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Emerging preclinical animal models for FSHD

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Abstract

Facioscapulohumeral dystrophy (FSHD) is a unique and complex genetic disease that is not entirely solved. Recent advances in the field have led to a consensus genetic premise for the disorder, enabling researchers to now pursue the design of preclinical models. In this review, we explore all available FSHD models (DUX4-dependent and -independent) for their utility in therapeutic discovery and potential to yield novel disease insights. Due to the complex nature of FSHD, there is currently no single model that accurately recapitulates the genetic and pathophysiological spectrum of the disorder. Existing models are limited to emphasize only specific aspects of the disease, thus highlighting the need for more collaborative research and novel paradigms to advance the translational research space of FSHD.

Keywords

Facioscapulohumeral dystrophy; muscular dystrophy; DUX4

Facioscapulohumeral muscular dystrophy (FSHD)

Muscular dystrophies are a genetically inherited group of diseases that result in progressive skeletal muscle weakness and wasting. Facioscapulohumeral dystrophy (FSHD) is the most common autosomal dominant form of muscular dystrophy, affecting approximately 1 in 8,000 individuals worldwide [1]. The condition was first described in 1885 [2], outlining a characteristic asymmetric pattern of weakness involving the facial (facio), shoulder

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Conflicts of interest

The authors declare no conflicts of interest.

(scapula) and upper arm (humeral) muscles (Box 1, Figure I). Clinical symptoms usually manifest within the second or third decade of life, with an estimated penetrance of ~80% by the age of 30 years [3]. A fraction of affected patients also demonstrate hearing loss and potential vision loss from retinal vasculopathy (Coats disease) [4,5]. FSHD cases can be subcategorized into two groups, based on the underlying genetics of their disease. Patients with contractions of the 4q35 D4Z4 (see Glossary) are considered FSHD1, while FSHD2 is contraction independent. Although clinically indistinguishable from each other, approximately 5% of all FSHD cases are FSHD2 and have a distinct digenic inheritance pattern that includes aspects of the 4q35 D4Z4 array [6].

Progress in therapeutic discovery has largely been hampered by lack of suitable animal models and thus there are no specific treatments or cures for FSHD. However, recent key advances in the understanding of FSHD genetics and epigenetics, implicating misexpression of the DUX4 gene as being necessary for pathogenesis, have enabled the field to more accurately attempt recapitulating the FSHD pathogenic mechanism and begin to address the need for FSHD-like animal models to use in therapeutic development and preclinical testing.

Animal models of genetic diseases often do not recapitulate all aspects of the human condition (they all share one weakness: they are not, in fact, a human); however, that is not necessarily required for a model to be beneficial and informative. Animal models can be utilized to identify and/or confirm potential disease mechanisms and pathways, to identify therapeutic targets, to test therapeutic molecules and strategies, and ideally to determine if a putative therapy will have a beneficial impact on the disease pathophysiology. The FSHD field is in need of models addressing each of these areas and, due to the unusual nature of the disease locus, it is unlikely that any one model will be entirely suitable. In this manuscript, we discuss the strengths and weaknesses of emerging FSHD-like models and evaluate their utility as preclinical models for various therapeutic approaches. These models fall under two categories: those that are DUX4 centric; and those that are based on alternative gene candidates. Interestingly, recent data suggests that these two seemingly divergent approaches may actually intersect.

Molecular mechanism of FSHD

FSHD1 is an autosomal dominant gain-of-function disease with low penetrance and a highly variable clinical presentation and severity. The underlying criteria for developing clinically recognized FSHD1 and FSHD2 are complex, with both genetic and epigenetic requirements (Figure 1) [7,8]. Genetically, FSHD1 is linked to a highly polymorphic macrosatellite repeat composed of tandemly arranged D4Z4 repeat units (RU) residing at chromosome 4q35 (Figure 1) [9]. In healthy individuals, D4Z4 macrosatellite clusters vary between 11 and >100 RU [10], while contractions of one 4q D4Z4 array, retaining 1–10 RU, can lead to FSHD1 [11]. However, only contractions maintaining at least one D4Z4 RU in *cis* with a specific disease permissive A-type subtelomere distal to the D4Z4 are associated with FSHD1 [12,13]. Interestingly, several recent FSHD cases were reported to not involve the 4qA allele and may indicate the existence of rare alternate permissive alleles or suggest an altogether independent pathogenic mechanism in these rare cases [14,15]. Of the permissive A-type subtelomeric cases, ~5% of FSHD cases are contraction independent (FSHD2) [16].

Therefore, the genetic requirements to develop FSHD, at least one intact D4Z4 RU at 4q35 in *cis* with a disease permissive A-type subtelomere, are together merely permissive and not necessarily pathogenic. Both forms of clinical FSHD also share an epigenetic requirement: the contracted 4q35 array (FSHD1) or both 4q35 arrays (FSHD2) are depleted in heterochromatic features and gain characteristics of euchromatin resulting in an epigenetic derepression of the region [17–19]. The epigenetic dysregulation is caused by a physical loss of regulatory heterochromatin (FSHD1) [20], or heterozygous loss-of-function mutations in the repressive chromatin-associated protein SMCHD1 (FSHD2) [6,16]. It is this epigenetic change in a disease permissive genetic context that distinguishes clinically affected FSHD subjects from asymptomatic subjects who merely have a permissive deletion [8,21,22]. Therefore, the epigenetic regulation of the region provides a potential therapeutic avenue.

Each D4Z4 RU contains an open reading frame (ORF) encoding the double-homeobox transcription factor DUX4 [23,24]. Two isoforms of DUX4 may be derived from this ORF, DUX4-fl (full-length) expressed in the germ line and pluripotent stem cells, and an alternatively spliced DUX4-s (short) which is expressed in some somatic cells [25]. Both of these mRNAs require a subtelomeric sequence distal to the array to provide the stabilizing polyadenylation signals (PAS) [12,24], which in somatic cells is provided by the permissive A-type subtelomere. FSHD skeletal muscle and cells express significantly more DUX4-fl than healthy control samples [12,25,26]. As a germ-line transcription factor, DUX4-fl misexpression in the somatic cell is capable of initiating a complex cascade of inappropriate gene expression that involves activation of early stem cell programs in post-mitotic cells [27]. Precise expression levels of DUX4-fl vary between subjects, but the general quoted estimate is that DUX4 activation occurs in approximately 1 in every 200–1000 myonuclei [25,26,28]. Although transient bursts of DUX4 transcription result in high levels of expression, these rare events amount to very low levels of total DUX4 in FSHD myogenic cells and muscle biopsies. Still, DUX4-fl target genes constitute the major transcriptional signature in FSHD and misexpression of DUX4-fl in muscle progenitor cells or post-mitotic cells is likely the key mediator of FSHD pathology [29–31]. Therefore, DUX4-fl splicing, polyadenylation, mRNA expression, protein function and downstream target genes are all potential targets for therapeutic intervention.

Meanwhile, pursuit of other FSHD candidate genes are ongoing, but are proving difficult to independently validate due in part to their variability in patient and control muscle samples [32,33]. These alternative FSHD gene candidates include the 4q35-localized genes *FRG1* [34], *FRG2* [35], *TUBB4Q* [36], *ANTI* [33], *FAT1* [37], *DUX4C* (a truncated DUX4-FL with a different C-terminal) [38], the DBE-T lncRNA [39], and multiple D4Z4 encoded ncRNAs [40,41] as well as other genes found to be misexpressed in some FSHD subjects, including mu-crystallin [42], *PITX1* [24,43], and several miRNAs [44,45]. The pathogenic role for these genes in FSHD is unknown, but expression of DBE-T, *FRG1*, *FRG2*, *PITX1* and *FAT1* have all recently been directly linked to DUX4-fl (DBE-T upstream and the remaining genes are direct targets) and they must be considered.

Challenges in modeling FSHD

Animal models are an indispensable tool in biomedical research, providing an essential platform for the process of scientific discovery and therapeutic testing that precede clinical trials. In the case of FSHD, there are several unique hurdles that make the generation of an FSHD-like animal model challenging. The most significant of which is that the D4Z4 macrosatellite encoding the DUX4 retrogene, the near consensus FSHD causal gene, is specific to old world primates [46], which negates the possibility of working with a ‘natural’ model of the disease in commonly used laboratory animal species. Attempts to model the disease in a non-primate species require the introduction of an exogenous genetic construct for DUX4, thus removing the natural regulatory context of the gene. Fortunately, the most common form of FSHD is a dominant gain-of-function disorder and thus potentially amenable to this approach. However, modeling FSHD in non-primate species that do not express endogenous DUX4 raises concerns of whether the same downstream gene targets and regulatory networks exist, and can be activated as a consequence of DUX4 misexpression to cause disease as in primates. Transcriptomic analyses demonstrate that induction of DUX4 expression differentially regulates the transcriptome of human and mouse cells [47]. In addition, many of the genomic targets of the DUX4-FL protein are repetitive elements and other retrotransposons that are similarly not conserved, with DUX4 binding generating a novel promoter and driving expression [48]. Thus, a given DUX4 target gene may be conserved, but the regulatory element bound by DUX4 may not be. FRG1 is one such example in which the human gene is regulated by DUX4 but the mouse gene lacks the DUX4 binding site and consequently is not regulated by DUX4 when expressed in murine cells [49]. One way to mitigate this concern is to undertake the study of DUX4 in a system containing the full repertoire of DUX4 targets, such as human cell lines. The study by Sharma *et al.* showed that the induction of DUX4 in a human line (rhabdomyosarcoma cells) was able to differentially regulate ~15 times the number of genes than in a mouse line (C2C12 cells) [47], suggesting DUX4’s propensity to cause increased de-regulation in a primate cell model. Notably, the top three gene pathways affected by DUX4 expression in human (inflammation, BMP signaling, oxidative stress) was also found to be different to mouse (p53 signaling, cell cycle regulation, cellular energy metabolism), thus highlighting possible complications of investigating DUX4 in non-primate models.

The DUX4 gene is in itself challenging to study due to its extremely low expression at the transcript level as well as its protein instability, in patient muscle cells and biopsies [28,29]. Dominant disorders are typically characterized by the systemic misexpression of mutant protein. However, since DUX4 transcription occurs in so few cells, it compounds attempts to both study and model in *in vivo* and *in vitro* systems. That is, in order to design a model that accurately recapitulates the disorder will require the engineering of a small population of cells to express the majority of DUX4 detected. Additionally, studies of DUX4, particularly those that seek to profile gene expression patterns of disease cases, are hampered by its low mosaic pattern of expression, whereby positive signals from rare DUX4-expressing cells are obscured by strong background signals emitted from the majority of non-DUX4 expressing cells. Attempts to forcefully over-express DUX4 in *in vitro* models have not been very informative for down-stream studies due to its overt

toxicity and apoptotic outcome. In contrast, such approaches have been useful in identifying direct targets and pathways of DUX4, such as MyoD down-regulation and p21 up-regulation [31], both of which are rapidly induced DUX4 changes mirrored in FSHD patient samples [30,50]. Future studies seeking to obtain an accurate and workable FSHD model will require fine-tuning of DUX4 expression levels.

Finally, there remains no conclusive evidence that DUX4-fl or any other candidate gene is the sole disease-causing factor in FSHD. Although DUX4 remains the consensus disease gene in the field, emerging evidence implicate the potential role of modifier genes that remain as yet unidentified. The genetic basis of existing FSHD animal models are based solely on the ectopic expression of DUX4. However, if DUX4 alone is not sufficient to recapitulate the disease pathology, we cannot work towards an all-encompassing disease model until these additional determinants are discovered. Still, single gene models will be useful for many important preclinical studies.

Due to the many challenges that underlie the study of a complex disorder such as FSHD, no single animal model currently exists that faithfully encompasses the genetic and physiological spectrum of the human pathology. In the next sections, we will describe as well as evaluate existing animal models of FSHD1, both in terms of their strengths and weaknesses. Summary features of each model are summarized in Table 1. There are currently no published models on FSHD2.

DUX4-based models

Early attempts to model FSHD based on DUX4 expression proved difficult. Expression of DUX4-fl transcript in embryonic xenopus and zebrafish indicated that DUX4 expression was extremely cytotoxic, similar to human cell culture [51–53]. Importantly, this showed a conservation of this putatively pathogenic function outside of primates, yet also suggested that the generation of a DUX4-based transgenic FSHD-like model could be quite difficult due to embryonic lethality. To date, there have been several prominent DUX4-centric models published, three mice and two zebrafish, each engineered through a different approach and producing different results.

AAV-DUX4 mouse

The mouse model published by Wallace *et al.* (AAV-DUX4) was based on bolus injection of DUX4 to transiently model DUX4 over-expression in localized muscle tissue [54]. The injection model was the first to demonstrate that DUX4 over-expression is capable of causing muscle damage in an *in vivo* adult mammalian system. Using AAV6 vectors to deliver DUX4 via a single intramuscular injection into 6–8 week old mice, widespread expression of DUX4 was detectable at one week post-injection. DUX4-transduced muscles were characterized by lesions of degenerating myofibers, infiltrating mononuclear cells, and were positive for the pro-apoptotic marker caspase-3. These myopathic effects of DUX4 were demonstrated to occur via a p53-dependent pathway, supported by the less severe phenotype associated with the injection of DUX4 to muscles of p53-null mice. Notably, such severe myopathic changes and signs of apoptosis are not normally characteristic of FSHD patient muscle. Functionally, DUX4-injected mice were determined to be weaker in

grip strength at 1–2 weeks post-injection, but recovered by 3 weeks, likely due to healthy myofiber replacement of degenerated DUX4-expressing myofibers. This is an optimistic finding that suggests the possibility of halting DUX4 expression during early stages of onset may potentially reverse DUX4-induced muscle damage in patients. Mechanistically, the attenuation of DUX4 toxicity associated with loss of tumor suppressor function is intriguing and warrants further investigation to identify potential therapeutic opportunities.

D4Z4-2.5 and 12.5 mice

The DUX4 transgenic models published by Krom *et al.* introduced a large segment of the human D4Z4 locus known to cause FSHD in humans, into the mouse genome [55]. The contracted locus harbors two and half copies of the D4Z4 unit (termed D4Z4-2.5 mouse), and is of the permissive haplotype background. A control mouse was generated alongside, which harbored a normal-sized locus consisting of twelve and a half D4Z4 units (termed D4Z4-12.5 mouse), and does not cause FSHD in humans.

DUX4 transcript expression was most abundantly detected in the testis of both D4Z4-2.5 and D4Z4-12.5 mice, mirroring male germ-line DUX4 expression in humans. In contrast, only the D4Z4-2.5 mice resulted in body-wide expression of the DUX4 transcript. Despite its low and variable expression amongst littermates, DUX4 was notably detected in the limb, trunk and head (FSHD affected muscle groups). Other non-muscle tissue in which DUX4 transcript expression was detected includes the cerebellum, eye and liver. This is in contrast to the D4Z4-12.5 mice, in which the DUX4 transcript could only be detected in the *tibialis anterior* and *pectoralis* muscle groups. Thus, tissue expression analysis confirmed that in mice, like humans, decreased D4Z4 copy number results in the inefficient repression of DUX4 in somatic tissue.

Further studies were performed to investigate the link between DUX4 transcript expression and subsequent protein expression levels in myogenic cultures derived from both mouse models. As expected, neither DUX4 transcript nor protein was detected in either myoblast or myotubes of cultures derived from the D4Z4-12.5 mice; but were present in sporadic nuclei of myoblasts derived from the D4Z4-2.5 mice. These sporadic DUX4-positive nuclei were demonstrated to be muscle specific, and did not further contribute to the fusion and differentiation into myotubes. Epigenetically, both mouse models recapitulate profiles for FSHD and normal tissue. The D4Z4 locus of the D4Z4-12.5 mice has high levels of CpG methylation, similar to unaffected individuals; conversely, the D4Z4 locus of the D4Z4-2.5 mice was associated with body-wide hypomethylation, which may underlie the expression of DUX4 in many tissues.

Remarkably, despite demonstrating all the hallmarks of FSHD on a molecular level, the D4Z4-2.5 mice did not show any noticeable muscle weakness or wasting, nor do they show morphological or histological abnormalities. Measurements typically used to characterize muscular dystrophy subjects such as increased creatine kinase, lower grip strength, Evan's blue dye uptake, and defective regeneration upon cardiotoxin injury, were unable to distinguish between the D4Z4-2.5 and control mice. The only notable phenotype is the onset of an eye abnormality in approximately half of the D4Z4-2.5 population between 8–12 weeks of age. This pathology manifests as a progressive keratitis and eventually leads to

blindness. It is said to be somewhat comparable to the weakness of the eyelid muscle experienced by FSHD patients, although different in etiology to the more commonly described retinal vasculopathy.

Despite the successful recapitulation of the genetics and epigenetics of FSHD in the D4Z4-2.5 mouse that ultimately lead to the ectopic expression of DUX4, the absence of a muscle phenotype suggests that there may be additional factors required for the pathogenesis of FSHD. The lack of a measurable muscle phenotype therefore does not make this mouse model ideal for therapeutic assessment. Instead, its utility lies in providing insight into aspects of epigenetic regulation and disease biomarkers associated with FSHD.

iDUX-2.7 mouse

A second attempt to generate a DUX4 transgenic mouse was recently published by Dandapat *et al.* [56]. This model, termed iDUX-2.7, is characterized by a doxycycline (dox)-inducible DUX4 transgene integrated into a euchromatic site on the X-chromosome upstream of HPRT, a ubiquitous tissue expression locus. The inserted transgene is a 2.7 kb genomic fragment consisting of the DUX4 ORF and a trailing 3' sequence derived from the distal D4Z4 repeat. A polyadenylation signal is conferred to the DUX4 transgene via the SV40 insertion vector used.

Unexpectedly, in the absence of dox, leaky expression of DUX4 RNA was detectable at low levels in many tissues (but none in muscle), resulting in male-specific embryonic lethality. Rare surviving males presented with numerous severe phenotypes resulting in a reduced lifespan of less than two months. Although indistinguishable from their littermate controls at birth, carrier males developed a skin phenotype by two weeks, and were significantly smaller in size by six weeks. Underweight carrier males had subsequently smaller muscles with fewer fibers, which were consequently weaker in strength. Interestingly, despite displaying a clear muscle phenotype, carrier male muscles did not appear overtly dystrophic, reminiscent of patient muscle pathology. Additionally, detectable expression of DUX4 transcript in the testis and retina resulted in defective gametogenesis and retinal telangiectasis, respectively. Female mice are reported to have no muscular dystrophy phenotype, and are biased towards inactivation of the DUX4 carrying chromosome.

Although severely affected iDUX-2.7 males may not be ideal for drug treatment studies due to their severity and short lifespan, cells derived from them may provide a useful means of evaluating DUX4 activity both *in vivo* and *in vitro*. The authors demonstrate a quantitative method of evaluating DUX4 effect on muscle regeneration *in vivo* by transplanting satellite cells from iDUX-2.7 into recipient mice treated with doxycycline. Recipient mice were subsequently shown to have impaired donor satellite cell ability to produce new muscle, hence providing a useful preclinical approach to screen for therapeutics that can potentially rescue the regeneration defect.

Summary of DUX4 mouse models

The AAV-DUX4, D4Z4-2.5 and iDUX-2.7 models of DUX4 expression each employ very different techniques in modeling FSHD. Each resulted in their unique set of measurable

phenotypes, with disappointingly few consensus features that recapitulate the human condition. The transient and localized nature of the AAV-DUX4 model, the short-lived status of the iDUX-2.7 model, and the lack of assessable muscle phenotype of the D4Z4-2.5, together make these models unsuitable for long-term muscular dystrophy drug studies.

With respect to physiological levels of DUX4, the AAV-DUX4 model may not be ideal, but the low body-wide detection of DUX4 transcript in the iDUX-2.7 and D4Z4-2.5 models more accurately mimics the low levels of DUX4 in patient skeletal muscle. Physiological levels of DUX4 that result in recapitulation of FSHD biomarkers are favorable; as are cell cultures obtained from the iDUX2.7 and D4Z4-2.5 models with the characteristic variegated expression pattern of DUX4 in patient cultures. Notably, DUX4 expression at the protein level was only detected in the AAV-DUX4 model, and not in any tissues of the iDUX-2.7 and D4Z4-2.5 models. The finding that the iDUX-2.7 model did not show leaky DUX4 RNA expression in skeletal muscle is surprising, and suggests the possibility that muscle defects could be secondary to other tissues in which DUX4 are expressed.

The integration site of a transgene is crucial for the physiological relevance of a model. Integration of the DUX4 transgene in both the iDUX-2.7 and D4Z4-2.5 models lacks the genomic context of the human disease, as neither are in subtelomeric regions. The natural context of DUX4 on the subtelomeric region of chromosome 4 may hold important structural and/or regulatory implications for the disease. An additional concern is that only one transgenic line was used for comparison between D4Z4-2.5 and D4Z4-12.5, and hence the reported epigenetic differences may be attributable to differences in the transgene integration site, as opposed to an intrinsic property of the D4Z4 contraction.

DUX4 zebrafish models

Zebrafish (*Danio rerio*) are an ideal organism for the study of vertebrate muscle development and disease because they are easy to assay for muscle abnormalities. They are also commonly used in genetic studies because they allow for the effect of gene up- or down-regulation to be rapidly assessed on the whole-organism. Despite the absence of a DUX4 ortholog in zebrafish, misexpression of human DUX4 during zebrafish development was shown to recapitulate several characteristic features of human FSHD in two independent zebrafish models [52,54].

Wallace *et al.* used the Tol2 transposon system to generate a transgenic zebrafish model that expresses DUX4 under a MHCK7 muscle specific promoter upon genomic integration. As the MHCK7 promoter was only reported to switch on during the third day post-injection, this model limits the study of DUX4 expression to late stages of embryonic development. By day 4, it was reported that approximately half of the injected embryos had gross body malformations and abnormal muscle histology. Asymmetric fin development and cardiac hypertrophy were also noted, although at an unreported frequency.

Alternatively, Mitsuhashi *et al.* injected DUX4-fl RNA at the one-cell stage to drive systemic and unregulated DUX4 expression from the beginnings of embryogenesis. This resulted in a more penetrant toxic phenotype, causing lethality in the majority of embryos within 24 hours. Subsequently, it was demonstrated that by titrating down levels of injected

DUX4 mRNA to $\sim 1\text{--}2 \times 10^5$ copies resulted in viable embryos. Similar to Wallace's model, viable embryos showed gross body abnormalities, marked by muscle degeneration. Birefringence and myosin immunostaining reveal disorganized skeletal muscle structure, which correlated with reduced swimming ability. In addition, FSHD-like asymmetric abnormalities of the eyes, ears, and fins were also observed.

Additional molecular studies performed by Mitsuhashi sought to compare differences between the functional roles of DUX4-s and DUX4-fl in zebrafish. Expectedly, injection of DUX4-s into developing embryos did not result in toxicity, and resulted in intact muscles that were functionally normal; supporting their detection in healthy human muscles. However, an interesting and novel finding is that DUX4-s can be used to out-compete DUX4-fl, significantly reducing its toxicity. This finding suggests that in addition to down-regulating DUX4-fl in FSHD muscle, another viable therapeutic option lies in the up-regulation of DUX4-s, the short non-toxic counterpart to DUX4-fl.

Through two different approaches, Wallace *et al.* and Mitsuhashi *et al.* reported corroborating phenotypes that together demonstrate the possibility of recapitulating aspects of FSHD in zebrafish. Minor differences in phenotype, penetrance and severity reported may be attributable to differences in timing and dosage of DUX4 expression in both models, thus highlighting the potential importance of both factors in the pathogenesis of FSHD. The manifestation of body-wide muscle degeneration in both is encouraging, as is their subsequent impaired swimming ability. Together, they provide an assayable high-throughput read-out of muscle structure and function suited to future drug screening studies. Notably, some discrepancies exist between phenotypes described in DUX4 zebrafish models to FSHD in humans. These include the apparent developmental origin of the fin, eye and ear abnormalities; presumably brought upon by exposure to high levels of DUX4 during embryogenesis. Additionally, the systemic nature of muscle degeneration reported in both zebrafish models is also inconsistent with human FSHD, where specific muscle groups are affected while others are spared.

DUX4-independent mouse models

Independent efforts to concurrently explore other FSHD gene candidates proximal to the 4q35 region have been attempted. Gabellini *et al.* published on transgenic over-expression mouse models for *FRG1*, *FRG2* and *ANTI* [57], while Caruso *et al.* generated a muscle-specific knock-out mouse of *Fat1* [37]. To explore the possible pathogenic role of *Pitx1*, a DUX4 transcriptional target that is specifically upregulated in FSHD patients [24], Pandey *et al.* generated a conditional over-expression mouse of *Pitx1* [43]. Meanwhile, a gene-independent mouse model based on xenograft of FSHD patient muscle provides an alternative option to candidate gene approaches in modeling FSHD [58].

FRG1 mouse

As a compelling candidate alternative to DUX4, Gabellini *et al.* reported that mice over-expressing *FRG1* (but not *FRG2* or *ANTI*) resulted in characteristic phenotypes reminiscent of FSHD patients [57]. *FRG1* is a multifunctional RNA-binding protein primarily localized to the sarcomere in skeletal muscle and involved in nuclear shuttling of mRNAs and actin

bundling. Mice greatly overexpressing *FRG1* showed an increase in spinal curvature, dystrophic features, and proliferation of collagen fibers accompanied with reduced body mass and exercise intolerance. These features correlated with increasing amounts of *FRG1* transgene expression. Fiber atrophy of selective muscle groups, and absence of a sarcolemmal defect in the *FRG1* mouse are also consistent with distinctive features of FSHD pathophysiology. On a molecular level, splicing of two candidate genes (*TNNT3* and *MTMR1*) known to be abnormal in myotonic dystrophy, were also directly affected in the *FRG1* mouse from as early as 4 weeks of age. Although abnormalities in cellular splicing machinery can potentially affect thousands of genes, the identification of aberrant splicing associated with *TNNT3* and *MTMR1* present promising mechanistic leads due to their association in regulating muscle contractility and atrophy, respectively. However, it is also important to consider the possibility that over-expressing an RNA-binding protein using a strong skeletal actin promoter may cause serious RNA-processing defects, thus possibly explaining the muscle-specific phenotype detected only in the highest-expressing transgenic line.

Clinically, *FRG1* over-expression in FSHD remains controversial, with a range of studies reporting a significant increase [33,59], a modest increase [60] to no increase [24,30,61,62] compared to controls. Nevertheless, *FRG1* was recently identified as a transcriptional target of DUX4, thus potentially linking *FRG1* overexpression in FSHD to DUX4 misexpression and disease pathogenesis [49]. Taken together, the collective evidence so far points to a pathogenic role for *DUX4* and potentially *FRG1* in FSHD. Reports that the mouse *Frg1* gene does not contain putative DUX4 binding sites offers one explanation as to why the D4Z4-2.5 mouse is unable to recapitulate the full spectrum of phenotypes associated with FSHD [49].

Fat1 mouse

Caruso *et al.* generated a knock-out mouse model of *Fat1*, a protocadherin gene located 3.6Mb upstream of 4q35 [37]. FAT-like cadherins are involved in tissue morphogenesis through regulation of cell polarity and adhesion. Caruso *et al.* demonstrated that during development, *Fat1* controls the shape of muscles in the facial and scapulohumeral regions through modulation of myoblast polarity, and that *Fat1* deficiency results in regionalized muscle and non-muscle abnormalities consistent with FSHD. Affected muscle groups include the trapezius, rhomboid, *pectoralis major*, *latissimus dorse* and *cutaneous maximus*; showing signs of fiber necrosis, inflammatory infiltration, disrupted sarcomeres and reduced muscle mass. Non-muscle phenotypes in *Fat1*-deficient mouse include retinal vasculopathy and abnormal inner ear patterning, consistent with those reported in FSHD patients. In support of their mouse data, they report a muscle-specific reduction of *FAT1* in human foetal FSHD biopsies and identified a deleterious single nucleotide polymorphism (SNP) in the regulatory enhancer of *FAT1* that segregates with FSHD cases. There have been no other reports of altered *FAT1* expression in FSHD tissue. Interestingly, a recent report links splicing variants in *FAT1* to patients with FSHD-like symptoms in the absence of D4Z4 contraction, 4q hypomethylation or SMCHD1 variants [63]. Taken together, these observations suggest a potentially important role for *FAT1* tissue-specific deregulation in the pathophysiology of FSHD.

Pitx1 mouse

The homeobox transcription factor *Pitx1*, was one of the first identified transcriptional targets of DUX4 [24]. *Pitx1* knockout mice exhibit structural changes in their hindlimbs [64] and mutations that abolish PITX1 expression in humans can cause asymmetric lower-limb malformations [65]. To investigate the potential pathogenic role of *Pitx1* misexpression in FSHD, Pandey *et al.* generated a conditional over-expressing *Pitx1* mouse [43], where PITX1 over-expression in skeletal muscle is activated in the absence of doxycycline administration. PITX1 over-expression was activated at 10 weeks of age, and after 5 weeks, transgenic mice were shown to have reduced mass of selected muscle groups (see Table 1). Histology of PITX1 over-expressing muscle revealed myofiber atrophy, necrotic and centrally nucleated fibers, and inflammatory infiltration. Average fiber size in affected muscles was reduced by ~40%. Grip strength tests performed before and after activation of PITX1 over-expression showed significant muscle weakness after 3 weeks of over-expressing PITX1. Notably, p53 upregulation was detected in affected muscles of *Pitx1* transgenic mice. This finding supports the potential role of p53 in FSHD reported in other studies [54], and paints a potential cascade involving the activation of DUX4, PITX1 and p53 to induce muscle pathology in FSHD.

Xenograft mouse

In an attempt to generate an FSHD mouse model independent of candidate genes, Zhang *et al.* explored the feasibility of using a human to mouse muscle xenograft as a model of FSHD [58]. Xenografts of whole-mouse muscles from the mdx model into an immunodeficient mouse have proven to be successful in retaining their dystrophic phenotype, thus laying the foundation for engraftment potential of diseased human muscle. The humanized mouse model of FSHD is the first to demonstrate the feasibility of this novel xenograft approach for modeling a myopathy, using both fresh and autopsy-derived human muscle samples.

In this model, bicep muscles from FSHD patients (expressing DUX4-fl) and their unaffected family members were collected and grafted into immunodeficient mice, recapitulating both diseased and normal muscles, respectively. Grafted muscles were transplanted into the anterior compartment of the hind-limb of recipient mice, where they became vascularized and survived through 41 weeks post-transplantation. Expression analysis of 15 genes demonstrated to be differentially expressed in FSHD compared to control biopsies were recapitulated in xenografts, demonstrating that FSHD biomarkers can be maintained in a host environment for further studies.

Although the recipient mouse is unable to recapitulate FSHD systemically, the xenograft approach is especially useful for studies where in-depth molecular and cellular outcomes are sought. Given the localized nature of the xenograft model, it cannot be used to assess whole body functional outcomes in a disease setting. Instead, the xenograft approach provides a more physiologically relevant milieu under which disease biomarkers and regenerative processes associated with FSHD can be monitored over time. The novelty of this approach lies in its utility to test the potential effects of a candidate therapeutic on a 'living and breathing' patient sample, particularly useful toward the end stage of a translational pipeline.

Concluding remarks

Over a century since its first description, clinical studies of FSHD have finally entered a new era of translational research. This encouraging leap is only made possible due to substantial developments in the understanding of FSHD genetics that now enable partial replication in animal models. In an age with advanced drug synthesis technologies at hand, and where therapeutic options are emerging for other forms of muscular dystrophy; we need methods to enable assessment of their beneficial impact for FSHD. Central to this goal is to have at hand animal models for preclinical efficacy testing prior to human clinical trials. Due to the complexity of FSHD, it is no surprise that existing models for FSHD remain sub-optimal and fall short of being capable of assessing therapeutic efficacy.

DUX4 is our best therapeutic target to date; its myotoxicity and muscle damaging capacity remains our most substantial insight to understanding the disease mechanism of FSHD. However, the lack of supporting evidence to link our molecular findings with patient pathology suggests there may be other genes or pathways at play that are yet to be discovered. As the search for these additional factors continue, we must seek to better understand the link between molecular and body-wide consequences in FSHD. This is crucial if we are to devise functional assays to accompany our animal models in an effort to identify therapeutic efficacy of drugs and their potential translation into the clinic.

As we continue to refine our disease models for therapeutic purposes, we should also strive to learn from the limitations of existing models, and keep in mind other unresolved issues (see Box 2) that we can address with our next generation models. As we have learned, each attempt at an FSHD-like animal model emphasizes different aspects of FSHD with its characteristic strengths and weaknesses. It is undoubtedly difficult to incorporate all desirable features into a single model, due to constraints posed by current genetic engineering technologies and intrinsic species limitations.

To model FSHD it is both advantageous and likely necessary to express a candidate gene from an inducible locus over one that is constitutively expressed. This enables regulation of both dosage and timing of its expression, which is particularly suited to studies of FSHD where the contribution of each remains to be explored. Gene dosage is important to ensure physiological level of expression, such that phenotypic effects are not due to over-expression artifacts; whereas regulating expression timing will shed insight into aspects of disease onset and progression. Future animal models of FSHD will greatly benefit from robust engineering of conditional transgenes that will allow for more in-depth experiments into affected tissues and disease onset/progression.

In light of the primate specific origins of DUX4 as well as the lack of a muscle phenotype in the D4Z4-2.5 mouse model, another issue of concern is whether FSHD can be recapitulated, if at all, in non-primate species. We need to consider the possibility that FSHD cannot be modeled in its entirety, but that we are limited to highlighting only some aspects as of the disease; which explains why our attempts thus far have fallen short in yielding an optimal model. Encouragingly, the substantial overlap in differentially expressed genes from both mouse and human studies suggests that DUX4 has the capacity to act as a transcriptional

activator, binding similar motifs in mouse as in humans. But whether this overlap is sufficient to encompass the pathogenic gene spectrum will require a systematic dissection and cross-referencing of results across multiple species.

Asymptomatic human carriers of FSHD, individuals, often family members of FSHD patients, that carry the genetics of FSHD but do not manifest symptoms may provide the necessary insight to generate better models [66]. Understanding the variables that contribute to the incomplete penetrance or delayed onset will lead to promising therapeutic avenues that will be relevant in a human context. Using reverse genetics of human variation to guide the process of drug discovery is a relatively new but highly successfully practice as it removes the added challenge of inter-species compatibility. Cohorts of asymptomatic human carriers of FSHD exist and should be investigated in greater detail, as what they can tell us will surpass anything we can hope to gain from a non-human animal model [22,67]. This underscores the need for clinicians and researchers to work together in thoroughly identifying and investigating these ‘cured’ human models of FSHD.

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Glossary

4qA	A major haplotype of chromosome 4 that confers permissiveness to FSHD. The 4qA haplotype is distinguished from other 4q haplotypes by the presence of a 6.2 kb beta-satellite repeat
ANT1	Adenine nucleotide translocator 1. An apoptotic inducing protein
D4Z4	A highly polymorphic macrosatellite repeat in chromosome 4q, ranging from 11-100 units in the unaffected population. Each D4Z4 unit is 3.3kb and contains a DUX4 open reading frame
DBE-T	A chromatin-binding non-coding RNA derived from a region proximal to the D4Z4 repeat. Detected in FSHD patients and not control alleles. Recruits members of the trithorax group to coordinate de-repression of 4q35 genes

DUX4C	A truncated DUX4-fl with a different C-terminal
<i>DUX4-fl</i>	Full-length DUX4 transcript derived from the open reading frame of the most telomeric D4Z4 unit. Consistently expressed in very low amounts in FSHD muscle biopsies and cells
<i>DUX4-s</i>	A truncated DUX4 transcript that utilizes a cryptic splice donor in the DUX4 open reading frame and lacks the carboxy-terminal region. Inconsistently expressed in very low amounts in both control and FSHD muscle biopsies and cells
<i>FAT1</i>	A protocadherin gene located centromeric to D4Z4 repeat array. FAT1 deficiency results in a similar pattern of muscle weakness to FSHD patient
<i>FRG1</i>	FSHD region gene 1. A nucleolar protein involved in RNA biogenesis
<i>FRG2</i>	FSHD region gene 2. A myogenic protein with a possible role in myogenesis
FSHD1	Facioscapulohumeral muscular dystrophy Type 1. The most common form of FSHD. An autosomal dominant muscular dystrophy linked to contraction of D4Z4 macrosatellite repeat in chromosome 4q, requiring a permissive 4qA haplotype
FSHD2	Facioscapulohumeral muscular dystrophy Type 2. A contraction-independent form of FSHD, requiring at least one permissive haplotype and a heterozygous loss-of-function mutation in the <i>SMCHD1</i> gene. FSHD 2 accounts for 5% of FSHD cases
Mu-crystallin	Also known as NADPH-dependent thyroid hormone binding protein. Selectively up-regulated in muscles of FSHD patients. A candidate protein in the pathogenesis of retinal and inner ear defects, as well as oxidative stress in FSHD
Polyadenylation signal (PAS)	DUX4 derived from the distal-most unit of D4Z4 is spliced to a polyadenylation sequence to stabilize the transcript. Internal copies of DUX4 are not polyadenylated
<i>PITX1</i>	Paired-like homeodomain transcription factor 1. Specifically up-regulated in FSHD patients and is a transcriptional target of DUX4
Retrogene	Intron-less genes that arise from duplication events involving the reverse transcription of mRNA from the parental gene source
<i>SMCHD1</i>	Structural Maintenance of Chromosomes Hinge Domain containing 1. <i>SMCHD1</i> is involved in gene repression by maintenance of CpG methylation. In FSHD2, the absence of <i>SMCHD1</i> causes derepression of the D4Z4 region

TUBB4Q

Tubulin, beta polypeptide 4, member Q. A pseudogene located 80 kb proximal to the D4Z4 region

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Box 1: FSHD is different to classical forms of autosomal dominant muscular dystrophies

FSHD is classed as an autosomal dominant (AD) muscular dystrophy, but bears several fundamental differences to classical forms of AD muscular dystrophies such as Limb Girdle Type 1 muscular dystrophies (LGMD1). Although both FSHD and LGMD1 result in progressive loss, wasting and atrophy of skeletal muscles, the specific muscle groups involved are unique to each. In LGMD1, muscle weakness is symmetric and involves the proximal muscles of the shoulder, thighs and pelvic region (Figure I) [68]; whereas in FSHD, muscle weakness is asymmetric and involves the face, shoulder and upper arm muscles (Figure I) [69]. Additionally, trunk and lower extremities often become affected with disease progression in FSHD; but the extraocular, pharyngeal and cardiac muscles are spared. Secondary clinical features include the potential for hearing and vision loss in FSHD; and cardiomyopathy in LGMD1. Both have a similar range of onset (childhood to middle age), with the majority becoming symptomatic during early adulthood. Muscle pathology of LGMD shows striking evidence of myofiber degeneration accompanied by elevated serum creatine kinase; however, FSHD muscle is only linked to minor myopathic changes, with evidence of inflammatory infiltrates specific to the perivascular region. The underlying genetic changes linked to LGMD1 are missense mutations that occur in structural (desmin, myotilin, lamin A/C), transmembrane (caveolin-3) and signaling (DNAJB6) proteins. Contrastingly, FSHD is not linked to nucleotide mutations but instead, a contraction of the D4Z4 macrosatellite repeat region, and the subsequent misexpression of subtelomeric gene(s) in chromosome 4.

Figure I: A comparison of clinical features between LGMD and FSHD (Affected muscle groups are shown in red). **(a)** FSHD is characterized by asymmetric weakness of the facial, shoulder and upper arm muscles. Weak abdominal and lower leg muscles are also a frequent feature. **(b)** Autosomal dominant LGMD is characterized by weakness of proximal muscles - shoulders, upper arms, pelvic region and thighs.

Box 2: Outstanding questions

- What is the most relevant phenotypic read-out, biomarker or assay for assessing FSHD therapies?
- To what extent does misexpression of DUX4 explain the spectrum of FSHD disease pathophysiology?
- How can such a low expressing protein cause such a devastating body-wide disease phenotype?
- Is there a single dominant pathway or multiple pathways that explains FSHD?
- What dictates the dosage and timing of DUX4 expression bursts?
- What is the biological role of DUX4 in somatic tissue?
- In which tissues does DUX4 misexpression contribute to disease phenotype?

Highlights

- FSHD is a complex disease with primate-specific genetic and epigenetic components.
- We reviewed DUX4-dependent and -independent mouse models of FSHD.
- We also reviewed two DUX4-dependent zebrafish models of FSHD.
- Current models do not fully encompass the genetics and pathophysiology of FSHD.

Figure 1.

The molecular and genetic mechanisms of FSHD type 1 and type 2. Normal, unaffected individuals carry 11–100 repeat units (triangles) within the highly condensed D4Z4 macrosatellite elements on the subtelomeric region of chromosome 4q35. Contraction of D4Z4 repeats in FSHD1 (less than 10 repeats) relaxes the chromatin structure and induces the expression of DUX4 from the distal-most repeat unit. DUX4 expressed from the non-permissive chromosomal allele without a poly(A) signal (red bar) does not become polyadenylated and are unstable; whereas polyadenylated DUX4 transcripts expressed from the permissive allele (green bar) are stable and translate into a toxic transcription factor, DUX4. SMCHD1 regulates D4Z4 methylation. In FSHD2, mutated SMCHD1 fails to methylate D4Z4 and to suppress DUX4 expression.

Table 1

Summary features of FSHD animal models^a

Model	Genetic/biological modification	Onset of phenotype	Expression pattern	Non-muscle phenotype	Muscle phenotype	Cellular phenotype	Chromatin structure	Ref
AAV6-DUX4 (mouse)	TA injection of AAV6-DUX4 into 6–8 week old C57BL/6 mice; low dose (8×10^8) and high dose (3×10^{10}).	Muscle damage detected 1 week post-injection. Unspecified lifespan.	DUX4 protein expression in TA muscle; cytoplasmic and myonuclei staining.	Unspecified	Degenerating myofibers and infiltrating mononuclear cells, reduced grip strength (high dose). Minor degeneration, smaller/variable-size fibers, increased central nuclei (low dose).	DUX4-induced cell death via a p53-dependent pathway.	Unspecified	[54]
D4Z4-2.5 (mouse)	Transgenic insertion of two and a half copies of D4Z4 from permissive haplotype of pathogenic allele.	Keratitis detected at 8–12 weeks. Unspecified lifespan.	DUX4 transcript detected in ES cells, tongue, testes, heart, dia, pec, mas, orb, qua, TA, gas. DUX4 transcript also detected in myoblast, myotubes.	Eye abnormality (keratitis) in >50%, leading to blindness.	No muscle weakness or abnormalities in morphology. Minor regeneration defect upon cardiotoxin injury.	Satellite-cell derived myoblasts with DUX4 positive nuclei fail to fuse into myotubes.	Hypomethylated (10–20%)	[55]
D4Z4-12.5 (mouse)	Transgenic insertion of twelve and a half copies of D4Z4 from permissive haplotype of pathogenic allele.	Unspecified. Presumed normal lifespan.	DUX4 transcript detected in ES cells and testes (lower than in D4Z4-2.5). Muscle expression in pec, qua, TA. Transcript not detected in myoblast and myotubes.	Unspecified	Unspecified	Unspecified	High methylation (60–90%) of D4Z4 units.	[55]
iDUX-2.7 (mouse)	Doxycycline-inducible DUX4 transgene on X-chromosome.	Abnormalities detected during embryogenesis. Majority were embryonic lethal. Surviving males lived <2 months.	Transcript level highest in retina, testis, brain; lower in skin, thymus, kidney, lung. DUX4 positive nuclei in myotube (5%) and myoblast (1.5%).	Male-specific dominant lethal. Surviving males were smaller in size with a skin phenotype, defective gametogenesis and retinal telangiectasia.	Smaller and fewer fibers, but not dystrophic. Weaker grip-strength.	Growth inhibition of DUX4 positive myoblast and differentiation of myotubes. DUX4 positive cells show impaired contribution to myogenic regeneration.	DUX4 transgene inserted into euchromatic region.	[56]

Model	Genetic/biological modification	Onset of phenotype	Expression pattern	Non-muscle phenotype	Muscle phenotype	Cellular phenotype	Chromatin structure	Ref
DUX4 transgenic (zebrafish)	Transgenic zebrafish expressing MHCK7 (muscle-specific) driven DUX4.	Day 4 post-fertilization. Unspecified lifespan.	MHCK7 activity in skeletal muscle 3rd day post-injection. Detected in myocardium.	Gross body malformations, asymmetrical undeveloped fins. Cardiac hypertrophy.	Absent sarcomeric banding, myofiber disorganization, undefined somite boundaries.	Unspecified	Unspecified	[54]
DUX4 RNA injection (zebrafish)	Microinjection of human DUX4-fl mRNA into one-cell stage.	Muscle disorganization from 24hrs post-injection. Surviving larvae had shortened lifespan.	Approximately 1 RNA molecule per 1000 cells before somitogenesis.	Less pigmentation of eyes, altered morphology of ears, fin abnormality	Disorganization of facial musculature, degeneration of trunk muscle, misaligned myosepta. Impaired swimming.	Absent localization of myogenic cells in the head region.	Not applicable	[52]
Xenograft (mouse)	Human muscle engraftment into immunodeficient mice.	Unspecified. Lifespan of 41 weeks post-transplantation.	DUX4 expression was detected in xenograft extracted from mouse TA muscle.	Unspecified	Unspecified	FSHD biomarker profile maintained in xenograft.	Unspecified	[58]
FRG1 (mouse)	Transgenic insertion of <i>FRG1</i> driven by a human skeletal α -actin promoter. Three levels of over-expression: low, mid, high.	From 13 weeks. Unspecified lifespan.	Skeletal muscle	Spinal curvature correlated with level of <i>FRG1</i> expression.	Fiber size variability, necrosis, centralized nuclei. Excess collagen fibrils, selective muscle atrophy, reduced exercise tolerance in med/high mice.	Evidence of aberrant alternative splicing of specific pre-mRNAs.	Unspecified	[57]
FAT1 (mouse)	Knock-out of <i>Fat1</i> .	Abnormalities detected during embryogenesis. Approximately 50% die within 3 months.	Loss of Fat1 in Pax3-derived cells (premigratory muscles of the limbs, trunk migrating myoblasts, dorsal neural tube and neural crest).	Retinal vasculopathy, abnormal inner ear patterning.	Muscle weakness of the face (branchiomeric muscles), and scapulohumeral region (cutaneous maximus, humeral muscles).	Altered myoblast migration polarity.	Unspecified	[37]
Pitx1 (mouse)	Transgenic over-expression of <i>Pitx1</i> induced in the absence of doxycycline.	3–5 weeks post activation of <i>Pitx1</i> over-expression.	<i>PITX1</i> protein was ~3 fold higher in skeletal muscle.	None	Reduced muscle mass of TA, gas, bi, tri, del. Atrophic muscle fibers with necrosis and inflammatory infiltration. Weaker grip strength.	Upregulation of p53 in <i>PITX1</i> -expressing muscle groups.	Unspecified	[43]

Model	Genetic/biological modification	Onset of phenotype	Expression pattern	Non-muscle phenotype	Muscle phenotype	Cellular phenotype	Chromatin structure	Ref
FSHD patients (human)	D4Z4 contraction on a permissive 4qA haplotype.	Early adulthood, variable. Normal lifespan.	Polyadenylated DUX4 mRNA expressed at higher levels in FSHD muscle.	Retinal vasculopathy (Coats disease), hearing loss.	Asymmetric muscle weakness in the face and shoulders, gradually progresses into the trunk and leg muscles. Histology shows little myopathic changes. Evidence of endomysial inflammation.	Variiegated DUX4 expression in myonuclei. Nuclear changes signaling a pre-apoptotic cascade. Inhibitory muscle cell differentiation and skeletal muscle regeneration.	Reduced D4Z4 CpG methylation and chromatin compaction.	[7,70,71]

^a Abbreviations: AAV6, adeno-associated virus 6; bi, bicep; del, deltoid; dia, diaphragm; ES, embryonic stem; gas, gastrocnemius; mas, masseter; MHCK7, alpha-myosin heavy chain enhancer-muscle creatine kinase enhancer-promoter orb, orbicularis; pec, pectoralis; qua, quadriceps; TA, tibialis anterior; tri, tricep.