FSH Society Facioscapulohumeral Muscular Dystrophy [FSHD]
2014 International Research Consortium & Research Planning Meetings

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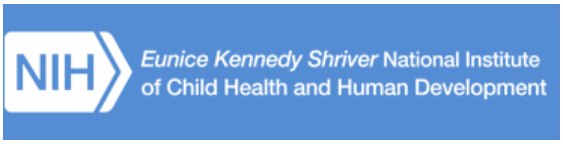

FSH Society Facioscapulohumeral Muscular Dystrophy [FSHD]
2014 International Research Consortium & Research Planning Meetings

Friday, October 17, 2014
8:00 a.m. – 6:00 p.m.
[Registration and breakfast begins 8:00 a.m.-]

Saturday, October 18, 2014
8:00 a.m. – 1:30 p.m.
[Registration and breakfast begins 8:00 a.m.-]

San Diego Marriott Marquis & Marina
San Diego Ballroom A, North Tower, Lobby Level
333 West Harbor Drive
San Diego, California 92101 USA

Co-Chairs:
Michael Altherr, PhD
Los Alamos National Laboratory, Los Alamos, New Mexico
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
Michael Altherr, PhD
David E. Housman, PhD
Stephen J. Tapscott, MD, PhD
Silvère van der Maarel, PhD

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NIH Eunice Kennedy Shriver NICHD Senator Paul D. Wellstone MDCRC for FSHD at University of Massachusetts Medical School

PREFACE

October 17, 2014
San Diego, California

Dear Colleagues,

Welcome to the FSH Society 2014 International Research Consortium & Research Planning Meetings!

This workshop brings together clinicians, scientists, 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 over the past three years and even in the past few 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 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, what are the critical gaps that remain, and where we need to focus. 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 if new areas should be considered.

This meeting is organized by the FSH Society and sponsored by the Association Française Contre les Myopathies (AFM), aTyr Pharma, Cytokinetics, FSHD Canada, FSH Society, FSHD Global Research Foundation, Genzyme, a Sanofi Company, Muscular Dystrophy Association (MDAUSA), the U.S. DHHS NIH Eunice Kennedy Shriver NICHD Sen. Paul D. Wellstone UMMS FSHD Muscular Dystrophy Cooperative Research Center. 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,

Michael Altherr, PhD
Los Alamos National Laboratory, Los Alamos, New Mexico & FSH Society Scientific Advisory Board

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

Friday, October 17, 2014

Registration & Breakfast	8:00 a.m.-9:00	
Welcome	9:00-9:05	Welcome William Lewis, Sr., Michael Altherr, Stephen Tapscott, Silvere van der Maarel
Review of 2013	9:05-9:20	**Review of 2013/2014 priorities** as stated by FSHD workshop in 2013 Moderators: Michael Altherr, Rune Frants, Stephen Tapscott
Platform Session 1	9:20-9:55	**Clinical Studies** (1x15 minutes; 2x10 minutes) Moderators: George Padberg, Rabi Tawil
	9:55-10:15	Discussion
Break	10:15-10:30	
Platform Session 2	10:30-11:30	**Genetics and epigenetics** (4x15 minutes) Moderators: Davide Gabellini, Richard Lemmers
	11:30- Noon	Discussion
Poster Introductions	Noon-1:00	Lunch and **Poster Viewing** [collect and have **Lunch**]
Platform Session 3	1:00-1:50	**Molecular mechanisms** (2x15 minutes; 2x10 minutes) Moderators: Louis Kunkel, Rune Frants
	1:50-2:15	Discussion
Break	2:15-2:30	
Platform Session 4	2:30-3:40	**Models** (4x15 minutes; 1x10 minutes) Moderators: Peter Lunt, Yi-Wen Chen
	3:40-4:00	Discussion
Break	4:00-4:15	
Platform Session 5	4:15-5:00	**Therapeutic studies** (3x15 minutes) Moderators: Lynn Hartweck, Joel Chamberlain
	5:00-5:30	Discussion
Assembly Session	5:30-6:00	**Discuss charge for Saturday's meeting to define 2014/2015 research priorities, future directions, etc.** Moderators: Michael Altherr, Rune Frants, George Padberg, Stephen Tapscott, Silvere van der Maarel
Adjourn	6:00 p.m.	

Saturday, October 18, 2014

Registration & Breakfast	8:00 a.m.-9:00	
Welcome	9:00-9:05	**Welcome** William Lewis, Sr., Michael Altherr, Stephen Tapscott, Silvere van der Maarel
Discussion and planning	9:10-12:30	**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 Saturday 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 2014/2015.

	9:10-12:20	**Discussions** Moderators: Michael Altherr and Rune Frants
	12:20-12:30	**Finalizing listing of items, areas and priorities**
Lunch	12:30 -1:30 p.m.	
Adjourn	1:30 p.m.	

NOTES ON TALKS AND POSTERS

	First Author	Presenting Author	Topic	
Platform Session 1				
Clinical Studies Chairs: George Padberg, Rabi Tawil				
9:20-9:35 a.m.	Statland	Statland	Relevant outcome measures	
9:35 -9:45 a.m.	*Smith	Smith	Testing for FSHD2 in high-volume	
9:45-9:55 a.m.	*Strom	Boyar	Molecular combing FSHD1	
9:55-10:15 a.m.			Discussion	
Platform Session 2				
Genetics and Epigenetics Chairs: Davide Gabellini, Richard Lemmers				
10:30 -10:45 a.m.	Calandra	Deidda	New methylation assay for FSHD	
10:45-11:00 a.m.	Lemmers	Lemmers	CpG methylation at D4Z4	
11:00-11:15 a.m.	Zeng	Yokomori	Role D4Z4 chromatin DUX4 ORF	
11:15 -11:30 a.m.	Balog	Balog	Epigenetic requirement of DUX4	
11:30 -Noon			Discussion	
Platform Session 3				
Molecular mechanisms Chairs: Rune Frants, Louis Kunkel				
1:00:1:15 p.m.	Gabellini	Gabellini	Molecular char. of DBE-T lncRNA	
1:15-1:30 p.m.	Eidahl	Eidahl	Protein-protein interact of DUX4	
1:30-1:40 p.m.	*Annseau	Coppee	DUX transcript. factors cytoplasm	
1:40-1:50 p.m.	*Thijssen	Thijssen	DUX4 induces FRG2	
1:50-2:15 p.m.			Discussion	
Platform Session 4				
Models Chairs: Peter Lunt, Yi-Wen Chen				
2:30-2:45 p.m.	Mariot	Dumonceaux	Early late correlation FAT1	
2:45-3:00 p.m.	Zavaljevski	Chamberlain	"On demand" model of FSHD	
3:00-3:15 p.m.	Caron	Schmidt	Human stem cell model FSHD	
3:15-3:30 p.m.	Dandapat	Hartweck	Inducible transgene encode DUX4	
3:30 -3:40 p.m.	*Morere	Magdinier	hiPSCs model FSHD1 and FSHD2	
3:40 -4:00 p.m.			Discussion	
Platform Session 5				
Therapeutic Studies Chairs: Michael Kyba, Joel Chamberlain				
4:15-4:30 p.m.	Pandey	Chen	Mol. factors that affect DUX4 expr	
4:30-4:45 p.m.	Sverdrup	Sverdrup	Inhibition BET Blocks DUX4	
4:45-5:00 p.m.	Wallace	Wallace	DUX4 targeted RNAi-therapy	
5:00 -5:30 p.m.			Discussion	
Posters [	Charron	Coppee	WNT pathway alterations	Poster
	Derenne	Derenne	method naked DNA delivery FSHD	Poster
	Kazakov	Kazakov	Dropped head in FSHD	Poster
	Lunt	Lunt	UK FSHD-Registry	Poster
	Tierney	Sacco	STAT3 signaling controls satellite cell	Poster
	Wauters	Coppee	Memb. proteins FSHD biomarkers	Poster]

Talks that are asterisked (*) indicate a shorter 10 minute presentation

Priorities as stated by FSHD Research Community for FSHD Research in 2014 at the 2013 FSH Society FSHD International Research Consortium, held October 21-22, 2013 at the David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts

Summary of Priorities and General Discussion, Tuesday October 22, 2013

DUX4

The unanimous conclusion of the general discussion was that overexpression of the toxic transcription factor Dux4 is at the root of FSHD1 and FSHD2. Expression of flDUX4 mRNA (and protein) is dependent on two conditions: 1. A specific DUX4 haplotype containing a poly-A site and 2. An open chromatin structure, due to D4Z4 repeat contraction-dependent (FSHD1) or contraction-independent (FSHD2) mechanisms, the latter due to mutations in the SMCHD1 gene encoding a chromatin modulating enzyme. There are indications for further genetic heterogeneity, thus additional gene defects causing FSHD.

The chromatin relaxation of the DUX4 region (close to the #4q telomere) can induce additional gene expression effects in cis (#4) and trans (other chromosomes).

As Dux4 is a transcription factor, the over-expression can trigger a cascade of downstream molecular pathways contributing to the large variability in the clinical phenotype and natural history of FSHD.

Conclusion: DUX4 expression is necessary but not always sufficient to cause FSHD. Research should focus on upstream and downstream molecular pathways and mechanisms as they form the most plausible intervention targets.

Disease models

The field needs improved and specific in vivo (animal) models for mechanistic and intervention studies. At this stage it is not sensible to give strict recommendations.

Inducible (conditional) models seem necessary to dissect spatial and temporal effects of the DUX4 pathway. For specific questions, simpler models, like zebra fish may have unique potential.

Various xenograph models aiming at generating human muscle in mouse muscle are promising, but strongly dependent on availability of human muscle biopsies or cell lines. In other muscle disease fields, AAV mediated “gene therapy” has proven its value. Availability of higher vertebrate models (e.g. dog, primates etc.) may be helpful to study intervention effects prior to human trials.

Intervention

Is there a magic bullet for FSHD treatment? Although the DUX4 over expression is crucial, various biological and chemical (pharma) strategies can be envisaged to intervene with the overall expression mechanism, directly by targeting the mRNA or the protein, or indirectly by modulating the transcription machinery, including the chromatin structure.

As the ultimate goal is to influence the clinical phenotype and disease progression, intervention in specific molecular subpathways of the DUX4 cascade may form alternative strategies.

Clinical studies and trial readiness.

There is an urgent need for better understanding of the natural history of FSHD, e.g. the clinical heterogeneity between and within families, the asymmetric expression of the disease, the cause and consequence of inflammation, and the effect of physical and cognitive training, etc.

The FSHD field is working hard to establish patient databases with detailed clinical and genetic information. Equally, the development of sensitive quantitative clinical monitoring methods to follow intervention trials has a high priority.

It is important to closely follow the situation in related fields, e.g. Duchenne, etc.

The 10 Priorities

- The DUX4 interactome
- Understanding DUX4 manifestation and variation
- Additional genetic heterogeneity; non-FSHD1 and 2
- Disease models
- Well documented Natural history with reliable endpoints; modulating mechanisms/genes
- Increasing data depth of patient databases with extensive (follow-up) clinical data
- Prepare for clinical trials: reliable and meaningful outcome measures; with access to discreet patient populations and disease mechanism of action classes.
- Therapy; proof-of-principle experiments
- Focus on translational research; from clinic to bench and back
- Understanding pathophysiology of FSHD: connection to DUX4, heterogeneity, asymmetry, role of inflammation; infiltrates and etiology

	first author	presenter	topic	preference
1.	Statland	Statland	Relevant outcome measures	spoken
2.	Smith	Smith	Testing for FSHD2 in high-volume	spoken
3.	Strom	Boyar	Molecular combing FSHD1	spoken
4.	Calandra	Deidda	New methylation assay for FSHD	spoken
5.	Lemmers	Lemmers	CpG Methylation at D4Z4	spoken
6..	Zeng	Yokomori	role D4Z4 chromatin DUX4 ORF	spoken
7.	Balog	Balog	Epigenetic requirement of DUX4	spoken
8.	Gabellini	Gabellini	Molecular char. of DBE-T lncRNA	spoken
9.	Eidahl	Eidahl	Protein-protein interact of DUX4	spoken
10.	Ansseau	Coppee	DUX transcript. factors cytoplasm	spoken
11.	Thijssen	Thijssen	DUX4 induces FRG2	spoken
12.	Mariot	Dumonceaux	Early late correlation FAT1	spoken
13.	Zavaljevski	Chamberlain	“On demand” model of FSHD	spoken
14.	Caron	Schmidt	human stem cell model FSHD	spoken
15.	Dandapat	Hartweck	inducible transgene encode DUX4	spoken
16.	Morere	Magdinier	hiPSCs model FSHD1 and FSHD2	spoken
17.	Pandey	Chen	Mol. factors that affect DUX4 expr	spoken
18.	Sverdrup	Sverdrup	Inhibition BET blocks DUX4	spoken
19.	Wallace	Wallace	DUX4 targeted RNAi-based therapy	spoken
20.	Charron	Coppee	WNT pathway alterations	poster
21.	Derenne	Derenne	method naked DNA delivery FSHD	poster
22.	Kazakov	Kazakov	Dropped head in FSHD	poster
23.	Lunt	Lunt	UK FSHD-Registry – first 16 mos.	poster
24.	Tierney	Sacco	STAT3 signal controls satellite cell	poster
25.	Wauters	Tassin	Memb. proteins FSHD biomarkers	poster

1. **Clinically Relevant Outcome Measures for Facioscapulohumeral Muscular Dystrophy**

Jeffrey Statland, MD [1], Katy Eichinger, PT [2], Colleen M. Donlin-Smith, MA [2], Chad Heatwole, MD [2], and Rabi Tawil, MD [2]

1 University of Kansas Medical Center, Kansas City, Kansas
2 University of Rochester Medical Center, Rochester, New York

Background: As we move towards investigating new therapeutics for Facioscapulohumeral muscular dystrophy (FSHD) it is important to have the tools in place to conduct efficient, high quality clinical trials. Different outcome measures will be important at different phases in the drug development process: biomarkers for early phase and proof of concept studies, and functional and patient-reported outcomes for later phase studies.

Methods: We are conducting a prospective 12 month observational study in 40 genetically confirmed FSHD participants to determine the reliability, concurrent validity, and responsiveness to FSHD disease progression of 1) an FSHD-specific patient-reported outcome measure; 2) an FSHD-specific clinical outcome measure (FSH-COM); and 3) a non-invasive biomarker of muscle structure, electrical impedance myography (EIM). Here we present baseline data (n=20) for the FSH-COM and EIM. Reliability is determined by the intra-class correlation coefficient (ICC). The relationships between different clinical measures were performed using Pearson correlation coefficient (r). The minimally detectable change is defined as the smallest change at which we are 90% confident the change is not due to measurement error (MDC90). Sample size estimates were for two independent samples with equal variance, two tailed, at 80% power and a level of significance of 0.05.

Results: The FSH-COM shows excellent reliability with an intra-class correlation (ICC) coefficient of 0.97 (95% lower confidence limit [LCLM] 0.92). EIM showed reliability range (ICC) between 0.76-0.99 for major muscle groups, but was less reliable for facial muscles. The FSH-COM has moderate to strong linear relationships to clinical severity scores (clinical severity score r=0.67, P=0.002; FSHD clinical score r=0.78, P<0.001). EIM reactance measurement at 100 kHz of the right biceps (EIM RB) showed moderate to strong relationships to the FSH-COM (r=-0.52, P=0.03), the FSHD clinical score (r=-0.67, P=0.002), and time to doff or don a jacket (r=-0.51, P=0.02). The MDC90 for the FSH-COM is 4.8, this represents a 20.5% change from the baseline mean. The number of patients needed per treatment group to detect a difference at least as large as the MDC90 in the FSH-COM is 100 per group. The number of patients needed to detect a similar %change in the baseline EIM RB is 21 per group.

Conclusion: Initial evaluation of baseline data shows excellent reliability for the FSH-COM. EIM shows acceptable to excellent reliability for major muscle groups. Both demonstrate concurrent validity when compared to related FSHD disease measures. EIM for individual muscles (e.g. right biceps) shows potential sample size efficiency for early phase studies.

Study supported by FSH Society grants (FSHS-22013 and FSHS-82012), and National Institutes of Health (NIAMS) U01AR065119 Development of a Clinically Relevant Outcome Measure for FSHD Therapeutic Trials. JS support provided by KUMC CTSA award KL2TR000119.

2. **Testing for FSHD2 in a high-volume FSHD diagnostic service setting: pyrosequencing-based hypomethylation assay and *SMCHD1* sequencing - the Bristol experience**

Debbie Smith, Jennifer Whitfield, Rebecca Whittington, Susanne O'Shea, Peter Lunt, Maggie Williams

Bristol Genetics Laboratory, Southmead Hospital, Bristol, BS10 5NB, United Kingdom

Debbie.Smith@nbt.nhs.uk

Bristol Genetics Laboratory has provided a UKGTN (United Kingdom Genetics Testing Network) specialist diagnostic service for FSHD since 1992, and processing over 400 referrals annually (diagnostic, exclusion, predictive and prenatal referrals) from the UK and abroad. In a seven year audit of 2047 diagnostic FSHD referrals, 37% showed a contraction of the D4Z4 repeat at 4q35 including 10 individuals with two pathogenic 4qA fragments, where further haplotyping was required. 111 patients were referred for extended deletion testing and a deletion of the p13E-11 probe region was confirmed in 4 patients. The clinically typical negative patients (4.8% of BGL diagnostic referrals) are candidates for FSHD2 testing.

Building on the work of Lemmers *et al.* (2012) and Hartweck *et al.* (2013), we have developed a novel pyrosequencing assay for quantification of methylation using 10 sites within DR1. A pilot UK cohort of 15 clinically typical FSHD1 negative patients (assessed by clinical proforma) and recruited/consented by geneticists/neurologists were assessed for hypomethylation by bisulphite conversion and pyrosequencing, followed by *SMCHD1* sequencing. 9/15 patients had hypomethylation (defined by a 42% methylation cut off level) and candidate *SMCHD1* mutations were identified in all 9 patients (3 missense, 1 non-sense, 1 duplication, 2 deletions, 2 potential splice site). 3 patients without hypomethylation were sequence negative. Family studies are on-going.

The pilot study has informed diagnostic service provision and a UKGTN gene dossier application has supported successful commissioning of testing for FSHD2 for the UK from April 2014 facilitating direct translation of this work into clinical service. A further 20 patients have been tested for FSHD2, including 3 requests for predictive testing.

We present our pyrosequencing assay method and the results of the FSHD2 cohort study, set in the context of an audit of FSHD testing, including some interesting cases which highlight the clinical utility of the testing pipeline in unlocking the complexity for diagnostic interpretation which can sometimes be encountered.

3. **Molecular Combing for Facioscapulohumeral Dystrophy Type 1: Benefits of Direct Visualization of DNA Fibers**

C.M. Strom[1], J.C. Wang[1], X.J. Yang[1], B.H. Nguyen[1], V. Sulcova1, P. Chan[1], Y. Liu[1], A. Anguiano[1], J.J. Higgins[2], F.Z. Boyar[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 subtelomere of chromosome 4q. Two common haplotypes exist at the 4q locus: 4qA and 4qB. D4Z4 macrosatellite repeat motifs are also present on chromosome 10q (10qA and 10qB). Differentiation among alleles is important for diagnosis, as contractions of the 4qA but not the 4qB, 10qB, or 10qB allele are associated with FSHD.

Prior to the advent of molecular (DNA) combing, molecular diagnosis relied on a series of pulsed-field Southern blots. Molecular combing is a technique in which DNA is uniformly stretched and then hybridized with gene-specific probes of various colors to create a molecular bar code. We developed and validated a molecular combing test to identify and measure contractions of the 4qA allele.

Here we report the first 44 suspected FSHD cases tested with the molecular combing assay in our laboratory. In all 44, we were able to unambiguously identify all 4q and 10q alleles and determine the size of the D4Z4 repeat. Of the 44 samples, 13 (30%) were clearly affected, with 4qA repeat sizes of 3 to 8; 28 (63%) were clearly normal, with 4qA repeat sizes of 12 to 68; and 3 (7%) had borderline results, with 4qA repeat sizes of 10 to 11. Two patients had a 4qB contraction and 15 had 10qA or 10qB contractions.

We conclude that a molecular combing assay for FSHD is capable of determining the 4qA repeat size in clinical samples and differentiating the 4qA allele from 4qB, 10qA, and 10qB alleles.

Since the molecular combing assay reports the copy number of 4qA repeat, it will allow correlation of repeat number with the timing of disease onset.

4. **Development of a new methylation assay for FSHD diagnosis**

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

[1] Institute of Cell Biology and Neurobiology C.N.R.- Rome Italy
[2] Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands.
[3] Institute of Neurology, Catholic University School of Medicine, Rome, Italy.

FSHD is linked to chromosomal 4q35 region, that contains a D4Z4 array of up to 200 units. The most common form, autosomal dominant FSHD1, is caused by a contraction of the 4q D4Z4 array to less than 11 units, whereas FSHD2 is caused by reduced levels of functional SMCHD1 protein (Structural maintenance of chromosomes flexible hinge domain-containing 1). Although with different mechanisms, both genetic defects lead to DNA hypomethylation at D4Z4 on 4qter causing chromatin relaxation. This genomic modulation provides a transcriptionally permissive chromatin environment that is associated with the expression of DUX4 gene, the best candidate FSHD gene, enclosed within each D4Z4 unit. DUX4 expression requires also the presence of a polyadenylation signal (PAS) distal to the last D4Z4 unit that stabilizes the DUX transcript. There are two different allelic forms of the region distal to the D4Z4 array, A and B. Although a D4Z4 array followed by an A "Telomere" is also present on 10q, a functional PAS sequence has been identified almost exclusively on 4qA alleles.

Ultimately, vast majority of FSHD1 and FSHD2 subjects show hypomethylation at D4Z4 region associated with an A allele containing a functional PAS. Currently, FSHD diagnosis is based on the identification of shortened D4Z4 4q arrays (in FSHD1) or the presence of mutations in SMCHD1 (in FSHD2) and the assessment of the A/B genotype of 4q alleles and eventually by methylation analysis at the D4Z4 units.

Our study aims at developing a new diagnostic test based on the methylation analysis of CpGs in the region distal to the D4Z4 array.

Indeed, we considered that DNA methylation changes in this distal region may correlate with DUX4 expression more effectively than changes in the proximal units. Therefore, we evaluated the relationship between the DNA methylation, disease status, repeat size and clinical severity by analyzing CpGs located in the close proximity of the polyadenylation signal. We have been able to design bisulfite sequencing assays that allow the analysis of 10 CpGs in the close proximity of the PAS. Results using a 4qA PAS-specific assay in a group of 38 FSHD1, 14 FSHD2 and 18 control samples show significant differences between affected and unaffected subjects, supporting the potential usefulness of this assay as a diagnostic tool for FSHD diagnosis.

5. **Inter-individual Differences in CpG Methylation at D4Z4 Correlate with Clinical Variability in FSHD**

Richard JLF Lemmers[1], Jelle J Goeman[2], Patrick J van der Vliet[1], Merlijn P van Nieuwenhuizen[3], Judit Balog[1], Sabrina Sacconi[4], George W Padberg[5], Stephen J Tapscott[6], Rabi Tawil[7], Bert Bakker[3], Silvère M van der Maarel[1]

[1] Department of Human Genetics, Leiden University Medical Center, Leiden, Netherlands
[2] Biostatistics, Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands
[3] Department of Clinical Genetics, Leiden University Medical Center, Leiden, Netherlands
[4] Centre de référence des Maladies neuromusculaires and CNRS UMR6543, Nice University Hospital, Nice, France
5 Neurology, Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands
[6] Division of Human Biology, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA
[7] Department of Neurology, University of Rochester, Rochester, NY, USA

FSHD is caused by contractions of the D4Z4 repeat array on chromosome 4 to 1-10 units (FSHD1), or by mutations in the D4Z4-binding chromatin modifier SMCHD1 (FSHD2). Both situations lead to a partial opening of the D4Z4 chromatin structure, and facilitate transcription of D4Z4-encoded polyadenylated DUX4 mRNA in skeletal muscle. The opening of the chromatin structure is marked by a partial loss of CpG methylation in the promoter region of the *DUX4* gene. Clinically, FSHD is marked by a high inter- and intra-familial variability that is largely unexplained. This clinical variability can be seen in both FSHD1 and FSHD2, suggesting a role of other factors besides the contracted D4Z4 repeat array or the SMCHD1 mutation.

We measured CpG methylation at the FseI site of D4Z4 in a large cohort of control, FSHD1 and FSHD2 individuals and found a significant correlation with the D4Z4 repeat array size. After correction for repeat array size, we show that the variability in clinical severity in FSHD1 and FSHD2 individuals correlates with the level of D4Z4-CpG methylation. In FSHD1, for individuals with D4Z4 repeat arrays of 1-6 units, the clinical severity mainly depends on the size of the D4Z4 repeat. However, in individuals with arrays of 7-10 units, the clinical severity also depends on other factors that regulate D4Z4 methylation since affected individuals, but not non-penetrant mutation carriers, have a greater reduction of D4Z4-CpG methylation than can be expected based on the size of the pathogenic D4Z4 repeat array. In FSHD2 this epigenetic susceptibility depends on the nature of the *SMCHD1* mutation in combination with D4Z4 repeat array size with dominant negative mutations being more deleterious than haploinsufficiency mutations. With this concept of epigenetic susceptibility, we are uncovering the basis of the marked variability in disease onset and progression.

6. **Genetic and epigenetic characteristics of FSHD-associated 4q and 10q D4Z4 that are distinct from non-4q/10q D4Z4 homologs**

Weihua Zeng,[1,2*] Yen-Yun Chen,[1*] Daniel A. Newkirk,[1] Beibei Wu,[3] Judit Balog,[4] Xiangduo Kong,[1] Alexander R. Ball, Jr.,[1] Simona Zanotti,[5] Rabi Tawil,[6] Naohiro Hashimoto,[7] Ali Mortazavi,[2] Silvère M. van der Maarel,[4] and Kyoko Yokomori[1§]

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Facioscapulohumeral dystrophy (FSHD) is one of the most prevalent muscular dystrophies. The majority of FSHD cases are linked to a decreased copy number of D4Z4 macrosatellite repeats on chromosome 4q (FSHD1). Less than 5% of FSHD cases have no repeat contraction (FSHD2), most of which are associated with mutations of SMCHD1. FSHD is associated with the transcriptional derepression of DUX4 encoded within the D4Z4 repeat, and SMCHD1 contributes to its regulation. We previously found that the loss of heterochromatin mark (i.e. histone H3 lysine 9 tri-methylation (H3K9me3)) at D4Z4 is a hallmark of both FSHD1 and FSHD2. However, whether this loss contributes to DUX4 expression was unknown. Furthermore, additional D4Z4 homologs exist on multiple chromosomes, but they are largely uncharacterized and their relationship to 4q/10q D4Z4 was undetermined. We found that the suppression of H3K9me3 results in displacement of SMCHD1 at D4Z4 and increases DUX4 expression in myoblasts. The DUX4 open reading frame (ORF) is disrupted in D4Z4 homologs and their heterochromatin is unchanged in FSHD. The results indicate the significance of D4Z4 heterochromatin in DUX4 gene regulation and reveal the genetic and epigenetic distinction between 4q/10q D4Z4 and the non-4q/10q homologs, highlighting the special role of the 4q/10q D4Z4 chromatin and the DUX4 ORF in FSHD.

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7. **Epigenetic requirement of DUX4 expression in muscle cells**

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Recent studies have identified DUX4 (Double Homeobox 4) as a key protein in the pathomechanism of FSHD. DUX4 is a germline transcription factor and its ectopic expression in muscle initiates germline and early developmental transcriptional programs, which eventually lead to muscle cell death. The typical bursts of DUX4 expression in a subset of myonuclei are associated with local chromatin relaxation is caused either by contraction of the D4Z4 macrosatellite array to a size of 1-10 units (FSHD1), or by mutations of SMCHD1 (FSHD2). Mouse Smchd1 was first identified in a ENU mutagenesis screen as a factor involved in repeat mediated epigenetic silencing. We aimed to elucidate the epigenetic mechanism leading to DUX4 transcription during in vitro myoblast differentiation. We performed expression analysis by RT-qPCR of 6 control, 5 FSHD1 and 9 FSHD2 myoblast-myotube pairs and determined the D4Z4 histone profile by ChIP-qPCR. Next we examined the effect of modulation of known D4Z4 chromatin repressors including SUV39H1, cohesion components SMC3 and RAD21, and SMCHD1 in control myoblasts on DUX4 expression. Our results are supportive of a model where SMCHD1 is a strong epigenetic regulator of DUX4 in muscle cells.

8. **Molecular characterization of DBE-T lncRNA driving FSHD muscular dystrophy**

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Up to two thirds of the human genome is composed of repetitive sequences, which are dynamically transcribed producing a vast pool of non protein-coding RNA (ncRNA) molecules. We contributed to this field with the discovery of DBE-T, the first activating long ncRNA (lncRNA) involved in a human genetic disease: facioscapulohumeral muscular dystrophy (FSHD).

FSHD, one of the most important neuromuscular disorders, is an autosomal dominant disease with a strong epigenetic component. The disease is associated with a reduced copy number of the D4Z4 macrosatellite repeat. We found that DBE-T is a chromatin-associated lncRNA that mediates a Polycomb to Trithorax epigenetic switch at the FSHD locus, driving chromatin remodeling and transcription of FSHD candidate genes.

Here, I will summarize our recent results regarding the dynamic expression of DBE-T during hESC differentiation, in FSHD mouse models and in FSHD patients. The regulation of DBE-T expression by D4Z4 copy number, the mechanism responsible for DBE-T tethering to chromatin and the identification of DBE-T functional domains will be also discussed.

9. **Protein Chemistry and Protein-Protein Interactions of DUX4**

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The ultimate goal of our research is to develop treatment strategies for Facioscapulohumeral muscular dystrophy (FSHD). With the emergence of DUX4 as an important pathogenic insult underlying FSHD, we now have a viable target for therapy. Nevertheless, therapy development is still burdened by the lack of knowledge about how DUX4 contributes mechanistically to FSHD onset. Defining the biology and biochemistry of DUX4 as it relates to FSHD is a critical unmet need in the field. DUX4 contains two N-terminal homeodomains, which are responsible for its ability to bind specific DNA sequences. The DUX4 transactivation domain, consisting of C-terminal residues 160-424, is essential for inducing muscle toxicity, however the mechanisms by which these residues mediate DUX4 activity are unknown. Our central hypothesis is that the DUX4 transcription factor is involved in protein-protein interactions, or subject to post-translational modifications (PTMs), that influence its ability to induce toxicity in muscle cells and ultimately contribute to FSHD. We propose that the DUX4 C-terminal domain is involved in the recruitment of proteins that influence its ability to transactivate normal and toxic genes. We are examining DUX4's preference for particular protein domains, as well as its ability to act as a ligand for known protein interaction domains. In preliminary data, we identified several candidate DUX4-interacting proteins and multiple PTM-modified DUX4 residues. Our goal here is to study DUX4 protein chemistry and identify critical interactions between DUX4 and its candidate binding partners. Ultimately we hope to establish a framework for developing therapeutic strategies that disrupt critical DUX4 protein interactions that may be required for its toxic effects in FSHD muscles.

10. **Unexpected role for DUX transcription factors into the cytoplasm**

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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, a potent transcription factor that causes the FSHD muscular dystrophy. In the present study we identify skeletal muscle partners of either DUX4, DUX4c (an identical protein except for the end of the carboxyl-terminal domain) or DUX1 (limited to the double homeodomain). We first used the yeast two-hybrid system (Y2H) to screen an adult human skeletal muscle cDNA library with DUX1 or DUX4c that presented no or weak transcriptional activity in this system. We also expressed HaloTag-DUX4 or DUX4c fusion proteins in human muscle cells, co-purified them by affinity chromatography with their protein partners and identified these by mass spectrometry. We unexpectedly found desmin, an intermediate filament protein, other cytoskeletal proteins involved in actin-bundling or myofibrilar organization besides RNA-binding proteins involved in muscle-specific mRNA splicing and translation. Most of these partners play major roles in cytoskeletal organization during muscle differentiation. We confirmed DUX interaction with desmin as well as other partners involved in mRNA splicing by in vitro GST pull down, immunoprecipitation or by in situ proximal ligation assay in muscle cells. The functionality of these interactions was underscored by the observation of DUX4/DUX4c nucleo-cytoplasmic translocation upon myoblast fusion. Moreover, DUX4c and desmin immunostaining partially co-localized in transversal muscle sections of patients affected with Duchenne Muscular Dystrophy.

Our data suggest cytoplasmic functions for the numerous DUX gene products and identify novel toxicity pathways besides transcription activation for DUX4/DUX4c in FSHD muscles.

11. **DUX4 induces FRG2 expression by directly activating its promoter in facioscapulohumeral muscular dystrophy**

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Based on a chromatin spreading or looping model, several genes centromeric to the D4Z4 repeat array have been considered as FSHD candidate genes. Initial reports showing deregulation of some of genes, including *FRG1* and *ANT1*, could not be confirmed in independent studies. However, *FRG2,* located most proximal to the D4Z4 macrosatellite repeat, was reproducibly shown to be induced in differentiating myoblast cultures derived from FSHD patients. However, the function of FRG2 and its possible role in FSHD pathogenesis has remained unresolved. Sporadic activation of the DUX4 retrogene, encoded within the D4Z4 macrosatellite repeat array, has emerged as the most likely disease model over the recent years. DUX4 functions as a double homeobox transcription factor and activates a specific set of genes upon expression in muscle cells. We now show that FRG2 is one of the direct target genes of DUX4 protein. Overexpression of DUX4 in proliferating primary myoblasts or fibroblasts results in a significant activation of FRG2 transcription. Moreover, we identified DUX4 binding at the FRG2 promoter by chromatin immunoprecipitation followed by deep sequencing. We confirmed the transactivation effect of DUX4 on the FRG2 promoter by luciferase reporter assays. We observed a significant increase of luciferase expression upon co-transfection of DUX4, which was completely ablated upon removal of the DUX4 binding sites in the FRG2 promoter sequence. In conclusion we show that the long known FSHD specific upregulation of FRG2 can be explained by the protein activity of DUX4, emphasizing its central role in FSHD pathogenesis

12. **Selectivity of early and late affected muscles in FSHD is correlated with *FAT1* expression, which is independent of DUX4**

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Facio-Scapulo-Humeral muscular Dystrophy (FSHD) is one of the most common inherited muscular diseases and is linked to either contraction of D4Z4 repeats on chromosome 4 or to mutations in the SMCHD1 gene on chromosome 18, leading in most cases, to the aberrant expression of the transcription factor DUX4. However, it remains difficult to correlate these genotypes with the phenotypes observed in patients such as atrophy of specific muscle groups, asymmetry, as well as non-muscular symptoms. Because we have recently shown that a transgenic mouse with a down-regulated expression of *Fat1* recapitulates most of the phenotype observed in FSHD, we investigated expression of the human *FAT1* gene in FSHD. We observed that *FAT1* is expressed at lower levels in both adult FSHD1 and FSHD2 muscles as compared to control muscles. Remarkably, *FAT1* expression level does not seem to be driven by DUX4 since (i) *DUX4* mRNA down-regulation mediated by shRNA does not influence *FAT1* expression levels and (ii) levels of *FAT1* mRNA were identical when comparing myotubes from

contracted and non-contracted clones isolated from mosaic patients, suggesting that the reduced level of *FAT1* seen in FSHD patients is not linked to DUX4 expression, but rather to an individual intrinsic expression level. Finally, by comparing the endogenous expression level of *FAT1* mRNA in 64 muscle biopsies isolated from 16 control fetuses, we observed lower expression levels of *FAT1* in the muscles which represent early targets in FSHD patients compared to muscles affected at later stages, strongly suggesting a major role of FAT1 in targeting the muscle groups early affected in FSHD. We propose a new model for FSHD in which FAT1 levels may play a role in determining which muscles will exhibit early and late onset whereas DUX4 may rather slowly worsen the muscle phenotype.

13. **Development of an "on demand" model of FSHD in adult muscle**

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Contraction of the D4Z4 macrosatellite repeat on chromosome 4q35 results in low-level DUX4 mRNA expression in skeletal muscles and is required for FSHD1 disease manifestation. DUX4 protein has not been detected in FSHD muscle, although its low-level expression is assumed to be the catalyst of pathophysiology. Reproducing the presumably low DUX4 protein expression in mice to recapitulate the phenotypic features of FSHD in adult mouse muscle has proven challenging. We have developed an adeno-associated viral vector (AAV) that can deliver a DUX4 gene expressed from its own promoter to the muscles of wild type mice. In order to further characterize this model, which could enable "on demand" phenotypic induction via AAV DUX4, we refined virus production methods for reproducibility and also developed a method for DUX4 localization in cryosections. Histological analyses of injected muscles revealed AAV DUX4 dose dependent tissue damage with ultrastructural properties similar to FSHD muscle, including focal lesions, split fibers, and regions lacking muscle fibers. Reduction of the level of empty vector particles in the AAV preparations limited background damage from viral proteins. Injection of AAVs expressing a mutant DUX4 protein did not lead to muscle damage, nor did injections of vectors lacking genomes. Our results indicate that AAV delivery of DUX4 can be used to generate a reproducible mouse model, both for understanding the downstream effects of DUX4 expression and for use in testing candidate therapies aimed at preventing and reversing the muscle damage.

14. **A human stem cell model of facioscapulohumeral muscular dystrophy (FSHD)**

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Facioscapulohumeral muscular dystrophy (FSHD) presents a major unmet clinical need with no specific treatments being available. While significant progress was made in recent years to understand the complex genetics and epigenetics of the disease, research and drug discovery efforts would benefit from better model systems that overcome limitations of animal models and biopsy material. In order to develop an accurate human, scalable and consistent cellular disease model we created 4 human embryonic stem cell lines from donated pre-implantation genetic diagnosis (PGD) embryos carrying the FSHD type 1 chromosomal deletion. We previously reported that we used several rounds of high-content screening to establish a unique 3-step protocol for the differentiation of human pluripotent stem cells to skeletal muscle. To our knowledge, this is the world's first process that allows simple and robust industrial-scale cell production, achieving high yields of functional myotubes without any cell sorting, selection or genetic manipulation. We investigated the muscle phenotypes from 3x FSHD, 1x Becker muscular dystrophy and 3 normal control cell lines. FSH cell lines formed muscle at similar efficiencies than normal controls while Becker stem cells showed impaired differentiation and disorganized myotube morphology. However, we demonstrated exclusive expression of Dux4 in FSH-affected myotubes and found consistent morphological differences indicating atrophy and reduced myoblast fusion. We further analyzed expression profiles by mRNA microarrays and demonstrated profound changes to genes regulating the cell cycle, myogenesis and extracellular matrix interactions. Based on these results we are now developing a phenotypic high-content screening assay for drug development, and we believe that our unique cellular model will be a useful resource for FSHD and other muscular dystrophy research.

15. **A new mouse model for FSHD**

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To understand the effect of DUX4 in vivo, and to generate a model in which to test anti-DUX4 therapeutics, we have introduced a doxycycline (dox)-inducible transgene encoding DUX4 and 3′ genomic DNA into a euchromatic region of the mouse X chromosome. Although this mouse was intended to provide doxycycline-inducible phenotypes, through expression of DUX4, we found that the transgene alone, without dox, was lethal in males. Females were runted and presented a skin pathology in the absence of dox. This is due to low level leaky expression of the DUX4 gene, as transcript can be detected by PCR in most tissues with the most consistent detection in neural tissues including retina, and in testis. We could not detect the protein in the absence of dox. Although males are not usually born live, occasional males survive to term. These animals are much more severely affected than their female counterparts, and remarkably, display a retinal vascular pathology similar to that seen in FSHD. In addition, they show a defect in spermatogenesis. Muscles from these animals were composed of smaller fibers and were weaker, but proportionally so, and no dystrophy was present prior to death, invariably before 6 weeks of age. We performed ex vivo culture experiments with myoblasts and muscle fibroadipogenic progenitors (FAPs) isolated from the iDUX4 males, and found that high levels of dox caused cell death of myoblasts and was growth inhibitory to fibroblasts. Low non-toxic levels of dox impaired differentiation of myoblasts in vitro, but had no effect on adipogenic differentiation of muscle FAPs. To evaluate the effect of DUX4 expression during regenerative repair in vivo, we crossed the Pax7-ZsGreen satellite cell marker into this strain and performed transplants of satellite cells from live-born males into female NSG-mdx4Cv mice,

and treated recipients with varying doses of dox. By using dystrophin staining to quantify engraftment, we observed a consistent and easily quantifiable dose-dependent reduction in regeneration by transplanted satellite cells. These data show that DUX4 expression, even at low levels, is inhibitory to regenerative myogenesis. We further propose that the quantification of donor-derived myofibers generated by transplanted iDUX4 satellite cells can serve as a useful in vivo test system for therapeutics directed against the DUX4 protein or transcript.

16. **Creation of a repertoire of human induced pluripotent stem cells for modeling type 1 and 2 Facio-Scapulo-Humeral Dystrophy**

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Abstract

Human induced pluripotent stem cells provide a valuable platform for investigating disease mechanism in vitro. In this context, we created a repertoire of induced pluripotent stem cells from patients clinically affected with Facio-Scapulo-Humeral Dystrophy. In most cases (95%), this pathology is linked to shortening of an array of macrosatellite elements at the distal 4q35 locus. In 2-3% of patients, the pathology is associated with mutation in the SMCHD1 gene while in the remaining 2-3%, the cause of the pathology remains undetermined. At the molecular level, the pathology is associated with hypomethylation of the D4Z4 repetitive sequence and activation of the DUX4 retrogene.

We have produced and characterized a collection of hiPSCs clones corresponding to the different types of patients with FSHD and investigated the D4Z4 methylation dynamics and DUX4 induction after reprogramming and differentiation. In parallel, we delopped a rapid and efficient method for differentiation toward the skeletal muscle lineages. Implication for disease modeling will be discussed. In conclusion, this repertoire provides us with new tools for investigating D4Z4 pathogenesis and the development of therapeutic or testing of therapeutic strategies.

17. **Molecular factors that affect DUX4 expression and phenotypes of FSHD muscle cells in culture**

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Previous studies have reported various phenotypes of FSHD muscle cells in culture, which include those that are indistinguishable from healthy control myoblasts as well as abnormal phenotypes, such as a necrotic appearance of undifferentiated myoblasts and atrophic/disorganized phenotypes of differentiating myotubes. In this study, we investigated effects of several factors that can potentially affect DUX4 expression and/or myotube phenotypes in culture. These include serum and steroid that have been commonly used in cell culture; and few additional pharmaceutical and natural compounds. The factors tested were 1) serum and serum replacer (KOSR) at different concentrations; 2) steroids, including dexamethasone and VBP15 (a steroidal analogue which retains anti-inflammatory activity but lack adverse GRE-mediated transactivation); 3) three polyphenols (fisetin, punicalagin and gallotannin) which can potentially affect DUX4 expression and/or myotube phenotypes. Our findings showed that the concentration of the serum replacer affected DUX4 expression and myotube phenotypes. Both dexamethasone and VBP15 suppressed DUX4 expression in culture. The DUX4 suppressive effect of fisetin was in a dose-dependent manner. Fisetin showed suppressive effects of DUX4 expression in all five concentrations tested, including 10 µM (-1.2 fold, $p<0.05$), 50 µM (-1.6 fold, $p<0.01$), 200 µM (-5.1 fold, $p<0.01$), 500 µM (-8.3 fold, $p<0.01$) and 800 µM (-10.4 fold, $p<0.01$). The fisetin (tested at 50 µM) also showed a positive effect on myotube differentiation. Finally, there were dose-dependent effects on myotube differentiation in cells treated with punicalagin and gallotannin. The results show that culture conditions significantly affect the expression of DUX4 and myotube phenotypes. The study also identified pharmaceutical agents and natural compounds that modulate expression of DUX4 and improve myotubes phenotypes, which can be further tested for their therapeutic potentials for treating FSHD.

18. **Inhibition of BET Proteins Blocks DUX4 Expression FSHD Muscle Cells**

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Facioscapulohumeral Musclular Dystrophy (FSHD) is characterized by epigenetic changes resulting in the aberrant expression of the DUX4 gene in muscle. The goal of this research is to identify druggable targets for the promotion of epigenetic silencing and/or prevention of DUX4 expression. We have identified the bromodomain and extra-terminal (BET) family of proteins as key targets involved in DUX4 expression. Selective inhibitors of BET proteins (BETi) block expression of DUX4 and its downstream targets in FSHD myoblasts and myotubes. To evaluate the therapeutic potential of BETi, we have analyzed the expression of BET family protein in normal and FSHD muscle cells in vitro and used siRNA knockdowns to determine which family member is most critical for DUX4 expression. In FSHD1 and FSHD2 myoblasts, BRD4 appears to play the dominant role in sustaining DUX4 expression. In addition, we have explored the relationship between exposure of FSHD muscle cells to BET inhibitors and the transcriptional response of DUX4 and its targets as well as specific markers of myoblast and myotube cell lineages. A 24 hour exposure to BETi results in sustained (>72h) decreases in DUX4 target gene expression while allowing recovery of lineage-specific gene expression in FSHD myoblasts (e.g. MYOD1) and myotubes (MYH2). This information is being applied to pharmacokinetic evaluation of BETi in mice with the goal of providing sustained muscle exposures predicted to elicit decreased DUX4 expression in a mouse model of DUX4 de-repression based on in vitro exposure-response relationships.

19. **Developing a DUX4-targeted RNAi-based gene therapy for FSHD**

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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. In these studies an artificial microRNA targeted near the Hox region of DUX4 was co-delivered with pathogenic levels of DUX4 via adeno-associated viral (AAV) vectors. The treatment effectively reduced DUX4-induced toxicity including prevention of fiber degeneration and loss of grip strength in mice. The goal of this current study is to continue down a pre-clinical path by addressing three major concerns of the first generation miDUX vector including 1) removal of the GFP reporter gene, 2) including a second miRNA specifically targeting full length DUX4 in case DUX4 short is necessary for normal function, and 3) determine the safety of miRNA therapy to muscle by delivering high doses for toxicity studies. These continued studies provide necessary data for translating an RNAi-based therapy for FSHD.

20. [P] **WNT pathway alterations in FSHD**

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DUX4 is a potent transcription factor that initiates a large gene deregulation cascade in FSHD muscle cells but most of the cellular pathways causing the pathology are still unknown. In addition, the homologous DUX4c protein is also induced in FSHD and probably contributes to the pathology. Different non-muscular symptoms have been reported in patients affected with FSHD which could be related to perturbations of the canonical/beta-catenin and/or the non-canonical planar cell polarity (PCP) WNT pathways (1). These pathways play essential roles in muscle development and regeneration.

In the present study, we analyzed whether the WNT pathway was altered in FSHD muscle cells. We first observed activation of a reporter gene for the canonical WNT pathway in FSHD and in DUX4- or DUX4c- overexpressing myoblasts. We then profiled mRNA expression for 84 genes related to the WNT pathway in control and FSHD proliferating or differentiating myoblasts. In healthy individuals, we identified new genes involved at the onset of muscle differentiation. The FSHD myotube present either an atrophic (aFSHD) or a disorganized (dFSHD) phenotype in a proportion that differs from one primary myoblast line to another. The aFSHD myoblasts presented the strongest WNT pathway mRNA deregulations with decreases in mRNAs involved in proliferation and in the non-canonical PCP pathway (CCND1, AP1 complex, etc.). In contrast, the dFSHD myocytes/myotubes presented the strongest WNT pathway mRNA deregulations with increases in mRNAs encoding antagonists or involved in cell proliferation (FRZB, CCND1, etc.) and decreases in mRNAs encoding agonists such as WNT10A. Some of the deregulated genes were confirmed at the protein level. Following myoblast treatment with an siRNA against beta-catenin the aFSHD myotubes formed upon differentiation were enlarged (similar to dFSHD) while the dFSHD myotubes were thinner (similar to aFSHD).

In conclusion, we identified WNT pathway perturbations in both FSHD myotubes phenotypes but even if some WNT components were similarly altered (agonists/antagonists, receptors/co-receptors, co-regulator of β-catenin transcriptional activity) we also identified several ones presenting different changes between aFSHD and dFSHD myoblasts and myotubes. Targeting the WNT pathways therefore seems an attractive target in therapeutic strategies for FSHD with the caution that the most important components to target still have to be identified and will most probably differ according to the pathological myotube phenotype.

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21. [P]. **Comparison of two methods of naked DNA delivery in mice: which one is the most appropriate in the FSHD context?**

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Potential therapeutic agents (AOs, siRNA) we have developed with the aim to inhibit DUX4 expression in muscles are promising and could contribute to the development of therapeutic approaches for FSHD (1). The development of animal models to assess these antisense agent toxicity and efficacy constitutes a next step towards clinical trials for FSHD. However no transgenic mouse expressing DUX4 and presenting a myopathy is available. A transgenic mouse that carries the D4Z4 repeat array from an FSHD allele or a control allele was described. However these mice do not develop a myopathy (2). Another mouse model with a doxycycline-inducible DUX4 transgene on the X chromosome presents muscle differentiation and retina defects typical of FSHD but high male mortality makes it difficult to use for AO screening (3). A different murine model is obtained by injection of a viral expression vector for DUX4 (AAV.DUX4) in the TA (Tibialis Anterior) muscle. This procedure causes a local myopathy with several pathological features of FSHD (4). However, the development of alternative models avoiding the use of a viral vector remains necessary.

Models based on naked DNA injection have several advantages as regards to other existing models (ease of naked DNA production, reproducible transgene expression pattern, no immune response following repeated injections). Therefore, as a non-transgenic, non-viral alternative for in vivo models, we have established methods allowing the expression of a transgene into muscles of the lower limb in the mouse. In the present study, two routes of administration were confronted for the first time. Naked DNA has been injected either (i) hydrodynamically into the saphenous vein of the limb transiently isolated by a tourniquet (IVHD; 5-6) or (ii) intramuscularly in the TA, followed by an electroporation (IMEP; 7). The pcDNA-His-LACZ reporter vector was selected for the injections in the aim to easily monitor its expression by the detection of beta-galactosidase enzymatic activity on muscle tissue sections after incubation with its XGal substrate yielding a bright blue product. The plasmid was prepared endotoxin free for the injections (Plasmid Factory, Bielefeld, Germany). Different DNA doses and time-points were tested in triplicate in IMEP and IVHD groups. The two injection methods lead to very different expression patterns. The IMEP resulted in a strong B-gal expression in fibers localized close to the injection site, while the IVHD caused expression in scattered fibers among the different lower limb muscles.

In conclusion, IVHD and IMEP naked DNA delivery yield different transgene expression levels and patterns which could both be useful in the context of an FSHD model. Indeed, the IMEP leads to a strong and local transgene expression which could allow an easier scoring of AO-mediated DUX4 suppression. The IVHD results in a pattern expected in FSHD patient muscles

based on DUX4 stochastic expression observed in primary myoblast and myotube cultures. The IVHD model will thus be preferred for an investigation of pathophysiological mechanisms induced by DUX4 expression.

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22. [P] **Dropped head in FSHD patients without atrophy and weakness of neck extensor muscles**

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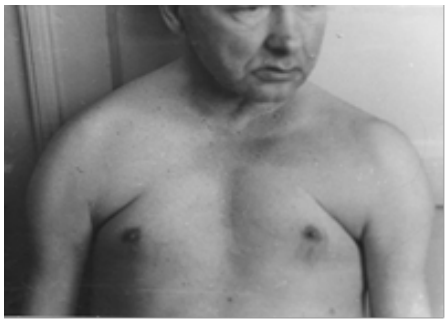
Some authors previously described FSHD patients showing conspicuous dropped head caused by severe wasting of posterior neck muscles (Ichikawa et al. 1996). Analyzing muscle status of 200 FSHD patients we could find slight or moderate weakness of neck extensor muscles only in 4 of them. However some patients with severe muscle atrophy and weakness or loss of muscles fixing the scapulae showed visible dropped head due to abnormal posture of their shoulder girdle, that is sharp displacement of the shoulder girdle downwards and forwards with compensatory cervical spine flexion. The posterior neck muscles were normal. In several years the cervical spine kyphosis and contractures of shoulder internal rotator muscles developed in these patients. Abnormal neck posture may be a characteristic FSHD feature for some patients with severe deformation (abnormal posture) of the shoulder girdle due to severe affection of muscles fixing the scapulae without atrophy and weakness of neck extensor muscles.



Observation 47, pedigree 15, IV-3, 63 y.o. male

Dropped head (or head ptosis) due to severe deformation of the shoulder girdle: sharp displacement of the shoulder girdle downwards and forwards, the clavicles have horizontal position, the axial folds go atypically from outwards to the middle of sternum, “sunken” chest (due to almost completely loss of muscles fixing the scapulae and pectoralis major). Slight atrophy of the upper lip and protrusion of the lower lip are seen due to acting of depressor labii inferioris muscle. Cervical spine kyphosis and contractures of subscapular and teres major muscles developed in this patient as well. Neck extensor muscles were normal.

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23. [P]. **The UK FSHD-Registry – the first 16 months**
www.FSHD-registry.org/uk

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Introduction
In the UK, a national registry for FSHD has been operational since May 2013. Its primary aim is to enable research in FSHD, including clinical trials, to be achieved more rapidly, and more easily. By Sept 2014, 417 people with FSHD from across the UK have voluntarily joined, entering their own demographic and clinical data into the register.

How do people with FSHD hear about the Registry ?
People may first learn of the Registry through attending a specialist muscle clinic, whose staff are linked in to the UK Neuromuscular network, or through contact with the UK FSH-MD support group or the Muscular Dystrophy Campaign. A mention of the registry may also be included with diagnostic laboratory reports, while the Registry web-site address (www.FSHD-Registry.org/uk) will hopefully come up with search terms commonly used by someone seeking information about FSHD.

The registry in perspective
The 417 participants(to date) account for an estimated 14% of all people with FSHD in the UK (based on prevalence of 1/20,000). Of these, 230 joined in the first 2 months, since when the joining rate has run at around 12-13 per month. This ongoing rate of 150 new recruits per year represents 2.5/M population per year, and is still well in excess of a new diagnosis rate for FSHD which can be estimated at 0.67/M population per year (1/20,000 prevalence with natural renewal turnover expected of around 1/80th of total cases per yr).

Data entry and access
The clinical data entered includes self-reporting of age and site of onset of symptoms, functional disability, scapular fixation surgery and symptoms of pain. Each person names their clinical specialist, who is invited to corroborate the diagnosis and enter the diagnostic

DNA details
The Registry is intended to be accessible for research studies in the UK or abroad. There is a registry steering committee to whom all applications for research which would involve individualised participation or anonymous pooled information must first be submitted for approval. For studies which are approved, the process of recruiting and consenting individual participants is channelled through the registry curator, enabling the researcher then to be able to liaise further as appropriate. For example, a Canadian-based international study of infantile and early childhood-onset FSHD has been able to recruit 10 participants from the registry.

Data summary

The registry curator (Libby Wood) produces a regular newsletter which summarises overall data from the registry. Key indicators include the age and sex distribution (52%M:48%F, with 41% aged <40y or >70y); hearing loss (18% overall, in 1/3rd of whom this starts at <40y); and wheelchair use (18% full-time, but 45% part-time). Recognised retinal vascular disease is rare (2%), but respiratory problems are reported in 12%. Persistent daily pain is experienced by 92%, for 2/3rds of whom this is in the upper limb, with 53% describing this as distressing or worse (17% 'horrible' and 11% 'excruciating'). Scapular fixation has been performed in 10%, for whom 74% were happy (30%) or very happy (44%) with the result, allowing improvement in the ability to raise the arm.

Linking with other Registries

There are now FSHD Registries in at least 9 countries across the World (UK, USA, Czech Rep., Italy, Egypt, Canada, France, Netherlands, Australia), collecting the same core dataset; therefore providing an opportunity for an international network coordinated through Treat-NMD (www.treat-nmd.eu) . The primary aim for all of these is to facilitate research, enabling clinical trials to be achieved more rapidly, and more easily.

24. [P]. **STAT3 signaling controls satellite cell expansion and skeletal muscle repair**

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The progression of disease- and age-dependent skeletal muscle wasting results, in part, from a decline in the number and function of satellite cells (SC), the direct cellular contributors to muscle repair. However, little is known about the molecular effectors underlying SC impairment and depletion. Elevated levels of inflammatory cytokines, including Interleukin-6 (IL6), are associated with both age and muscle wasting conditions. Signal Transducer and Activator of Transcription 3 (STAT3), a downstream effector of IL6, is also elevated with muscle wasting and has been implicated in the regulation of self-renewal and stem cell fate in several tissues. Here we show that IL6-activated Stat3 signaling regulates SC behavior, promoting myogenic lineage progression through Myod1 regulation. Conditional ablation of Stat3 in Pax7-expressing SC resulted in increased expansion during regeneration, but compromised myogenic differentiation prevented their contribution to regenerating myofibers. In contrast, transient Stat3 inhibition promoted SC expansion and enhanced tissue repair in both aged and dystrophic muscle. Finally, the effects of STAT3 inhibition were conserved in human myoblasts. The results of this study indicate that pharmacological manipulation of STAT3 activity can be used to counteract the functional exhaustion of SC, thereby maintaining the endogenous regenerative response and ameliorating muscle-wasting diseases.

25. [P]. **Membrane proteins: putative FSHD biomarkers?**

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The identification of FSHD biomarkers and their validation are essential to evaluate the therapeutic approach developed by our laboratory i.e. antisense tools preventing DUX4 expression1. These FSHD biomarkers which belong to the DUX4 deregulation cascade should return to healthy control values upon DUX4 suppression.

Fusion of FSHD primary myoblasts yields varying proportions of myotubes presenting either an atrophic or disorganized phenotype2. Our laboratory has compared the proteome of primary FSHD and control myotubes at day 4 of differentiation3. FSHD myotubes of both phenotypes presented a disturbance of several caveolar proteins such as PTRF (Cavin-1, Polymerase I and Transcript Release Factor), SDPR (Cavin-2, Serum Deprivation Response protein) and MURC (Cavin-4, MUscle Related Coiled-coil protein). Caveolae, considered as a subset of lipid rafts, are characterized by their own lipid and protein composition. Lipid rafts contain small clusters of GPI-anchor proteins, also increased in FSHD atrophic myotubes, such as Thy1 (Thy-1 membrane glycoprotein). Some proteins of the membrane repair complex were also deregulated such as AHNAK (Neuroblast differentiation-associated protein), MG53 (Mitsugumin 53) known to interact with PTRF, Dysferlin and CAV33. We have begun to validate these deregulations by western blot and immunofluorescence but this study would require primary myoblasts which are in limited supply. We decided in a first approach to use human immortalized myoblast clones derived from a mosaïc individual4. We received 3 clones (kindly provided by S. Van der Maarel and V. Mouly, Institute of Myology, Paris): a control line (54-6), and 2 lines expressing different amounts of DUX4 mRNA (54-A5 and 54-12). We have grown these cells and differentiated them for 4 days with either of two differentiation media. The first one contained a reduced amount of fetal bovine serum (2%) and the other one was complemented with insulin and apotransferrin. The cells were seeded at 2,5x105 cells/dish using 35mm matrigel-coated dish. At day 4 after induction of differentiation, cells were immunostained with an anti-troponinT (a differentiation marker) antibody and nuclei labelled with DAPI. The fusion index (MFI) was evaluated in ten fields per culture and the width of the myotubes was mesured at their largest place. Depending on the clone and the medium, we have observed distinct myotube phenotypes. The 54-12 myotubes present an atrophic phenotype and the 54-A5 myotubes presented many clusters of nuclei in the differentiating medium with insulin/apotransferrin as observed in disorganized primary myotubes.

Thus, we could reproduce the two phenotypes typical of FSHD primary myotubes and this cell model is being used for the validation of the deregulated proteins identified in primary myotubes.

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