FSH Society Facioscapulohumeral Muscular Dystrophy [FSHD]
2015 International Research Consortium & Research Planning Meetings

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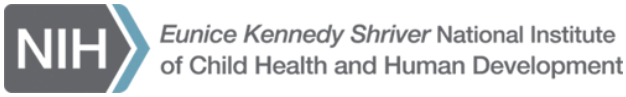

FSH Society Facioscapulohumeral Muscular Dystrophy [FSHD]
2015 International Research Consortium & Research Planning Meetings

Monday, October 5, 2015
8:00 a.m. – 6:00 p.m.
[Registration and breakfast begins 8:00 a.m.-]

Tuesday, October 6, 2015
8:00 a.m. – 1:30 p.m.
[Registration and breakfast begins 8:00 a.m.-]

The Westin Boston Waterfront Hotel, Marina Ballroom
425 Summer Street, Boston, MA 02210 USA

Co-Chairs: **David E. Housman**, PhD
Massachusetts Institute of Technology, Cambridge, Massachusetts
Stephen J. Tapscott, MD, PhD
Fred Hutchinson Cancer Research Center, Seattle, Washington
Silvère van der Maarel, PhD
Leiden University Medical Center, Leiden, the Netherlands

Organizers: **Daniel Paul Perez**
FSH Society, Lexington, Massachusetts
David E. Housman, PhD
Stephen J. Tapscott, MD, PhD
Silvère van der Maarel, PhD

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Muscular Dystrophy Campaign United Kingdom
NIH Eunice Kennedy Shriver NICHD Senator Paul D. Wellstone MDCRC for FSHD at University of Massachusetts Medical School
Regeneron Pharmaceuticals
Sarepta

PREFACE

October 5, 2015
Boston, Massachusetts

Dear Colleagues,

Welcome to the FSH Society 2015 International Research Consortium & Research Planning Meetings!

This workshop brings together clinicians, scientists, industry, patient representatives and policy makers to discuss the latest developments in facioscapulohumeral muscular dystrophy (FSHD). For twenty years this gathering has provided the FSHD community with a forum to present and discuss new findings, reinforce collaborative efforts, facilitate new initiatives, and coordinate research and clinical activities.

Impressive scientific progress has been made in recent years and months in our understanding of the disease. This is a critically important time for the community to convene and discuss new data and advances in FSHD; discuss strategies to verify and independently corroborate the findings; discuss focusing efforts and resources in the preclinical gap and translational phase of research; improve diagnostic techniques and criteria for FSHD; and consider and evaluate with industry how to move forward with new and existing therapies for the disorder.

Over the two days, we will revisit the priority areas identified at last year's meeting, and discuss what we have achieved, evaluate the gaps that need addressing, and where we need to focus and invest intellectual, scientific and financial resources. By the end of day two we should be able to identify whether any of last year's priority areas should change or be modified, and outline a new list/set of priority areas to be considered.

This meeting is organized by the FSH Society and sponsored by Acceleron Pharma, Association Française Contre les Myopathies (AFM), aTyr Pharma, BioMarin Pharmaceutical, Facio-Therapies, FSHD Canada, FSH Society, FSHD Global Research Foundation, Genomic Vision, Genzyme, a Sanofi Company, Idera Pharma, Muscular Dystrophy Association United States (MDAUSA), Muscular Dystrophy Campaign United Kingdom, NIH Eunice Kennedy Shriver NICHD Senator Paul D. Wellstone MDCRC for FSHD at University of Massachusetts Medical School, Sarepta, and, Regeneron Pharmaceuticals. We thank our sponsors for their generous financial support.

It is truly a pleasure to come together to accelerate solutions for FSHD. Thank you for your extraordinary efforts and hard work on behalf of patients and their families.

Sincerely,

David E. Housman, PhD
Massachusetts Institute of Technology, Cambridge, Massachusetts & FSH Society Scientific Advisory Board

Stephen J. Tapscott, MD, PhD
Fred Hutchinson Cancer Research Center, Seattle, Washington

Silvère van der Maarel, PhD
Leiden University Medical Center, Leiden, the Netherlands

Daniel Paul Perez
FSH Society, Lexington, Massachusetts

Monday, October 5, 2015

Registration & Breakfast	8:00 a.m.-8:30	
Welcome	8:30-8:40	**Welcome** David Housman, Daniel Perez, Stephen Tapscott, Silvere van der Maarel
Review of 2014	8:40-9:00	**Review of 2014/2015 priorities** as stated by FSHD workshop in 2014 Moderators: Michael Altherr, Stephen Tapscott
Platform Session 1	9:00-10:15	**Clinical Studies; Genetics & epigenetics** (5x15 minutes) Moderators: Silvere van der Maarel, Jeffrey Statland
	10:15-10:45	Discussion
Break	10:45-11:00	
Platform Session 2	11:00-12:15	**Molecular mechanisms** (5x15 minutes) Moderators: Charles Emerson, Jr., Alberto Luis Rosa
	12:15-12:45	Discussion
Poster Introductions & Lunch	12:45-2:00	Lunch and **Poster Viewing** [collect and have **Lunch**]
Platform Session 3	2:00-3:30	**Models** (6x15 minutes) Moderators: David Housman, Yi-Wen Chen
	3:30-4:00	Discussion
Break	4:00-4:15	
Platform Session 4	4:15-5:00	**Therapeutic studies** (3x15 minutes) Moderators: Louis Kunkel, Michael Kyba
	5:00-5:30	Discussion
Assembly Session	5:30-6:00	**Discussion and Review of Tuesday Agenda** Moderators: David Housman, Stephen Tapscott, Silvere van der Maarel, Michael Altherr, and Daniel Perez
Adjourn	6:00 p.m.	

The FSH Society (Facioscapulohumeral Muscular Dystrophy) is an independent, non-profit 501(c)(3) and tax-exempt U.S. corporation organized to address issues and needs specifically related to facioscapulohumeral muscular dystrophy (FSHD). Contributions and financial donations are acknowledged for tax purposes. All inquiries should be addressed to: FSH Society, Daniel Paul Perez, 450 Bedford Street, Lexington, MA 02420. Phones: (781) 301-6650 and (781) 275-7781, fax: (781) 862-1116, e-mail: daniel.perez@fshsociety.org, website: http://www.fshsociety.org

Tuesday, October 6, 2015

Registration & Breakfast 8:00 a.m.-8:30

Welcome 8:30-8:35 **Welcome**
David Housman, Daniel Perez, Stephen Tapscott, Silvere van der Maarel

Discussion/Planning 8:35-12:30 **International "lab meeting"**

Planning and problem solving sessions

Moderated discussion sessions with entire group of attendees based on data presented at day 1. Co-chairs and organizers will meet Tuesday morning before the session to help identify specific topics of interest to lead the discussion around. The goals are to 1.) help identify and troubleshoot bottlenecks; and, 2.) define the research/clinical priorities for the next year 2015/2016.

8:35-12:30 **3 Discussions**
Overall Moderators: Michael Altherr, David Housman, Stephen Tapscott, Silvere van der Maarel

8:35-10:15 **Discussion 1 aimed at educating FSHD community on issues important to industry**
Moderators/panel (may change slightly): Bruce Wentworth, Melissa Ashlock, David Dasberg, Ken Attie, Tim Miller, and others
Possible topics Issues that come to mind:
Important points to consider in academic-industry collaborations.
Drug targets and validating drug targets.
Importance (or lack thereof) of animal models.
Clinical trial design: measures of efficacy, Phase 2 vs. Phase 3.
Natural History.
Therapeutic Index.
Opportunity to restore muscle health (in addition to disease stabilization) and ways to know in a clinical trial.
Biomarkers.
Uniform Clinical Assessment.

10:15-10:45 **Discussion 2 on FSHD Champions Initiative**
Moderators/panel: June Kinoshita, Gregory Block
10 minute presentation, and then 20 minutes Q&A to encourage the audience to let the Champions know what they need and what works and does not.

10:45-12:30 **Discussion 3 to identify and troubleshoot bottlenecks; and, define the research/clinical priorities going forward**
Moderators: Michael Altherr, David Housman

12:30-12:45 **Finalizing listing of items, areas and priorities**

Lunch 12:45 -1:30 p.m.

Adjourn 1:30 p.m.

1:30-6:00p.m. **Champions Annual Meeting** [for the Champions group only]
Carlton Room (On the Conference Level -- one level up from the Lobby Level)

NOTES ON TALKS AND POSTERS

	First Author	Presenting Author	Topic
Platform Session 1			
Clinical Studies & Genetics and Epigenetics Chairs: Silvere van der Maarel, Jeffrey Statland			
9:00 – 9:15 a.m.	Owen	Owen	DNA Combing as a First-Tier Genetic Testing
9:15 – 9:30 a.m.	Eichinger	Eichinger	6MWT as an Outcome Measure
9:30 – 9:45 a.m.	Heatwole	Heatwole	Reliably Measuring Patient-Reported Disease Burden
9:45 – 10:00 a.m.	Lemmers	Lemmers	Hemizygosity for SMCHD1 in FSHD2 conseq. 18p CNV
10:00 – 10:15 a.m.	Calandra	Deidda	Advances in the development of a methylation assay
10:15 – 10:45			Discussion
Platform Session 2			
Molecular mechanisms Chairs: Charles Emerson, Jr., Alberto Luis Rosa			
11:00 – 11:15 a.m.	Ansseau	Ansseau	Translocation of DUX4 and DUX4c during myoblast diff
11:15 – 11:30 a.m.	Banerji	Banerji	PAX3/7 target gene repression is a hallmark of FSHD
11:30 – 11:45 a.m.	Eidahl	Eidahl	Protein Chemistry & Protein-Protein Interactions DUX4
11:45 - Noon a.m.	Knopp	Zammit	DUX4 inhibits satellite cell myogenesis by inducing
Noon – 12:15 a.m.	Jagganathan	Jagannathan	Proteomic DUX4-expressing muscle cells reveals
12:15 – 12:45 a.m.			Discussion
Platform Session 3			
Models Chairs: David Housman, Yi-Wen Chen			
2:00 – 2:15 p.m.	de Ryhove	Belayew	D4Z4-Encoded Proteins in the Osteogenic Diff.
2:15 – 2:30 p.m.	Hall	Chamberlain	Immunodetection of DUX4 protein protein expression
2:30 – 2:45 p.m.	Zhang	Zhang	DUX4-targeting antisense therapy dev human xenogra
2:45 – 3:00 p.m.	Eidahl	Giesige	Func. overlap between mouse Dux and human DUX4
3:00 – 3:15 p.m.	de Greef	de Greef	Haploinsufficiency of Smchd1 exacerbates
3:15 – 3:30 p.m.	Kyba	Kyba	Cell and animal models for studying DUX4 in FSHD
3:30 – 4:00 p.m.			Discussion
Platform Session 4			
Therapeutic Studies Chairs: Louis Kunkel, Michael Kyba			
4:15 – 4:30 p.m.	Marsollier	Marsollier	Targeting 3'end elements involved in mRNA
4:30 – 4:45 p.m.	Wallace	Wallace	Toxicology for DUX4-targeted microRNAs
4:45 – 5:00 p.m.	Sverdrup	Sverdrup	BET Protein Inhibition as a Therapeutic Strategy
5:00 – 5:30 p.m.			Discussion
Posters [	Block	Block	Global Initiatives to Accelerate FSHD Research
	Chen	J. Chen	Antisense oligo-dependent knockdown
	Chen	YW Chen	Reduction of circulating glutathione peroxidase
	Choi	Choi	Human FSHD myogenic cell model from iPSC
	DeSimone	Desimone	FSHD-modifying multifunctional C1QBP protein
	Gatica	Rosa	Nuclear DUX4 corepressor activity progesterone NR
	Glasser	Attie	Phase 1 dose escalation study of ACE-083
	Hamanaka	Hamanaka	Features of FSHD2 with SMCHD1 mutation in Japan
	Homma	Homma	New view of DUX4-induced pathology encompasses
	Kazakov	Kazakov	Relationship between FSHD1 and FSL type 2
	Lek	Lek	Genome-scale CRISPR knock-out screen
	Shi	Shi	iPSC model of FSHD myogenesis
	Statland	Statland	An Instrumented Timed Up and Go in Patients
	Udaka	Udaka	Combined single fiber physiology and proteomic]

Priorities as stated by FSHD Research Community for FSHD Research in 2015 at the 2014 FSH Society FSHD International Research Consortium, held October 17-18, 2014 in San Diego, California

Priorities Defined For 2015
As defined by the FSHD clinical and research community

I. Genetics

The vast majority of clinically diagnosed FSHD patients can be genetically classified as FSHD1, due to D4Z4 repeat contraction on chromosome 4, or FSHD2, due to mutations in the SMCHD1 gene on chromosome 18. Other forms of FSHD2 may also exist as <15% of FSHD2 cases cannot be explained by SMCHD1 mutations. Both forms converge to a common molecular pathway characterized by D4Z4 repeat chromatin relaxation and DUX4 expression in amongst other muscle. It was discussed as to whether this is the operational definition of FSHD and a consensus arrived at. It was agreed on that there are rare FSHD-syndromes possibly without these epigenetic and molecular hallmarks and that maybe caused by other genes and mechanisms – it is known that mutations in various muscular dystrophy genes can yield FSHD-like symptoms. It was considered important to collect and carefully characterize these patients clinically and genetically. Samples could be handled by the U.S. NIH Wellstone and Fields Centers. To facilitate access to information on FSHD mutations, it was recommended to submit data to the Leiden Open Variation Database (LOVD) mutation database, hosted by Leiden (http://www.lovd.nl/3.0/home/ curator Dr. Richard Lemmers). The options to include relevant clinical data will be explored.

II. Mechanisms and targets

The discussion focused on the mechanism of DUX4 expression (bursts), including up-and downstream steps. Although there is much evidence for stochastic expression bursts of various genes, (muscle-specific) factors may specifically facilitate bursts of DUX4 in adult muscle - normally expressed only in embryonic tissue. The significance of DUX4-inducedlink with apoptosis is not understood, though it has been shown that the RNA (and protein) spreads to multiple nuclei in the same fiber. Why FSHD preferentially affects skeletal muscle, whether reflecting the DUX4 expression or toxicity, is particularly toxic in muscle is poorly understood and in need of further work as it might reveal interesting intervention targets.

III. Models

During the past several years, various models have been generated, most of them focusing on DUX4. In the past year, the most intriguing ones are: 1). the long-awaited inducible mouse model. This model shows low expression through leakage in the un-induced state, leading to lethality. No muscle phenotype was detected. 2). Various virus viral delivery-based models have been reported. Depending on amongst other the delivery system, these models can produce burst-like expression patterns of small numbers of myonuclei expressing DUX4. 3). Two labs reported on the generation of stem cells, embryonic and induced ones. Although in an early stage, this approach might prove very interesting also for fundamental studies on DUX4 and chromatin structure.

Priorities Defined For 2015
As defined by the FSHD clinical and research community (continued)

IV. Patients - trial preparedness

For FSHD phenocopies (non-D4Z4 or SMCHD1 mutated) all agreed that differential diagnosis has to be ruled out (by muscle biopsy and genetics) and it was advised to put these cases in an international repository. On trial readiness the audience suggested to reach for a worldwide agreement on a severity score and a standpoint on the FSH-com and a patient reported outcome in order to be able to compare clinical trials more easily. Other suggestions that were briefly discussed: 1). to study which methylation assay would separate best patients from controls, 2). the need for more groups to study biomarkers in order to select the best ones for follow-up of patients, 3). more studies on Electrical Impedance Myography (EIM) are needed to find the shortest time interval to demonstrate significant changes in muscles, and 4). integration of MRI in the clinical discussions of FSHD is needed.

	first author	presenter	topic	assignment
1.	Owen	Owen	DNA Combing as a first-tier test	spoken
2.	Eichinger	Eichinger	6MWT as an outcome measure	spoken
3.	Heatwole	Heatwole	Reliably measure patient-reported	spoken
4.	Lemmers	Lemmers	Hemizygosity SMCHD1 18p CNV	spoken
5.	Calandra	Deidda	Advances in the methylation assay	spoken
6.	Ansseau	Ansseau	Translocation DUX4 / DUX4c	spoken
7.	Banerji	Banerji	PAX3/7 target gene repression	spoken
8.	Eidahl	Eidahl	Protein-Protein Interactions DUX4	spoken
9.	Knopp	Zammit	DUX4 inhibits satellite myogenesis	spoken
10.	Jagganathan	Jagannathan	Proteomic DUX4-expressing reveals	spoken
11.	de Ryhove	Belayew	D4Z4-Encoded proteins osteogenic	spoken
12.	Hall	Chamberlain	Immunodetection of DUX4 protein	spoken
13.	Zhang	Zhang	DUX4 antisense xenograph	spoken
14.	Eidahl	Giesige	Mouse Dux and human DUX4	spoken
15.	de Greef	de Greef	Haploinsufficiency of Smchd1	spoken
16.	Kyba	Kyba	Cell and animal models for DUX4	spoken
17.	Marsollier	Marsollier	Targeting 3'end elements mRNA	spoken
18.	Wallace	Wallace	Toxicology DUX4-target microRNAs	spoken
19.	Sverdrup	Sverdrup	BET Protein Inhibition as strategy	spoken
20.	Block	Champions	Global initiatives to accelerate	poster
21.	Chen	J. Chen	Antisense oligo-dep. knockdown	poster
22.	Chen	Y.W. Chen	Reduction glutathione peroxidase	poster
23.	Choi	Choi	Myogenic cell model from iPSC	poster
24.	DeSimone	Desimone	C1QBP-targeting Inhibits DUX4	poster
25.	Gatica	Rosa	DUX4 corepressor progesterone	poster
26.	Glasser	Attie	Phase 1 study of ACE-083	poster
27.	Hamanaka	Hamanaka	Genetic FSHD2 SMCHD1 Japan	poster
28.	Homma	Homma	New view of DUX4-induced path	poster
29.	Kazakov	Kazakov	FSHD1 and type 2	poster
30.	Lek	Lek	Genome-scale CRISPR knock-out	poster
31.	Shi	Shi	iPSC model of FSHD myogenesis	poster
32.	Statland	Statland	Instrumented timed up and go	poster
33.	Udaka	Udaka	Single fiber physiology proteomic	poster

1. **DNA combing as a first-tier genetic testing for facioscapulohumeral dystrophy type 1: A cohort of 155 patients**

R Owen[1], F.Z. Boyar1, J.C. Wang[1], X.J. Yang[1], B.H. Nguyen[1], V. Sulcova[1], P. Chan[1], Y. Liu[1], A. Anguiano[1], C.M. Strom[1]

[1] Quest Diagnostics Inc., Nichols Institute, San Juan Capistrano, California 92690, USA
[2] Quest Diagnostics Inc., Athena Diagnostics, Worcester, Massachusetts 01605, USA

Facioscapulohumeral dystrophy (FSHD) is the third most common muscular dystrophy. Type-1 FSHD is due to a contraction of the D4Z4 macrosatellite repeat motif on the 4qA allele. Shortening of 4qB, 10qA or 10qB allele is not associated with FSHD. DNA combing analysis hybridizes multi-color DNA probes onto uniformly stretched DNA fiber and accurately identifies the 4qA and other alleles. In this cohort of 155 patients, 147 (95%) had successfully generated enough DNA fibers for analysis (including two cases with mosaicism). The average number of DNA fibers obtained for each patient was 54 (~13 for each allele). A total of 62 cases (positive rate = 42%) had either abnormal (51 cases, equal or less than 8 repeats at 4qA) or borderline results (11 cases, 9-11 repeats). For abnormal cases with 2-4 repeats at 4qA, the average age was 29 years old when referred to us for analysis. In contrast, the cases with 5-8 repeats at 4qA were 53 years of age on average. Among the 85 cases with normal result, 35 (41%) had either contracted 4qB (7 cases) or shortened 10qA allele (28 cases). Based on the results of this cohort, we conclude that DNA combing is recommended to be offered as the first-tier genetic testing for FSHD because of its accuracy of identifying the repeat number of 4qA allele, and capability of detecting FSHD1 rearrangements and mosaicism. Furthermore, the precise measurement of the D4Z4 repeat motif by DNA combing may help correlating the size of the contracted 4qA allele with the timing of FSHD1 onset.

2. **The 6MWT as an outcome measure in FSHD**

Katy Eichinger, PT, DPT, NCS[1], Rabi Tawil, MD[1], Chad Heatwole, MD, MS-CI[1], Susanne Heininger, RN, BS, BSN[1], Jeffrey Statland, MD[2]

[1] Neuromuscular Disease Unit, Department of Neurology, University of Rochester Medical Center, Rochester, New York USA
[2] Department of Neurology, University of Kansas Medical Center, Kansas City, Kansas USA

Background: The 6 Minute Walk Test (6MWT) is an outcome measure used in therapeutic trials in various neuromuscular disorders. Here we determine the test-retest reliability, variability, and the relationship to known disease severity measures of the 6MWT in genetically defined and clinically affected individuals with facioscapulohumeral dystrophy (FSHD).

Methods: Individuals with FSHD participating in studies at the University of Rochester performed measures of mobility and strength at baseline, 1-3 weeks, 6 months and 12 months. The 6MWT, the Timed Up and Go and the 30' go test were performed as measures of mobility. Strength was assessed using standard manual muscle testing procedures. Reference equations were used to calculate the percent predicted distance walked during the 6MWT. Regression analysis was used to assess the independent variables contributing to the variance in the 6MWT distance.

Results: 33 individuals (64% male) with a mean age of 52.8 (range= 22-68) performed mobility and strength measures. The mean distanced walked during the 6MWT was 382.8 (range= 61.3-653.7; standard deviation (sd) = 134.7). The interclass correlation coefficient (ICC) for the 6MWT was 0.99. The 6MWT was correlated with lower extremity strength ($r=0.789$; $p<0.0001$) the 30' go ($r= 0.951$; $p<0.0001$) and the FSHD Clinical Score ($r=0.696$; $p<0.001$). The mean percent-predicted distance walked during the 6MWT was 66%. When age and gender were controlled for, lower extremity strength accounted for 47% of the variation in the distance walked during the 6MWT.

Conclusions: The 6MWT is a reliable measure of functional capacity for individuals with FSHD. Individuals with FSHD walk shorter distances during the 6MWT than predicted by normative data. Lower extremity strength is a significant contributor to the distance walked during the 6MWT. Assessing the sensitivity of the 6MWT will be important in determining its utility of as an outcome measure for future trials.

3. **Reliably measuring patient-reported disease burden: An update of the performance of the facioscapulohumeral muscular dystrophy health index (FSHD-HI)**

Chad Heatwole[1], MD, MS-CI, Kate Eichinger, PT, DPT, NCS[1], Susanne Heininger,RN,BS,BSN[1], Nuran Dilek, M.S[1], Bill Martens, BA[1], Mike McDermott, PhD[1], Rabi, Tawil, MD[1], Nicholas Johnson, MD[2], Richard T. Moxley, III, MD[1], Jeffrey Statland, MD[3]

[1] Neuromuscular Disease Unit, Department of Neurology, University of Rochester Medical Center, Rochester, New York USA
[2] Departments of Neurology, Pediatrics, and Pathology, University of Utah, Salt Lake City, Utah USA
[3] Department of Neurology, University of Kansas Medical Center, Kansas City, Kansas USA

Introduction: Responsive, reliable, valid, and clinically relevant outcome measures are required to conduct therapeutic trials involving FSHD patients. The FSHD-HI is a disease-specific patient-reported outcome measure developed using input from 238 FSHD patients. The FSHD-HI measures overall disease-burden and includes 14 subscales that individually measure the areas of health reported by FSHD patients to have the greatest impact on their lives.

Methods: We evaluated the test-retest reliability of the FSHD-HI and each of its subscales. Participants completed the FSHD-HI at baseline and subsequently within 3 weeks. Reliability testing of the FSHD-HI was performed as an aim of the Clinically Relevant Outcome Measures for FSHD (CROMFiSH) study at the University of Rochester. Our interim analysis included the first 22 (of 40) participants to complete the FSHD-HI in the CROMFiSH study.

Results: Each FSHD participant serially completed the FSHD-HI. The test-retest reliability of the total FSHD-HI was high with an intraclass correlation coefficient (ICC) of 0.945. Of the FSHD-HI subscales, the highest ICCs were demonstrated with the pain subscale (0.934), the mobility and ambulation subscale (.914), the hand and finger function subscale (.902), and the fatigue subscale (.887).

Conclusion: The FSHD-HI is a reliable disease-specific outcome measure designed using extensive FSHD patient input. Ongoing studies will provide additional information regarding the responsiveness, minimal clinically important difference, and known group validity of the FSHD-HI and its subscales in the FSHD population.

4. **Hemizygosity for SMCHD1 in FSHD2 and the consequences of 18p CNV**

Richard J.L.F. Lemmers[1], Judit Balog[1], Patrick J. van der Vliet[1], Marlinde L. van den Boogaard[1], Colleen M. Donlin-Smith[2], Sharon P. Nations[3], Claudia A.L. Ruivenkamp[4], Patricia Heard[5], Stephen Tapscott[6], Jannine D. Cody[5], Rabi Tawil[2] and Silvère M. van der Maarel[1]

[1] Department of Human Genetics, Leiden University Medical Center, Netherlands
[2] Neuromuscular Disease Unit, Department of Neurology, University of Rochester Medical Center, Rochester, New York USA
[3] Department of Neurology, University of Texas Southwestern Medical Center, Dallas, Texas USA
[4] Department of Clinical Genetics, Leiden University Medical Center, Netherlands
[5] University of Texas Health Science Center at San Antonio, Chromosome 18 Research Center, San Antonio, Texas USA
[6] Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington USA

Facioscapulohumeral muscular dystrophy type 2 (FSHD2) is characterized by D4Z4 CpG hypomethylation and is most often caused by heterozygous mutations in the chromatin modifier structural maintenance of chromosomes flexible hinge domain-containing protein 1 (SMCHD1). SMCHD1 is most likely involved in the maintenance of D4Z4 methylation and the gene encoding SMCHD1 maps to chromosome 18p at 3 Mb from the telomere. In approximately 15% of the FSHD2 families we could not identify a mutation in the SMCHD1 gene. Additional genetic studies identified two FSHD2 families with a 1.2 Mb deletion encompassing five genes, amongst which SMCHD1. Similar to haploinsufficiency mutations, hemizygosity for SMCHD1 resulted in a milder methylation defect at D4Z4 than dominant negative SMCHD1 mutations. Copy number variations of chromosome 18 are viable and our studies show that almost all 18p deletion syndrome (18p-) cases have, like these FSHD2 families, only one copy of SMCHD1. Our finding therefore raises the possibility that 18p- cases are at risk of developing FSHD. Detailed genotyping including D4Z4 CpG methylation and repeat array size analysis in 18p- individuals suggests that approximately 1:8 18p- cases may have an increased risk of developing FSHD. Our studies suggest that D4Z4 CpG methylation and DUX4 expression is SMCHD1 dosage sensitive. To address this, we combined D4Z4 CpG methylation analysis in trisomy 18 and tetrasomy 18p individuals with ectopic expression of SMCHD1 in FSHD1 muscle cell cultures. The results will be presented at the workshop.

5. **Advances in the development of a methylation assay for FSHD**

Calandra P.[1], Cascino I.[1], Lemmers R.J.[2], Teveroni E.[1,3], Ricci E.4, Galluzzi G.[1], Monforte M.[4], Tasca G.[5], Moretti F.[1], van der Maarel S.M.[2], Deidda G.[1]

[1] Institute of Cell Biology and Neurobiology, National Research Council of Italy, Monterotondo (Rome), Italy
[2] Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands
[3] Institute of Pathology, Catholic University School of Medicine, Rome, Italy
[4] Institute of Neurology, Catholic University School of Medicine, Rome, Italy
[5] Don Carlo Gnocchi Onlus Foundation, Milan, Italy

Facioscapulohumeral muscular dystrophy is a disease linked to an epigenetic defect in the chromosome 4q subtelomere. This dystrophy is caused by contraction of the D4Z4 macrosatellite array on chromosome 4qter in FSHD1, or by functional impairment of SMCHD1, a chromatin modifier binding to D4Z4, in FSHD2. Both genetic defects lead to D4Z4 DNA hypomethylation associated with the inappropriate expression in skeletal muscle of the D4Z4-encoded DUX4 transcription factor in the presence of a polymorphic polyadenylation signal (PAS) distal to the last D4Z4 unit (4qA). Currently, diagnosis by methylation analysis has two main limits: the interference of non-pathogenic arrays and the lack of information about the presence of the DUX4-PAS. Importantly, the methylation status of the DUX4-PAS critical region has not been thoroughly investigated. We investigated the DNA methylation levels of the region immediately distal to the D4Z4 array critical to FSHD development and encompassing 10 CpGs in PAS-positive alleles. Comparison of FSHD1, FSHD2 and control subjects showed highly significant differences of methylation levels in all CpGs tested. Noteworthy, one of these CpGs (CpG6) was able to discriminate the affected individuals with a sensitivity 0.95 in a cohort of 112 samples, supporting the potential usefulness of this assay for FSHD diagnosis. Moreover, our study evidenced a relationship between PAS-specific methylation and the severity of the disease. These data point to CpGs distal to the D4Z4 array as a critical region that summarizes multiple factors affecting epigenetics of FSHD. Additionally, methylation analysis of this region may allow the establishment of a rapid and sensitive tool for FSHD diagnosis.

6. **The translocation of DUX4 and DUX4c during myoblast differentiation allows their association with nucleo-cytoplasmic proteins associated with mRNP granules**

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Hundreds of double homeobox (DUX) genes map within dispersed 3.3-kb repeated elements and encode DNA-binding proteins. Among these, we identified DUX4 and DUX4c (identical to DUX4 except for the end of the carboxyl terminal domain) which are induced in FSHD muscles. In the present study, we performed yeast two-hybrid screens and protein co-purifications with HaloTag-DUX fusions or GST-DUX4 pull-down to identify protein partners of DUX4 and DUX4c. Unexpectedly, we identified and validated (by co-IP, GST pull-down and in situ Proximal Ligation Assay) interaction of DUX4 or DUX4c with type III intermediate filament desmin in the cytoplasm and at the nuclear periphery. Desmin filaments link adjacent sarcomeres at the Z-discs, connect them to sarcolemma proteins, interact with mitochondria, and also contact the nuclear lamina and contribute to positioning of the nuclei. Nuclei and mitochondria positioning are altered in FSHD muscles. Another Z-disc protein, LMCD1, was also validated as a DUX4 partner. We also validated (by co-IP or in situ PLA) several RNA-binding proteins such as C1QBP, SRSF9, RBM3, FUS and SFPQ that are involved in mRNA splicing and translation. FUS and SFPQ are nuclear proteins, however their cytoplasmic translocation was reported in neuronal cells where they associated with ribonucleoparticles (RNPs). The functionality of DUX4-DUX4c interactions with cytoplasmic proteins is underscored by the observation of DUX4/DUX4c nucleo-cytoplasmic translocation upon myoblast fusion and their co-localization with some cytoplasmic DAPI-positive spots. Several validated or identified DUX4/4c partners are also contained in mRNP-granules such as C1QBP (which associates with SRSF9), NONO (which associates with SFPQ), IGF2BP1, IGF2BP3, DHX9 (an ATP-dependent RNA helicase A), ELAVL1, HNRNPU, HNRNPH1, HNRNPQ, PABPC1, PABPC4, PCBP2, nucleolin and several ribosomal

proteins (Protein-protein interaction databases at UniProtKB). These complexes contain untranslated mRNAs, travel along microtubules and bring mRNAs to subcellular areas (such as the plasma membrane), where their translation is required at specific times in muscle differentiation. Interestingly, we sometimes observed DUX4 at the cell membrane. Large muscle RNPs were recently shown to exit the nucleus via a novel mechanism of nuclear envelope budding. We observed DUX proteins associated with similar nuclear buds following DUX4 or DUX4c overexpression. In conclusion, in addition to their transcriptional activities, DUX4 and DUX4c overexpression in FSHD muscle cells might disturb cytoskeletal dynamics and mRNA splicing, location and translation by their association with proteins regulating these processes. Further investigations are on-going to confirm such a function that could be common to hundreds of DUX proteins.

7. **PAX3/7 target gene repression is a hallmark of FSHD muscle**

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Although DUX4 is detectable in about 1/1000-1/8000 FSHD myoblasts, a mechanistic understanding of how DUX4 expression causes the clinical symptoms of FSHD remains enigmatic. Two major hypotheses on DUX4 induced molecular pathogenesis have been advanced: (1) the more widely accepted idea that DUX4 target gene expression drives FSHD [e.g. Yao et al. (2014) Hum Mol Genet 23: 5342-52], or (2) DUX4 affects PAX3/7 target genes to cause FSHD [Bosnakovski et al. (2008) EMBO J 27: 2766-79]. These theories have not been rigorously evaluated, or indeed, directly compared, and this has clear ramifications for understanding FSHD molecular pathogenesis and the development of therapy.

Here, we constructed a robust framework to evaluate the two hypotheses. We collated a data set comprising 21 publicly available transcriptomic studies as well as generating a novel microarray analysing gene expression changes directed by Pax7, Dominant-negative Pax7 and PAX7-FOXO1A in muscle stem cells. By performing a meta-analysis, we found that PAX3/7 target gene repression is a clear hallmark of FSHD muscle, whilst DUX4 target gene expression is not a significant discriminator. Thus the dominant impact of DUX4 on FSHD muscle is the regulation of PAX3/7 target genes, rather than induction of its own target gene set.
This data set also allowed us insight into other FSHD enigmas. (1) The FSHD-affected muscle distribution may be explained by differential PAX3/7 target gene expression. (2) Age of onset of FSHD can be understood by a dramatic de-repression of the D4Z4 macrosatellite due to telomere shortening (building on the work of Stadler et al. (2013) Nat Struct Mol Biol 20: 671-8). (3) Oxidative stress sensitivity in FSHD can be explained by HIF1(alpha) over-expression, building on our previous work [Banerji et al. (2015) J R Soc Interface 12: 20140797].

8. **Protein chemistry and protein-protein interactions of DUX4**

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OBJECTIVE: Facioscapulohumeral muscular dystrophy (FSHD), the third most prevalent muscular dystrophy, is an autosomal dominant disorder that most commonly causes progressive weakness in muscles of the face, shoulder girdle, and limbs. FSHD was formally classified as a major form of muscular dystrophy in 1954, but the pathogenic events leading to the disease have only recently started coming into focus. Numerous studies now support that FSHD disease pathogenesis involves aberrant expression of the DUX4 gene, which encodes a myotoxic transcription factor. The emergence of DUX4 represented a momentum shift in the FSHD field as it provided an important target for therapy design. Indeed, as FSHD is currently untreatable, developing effective therapies is a critical need in the field. We hypothesized that an FSHD treatment should center on inhibiting DUX4 activity. The overall objective of this study is to identify, characterize, and ultimately inhibit DUX4 by exploiting biochemical properties including protein binding partners, functional residues and protein modifications that may contribute to its toxicity in FSHD muscle.

BACKGROUND: The DUX4 protein consists of two functional domains, the N-terminal double homeodomain and a C-terminal transactivation domain, both of which are essential for inducing toxicity. The homeodomains facilitate specific DNA binding, while the mechanistic role of the C-terminal domain remains unknown. DUX4 likely activates gene expression and is regulated in a tissue- and temporal-specific manner resembling other better-studied transcription factors. Protein interactions, functionally significant amino acids and protein post translational modifications (PTMs) could directly alter DUX4's transcriptional roles by changing it subcellular localization, stability or affinity for DNA and other cofactors. Our primary goal was to identify binding partners of DUX4, elucidate the functional role of C-terminal residues and determine whether DUX4 is post translationally modified. Ultimately, we will determine how these three biochemical aspects contribute to DUX4 associated toxicity.

RESULTS: We have continued to focus on three ways that DUX4 protein function can be altered and will present an update on our findings. First, we have validated binding partners of DUX4 in HEK293 and human myoblasts. The functional implication of DUX4's association with some of these binding partners is underway. Second, we have identified residues of the extreme C-terminus that are critical for toxicity and have designed experiments to determine whether this region is involved in protein-protein interactions. Third, we have begun mapping DUX4 PTMs in a variety of cell types and find modification events occur in both the DNA binding and transactivation domains which include acetylation, methylation and phosphorylation. We will present studies designed to elucidate functional contributions of DUX4 modifiable residues.

CONCLUSION: Defining the role of DUX4's interaction proteome, functionally significant residues, and DUX4 PTMs will help us understand its protein function and regulation. These three biochemical aspects of DUX4 could alter ligand binding affinity, protein stability and subcellular localization and therefore be exploited to design FSHD therapies that inhibit DUX4 activity and prevent myotoxicity.

9. **DUX4 inhibits satellite cell myogenesis by inducing a 'Stem-cell Like' transcriptome**

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Facioscapulohumeral muscular dystrophy (FSHD) affects specific skeletal muscles, resulting in significant morbidity. FSHD is associated with a contracted, DNA/CpG hypomethylated, D4Z4 repeat region in the subtelomere of chromosome 4q (FSHD1), which, in combination with 4q allelic variants, allows stable transcription of a Double Homeobox 4 (DUX4) retrogene. There is an open reading frame for the DUX4 transcription factor in each D4Z4 unit. Here, we analysed skeletal muscle regeneration in transgenic mice carrying a human D4Z4 genomic region from a contracted pathogenic FSHD1 allele, or carrying a 'healthy' sized allele. DUX4 was transiently induced during muscle regeneration only in mice carrying a contracted D4Z4 repeat. FACS revealed that DUX4 expression was associated with differentiating myoblasts. A panel of DUX4 constructs, including constitutively active, dominant-negative and truncated versions, revealed that DUX4 is a potent activator of transcription that acts to inhibit satellite cell proliferation and differentiation. Sensitive information theoretic transcriptomic analysis showed that DUX4 orchestrates a more "stem cell-like" transcriptome, and inhibits myogenesis. Thus, impaired muscle maintenance/repair by satellite cells likely contributes to FSHD pathology.

10. **Quantitative proteomic analysis of DUX4-expressing muscle cells reveals widespread post-transcriptional regulation**

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FSHD is caused by the misexpression of a germline transcription factor, DUX4, in muscle cells. Upon expression, DUX4 activates germline antigens, retrotransposons and repetitive elements, inhibits innate immunity and compromises cellular RNA quality control - ultimately leading to cell death via apoptosis. Though most studies on DUX4 have focused on its effect on the cellular transcriptome recent studies suggest that the RNA and protein levels in DUX4 expressing cells may be decoupled. For example, DUX4 expressing cells show rapid proteosome-mediated degradation of UPF1, a member of the RNA quality control machinery. Moreover, the resultant failure in RNA quality control stabilizes scores of aberrant RNAs and whether or not such RNAs are translated to make dysfunctional or neofunctional proteins is unknown. Hence, our understanding of the gene regulatory network of DUX4 would be incomplete without a thorough analysis of proteome-level changes induced by DUX4, in addition to the transcript-level changes. Here, we use paired RNA-seq and quantitative SILAC-based mass spectrometry of human myoblasts inducibly expressing DUX4 to identify proteins regulated at the post-transcriptional level as well as determine the impact of failed RNA quality control on the cell's proteome.

While there is overall correlation between the RNA and protein level of those genes that are most highly induced by DUX4, we see several instances where the RNA and protein level do not correlate, or even display a negative correlation. For example, several extracellular matrix components are induced at the RNA level, but severely downregulated at the level of protein. Certain genes that do not show any change at the RNA level show significant up- or down-regulation at the protein level, including several genes involved in RNA processing, indicating that DUX4 directly or indirectly modulates the translational output of such mRNAs and/or the half-life of the corresponding proteins. DUX4 expression also triggered widespread production of novel peptides and truncated proteins derived from NMD-targeted mRNAs, suggesting that NMD inhibition could be a contributing factor to muscle immunogenicity in FSHD as well as trigger proteotoxicity. In summary, our results uncover new layers of gene regulation by DUX4 that could form the basis for novel therapeutic avenues for FSHD.

11. **The role of D4Z4-encoded proteins in the osteogenic differentiation of mesenchymal stromal cells isolated from bone marrow**

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The Double Homeobox 4 (DUX4) gene is inappropriately activated in FSHD muscle cells. DUX4 is also a retrogene that is normally expressed in germline cells and is submitted to repeat-induced silencing in adult tissues. DUX4 mRNAs have been detected in human embryonic and induced pluripotent stem (hES and iPS) cells. In collaboration with Dr. Laurence Lagneaux's group (Institut J. Bordet, ULB), we investigated whether DUX4 could be expressed in human mesenchymal stromal cells (hMSCs). This group has developed different techniques to isolate hMSCs from bone marrow but also to differentiate them along several lineages (osteoblasts, chondroblasts, adipocytes,…).

We have unexpectedly found that DUX4 expression was induced upon hMSC differentiation to osteoblasts. This process involved 52-kDa DUX4 known in FSHD muscles and a new longer protein form (58 kDa). The 52-kDa DUX4 protein is express in the undifferentiated and differentiated cells while the 58-kDa DUX4 protein is express from day 8 following the induction of the osteogenic differentiation. During osteogenic differentiation of human embryonic stem cells (hESC) that carry the FSHD genetic defect, a DUX4 mRNA with a more distant 5' start site that presented a 60-codon reading frame extension was characterized and encoded the 58-kDa protein (DUX4M60). Transfections of hMSCs with an antisense oligonucleotide targeting DUX4 mRNAs decreased both the 52- and 58-kDa protein levels and confirmed their identity. Gain and loss of function experiments in hMSCs suggested these DUX4 proteins had roles in osteogenic differentiation as evidenced by the alkaline phosphatase activity and calcium deposition. The differentiation was delayed by 58-kDa DUX4 expression, but it was increased by 52-kDa DUX4 showing opposite role in osteoblastic differentiation.

These data indicate an opposite role for DUX4 protein forms in the osteogenic differentiation of hMSCs.

Several therapeutic approaches for FSHD are being developed that aim to interfere with DUX4 expression. Our present study indicates essential functions in MSC differentiation that should

not be suppressed and demonstrates the need for specific muscle targeting of DUX4-suppressing agents.

de la Kethulle de Ryhove L, Ansseau E, Nachtegael C, Pieters K, Vanderplanck C, Geens M, Sermon K, Wilton SD, Coppée F, Lagneaux L, Belayew A. - The Role of D4Z4-Encoded Proteins in the Osteogenic Differentiation of Mesenchymal Stromal Cells Isolated from Bone Marrow. - Stem Cells Dev. 2015 Aug 26.

12. **Immunodetection of DUX4 protein in an FSHD muscle biopsy links protein expression to a phenotype shared with the rAAV-DUX4 mouse model**

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A major hurdle for understanding the effects of *DUX4* expression *in vivo* is reproduction of the low level *DUX4* expression in mice. One approach to circumvent either the overt toxicity or lack of a muscle phenotype in *DUX4* transgenic models is to modulate expression of *DUX4* in wild-type adult mouse muscle. We have presented evidence that it is possible to produce a range of phenotypic changes in mouse muscle consistent with FSHD through intramuscular injection of a recombinant adeno-associated viral vector (rAAV) carrying a human *DUX4* gene and promoter. The phenotypic changes resulting from *DUX4* expression include variations in fiber size, focal areas of fiber necrosis and mononuclear cell infiltrates, increased internal nuclei, angulated fibers, and fiber splitting. In order to explore the relationship of DUX4 protein expression to muscle histological changes we developed an immunohistochemistry-based method for DUX4 protein detection in cryosections from rAAV-*DUX4* IM injected mouse TA muscles. Analysis of serial sections shows juxtaposition of DUX4 protein expression in myonuclei with myofiber necrosis and physically links DUX4 protein to toxicity *in vivo*. Successful antibody detection of DUX4 protein in mouse muscle prompted us to examine FSHD muscle biopsy cryosections for DUX4 protein. We report the first detection of DUX4 protein in human muscle using immunofluorescence confocal microscopy. The DUX4 positive FSHD myofiber displays a 'splitting' phenotype consistent with muscular dystrophy and the same fiber splitting phenotype is physically associated with DUX4 expression in rAAV-*DUX4* transduced wild-type mouse myofibers *in vitro* and with rAAV-*DUX4* IM injection of the mouse TA muscle *in vivo*. We are collecting more FSHD muscle biopsies for a more extensive analysis of DUX4 protein and its relationship to disease. Relating DUX4-associated phenotypes in the rAAV-*DUX4* mouse and in FSHD muscle biopsies serves to further validate our model as a platform for investigating the mechanism of disease, for identification of additional therapeutic targets, and for testing candidate therapies dependent on *DUX4* gene expression.

13. **DUX4-targeting antisense therapy development in a human muscle xenograft model for FSHD**

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We have successfully established a human skeletal muscle xenograft model for FSHD by transplanting FSHD affected donor biceps muscle into the anterior tibial compartment of immunodeficient NRG mice hind limbs. The grafted muscle from fresh biopsy donors and autopsy donors was regenerated by graft myocytes, re-vascularized by graft and host blood vessels, and re-innervated by host motor neurons. Currently, the loss of transcriptional repression of Double Homeobox Protein 4 (DUX4) in the D4Z4 macrosatellite repeats at chromosome 4q35 is believed to be the causative genetic defect of FSHD. In our human muscle xenografts, DUX4 expression as well as FSHD biomarker profile mirror those of the donor muscle tissue, making this model a feasible and valid preclinical tool to predict the response to novel therapeutics for FSHD. Recently, antisense oligonucleotides (AONs) which are small (20–30 nucleotides), single-stranded pieces of chemically modified DNA or RNA that can target gene transcripts have shown great promise in therapy development for neuromuscular diseases. AONs have now progressed to the clinical trial phase for Duchenne muscular dystrophy (DMD), spinal muscular atrophy (SMA) and myotonic dystrophy. We report a proof-of-concept study to investigate the therapeutic potential of DUX4 targeting AON therapy for FSHD in our human muscle xenograft model and show promising knockdown of DUX4 expression and FSHD biomarker profile

14. **Defining the functional overlap between mouse Dux and human DUX4**

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D4Z4 repeats are present in at least 11 different mammalian species, including humans and mice. Each D4Z4 repeat contains an open reading frame (ORF) encoding a Dux (double homeodomain) family transcription factor. In humans, aberrant expression of the D4Z4 ORF called DUX4 is associated with pathogenesis of autosomal dominant Facioscapulohumeral muscular dystrophy (FSHD). DUX4 is toxic to numerous cell types of different species, and DUX4 over-expression in vivo caused dysmorphism and developmental arrest in frogs and zebrafish, embryonic lethality in transgenic mice, and dose-dependent lesions in adult mouse muscle. Because DUX4 is a primate-specific gene, questions have been raised about the biological relevance of these over-expression studies in non-primate model organisms, as DUX4 toxicity could be related to non-specific cellular stress induced by over-expressing a Dux family transcription factor in cells or organisms that did not co-evolve its naturally regulated transcriptional networks. To begin addressing this question, we used identical methods to over-express several different Dux family transcription factors from humans and mice in vitro and in vivo, including mouse Dux, which is a paralog of DUX4 that emerged independently from a common progenitor. We found that double homeodomain transcription factors are not universally toxic, and only the D4Z4-resident mouse Dux gene caused similar toxic phenotypes as human DUX4 in vitro and in vivo. Furthermore, using ChIP-seq, we found that DUX4 and Dux have similar consensus binding sites and regulate some common pathways, including those involved in cell death. These data are consistent with an expected phenotype in paralogous genes, which diverge from a common progenitor to acquire new roles but can also maintain some common primordial functions as well. Thus, our data support the hypothesis that mouse Dux and human DUX4 are at least partially functionally homologous, which would suggest that mice could be sufficient proxy for studying DUX4 over-expression and FSHD in the absence of a more relevant primate model.

15. **Haploinsufficiency of Smchd1 exacerbates the phenotype of a transgenic FSHD1 mouse model**

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Individuals with facioscapulohumeral muscular dystrophy (FSHD) have a partial failure of DUX4 somatic repression resulting in the presence of DUX4 protein in a small number of muscle nuclei. Failure in repeat-mediated epigenetic repression of DUX4 is caused by contraction of the D4Z4 repeat array to 1-10 units (FSHD1) or by heterozygous mutations in the structural maintenance of chromosomes hinge domain 1 (SMCHD1) gene (FSHD2). SMCHD1 is a chromatin repressor that binds to the D4Z4 repeat array in order to maintain a repressive chromatin structure in somatic tissue. SMCHD1 mutations have also been identified in FSHD1 families; patients that carry both a contracted D4Z4 repeat array and a SMCHD1 mutation present with a more severe phenotype than relatives carrying only a contracted D4Z4 repeat array or a SMCHD1 mutation. In agreement, lower D4Z4 DNA methylation levels and enhanced DUX4 levels are measured in myotubes of FSHD1 patients upon SMCHD1 knockdown.

We have generated transgenic mice with an FSHD-sized D4Z4 repeat array of 2.5 units. These D4Z4-2.5 mice, like FSHD1 patients, fail to efficiently suppress DUX4 in somatic cells. As a consequence, sporadic muscle nuclei express relatively abundant amounts of DUX4 protein. However, the consequences of DUX4 expression in mice seem different; DUX4 can induce the expression of only a fraction of coding and noncoding transcripts in the mouse genome compared to the human genome. Apart from an uncharacterized eye phenotype, D4Z4-2.5 mice appear healthy and do not present with a muscular dystrophy phenotype.

To evaluate the modifier role of Smchd1 on disease severity, we have crossbred our D4Z4-2.5 mice with mice that are haploinsufficient for Smchd1 (MommeD1 mice). While D4Z4-2.5/MommeD1 mice are born at the expected Mendelian ratio, they are smaller, have reduced body weight, and die at 3-4 weeks of age. Further, the D4Z4-2.5/MommeD1 mice develop a severe skin and eye phenotype; from day 10, hyperkeratosis becomes apparent at the nose and paws; between day 11 and 14, the eyes of these mice do not open. Histopathological examination revealed that the muzzle epidermis, the epidermis within the external and middle ears, and the eyelids displayed irregular acanthosis and thickened stratum cornea. In addition, inflammatory infiltrates were observed in these tissues, in the stomach, and in the liver. In line with this, we measured increased transcript levels of inflammatory markers in tissues of the

D4Z4-2.5/MommeD1 mice. The epidermal and inflammatory phenotypes seen in D4Z4-2.5/MommeD1 mice were also found in D4Z4-2.5 mice, albeit in a milder form.

In conclusion, Smchd1 haploinsufficiency aggravates the phenotype of our transgenic FSHD1 mouse model. While the penetrance of the skin and eye phenotype was complete in D4Z4-2.5/MommeD1 mice, considerable variation in severity was observed, and DUX4 transcript levels showed both tissue-specific and mouse-specific sensitivity for reduced Smchd1 levels.

16. **Cell and animal models for studying DUX4 in FSHD**

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Using conditional inducible systems, we have developed various cellular and animal model systems to study DUX4. We present recent progress on using these models to study the functional activities of the DUX4 protein, and physiological effects of DUX4, and discovery of inhibitors of DUX4.

17. **Targeting 3'end elements involved in mRNA processing is an efficient therapeutic strategy for FacioScapuloHumeral Dystrophy**

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FacioScapuloHumeral Dystrophy (FSHD) is caused by a loss of epigenetic marks within the D4Z4 macrosatellite located in the sub-telomeric region of chromosome 4 leading to chromatin relaxation, aberrant expression of the DUX4 transcription factor and a cascade of gene deregulations. It was previously shown than targeting DUX4 mRNA using antisense oligonucleotides (AOs) is an efficient strategy to silence DUX4. AOs are chemically modified single-stranded DNA, RNA or chemical analogue molecules which are able to modulate the expression of a specific targeted gene and during the past 10 years, synthetic AOs have emerged as a promising strategy for drug therapy of genetic disorders affecting skeletal muscles such as the muscular dystrophies and motor neuron diseases. However, the use of AOs in the muscular dystrophies was mainly restricted to targeting splicing enhancer and suppressor elements to force exon exclusion or inclusion.

The aim of our study was to determine whether or not other components of DUX4 pre-mRNA could be targeted by AOs to reduce DUX4 expression. We decided to focus on key 3'-end elements involved in DUX4 mRNA processing. These key 3'-elements are the poly(A) signal and the U/GU rich sequence, which are implicated in the cleavage and polyadenylation of the fundamental 3'-UTR end processing steps for the maturation of the vast majority of eukaryotic mRNAs. Indeed, in most cases, co-transcriptional 3'UTR maturation is required for nuclear export, stability of the mRNA and efficient translation, and consequently could represent interesting targets for suppression of gene expression.

Here we describe a new therapeutic AO-based approach using phosphorodiamidate morpholino oligomers (PMOs) for FSHD targeting the key elements of 3'-end processing. We observed that targeting DUX4 3'-end key elements in myotubes leads to an efficient extinction of DUX4 mRNA and prevents aberrant expression of genes downstream of DUX4. Importantly, redirection of DUX4 mRNA polyadenylation site in downstream exons was not observed. Targeting 3'-end elements using AOs is thus an efficient strategy for DUX4 gene silencing with important clinical therapeutic potential.

18. **Toxicology for DUX4-targeted microRNAs**

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The emergence of DUX4 as the pathogenic insult in FSHD now makes developing targeted therapeutic strategies for treatment possible. Previously our lab demonstrated proof of principle for the use of a DUX4-targeted RNAi-based gene therapy. We have pursued two artificial microRNAs with the greatest therapeutic efficacy determined by co-delivery with pathogenic levels of DUX4 via adeno-associated viral (AAV) vectors. To continue down a translational path this study was designed to determine the safety of miRNA therapy to muscle by delivering high doses for toxicity studies. Both local intramuscular injections and systemic isolated limb perfusions were performed. Acute (3 week) and long term (5 month) studies have revealed safe sequence and dosing parameters for the miRNA therapies. To further circumvent off target organ toxicity we have optimized these vectors by replacing the ubiquitous U6 promoter with the muscle specific promoter tMCK. The safety parameters along with the promoter-restricted expression provide necessary data for translating an RNAi-based therapy for FSHD.

19. **BET protein inhibition as a therapeutic strategy in FSHD**

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Facioscapulohumeral dystrophy (FSHD) is characterized by epigenetic changes resulting in aberrant expression of the DUX4 retrogene in skeletal muscle. Since DUX4 expression is likely to be a major cause of the muscle pathology in FSHD, we have pursued the therapeutic strategy of identifying drug targets for the promotion of epigenetic silencing and prevention of DUX4 expression. We have previously identified the bromodomain and extra-terminal (BET) family of proteins as key targets involved in DUX4 expression and demonstrated that selective inhibitors of BET proteins (BETi) block DUX4 expression in FSHD myoblasts and myotubes. Remarkably, 24 h of compound exposure resulted in a sustained (>72h) decrease in DUX4 expression. This "memory" effect was mediated by histone deacetylase activity, as it was blocked by class I HDAC inhibitors. Furthermore, the increased DUX4 expression that occurs when FSHD cells are exposed to HDAC inhibitors is completely blocked by BETi, suggesting that BET proteins play a key role in mediating acetylation-dependent DUX4 transcription. We are continuing to explore the potential therapeutic value of BETi for FSHD using mouse models that replicate the epigenetic de-repression of the human D4Z4 locus. The goal is to provide sustained muscle exposures of BETi predicted to elicit decreased DUX4 expression based on in vitro exposure-response relationships.

20. [P] **Global initiatives to accelerate FSHD research**

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The FSHD Champions are an international alliance of organizations that fund FSHD research and advocate for patients with this disease. The alliance was formed to educate, support, and advance each organization's mandate of improving the lives of FSHD patients. The Champions are currently spearheading a number of efforts to [i] strategically leverage the knowledge and efforts of each member's programs in an international context, [ii] reduce duplication of effort, and [iii] remove global barriers to the advancement of therapies for FSHD. Champions programs include efforts to coordinate existing patient registries toward a global harmonized registry, streamline grant application processes, and establish basic research and clinical priorities. Initial traction toward these goals has identified key challenges for stakeholders, from individual labs to global consortia.

21. [P]. **Antisense oligo-dependent knockdown of FSHD biomarkers in myotube cultures**

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The transcriptional biomarker profile of FSHD muscle and cells features elevated levels of the transcription factor DUX4 and its downstream targets when compared to control profiles. We developed and tested morpholino-based antisense oligos (ASOs) targeting the DUX4 transcript. Highly stable, morpholinos act by binding target sequences with high affinity and using steric blocking to inhibit translation or change splicing patterns. Myotube cultures from FSHD subjects were treated with DUX4 or control morpholinos for four days. Cells were harvested for RNA isolation and RT-qPCR analysis, as well as for DUX4 immunohistochemistry. Expression of FSHD biomarkers (MBD3L2, TRIM43, ZSCAN4) were found to be consistently down-regulated with morpholino #GZ10. DUX4 protein expression was similarly reduced. Cell toxicity was not observed in cultures treated with a range from 0 to 500 uM GZ10. To evaluate global and off-target effects, FSHD and control myotube cultures treated with GZ10 or standard control morpholinos were analysed by RNAseq. DUX4-dependent FSHD biomarkers were consistently down-regulated in GZ10-treated FSHD cultures, and no consistent off-target effects were observed. In conclusion, we have identified a morpholino-based ASO that blocks DUX4 expression and function in FSHD myotubes in vitro.

This study was supported by the FSH Society, NICHD-UMMS Wellstone MDCRC, and Genzyme.

22. [P] **Reduction of circulating glutathione peroxidase activity in individuals with facioscapulohumeral muscular dystrophy (FSHD)**

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The purpose of this study is to identify protein biomarkers in peripheral blood samples from patients with early onset FSHD. Samples were obtained through two studies from the Cooperative International Neuromuscular Research Group (CINRG). For initial discovery we used serum samples from 3 patients with FSHD (males, 7-14yrs), 3 age- and gender-matched controls and 12 patients with Duchenne muscular dystrophy (DMD). Mass spectrometry based proteome profiling identified 11 elevated proteins (3 folds increase) and 103 proteins reduced more than 3 fold in the FSHD group relative to healthy volunteers. One of the altered proteins, glutathione peroxidase 3 (GPX3), was specific to FSHD and showed significantly lower level in FSHD samples (1 hit) compared to both healthy (10 hits) and DMD (13 hits) samples. Decrease in GPX3 was further validated by a GPX functional assay using 13 FSHD (8 females, 12-51yrs; 5 males, 13-36 yrs) and 13 age- and gender-matched controls. The results confirmed that GPX function was significantly lower (1.5 fold, $p<0.05$) in FSHD plasma samples compared to age- and gender-matched healthy controls. In addition, the GPX function was lower ($R2=0.4$, $p<0.05$) in patients with more severe disease, as defined by the FSHD clinical severity scale. GPX3 is the major antioxidant protein in circulation and was reported to express at lower level in FSHD myoblasts. The findings suggest that GPX3 is a potential biomarker for FSHD.

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23. [P]. **Development of a human FSHD myogenic cell model from iPSC**

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The pathogenesis of facioscapulohumeral muscular dystrophy (FSHD) pathogenesis is complex and not yet fully understood. Recent detailed genetic studies have significantly increased our knowledge of this enigmatic and multifaceted disorder, suggesting aberrant gene expression during very early myogenesis. The unprecedented opportunities provided by human induced pluripotent stem cells (hiPSCs) could offer a novel insight on FSHD pathogenesis. We have developed a new defined myogenic specification system using chemical compounds, followed by FACS purification of myoblasts. Using this protocol, we have generated multiple batches of myoblasts from one FSHD-hiPSC line. Using primer sets specific for DUX4, MBD3L2, TRIM43, ZSCAN4 and CCNA1, we performed comparative gene expression profile analysis (the isolated myoblasts, parental myoblasts and their undifferentiated hiPSCs), which shows inconsistent results among the batches. We believe this issue is caused by either the parental myoblasts (used for hiPSC generation) have low levels of DUX4 expression. Currently, we are generating additional hiPSC lines from myoblasts with higher levels of DUX4 expression, along with myoblasts of unaffected siblings obtained from the Wellstone FSHD biobank. In addition, a genetic reporter system will be applied to the new FSHD- and control-hiPSC lines, including MESOGENIN1::GFP (a marker gene for somite stage) as well as PAX7::GFP (a putative skeletal muscle stem cells) with CRISPR/Cas9 system. Our FSHD human cell model will shed light on the stage-specific genetic regulation during early myogenesis.

24. [P]. **A C1QBP-targeting compound inhibits DUX4-target gene expression**

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Development of FSHD is linked to the de-repression and stabilization of the DUX4 gene contained within the D4Z4 repeats on chromosome 4. However, individuals have been identified who express DUX4 in their muscle biopsies, but who do not show any clinical symptoms of the disease. It therefore seems that while DUX4 expression may be necessary for the development of the FSHD, it is not sufficient. This suggests that there may be other factors that modify the FSHD phenotype. We hypothesized that an FSHD-modifying factor would physically interact with and modify the function of DUX4, and we therefore took a proteomic approach to screen for DUX4-interacting proteins that may act as disease modifiers. We identified the multifunctional C1QBP protein as one such candidate. C1QBP is known to regulate several of the processes that DUX4 affects, including gene expression, oxidative stress, and apoptosis. We have observed that C1QBP is dynamically regulated in myogenic cells. It is primarily localizes to ribbon-like structures outside of the nucleus in myoblasts, but appears to relocate to the nuclear periphery when they are allowed to fuse into myoblasts. Expression of DUX4 causes increased C1QBP accumulation in the nucleus, supporting the hypothesis that DUX4 and C1QBP form functionally relevant interactions. Importantly, C1QBP is known to bind to the intra- and extra-cellular signaling molecule hyaluronic acid (HA), which can regulate its phosphorylation state. We have found that that decreasing intracellular HA by treating cells with 4-methylumbelliferone (4MU), an inhibitor of HA synthesis, results in a sharp decline in DUX4-target gene expression, both under endogenous conditions in myotubes, and when DUX4 is overexpressed in myoblasts.

This suggests that C1QBP is a modifier of DUX4 activity and potentially identifies 4MU as an FSHD therapeutic.

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25. [P]. **Nuclear location of DUX4 is required for its corepressor activity on the progesterone nuclear receptor**

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Our laboratory first presented evidence indicating that DUX4 is a negative co-regulator of the human progesterone receptor (PR) (1). We also showed that progesterone protects cultured cells from DUX4-mediated cytotoxicity (1,2). DUX4 is normally expressed in gonadal tissues of healthy individuals (3), coincidental with a potential role for DUX4 in the endocrine pathway. Because DUX4 is a toxic protein, we hypothesize that cells from germinal tissues have specific regulatory and protective mechanism allowing normal expression of the DUX4 gene and bypassing the toxic effect of DUX4, respectively. The hypothetical protective mechanism(s) would not be present in tissues where ectopically/improperly expressed DUX4 leads to cell death. We have previously shown that DUX4-mediated toxicity is dependent on the subcellular location of DUX4 (4). In this work we explored if alternative subcellular locations of DUX4 regulates/modifies its coregulatory activity on the PR. Mutations at the more relevant NLSs from DUX4 (i.e. NLS1 and NLS2) partially delocalized DUX4 from the cell nucleus (4) and were analyzed in these studies. It was observed that DUX4 with altered transit to the nuclei lose its corepressor activity on the PR. Thus, re-located DUX4 (i.e. mostly cytoplasmic) does not have any effect on the activity of the PR. These results indicate that the corepressor effect of DUX4 on the activity of the PR is exerted at the cell nucleus. DUX4-NLS mutants carrying the NLS from the virus SV40 would allow to re-direct DUX4 to the nuclei to analyze if the DUX4 NLS sequences per se participate in the DUX4 regulatory activity on the PR. To explore if alternative macromolecular structures of DUX4 disturb its activity on the PR, DUX4 fusions to GFP were analyzed. All the studies were performed on a reconstructed PR gene reporter system, using HepG2 cells, as well as on breast cancer cells endogenously expressing the PR. In this work we also analyzed the protective effect of progesterone on the toxicity of DUX4 NLS mutants. The toxic effect of DUX4 mutants was analyzed using a modified assay described in our laboratory (2) and quantified using FACS. Results from these experiments indicate that progesterone synergize the low-toxicity of DUX4 NLS mutants. Taken together these studies strongly support our previous contention about the negative co-regulatory activity of DUX4 on the PR as well as the protective effect of progesterone on DUX4 toxicity. These results are relevant to both the normal and pathological function of DUX4 as well as the future rational therapies in FSHD.

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26. [P]. **A Phase 1 dose escalation study of ACE-083, a locally acting muscle building agent, in healthy volunteers**

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Background: ACE-083 is an investigational protein therapeutic that acts as a ligand trap for myostatin (GDF8) and other ligands in the TGF-β superfamily. These ligands are negative regulators of muscle growth and repair. In wild-type mice, twice weekly injection of ACE-083 into the left gastrocnemius muscle for a total of 4 weeks led to localized, dose-dependent muscle volume increases due to muscle hypertrophy. These changes were also associated with an increase in absolute force in the muscle. There was no effect observed on select uninjected muscle volumes. In an MDX mouse model of muscular dystrophy using mice treated with the same ACE-083 dosing regimen administered into the left gastrocnemius muscle, a similar increase in muscle volume was observed compared to the contralateral side. These data support the clinical evaluation of ACE-083 as a locally-acting muscle building agent.

Methods: This is a single-center, randomized, double-blind, placebo-controlled, dose escalation study in healthy post-menopausal women. The primary objective of the study is to characterize the safety and tolerability of single and repeated doses of ACE-083 in this healthy volunteer population. Secondary objectives include estimation of systemic exposure and evaluation of pharmacodynamic effects, including changes in muscle volume as measured on MRI and changes in strength as measured by hand-held dynamometer and Biodex fixed system. Five cohorts of 8 subjects each were randomized to receive ACE-083 (n=6) or placebo (n=2), administered as 2 or 4 injections along the length of the right rectus femoris muscle: Cohorts 1-3 received 50, 100, 200 mg of ACE-083, respectively on Day 1, and Cohorts 4-5 received 100, 200 mg of ACE-083, respectively, on Days 1 and 22. The study will also evaluate the effects of ACE-083 (100 mg, 150 mg) injected into the right tibialis anterior muscle in two additional repeated-dose cohorts (Cohorts 6-7).

27. [P]. **Genetic features of FSHD2 with SMCHD1 mutation in Japan**

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FSHD1 is caused by contraction of D4Z4 repeat array on chromosome 4. In Europe, D4Z4 repeat array is from 1 to 10 units in FSHD1 population, while from 11 to 100 units in control population. In Asia, D4Z4 repeat array is from 1 to 6 units in FSHD1 population.
FSHD2 is caused by heterozygous mutations in SMCHD1 and mild contraction of D4Z4 repeat array. In Europe, 30% of SMCHD1 mutations disrupts ORF (D-ORF) and 70% preserves ORF (P-ORF), and D4Z4 repeat in FSHD2 is 11 to 71 units (median 13). In Asia, genetic features of FSHD2 are not known. Therefore, we investigated SMCHD1 mutation and size of D4Z4 repeat array in Japanese FSHD2 population.

In 317 Japanese FSHD patients with more than 6 D4Z4 units, we identified 20 patients with DNA hypomethylation on D4Z4 repeat array by bisulfite pyrosequence. Among the 20 patients, we identified ten novel and one known SMCHD1 mutation by Sanger sequence in 13 patients from 11 unrelated families: D-ORF and P-ORF mutation comprise 64% and 36%, respectively. In the 11 mutations, 10 mutations were heterozygous, while 1 mutation was homozygous. We found 9 to 14 D4Z4 units (median 13) in ten unrelated patients with heterozygous mutations, while more than 14 D4Z4 units in one patient with homozygous mutation.

In conclusion, we identified 13 Japanese FSHD2 patients with ten novel and one known SMCHD1 mutation and found that D-ORF mutation might be common in Japanese FSHD2 population.

28. [P]. **A new view of DUX4-induced pathology in FSHD encompasses both homeodomain-1-dependent and -independent mechanisms**

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BACKGROUND: Myopathology in facioscapulohumeral muscular dystrophy (FSHD) appears to be due to aberrant expression of a full length isoform of DUX4 (DUX4-FL), which is a transcription factor with two homeodomains. We previously reported that DUX4-FL expression in human myotubes inhibits protein turnover, alters ubiquitination, activates caspase-3, and leads to nuclear aggregation of TDP-43 and DUX4-FL itself. In contrast, these changes were not induced by the short DUX4-S isoform that binds DNA through the two homeodomains but lacks the C-terminal transcription activating domain. Though the transcription factor activity of DUX4-FL is vital for cytotoxicity, previous work left open the possibility that a subset of DUX4-FL-induced pathological changes might be independent of homeodomain-mediated DNA binding.

OBJECTIVES: Our goal was to determine which DUX4-FL-induced pathological changes were independent of homeodomain-1.

METHODS: Using human myogenic cell cultures, we compared the effects of BacMam-mediated expression of DUX4-FL, DUX4-S, and muthox1-DUX4-FL (in which the first homeodomain was mutated to inhibit DNA-binding). We assayed caspase-3 activation, protein ubiquitination, and aggregation of TDP-43 and DUX4-FL itself.

RESULTS: Though expression of muthox1-DUX4-FL did not activate caspase-3, as also found by others, or lead to altered protein ubiquitination, it did lead to nuclear aggregation of TDP-43 and DUX4 itself. In addition, aggregation of TDP-43 and DUX4 was still found when DUX4-S was co-expressed with DUX4-FL or muthox1DUX4-FL, but the DUX4-FL-induced changes in protein ubiquitination and caspase-3 activation were prevented by co-expression with DUX4-S.

CONCLUSIONS: Our results show that altered protein ubiquitination and caspase-3 activation are dependent on homeodomain-1, presumably via DNA binding and transcription activation. In contrast, aggregation of TDP-43, a protein implicated in ALS, and DUX4-FL itself occurs independently of homedomain-1. These studies thus lead to a new view of DUX4-induced myopathology in which abnormal protein aggregation in myonuclei is independent of homeodomain-1-mediated DNA binding.

29. [P]. **The relationship between FSHD1 and facio-scapulo-limb, type 2 (or the facio-scapulo-peroneal) muscular dystrophy 4q35-linked**

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To my mind, the facio-scapulo-humeral muscular dystrophy type 1 (FSHD1) in its clinical picture corresponds to facio-scapulo-limb muscular dystrophy type 2 (FSLD2), characterized by the descending distribution of muscle weakness with a “jump”, with initial facio-scapulo-peroneal (FSP) phenotype which I described in 1971 (Kazakov, 1971, 1995, Kazakov et al. 1974, 1995, 2012).

This idea was well confirmed by the opinions of other authors who supposed that the facioscapuloperoneal topography of muscle weakness (early involvement of some facial, shoulder girdle, pectoral and anterior tibial muscles) indicates the specific sign of FSHD at the initial stage of the development of the disease (Tyler, Stephens, 1950; Zundell, Tyler, 1965; Chyatte, Vignos, Watkins, 1966; Vignos, 1967).

The same opinion was expressed by Ricker and Mertens (1968), who noted early and severe peroneal group muscles atrophy which was common for the facio-scapulo-humeral muscle dystrophic patients, nevertheless this clinical feature was rarely indicated. On the other side, descending muscle’s involvement starting from the shoulder girdle, and then spreading gradually to the trunk, and then to pelvic muscles, as it is usually described by the majority of authors, doesn’t happen in FSHD as a rule. On the contrary, this form is characterized by “jumping” of the atrophies from the shoulder girdle muscles to the peroneal group of the lower leg muscles at the initial stage of the disease.

Serratrice (1969), Padberg (1982) and Tawil, Maarel (2006) have the same opinion. The last authors wrote: “FSHD is suspected in individuals with the following: weakness that predominantly involves the facial, scapular stabilizer, and foot dorsiflexor muscles...”.
Thus it is quite possible that FSHD1 is the same disease as a facio-scapulo-peroneal muscular dystrophy linked with chromosome 4q35. The best name for it is facio-scapulo-limb muscular dystrophy, type 2 (FSLD2), the descending variety with a “jump”, with initial facio-scapulo-peroneal phenotype; autosomal dominant (Erb, Landouzy and Dejerine). The facioscapuloperoneal or (facio)scapuloperoneal syndrome constitutes merely a stage in the development of FSLD2 (or FSHD1). On my opinion, this hypothesis is solving a knotty problem about nosological place of facio-scapulo-peroneal muscular dystrophy first described by Oransky in 1927, and Davidenkov, Kulkova in 1938.

I suppose that classical descending FSHD which I called the facio-scapulo-limb muscular dystrophy type 1 (FSLD1), a gradually descending variety with initial facio-scapulo-humeral phenotype; autosomal dominant (Duchenne de Boulogne) differs from the FSLD2 (or FSHD1) by

the pattern of muscle's involvement. However the nosological place of FSLD1 remains unknown.

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30. [P]. **Genome-scale CRISPR knock-out screen to identify genetic modifiers of FSHD**

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Background. Facioscapulohumeral dystrophy (FSHD) is a complex autosomal dominant muscular dystrophy with an incomplete genetic picture. The likely mechanism of disease is linked to epigenetic changes at the 4q35 locus, causing misexpression of the retro-transposon derived gene, DUX4. The DUX4 gene encodes a transcription factor whose expression is rare, but extremely toxic. Misexpression of DUX4 is hypothesized to induce inappropriate expression of downstream genes to cause disease pathogenesis. How DUX4 and its target genes cause disease remain unknown, but clinical evidence suggests that DUX4 misexpression is not an exclusive determinant of FSHD.

Objectives. We aim to identify genes whose loss-of-function can modulate FSHD disease severity through suppression of DUX4 toxicity. These genes will allow us to better understand why individuals with permissive FSHD alleles do not show symptoms (non-manifesting carriers), and why there is phenotypic variability between affected members of the same family.

Methods. We have utilized a genome-wide CRISPR knock-out (GeCKO) library to create a cell population of single gene knock-outs. These cells were subsequently transduced with a baculovirus expressing DUX4 to induce widespread toxicity. Cells that were able to overcome DUX4 toxicity will undergo sequencing to identify their CRISPR gene knock-out target and will be validated in functional studies.

Results. Using the lenti-CRISPR library, we have successfully edited HEK293T cells to introduce loss-of-function mutations in ~20,000 human genes. Infection of DUX4 baculovirus into our CRISPR-edited cells induced global toxicity and cell death within 48hrs. We observed that there were cell colonies within our population of single gene knock-outs that were resistant to the toxic effects of DUX4 and continued to proliferate.

Conclusions. Our preliminary data demonstrates that there are cells likely to contain CRISPR-targeted loss-of-function mutations in specific genes that can ameliorate DUX4 pathogenicity. Identification of these candidate genes will shed light on FSHD disease modifiers and provide valuable targets for therapeutic intervention.

31. [P]. **An iPSC model of FSHD myogenesis**

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Facioscapulohumeral muscular dystrophy (FSHD) is associated with deletions or hypomethylation of the chromosome 4 D4Z4 repeat region, leading to misexpression of the transcription factor DUX4 and DUX4---dependent target genes in adult muscle and differentiated myogenic cells. Our objective is to develop induced pluripotent stem cell (iPSC) models of FSHD to investigate the developmental mechanisms regulating DUX4 misexpression.

Isogenic myogenic (CD56+) and nonmyogenic (CD56---) cells derived from biceps muscle biopsies from three FSHD subjects and three control family members were reprogrammed using the CytoTune iPS Sendai Reprogramming Kit (Invitrogen). Multiple iPSC lines, confirmed pluripotent by teratoma and embryoid body assays, were subjected to a gene--- free, three---stage myogenic induction protocol developed by Genea BioCells.

iPSC lines derived from both myogenic and nonmyogenic cells underwent efficient induction of skeletal myogenesis in response to the Genea protocol, as assessed by high level expression of MYOD and muscle differentiation biomarkers. In contrast to differentiated parental myogenic cells, differentiated FSHD iPSCs expressed low levels of the adult myosin MYH1 relative to the embryonic MYH8 isoform, and much lower levels of DUX4---fl target genes. This was observed for iPSC derivatives regardless of their having a myogenic or nonmyogenic cell of origin. However, high level expression of DUX4--- dependent target genes could be achieved by collecting “reserve cell” populations after completion of the Genea differentiation protocol. These cells were highly proliferative, expressed MYOD, and fused into multi---nucleated myotubes with elevated biomarker expression.

Our findings provide evidence that the Genea Biocells gene---free myogenesis protocol activates an embryonic myogenic program that is developmentally delayed in DUX4---dependent FSHD biomarker activation. This regulatory block can be overcome by cell culture manipulations. In conclusion, we found that iPSC lines can be used to investigate the developmental mechanisms regulating DUX4 & target gene misexpression in FSHD.

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32. [P]. **An Instrumented timed up and go in patients with facioscapulohumeral muscular dystrophy**

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Background: Timed functional tasks measure the time to complete activities like going 30 feet, getting up from a chair, or ascending 4 stairs, however, are not sensitive to disease progression in facioscapulohumeral muscular dystrophy (FSHD) over periods as long as 3 years of follow up. Further quantifying functional motor tasks may reveal a continuum of motor disability that predicts, only when it reaches a threshold value, future motor dysfunction. Here we use a commercially available portable wireless gait system to instrument a timed up and go.

Methods: This is part of an ongoing pilot study of 20 genetically confirmed and clinically affected FSHD participants at the University of Kansas Medical Center. We utilized 6 wireless sensors (Opal sensors, APDM, Portland, OR) which measure acceleration and angular velocity to assess dynamic parameters of movement that include temporal and spatial parameters of gait, limb range of motion, trunk sway, and transitions from seated to standing and standing to seated. Patients performed the timed up and go test, a standardized clinical test of strength and balance that requires the patient to rise from a seated position, walk a defined distance, turn 180 degrees, then walk back to a chair and sit down. Participants returned within 2 weeks to repeat testing to determine test-retest reliability of the wireless sensor metrics. Temporal and spatial parameters of wireless sensor gait metrics were compared to other measures of disease severity, including a clinical severity score and the 6 minute walk test.

Results: We present preliminary data on 8 participants (3 men, 5 women, mean age 56.5 years, 4 mild/moderately affected, and 4 moderate/ severely affected), with reliability testing available for 5 participants. All gait parameters in FSHD subjects were altered, and there was a trend towards decreasing performance with increasing disease severity. Reliability was good to excellent for spatial and temporal gait metrics (ICC 0.85-0.99). Range of motion in arms and legs showed asymmetry in FSHD. Spatial and temporal gait metrics had moderate to strong correlations to 6 minute walk test distances (Pearson correlation: r=0.76-0.96).

Conclusions: Instrumented timed functional tasks using a portable wireless gait system are simple to perform, take about 10 minutes per session, are reliable, abnormal in FSHD, and appear to be able to distinguish between participants with differing disease severities.

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33. [P]. **Combined single fiber physiology and proteomic analyses of FSHD muscle**

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The pathophysiology of Facioscapulohumeral Dystrophy (FSHD) is poorly understood and understudied. To address this need, we have developed a combined single fiber muscle physiology and proteomics platform to define the underlying molecular pathology of FSHD. Single fiber physiological methods have been utilized to assess and compare the contractile and elasticity properties of “skinned” muscle fibers prepared from surgically isolated biceps muscle from manifesting and non-manifesting FSHD subjects, unaffected family members and subjects with unrelated muscle diseases. These approaches also have been applied to xenoengrafted FSHD biceps muscle to investigate FSHD disease progression during fiber differentiation and maturation and to develop therapeutics targeted to treat FSHD disease pathology in an animal model. Disease-associated physiological processes are being correlated with quantitative disruptions in muscle proteins and isoform expression and in protein phosphorylation at the level of single fibers. Our initial studies have focused on Ca2+ sensitivity of contraction and passive force as physiological biomarkers of early FSH disease onset.

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