]. Most individuals with FSHD1 have between 4–7 repeats and tend to have, as a group, more moderate disease. Contractions with 8–10 repeats have later onset, milder disease, and a higher frequency of non-penetrance (not developing symptoms). Despite the relationship between disease severity and repeat size, the wide intra-familial variability points to the presence of other factors influencing disease severity.

The remaining 5% of patients with FSHD have FSHD type 2 (FSHD2). FSHD2 is a digenic disease requiring the co-occurrence of two events: 1) at least one 4q35 D4Z4 with an A polymorphism and a contracted array, and 2) mutation in a gene that plays a role in the epigenetic repression of the D4Z4 repeats. Whereas the contraction of the D4Z4 array is the main reason for derepression of that D4Z4 array (in *cis*) for FSHD1, the mutations in FSHD2 result in derepression of D4Z4 repeats in *trans*, (of all D4Z4 arrays even the ones on chromosome 10 in addition to those on chromosome 4). About 95% of individuals with FSHD2 have concomitant mutation in the *SMCHD1* gene. SMCHD1 protein is involved in DNA hypermethylation and plays a role in X-inactivation [10–13]. The *SMCHD1* mutation results in chromatin hypomethylation of the repeats on chromosome 4 resulting in DUX4 expression from the 4q35 with a permis-

sive A polymorphism. Another gene implicated in FSHD2 is DNA (cytosine-5-)-methyltransferase 3 beta (*DNMT3B),* a methyltransferase gene responsible for epigenetic repression [14]. Heterozygous mutations in *DNMT3B* also result in hypomethylation; but only those patients with relatively short 9–13 units of D4Z4 on a permissive allele develop FSHD. Most recently, a homozygous mutation in a third gene, Ligand-Dependent Nuclear Receptor Interacting Factor 1 (*LRIF1* [also known as *HBiX1*]), was found in a patient with an FSHD phenotype and hypomethylation of the D4Z4 repeats consistent with FSHD2 [15]. The number of D4Z4 repeats in FSHD2 is on the lower range of what is considered normal, possibly less than 16 [16]. There is also an overlap between FSHD1 and FSHD2 where the penetrance of symptoms in patients with 8–10 repeats is increased due to hypomethylation at the D4Z4 array by mutations in *trans* such as *SMCHD1* [16]. There is no differences between FSHD1 and FSHD2 clinical phenotypes [17].

Normal function of DUX4 in humans

DUX4 is highly conserved among primates such as humans, chimpanzees, and orangutans. DUX4 is a transcription factor important in zygotic genome activation at the two-/four-cell embryo stage as well as in regulation of spermatagonia in adult male testes [18–20]. It is not clear how DUX4 is activated during embryogenesis, however, DUX4 is produced from the most distal monomer of both 4q35 and 10q26 in the embryo [19]. Downstream DUX4 targets are expressed in human testis [21]. Other that testes, DUX4 is known to be expressed in the thymus but has not been shown to be in any other somatic cells [22].

Pathophysiologic consequences of DUX4 expression in human skeletal muscle

DUX4 expression in skeletal muscle tissue activates a similar transcriptional program as in the embryo—inducing many of the DUX4 target genes before zygotic genome activation at the four-cell stage of the human embryo [18]. However, DUX4 protein is toxic to muscle fibers or cultured myocytes [12, 23–26]. In FSHD patient-derived myoblasts, DUX4 is expressed in stochastic bursts in a small number of myonuclei as opposed to a steady low-level expression [19, 27]. With differentiation into myotubes, DUX4 expression is more pronounced

resulting in cell death [28]. Once activated, DUX4 induces a number of genetic programs that lead to initiation of the inflammatory cascade, muscle atrophy, oxidative stress, and disrupted myogenesis [21, 28–32]. The expression of these genes is undetectable or nearly undetectable in control muscle samples but increased in FSHD1 and 2 muscle samples or *DUX4*-transfected cell lines [33].

THERAPEUTIC APPROACHES

A number of non-targeted therapeutic interventions were tried in FSHD. These include an open label trial of prednisone [34], several randomized control trials of albuterol [35–37], an intravenous myostatin inhibitor (MYO-029) [38], and a trial of oral antioxidants [39]. In none of those studies did the primary outcome measure show positive results. A more recent study evaluated the effects of an intramuscularly-administered myostatin inhibitor, ACE-083, in FSHD [40]; however, the phase 2 study was stopped as no functional benefit was demonstrated despite increasing muscle mass. One could speculate that muscle mass was only increased in good muscle and the lack of recovery of already-affected muscle prevents an improvement in functionality; this seems to be a lesson for not just FSHD but also myostatin inhibitors in other muscle diseases such as inclusion body myositis.

More targeted approaches are now possible. FSHD is an attractive target pharmaceutically because it is a relatively common muscle disease. Moreover, whereas most muscular dystrophies result from loss-of-function mutations in genes coding for critical skeletal muscle proteins, FSHD is the result of the deleterious gain-of-function due to the expression of a gene not expressed in somatic cells. Consequently, effective blocking of DUX4 expression could potentially be curative in FSHD. Possible therapeutic approaches include: 1) epigenetic silencing of the D4Z4 repeats; 2) blocking *DUX4* mRNA production; 3) targeting one of several identified downstream pathologic pathways triggered by *DUX4* expression. (For overview, see Figure 2.)

Targeting DUX4 upstream

Multiple approaches have shown decreased *DUX4* expression by either enhancing epigenetic repression of D4Z4 repeats or inhibiting upstream signals. Enhancing epigenetic repression can be achieved by targeting methylation, SCHMD1 activity, or other signals that repress the chromatin.

Delivering non-coding RNAs of the D4Z4 repeats into muscle cells may help with regulation of D4Z4 repeats by facilitating DICER/AGO-dependent epigenetic silencing of the D4Z4 repeat arrays [41, 42]. *SMCHD1* overexpression in FSHD1 and FSHD2 myotubes suppresses *DUX4* expression [43] and small molecules are being developed to augment SMCHD1 activity. The caveat would the unintended effects of increased SMCHD1 activity as it regulates and inactivates other loci such as the X chromosome. Viral delivery of *SMCHD1* under a muscle-specific promoter could be an alternative but would not be as elegant of a solution as a small molecule whose effect one can titrate.

For patients with FSHD2 secondary to *SCHMD1* mutations, genome editing of intronic mutation was attempted in muscle cell culture and could be a therapeutic approach in patients with FSHD2 secondary to *SCHMD1* mutations [44]. However, it is limited by the current low efficiency rate of genome editing in a whole organism. Other signals that repress the chromatin through histone modification such as inhibiting acetylation of histone or increasing methylation by the polycomb repressive complex 2, may suppress DUX4 expression [27]. Agents that increase the activity and/or expression of NuRD/MBD2 and/or MBDl/CAF-1 complex members that modify the chromatin/histone have also been patented for the treatment of FSHD.

Increasing methylation results in repression of the D4Z4 region, and molecular therapies that increase DNA methylation through DNMT3B may inhibit *DUX4* expression. DNMTs utilize S-Adenosylmethionine as a co-factor, which is synthesized from methionine using co-factors such as folate, choline, betaine, and vitamins B_2, B_6 and B_{12}. However, indiscriminate dietary supplementation has not been documented to effect epigenetic modification beyond the perinatal period [45] and therefore unlikely to increase DNMT3B activity in muscle specifically. This was borne out in a small study utilizing folic acid and methionine to try to increase methylation [46].

In a screen of immortalized myoblasts derived from patients with FSHD1 or FSHD2 transfected with a reporter of *DUX4* activity, inhibitors of bromodomain and extra-terminal domain (BET) family of proteins were found to suppress *DUX4* activity by blocking binding of BET family proteins to acetylated histones allowing class I histone deacetylases
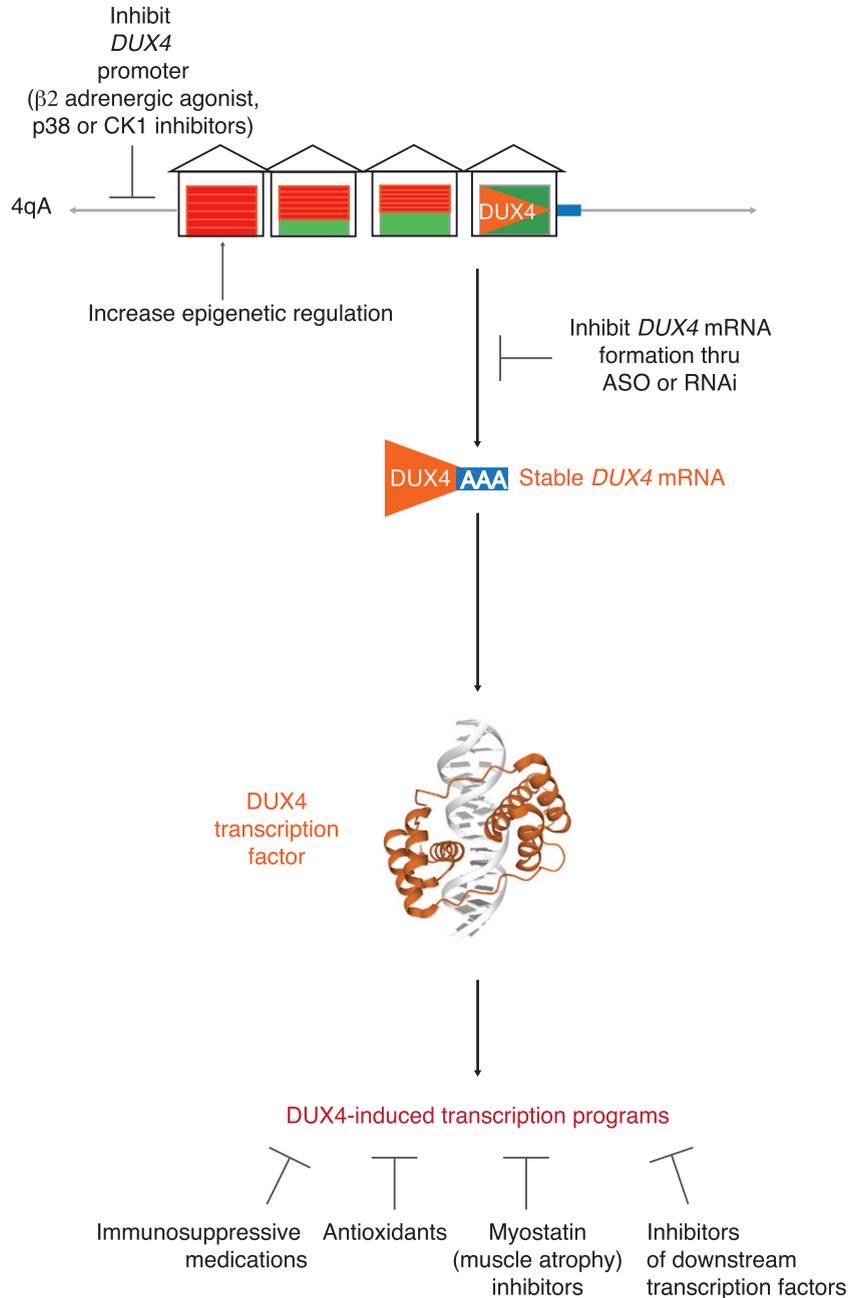
Fig. 2. Targeting DUX4. Possible targeted therapeutic approaches to FSHD include: 1) epigenetic silencing of the D4Z4 repeats; 2) blocking *DUX4* mRNA production by inhibiting DUX4 promoter or DUX4 mRNA formation; 3) targeting one of several identified downstream pathologic pathways triggered by *DUX4* expression.

(HDACs) to suppress *DUX4* expression [47].

Other potential drugs identified by screening immortalized FSHD patient-derived myoblasts are beta2 adrenergic receptor agonists, such as clenbuterol and albuterol, which decreased *DUX4* mRNA synthesis [47]. Interestingly, β2 adrenergic agonists are known to be powerful anabolic agents that trig-ger skeletal muscle hypertrophy and have been tried for treatment of muscle wasting as well as FSHD (for review see Joassard et al. [48]). They activate adenylyl cyclase to increase cellular cyclic adenosine monophosphate (cAMP) levels and subsequently the protein kinase A (PKA) pathway. Clinical trials of β2 adrenergic agonists (albuterol/salbutamol) failed

to improve strength or function [36, 37]. However, Kissel et al. reported that albuterol caused a modest, dose-dependent, but statistically significant increase lean body mass compared to placebo. Whether clenbuterol, a more potent β2 adrenergic agonist, can achieve a more marked increase in lean body mass resulting in improved strength or function remains to be seen.

A systematic search for small-molecule inhibitors of the PKA pathway led to the discovery of p38α/β inhibitors as inhibitors of DUX4 expression in immortalized myoblasts [49]. In xenograft transplant, RNA levels for *DUX4* and its targets were decreased by approximately 80% with the p38α/β inhibitor losmapimod. P38 is a subgroup of the mitogen-activated protein kinases (MAPKs) that regulates multiple processes. Of the four family members, p38α is ubiquitously expressed, p38β is more restricted in expression and expressed at a lower level in muscle [50]. When muscle satellite stem cells are activated and divide, one cell with active p38α/β becomes the proliferating daughter cell (myoblast) and the other cell with inactive p38α/β replenishes the quiescent satellite stem cell pool. Further down the myogenesis process, p38α/β MAPKs are also important for activating transcriptional and epigenetic programs that result in differentiation of the myoblast into the myocyte [51]. Losmapimod appears to work in the myoblasts and the differentiated myocytes.

However, one must take into context the ubiquity of the p38 pathway with p38α being expressed in most cells. Tissue-specific knockout of p38α show that it is important for proliferation of lung epithelial cells and cardiomyocytes [52, 53]. Activation of p38 leads to activation of inflammatory cytokines. This led to therapeutic trials of losmapimod in chronic obstructive lung disease, and pain, none of which showed a beneficial effect of losmapimod [54–56]. Currently, Fulcrum Therapeutics is conducting a phase II trial to address whether p38α/β inhibitors decrease *DUX4* expression and preserve muscle bulk on MRI [57]. However, the ubiquitous role of p38 in cell functions will make long-term surveillance important.

Another compound that inhibits DUX4 expression is casein kinase I (CK1) inhibitors which suppress DUX4 expression in FSHD myotubes and in the xenograft model [58]. The authors consider their drug-screening assay to be superior as it can simultaneously measure DUX4 repression and myoblast fusion index at various drug concentrations. Since DUX4 expression occurs when myoblasts fuse into myotubes, a drug inhibiting myoblast fusion will result in a false positive measure of DUX4 inhibition.

Another therapeutic approach to inhibit DUX4 upstream is to target the promoter of DUX4 on 4q. Himeda et al. fashioned a dominant negative inhibitor with a catalytically dead Cas9 loaded with a guide RNA to the promoter region of DUX4 linked to a protein that blocked transcription activation (the catalytically dead Cas9) [59]—suggesting a clever way to utilize our understanding of the 4q35 genetic architecture to block DUX4 expression.

Any approach to DUX4 repression should consider possible off-target effects on tissues that normally express DUX4, such as the thymus and testes. DUX4 repression in the thymus is not likely to cause untoward side effects but possible effects on spermatogenesis need to be considered.

Targeting DUX4 directly at the RNA level

Targeting DUX4 directly at the RNA level is appealing because it targets a transcript that should not be expressed and there is well-described chemistry, antisense oligonucleotide (ASOs) or inhibitory RNA (RNAi) therapies, to target the RNA.

Inhibitory RNA (RNAi) therapies using small interfering RNA (siRNA) were used to target the 3′ untranslated region transcribed from pLAM [60], the coding region [21], well as the region upstream of the DUX4 transcription start site [41]. The last is an endogenously produced siRNA that may be part of the cell's regulatory mechanism of the D4Z4 region. One of the limitations of RNA interference approach is its high dose cytotoxicity derived from its off-target effects [61, 62].

Similarly, ASOs have been successful in targeting portions of the 3′ untranslated region of the DUX4 pre-mRNA to inhibit the polyadenylation in immortalized FSHD cells [63], myotubes derived from FSHD muscle cultures and xenografts [64]. A polyadenosine tail is extended on the pre-mRNA and is beyond the polyadenylation signal and not encoded in the D4Z4 DNA. ASOs interfere with transcript termination and 3' end processing to cause mRNA degradation and decrease DUX4 protein expression. ASOs have also been made to interfere with DUX4 mRNA splicing [65].

Locked nucleic acid (LNA) gapmer antisense oligonucleotides also have been engineered to bind to DUX4 mRNA and be knocked down through RNAse H-mediated degradation and shown to be successful in tissue culture and injection into mouse models [66].

However, these types of technologies are bedeviled by the electrostatic nature or bulk of the compounds which prevents efficient uptake through the lipid bilayer and the muscle cells when delivered systemically; as have been found in myotonic dystrophy and Duchenne muscular dystrophy [61, 62]. Some of this is being addressed by using adeno-associated virus (AAV) as a delivery vector. AAV vectors delivering artificial microRNAs targeting the *DUX4* mRNA were able to direct the transcript toward an RNAi degradation pathway [67]. In addition, ASOs modified with a carrier that targets muscle cells and facilitates uptake are being tried. Finally, modifying the chemistry of the backbone could be beneficial as locked nucleic acid (LNA) gapmer antisense oligonucleotides.

Targeting downstream effects of DUX4

DUX4 is a powerful inducer of myriad genetic programs that lead to initiation of the inflammatory cascade, muscle atrophy, oxidative stress, disrupted myogenesis. As a transcription factor, DUX4 uncovers a vast, complex gene regulatory network. Inhibition of DUX4 can be achieved by utilizing DNA aptamers, short oligonucleotides, engineered bind to the DNA binding site of DUX4 and thus inhibiting DUX4 from binding to its transcriptional activator sites [68].

To exert its transcriptional activity, DUX4 recruits histone acetyltransferases (HATs) p300 and CBP (CREB binding protein) [69]. Selective inhibitors of p300 can inhibit the transcriptional activity of DUX4 in cell culture [70]. One such transcription factor is PITX1, another double homeobox transcription factors that activates pathways that lead to muscle atrophy (through atrogin-1 and MuRF-1) and inflammatory features [30]. *PITX1* suppression with ASOs can ameliorate the pathological features of the muscle-specific *PITX1* transgenic mouse [71].

DUX4 induction in FSHD myoblast model results in accumulation of the glycosaminoglycan hyaluronic acid and mediates a few of the downstream pathways that DUX4 is known to activate [72]. 4-methylumbelliferone, a well-characterized competitive inhibitor of HA biosynthesis prevents DUX4-induced accumulation of hyaluronic acid and subsequent downstream pathways. It is an already approved drug in Europe and Asia called "hymecromone" where it is used to treat biliary spasm.

Oxidative stress, with its resultant production of free radicals and reactive oxygen species, results in cellular damage can be important in the pathophysiology of FSHD and several known antioxidants have been identified to inhibit DUX4-induced toxicity in myoblasts [73]. A small subset of antioxidants were studied in adults with FSHD [39] and no follow-up studies have since been done to look at more specific antioxidants.

It is not clear, however, that inhibiting specific downstream pathways of DUX4 will completely abrogate all the damage caused by the myriad genetic programs that DUX4 uncovers in muscle cells.

CLINICAL TRIAL READINESS IN FSHD

Work on various aspects of trial readiness is ongoing for the last decade as consensus on the FSHD disease mechanism was reached [74, 75]. Critical components of trial readiness include facilitating patient access to clinical trials, establishing research centers familiar with FSHD assessments, having a good understanding of the natural history of the disease and developing a multitude of relevant outcome measures for early and late phase trials.

Patient access to research studies is facilitated by the presence of a number of FSHD patient registries in the US and in several European countries [76–79]. The oldest is the National Registry for Facioscapulohumeral Dystrophy in the US which prospectively collected yearly clinical data on patients with FSHD for almost two decades, data that proved valuable in understanding aspects of functional progression [80].

The first prospective natural history study of FSHD followed 80 patients for up to three years [6]. The study was limited by absence of genetic testing in all subjects and evaluations were restricted to manual muscle testing and quantitative myometry. Nevertheless, both outcome measures showed a slight 12-month decline in strength. A large, multi-national, natural history study, the ReSolve study, is currently in its third year and will exam a change in a variety of outcome measures over a span of 24 months [81]. These outcome measures include, in addition to strength testing, a composite functional outcome measure (FSHD-COM), reachable workspace as a quantitative measure of shoulder function an FSHD-specific patient reported health index (FSHD HI) (90, 91, 92, 93). Additionally, DEXA scan to assess changes in lean body mass and electrical impedance myography (EIM), a measure of muscle composi-

tion, to look for changes in individual muscles are being investigated as potential biomarkers [82]. Separate studies have investigated the utility of MRI and muscle ultrasound as a biomarker in FSHD [83–86].

Early phase 2 trials seeking to test safety and target engagement of DUX4 will require either a tissue or validated circulating biomarkers. To date, there are no validated FSHD circulating biomarkers [87, 88]. However, as *DUX4* is expressed stochastically and at very low levels, it is difficult to measure quantitatively in muscle samples. Fortunately, as *DUX4* is a transcription factor, a large number of genes are turned on and act as reliable surrogates of *DUX4* activity. [21, 33, 47, 89]. Moreover, quantitating a subset of four *DUX4*-regulated genes (*LEUTX, KHDC1L, PRAMEF2* and *TRIM43*) may increase sensitivity of detecting *DUX4* activity. A recent study, using MRI to select muscles for biopsy showed that muscles with T2 STIR positive changes and fatty infiltration on T1 sequences showed the highest levels of *DUX4*-target expression [90]. These findings suggest MRI guidance is crucial in the selection of the optimal muscle to biopsy in early phase 2 trials. This concept is being validated in the current losmapimod trials, as is the use of downstream *DUX4*-targets as a marker of DUX4 activity.

CONCLUSION

For almost two decades, the underlying disease mechanism in FSHD, one of the most common muscular dystrophies, was an enigma. With consensus reached on disease mechanism, targeted treatments are now possible resulting in heightened interest from pharmaceutical companies. Simultaneously, active clinical research studies are reexamining FSHD natural history, vetting a number of novel disease-specific clinical outcome measures as well as imaging, circulating and tissue biomarkers.

FINANCIAL DISCLOSURES

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