

Priorities as stated by FSHD Research Community for FSHD Research in 2017/2018 at the 2016 FSH Society FSHD International Research Consortium, held November 10-11, 2016 in Boston, Massachusetts

Statement of FSHD Scientific/Research Priorities 2017

By consensus of the 2016 FSHD International Research Consortium

I. Clinical and therapeutic studies.

- There is a need for surrogate outcome biomarkers now that trials are becoming reality.
- Need for validated outcome measures - preferably internationally standardized.
- Additional natural history studies are required.

Highlighted comments from the group:

“Think a little bit about the issues that are posed by when therapeutic ‘A’ is actually in use how it might impact on the design and implantation of clinical trials. For Huntington's Disease, clinical studies which use the UHDRS, the Universal Huntington's Disease Rating Scale, rely heavily on movement. So that in fact if the use of tetrabenazine, which inhibits movement, is now allowed into the clinical trial, which may have to be, because it's an approved therapeutic which has become the standard of care, now what you've done is to dramatically diminish the dynamic range that is available to your therapeutic.”

“So all of these outcomes discussed are going to become increasingly important as we move through the clinical development process, we need good data from them, as we can't really convince regulators that these are good outcome measures in the clinic that are clinically meaningful and should be approvable. The more people that start using these measures, the better, and, obviously, in a nicely longitudinal way, that's even better.”

II. Genetics and epigenetics.

- Need to focus on the uniformity in the genetic testing and the subgrouping of patients as so far as that is possible for trial readiness.
- Further understanding of the epigenetic regulation of the repeats helps us to better understand the disease process and the disease mechanism and to identify therapeutic targets.
- The search for modifiers of the disease mechanism needs to be continued as this can explain variability and identify new therapeutic targets.
- Consistent measures of (epi)genetic changes are needed.

Highlighted excerpts from group discussion:

“Consider Request for Applications (RFA) from funding agencies related to one or more these priorities. Consider Sub-meeting(s) that certainly addresses each of these areas, sometime in the next 7 or 8 months. Essentially the establishment of a central equivalent of World Anti-Doping Agency (WADA) for the Olympics or something like that so that uniformity in the genetic testing is achieved and the sub grouping of FSHD patients can be done, done under uniform conditions.”

III. Molecular mechanisms.

- Need to understand genetic toxicity in FSHD. There is a gap in our knowledge between DUX4 cellular toxicity and pathophysiological processes in FSHD.
- We need to understand the regulation and identity of DUX4. We need to know how to silence it, and how much to silence it.
- Refine relationship to other markers and correlation between the expression and activity, transcriptional activity of DUX4 with some of the markers that we currently have.

Highlighted excerpts from group discussion:

“A lot of consensus that the expression of DUX4 probably its activity in the nucleus mediated through binding of the DNA possibly through its transcriptional activity is really the major cause of the disease. So there’s consensus if you knew how to epigenetically silence it, silence the RNA, silence the transcriptional activity that’s a good process.”

“Need to open big black box in terms of what the real pathophysiology is, is it transcriptional toxicity from inducing apoptosis, is it RNA toxicity, protein toxicity -- that box really intellectually needs to be filled in. It may not need to be filled in to continue at present with developing therapies – but none-the-less this understanding is critical and essential.”

“Relationship to other markers. The next priority is to really start to correlate between the expression and activity, transcriptional activity of DUX4 with some of the markers that we have, how do the molecular markers correlate with disease muscle, how does the MRI correlate with the markers, and how can we measure disease progression in a mechanism that does not require years long functional assays, but might be focused to a specific marker or a specific muscle group.”

IV. Models.

- There is no ideal model; each model will serve its own needs.
- Create a focus to ensure that we are measuring the same kinds of things, that it does translate into a usable tool for our therapeutic industry. Establish meetings of the consortium of laboratories that are working on mouse/animal models.
- Need for further development, characterization and use of variety of animal models.
- Xenograft models -- real human muscle represents the true disease state either patients or grafts
- More emphasis on cellular models (e.g. iPSC) -- all aspects of all models.

Highlighted excerpts from group discussion:

“Need to create a nucleating focus to ensure that we are measuring the same kinds of things, that it does translate into a usable tool for our therapeutic industry brethren to ensure that these things can move as quickly as possible into testing paradigms in that way.”

“We need to consider all aspects of all models. Cell-based, again, are the kinds of things that lend themselves to high throughput assays. Our therapeutic industrial partners might look to engage in those kinds of high throughput assays using a variety of cells. If stem cells, either induced or embryonic, were useful in this. Consideration of this potentially being a developmental phenomena with a later in life trigger after some sub-population of cells has been set up is disquieting, but I do think those models might actually provide some insight into that as well.”

“Meetings of the consortium of laboratories that are working on mouse models I believe is very valuable and almost essential and I would argue that the various commercial entities that are attempting to enter the FSHD therapeutics space should be involved in attendance and I would argue support at least the meetings of the consortium if nothing else, because I think this is a very simple way in which the therapeutic development can (a) be accelerated, and (b) to some extent, de-risk or lower the risk.”

V. Therapeutic studies.

- Clinical trials are on the horizon, meaning that the community needs to be prepared (clinical trial preparedness).
- FSHD models need to be available to address drug delivery and efficacy in preclinical trials.
- For clinical trial preparedness registries need to be assembled and harmonized.
- For clinical trial preparedness registries biomarkers (e.g. MRI or molecular markers) need to be identified and validated.
- For clinical trial readiness validated patient relevant functional outcome measures need to be available.

Highlighted excerpts from group discussion:

“In addition to testing our compounds, though, some models that really recapitulate the disease in their progression can give us insight into when we might consider treating, how early in the course of the disease we may need to treat in order to see the changes that we like to drive into the clinic. The other information it might give us is the duration of treatment that may be required to impact the disease. So if you were to have a model that recapitulates the course of the disease relatively accurately, using the endogenous gene and potentially even using the endogenous locus regulation region, that could be highly valuable in understanding not just how much to treat with, the dose, but the duration, and the time of initiation.”

“Precisely how you deliver, how you formulate, how you get the conceptual entity to the effective therapeutic use of the entity requires something that you can test. Now even need to address formulation and delivery issues and half life issue, PK, PD, all that stuff. You can do some of that in normal animals, but it really begs the question if the delivery to an affected tissue is different from the delivery to a normal tissue and that, for example, might be relevant, let’s say, in the muscular dystrophies we know some of the issues in delivery to Duchenne muscle and that’s been an issue, I think, in some of this clinical work that’s been done. So I agree with you completely, we have to think about and have ready thoughtful understanding of how we’re going to develop both understanding of delivery modalities and understand PK and PD and everything else about the use of a therapeutic intervention, whatever it is.”

VI. Workshops.

Ideas for workshops that came up during parts of the discussion (no specific ranking).

- Workshop 1 -- Mouse models consortia/meeting powered by industry.
- Workshop 2 -- Uniformity in the genetic testing as so far as that is possible (the establishment of a central equivalent of WADA for the Olympics or something like that where, done under uniform conditions).
- Workshop 3 -- The subgrouping of patients (a further understanding of the epigenetic regulation of the repeats helps us to better understand the disease process and the disease mechanism).
- Workshop 4 -- Surrogate outcome biomarkers.

First Author	Presenting Author	Title
Banerji	Banerji	Dynamic transcriptomic and morphological analysis of FSHD atrophic myogenesis reveals a correctable defect in mitochondrial biogenesis
Brand	Brand	Lessons in Oligogenetics and Pleiotropy: Identical SMCHD1 Alleles can be Associated with Arhinia, Bosma Syndrome, FSHD2, Comorbidities, or No Phenotype at All
Calandra	Deidda	Large-scale methylation analysis in facioscapulohumeral muscular dystrophy (FSHD)
Cammish	Orrell	The UK FSHD Patient Registry: A Key Tool in the Facilitation of Clinical Research
Campbell	Campbell	Identifying mechanisms that regulate DUX4 and the D4Z4 macrosatellite repeats
Chang	Chang	Testing the potential for comorbidity of FSHD with arhinia using inducibility of DUX4 expression in dermal fibroblasts
Chen	Hayward	Single-cell Transcriptome Heterogeneity in Myogenic Cells from Individuals with FSHD
Chen	Chen	Systemic delivery of LNA gapmers targeting DUX4 improved muscle function in FLEXDUX4 mice
Choi	Choi	Establishment of FSHD-PAX7 genetic reporter lines to study function of muscle stem cells in FSHD
Choi	Lim	Modular platform for the myogenesis of human embryonic stem cells by using multiple genetic reporter lines
Ciskewski	Popplewell	Novel epigenetic mechanisms regulating DUX4 expression
Claus	Claus	Direct interaction of DUX4/4c with the multifunctional protein C1QBP
Coulis	Coulis	Overexpression of DUX-4 induces muscle Tregs: A potential role for the immune system in FSHD
Cruz	Clarke	Protein kinase A activation inhibits DUX4 gene expression in myotubes from patients with FSHD
Daman	Daman	An FSHD cell xenograft assay for drug development
de Morrée	de Morrée	U1 snRNA controls alternative polyadenylation of Pax3 in muscle stem cells
Denny	Denny	High-Density Lipoproteins protect against DUX4-mediated damage in a lentiviral model of FSHD
DeSimone	DeSimone	Identification of a DUX4-interacting protein and the hyaluronic acid pathway as novel therapeutic targets for FSHD
Dion	Robin	Implication of SMCHD1 in D4Z4 epigenetic dynamics: lesson from iPSCs
Eidahl	Eidahl	Regulation of Facioscapulohumeral muscular dystrophy candidate protein DUX4
Giesige	Giesige	AAV.RNAi and follistatin gene therapy development in the TIC-DUX4 Mouse Model of FSHD
Hamel	Hamel	MRI Correlates to Electrical Impedance Myography in Facioscapulohumeral Muscular Dystrophy
Han	Han	Longitudinal study of Kinect-based upper extremity reachable workspace in FSHD
Hiramuki	Hiramuki	A mapping study of SMCHD1 identifies the region of nuclear localization, dimerization, and protein cleavage
Homma	Homma	DUX4 alters mRNA splicing of TDP-43 target genes
Horlings	Horlings	Clinical outcome measures, muscle imaging and (epi)genetic testing in a large cohort of FSHD patients
Hupper	Clarke	A low molecular weight compound screen in FSHD patient myotubes identifies modulators of Dux4 activity and novel mechanisms of action
Jansz	Blewitt	The epigenetic repressor, FSHD2 gene and FSHD1 modifier SMCHD1 functions by mediating long range chromatin interactions
Jones T	Jones T	The FLEXDUX4 transgenic mouse can be used to develop FSHD-like mouse models with pathophysiology ranging in severity

First Author	Presenting Author	Title
Kazakov	Kazakov	Some problems connected with AD FSHD classification
Kyba	Kyba	Structural and functional studies on DUX4 in human myogenesis
Lemmers	Lemmers	Cis D4Z4 repeat duplications associated with FSHD2
Lopez	Lopez	Autologous stem cell treatment in FSHD. Preliminary report
Lu-Nguyen	Lu-Nguyen	In vivo assessment of antisense therapy for Facioscapulohumeral muscular dystrophy
Lunt	Lunt	No evidence for altered incidence of cancer in FSHD
Mariot	Dumonceaux	Myostatin expression in neuromuscular diseases: challenges and hopes
Maruyama	Maruyama	Development of LNA and 2'-MOE Gapmers to Treat Facioscapulohumeral Muscular Dystrophy
Mohassel	Shaw	Deep neuromuscular phenotyping of arhinia patients with SMCHD1 mutations reveals a mild myopathy distinct from FSHD2
Mueller	Mueller	Xenografting Human Muscle Stem Cells into Mice to Study FSHD
Nguyen	Magdinier	Genetic variability and identification of complex genotypes in FSHD patients by Molecular Combing
Pakula	Pakula	The role of estrogen regulation in FSHD-1
Rashnonejad	Rashnonejad	AAV.U7-snrRNA-mediated exon skipping of the toxic DUX4 gene as a promising therapeutic approach for facioscapulohumeral muscular dystrophy
Rickard	Schmidt	GBC0905: A Novel Targeted Therapeutic Agent to Treat Facioscapulohumeral Muscular Dystrophy
Robertson	Robertson	Measurement of evidence of DUX4 as a proof of concept biomarker for FSHD clinical trials
Rojas	Rojas	Pharmacological inhibition of DUX4 expression rescues FSHD pathophysiology in FSHD skeletal muscle myotubes
Saad	Saad	The natural microRNA miR-675 reduces DUX4 expression and toxicity in vitro
Sacconi	Sacconi	FSHD1 and FSHD2 form a disease continuum
Sanson	Sacconi	Self-report questionnaire vs. clinical evaluation form in the French National FSHD Registry: a statistical comparison
Statland	Statland	Preliminary Results from a Dose-Escalation Phase 2 Study to Evaluate ACE-083, a Local Muscle Therapeutic, in Patients with Facioscapulohumeral Muscular Dystrophy
Teveroni	Moretti	Set-up of an in vivo model of facioscapulohumeral muscular dystrophy (FSHD) based on human perivascular cells
van den Heuvel	van den Heuvel	Single-cell RNA-sequencing in Facioscapulohumeral muscular dystrophy disease etiology and development
van der Stoep	van der Stoep	Evaluation of FSHD1 testing in diagnostics using FiberVision Molecular Combing technology
Zhang, VW	Zhang, C	Accurate molecular diagnosis of Facioscapulohumeral muscular dystrophy in a cohort of 37 Chinese patients
Zheng	Zheng, Y	A case of first trimester prenatal diagnosis for FSHD1 using Karyomapping and single-molecule optical mapping

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Lessons in oligogenetics and pleiotropy: Identical SMCHD1 alleles can be associated with Arhinia, Bosma Syndrome, FSHD2, co-morbidities, or no phenotype at all

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Arhinia, or the complete absence of an external nose, is a rare congenital malformation with fewer than 100 cases reported to date. Nearly all patients demonstrate additional craniofacial defects, including mid--face and sinus hypoplasia, nasolacrimal duct atresia, choanal atresia, and cleft or high--arched palate. A subset also have co--morbid ocular defects (e.g., colobomatous microphthalmia, cataracts) and neuro--reproductive failure, a triad called Bosma Arhinia Microphthalmia syndrome (BAM;; OMIM 603457). We established an international consortium to aggregate a cohort of 38 patients with arhinia (22M, 16F; ; 24% of all reported cases and 19 new cases) and performed whole--genome, whole--exome (WES), and targeted sequencing to identify the etiology of this disorder. We compared the rare mutation burden for each gene in the genome in our arhinia patients to WES data from 60,706 control subjects from the Exome Aggregation Consortium (ExAC) and identified a single gene, SMCHD1, that exceeded genome--wide significance for enrichment of rare mutations in arhinia patients versus unaffected controls ($p = 2.9 \times 10^{-17}$). We identified rare, missense SMCHD1 variants in 86.1% of arhinia patients;; six were recurrent and none were observed in ExAC. Intriguingly, all arhinia--specific mutations were clustered within the 5' ATPase domain of SMCHD1 (exons 3, 5, 6, 8--13), and analyses using regional models of evolutionary constraint revealed that this region (exons 1--19) is under strong evolutionary constraint, whereas the 3' end of the gene (exons 19--48) is not.

SMCHD1 encodes a master epigenetic repressor that maintains X--inactivation and silences autosomal repeat sequences. Loss--of--function mutations in SMCHD1 cause fascioscapulohumeral muscular dystrophy type 2 (FSHD2) through a complex oligogenic trans--acting mechanism that requires hypomethylation of the 4q35 D4Z4 repeat array, a permissive 4qA haplotype, and a truncated D4Z4 repeat. Variants associated with FSHD2 span the entire gene and include truncating mutations, whereas variants associated with arhinia are exclusively missense mutations and cluster tightly around the ATPase domain. However, many previously reported FSHD2-- specific mutations are in the same exons (n=23) or even the same nucleotide position (n=3)

as mutations identified in patients with arhinia, suggesting common functional consequences of these mutations. Moreover, we have now confirmed 1 individual with arhinia and a late-onset, slowly progressive myopathy suggestive of FSHD2. Like most complex genetic traits, the genotype-phenotype correlations are not perfect: 1) individuals harboring identical alleles, or alleles within the same exons, may present with arhinia, BAM, FSHD2, and other comorbidities, and 2) transmitted alleles range in penetrance from complete arhinia to nasal hypoplasia to no dysmorphism at all. We further investigated the mechanistic overlap between arhinia and FSHD2 by measuring 4q35 D4Z4 methylation in 23 arhinia patients and 22 unaffected family members, revealing that 74% of arhinia patients with an SMCHD1 variant had D4Z4 hypomethylation characteristic of FSHD2, whereas arhinia patients without a rare missense variant in SMCHD1 had normal methylation. We found slight reductions but no statistically significant differences in overall SMCHD1 mRNA or protein expression in arhinia patients vs. unaffected family members. These data therefore demonstrate a diverse phenotypic spectrum associated with SMCHD1 mutations that includes arhinia, ocular and reproductive defects, and/or muscular dystrophy, and serve as an example of how identical mutations can manifest as diverse phenotypes depending on genetic background and potentially environmental exposures. Importantly, these data reveal that arhinia is not a Mendelian trait but a complex genetic trait. Thus, simplistic genetic models and any expectation of a one-to-one correlation between alleles and phenotypes need not apply.

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Deep neuromuscular phenotyping of arhinia patients with *SMCHD1* mutations reveals a mild myopathy distinct from FSHD2

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Background: Loss-of-function mutations in *SMCHD1* in combination with a permissive 4q35 allele cause fascioscapulohumeral dystrophy type 2 (FSHD2). Heterozygous missense mutations in *SMCHD1* (some of which are identical to those causing FSHD2), have also been implicated in congenital arhinia (absent external nose), a seemingly unrelated disorder that is often associated with ophthalmic and reproductive defects, but not with muscular dystrophy.

Methods: We performed comprehensive neuromuscular phenotyping and genotyping studies in nine patients with either congenital arhinia, a bifid nose, or nasal hypoplasia. Patients underwent a physical exam, muscle ultrasound and MRI, and blood draw to measure creatine kinase. Patient fibroblasts (n=2) were transdifferentiated into a myogenic lineage and DUX4-fl expression was measured and compared with expression in primary myoblasts from an FSHD patient with an *SMCHD1* missense mutation. We determined each patient's risk for FSHD2 based on standard genetic testing: Southern blot, 4q35 haplotyping, D4Z4 methylation (using methylation-sensitive restriction enzyme digestion), and *SMCHD1* sequencing.

Results: Seven of the nine patients had rare, heterozygous missense mutations in exons 3-11 of *SMCHD1*, and a subset (n=5/7; 1 male, 4 females; age 17-30 yrs) met all genetic criteria for FSHD2 (i.e., *SMCHD1* mutation, 4qA haplotype, D4Z4 methylation 4-24%). While none of the patients endorsed typical signs and symptoms of FSHD2 such as facial or periscapular weakness, chronic fatigue, or muscle pain, these five patients consistently demonstrated: 1) mild, symmetric weakness involving proximal and truncal muscles (neck flexion, hip flexion, knee flexion), and 2) a granular pattern of mildly increased echogenicity on ultrasound confined to the hamstrings, medial gastrocnemius, and paraspinal muscles. Corresponding changes, such as small foci of fatty replacement, were also observed on MRI in several patients. Serum CK was within normal limits (64-220 IU/L). DUX4-fl mRNA was detected in MyoD-transduced fibroblasts from two arhinia patients at levels comparable to that in FSHD2 primary myotubes (see also poster by Chang et al.).

Conclusion: Arhinia patients who meet all genetic criteria for FSHD2 demonstrate a mild myopathy with a specific anatomical distribution that is distinct from FSHD. The underlying cause for this divergent neuromuscular phenotype is unclear but may provide an opportunity to identify disease-modifiers unique to each condition and therapeutic targets for DUX4-mediated myopathies.

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The epigenetic repressor, FSHD2 gene and FSHD1 modifier SMCHD1 functions by mediating long range chromatin interactions

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Over the past six years, it has become apparent that heterozygous loss of function mutations in the epigenetic repressor SMCHD1 cause FSHD2, and correlate with more severe disease in FSHD1. By contrast, we recently reported that heterozygous gain of function mutations in SMCHD1 are found in the rare craniofacial development disorder, Bosma arhinia and microphthalmia. For these reasons, we have been interested to understand when and where Smchd1 plays a role, and SMCHD1's molecular mechanism of action. Here we reveal that Smchd1 is a novel regulator of long-range chromatin interactions, and add it to the canon of epigenetic proteins required for *Hox* gene regulation. The effect of losing Smchd1-dependent chromatin interactions has varying outcomes dependent on chromatin context. At autosomal targets transcriptionally sensitive to Smchd1 deletion, we find increased short-range interactions and ectopic enhancer activation. However, the inactive X chromosome is transcriptionally refractive to Smchd1 ablation, despite chromosome-wide increases in short-range interactions. There we observe spreading of H3K27me3 domains into regions not normally decorated by this mark. Previously we have found increased Ctf binding at autosomal Smchd1 targets in the absence of Smchd1. Together these data suggest Smchd1 has the capacity to insulate the chromatin, thereby limiting access to other chromatin modifying proteins. Interestingly, H3K27me3 has been observed to be increased at *D4Z4* in cells from FSHD2 patients, and CTCF is reportedly increased at the repeat in FSHD patient cells, raising the possibility that SMCHD1 may also play an insulator role at *D4Z4*. It has also been of interest how Smchd1 is targeted to chromatin in order to elicit its insulating effects, and here we have uncovered an intriguing link with polycomb repressive complex 1 (PRC1).

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Genetic variability and identification of complex genotypes in FSHD patients by Molecular Combing

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Facio Scapulo Humeral dystrophy (FSHD), the third most frequent neuromuscular dystrophy is linked to the subtelomeric locus 4q35 and associated with different genetic variants of non-coding regions. In 95% of cases, a reduction in the number of D4Z4 macrosatellite units on a A-type allele is associated with FSHD. The loss of these elements of repeated DNA is associated with a decreased DNA methylation. For 5% of patients, the reduction in the number of D4Z4 is not observed (FSHD2). A large proportion of these patients, however, have a decrease in D4Z4 methylation linked in some cases to mutation in the *SMCHD1* gene.

In most laboratories, the molecular diagnosis is based on the Southern blot technique, used to determine the number of repeated D4Z4 units. However, this technique has some limitations and a number of cases remain difficult to interpret. To circumvent this difficulty, we have developed an alternative diagnostic approach based on the molecular combing technique (Nguyen et al., 2010). Using this highly resolutive methodology, we explored 895 individuals for diagnosis, confirmation or exclusion of FSHD. In 9% of cases, we identified the existence of new complex rearrangements of the disease-related region 4q35 or the 10q26 region homologous to 98%. In these patients, we systematically analyzed the genomic variants associated with FSHD (subtelomeric qA or qB alleles), D4Z4 methylation and presence of *SMCHD1* mutations. Our results show that there is no systematic correlation between variation of *SMCHD1*, hypomethylation of D4Z4 and presence of clinical signs of the disease and in some patients, these complex remodeling are the only abnormalities associated with the pathology. All these results therefore raise additional questions on molecular diagnostics, especially in complex cases and genetic counseling.

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***Cis* D4Z4 repeat duplications associated with FSHD2**

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Abstract

Two genetic forms of facioscapulohumeral muscular dystrophy have been identified, FSHD1 and FSHD2. Both forms are associated with D4Z4 repeat array chromatin relaxation and somatic derepression of *DUX4*, of which a full copy is encoded by the macrosatellite repeat D4Z4 and adjacent sequences on 4qA chromosomes. Normally, the D4Z4 repeat on 4qA alleles varies between 8-100 units, and in FSHD1 D4Z4 chromatin relaxation is mainly caused by a repeat contraction to sizes between 1-10 units. FSHD2 is digenic, with D4Z4 chromatin relaxation typically being caused by heterozygous pathogenic variants in the chromatin modifier SMCHD1 in combination with 8-20 D4Z4 units 4qA alleles. The same combination with >20 units 4qA alleles typically does not result in disease or in a much milder phenotype. In this study, we identified 11/79 (14%) FSHD2 patients with unusually large 4qA alleles of 21-70 D4Z4 units. By a combination of Southern blotting and molecular combing we show that 8/11 (73%) of these unusually large 4qA alleles represent duplication alleles in which the long D4Z4 repeat arrays are followed by a small FSHD-sized D4Z4 repeat array duplication. We also show that these duplication alleles are associated with *DUX4* expression and disease presentation only when combined with a pathogenic variant in SMCHD1. This duplication allele frequency is significantly higher than in control individuals (2.9%) and FSHD1 patients (1.4%). We conclude that *cis* duplications of D4Z4 repeats explain *DUX4* expression and disease presentation in FSHD2 families with unusual long D4Z4 repeats on 4qA chromosomes.

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Identifying mechanisms that regulate DUX4 and the D4Z4 macrosatellite repeats

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Mis-expression of the transcription factor double homeobox 4 (DUX4) in skeletal muscle causes facioscapulohumeral dystrophy (FSHD). To agnostically identify regulators of the D4Z4 macrosatellite repeat array which houses the DUX4 locus, we carried out CRISPR-based locus-specific proteomics in normal human myoblasts. This approach identified over 250 D4Z4-associated proteins and, when combined with gene depletion, revealed that the Nucleosome Remodeling Deacetylase (NuRD) and Chromatin Assembly Factor 1 (CAF-1) complexes are necessary for DUX4 repression in human skeletal muscle cells and induced pluripotent stem cells. Furthermore, we showed that DUX4-induced expression of MBD3L proteins partly relieved this repression in FSHD muscle cells. Together, our findings characterize NuRD and CAF-1 as novel mediators of DUX4 chromatin repression and suggest a mechanism for the amplification of DUX4 expression in FSHD muscle cells.

7.
[O]

U1 snRNA controls alternative polyadenylation of Pax3 in muscle stem cells

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FSHD is caused by chromatin relaxation and an allele-specific polymorphism in the Dux4 gene that creates a polyadenylation site that protects the Dux4 transcript from degradation by adding a poly(A) tail. To identify potential strategies to block the polyadenylation site in Dux4, we studied how a cell selects or ignores a specific polyadenylation site. The Pax3 gene has four functional polyadenylation sites in the 3'UTR. Muscle stem cells in the diaphragm mainly use one of two proximal polyadenylation sites to express short Pax3 isoforms, while muscle stem cells in the lower hind limb muscles use one of two distal polyadenylation sites to express long isoforms. We now identify U1 snRNA as the factor that prevents the selection of proximal polyadenylation sites in Pax3 in limb muscle stem cells. Knockdown of U1 snRNA induced a switch to short Pax3 isoforms in vivo and ex vivo, whereas U1 overexpression led to the expression of more long Pax3 isoforms. Pax3 is regulated by microRNA206, which binds to the Pax3 3'UTR. The short Pax3 isoforms lack the binding sites for miR206. Consistently, the induction of more short Pax3 isoforms led to resistance to microRNA206 and increased levels of Pax3 protein. Moreover, using antisense morpholino oligonucleotides complementary to the U1 snRNA binding sites on the Pax3 transcript we could similarly increase the expression of short Pax3 isoforms and Pax3 protein. Our results show that modulation of U1 snRNA is an effective approach to modulate the selection of a polyadenylation site.

8.
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Dynamic transcriptomic and morphological analysis of FSHD atrophic myogenesis reveals a correctable defect in mitochondrial biogenesis

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Myoblasts from FSHD patients can display an atrophic myotube phenotype and a reduction in skeletal muscle myosin content. However, molecular mechanisms driving FSHD atrophic myotube formation are poorly understood, limiting therapeutic development. Here we perform high-throughput imaging of in vitro myogenesis, generating 8640 images. By developing novel software we construct a morphometric staging, identifying 8 critical timepoints where FSHD and control myogenesis differ. We then perform time course transcriptomics at these time points, generating 60 RNAseq samples. By performing multivariate regression analysis, we identify numerous dysregulated pathways in FSHD, including misregulation of apoptosis, epigenetic processes and canonical Wnt signalling. Importantly, the temporal depth of our data allowed us to investigate genes dynamically repressed during FSHD myogenesis, which would otherwise be undetectable on conventional differential expression analysis. In doing so we identify clear repression of genes involved in mitochondrial biogenesis. We demonstrate that knockdown of one of these genes is sufficient to generate atrophic myotubes from healthy myoblasts. Moreover, supplementing FSHD myoblast cultures with a safe agonist of another of these genes, available as a food supplement, is sufficient to rescue the FSHD atrophic myotube phenotype. Thus we provide a detailed analysis of the morphological and molecular processes driving FSHD atrophic myoblasts and identify novel therapeutic targets for rapid translation.

9.
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Structural and functional studies on DUX4 in human myogenesis

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Loss of silencing DUX4 causes facioscapulohumeral muscular dystrophy (FSHD), but the pathological mechanism is not understood. DUX4 contains tandem Paired-type homeodomains at its N-terminus, and a p300-dependent transcriptional activation domain at its C-terminus. Low levels and sporadic expression of DUX4 have been reported in FSHD myoblast cultures. At high levels, DUX4 induces apoptosis; the effects of low level DUX4 expression on human myogenic cells are not well understood. Dystrophy due to rare sporadic bursts of high DUX4 expression leading to fiber loss is difficult to reconcile with the tremendous regenerative potential of human skeletal muscle and the total degeneration seen in certain FSHD-involved muscles.

We are pursuing a structural understanding of the domains of the DUX4 protein and a functional understanding of the cell physiological effects of this transcription factor on myogenic progenitors. To address the lack of structural data on DUX4, we have obtained the crystal structure of the DUX4 tandem homeodomains bound to DNA. Despite being related by gene duplication and more similar to each other than to any other homeodomains, the two DUX4 homeodomains recognize different DNA sequences. This difference in sequence specificity is unique to the primate lineage (i.e. mouse Dux homeodomains recognize the same core sequence) and is attributable to specific residues outside of the recognition helix of each homeodomain. We discuss the implications of our data explaining the distinct sequence preferences of human DUX4 and mouse Dux.

In human myoblasts modified with a conditional titratable DUX4 genes, a large set of human myogenic genes is rapidly deregulated by DUX4, including MYOD1 and MYF5. Human myoblasts expressing low nontoxic levels of DUX4 are impaired from differentiating into myotubes in vitro. Surprisingly, inhibition of differentiation does not require the activation domain. To explore mechanisms of downregulation, we focused on MYF5, which lacks nearby DUX4 binding sites. Remarkably, a prominent ChIP-seq peak resides within the MYF5 -118 kb enhancer. When DUX4 binds at this site, it directs enhancer activity towards an enhancer-proximal TSS for a noncoding RNA we name DIME (DUX4-induced MYF5 enhancer) transcript. Induction of DIME expression does not require the DUX4 transcriptional activation domain, further supporting that binding of DUX4 to the -118 kb enhancer redirects its activity away from MYF5 and towards DIME. These data highlight the anti-myogenic properties of DUX4 in human myogenic progenitor cells, and provide an example of enhancer disruption in the regulation of MYF5.

10.
[O]

Regulation of Facioscapulohumeral muscular dystrophy candidate protein DUX4

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The *DUX4* gene associated with Facioscapulohumeral muscular dystrophy (FSHD) encodes a transcription factor protein that is toxic when expressed in numerous organisms. We hypothesized that one avenue for developing an FSHD treatment could involve inhibiting DUX4 protein activity. However, little is known about the mechanisms that may confer toxic properties to the DUX4 protein. We hypothesized that characterizing the biochemical properties of the DUX4 protein would provide fundamental information required to ultimately design FSHD therapies aimed at inhibiting DUX4 toxicity at the protein level. Our objectives were to identify cofactor proteins and post-translational modifications that may contribute to DUX4 toxicity in FSHD muscle.

Identification of DUX4 cofactor proteins: To identify proteins that may interact with DUX4 transiently (e.g. kinases) or indirectly (e.g. transcriptional complex) we performed Rapid Immunoprecipitation Mass Spectrometry of Endogenous Proteins. We have identified a list of 22 human kinases that phosphorylate DUX4 *in vitro*. Currently, we are monitoring DUX4 toxicity in the presence of cells overexpressing each of these human kinases to determine whether protein toxicity is altered. Our RIME analysis identified one modifying enzyme class that when inhibited by small molecules alters DUX4 toxicity in both HEK293 and human myoblasts transfected with CMV.DUX4. In addition, we monitored the effects of these inhibitory compounds on DUX4 expressed in a doxycycline-inducible human myoblast line. Although the mechanism of DUX4 inhibition has yet to be determined, we have experiments underway that monitor DUX4 protein stability and its methylation status.

DUX4 post-translational modifications: We performed high resolution mass spectrometry (MS) to identify DUX4 post-translational modifications (PTMs) in HEK293 cells. Our modification search included phosphorylated, methylated, and acetylated residues. We then targeted these modified DUX4 residues by mutagenesis to determine the impact of modified residues on DUX4 toxicity and function in both HEK293 cells and human myoblasts. Our results revealed the importance of methylated residue Arg 71 located in HOX1 domain. We will report the results of an identical MS analysis of DUX4 isolated from human myoblasts to determine whether the PTM signature varies from DUX4 isolated from HEK293 cells. Importantly, we have included additional modification types, such as sumoylation and ubiquitination.

Defining the DUX4 interactome and PTMs will help us understand the factors regulating DUX4 protein activity, such as binding affinity or nuclear localization. Ultimately, this work has allowed us to identify a pattern of PTMs that could be regulating DUX4 protein function, as well as a DUX4 modifying enzyme that when inhibited prevents DUX4-induced toxicity.

11.

[O]

Identification of a DUX4-interacting protein and the hyaluronic acid pathway as novel therapeutic targets for FSHD

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Expression of the FSHD disease gene *DUX4* is associated with a number of cellular pathologies, including cytotoxicity, that likely underlie the pathology of the disease. Unfortunately, the pathway(s) by which the *DUX4* protein causes these cellular pathologies remains uncertain. To identify pathways that may mediate *DUX4*-induced pathology, we screened for *DUX4*-interacting proteins using a co-IP/mass spec approach. We identified the hyaluronic acid (HA)-binding protein C1QBP, also recently identified in a yeast two-hybrid screen, as a potential *DUX4*-interacting protein. C1QBP has roles in several of the pathways that are disrupted in *DUX4*-expressing cells, both in the nucleus, where it regulates gene expression and pre-mRNA splicing, and in the mitochondria, where it regulates response to oxidative stress, glycolysis, and apoptosis, suggesting that it may mediate *DUX4* pathology. We found that *DUX4* expression caused mis-regulation of C1QBP and the mitochondria, leading loss of their normally perinuclear localization. Loss of C1QBP/mitochondrial regulation correlated with the appearance of additional pathologies, including the formation of dsRNA granules, mis-regulation of the ALS-associated FUS protein, appearance of double-stranded DNA breaks, and activation of caspase 3/7 and apoptosis. Critically, depleting cellular stores of HA using 4-methylumbelliferone (4MU), a competitive inhibitor of HA biosynthesis, maintained proper C1QBP/mitochondrial localization and prevented the appearance of each of these pathologies and induction of toxicity, establishing a central role for HA or HA-dependent signaling pathways in toxicity. Surprisingly, while 4MU has a profound impact on *DUX4*-induced pathology and toxicity, it has only a moderate and selective effect on the expression of *DUX4*-target genes, suggesting that toxicity is primarily caused by interactions between *DUX4* and the HA pathway, rather than by *DUX4*'s transcriptional activity. This establishes the HA biosynthetic pathway and HA-dependent signaling pathways as novel targets for the development of FSHD therapeutics.

12.

[O]

Myostatin expression in neuromuscular diseases: challenges and hopes

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Muscle atrophy and weakness is one of the biggest issues in neuromuscular patients. Because myostatin is a negative regulator of muscle mass, numerous studies have tried to inhibit its expression in order to increase muscle mass. In different mouse models of muscular diseases such as in the mdx mouse model for Duchenne Muscular Dystrophy, studies were encouraging and an improvement of muscle function was observed. Similarly, clinical trials on control individuals have shown an increase of muscle mass and function. However, so far, the results were disappointing in neuromuscular patients and in DMD patients in particular. We recently demonstrated that in patients showing an important muscle atrophy, the myostatin pathway is intrinsically down-regulated to counter balance the wasting process. Patients affected by a less atrophying disease such as FSHD, show a high heterogeneity among patients. These data may thus explain the poor clinical efficacy of anti-myostatin approaches in several of the clinical studies, may inform patient selection and stratification for future trials.

Moreover, this myostatin down regulation was shown to be reversible in MTM1-deficient myotubular myopathy upon gene transfer. Myostatin expression could thus be a reliable biomarker for treatment efficacy in atrophying neuromuscular diseases.

Here we show that components of the myostatin pathway are robust and reliable circulating biomarkers of drug efficacy in a gene therapy approach for dystrophin deficiency. Using an AAV8-microdystrophin vector in the GRMD dog model of Duchenne Muscular Dystrophy, we demonstrate that the intrinsic loss of myostatin production in GRMD muscle can be partially corrected by AAV8-microdystrophin transfer in a dose-dependent manner.

Myostatin levels are thus the first quantifiable biomarker allowing the non-invasive monitoring of treatment efficacy by providing a measure, which can determine the overall degree of the gene therapy efficacy as well as a longitudinal monitoring tool to follow eventual decrease of the therapeutic effect. This biomarker may therefore also be useful in future to judge the effect of combined therapy approaches non-invasively. Because circulating myostatin levels represent the product of general muscle health and activity we predict that myostatin pathway monitoring can be used to judge therapy efficacy in a wide range of neuromuscular diseases, which are associated with muscle wasting such as FSHD.

13.

[O]

The natural microRNA *miR-675* reduces DUX4 expression and toxicity *in vitro*

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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant muscle disease associated with progressive weakness in muscles of the face, shoulder-girdle and arms. FSHD arises from an epigenetic defect that ultimately causes aberrant expression of the transcription factor DUX4 in skeletal muscles. DUX4 is toxic to muscle and numerous non-muscle cell types, and causes differentiation defects, muscle atrophy, oxidative stress and cell death. However, FSHD symptoms are often variable from person to person, and there may be also variability in severity of symptoms, rate of progression and age at onset, even in families with several affected relatives. Asymmetry is often seen, where a person may have more muscle weakness on one side of the body versus the other. Although DUX4 is toxic, some cells and tissues seem to resist its damaging effects. We hypothesize that FSHD variability and the differential toxicity of DUX4 are linked; it is possible that the toxic effects of DUX4 may be reduced in cells or muscles that are spared in FSHD. However, the mechanisms by which some cells might resist DUX4 damage are unclear. In this work, we investigate the hypothesis that natural microRNAs could reduce DUX4 expression, reduce its toxicity, and potentially slow FSHD progression. *H19* is a maternally imprinted gene encoding a long non-coding RNA (lncRNA) that promotes muscle regeneration and differentiation through the function of its two encoded miRNAs (*miR-675-5p* and *-3p*). In this work, we investigated the potential role of *H19* and its miRNAs to counteract DUX4 pathogenicity. We show that *miR-675-5p* is capable of reducing DUX4-gene expression and associated cytotoxicity *in vitro*. We also show that *miR-675-5p* acts directly on DUX4 by binding to *DUX4* mRNA therefore reducing DUX4 protein expression. The expression of endogenous *miR-675* was variable between different affected and non-affected muscle cell lines. In particular, we show that *miR-675* expression is inversely correlated with *DUX4* expression when comparing three characterized FSHD cell lines (15Abic, 17Abic and 18Abic). With the lowest *DUX4* expression, 15Abic myoblasts and myotubes had the highest *H19* and *miR-675* expression. Furthermore, in differentiated 15Abic myotubes, a decrease in *miR-675* expression was associated with an increase in the expression of *DUX4* and its biomarkers. We propose that the increased *H19* expression in differentiated 15Abic myotubes plays a key role in maintaining relatively high *miR-675* expression compared to the more affected cell lines leading to a reduced *DUX4* expression. We therefore anticipate that by increasing *H19* or *miR-675* expression in our newly characterized TIC-DUX4 mouse model we would observe a reduction in muscle toxicity and an increase in muscle strength. This proof-of-principle triggered us to perform a functional screen to identify the full set of natural DUX4-targeted miRNAs that could function as potential miRNA therapeutics or drug targets in FSHD. This screen is an ongoing project in the lab.

14.
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Single-cell transcriptome heterogeneity in myogenic cells from individuals with FSHD

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A more complete understanding of the cellular and molecular events underlying muscle damage and inflammatory responses is needed to define effective therapeutic targets for FSHD. We hypothesize that single-cell transcriptome analysis of cells recovered from FSHD muscle biopsies may provide novel insights regarding the pathophysiology of muscle damage in FSHD and the identification of FSHD biomarkers to monitor disease progression in future clinical trials. Our initial goal was to show feasibility of single-cell RNA-seq (scRNA-seq) to distinguish between primary myogenic and non-myogenic cells in undifferentiated proliferating cell cultures. In a pilot experiment, we obtained low-passage cells from FSHD biceps muscle and age-matched controls and performed scRNA-seq using the Drop-seq method. Library samples were run on one lane on an HS4K instrument, which generated 280-350 M reads (performed in duplicate). Each mRNA read was mapped both to the barcoded cell-of-origin and to the gene-of-origin, and each capture oligo also contained a unique molecular identifier (UMI) to enable accurate quantitation of mRNA molecules. Reads were aligned to the human reference genome GRCh38.88 using STAR, and a digital gene expression (DGE) matrix was extracted for each aligned library. Consensus clustering and expression analyses were performed using Seurat v2.03. We confirmed that proliferating myogenic cultures from FSHD and control muscle biopsies did not express detectable DUX4 mRNA or its target genes, as expected since myogenic differentiation is required to express appreciable DUX4. t-distributed stochastic neighbor embedding (t-SNE) visualization identified a large predominantly myogenic cluster and two smaller clusters with non-myogenic expression patterns. Within the myogenic clusters, a subset of cells expressed the satellite cell markers PAX3 and PAX7, while other cells expressed markers of myogenic commitment (MYF5 or MYOD1), but very few cells expressed markers of muscle differentiation (e.g. MYOG and TNNI1) in these proliferating cultures. Several mRNAs were enriched specifically in the non-myogenic population, including tissue factor pathway inhibitor 2 (TFPI2), proenkephalin (PENK), procollagen C-endopeptidase enhancer (PCOLCE), and collagen type VI alpha 3 chain (COL6A3), while others were depleted, including leucine-rich repeat containing 17 (LRRC17) and TRIM55/MURF-2. We did not observe clearly distinct gene expression signatures for FSHD vs. control cells in the undifferentiated state in the absence of DUX4 expression. These results suggest that the single-cell RNA-seq approach and its evolving tools will allow us to identify relevant cellular subpopulations within a heterogeneous FSHD muscle sample that cannot be captured by bulk RNA-seq analysis.

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15.

[O]

Single-cell RNA-sequencing in Facioscapulohumeral muscular dystrophy disease etiology and development

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Facioscapulohumeral muscular dystrophy (FSHD) is characterized by the sporadic expression of the germline and cleavage-stage transcription factor DUX4 in myonuclei of affected muscle. Its presence in muscle activates a cascade of muscle disrupting events eventually leading to muscle atrophy and apoptosis of affected cells. Yet, with an estimated ratio of 1:100 - 1:1000 nuclei expressing DUX4 in primary myotube cultures, transcriptome analyses have systematically been challenged by the majority of nuclei being DUX4^{neg}, thereby weakening the DUX4 transcriptome signature. Moreover, DUX4 has been shown to be expressed in a highly dynamic burst-like manner, likely resulting in the detection of the downstream cascade of events long after DUX4 expression has faded. Identifying the FSHD transcriptome in individual cells and thereby unraveling the dynamic cascade of events leading to FSHD development, may overcome some of these challenges.

We employed single-cell RNA-sequencing (scRNAseq) combined with pseudotime trajectory modeling to study FSHD disease etiology and cellular progression in human patient-derived primary myocytes. We identified a small FSHD-specific cell population in all tested FSHD patient-derived primary cultures and detected promising new genes that are likely related to both direct and indirect effects of DUX4. Furthermore, capitalizing on the heterogeneity in cellular state in FSHD cell cultures, we performed pseudotime trajectory modeling to generate a detailed FSHD cellular progression model, reflecting both the early burst-like DUX4 expression as well as the downstream activation of various pathways previously implicated FSHD. We expect that pseudotime trajectories like our FSHD pseudotime model hold invaluable information not only for studying disease etiology, development and progression, but also for biomarker identification and therapeutic target selection

16.
[O]

AAV.RNAi and follistatin gene therapy development in the TIC-DUX4 Mouse Model of FSHD

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Autosomal dominant Facioscapulohumeral muscular dystrophy (FSHD) is among the most prevalent muscular dystrophies, estimated to affect 1 in 8,333-20,000 individuals worldwide. FSHD is linked to aberrant expression of the *DUX4* gene, which encodes a myotoxic transcription factor. Since DUX4 is extremely toxic, animal model development has been difficult, but progress has been made, revealing that tight regulation of DUX4 expression is critical for creating a viable model that develops myopathic features that are useful as therapeutic outcome measures. Here we report an inducible FSHD mouse model – called TIC-DUX4 - that utilizes Tamoxifen (TAM)-Inducible CRE recombinase to turn on DUX4 in skeletal muscle. Uninduced TIC-DUX4 (i.e. DUX4-off) mice are born in Mendelian ratios, develop normally to adulthood, and are indistinguishable from wild-type animals. Induced animals display significantly reduced skeletal muscle force, impaired open field activity, muscle wasting, and histological indicators of muscular dystrophy, including increased central nuclei and inflammation. Importantly, these phenotypes are tunable; myopathy progresses slowly over many months at low doses of TAM, while high doses can be used to rapidly induce widespread myopathic phenotypes within 2 weeks. We are now using this model to test DUX4-targeted gene therapies and myostatin inhibition to prevent DUX4 induced muscle weakness and increase muscle strength respectively. To directly target DUX4 expression, we utilized the RNAi pathway by AAV delivery of a DUX4-targeted microRNA. This provided long-term protection from DUX4-associated damage in old induced TIC-DUX4 mice. We also tested AAV delivery of follistatin by directly injecting virus into tibialis anterior and gastroc muscles of TIC-DUX4 mice. Follistatin expression significantly increased muscle mass and total muscle strength in the presence of DUX4 expression after 8 weeks of induction. These data will support translation of gene therapies for FSHD, and the TIC-DUX4 mouse model will be useful for testing other FSHD therapies as they emerge.

17.

[O]

An FSHD cell xenograft assay for drug development

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Facioscapulohumeral muscular dystrophy (FSHD) is a commonly diagnosed form of muscular dystrophy that affects both children and adults. Inherited in an autosomal dominant manner, FSHD is characterized by the frequently asymmetric, progressive weakening of muscles in the face, back and shoulder girdle. Misexpression of the skeletal muscle gene DUX4 has been implicated as the cause of muscle pathology in FSHD, however, no therapy or cure has been approved thus far. In order to advance potential therapeutics to clinical trials, we must first determine their efficacy using an in vivo model of disease. To that end, the Emerson lab has developed and optimized a robust cell xenograft assay to determine the effectiveness of potential FSHD therapeutics. Our model combines hindlimb irradiation of immunodeficient mice with BaCl₂-induced muscle injury to create a niche for the engraftment of primary, patient derived FSHD or unaffected control myoblasts. The xenoengraftment is highly reproducible and our power analysis suggests that drug testing can be accomplished efficiently. Pilot studies have produced quantifiable levels of DUX4 as well as DUX4 target genes in FSHD engrafted muscles compared to unaffected controls after a short 2 and 4-weeks of xenoengraftment. We are currently working with several industry collaborators as well as UMMS colleagues in the RNA Therapeutics institute to assay promising FSHD therapeutics using our xenograft model for drug development.

18.
[O]

Systemic delivery of LNA gapmers targeting DUX4 improved muscle function in FLExDUX4 mice

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Antisense oligonucleotides (AONs) selectively hybridize to their RNA targets via Watson-Crick base pairing and have been used for modulating gene expression via exon skipping or knocking down specific RNA targets. AON therapy has shown promise for treating an array of disorders however several problems associated with the AONs yet to be improved. Locked nucleic acids (LNAs) gapmers is one of the AONs that have higher affinity and stability, meanwhile retain the ability to elicit RNase H activity for RNA degradation. Using cell assays, we have identified several LNA gapmers successfully reduced DUX4 expression in vitro. In this study, we examined one of the LNA gapmers by either local delivery (intramuscular injections; i.m.) or systemic delivery (subcutaneous injections, s.c.) to the DUX4-expressing FLExDUX4 FSHD-like mouse model. Our results showed that the fluorescein-labeled LNA gapmers were present in skeletal muscles 24 hours after either one i.m. or one s.c. injection. Three i.m. injections (20ug) into the tibialis anterior muscles significantly reduced DUX4 transcripts in the muscles of the FLExDUX4 mice. Importantly, the same result was observed with systemic delivery, using three s.c. injections (20mg/kg) of the LNA gapmers. In a long-term 10-week trial, s.c. injections (20mg/kg) twice a week significantly reduced DUX4 expression. In addition, muscle function measured by grip strength testing showed functional recovery after the treatment. Our findings showed that the LNA gapmers targeting DUX4 delivered to skeletal muscles by systemic delivery significantly reduced DUX4 transcripts. The reduction of DUX4 was accompanied by recovery of muscle functional deficits in the FLExDUX4 mice. These data support the use of LNA gapmers, delivered either locally or systemically, as a viable therapeutic approach to FSHD.

19.
[O]

One step forward in clinical trial readiness for FSHD Clinical outcome measures, muscle imaging and (epi)genetic testing in a large cohort of FSHD patients

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Background

The 2015 FSHD Workshop on clinical trial preparedness (Tawil et al, Neuromuscular Disorders 2016) concluded that more research is needed on validation of clinical outcome measures, longitudinal imaging studies, and collection and banking of biomarker samples, especially in large cohorts of FSHD patients. We present here the data of the baseline measurement of a large longitudinal cohort of FSHD patients.

Methods

We included 203 FSHD patients for extensive clinical testing, including various questionnaires, the Motor Function Measure, 6-minute walk test, Ricci score, FSHD clinical score, spirometry, manual muscle testing and dynamometry of various muscle groups. 140 patients, including 9 asymptomatic gene carriers, were examined by muscle MRI as well and MRI fat fractions were correlated with clinical outcome measures to determine whether muscle MRI can be a useful biomarker in FSHD. Furthermore, 148 FSHD1 mutation carriers from 49 different families were included for extensive genetic testing to determine the influence of (epi)genetic factors (D4Z4 repeat array size, D4Z4 methylation) on the clinical variability of FSHD.

Results

The mean fat fraction of the leg musculature correlated strongly with the motor function measure, FSHD clinical score, Ricci score, and 6-minute walking test (correlation coefficients from -0.701 up to -0.845). Fat fraction per muscle group correlated well with corresponding muscle strength (correlation coefficients up to -0.82). Muscle MRI was able to detect fatty infiltration in early stage disease and in patients without leg muscle weakness. The explained variance in disease severity by familial factors, excluding the D4Z4 repeat array size, was approximately 40%. The explained variance by the D4Z4 repeat array size alone was limited (approximately 10%) and varied per body region. Asymptomatic gene carriers had longer repeat array sizes compared to symptomatic individuals (7.3 vs 6.2 units, $p=0.001$). The D4Z4 methylation corrected for repeat array size (Delta1 score) was higher in asymptomatic gene carriers compared to affected individuals (0.96 vs -2.78, $p=0.048$).

Conclusion

The 1st measurement of this large longitudinal study on FSHD shows that muscle MRI is a promising biomarker for disease severity in FSHD with strong correlations to all clinical outcome measures and is able to detect muscle involvement before symptoms occur. It showed that the D4Z4 repeat array size and D4Z4 methylation contribute to disease severity and penetrance, but unidentified disease modifying factors must be involved as well. Follow-up data will further deepen our knowledge on the natural history of and the influence of (epi)genetic factors on disease progression.

20.
[O]

FSHD1 and FSHD2 form a disease continuum

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Facioscapulohumeral dystrophy (FSHD) is caused by derepression of the D4Z4-encoded transcription factor DUX4 in skeletal muscle. Historically, two forms of FSHD are known: FSHD1, being associated with D4Z4 repeat contractions to a size of 1-10 units, and FSHD2, typically caused by pathogenic variants in *SMCHD1*. This study investigates the clinical correlations (CSS, MMT, Brooke and Vignos scores) with the genetics (D4Z4 repeat size and haplotype; *SMCHD1* variant status) and epigenetics (methylation of DR1) of FSHD1 and FSHD2.

In this study we clinically, genetically and epigenetically characterized a cohort of patients referred to our centers because of a clinical suspicion of FSHD. Of 103 patients, four FSHD1 patients having complex 4q rearrangements such as deletions, translocations or mosaicism, three FSHD2 subjects with an undetermined number of D4Z4 RU (>11) linked to technical issues and twelve patients excluded for FSHD, were not included in the statistical analyses. Then, we analyzed 57 patients who had classical FSHD1 defined by a D4Z4 repeat contraction to 1-10 RU on a disease permissive 4qA allele, 20 who had FSHD2 defined by a clinical phenotype consistent with FSHD, the presence of at least one 4qA chromosome, and D4Z4 hypomethylation in the absence of a D4Z4 repeat contraction. Interestingly, we identified seven patients who displayed genetic characteristics of FSHD1 and FSHD2, all carrying repeats of 9-10 D4Z4 units and a pathogenic *SMCHD1* variant (FSHD1+2). We confirmed the inverse correlation between repeat size and CSS, Brooke and Vignos scores and

the positive one with MMT in FSHD1 but did not find correlations between the D4Z4 DR1 methylation level and any of the clinical parameters. FSHD1 patients with repeat sizes of 8-10 units generally showed the mildest disease by all clinical scales, FSHD2 and FSHD1+2 patients being more severe than this previous subpopulation. The overlap between controls, FSHD1 and FSHD2 patients in the 8-10 D4Z4 repeat units range suggests that FSHD1 and FSHD2 form a disease continuum. We can conclude that previously established repeat size threshold for FSHD1 (1-10 repeat units) and FSHD2 (11-20 repeat units) needs to be reconsidered.

21.
[O]

MRI correlates to Electrical Impedance Myography in facioscapulohumeral muscular dystrophy

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Introduction: Electrical impedance myography (EIM) is a noninvasive technique to measure muscle composition in facioscapulohumeral muscular dystrophy (FSHD) and correlates with disease severity measures. The relationship between EIM and muscle MRI parameters is not established.

Objective: To determine associations of EIM parameters to muscle MRI in patients with FSHD.

Methods: 20 patients with FSHD were evaluated at 2 centers. EIM parameters including resistance, reactance, and phase at 50, 100, and 211 kHz were recorded from bilateral vastus lateralis, tibialis anterior, and medial gastrocnemius muscles and compared to MRI skin and subcutaneous fat thickness (SC), T1 muscle score from T1-weighted MRI, quantitative muscle fat fraction (FF), clinical severity score, muscle strength and 6-minute-walk-test.

Results: Resistance was reflected in MRI SC across all frequencies ($|r|: 0.88-0.89$) while reactance was sensitive to MRI SC at higher frequencies ($|r|: 0.62-0.78$). The MRI T1 muscle score and FF correlated to clinical disease severity measures ($|r|: 0.63-0.81$) and to reactance and phase, but not resistance. Phase showed frequency dependent strong associations with FF ($|r|: 0.64-0.77$) and moderate association with MRI T1 muscle score ($|r|: 0.43-0.56$). 50 Hz reactance demonstrated the strongest association with both FF and MRI T1 muscle score ($|r|: 0.71-0.74$).

Conclusions: This study establishes the correlation of structural changes in FSHD measured by MRI and EIM. While reactance and phase both correlate with MRI T1 muscle score and FF, reactance 50 was most sensitive to changes in muscle structure. These results support the role of EIM as a potential biomarker in FSHD clinical trials.

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22.

[O]

Accurate molecular diagnosis of Facioscapulohumeral muscular dystrophy in a cohort of 37 Chinese patients

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Aim

To investigate the accuracy of molecular combing, MC, in the genetic diagnosis of Chinese patients suspected of FSHD.

Methods

Single-molecular FISH was used to detect and analyze D4Z4 repeat units in 37 patients from China.

Results

The results showed that D4Z4 repeat units in the 4qA region of 35 confirmed FSHD patients was ranged from 2 to 7, with an average of (4.229±1.031). The results of 7 FSHD patients and 2 clinically suspected FSHD patients were: Cases 1, 2, 3 were from the same family and were brothers and sisters. D4Z4 repeat units of patient 1, 2, 3 in the 4qA region were 5, (6; 32) and (5; 32), respectively, and their D4Z4 fragment sizes were 17.5 Kb, (18.3; 106.0) Kb and (17.9; 104.9) Kb, respectively. D4Z4 repeat units of cases 1, 2, 3 were likely be inherited from their mother. D4Z4 repeat units in the 4qA region of case 4 was (2; 22), and the fragment size was (6.6; 71.9) Kb. D4Z4 repeat units in the 4qA region of cases 5, 6 were 4 and 5, respectively, and the fragment sizes of them were 11.7 Kb and 17.3 Kb, respectively. Case 7 was a 55-year-old woman, who was the eldest female patient among this cohort. D4Z4 repeat units in the 4qA region of case 7 was (5; 68), and the fragment size was (18.1; 224.0) Kb. Cases 8 and 9 were 2 clinically suspected FSHD patients. D4Z4 repeat units in the 4qA region were (14; 50) and 33, respectively, and the fragment size were (44.6; 164.8) Kb and 110.2 Kb, respectively. D4Z4 repeat units in the 4qA region of cases 8 and 9 were more than 10.

Conclusion

There is a high degree of inter- and intra-family clinical heterogeneity in patients with FSHD, and single-molecular FISH can accurately resolve D4Z4 repeat units of four different haplotypes, namely 4qA, 4qB, 10qA and 10qB. The results of single-molecular FISH can be accurate to one D4Z4 repeat unit and 1 Kb D4Z4 fragment sizes, which can greatly improve specificity and sensitivity. Therefore, single-molecular FISH currently can provide accurate molecular diagnostics for FSHD patients.

23.

[O]

A case of first trimester prenatal diagnosis for FSHD1 using Karyomapping and single-molecule optical mapping

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The DUX4 region, which causing FSHD1, is relatively complex, leading to the difficulty of FSHD1 prenatal diagnosis. Single-molecules optical mapping (SMOP) is a technique for constructing ordered, genome-wide, high-resolution restriction maps from single, stained molecules of DNA, and can accurately detect the number of D4Z4 repeats and 4qA/B allele. Karyomapping—genome wide parental haplotyping using high density single nucleotide polymorphism (SNP) genotyping, providing a comprehensive method for linkage-based diagnosis of any single gene defect, needs less amount of DNA, simpler and faster in fetal sample than Southern Blot. In our study, we recruited a FSHD1 family, which was 11 weeks of pregnant. Firstly, we used SMOP for molecular diagnosis of proband, and the result showed that 4 D4Z4 repeats with 4qA allele coming from chromosome 4 is pathogenic gene for FSHD1. Second, we performed linkage-based diagnosis for fetus, proband, his mother and wife by Karyomapping and molecular diagnosis for fetus by SMOP. The results indicated that the fetus inherited the pathogenic chromosome from proband and also had 4 D4Z4 repeats with 4qA allele. Third, follow-up result of aborted fetus tissue was same as the result of fetal chorionic villus sample by SMOP and conformed that it is feasible and accurate for prenatal diagnosis of FSHD1 through karyomapping combined with SMOP. This case study illustrates a new way of FSHD1 prenatal diagnosis.

24.
[O]

Preliminary results from a dose-escalation Phase 2 study to evaluate ACE-083, a local muscle therapeutic, in patients with facioscapulohumeral muscular dystrophy

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ACE-083 is a locally acting muscle therapeutic based on follistatin that binds myostatin and other muscle regulators. ACE-083 increased muscle mass and force in mouse models of neuromuscular disease and increased muscle mass in healthy volunteers. Facioscapulohumeral dystrophy (FSHD) is characterized by weakness in various muscles, including the upper arm and lower leg.

We are conducting a 2-part trial of ACE-083 in patients with FSHD; Part 1 is open-label, ascending dose and Part 2 is double-blind, placebo-controlled. Here we report results from Part 1. ACE-083 (3 cohorts, 150-240 mg) is injected into either the tibialis anterior (TA) or biceps brachii (BB) muscle q3w (5 doses), unilaterally or bilaterally. The primary objective of Part 1 is safety, and objectives for Part 2 include muscle volume, strength, function, and quality of life.

Data were available as of 28Mar2018 for Cohorts 1 and 2. For the TA Cohorts 1 and 2 (n=12), median age (range) was 46 yr (19-63), median (range) duration of symptoms was 26 yr (4-35), and median (range) baseline fat fraction (FF) was 36% (12-82). For the BB Cohorts 1 and 2 (n=12), median age (range) was 53 yr (20-69), median (range) duration of symptoms was 22 yr (4-55), and median (range) baseline FF was 14% (6-79).

Adverse events included transient injection site reactions and myalgia, primarily grade 1-2; there were no serious AEs. One patient experienced a related grade 3 non-serious adverse event of lower leg intramuscular swelling.

At Day 106, 3 weeks after last dose, the mean (SEM) percent change in total muscle volume (TMV) in the TA was 8.1 (3.5) for the 150mg cohort (n=6), 16.8 (3.0) for the 200mg cohort (n=6), and 2.0 (1.6) for pooled untreated sides (n=12). The mean (SEM) percent change in TMV in the BB was 8.2 (6.0) for the 150mg cohort (n=6), 18.1 (7.5) for the 200mg cohort (n=6) and 6.8 (4.6) for the pooled untreated sides (n=11). Mean (SEM) absolute change in FF in the TA was -4.5% (3.0) for the 150mg cohort (n=5), -5.0% (2.8) for the 200mg cohort (n=6), and -0.1 (0.5) for pooled untreated (n=11); minimal mean changes in FF were observed in the BB cohorts.

ACE-083 treatment was generally safe and well tolerated in patients with FSHD and resulted in increased muscle volume and decreased fat fraction. These preliminary findings support continued investigation of ACE-083 in neuromuscular disorders.

25.

[O]

Pharmacological inhibition of DUX4 expression rescues FSHD pathophysiology in FSHD skeletal muscle myotubes

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Facioscapulohumeral dystrophy (FSHD) is caused by mutations that lead to loss of repression at the D4Z4 macrosatellite repeats on chromosome 4 and increased expression of the homeobox transcription factor DUX4. Aberrant expression of DUX4 in skeletal muscle results in activation of an early embryo development transcriptional program that leads to death of muscle fibers. While some progress toward understanding the signals driving DUX4 expression has been made, the factors and pathways involved in the transcriptional activation of this gene remain largely unknown. Using optimized in vitro myotube culture systems and highly sensitive assays to screen a custom chemical probe library, we identified several small molecules against a single target protein that reduced the expression of DUX4 and its downstream genes in multiple patient-derived FSHD myotubes representing diverse disease genotypes and phenotypes. We found a similar reduction in DUX4 and DUX4-dependent gene expression when we used siRNAs and sgRNAs to reduce the expression of this target protein in FSHD myotubes. RNA-seq studies in small molecule-treated FSHD myotubes revealed that a small number of genes were differentially affected, ~75% of them direct targets of DUX4. In contrast, no changes in gene expression of myogenin or other myogenic factors were observed nor were there quantifiable changes in myogenic fusion. Further pharmacologic characterization of these molecules showed potent and selective inhibition of the target pathway, DUX4, reduction of the detrimental early embryonic gene expression program, and profound inhibition of apoptotic cell death in multiple patient derived primary and immortalized FSHD myotubes. These observations provide us with the rationale to continue our efforts to test the therapeutic benefit of reduction of DUX4 in FSHD patients.

26.

[O]

A low molecular weight compound screen in FSHD patient myotubes identifies modulators of Dux4 activity and novel mechanisms of action

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Facioscapulohumeral muscular dystrophy (FSHD) is among the most prevalent of the adult-onset muscular dystrophies. FSHD causes a loss of muscle mass and function, resulting in severe debilitation and reduction in quality of life. Currently only the symptoms of FSHD can be treated and with minimal benefit. The genetic, epigenetic, and molecular mechanisms triggering FSHD are now quite well understood, and it has been shown that expression of the transcriptional regulator *double homeobox 4 (DUX4)* is necessary for disease onset and is largely thought to be the causative factor in FSHD. Therefore, we sought to identify compounds suppressing Dux4 activity in a phenotypic screen using FSHD patient-derived muscle cells, a zinc finger and SCAN domain-containing 4 (ZSCAN4)-based reporter gene assay for measuring Dux4 activity, and ~3000 small molecules. This effort identified molecules that reduce *DUX4* gene expression and hence Dux4 activity. The majority of active compounds were molecules that increase cAMP levels and activate PKA thereby reducing *DUX4* expression however one hit-class in particular was able to lower Dux4 dependent gene expression independent of the cAMP-PKA signaling pathway. The activity of these compounds represents a novel mechanism effective at reducing Dux4 activity. They are both potent and effective and will prove to be valuable tools in the identification of additional signaling pathways capable of lowering Dux4 activity in FSHD.

27.

[O]

Protein kinase A activation inhibits *DUX4* gene expression in myotubes from patients with facioscapulohumeral muscular dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is among the most prevalent of the adult-onset muscular dystrophies. FSHD causes a loss of muscle mass and function, resulting in severe debilitation and reduction in quality of life. The genetic, epigenetic, and molecular mechanisms triggering FSHD are now quite well understood, and it has been shown that expression of the transcriptional regulator *double homeobox 4* (*DUX4*) is necessary for disease onset and is largely thought to be the causative factor in FSHD. During a screen for low molecular weight compounds capable of lowering Dux4 activity we identified several classes of compounds that reduce *DUX4* mRNA expression in FSHD patient primary myotubes by increasing cAMP levels. These included β -2 adrenergic receptor agonists and phosphodiesterase inhibitors, as well as other putative Gs coupled GPCR ligands. Using a mix of chemical, biochemical and genetic tools, we have found that cAMP production reduces *DUX4* expression through a protein kinase A-dependent mode of action in FSHD patient myotubes. These findings increase our understanding of how *DUX4* expression is regulated in FSHD and point to potential areas of therapeutic intervention.

28.

[O]

In vivo assessment of antisense therapy for Facioscapulohumeral muscular dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) development requires both genetic and epigenetic alterations. The presence of a permissive pLAM polyadenylation signal distal to the last D4Z4 repeat is generally accepted as being required for the stabilization of DUX4 transcripts and translation of the pathogenic DUX4 protein. Based on advanced bioinformatics tools analysis, we designed over 40 antisense oligonucleotides (AOs) targeting key 3'UTR elements of the DUX4 mRNA, aiming to downregulate DUX4 expression. We selected the 5 most potentially active AOs and examined their inhibitory efficiencies in immortalized muscle cell lines from FSHD patients and matched controls. AOs reducing DUX4 mRNA in patient cell cultures, without or with AAV vector-mediated DUX4 overexpression, to the level of healthy controls were subsequently studied in in vivo. The optimal AOs conjugated to cell-penetrating elements were intravenously delivered in mice injected intramuscularly with AAV vectors expressing DUX4. We report here the results of electrophysiological muscle force measurements, assessment of DUX4 RNA and protein levels, and muscle histological analyses, as well as valuable lessons learned from the study.

29.

[O]

GBC0905: A novel targeted therapeutic agent to treat facioscapulohumeral muscular dystrophy (FSHD)

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Genea Biocells US Inc.

FSHD is caused by aberrant expression of the primate-specific DUX4 transcription factor that triggers a toxic transcriptional cascade believed to be mediated by the epigenetic machinery in myotubes. This complex pathogenic mechanism for muscle weakness is challenging to recapitulate in non-human cell types and thereby necessitates development of a cell-based human myogenesis model for optimal therapeutics development. Our team studied multiple human pluripotent stem cell lines (hPSC) in vitro-differentiated to skeletal muscle to identify DUX4, DUX4-activated target genes, and DUX4-dependent cellular phenotypes. We conducted a series of diverse primary and secondary assays including assessment of DUX4 and reduction of the expression of its target genes, DUX4-induced apoptosis, and signaling mediated by specific molecular networks. We identified a small molecule, GBC0905 which reduces DUX4 expression in a mechanistically relevant and dose-dependent manner in both FSHD-affected hPSC-derived skeletal muscle and primary muscle biopsies from FSHD-affected donors. Furthermore, GBC0905 rescues DUX4-dependent cellular pathologies in FSHD-affected skeletal muscle, including the activation of multiple DUX4 target genes, apoptosis induction, and subsequent cell death, without impairing myogenesis and myotube formation. Notably, the molecule displays favorable single- and repeated dose pharmacokinetic and safety profile upon oral administration in murine models. In this presentation, we will summarize our current progress, including both ex vivo and in vivo evaluation of GBC0905 as we move toward IND approval and clinical studies.

30.
[P]

Large-scale methylation analysis in facioscapulohumeral muscular dystrophy (FSHD)

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FSHD forms are associated to D4Z4 DNA hypomethylation on 4qA allelic chromosomes. These epigenetic changes provide a transcriptionally permissive chromatin environment leading to the production of a pathogenic protein DUX4. We have shown that the epigenetic changes in FSHD, can be investigated using methylation assays specific for permissive chromosomes 4qA (PAS+). Here, we report the analysis of a large cohort of individuals consisting of 287 FSHD1, 53 FSHD2 and 165 control subjects. This analysis showed highly significant difference of methylation levels between affected and control subjects further supported by strong correlation with the number of D4Z4 repeats both in FSHD1 and FSHD2.

The different haplotype distribution between affected and control groups as well as particular methylation levels of some haplotypes suggested that diagnosis by methylation analysis can be improved by developing haplotype specific assays. To this aim, we developed haplotype specific assays and additional assays that allow precise genotyping. Furthermore, we set up bisulfite methylation assays to discriminate FSHD1 from FSHD2. Finally, assessment of methylation of intra-D4Z4 region and another SMCHD1 target sequence allowed evaluation of the effect of SMCHD1 mutations.

Our results can allow the development of an integrate platform of methylation and sequencing assays to accelerate and improve the diagnostic procedure of FSHD.

31.
[P]

The UK FSHD Patient Registry: a key tool in the facilitation of clinical research

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Background

The UK FSHD Patient Registry is a patient driven, clinician verified tool designed to support clinical research. The registry shares a common dataset with many registries worldwide and is able to collect symptomatic information longitudinally. This online patient driven registry aims to facilitate and accelerate planning and recruitment of clinical trials. Patient reported outcomes are entered into a secure online portal that are then combined with clinician verified genetic details. Core clinical and genetic information has been collected through the registry with additional data about pain (MPQ-SF) and quality of life (INQoL).

Results

Between May 2012 and May 2018 over 870 people have registered, 93% with a clinical diagnosis of FSHD1. There is an almost even distribution of patients registered from both sexes and 61% of patients were between 31 and 60 years old. Muscle weakness was widely reported with periscapular shoulder weakness occurring most frequently (84%) followed by weakness of the hip girdle (65%), facial muscles (65%) and foot dorsiflexor (63%). Onset of facial weakness was reported to occur before the age of 30 in 44% of cases, earlier than weakness in other areas. The use of a wheelchair was reported to begin most often after the age of 30 (80%). Hearing loss was reported in 14% of patients mostly (64%) in those over 60.

Conclusion

A broad spectrum of patients have registered providing a new insight into the FSHD population in the UK. The Registry aims to help in the planning and recruitment of research. Sharing a common dataset with a growing number of FSHD registries around the world will allow the registry to achieve this locally and internationally. The registry is well placed to inform future clinical research and help develop of standards of care.

32.

[P]

Testing the potential for comorbidity of FSHD with arhinia using inducibility of DUX4 expression in dermal fibroblasts

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Facioscapulohumeral muscular dystrophy type 2 (FSHD2) has complex genetics and epigenetics characterized by mutations in the SMCHD1 gene (structural maintenance of chromosomes flexible hinge domain containing 1) and at least one intermediate-sized D4Z4 array (~11-28 repeat units) on chromosome 4q in cis with an FSHD-permissive 4qA haplotype. These conditions allow for the pathogenic expression of DUX4 in skeletal muscle. Recently, mutations in SMCHD1 have also been observed in arhinia, a rare congenital disorder characterized by the lack of an external nose and often associated with eye and reproductive defects. Interestingly, muscular dystrophy or muscle weakness has not been previously associated with arhinia, despite the overlap of some SMCHD1 mutations between FSHD2 and arhinia. Herein, we aim to further understand the underlying mechanisms contributing to these two distinct clinical phenotypes caused by mutations in the same gene, and sometimes the same exact mutation. Our previous analysis showed the D4Z4 DNA methylation profiles of 4q chromosomes in arhinia are characteristic of those found in FSHD2, which prompted us to ask if FSHD2 exists in the arhinia population, or if arhinia patients might be at risk for developing FSHD2 as they age. To explore this potential for FSHD2 comorbidity among arhinia patients, we sought to determine if cells from arhinia patients express DUX4. Given that somatic expression of DUX4 is exclusive to skeletal muscle cells in FSHD, and since muscle biopsies are difficult to obtain, we collected skin samples from arhinia patients and

then used either MyoD overexpression or drug treatments known to induce a myogenic-like state to allow for DUX4 expression. This enabled us to assess if individual arhinia patients have the genetic and epigenetic conditions required to express DUX4 and potentially develop FSHD2. As a proof of concept, introduction of MyoD or the combined treatment with 5-azacytidine (ADC) and trichostatin A (TSA), which induces expression of endogenous MyoD in fibroblasts, induced expression of DUX4-fl mRNA in fibroblasts from FSHD2 patients, but not healthy control subjects. Interestingly, MyoD expression or ADC and TSA treatment also induced significant DUX4-fl mRNA expression in fibroblasts derived from arhinia patients with FSHD2 genetic and epigenetic profiles, but not from those with a non-permissive haplotype or from healthy relatives. Thus, we show that arhinia patients meeting the genetic and epigenetic criteria for FSHD2 can, in fact, express DUX4 in myogenic cells and therefore have the potential to develop FSHD

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[P]

Establishment of FSHD-PAX7 genetic reporter lines to study function of muscle stem cells in FSHD

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Facioscapulohumeral muscular dystrophy is one of the most common muscular dystrophy, and has a progressive symptom of muscle weakness. In FSHD patients, it is unclear which cell types express DUX4, and it is extremely difficult to find any DUX4 immunoreactive cells in human primary myoblast cultures. A recent study showed that Dux4 is transiently expressed during skeletal muscle regeneration, and Dux4 maintains Pax7 expression through transcriptional activation of target genes. Satellite cells give rise to a large pool of myoblasts that subsequently fuse and differentiate to produce the multinucleated myotube. So, we hypothesize that DUX4 can be expressed in PAX7 expressing cells of FSHD iPSCs, or at least PAX7 expressing cells should be the correct cell type to study FSHD molecular pathogenesis. We generated 'knock-in' PAX7::EGFP FSHDhiPSC and PAX7::EGFP control-hiPSC lines to isolate putative skeletal muscle stem/progenitor cells, followed by detailed cellular and molecular analysis. These approaches will give us new insights on FSHD disease mechanisms

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[P]

Modular platform for the myogenesis of human embryonic stem cells by using multiple genetic reporter lines

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Stem cell biology for genetic engineering and studying human development with human pluripotent stem cells (hPSCs), including embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), is providing unprecedented opportunity. The ability to generate skeletal muscle in vitro opens up new avenues for deciphering essential, but poorly understood aspects of skeletal myogenesis such as satellite cell formation and myoblast differentiation. We have recently developed a directed differentiation protocol for skeletal muscle induction of hESCs with two chemical compounds. In addition, we have generated four independent genetic reporter lines. During the differentiation with multiple genetic reporter lines, we uncovered find novel transcription factors to contribute to human embryonic myogenesis with time series gene expression profiles. Our comprehensive transcriptional analysis of hESC-derived myogenic specification process will provide a unique insight including defining discrete stages of in vitro myogenesis and novel transcription factors.

35.

[P]

Novel epigenetic mechanisms regulating DUX4 expression

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Compelling evidence indicates that DUX4 expression plays a key role in FSHD disease pathogenesis. We have identified novel nucleic acid regulatory elements, namely G-quadruplexes (GQs), in the DUX4 genomic locus and transcript using in silico analysis. Due to their characteristic structural and thermodynamic properties, GQs have become an attractive target in a number of other disease models and we have therefore examined the potential of a novel GQ-related therapeutic approach to knockdown abhorrent DUX4 expression as a potential treatment for FSHD. We have used state-of-the-art techniques to: (1) understand the function of GQs in each of the important elements involved in DUX4 expression, including enhancers, promoter, splicing and 3'UTR using an GQ-interacting small molecule; (2) pharmacologically test the most active GQ-modulating candidate in an FSHD animal model based on DUX4 gene transfer. We show that the promoter-associated GQ structures act to switch off the promoter, since transcript expression is significantly reduced upon treatment of FSHD patient cells with a GQ-stabilising ligand in a dose-dependent manner. This is accompanied by a dose-dependent decrease in the expression levels of the downstream genes (ZSCAN4, TRIM43 and MBD3L2), that are normally switched on by DUX4. This is borne out in a gene addition model of the disease where mice injected intramuscularly with AAVs overexpressing DUX4. When systemically treated with the GQ-stabilizing ligand, significantly reduced levels of DUX4 protein expression are seen that is associated with an improvement in muscle function. This would suggest that manipulation of GQs within the DUX4 gene could hold provide a novel therapeutic option for FSHD.

36.

[P]

Direct interaction of DUX4/4c with the multifunctional protein C1QBP

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DUX4 (**DoU**ble homeobo**X4**), the FSHD causal gene, is a nuclear transcription factor. Its overexpression in myoblasts induces a cascade of gene expression that interferes with many pathways leading to typical FSHD features such as muscles wasting, inflammation, decreased innate immune response, and oxidative stress. However, other FSHD muscle cell features are not yet understood such as its clusters of myonuclei and cytoskeletal defects. DUX4c, a DUX4 homologue, is identical in the first 342 amino acid residues but is 50-residues shorter than DUX4. Interestingly, DUX4c is expressed at low level in healthy muscles but greatly increased in FSHD ones. It could act as an FSHD modifier gene.

FSHD is a gain-of-function pathology. DUX4 expression is stochastic, has irreversible downstream effects and a short half-life. Therefore, the time window to target it is difficult. Current therapeutic developments focus on the inhibition of its expression or activity. However, in view of DUX4 pleiotropic effects, combinatory treatments acting at different level (similarly to tri-therapy in AIDS) might overcome the development or progression of the disease. Therefore, a better understanding of the impact of DUX4/4c overexpression, in healthy and pathological muscles is needed. For this purpose, our lab in collaboration with S. Harper's group, searched to identify DUX4/4c protein partners using several methods as well as different cell types, and surprisingly found a lot of RNA-binding proteins and also cytoplasmic proteins (Anseau et al., 2016). To further confirm the specificity of these interactions, we now used cross-linking and mass-spectrometry to covalently capture DUX4/4c bound to its protein partners. This new method also allows to define the quaternary structures of these protein complexes.

Our first results are in keeping with our previous published data. Moreover, we found that C1QBP (complement component 1Q subcomponent-binding protein), a multifunctional and multicompartamental protein (we already validated as a DUX4 partner, Anseau et al. 2016), interacts directly with DUX4c. Here, cross-links between C1QBP and DUX4c are found in two regions that are identical in DUX4. In addition, the observed C1QBP internal cross-links are in agreement with its known X-ray structure. C1QBP is involved in apoptosis, innate immunity, mRNA processing, mRNA splicing, ribosome biogenesis and transcriptional regulation.

As we postulate that DUX4 interferes with a normal role of DUX4c in skeletal muscle, a clear knowledge of the DUX4 or DUX4c interactome will help to find new perspectives for therapeutic strategies for FSHD without inhibition of muscle functions: either by the development of interfering molecules that suppress/decrease the DUX4 interactions involved in toxic pathways or by the use/ development of molecules specifically targeting DUX4-downstream pathways or combined therapies.

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37.

[P]

Overexpression of DUX-4 induces muscle Tregs: A potential role for the immune system in FSHD

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Muscular dystrophy includes asynchronous cycles of muscle degeneration and regeneration leading to chronic inflammatory responses that further damage muscle and impair regeneration. Facioscapulohumeral dystrophy (FSHD) is one of the most common dystrophy in adults with the primary cause of the pathology resulting from activation of a normally repressed retrogene called DUX-4. Although chronic inflammation is known to modulate muscle degeneration in certain forms of muscular dystrophy, there are few studies investigating the impact of the immune response on FSHD. In mouse models of Duchenne muscular dystrophy, the immune response plays a dual role by promoting injury through chronic activation of M1- like macrophages and facilitates muscle regeneration by inducing M2-like macrophages. Despite the recognition that the M1:M2 macrophage balance regulates the severity and progression of muscular dystrophy, it remains unclear what regulates this balance. Work from our lab and others recently revealed that Regulatory T cells (Tregs) modulate macrophage activation and suppress the severity of muscular dystrophy. Herein, the induction of FSHD, through overexpression of DUX-4 in skeletal muscle, revealed the existence of both M1- and M2-like muscle macrophage populations. Moreover, DUX-4 overexpression increased muscle Tregs, suggesting that activation of Tregs might be a response to injury to reduce muscle inflammation and promote regeneration. Our preliminary studies provide the premise for studying the role of the immune system in FSHD; understanding how Tregs suppress muscle inflammation will reveal pathways that may be targeted for novel and safer anti-inflammatory therapy for treating FSHD

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[P]

High-Density Lipoproteins protect against DUX4-mediated damage in a lentiviral model of FSHD

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Facioscapulohumeral muscular dystrophy 1 (FSHD1) is a skeletal muscle weakness and wasting disease caused by a genetic mutation on chromosome 4q35 combined with hypomethylation of the D4Z4 repeat region. This mutation and hypomethylation results in the expression of DUX4, a protein primarily responsible for the pathology of FSHD1, including increased oxidative stress, apoptosis and disturbed myotube formation. There is no cure and currently no effective therapy for FSHD1, however high-density lipoprotein (HDL) research has demonstrated antioxidant and anti-apoptotic properties, protecting against cellular damage in a variety of disease milieus. We hypothesised that HDL treatment may be effective against DUX4-induced oxidative stress and cell death in skeletal muscle. Lentiviral transduction results in chronic DUX4 expression where DUX4 can diffuse to nearby nuclei resulting in greater DUX4 expressing nuclei, a model of disease progression proposed for this disease. C2C12 cells were transduced with DUX4 under standard culture conditions and after 32 hours of exposure to DUX4 damage were treated with either 21 μ M HDL, or as control, 30 μ M low-density lipoprotein (LDL), for 16 hours. DUX4 was highly expressed in transfected cells, with no expression in control transfected cells (determined by mRNA and immunocytochemistry) and it was this expression of DUX4 which induced the oxidative stress phenotype. HDL, but not LDL, -treatment was able to alleviate DUX4-induced oxidative stress and cell death by 62.3% and 65.6%, respectively, whilst improving the formation of skeletal muscle myotubes. These results suggest that HDLs, or a HDL-based therapy could now be considered for development as a treatment to preserve and protect skeletal muscle in FSHD1 patients.

39.

[P]

Implication of SMCHD1 in D4Z4 epigenetic dynamics: lesson from iPSCs

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FacioScapulohumeral Dystrophy (FSHD) is characterized by the progressive loss of facial, scapulo-humeral and anterior forelegs muscles. This specific weakening correlates with methylation changes at the 4q35 locus. In most cases (FSHD1, 95%) hypomethylation of the D4Z4 macrosatellite is moderate and associated with a reduction of those repeats. In the majority of the remaining cases (FSHD2), there is no contraction of the repeats but a significant hypomethylation of the array along with mutations in *SMCHD1*. Interestingly, *SMCHD1* mutations have recently been involved in a drastically different pathology; the Bosma Arhinia Microphthalmia (BAMS). BAMS individuals present important cranio-facial abnormalities without any muscular dystrophies. These recent observations highlight the complexity of mechanisms at play in FSHD, and reveal the crucial need of characterization of *SMCHD1* function before offering therapeutic strategies for FSHD. To understand the involvement of *SMCHD1* in the etiology of FSHD and BAMS, we developed a strategy based on the use of human induced pluripotent stem cells (hiPSCs) from FSHD1, FSHD2 and BAMS patients. We followed the dynamic methylation of the D4Z4 array after reprogramming and muscle differentiation by Bisulfite modification of DNA combined to deep sequencing (BisSeq) together with DUX4 expression. Our approach reveals active remethylation of D4Z4 upon reprogramming followed by demethylation during differentiation. Methylation changes are moderate in FSHD2 and BAMS suggesting a role for *SMCHD1* in this process. D4Z4 methylation is not correlated with DUX4 expression. However, *SMCHD1* is not sufficient for the maintenance of the associated methylation in differentiated cells as shown upon somatic *SMCHD1* invalidation. Altogether our findings highlight the key role of *SMCHD1* in epigenetic features at the pluripotent stage. *SMCHD1* target genes identified by RNA-Seq will be discussed.

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[P]

Longitudinal study of Kinect-based upper extremity reachable workspace in FSHD

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Recently, Kinect sensor-based upper extremity reachable workspace (RWS) has shown promise as a useful outcome measure in FSHD. A three-dimensional (3D) motion sensor based measure of an individual's reachable workspace has provided both quantitative and finely-granular data with excellent reliability and validity when compared to other traditional outcome measures used in FSHD research.

Because of the slowly-progressive nature of the disease as well as the wide-spectrum of phenotype that can be seen in FSHD, development of an outcome measure sensitive enough to monitor disease progression over time and detect efficacy of potential therapeutics in clinical trials has been an important priority. Since FSHD presents with stereotypical shoulder girdle muscle weakness, proximal upper extremity range of motion is impaired, and subsequently the arm reachability function is also impacted significantly. Use of Kinect sensor to track arm motion, and then reconstructing an individual's reachable workspace has provided a potentially valuable clinical outcome measure that could be useful in monitoring disease progression as well as determining the effectiveness of therapeutics in future clinical trials.

Here, we further extend our initial work with Kinect reachable workspace outcome measure in FSHD by following a cohort of 18 FSHD subjects (7 women, 11 men) longitudinally over a period of 5 years. At each time point of clinical evaluation, reachable workspace, FSHD evaluation scale (as well as scapular and upper limb scores), and Brooke scale scores were assessed for each individual. At the start of study, majority of subjects were ambulatory (94.4%) with average total FSHD evaluation score of 8/15. We tracked the longitudinal changes in reachable workspace through linear mixed-effect statistical analysis for each quadrant (designated as Q1-Q4 representing upper medial, lower medial, upper lateral, and lower lateral quadrants respectively) as well as the total reachable workspace.

Overall, the total reachable workspace declined by approximately 4% over the duration of the follow-up, and this was not statistically significant ($p=0.144$). The reachable workspace change over time in the lower quadrants (Q2 and Q4) were likewise not statistically significant ($p=0.319$ and $p=0.334$, respectively). However, the upper lateral quadrant (Q3) reachable workspace contracted significantly by nearly 15% ($p=0.0001$), and the upper medial quadrant (Q1) reachable workspace declined by about 7%, approaching statistical significance ($p=0.063$). Both the Brooke and the FSHD evaluation scale scores did not show any significant change over the duration of the follow-up ($p=0.988$; $p=0.310$ respectively).

Perhaps not surprisingly, the reachable workspace in the upper quadrants declined most notably over the years rather than the lower quadrants, which remained essentially unchanged. However, the study demonstrates that in FSHD, the majority of arm reachability functional loss occurs in the upper lateral quadrant (Q3). This longitudinal natural history study of a small FSHD cohort found that Kinect-based reachable workspace outcome measure is capable of effectively monitoring the decline in upper extremity function over prolonged timeframe (years) and is also sensitive enough to track slowly-progressive upper extremity functional change that is often encountered in FSHD.

41.

[P]

A mapping study of SMCHD1 identifies the region of nuclear localization, dimerization, and protein cleavage

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DUX4 is abnormally expressed in skeletal muscle of facioscapulohumeral muscular dystrophy type 1 (FSHD1) and type 2 (FSHD2). SMCHD1 is responsible for the DUX4 expression as a disease modifier and a causative gene for FSHD1 and FSHD2, respectively. Although a variety of mutations in SMCHD1 have been identified in FSHD, how these mutations are different from wild-type SMCHD1 is poorly understood. To better understand the character of SMCHD1 in muscle cells, we performed a mapping study using lenti-viral vectors that express Flag-tagged full-length or different mutant SMCHD1. We identified regions of nuclear localization, dimerization between wild-type and mutant SMCHD1, and protein cleavage. Moreover, we compared DUX4 expression with these different mutants in FSHD myoblasts and confirmed that some mutants altered DUX4 expression. These findings could help to understand the difference between wild-type and mutant SMCHD1.

42.

[P]

DUX4 alters mRNA splicing of TDP-43 target genes

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Background. Facioscapulohumeral muscular dystrophy (FSHD) is caused by abnormal expression of the full-length DUX4-FL protein. Expression of DUX4-FL induces misexpression and alters the splicing pattern of genes involved in RNA processing, splicing, surveillance, and ubiquitination among others. Our published work demonstrated that DUX4-FL expression induces abnormal nuclear aggregation of TAR DNA-binding protein 43 (TDP-43), fused in sarcoma (FUS), and SC35 (SRSF2; arginine/serine-rich splicing factor 2). Such aggregation of RNA-binding proteins is usually indicative of loss-of-function and is found to contribute to pathogenesis of other neurological and neuromuscular disorders, including amyotrophic lateral sclerosis (ALS). The aim of this study was to determine if TDP-43 loss-of-function is induced upon DUX4-FL expression and thus could contribute to FSHD pathology.

Methods. We examined splicing patterns in genes that were identified by others as direct and potentially important targets of TDP-43 for pathology: FNIP1 (folliculin interacting protein 1), BRD8 (bromodomain containing protein 8), MADD (MAP kinase-activating death domain), and POLDIP3 (polymerase delta interacting protein 3). We isolated total RNA from human myotubes with or without BacMam-mediated expression of DUX4-FL and also from muscle biopsies obtained from FSHD and control donors. Splicing patterns were analyzed by RT-PCR.

Results. In DUX4-FL-expressing myotubes, we found increases in BRD8 exon 20 inclusion, POLDIP3 exon 3 exclusion, and FNIP1 exon 7 exclusion. Muscle biopsies from some FSHD patients also showed increases in FNIP1 exon 7 exclusion. These splicing alterations closely mimicked the splicing changes seen upon TDP-43 knockdown/loss-of-function in other cell types and are consistent with changes observed in patients with distal hereditary motor neuropathy and myofibrillar myopathy.

Conclusions. These results suggest that DUX4-FL expression affects splicing in TDP-43-regulated genes, possibly through TDP-43 loss-of-function. As a result, DUX4-FL-induced TDP-43 dysfunction could contribute to impaired RNA homeostasis, and contribute to the myopathology in FSHD.

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[P]

The FLExDUX4 transgenic mouse can be used to develop FSHD-like mouse models with pathophysiology ranging in severity

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Muscle weakness in facioscapulohumeral muscular dystrophy (FSHD) patients typically becomes noticeable in the second or third decade of life, followed by a progressive decline over one's lifetime. The aberrant expression of the DUX4 gene is the key mediator of all forms of FSHD and its expression correlates with presentation and severity. Thus, DUX4 expression is a prime target for therapeutic intervention and FSHD models should be based on DUX4. Previously, we reported the successful generation of the FLExDUX4 transgenic mouse, which contains a single copy inverted and floxed DUX4-fl transgene. When exposed to cre, the DUX4 transgene recombines into the sense orientation and expresses DUX4-fl under control of the ROSA26 promoter. Here we show that mating the FLExDUX4 mouse with the ACTA1-MerCreMer tamoxifen-inducible cre mouse produces double transgenic animals, a versatile highly tunable phenotypic FSHD-like mouse model, that can represent four levels of FSHD-like severity based on increasing DUX4 expression levels. Overall, the flexible design of this mouse model allows the investigator to initiate DUX4 expression in either young or adult animals as well as to control the degree of pathology and rate of disease progression, allowing for the assessment of prevention, inhibition, or reversal of pathology, as desired. Thus, the model can be used to distinguish early, initiating events in the pathogenic cascade from those cumulative, chronic or secondary effects. Importantly, these mice develop measurable pathology even with very low mosaic levels of DUX4 expression, similar with FSHD muscle, therefore can avoid potential artifacts associated with very high and non-physiological levels of DUX4 studied in other systems. In respect to preclinical testing of potential therapeutics, the model can essentially be tailored to the type of therapeutic being tested or pathway being studied. The FLExDUX4 line of cre-inducible DUX4-fl mice is available from JAX labs (Catalog #028710).

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[P]

Some problems connected with AD FSHD classification

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In my mind the problem with the classification of the AD facio-scapulo-humeral muscular dystrophy isn't fully worked out up to date. In modern articles and books on neuromuscular disorders in the chapter devoted to FSHD many authors wrote that this very muscular dystrophy is presented by an early affection of the face, interscapular and humeral muscles and sometimes later – of peroneal group (anterior tibial muscles). Thus, these authors present and describe inaccurately the facioscapuloperoneal muscular dystrophy, but not the facioscapulohumeral one which is a gradually descending type of MD.

As a rule in facioscapuloperoneal MD the upper arm muscles are not affected or slightly affected for a very long period of time. In some patients mild atrophy of the upper arm muscles predominate over their weakness. In these patients the quadriceps femoris, gluteus medius and in some extent iliopsoas muscles are usually preserved. During progression of the disease posterior thigh muscle, gluteus maximus and abdomen muscles become involved. Thus, our clinical, CT and MRI studies show that in the patients observed the lower limb muscles are widely involved. In this context the inaccuracy of the term “facio-scapulo-humeral” or “facio-scapulo-peroneal” muscular dystrophy becomes evident. That's why the name “facio-scapulo-limb muscular dystrophy, type 2 (FSLD2), a descending type with a “jump” with initial FSP or (F)SP phenotypes, autosomal dominant” would be more correct.

However, for the earlier clinical diagnosis of FSLD2 (the same disease as FSHD1 is quite possible) a typical pattern of the muscle weakness helps: slight asymmetrical atrophy of some portions of orbicularis oris muscle which is well marked in puckering the lips to whistle and /or the orbital part of the orbicularis oculi which is well marked in screwing up eyes; slight asymmetrical atrophy of the internal part of sternocostal portion of pectoralis major muscle is often seen as well as a slight local hollow in parasternal region; slight atrophy and weakness of the lower and middle parts of trapezius and serratus anterior muscles are seen at the level of lower angles and vertebral margins of scapulae, which slightly recede from the chest. The shoulders displacement downwards is evident, clavicles are posed horizontally and axial folds go atypically from outwards to the clavicle's midline. The presence of “spindle-shaped” forearms indicates a sign of atrophy or loss of the brachioradial muscle (by Duchenne, 1855). Atrophy of latissimus dorsi muscles is confirmed by weak coughing push which you can feel by hands. Slight weakness of tibialis anterior muscles is well marked if the patient cannot stand up fully at his one heel. All these early signs of FSLD2 the patient himself, his relatives and doctor don't note as a rule. Besides, the FSLD2 is characterized mostly by slow disease progression.

Then the question arises. Where is the classical gradually descending type of facio-scapulo-humeral muscular dystrophy with early affection of face, upper arm, shoulder girdle, trunk, abdomen, pelvic girdle, thigh muscles and at the late stage of the disease – peroneal group of muscles (not always). Duchenne de Boulogne called this MD a “Progressive hereditary muscular atrophy with fatty degeneration beginning with a face in adults or in youth or in children- 1855” and “Progressive hereditary fatty muscular atrophy with affection of some muscles of face, trunk and limbs in adults, children and in congenital period, hereditary - 1861”. The photography of these patients Duchenne presented in his famous books (1855, 1861, 1872) and in Album de photographies pathologiques -1862 (Fig.1, 2 and 16) [1-4].

Possible classification of FSLD

Facio-scapulo-limb MD type 2 (FSLD 2) or possibly the same disease as the facio-scapulo-humeral MD type 1 (FSHD1)

The form of MD	Inheritance	Chromosome, gene symbol, mutation
a. FSLD2 (FSHD1): descending with a “jump” from the face, shoulder girdle to tibialis anterior muscles with initial facioscapuloperoneal phenotype (Erb, Landouzy and Dejerine) (mild duration). The short name is the facioscapuloperoneal MD	AD	Chr. 4q35 DUX 4 gene, deletion D4Z4 in chromosome 4q35
b.FSLD2a (FSHD2 may be the same phenotype as in FSHD1)	AD	Chr. 18p11.32 SMCHD1 modifier gene, hypomethylation D4Z4 in chromosomes 4q & 10q
c. FSLD1 (FSHD3??): Classical gradually descending type with initial facioscapulohumeral phenotype (Duchenne de Boulogne) (severe duration). The short name is the facioscapulohumeral MD	AD	4q35? 10q26? Where is the gene?

Problem important!! May be this type of muscular dystrophy (FSLD1, a gradually descending type) doesn't exist at the present time as a nosological entity due to severe multiply mutations which get out this type of MD from the human population?!



Duchenne (de Boulogne). De l'électrisation localisee; 1st ed. Bailliere, Paris 1855.

The same patient M.B. presented as an Observation XXVIII, figures 57, 64 in 1855; figures 73, 74 in 1861 and observation LXXX, figures 124, 125 in 1872 [5, 6].

“Progressive fatty muscular atrophy with a very severe atrophy of the orbicularis oris muscle, an almost full absence of the pectoralis major, trapezius (all parts), rhomboideus, levator scapula and biceps brachii muscles on the left side and triceps brachii - on the right side. The brachioradialis muscles were absent on the both sides. Severe atrophy of the abdominal and trunk muscles, flexors and especially extensors muscles on the thighs. The lower legs and feet muscles were preserved.” (1-4).

In my opinion, this patient presents the classical hereditary gradually descending type of FSHD (the same disease as FSLD1).

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Kazakov V.M.

45.
[P]

Autologous stem cell treatment in FSHD. Preliminary report

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Two studies of treatments with autologous stem cells (SC) are reported, one compassionate and the other exploratory. A first compassionate study of SC treatment from the bone marrow in three patients with FSHD (2013-2015) indicated that the procedure is safe, and suggested signs of clinical improvement. A second exploratory clinical trial was initiated with 10 patients (only 5 have been treated so far), using the WHO evaluation system (ICF, International Classification of Functioning, disability and health); it found a reduction in average outcome score from 50.25 +/- 23.3 points to 34.75 +/- 16.5 points. Both studies suggest an improvement in the functionality of FSHD patients treated with stem cells.

46.

[P]

No evidence for altered incidence of Cancer in FSHD

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Introduction:

The recent findings that:

i) - the expression profile of FSHD myoblasts includes a significant enrichment for over-expression of cancer-related genes (Dimitriev *et al.* 2014),

ii) - over-expression of DUX4 in myoblasts from human or mouse can lead to upregulation of RET-signalling which is also seen in some cancer pathways, and can be blocked by the ant-cancer RTK (receptor tyrosine kinase) inhibitor-drug Sunitinib (Knight *et al* 2016).

....will raise the question for people with FSHD as to whether there could be any link between FSHD and cancer, and particularly whether the cancer incidence may be increased.

Until now, there have been only isolated case reports of the coincidence of tumours with FSHD (Kazakov 2009)(Yazici *et al* 2013). It is therefore helpful to look at this question more systematically in people and their relatives with FSHD.

Methods :

Detailed family history charts collected in the late 1980s during home visits to 182 people affected with FSHD from 33 families in the UK, have been reviewed, in order to abstract information on the causes of death in 163 deceased relatives reported at that time. The deaths were spread across the decades of the 20th century (up to 1990), although 70% had occurred in the latter 30years (1960-90).

Results :

Overall, initially classifying the 163 deceased relatives as 68 definite, probable or possible affected, vs. 95 definite or probable unaffected, cancer was reported as a cause of death in 19% (13/68) and 29% (28/95) respectively (Chi sq. = 2.25; p=0.13; n.s.), although there were 46 relatives where no cause of death (except in some cases debility from FSHD itself) was given. Also only 2 cases of cancer were recorded in then living family members: being 1 in 182 affected people seen (median age 34 yrs), and 1 in >130 unaffected family members or spouses seen.

Restricting analysis to the 102 deceased relatives where the affected or unaffected FSHD status seemed certain from the history, and where in each case a cause of death was reported by the family, revealed this to be cancer in 28% (8/29) of affected relatives, and in 38% (28/73) of unaffected relatives (Odds ratio 0.72; Chi sq. = 1.05, p=0.30 ; n.s.). The median age at death for each group was similar (57yrs vs. 55yrs respectively). This data can be compared with current UK national statistics (for 2016) showing that cancer accounts for 28% of all deaths in the UK.

Conclusion :

While this retrospective and rather ad hoc review may be subject to multiple different biases in ascertainment and accuracy of the data, it reassuringly finds no evidence to suggest any clinical or epidemiological association between FSHD and cancer, other than likely chance occurrence. The odds ratio of 0.72 (which also is not significant), equally cannot be taken to suggest a reduced incidence of cancer in FSHD given that the 28% rate matches the background population data.

47.

[P]

Development of LNA and 2'-MOE Gapmers to treat facioscapulohumeral muscular dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is caused by aberrant expression of DUX4 in muscles. DUX4 is normally expressed during early development and silenced after differentiation. Because ectopic expression of DUX4 induces FSHD in muscles, a promising therapeutic strategy is to decrease the activity of DUX4 in the patients. To accomplish this goal, we designed gapmers, antisense oligonucleotides composed of a central DNA segment flanked by locked nucleic acids (LNA) or 2'-O-methoxyethyl (2'-MOE) chemistry. Gapmers hybridize to target mRNAs and lead to cleavage of the RNA/DNA hybrid, which induces mRNA degradation by RNase H and reduces the targeted gene expression. We demonstrated that DUX4 mRNA expression was downregulated in the gapmer-treated immortalized FSHD patient-derived muscle fibers, with up to nearly 100% knockdown observed. Also, the expression of DUX4 downstream genes, ZSCAN4, TRIM43, and MBD3L2, was significantly decreased in the treated cells. These data indicate the promising potential our gapmers have for FSHD treatment. We confirmed that intramuscular injection of the gapmers reduced DUX4 expression in FSHD model mice. Further assessment of therapeutic efficacy and safety in vivo using FSHD mouse model will be conducted. In the long run, our study should help identify a drug candidate for FSHD therapy that could potentially enter human clinical trials.

48.

[P]

Xenografting human muscle stem cells into mice to study FSHD

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Aberrant expression of DUX4, a gene unique to humans and primates, causes Facioscapulohumeral Muscular dystrophy (FSHD), yet the pathogenic mechanism is unknown. Transgenic mouse and overexpression models have largely failed to reproduce the FSHD phenotype, therefore studies of endogenously expressed DUX4 have been limited to patient biopsies and myogenic cell cultures. Our laboratory developed a method to xenograft immortalized human muscle precursor cells from patients with FSHD and controls into the tibialis anterior muscle of immune-deficient mice, generating pure human muscle xenografts. We found that intermittent neuromuscular electrical stimulation increases myofiber size, quantity, and quality within xenografts. We also showed that FSHD cells mature into organized and innervated human muscle fibers, and also reconstitute the satellite cell niche within the xenografts. Recently, we modified the method to produce chimeric human/mouse xenografts to study genetic dysregulation by way of nuclear translocation of human proteins from human to mouse myonuclei. We are using these models to test the hypothesis that DUX4 overexpression is responsible for activating pathogenic gene targets which alter muscle physiology, inducing local muscle wasting, and leading to FSHD. Our results show that xenografts express DUX4 and DUX4-gene targets in a DUX4-dependent manner. Furthermore, the contractile protein composition of FSHD xenografts is distinctly different from controls. Finally, results from our cHMx show that several mouse genes are upregulated in response to DUX4 expression from human myonuclei, indicating that DUX4 induces a nuclear translocation cascade which may explain how relatively low expression of DUX4 causes the debilitating effects we observe in patients with FSHD.

49.

[P]

The role of estrogen regulation in FSHD-1

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Facioscapulohumeral muscular dystrophy-1 (FSHD-1) is the most prevalent muscular dystrophy affecting 1:8000, both children and adults. The disease is caused by partial deletion of 4q35 D4Z4 subtelomeric repeat that causes the activation of the DUX4 (double homeobox 4) locus, which eventually leads to misexpression of toxic DUX4 protein in skeletal muscles. DUX4 as a transcription factor has a DNA binding domain that is similar to transcription factors (TFs) of Paired Domain (i.e. Pax3, Pax7 and Pax6) and homeodomain classes (i.e. HOX). Recently, it has been shown that Pax3 and Pax7, when coexpressed with DUX4 in mouse myoblasts, rescue cell viability through competition for DNA binding sites. To monitor that process *in vivo* at the early stages of development, we have injected human DUX4 and zebrafish isoform Pax3a mRNAs into zebrafish embryos. We found that the zebrafish muscle phenotype worsened when Pax3a and DUX4 were coinjected, but we did not observe significant differences in zebrafish survival. This led us to hypothesize that DUX4 and Pax3 share the same target genes and that hyperactivation of those genes might disturb normal embryo development. To validate our hypothesis, we have performed ChIP-Seq experiments (analysis of DUX4 and Pax3a genomic occupancy *in vivo*) at 12h of embryo development and found 90 regions that DUX4 and Pax3a overlap for binding. Furthermore, we have discovered enrichment of the motif of the Estrogen Receptor-like (ER-like) TF DNA binding site at the DUX4 occupied sites (i.e., ChIP-seq summit regions), which concurs with the previously reported role of the Estrogen Receptor beta in FSHD. Taken together, we have shown here that the zebrafish model is a valuable tool to monitor the early stages of FSHD-1 development (which is not feasible in humans) and may complement well the research hypotheses driven from human or mouse studies.

50.
[P]

AAV.U7-snrRNA-mediated exon skipping of the toxic DUX4 gene as a promising therapeutic approach for facioscapulohumeral muscular dystrophy

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Facioscapulohumeral Muscular Dystrophy (FSHD) is an autosomal dominant disorder associated with progressive muscle wasting and weakness, typically in the face, shoulder girdle, and arms. However, this pattern of muscle involvement is not universal, and some patients may develop weakness in other muscles of the body, possibly leading to wheelchair dependence and caregiver assistance. There are currently no treatments that alter the course of FSHD and therapy development is an unmet need in the field. The pathogenic mechanisms underlying FSHD have only become clear during the last decade. FSHD arises from epigenetic changes that de-repress the *DUX4* gene in muscle. The full-length isoform of *DUX4* causes cell death and muscle toxicity, while a second isoform (*DUX4*-short; *DUX4*-s) is non-toxic. We hypothesize that FSHD therapies should therefore center on inhibiting full-length *DUX4* expression. In this study, we developed a *DUX4* exon-skipping strategy designed to bias *DUX4* splicing in favor of the non-toxic *DUX4*-s isoform. To do this, we designed several U7-snrRNAs targeting different parts of *DUX4* gene (called U7-*DUX4*) and demonstrated their ability to suppress fulllength *DUX4* and prevent cell death *in vitro*. To increase the efficiency of *DUX4* exon skipping, the most effective U7-*DUX4*s were chosen based on western blot and caspase assay, and then multiple copies were inserted into same pro-viral plasmid vector. As of this writing, additional studies are underway, including testing the increased efficiency of our new constructs to suppress *DUX4* long-term *in vitro*, making AAV-U7-*DUX4*s to investigate their capabilities to prevent *DUX4* toxicity in our new mouse model, and improve histopathological, functional, and molecular outcomes. We are also performing dose-escalation experiments for efficacy and toxicology outcomes. This study provides proof-of-concept for reducing *DUX4* toxicity using U7-snrRNA exon skipping, and has implications for future FSHD gene therapy, both as an individual treatment and combined with AAV.RNAi targeting of *DUX4*.

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Keywords: facioscapulohumeral muscular dystrophy, *DUX4*, U7-snrRNA, exon skipping, splicing, toxic, Muscle.

51.
[P]

Measurement of evidence of DUX4 as a proof of concept biomarker for FSHD clinical trials

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Facioscapulohumeral muscular dystrophy (FSHD) is the third most common muscular dystrophy. It is caused by mutations that lead to loss of repression at the D4Z4 macrosatellite repeats on chromosome 4 and expression of the homeobox transcription factor DUX4. Aberrant expression of DUX4 in skeletal muscle is the root cause of FSHD by activating an early embryonic developmental transcriptional program that leads to death of muscle fibers. We identified small molecule inhibitors that reduce expression of DUX4 and its downstream transcriptional gene program in human FSHD myotubes. Our goal is to identify a skeletal muscle biomarker to measure treatment-related reductions of DUX4 in skeletal muscle needle biopsies. For this, we developed highly sensitive assays to detect DUX4 protein and mRNA and used RNA sequencing and qPCR to assay the DUX4-regulated transcriptional gene program. DUX4 protein was measured with a sandwich electro-chemiluminescent immunoassay applied to human FSHD myotubes, skeletal muscle from DUX4 transgenic animals, and skeletal muscle needle biopsies from FSHD patients. *In vitro* we evaluated DUX4 protein and mRNA levels in response to a small molecule inhibitor of the DUX4 program. We also evaluated the impact of the small molecule inhibitor on the DUX4-regulated gene transcription program. The results showed that DUX4 protein and mRNA are present at very low levels in FSHD skeletal muscle and are difficult to quantitate in a reliable manner. In contrast, the DUX4-regulated gene transcript program can readily be measured in FSHD myotubes. We propose to measure DUX4-regulated target genes for the proof of concept molecular biomarker in skeletal muscle needle biopsies in the future clinical trial with the Fulcrum development candidate for FSHD.

52.

[P]

Self-report questionnaire vs. clinical evaluation form in the French National FSHD Registry: a statistical comparison

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Despite being one of the most prevalent dystrophies, Facioscapulohumeral dystrophy (FSHD) is an orphan disease in several respects. FSHD has no treatment and the natural history of the disease is not complete yet. In order to expand the knowledge on the disease, and develop standard of care, National registries on FSHD have been set up over the past ten years in Europe, Northern America, Australia and New Zealand. Data from most registries are gathered through medical evaluation, which relies on the willing participation of medical practitioners. On the other hand, some databanks are fed with data provided by patients themselves. However, as the English registry showed, answers provided by patients are sometimes unreliable. The French National Registry on FSHD combines both sources of data: a clinical evaluation form (CEF) filled by the neurologist and a self-report questionnaire (SRQ) completed by the patient (all forms are available on the registry webpage, fshd.fr). Aside from helping increase the number of patients included in the database, the strategy of the French registry has been devised so as to increase the quality of the data collected. Indeed, through a comparison between the two types of forms, the dual source allows for an evaluation of data accuracy. Such a statistical comparison between pairs of forms collected at close time points was thus made for 281 patients. The cohort has average age 59.5 and is divided into 131 women and 150 men. Kappa or ICC values were calculated to determine the correlation between answers provided in the CEF and the SRQ. The statistical analysis showed that answers related to a quantitative or objective content show a better agreement than questions involving feelings, interpretation or medical technicalities. The former are thus best answered by patients themselves: the lighter the questionnaire, the more probably the neurologist will use it. The disagreement observed in other questions can be explained by errors from one party or the other. As most of the questions concerned are medical questions, it is safe to assume that these questions should be best left to the medical practitioners. Patients naturally still provide a better answer when it comes to feelings. As a conclusion, patients provide a more accurate answer than medical practitioners in a number of cases. However, most medical questions should be avoided in an SRQ; although questions based on easily understandable objective criteria such as the Vignos scale are well answered by patients. The comparison highlights which questions are best answered by either the neurologist or the patient. Such results form the basis for tailoring an optimized collection form, where the questions are addressed to only one rater (the patient or the neurologist).

This work was supported by AFM.

53.

[P]

Set-up of an in vivo model of facioscapulohumeral muscular dystrophy (FSHD) based on human perivascular cells

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DUX4, the best candidate gene of FSHD, is specific of human cells with mouse models expressing variant forms of DUX family. Moreover, human and mouse muscle express a different pattern of hormone receptor. To study the muscle regenerative potential of FSHD individuals and the effect of hormones as well as other substances in this process, we set up an in vivo model based on human muscle precursor cells, perivascular cells or pericytes (PVCs). These cells when derived from intra-muscle blood vessel have the ability to differentiate in myotube.

We show that DUX4 is active in these cells and is able to induce its target genes and to impair muscle differentiation. Moreover, PVCs express an estrogen receptor pattern similar to that of human muscle cells. Accordingly, estrogens are able to reduce DUX4 transcriptional activity. Of importance, this reduction occurs only in differentiating PVCs whereas 17- β estradiol is ineffective towards DUX4 transcriptional activity during cell proliferation, thus recapitulating previous results obtained with myoblasts from FSHD patients. To evaluate PVCs survival, growth and differentiation, we analysed implants of PVCs in different districts of NSG mice: subcutaneous back, subcutaneous muscle and intramuscular. The results demonstrate that PVCs growth is similar in the different tissue districts whereas their muscle differentiation is increased when injected into injured muscle. Moreover, two different hydrogels were compared for *in vivo* PVCs implantation: a hydrogel consisting of photopolymerizable polyethylene glycol fibrinogen (PEG-FB) and the Matrigel a gelatinous protein mixture derived from Engelbreth-Holm-Swarm (EHS) mouse sarcoma. The PEG-FB resulted less able to sustain PVCs growth and differentiation.

Finally, we tested different markers to follow PVCs growth/survival and DUX4 activity. Cherry signal resulted the more stable over time whereas luciferase signal under the promoter of ZSCAN gene resulted more effective in monitoring DUX4 activity when compared to RFLP4b gene.

Overall, these experiments have allowed to set up a model that will be used to monitor in vivo DUX4 function in muscle regeneration and to test the effect of different factors on it.

54.
[P]

Evaluation of FSHD1 testing in diagnostics using FiberVision Molecular Combing technology

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The molecular genetic basis of Facio Scapulo Humeral muscular dystrophy (FSHD) is complex. FSHD type 1 (FSHD1), accounting for 95% of FSHD cases, results from a contraction of the macrosatellite repeat D4Z4 in the subtelomeric region of chromosome 4q35. The D4Z4 contraction is only pathogenic when it segregates in cis with the permissive 4qA allele on chromosome 4. This particular 4qA haplotype harbours a polyadenylation (poly-A) sequence which is required to produce stable DUX4 messenger RNA.

Molecular genetic diagnostic testing of FSHD1 requires the length measurement of the D4Z4 repeat region on the tip of chromosome 4q35 and is performed in most labs using DNA restriction enzyme (RE) digestions and subsequent Southern blot technology. Affected individuals, possess 1–10 D4Z4 repeats, yielding DNA digested fragments 10–38 kb in size. Non-pathogenic D4Z4 repeats are also present on the tip of chromosome 10, therefore specific RE-digestions are required to distinguish repeat lengths between chr.4 and chr.10. Measurement of the size of D4Z4 repeat array and its contraction on chromosome 4q35 forms the basis for genetic testing in FSHD. Considering, beforehand the diagnosis FSHD is established by an expert neurologist, most diagnostic labs routinely measure the D4Z4 repeat sizes on chromosome 4 without determining the A or B allelic variants. However D4Z4 repeat sizes ranging from 1 to 10 that lack the permissive 4qA allele do occur in the general healthy population (>1%), indicating the limitation and a potential misdiagnosis (in case of a less pronounced FSHD phenotype) by this method.

FiberVision® Molecular Combing technology from Genomic Vision is a different method that does allow the detection of both the D4Z4 repeat length and a permissive 4qA haplotype in cis in one assay. The locus specific Fiber probes are designed to characterize the 3.3kb-D4Z4 repeat-containing loci on chromosomes 4 and 10 by fluorescent hybridization on combed DNA extracted from blood samples. Considering the combined testing possibility for both D4Z4 repeat length and the 4qA allele and the further improved assay set up and dedicated software application of this method, we evaluated the analytical performances of this FiberVision® Molecular Combing method using 40 controls and 40 true positive FSHD1 samples in a blind study and also compared the detection accuracy with the Pulsed field gel electrophoresis (PFGE) results. The latter method being more accurate than the Southern blot approach in detecting all D4Z4 repeat allele lengths on chromosome 4 and 10, including also hybrids and mosaic alleles, and the presence of the 4qA haplotype per sample. An overview of the results and the performance, including diagnostic accuracy of the Molecular Combing method will be presented.

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