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Invited Review

INVITED REVIEW

Facioscapulohumeral dystrophy: activating an early embryonic transcriptional program in human skeletal muscle

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Abstract

Facioscapulohumeral dystrophy (FSHD) is the third most prevalent muscular dystrophy. A progressive disease, it presents clinically as weakness and wasting of the face, shoulder and upper arm muscles, with later involvement of the trunk and lower extremities. FSHD develops through complex genetic and epigenetic events that converge on a common mechanism of toxicity with mis-expression of the transcription factor double homeobox 4 (DUX4). There is currently no treatment available for FSHD. However, the consensus that ectopic DUX4 expression in skeletal muscle is the root cause of FSHD pathophysiology has allowed research efforts to turn toward cultivating a deeper understanding of DUX4 biology and the pathways that underlie FSHD muscle pathology, and to translational studies aimed at developing targeted therapeutics using ever more sophisticated cell and animal-based models of FSHD. This review summarizes recent advances in our understanding of FSHD, including the regulation and activity of DUX4 in its normal developmental roles as well as its pathological contexts. We highlight how these advances raise new questions and challenges for the field as it moves into the next decade of FSHD research.

Introduction

Facioscapulohumeral dystrophy (FSHD) affects approximately 1 in 10 000–20 000 individuals worldwide (1–3). Symptoms typically appear during the second or third decade of life as asymmetric weakness of the facial (facio), shoulder (scapulo) and upper arm (humeral) muscles, and progress to affect nearly all skeletal muscle groups (4). However, there are also more acute pediatric onset forms of FSHD (5). Non-muscular symptoms are rare but include high-frequency hearing loss and retinal vascular disease (6,7), and are usually associated with the more severe forms of FSHD (8). Disease progression and severity is widely variable, with ∼20% of mutation carriers asymptomatic while another ∼20% require use of a wheelchair (8).

There are two clinically indistinguishable but genetically distinct forms of FSHD. The most common, FSHD type 1 (FSHD1), is autosomal dominantly inherited and caused by deletion of a subset of D4Z4 macrosatellite repeats on chromosome 4q35 that result in an array of fewer than 11 units (9,10). Additionally, approximately 5% of individuals have FSHD type 2 (FSHD2), which results from mutations in regulators of the D4Z4 repeat

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array, such as SMCHD1 or DNMT3B (11,12). Both forms of FSHD require a specific permissive haplotype of chromosome 4 that contains a polymorphism that creates a polyadenylation signal distal to the D4Z4 array (13). Despite the different genetic etiology, both FSHD1 and FSHD2 are caused by a common pathophysiological mechanism: epigenetic de-repression of the D4Z4 array allowing mis-expression of the double homeobox 4 (*DUX4*) retrogene encoded within each unit of the D4Z4 repeat (8,14).

DUX4 is a transcription factor that is expressed in the luminal cells of the testis, most likely the spermatogonia and primary spermatocytes, but silenced in most somatic tissues, including skeletal muscle, through repeat-mediated epigenetic repression (15,16). Although expression of DUX4 in skeletal muscle and many other cell types causes cell death (17–20), questions remain concerning the pathophysiologic consequences of DUX4 expression and the regulation of DUX4 expression in development and in FSHD. Further understanding of these mechanisms should guide new potential therapeutic targets for FSHD.

Previous reviews have detailed the history of mapping the FSHD loci, the underlying genetic mutations and their epigenetic consequences, and the determination that mis-expression of DUX4 in skeletal muscle is the cause of FSHD (8,14,16,21–27). This review will focus on the regulation and activity of DUX4 (Fig. 1), particularly work done in the last 2 years that has revealed its normal developmental roles and further illuminated the pathological function of DUX4 in FSHD.

A Conserved Function for DUX4

In individuals unaffected by FSHD, the *DUX4* gene is transcriptionally silenced in most tissues of the body (8). However, our group reported in 2010 that DUX4 is normally expressed in the testis (15), and others have shown low levels of DUX4 in the thymus (28). More recent studies revealed that DUX4 is transiently expressed in four-cell human embryos where it functions to activate a cleavage-stage transcriptional program and might be a key regulator of zygotic genome activation (ZGA) (29,30). The mouse ortholog of DUX4 (Dux) was also shown to be expressed in the two-cell embryo co-incident with murine ZGA (31). Dux activates the mouse cleavage-stage gene program (30) and is necessary for proper embryonic development, as Dux-deficient embryonic stem cells show reduced conversion to the totipotent '2C-like' state (29) and Dux-depleted embryos fail to reach the morula stage (31). Overall, these new findings strongly support a functionally important role for human DUX4 and mouse Dux in early development. They also raise questions such as: what are the mechanisms by which DUX4/Dux are briefly activated and then rapidly silenced during cleavage-stage development? Are these same pathways utilized in other contexts where DUX4 is present (such as testis, thymus and FSHD muscle)? Why are DUX4/Dux not toxic in the embryo? And, do ancestral or related DUX genes (e.g. DUXA, DUXB, DUXC) function similarly?

The conserved function of DUX4 and Dux in early development is consistent with their analogous origins as retroposition events of the ancestral *DUXC* gene (32–34). However, DUX4 and Dux have diverged considerably at the sequence level, including within the homeodomains, resulting in slightly different DNA-binding motifs and the selective transcriptional induction of species-specific retrotransposons (30). This suggests an evolutionary pressure to maintain activation of the core ancestral gene network while allowing for divergence away from the activation of potentially deleterious retroelements. Understanding these complexities will inform the generation of FSHD model systems, which have generally expressed human DUX4 in non-primate species.

As has been suggested before (24), it is possible that improper expression of the DUX4 early developmental transcriptional regulatory network might be poorly tolerated in skeletal muscle, providing a link between the normal function of DUX4 and its role in the pathogenesis of FSHD. Indeed, it had already been well established that DUX4 activates germline genes, retroelements and immune modulators that define the pre-implantation embryo when mis-expressed in muscle (18,35,36). Whether or how this transcriptional activity might lead to cellular toxicity, and determining the precise mechanisms of toxicity, are areas of active research.

DUX4-Mediated Toxicity

The first report of DUX4 toxicity was from Alberto Rosa's group (17) and subsequent studies have demonstrated that DUX4 expression causes apoptosis in a multitude of human and mouse cells, including skeletal muscle, both *in vitro* and *in vivo* (18–20,37–40). However, the precise pathways responsible for DUX4-induced cytotoxicity are as yet unclear and several studies have identified different mechanisms, all of which might contribute to DUX4 toxicity.

p53

Earlier findings showed that DUX4 toxicity depends on p53 in mouse muscle (20). More recently, it was shown that this requirement for p53 is at least partly context dependent, since *p53* knockout in an inducible DUX4 mouse model and *P53* ablation in human myoblasts failed to mitigate DUX4-mediated toxicity (19,41). Due to differences in systems used and measurements of toxicity, however, the basis of the cellular context for the role of P53 in DUX4 cytotoxicity remains to be determined.

PAX3/PAX7

Another proposed mechanism of DUX4 toxicity is the impairment of myogenesis by competition of DUX4 with the homeodomain transcription factors PAX3 and PAX7, which are important for muscle development (38,42). In accordance with this hypothesis, it was recently shown that Pax3 or Pax7 overexpression, but not the overexpression of a panel of other related homeodomain factors, could inhibit DUX4 toxicity in mouse cells (43); and that suppression of PAX7 target genes is a hallmark of FSHD (44). Interestingly, a chimeric DUX4 protein with a Pax7 homeodomain substitution retained the ability to provoke cytotoxicity (43). As DUX4 toxicity requires both the ability to bind DNA as well as activate transcription (20,45,46), this finding suggests that there may be a core target or set of targets bound by both DUX4 as well as the DUX4-Pax7 chimeric protein that elicit cellular toxicity.

MYC

A recent genetics-based screen (19) identified two pathways that modulate DUX4-mediated toxicity: the MYC-mediated apoptosis pathway and the double-stranded RNA (dsRNA) antiviral pathway that includes EIF2AK2/PKR and RNASEL (discussed below). Intriguingly, MYC, which is known to cause apoptosis in certain contexts (47), was dramatically upregulated in

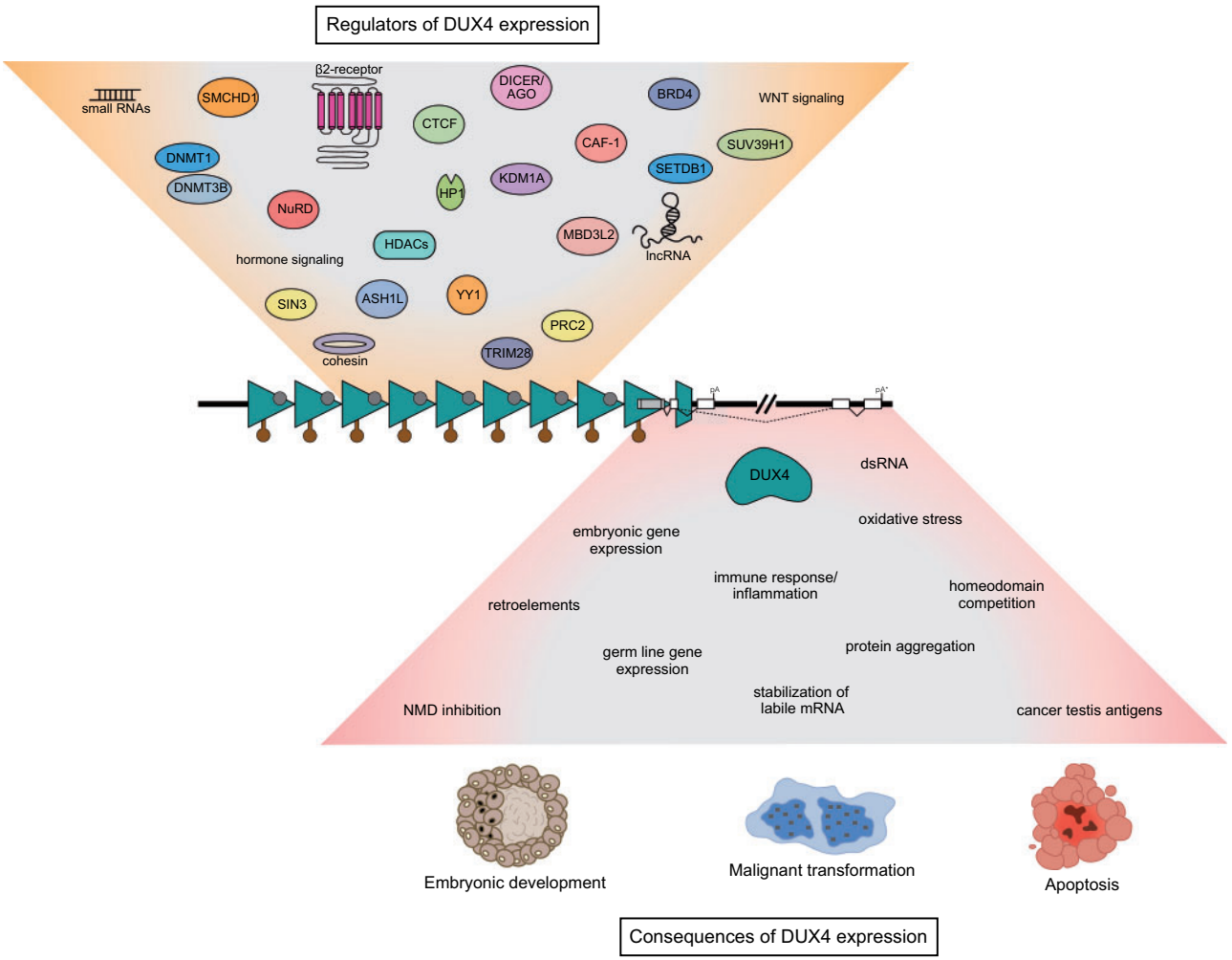

Figure 1. Causes and effects of DUX4 expression. The *DUX4* gene (white boxes with the open reading frame in gray) is encoded within each repeat unit (teal triangles) of the D4Z4 macrosatellite array. In healthy individuals the array is marked by high levels of CpG DNA methylation (brown lollipops) and transcriptionally repressive histone modifications (gray circles), which contribute to DUX4 silencing and are reduced in people affected by FSHD. Other factors and pathways known to regulate DUX4 expression are depicted in the tan funnel above the D4Z4 array. Production of stable DUX4 mRNA depends on the presence of a permissive haplotype located immediately distal to the last D4Z4 repeat that contains a functional polyadenylation site (pA), or on a polyadenylation sequence (pA*) found in downstream exons that is likely utilized in the germline. The expression of DUX4 protein is associated with many cellular phenotypes, which are outlined in the inverted pink funnel below the D4Z4 array. Depending on the context, DUX4 may or may not lead to normal development, inappropriate apoptosis or malignant transformation.

DUX4-expressing cells via mRNA stabilization (19). This mechanism was not specific to MYC, however, and appears to occur with other labile mRNAs such as FOSB and JUN (19), suggesting that DUX4 might regulate a set of genes involved in broadly altering a metabolic immediate-early response. As a consequence of MYC stabilization, DUX4 might also inhibit mitochondrial activity through the activation of MYC-targeted genes EGR1 and the gamma isoform of BCL2L11/BIM (BIMγ) (19). The enhanced half-life of normally labile mRNAs also suggests that DUX4 can provoke a general effect of globally decreased RNA quality control mechanisms, potentially related to the DUX4-mediated inhibition of the nonsense-mediated decay (NMD) pathway (48). This phenomenon of decreased RNA quality control may further play a role in DUX4-associated RNA/protein aggregation, described below. Whether DUX4 has similar effects in early development remains to be determined, but elevation of MYC and expression of BIMγ has been reported in germline cells (49,50) and pre-implantation embryos (51,52), and MYC RNA levels increase dramatically in human cleavage-stage embryos (see Supplementary Table 2 in 29).

Antiviral response pathways and RNA/protein aggregation

The genetic screen also demonstrated that knockdown of the dsRNA responsive, pro-apoptotic EIF2AK2/PKR and RNASEL at least partially rescued DUX4 toxicity (19). Further investigation revealed that DUX4-induced dsRNA forms aggregates that co-localize with the NMD-associated exon junction component EIF4A3, indicating that DUX4-induced dsRNAs might be spliced, and that EIF4A3 sequestration to DUX4-induced dsRNAs may partially explain NMD inhibition by DUX4, perhaps acting in concert with the DUX4-mediated UPF1 degradation described previously (48). Of interest, aggregation of a potential dsRNA-binding protein, TDP-43 (53) and FUS was also observed in DUX4-expressing cells (54), which parallels protein aggregation in other neuromuscular diseases such as amyotrophic lateral sclerosis. Together, these findings suggest that there may not be a specific target or set of targets that elicit DUX4 cytotoxicity, but rather that stabilization of otherwise labile mRNAs and/or the accumulation of dsRNAs by DUX4 results in RNA/protein

aggregation and ultimately the activation of apoptotic pathways. An important next step will be to identify the transcripts which make up DUX4-induced dsRNAs and to explore the consequences of the subset of mRNAs preferentially stabilized following DUX4 expression.

Conservation of toxicity

It was recently shown that human DUX4 and mouse Dux expression, but not the expression of other related homeodomain family members DUX1, DUX5, DUXA or Duxbl, is toxic to human and mouse cells (55). Because DUX4 and Dux are both toxic via mechanisms that depend on their transactivation and DNA-binding domains (20,45,46,55), it follows that a shared target gene, or set of target genes, may be necessary for the induction of DUX4/Dux toxicity. One option for uncovering the pathways necessary for DUX4 toxicity would therefore be to intersect downstream genes commonly targeted by each transcription factor. A recent study took this approach, comparing DUX4 and Dux binding genome-wide via chromatin immunoprecipitation coupled to high-throughput sequencing (55). With a multitude of shared targets, it remains difficult to ascribe a single target, or set of targets, as necessary for DUX4 and Dux toxicity (30,55). More perplexing is that DUX4 expression is toxic in distant, non-mammalian species such as zebrafish and *Drosophila melanogaster* (56–58). Because the double homeodomain family of genes arose in placental mammals (34), these species evolved without DUX factors and therefore likely have no conserved DUX4 target genes. Thus, it is currently unclear whether a specific DUX4 target gene or set of target genes is necessary for the multi-species DUX4 toxicity, or whether the potent transcriptional activity of DUX4 leads to an abundance of aberrant transcripts and proteins that form toxic aggregates.

DUX4 Mis-Expression in Non-FSHD Diseases

The FSHD modifiers SMCHD1 and DNMT3B not only regulate DUX4, but also have pleiotropic impacts when mutated. Missense mutations in *SMCHD1* were recently found in bosma arhinia microphthalmia (BAM) syndrome (59,60), and recessive mutations in *DNMT3B* are found in patients with immunodeficiency, centromeric instability and facial abnormalities (ICF) syndrome (61,62). In cases of ICF, mutant *DNMT3B* was associated with a hypomethylated D4Z4 array, making ICF patients more susceptible to DUX4 mis-expression and FSHD if they have the permissive 4q haplotype (12).

D4Z4 translocations to the IgH locus that result in the mis-expression of a truncated DUX4 protein in B-cells have recently emerged as the leading cause of acute lymphoblastic leukemia (ALL) in adolescents and young adults and contribute to leukemogenesis in mice (63–68). In a subset of sarcomas, a translocation fuses the DNA-binding portion of capicua (CIC) with the carboxy-terminal activation domain of DUX4 and results in aberrant gene expression (69). It is interesting to note that the DUX4 protein expressed in these two cancers is different than that found in normal development or in FSHD. In the cases of ALL, the truncated DUX4 transcript encodes a protein that lacks the DUX4 carboxy-terminal activation domain, an isoform that is largely transcriptionally inactive when expressed in skeletal muscle cells (35). In contrast, in the sarcomas, it is the activation domain of DUX4 that is fused to the DNA-binding domain of CIC, presumably activating the expression of genes that are normally repressed by CIC.

Regulators of DUX4 Expression

In most somatic tissues, the D4Z4 arrays appear to be silenced via multiple mechanisms, including DNA methylation, histone modifications and repressive chromatin proteins, which are disrupted in FSHD (11,12,56,70–77) (Fig. 1). Specifically, the D4Z4 repeats are typically CpG hypermethylated, wrapped in histones that contain tri-methylated histone H3 lysine 9 (H3K9me3) and histone H3 lysine 27 (H3K27me3) (repressive chromatin marks associated with heterochromatin), and bound by proteins (like YY1, HP1γ, EZH2 and cohesin) known to silence gene expression. However, a holistic understanding of *DUX4* gene regulation is lacking. For example, it is as yet unclear how DUX4 repression is established and maintained in somatic tissues, or how DUX4 is activated in the early embryo.

Recent work used CRISPR-based locus-specific proteomics to characterize proteins that bind the D4Z4 array (77). This approach, combined with gene depletion experiments, revealed that the nucleosome remodeling deacetylase (NuRD) and chromatin assembly factor 1 (CAF-1) complexes function to repress DUX4 expression in human skeletal muscle and induced pluripotent stem cells. Furthermore, these studies uncovered a role for the DUX4-induced expression of the MBD3L family of methyl-CpG-binding proteins (MBD3L2–5) in relieving NuRD complex silencing activity and amplifying DUX4 levels in FSHD muscle cells. Together with the earlier studies, it is becoming clear that D4Z4 silencing is multi-factorial and might have similarities to the silencing of other repetitive elements in the genome, such as mechanisms that repress retroelements (78).

Models of FSHD

Cell and animal models are indispensable for generating mechanistic understandings of human disease and for testing potential therapeutic approaches in anticipation of clinical trials. Vexingly, the unique complexity of FSHD has slowed the development of model systems that recapitulate the totality of the disease. However, models of specific aspects of FSHD have been developed that can be used to advance pre-clinical studies (8,79) (Fig. 2). The major transcriptional signature associated with FSHD muscle compared to controls is composed of genes regulated by DUX4 (80). Recent studies identifying the transcriptional signature in FSHD myocytes and the DUX4 signature in muscle cell lines engineered to express DUX4 showed a high similarity (18,37), and validates the tissue culture systems for aspects of the study of DUX4 biology and FSHD therapeutic development. Also, 114 lymphoblastoid cell lines from multigenerational FSHD families were recently characterized and present a possible new cellular model for FSHD investigation (81), as does the use of human pluripotent stem cells derived from FSHD patients (15,82) (Fig. 2A).

There have also been recent advances in the generation of DUX4 mouse models, including modifications which build upon previous systems in an attempt to more closely mimic FSHD pathology (79,83–85). The three most recent murine models, summarized in Figure 2B, all show skin phenotypes, and two show mosaic DUX4 expression and immune infiltrates in muscle as well as muscle wasting, recapitulating several features seen in FSHD. *In vivo* mouse models provide an opportunity to study cell non-autonomous effects (such as the role

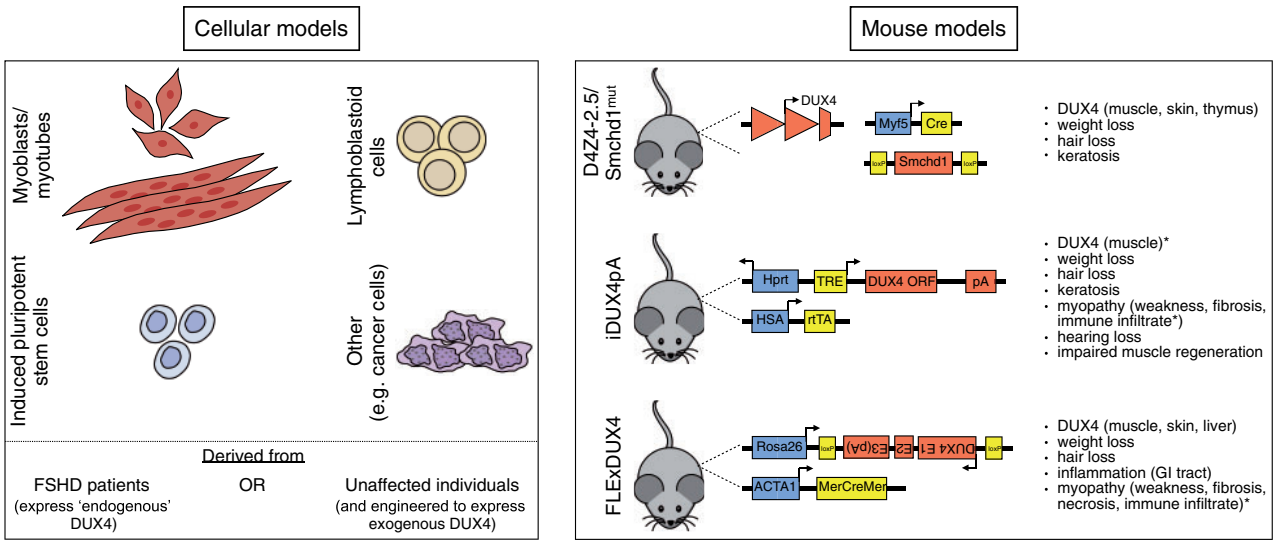

Figure 2. Cellular and murine models of FSHD. The normal and pathological functions of DUX4 are currently being investigated using the depicted cell (**A**) and murine (**B**) models. Note that DUX4 can be exogenously expressed in cell culture systems via transfection, transduction or stable integration of inducible transgenes. TRE, tetracycline response element; ORF, open reading frame; pA, polyadenylation signal; rtTA, reverse tetracycline-controlled transactivator; E1, exon 1; E2, exon 2; E3, exon 3; MerCreMer, Cre recombinase protein fused to two mutant estrogen-receptor ligand-binding domains; *, phenotype only apparent when DUX4 expression is induced.

Table 1. FSHD therapeutic development

Therapeutic	Mechanism(s) of action	Tested in patients?	References
Anti-inflammatory	Immunosuppression Inhibit pathologic processes downstream of DUX4	Yes	(101,102)
Antioxidant	Prevent oxidative stress Inhibit pathologic processes downstream of DUX4	Yes	(103–106)
Antisense RNA	Enhance D4Z4 repression Inhibit DUX4 expression Inhibit pathologic processes downstream of DUX4	No	(19,39,76,77,94–96,107,108)
BET bromodomain inhibitor	Enhance D4Z4 repression Inhibit DUX4 expression	No	(97)
Beta-2 adrenergic agonist	Increase muscle strength/mass Enhance D4Z4 repression Inhibit DUX4 expression	Yes	(97,109–111)
Calcium channel blocker	Restore calcium dysregulation Inhibit pathologic processes downstream of DUX4	Yes	(112,113)
Exercise	Increase muscle strength/mass	Yes	(114–116)
GSK3β inhibitor	Enhance D4Z4 repression Inhibit DUX4 expression	No	(40)
Myostatin inhibitor	Increase muscle strength/mass	Yes	(117,118)
Steroid	Increase muscle strength/mass	Yes	(119)
Tissue transplantation	Enhance muscle regeneration Block DUX4 spreading	Yes	(120,121)
Tyrosine kinase inhibitor	Enhance muscle regeneration Inhibit pathologic processes downstream of DUX4	No	(98)
Unknown	Enhance D4Z4 repression Inhibit DUX4 expression Block DUX4 activity Block DUX4 spreading Inhibit pathologic processes downstream of DUX4	No	(77,99)

of the immune system), making them invaluable for testing therapies. However, one major caveat for the current murine models is the use of human DUX4, which relies on the assumption that the divergence between human DUX4 and mouse Dux has maintained cross-species regulation of the FSHD-relevant mechanisms of pathology despite divergence in binding site motifs and cross-species transcriptional programs (30,55). Because of this divergence, it is possible that the expression of mouse Dux in mouse skeletal muscle might better recapitulate the full scope of the disease transcriptome and

mechanisms and provide a model that more broadly recapitulates FSHD.

FSHD Therapeutics

There are currently no effective treatments for FSHD, but therapeutic modalities are being actively explored (Table 1). Consequently, there is an urgent need to establish measurable clinical outcomes that are feasible for a trial timescale. The slowly progressive nature of FSHD has made finding functional outcomes that change predictably and rapidly enough a challenge. Tissue biomarkers may provide one practical approach, and a set of the DUX4 target genes most robustly expressed in FSHD muscle has been identified as candidate biomarkers that require further validation and correlation with clinical severity and progression (80). Serum biomarkers are also being explored (86,87). Additionally, there is much recent effort around using magnetic resonance imaging (MRI) to track FSHD disease progression, and studies have shown a correlation between short TI inversion recovery (STIR) positivity, likely a marker of inflammation, and progression to fibro-fatty infiltration (88–93), suggesting that MRI might be a valuable non-invasive mechanism to identify muscles with active disease.

Prior to the consensus that mis-expression of DUX4 is the root cause of FSHD, clinical trials investigated several drug classes hypothesized to improve overall FSHD muscle function but none showed a robust benefit to patients. Current approaches are more targeted, with the ultimate goal of pharmacological control of either *DUX4* gene expression or the activity of the DUX4 protein, and include antisense oligonucleotides that target DUX4 mRNA (76,94–96) and high-throughput screens to identify small molecules that block DUX4 expression or activity (97). Endeavors to inhibit downstream effectors of DUX4 (19,98), enhance DUX4-repressive proteins such as SMCHD1 and NuRD (77,99), inhibit activators of DUX4 such as ASH1L and MBD3L2 (70,77), and block the spread of DUX4 through the myofiber (48,77,100) are also avenues of future therapeutic progress. Those targets which are specific to repeat elements or are relatively narrowly expressed present the best options for development. Overall, having multiple FSHD therapeutic candidates in the drug development pipeline makes it imperative to actively prepare for clinical trials, including those that are designed to assess multiple therapies relative to each other.

Conclusions

The last two years have seen remarkable advances in our understanding of DUX4 biology. In addition to illuminating FSHD pathophysiology, the work highlighted here has significant implications for early embryo development, cellular reprogramming and cancer biology. We are hopeful that integrating the insights from scientists in these diverse fields will ultimately lead to a treatment or cure for FSHD.

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