FSH Society Facioscapulohumeral Muscular Dystrophy [FSHD] 2011 International Research Consortium & Research Planning Meetings

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FSH Society Facioscapulohumeral Muscular Dystrophy [FSHD] 2011 International Research Consortium & Research Planning Meetings

Monday, November 7, 2011
8:25 a.m. – 7:00 p.m.
&
Tuesday, November 8, 2011
8:30 a.m. – 2:00 p.m.

Boston Biomedical Research Institute
64 Grove Street, Watertown, Massachusetts 02472 USA

Co-Chairs: **Robert H. Brown, Jr., M.D., D.Phil.**
University of Massachusetts Medical School &
UMASSMemorial, Worcester, Massachusetts USA

Silvère van der Maarel, Ph.D.
Leiden University Medical Center, Leiden, the Netherlands

Organizers: **Daniel Paul Perez**
FSH Society, Inc.
Robert H. Brown, Jr., M.D., D.Phil.
Silvère van der Maarel, Ph.D.

Hosted By:
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Muscular Dystrophy Association United States (MDAUSA)
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PREFACE

November 7, 2011
Watertown, Massachusetts

Dear Colleagues,

Welcome to the FSHD International Research Consortium 2011. Thank you for coming. Thank you for participating. Thank you for sharing and collaborating.

We will begin this year's meeting with platform talks and poster sessions on day one. We encourage you to share your latest data and findings. Please keep platform presentations to 20 minutes including 5-10 minutes for questions and answers. Platform speakers please consider bringing a poster as well. This meeting is a working meeting with experts, developing future plans in the context of what we know now. In the interest of time, we would like to emphasize new data and ideas and to avoid restating data that has already been published. As you give your talk or present you poster please identify which of the "the top ten" the work addresses or has impact

We suggest that on day one we split the group in four smaller groups [Breakout Discussions 1] to discuss what we have achieved in the past year regarding the ten priorities? Then on day two we define in each group [Breakout Discussions 2] where we need to focus, what are the lacunae? Then during the last one and a half hours on day two a session where we report back to the group and have a plenary group discussion [Group Discussion 1]. By the end of the meeting we should be able to assess progress on these key areas. This should allow us to reassess these areas and associated deliverables to determine: what and if any of these should change or be modified, and if new areas should be considered for addition to the list.

Be sure to connect with funding agencies including the NIH at the workshop e.g FSH Society, AFM, MDA, Stichting FSHD and Carrino Foundation. The goal of this meeting is to create synergy to facilitate more progress on FSHD. It is essential to keep covering ground as quickly as we have over the past two years, to keep going in this direction, and for the entire community to work together at every level to communicate clearly on programs, developments and needs.

This meeting is organized and sponsored by the FSH Society, the U.S. DHHS NIH Eunice Kennedy Shriver NICHD Sen. Paul D. Wellstone BBRI FSHD Muscular Dystrophy Cooperative Research Center, the Association Française Contre les Myopathies (AFM), the Muscular Dystrophy Association (MDAUSA), Quest Diagnostics Athena Diagnostics and the FSHD Global Research Foundation. 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,

Dr. Robert H. Brown, Jr., M.D., D.Phil.
University of Massachusetts Medical School & UMASSMemorial, Worcester, Massachusetts USA

Silvère van der Maarel, Ph.D.
Leiden University Medical Center, Leiden, the Netherlands

Daniel Paul Perez
FSH Society, Inc., Watertown, Massachusetts, USA

The FSH Society, Inc. (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, Inc., Daniel Paul Perez, 11 Elmbrook Circle, Bedford, Massachusetts 01730 USA. Phones: (617) 658-7811 and (781) 275-7781, fax: (781) 275-7789, e-mail: daniel.perez@fshsociety.org, website: http://www.fshsociety.org

Day 1
Monday, November 7, 2011

Registration & Continental Breakfast	8:00-8:25-	
Welcome	8:25-8:30	
Platform Session 1	8:30-10:10	**Genetics & Epigenetics** (5x20 minutes)
Break & **Poster Session 1**	10:10-10:40	
Platform Session 2	10:40-12:00	**4Qter Genes** (4x20 minutes)
Platform Session 3	12:00-1:00	**Models** (3x20 minutes)
Lunch & **Poster Session 2**	1:00-2:20	[Cafeteria]
Platform Session 4	2:20-4:00	**Molecular Mechanisms** (5x20 minutes)
Break & **Poster Session 3**	4:00-5:00	
Breakout Discussions 1	5:00-5:55	**4 Breakouts** / Revisiting Priorities as Stated by the Group
Wrap Up Day 1	5:55-6:00	**Summary**
Reception @ BBRI	6:00-7:00	[Cafeteria]

Dinner on your own

Day 2
Tuesday, November 8, 2011

Registration & Continental Breakfast	8:00-8:30	
Platform Session 5	8:30-10:10	**Clinical Studies & Therapy** (5x20 minutes)
Break	10:10-10:30	
Breakout Discussions 2	10:30-11:30	**4 Breakouts** / Restating Top 10 Priorities for Next Year
Group Discussion 1	11:30-12:55	**Group Discussion** / Defining the 10 Priorities for Next Year
Wrap Up Day 2	12:55-1:00	
Lunch	1:00-2:00	[Cafeteria]

Adjourn

NOTES ON TALKS AND POSTERS

	First Author	Presenting Author	Topic	Session
Day 1				
Chairs: **Maarel & Tupler**				
8:30-8:50 a.m.	Lemmers	Lemmers	FSHD2	Genetics & Epigenetics
8:50-9:10 a.m.	Scionti	Tupler	Hetrozygotes	Genetics & Epigenetics
9:10-9:30 a.m.	Hartweck	Hartweck/Kyba	Methylation	Genetics & Epigenetics
9:30-9:50 a.m.	Cabianca	Gabellini	ncRNA	Genetics & Epigenetics
9:50-10:10 a.m.	Block	Block	Bidirectional	Genetics & Epigenetics
Chairs: **Harper & Tapscott**				
10:40-11:00 a.m.	T Jones	P Jones	DUX4	4Qter Genes
11:00-11:20 a.m.	Geng	Tapscott	DUX4	4Qter Genes
11:20-11:40 a.m.	Domire	Domire/Harper	DUX4	4Qter Genes
11:40-12:00 p.m.	Caruso	Helmbacher	FAT1	4Qter Genes
Chairs: **Emerson & Bloch**				
12:00-12:20 p.m.	Homma	JB Miller	Myogenic Cells	Models
12:20-12:40 p.m.	Zhang	Wagner	Xenograft	Models
12:40-1:00 p.m.	Kyba	Kyba	DUX4 Models	Models
Chairs: **Kunkel & Jones**				
2:20-2:40 p.m.	Sancisi	Morini	TropT	Molecular Mechanisms
2:40-3:00 p.m.	Stadler	Stadler	Telomere Length	Molecular Mechanisms
3:00-3:20 p.m.	Reed	Reed/Bloch	Mu-crystallin	Molecular Mechanisms
3:20-3:40 p.m.	Charon	Coppee	WNT Signaling	Molecular Mechanisms
3:40-4:00 p.m.	Harafuji	YW Chen	miRNA411	Molecular Mechanisms
Day 2				
Chairs: **Brown, Jr. & Wagner**				
8:30-8:50 a.m.	Heatwole	Heatwole	Cross Sectional	Clinical Studies & Therapy
8:50-9:10 a.m.	Statland	Statland	Disease Progression	Clinical Studies & Therapy
9:10-9:30 a.m.	Tyler	Chamberlain	FRG1&DUX4 RNAi	Clinical Studies & Therapy
9:30-9:50 a.m.	Wallace	Wallace/Harper	DUX4 RNAi	Clinical Studies & Therapy
9:50-10:10 a.m.	Lee	YW Chen	PITX1 morpholinos	Clinical Studies & Therapy
Posters				
[				
	Ansseau	Ansseau		Poster
	Barro	Barro		Poster
	J Chen	J Chen		Poster
	Kazakov	Kazakov		Poster
	Leidenroth	Leidenroth		Poster
	Leidenroth	Leidenroth		Poster
	Morree	Morree		Poster
	Multhaup	Multhaup/Kyba		Poster
	Rahimov	Rahimov		Poster
	Reed	Reed/Bloch		Poster
	Statland	Statland		Poster
	Tassin	Tassin		Poster
]				

Abstract numbers that are followed by a "[P]" in abstracts that follow denote poster presentation

NOTES ON BREAKOUT AND GROUP DISCUSSIONS

For the breakout sessions we propose that we evaluate the last year's 10 priorities by the following process: On day one we split the group in four smaller groups called, **Breakout Discussions 1,** held Monday 5:00-5:55p.m. to discuss what we have achieved in the past year regarding the priorities? Then on day two we define in each group called, **Breakout Discussions 2,** held Tuesday 10:30-11:30a.m., where we need to focus, and what are the lacunae?

During the last one and a half hours on day two Drs. **Rune Frants** and **Charles Emerson, Jr.** moderate the session where we report back to the group and have a plenary group discussion called, **Group Discussion 1,** held Tuesday 11:30a.m.-1:00p.m.

Group 1: Resources
Priorities 1 & 2
Moderators: **Drs. Lemmers and J. Chen**
1. **Shareable Protocols**
2. **Common and shareable materials and data by whole community**

Group 2: (Epi)genetics
Priorities 3 & 4 & 9
Moderators: **Drs. Stephen Tapscott and Silvere Van der Maarel and Davide Gabellini**
3. **Corroborate and verify DUX4 finding**
4. **FSHD alleles in context of population genetics need to be defined**
9. **Epigenetics / Genetics**

Group 3: Clinical trials
Priorities 5 & 6 & 10
Moderators: **Drs. Rabi Tawil and Robert H. Brown, Jr. and Rossella Tupler**
5. **Biomarkers**
6. **FSHD clinical evaluation scales/systems need be defined under one agreed standard**
10. **Clinical trials readiness**

Group 4: Model systems
Priorities 7 & 8
Moderators: Drs. TBA and **Daniel Miller**
7. **Working Groups / Mouse model working group consortium**
8. **Model systems for mechanistic, intervention work and advancement to clinical trials**

THE TOP TEN AREAS IDENTIFIED AS A PRIORITY FOR FSHD BY THE WORLD WIDE SCIENTIFIC COMMUNITY IN OCTOBER 2010

The international FSHD clinical and research community recently came together at the DHHS NIH NICHD Boston Biomedical Research Institute Senator Paul D. Wellstone MD CRC for FSHD. Almost 90 scientists working on FSHD globally met at the 2010 FSH Society FSHD International Research Consortium, held October 21-22, 2010.

The summary and recommendations of the group state that given the recent developments in our definition of FSHD and the potential that within one to two (1-2) years, evidence-based intervention strategies, therapeutics, and trials being planned and conducted. Our immediate priorities should be to confirm the DUX4 hypothesis, if valid then understand normal DUX4 function, and finally, understanding the naturally occurring variability should allow us to manipulate the disease in our favor. We need to be prepared for this new era in the science of FSHD, by accelerating efforts in the following areas:

1. **Shareable Protocols.** There is a need for access to FSHD research protocols and experimental methods by FSHD researchers internationally. Needed are available, clear and well defined research protocols to allow verification, standardization and corroboration of research findings and publications.
    a. All protocols should be gathered, standardized, and be publically available
    b. Sufficient detail that experiments can be reproduced
    c. Online @ FSH Society, Wellstone MD CRC, Fields

2. **Common and shareable materials and data by whole community.** There is a need for global and international biomaterials and data management. Needed are schemas to identify source and context of biomaterials and data, meaningful data identifiers, and easily accessible bio-repositories and data sources.
    a. FSHD materials should be shared, accessible and commercially produced when possible
    b. Well characterized materials need to be globally available
        i. Antibodies
            a. Proprietary pre-publication
            b. Proprietary post-publication
            c. Commercially made pre-publication
            d. Commercially made post-publication
        ii. Cellular
            a. Biopsies
            b. Mosaics
            c. iPS
            d. ESC
            e. Isogenic, sorted, immortalized
        iii. Mouse models /Animal
            a. Mouse – inducible / humanized mouse etc.
            b. Other species (Sheep)
        iv. Human
            a. FSHD patients and genetically related unaffected primary relations

i. FSHD1
ii. FSHD2

b. Unaffected controls
i. 4QA161 with short fragment

c. Model systems. Urgent need for more specific model systems for mechanistic, intervention work and advancement to clinical trials.
i. Cellular
a. Biopsies
b. Mosaics
c. iPS
ii. Animal
a. Mouse – inducible / humanized mouse etc.
b. Other species

d. Data and Datasets
i. genetic
ii. sequence
iii. gene expression
iv. proteomic

e. Shareable Materials and Data
i. all biomaterials gathered, standardized, and be publically available
ii. sufficient documentation with each
iii. online @ FSH Society, Wellstone MD CRC, Fields

3. Corroborate and verify DUX4 finding. This line of work will be instrumental to pinpoint the real identity of FSHD1A (chromosome-4-D4Z4-contraction-linked cases) and FSHD1B (chromosome-4-non-D4Z4-contraction-linked cases & non-chromosome-4-linked cases). This information will form the basis for evidence-based intervention. There is a need to verify and reproduce the DUX4 finding using multiple sites and patient materials.

a. different groups need to verify
b. explain the different pathophysiological pathways FSHD1/FSHD2
c. understand normal function of DUX4
i. developmental and embryogenesis
ii. stem cell, satellite cell, differentiation
iii. evolutionary
iv. conservation and propagation

4. FSHD alleles in context of population genetics need to be defined. There is a need to understand the normal function of the short DUX4 transcript in every human being and the abnormal function of toxic long-form of the DUX4 transcript.

a. Consortium or meeting needs be assembled
b. Determine the frequency in the population 4QA161 e.g. 1:200
c. Determine frequency of FSHD
d. Establish whether entire population has FSHD
e. Important to examine entire families include asymptomatic relations
f. Normal versus abnormal function of DUX4

5. **Biomarkers.** There is obvious need for monitoring intervention. There is the need to define biomarkers for clinical trials endpoints, to understand the FSH disease at multiple omics levels and to understand pathways and signaling of FSHD through "omics" analysis
    a. Systems biology
        i. transcriptomics, genomics, proteomics, metabolomics, lipidomics, etc.
        ii. DNA, mRNA, protein, metabolites
    b. In situ (RNA, protein) to detect cellular heterogeneity
    c. Industrially and FDA acceptable model systems and assays
    d. implications of DUX4 detection at levels of 1 cell in 1000

6. **FSHD clinical evaluation scales/systems need be defined under one agreed standard.** There is an important need to have a comprehensive and single clinical evaluation standard to allow a list of clinical identifiers and parameters to be assembled into a thorough and robust dataset. This can be applied to subsequent systems biology and –omics areas.
    a. Consortium or Meetings needs be assembled
    b. Multiple clinical evaluation scales need be assessed
    c. One system of measurement needs be defined
    d. Important phenotype/genotype correlations
    e. FSHD1, FSHD2 and asymptomatic carriers

7. **Working Groups / Mouse model working group consortium. There is a need for models** and methods to interpret the current mechanistic paradigm of FSHD and fidelity of current data. What is needed is an assessment of various modeling approaches. Short and near-term we need consensus on the limits and capabilities of current modeling approaches.
    a. Development and distribution
    b. Convene regular ongoing (monthly?) meetings to discuss mouse model work
    c. Initiate Webex/videoconferencing meetings to discuss mouse development
    d. Deposition of lines into national repositories

8. **Model systems for mechanistic, intervention work and advancement to clinical trials.** We need to be able to understand the limits of the both data and models. There is the need to address the complexity of FSHD in the context of mammalian systems. It becomes even more important to have a solid model and dataset to test.
    a. Cellular
        i. Biopsies
        ii. Mosaics
        iii. iPS
    b. Animal
        i. Mouse – inducible / humanized mouse etc.
            A. Knock outs
            B. Knock ins
            C. Conditionals
        ii. Other species (Chimp, Sheep)

9. **Epigenetics / Genetics.** There is the need to further evaluate FSHD1, FSHD2 and phenocopies using the genetic approach. Resolve and establish genes involved in the pathophysiology of

FSHD (DUX4, FRG1, FRG2 and other 4q35 loci, PitX1, Pax3, Pax7 and other impacted distributed loci, related cascades and pathways, etc.)

a. Modifying and protective factors for FSHD1 (large variation in symptoms)
b. Identify the FSHD2 mechanism/gene/pathway (common pathway with FSHD1)
c. Further work on the chromatin structure / function relationship
d. Haplotype sequencing

10. Clinical trials readiness. There is a need to revisit we know about the pathological progression and natural history of FSH disease. An international consensus/summary of pathology findings is needed as well as patient definition by clinical criteria vs. molecular criteria (phenocopies, incomplete penetrance, etc.)

a. Natural history studies on FSHD
b. Appropriate outcome measures and clinically meaningful endpoints measurements: primary and secondary, suitable for early phase trials and efficacy trials
c. Surrogate markers: Imaging (MRI,CT, DEXA) and/or biomarkers
d. Patient registries: National FSHD Registry, FSH Society, TREATNMD, others
e. Treatment modality considerations FSHD1/FSHD2 (ASO, AAV, small molecule, exon skip)
    i. open configuration caused by D4Z4 contraction
    ii. chromatin intact but hypomethylated / therapies to access chromatin
    iii. downstream pathways

1. [P]

DUX4 and DUX4c protein partners in muscle cells

Ansseau E[1], Mattéotti C[1], Gerbaux C[1], Yip C[1], Pire E[1], Vachaudez V[1], Cloet S[1], Tassin A[1], Leroy B[2], Wattiez R[2], Coppée F[1] and Belayew A[1]

[1]Laboratory of Molecular Biology and [2]Laboratory of Proteomics and Microbiology, University of Mons, 7000 Mons, Belgium

Our group has identified the *DUX4* gene in each D4Z4 repeat at 4q35 and shown its expression in affected but not control myotubes (Gabriels et al, 1999; Dixit et al, 2007; Kowaljow et al, 2007). We found that stable DUX4 mRNAs were transcribed from the distal D4Z4 element and extended to a polyadenylation signal in the flanking pLAM region (Dixit et al, 2007). This mRNA was confirmed by others who also identified additional transcripts (Snider et al 2009, 2010). This polyadenylation signal signal was shown to be necessary to develop FSHD (Lemmers et al, 2010). We also identified the homologous DUX4c gene located 42kb centromeric. It expresses a non toxic protein induced in FSHD myoblasts and probably involved in muscle regeneration by its capacity to activate myoblast proliferation (Ansseau et al, 2009).

In order to better understand the DUX4 and DUX4c protein functions we decided to identify their proteins partners in muscle cells (FSHD and control).

In a first approach we used the yeast two hybrid system. The first hybrid was a fusion between the GAL4 DNA binding domain (DBD) and the DUX4c protein which, in contrast to DUX4, did not present a transcriptional activity in yeast. The second hybrids were expressed as fusion proteins of the GAL4 transcription activation domain (AD) and the proteins encoded by a human adult skeletal muscle cDNA library. If a GAL4-AD -protein fusion expressed from the library interacted with DUX4c it would complement the DUX4c-GAL4-DBD fusion protein, enable transcription of the HIS3 gene and yeast survival on His^- medium. This library screening yielded 187 positive clones among which 150 were analyzed and 105 contained desmin cDNA fragments, suggesting a desmin interaction with DUX4c. We validated those result by GST-pull down and confirmed the co-immunolocalisation of desmin with DUX4c or DUX4 by the Olink procedure (Bioscience) in cell cultures.

In a second approach, we expressed the DUX4 or DUX4c protein in fusion with a HaloTag (Promega) in TE671 (human rhabdomyosarcoma) cells and we purified the tagged DUX4 or DUX4c proteins with their partners by affinity chromatography on HaloLink beads (Promega). A cleavage with the TEV protease was used to release DUX4 or DUX4c with its associated partners from the bead-linked Halotag. As a negative control we used an EGFP-HaloTag protein. The protein mixture was lysed by trypsin and the peptides identified by mass spectrometry (LC-MS/MS). In this way we identified partners involved in RNA splicing. We are validating their expression by immunodetection on western blots prepared with extracts of immortalized control and FSHD myoblasts. The partners interaction with DUX4 or DUX4c is being validated by co-immunolocalization with the Olink procedure.
Funded by the AFM (France), FSHD Stichting (NL), ABMM, FRIA, and FNRS (Belgium).

Ansseau et al *PLoS ONE* 4, e7482 (2009); Gabriels et al, *Gene* 236, 25-32 (1999).

Dixit et al, *Proc. Natl. Acad. Sci. U.S.A* 104, 18157-18162 (2007).
Kowaljow et al, *Neuromuscul. Disord* 17, 611-623 (2007).
Lemmers et al, *Science* 329, 1650-1653 (2010).
Snider et al *Hum. Mol. Genet* 18, 2414-2430 (2009) ; Snider et al *PLoS Genet* 6, e1001181 (2010).

2. [P]

Humanized mouse model for the study of FSHD

Marietta Barro[1], Daniel Zuch[1], Jennifer Chen[1], Kendal Hanger[1], Kathryn Wagner[2], Charles P. Emerson, Jr. [1]

[1] Senator Paul D. Wellstone Muscular Dystrophy Cooperative Research Center, and Program in Regenerative Biology, Boston Biomedical Research Institute, Watertown, Massachusetts USA
[2] The Center for Genetic Muscle Disorders, Kennedy Krieger Institute, Johns Hopkins School of Medicine, Baltimore, Maryland USA

Facioscapulohumeral muscular dystrophy (FSHD) is genetically caused by the contraction of D4Z4 DNA repeats located on chromosome 4 in 4q35. Although the genetic defect was identified 20 years ago, the exact molecular mechanism causing the disease is unknown, and there is currently no mouse disease model. To provide such a valuable tool, we are developing a humanized mouse model for FSHD, obtained by the engraftment of FSHD patient-derived myoblasts into mouse muscle. Engrafted human cells are able to form muscle fibers in the host mouse muscle, thus allowing pioneering studies in an in vivo context. Because of the dominant nature of FSHD, we hypothesized that the engrafted fibers will display a disease phenotype and recapitulate pathological molecular mechanisms associated with FSHD that will allow us to study the development of the disease. Our preliminary data showed that FSHD myoblasts injected into regenerating murine muscles successfully form myofibers.

Therefore, FSHD myoblasts can participate to murine muscle regeneration in vivo, establishing the feasibility of this project. Through the cell repository of the Boston Biomedical Research Institute (BBRI) Wellstone Center, we have the unique opportunity to access early passage myoblast cells from cohorts of FSHD probands and their appropriate controls, i.e., a first degree relative. We are grafting these standardized cultured cells into mouse muscle to obtain the humanized mouse model for FSHD, thereby generating a well-controlled in vivo model for the study of this enigmatic disorder. The very pressing issue in the field today is the verification of the current DUX4 model. The humanized mice produced will be used to investigate the hypothesis that DUX4 gene expression is a major cause of FSHD pathogenesis. In the obtained model, DUX4 expression will be evaluated during in vivo regeneration, and the consequence of its expression on fiber turnover and satellite cell renewal will be assessed. This work is contributing to the understanding of the role of DUX4 in vivo, thus providing a better understanding of FSHD pathogenesis.

3.

Asymmetric bidirectional transcription from the FSHD-causing D4Z4 array modulates DUX4 expression

Gregory J. Block, Lisa M. Petek, Divya Narayanan, Amanda Amell, James M. Moore, Natalia Rabia, Rabi Tawil, Galina Filippova, and Daniel G. Miller

[1] The University of Washington, Seattle, Washington
[2] Fred Hutchison Cancer Research Center, Seattle, Washington
[3] Department of Neurology, University of Rochester Medical Center, Rochester, New York

The DUX4 gene and upstream regulatory region are arranged as an array of directly repeating units, called D4Z4, on chromosomes 4 and 10 that vary in number between 10 and 200 copies. Loss of heterochromatin at D4Z4 is associated with DUX4 transcription in the context of chromosome 4 haplotype polymorphisms; however, additional regulatory constraints appear to govern DUX4 transcription. We hypothesized that bidirectional transcription at D4Z4 may regulate DUX4 transcription. To test this, reporter constructs were developed that contained the non-coding region of a single D4Z4 repeat flanked by sense and/or antisense reporter genes. We find the non-coding region of D4Z4 can initiate asymmetric bidirectional transcription in favor of antisense transcription in most cell types; however, maintenance of sense transcription can vary between cell types. Mutations generated in the non-coding region identified repressor and enhancer regions that contributed to the regulation of bidirectional transcription and altering D4Z4 to resemble the terminal partial repeat of D4Z4 abolished forward transcription to favor reverse transcription independent of polymorphisms within pLAM. The data support a model in which D4Z4 is constitutively transcribing, and that changes in downstream transcript processing, not transcriptional activation alone, result production of DUX4 protein.

4.

A chromatin-associated ncRNA regulates a Polycomb/Trithorax epigenetic switch in FSHD muscular dystrophy

Daphne S. Cabianca[1,2], Valentina Casa[1,2], Beatrice Bodega[3], Enrico Ginelli[3], Yujiro Tanaka[4], and Davide Gabellini[1]

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[3] Department of Biology and Genetics for Medical Sciences, University of Milano, Milano, Italy
[4] Genome Structure and Expression, Graduate School of Biomedical Science, Tokyo Medical and Dental University, Tokyo, Japan

Repetitive sequences account for more than 50% of the human genome. Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant disease associated to reduction in the copy number of the D4Z4 repeat mapping to 4q35. By an unknown mechanism, D4Z4 deletion causes an epigenetic switch leading to de-repression of 4q35 genes. Here we show that the Polycomb group of epigenetic repressors targets D4Z4 in healthy subjects and that D4Z4 deletion is associated to reduced Polycomb silencing in FSHD patients. We identify DBE-T, a long, chromatin-associated non-coding RNA produced selectively in FSHD patients that coordinates de-repression of 4q35 genes. DBE-T directly recruits the Trithorax group protein Ash1L to the FSHD locus, driving chromatin remodeling and 4q35 gene de-repression. This study provides insights into the biological function of repetitive sequences in regulating gene expression and on how mutations of such elements can influence the progression of a human genetic disease.

5.

Reduced expression of the planar cell polarity gene FAT1 as a causal mechanism for facioscapulohumeral dystrophy

Nathalie Caruso[1], Balàzs Herberth[1], Marc Bartoli[2], Julie Dumonceaux[3], Marie Lebossé[1], Stephane Roche[2], Flavio Maina[1], Nicolas Levy[2,4] and Françoise Helmbacher[1,#]

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Generation of skeletal muscles with forms adapted to their function is essential for normal movement. The process of muscle development involves long-range migration of muscle precursors, during which myoblasts coordinate their cellular polarity along migratory chains. Constitutive inactivation of the Planar Cell Polarity (PCP) gene Fat1 uncoupled individual myoblast polarity within chains, specifically altering the shape of groups of muscles in the shoulder and face. These shape abnormalities were predictive of regionalized muscle dystrophy in adult life, as evidenced in transgenic mice with a hypomorphic Fat1 allele. Unexpectedly, we noticed that the topography of muscle abnormalities in Fat1 mutant embryos and adult mice resembled that of human patients with facioscapulohumeral dystrophy (FSHD), a human hereditary myopathy that manifests as progressive weakening of muscles in the face, scapula and upper arms. Its most frequent form, FSHD1, is linked to pathogenic abnormalities at chromosome 4q35, including the contraction of a macrosatellite repeat array and polymorphisms activating a cryptic gene DUX4, thought to trigger the disease through gene regulatory changes. Strikingly, human FAT1 lies near the candidate gene region for FSHD1, and Fat1 mutant mice also show non-muscle defects characteristic of FSHD including retinal vasculopathy. We found that FAT1 expression was silenced in human foetal FSHD1 muscle in two cases with expected severe early onset FSHD. In addition, we showed that such silencing is achieved through epigenetic chromatin changes in the latter FSHD1 cases, or predicted genetically, by the deletion of a genomic regulatory element in rare atypical FSHD cases without D4Z4 contraction. Our data reveal a critical and unexpected role of collective migration polarity for muscle form and integrity. This led us to propose that the tissue-specific lowering of FAT1 expression may play a major role in FSHD pathogenesis (Caruso et al., Nature, revised, under review).

6.

Perturbation of the WNT signaling pathway in FSHD

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Our group has identified and characterized the double homeobox 4 (DUX4) gene within each D4Z4 unit of the 4q35 repeat array; expression of this gene in muscle cells is now considered as a key event to develop FSHD. DUX4 encodes a transcription factor that targets a number of genes, some of which encode other transcription factors thus causing a large deregulation cascade.

Some publications on gene expression profiles in FSHD have shown altered expression of inhibitors or components of the WNT signaling pathway. This pathway is involved in myogenesis, muscle regeneration, cell differentiation and angiogenesis. Retinovasculopathy which can be associated to FSHD may be also linked to the WNT pathway: for example Norrie disease is caused by a mutation of Norrin, a ligand of a WNT receptor (Fitzsimons RB, 2011).

In the OFF state of the WNT pathway, β-Catenin is phosphorylated, polyubiquitinated and degraded. Binding of a WNT ligand to its receptor will inhibit β-Catenin phosphorylation and degradation allowing its translocation to the nucleus where it activates transcription in association with the TCF/LEF trans factors.

We first investigated by transient expression experiments whether the WNT pathway was activated in FSHD: control and FSHD myoblasts were transfected by a luciferase reporter gene linked to TCF/LEF binding sites (TOP-FLASH and FOP-FLASH reporter vectors). We observed a 1.3 activation of the reporter in FSHD myoblasts. We then co-transfected control myoblasts with this reporter vector and a low amount of DUX4 expression vector. At 50ng of the expression vector we observed a 10X activation of the reporter.

Secondly we compared the expression of WNT pathway genes by qRT-PCR in FSHD and control primary myoblasts either in proliferation or differentiation. We used the SABiosciences PCR array targeting 85 mRNA encoding proteins specific to the WNT pathway at different levels: inhibitors, receptors, phosphorylation complex, downstream targets… We observed a down regulation of several mRNAs in FSHD cells during proliferation and differentiation, among which genes coding for proteins of the phosphorylation regulation complex of β-Catenin. In myotubes, we observed a decrease of the CCND3 mRNA (needed for differentiation) and an up regulation of the CCND1 mRNA (which favors cell cycling). These data suggest a perturbation of WNT pathway regulation that could contribute to the differentiation defect already described in FSHD muscle cells.

We acknowledge funding from the AFM (France) and the ABMM (Belgium). SC and CV had a FRIA (Belgium) graduate fellowship.

7. [P]

Building a repository of primary and immortalized myogenic cells derived from FSHD families

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One of the difficulties facing FSHD researchers is limited access to materials to model this complex disease. Available animal models, while highly intriguing and promising, have yet to be validated. In order to address this limitation, we have established primary CD56+ muscle cell strains from the biceps and deltoid muscles of FSHD subjects with mild to intermediate pathology and contractions of the D4Z4 repeat. These two muscles were selected given their tendency to exhibit different levels of FSHD pathology: while the biceps commonly is subject to muscle weakness, the deltoid muscle is frequently spared from pathology. In addition, parallel strains are being established from first-degree familial relatives to each FSHD subject, to serve as non-diseased controls to minimize variation due to genetic background. CD56+ cells from FSHD subjects and control family members express the skeletal muscle markers desmin and MyoD, and a subset of cells express Pax7. Upon serum starvation, cells upregulate expression of differentiation markers, including a number of myosin heavy chain isoforms, myogenin, and muscle creatine kinase. While we have successfully cultured cell strains to ~35 to 40 population doublings before onset of senescence, proliferation is nonetheless restricted; however, cells from a select number of family cohorts have been immortalized by viral infection with CDK4 and hTERT, and multiple clonal cell lines propagated. At present, primary biceps and deltoid cell strains (that have undergone approximately 20 population doublings) from six families, and immortalized cell lines from one family cohort are available for distribution.

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8. [P]

Toward therapeutics for FSHD: understanding mRNA processing

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FSHD develops in individuals who have BOTH a D4Z4 repeat contraction AND a PolyAdenylation Site (PAS) in the pLAM domain distal to the D4Z4 array. The D4Z4 repeat contraction results in relaxed chromatin, and allows for transcription of the DUX4 gene in the final D4Z4 repeat. However, it is the PAS in the pLAM domain that allows for cleavage of the pre-mRNA and subsequent polyadenylation. Only with a poly(A) tail in the 3'UTR, the Dux4 transcripts are stabilized and translated into protein. In individuals who have D4Z4 contractions but a single base change in the PAS, the cell does not recognize it as a PAS, preventing cleavage and polyadenylation. As a result the DUX4 transcript is unstable, no DUX4 protein is made, and the individuals are protected from getting the disease. Therefore, any intervention that prevents the addition of the poly(A) tail to the DUX4 transcript is a potential therapeutic approach for FSHD. Clearly, it is untenable to interfere with mRNA processing in general because of the toxicity to the cell. Therefore, understanding the mechanisms by which a cell can bypass a specific PAS would suggest a mechanism for selectively blocking the PAS in the pLAM domain in the DUX4 gene without generally affecting cellular mRNA processing. This would be an effective treatment for patients with FSHD. We will explore the regulation of PAS choice in order to discover the mechanisms by which a cell, given two PAS, can elect to choose one site and ignore another. If we understand the sequence elements that govern that process, we can design oligonucleotides that are both unique to that transcript and capable of blocking mRNA cleavage and polyadenylation without affecting mRNA processing in general. This would translate directly into a therapeutic approach to FSHD.

9.

DUX4 regulates expression of the pro-apoptotic gene, p63

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Understanding the molecular mechanisms underlying a disease is necessary for developing targeted therapeutic strategies. For FSHD, translational research was impeded because the pathogenic events required for diseased development were elusive and/or controversial. The landscape has changed recently with the emergence of DUX4 gene expression as an underlying pathogenic event in FSHD. We can now begin developing rational therapies that directly target DUX4 as well as downstream pathways it controls, since the DUX4 transcription factor likely initiates a cascade of gene expression changes that are incompatible with normal muscle homeostasis.

The objective of this study was to characterize prospective DUX4-controlled pathways. We hypothesized that this approach would help further define the pathogenic events involved in FSHD and identify new therapeutic targets. Toward this end, we used microarray data from DUX4-overexpressing muscle to identify potential transcriptionally regulated targets of DUX4. We found that several genes involved in apoptosis pathways were up-regulated, including p53-pathway members. The latter finding was consistent with our previous finding that DUX4-associated myopathy was p53-dependent in mice. Since DUX4 is a transcription factor, we therefore hypothesized that it could directly activate p53-pathway genes. In this study, we focused on one such gene, p63, because it was increased in DUX4-expressing muscles and cells, known to transactivate p53 target genes, and sensitize cells to apoptosis. Using EMSA, we found that DUX4 directly binds a putative homedomain site present within the human and mouse p63 promoters. We confirmed DUX4 modulation of p63 expression by cloning the mouse and human p63 promoter sequences upstream of a luciferase reporter and measuring activity following DUX4, or control, transfection in vitro. We found that DUX4, but not mutant DUX4, significantly increased p63 promoter-luciferase expression. Finally, using apoptosis assays, we found that a dominant negative form of p63 significantly reduced DUX4-associated cell death in vitro.

Together, these results suggest that p63 is a downstream target of DUX4, and may play an important role in DUX4-mediated cell death. As such, p63 could be a therapeutic target for FSHD, and we are currently investigating p63 expression, and that of other p53 pathway members, in biological material from human FSHD patients.

10.

DUX4 induces genes in skeletal muscle of individuals with facioscapulohumeral dystrophy

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Facioscapulohumeral dystrophy (FSHD) is one of the most common inherited muscular dystrophies. The causative gene remains controversial and the mechanism of pathophysiology unknown. We have shown that human DUX4 mRNA is normally expressed in the germline and epigenetically repressed in somatic tissues. The low levels of DUX4 mRNA in FSHD muscle represents inefficient epigenetic repression that results in a relatively high DUX4 expression in a small number of nuclei. We have now identified genes regulated by DUX4 in skeletal muscle cells. These genes are associated with germline and early stem cell development. The genes regulated by DUX4 are reliably detected in FSHD muscle but not in controls, providing direct support for the model that misexpression of DUX4 is a causal factor for FSHD. These findings suggest specific mechanisms of FSHD pathology and identify valuable candidate biomarkers for a disease that has been difficult to diagnose with genetic testing.

11.

Differential expression of miRNA-411 in FSHD myoblasts

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Previous studies in FSHD suggested defects in cell cycle regulation and differentiation of myotubes. In this study, we hypothesized that misregulation of microRNAs (miRNAs) in FSHD is involved in the disease mechanisms. To identify miRNAs misregulated in FSHD myoblasts, we performed miRNA expression profiling using TaqMan Human MicroRNA Array v1.0 (Applied Biosystems). The profiles of eight primary human myoblasts (four FSHD and four controls) at two time points, 0 hour and 48 hours post differentiation (PD) were analyzed, respectively. We identified 26 and 11 miRNAs significantly changed in expression in FSHD myoblasts at 0 and 48 hours PD, respectively. The expression change of miR-411 at the 0 time point was confirmed using TaqMan MicroRNA Assays (Applied Biosystems). To localize the miR-411 in myoblasts, we performed in situ hybridization with Locked Nucleic Acid (LNA)-Digoxigenin (DIG) labeled probes. The miR-411 was localized in the cytoplasm with stronger expression in the FSHD myoblasts. We further examined miR-411 expression in a mouse model of FSHD which over-expressing Pitx1 in skeletal muscles and localized the miR-411 expression in the atrophic fibers. Our results showed that miR-411 were up-regulated in FSHD primary myoblasts and may be involved in the muscle atrophy process in FSHD.

12.

Methylation within D4Z4 repeats is non-homogeneous with two domains: one sensitive and one insensitive to the methylation changes associated with FSHD2

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In the current hypothesis of the genetic basis of FSHD, two conditions are necessary for the disease. The first is inheritance of a permissive 161 chromosome 4 allele and the second is extensive demethylation of the D4Z4 repeat on that allele that together result in transcription of a polyadenylated stable Dux4 mRNA from the last repeat and translation of a toxic Dux4 protein leading to the disease. We sought to increase our understanding of the epigenetic mechanisms leading to demethylation of D4Z4 by testing DNA from patients of FSHD2, the form of the disease that had been previoulsy characterized as having D4Z4 demethylation of all alleles of chromosomes 4 and 10 at three methylation-sensitive DNA restriction enzyme sites. Using bisulfite sequencing of DNA from blood or myoblast cell lines from FSHD2 paitents, we measured methylation levels at 75 CpG sites across three disparate regions within D4Z4. Using these more detailed epigenetic maps, we found that methylation was non-uniform along D4Z4 with domains that were sensitive or insensitive to the methylation changes associated with FSHD2. One domain in the 5' region of D4Z4 had 94% less methylation in FSHD2 myoblasts. The 3' domain was not severely hypomethylated in FSHD2 and, surprisingly, contains the DUX4 open reading frame. These data challenge the simplistic notion that broad demethylation of D4Z4 causes FSHD and highlight regions of focused alterations that are likely to harbor regulatory domains.

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13.

What patients tell us: results from a national cross-sectional study of FSHD patients

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OBJECTIVE: To assess: 1) The most critical symptoms and disease manifestations in FSHD patients; and, 2) The modifying factors that affect these symptoms.

BACKGROUND: FSHD is a dominantly inherited multisystem disease capable of impairing the physical, mental, and social health of patients along with inflicting significant loss of muscle function. The identification of the symptoms and disease manifestations that are most significant to patients' health and the relationship between the severity of these manifestations and other patient characteristics are currently lacking for FSHD. These data are necessary to develop valid disease-specific patient-reported outcome measures for use in future FSHD clinical trials.

METHODS: We conducted individual qualitative interviews with twenty adult FSHD patients to identify the symptoms that potentially have the highest impact on patients' lives. Three hundred and twenty eight FSHD-affected individuals from the NIH National Registry subsequently participated in a large cross-sectional study to determine the frequency and relative importance of each symptom identified from the patient interviews. Responses were categorized by age, gender, education level, and duration of symptoms.

RESULTS: One thousand and seventy five quotes from participant interviews were used to identify 250 symptoms representing 15 themes of FSHD health. FSHD participants (n=328) provided over 48,000 symptom rating responses to address the relative frequency and importance of each FSHD symptom identified through participant interviews. Participants represented 46 states, Puerto Rico, Washington D.C., and Canada. Problems with shoulders or arms (96.9%), inability to do activities (94.7%), fatigue (93.8%), back, chest, or abdomen weakness (93.8%), and limitations with mobility or walking (93.6%) were the symptomatic themes with the highest frequency in FSHD. Participants identified problems with shoulders or arms and limitations with mobility or walking as the symptomatic themes with the greatest impact on their lives. Relationships were found between demographic data and the prevalence and relative impact of specific FSHD manifestations.

CONCLUSIONS: In this national cross-sectional trial patients with FSHD identified the symptoms of highest frequency and greatest impact. These symptoms, some under-recognized, vary based on patient age, gender, and duration of symptoms. Fortunately many, perhaps all symptoms, may be amenable to future therapeutic intervention.

14.

A unique library of myogenic cells from facioscapulohumeral muscular dystrophy subjects and unaffected relatives: Family, disease, & cell function.

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To explore possible mechanisms of pathology in facioscapulohumeral muscular dystrophy (FSHD), we generated a novel library of myogenic cells composed of paired cultures derived from FSHD subjects and unaffected first degree relatives. We prepared cells from biopsies of both biceps and deltoid muscles obtained from each of 10 FSHD and 9 unaffected donors. We used this new collection to determine how family background and disease affected patterns of growth and differentiation, expression of a panel of candidate and muscle-specific genes, and responses to exogenous stressors. We found that FSHD and unaffected cells had, on average, indistinguishable patterns of differentiation, gene expression, and dose-response curves to staurosporine, paraquat, hydrogen peroxide, and gluththione depletion. Differentiated FSHD and unaffected cultures were both more sensitive to glutathione depletion than proliferating cultures, but showed similar responses to paraquat, staurosporine, and peroxide. For stress responses, the sample size was sufficient to detect a 10% change in effect at the observed variability with a power of >99%. In contrast, for each of these properties, we found significant differences among cells from different cohorts, and these differences were independent of disease status, gender, or muscle biopsied. Thus, though none of the properties we examined could be used to reliably distinguish between FSHD and unaffected cells, we found that family of origin was an important contributor to gene expression patterns and stressor responses in cultures of both FSHD and unaffected myogenic cells.

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15.

Analysis of DUX4 mRNA and protein expression in muscles and myogenic cells from FSHD subjects and unaffected relatives

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Facioscapulohumeral muscular dystrophy (FSHD), the most prevalent myopathy afflicting children and adults, is predominantly caused by dominant deletions in the 4q35-localized macrosatellite D4Z4 repeat array. Recent studies have proposed that FSHD pathology is caused by the misexpression of the DUX4 (double homeobox 4) gene resulting in production of a cytotoxic protein, DUX4-FL, which was detected in FSHD but not unaffected control myogenic cells and muscle tissue. Here we report the analysis of DUX4 mRNA and protein expression in a large collection of myogenic cells and muscle biopsies derived from biceps and deltoids of FSHD affected subjects and their unaffected first-degree relatives. We found that DUX4-fl mRNA and/or protein was expressed in cells and muscle tissues from both unaffected and FSHD subjects. These results suggest that DUX4-fl expression per se is not sufficient for FSHD muscle pathology and raises the possibility that modifiers of DUX4-fl are determinants of FSHD muscle disease progression.

16. [P]

Facioscapuloperoneal (or facioscapulolimb, type 2) 4q35-linked muscular dystrophy

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The nosological place of scapuloperoneal muscular dystrophy with minimal/slight affection of the facial muscles (FSPD) is not fully defined: Is it an independent form or a variant of FSHD? We observed the pattern of muscle weakness in patients from nine 4q35 linked EcoRI/BlnI DNA fragment size 13-25 kb facioscapuloperoneal muscular dystrophy (FSPMD) families (under numbers 2, 5, 8, 13, 15, 18, 20, 9a and 13a). The 14 patients (9 men and 5 women) from these families were re-examined by V.K. after 27–29 years (5 patients aged 45, 52, 52, 55, 88), after 36-37 years (5 patients aged 41, 42, 63, 73, 73), after 43 years (1 patient aged 61), after 47 years (1 patient aged 68) and after 49 years (2 patients aged 62, 74). In the first examination the next phenotypes of muscle weakness were found: facio(scapular) (3 patients), (facio)scapular (1), facioscapular (1), (facio)scapuloperoneal (8) and facioscapuloperoneal-(humeral, slight affection) (1).

On re-examination (V.K.) these patients after 27-49 years the next phenotypes of muscle weakness were found: facio-scapulo-peroneal-femoro (posterior thigh muscles)-gluteo (gluteus maximus) (FSPFG) (4 patients), facio-scapulo-peroneal-femoro (posterior thigh muscles)-gluteo (gluteus maximus)- (humeral; biceps brachii) [(FSPFG(H)] (6 patients), facio-scapulo-peroneal-femoral (posterior thigh muscles) (2 patients) and (facio)scapuloperoneal (2 patients). Thus, in ten patients their phenotypes of muscle weakness were transferred in final FSPFG or FSPFG(H). However, the interscapular and peroneal group muscles were more severely affected than posterior group of thigh and gluteus maximus muscles. Two patients aged 42 and 73 years old on re-examination after 37 years had the same pure scapuloperoneal phenotype with minimal/slight weakness of isolated facial muscles or their parts. However, on MRI of lower limb muscles the severe involvement of some posterior thigh muscles and rectus femoris was found. Thus, in all observed patients the disease began with initial involvement of the face (in minimal/slight degree) and shoulder girdle muscles and some time later of the peroneal group (anterior tibial) muscles. However, the dystrophic process gradually was extended to the thigh muscles (posterior group, namely; the quadriceps were preserved in all patients), pelvic girdle muscles (gluteus maximus, namely; the gluteus medius were preserved in 13 patients) and not always on upper arm muscles (biceps brachii, namely; slightly weakened on the one side in four from 12 patients; in two patients these muscles were severe affected). In the patients having the final phenotype the abdominal muscles were involved after affection of the peroneal group muscles and the increased lumbar lordosis due to weakness of abdominal and gluteus maximus muscles but not the erector trunci ones was observed.

Our present clinical and MRI data, as well as our earlier investigations (1969-2009), allows to suggest that FSP muscular dystrophy is probably an independent form with “hard” static and dynamic pattern of muscle involvement and a mild course of the disease. All patients including those aged 68, 73, 73 and 74 years old could walk independently and climb the stairs with the aid of a railing excluding two patients aged 63 and 88 who could walk with aid of a stick on short distances only. However, in first patient the FSPMD associated with aortic aneurism and in second one - with diabetic polyneuropathy.

The term “facioscapulolimb muscular dystrophy, type 2 (FSLD2), descending with a “jump” with initial FSP phenotype” would be more correct. The FSP or (F)SP phenotype constitutes merely a stage in the development of FSLD2. We suppose that classical AD FSPMD (or FSLD2, a descending with a “jump” with initial FSP phenotype) is different from the classical AD FSHD (or FSLD1, a gradually descending with initial FSH phenotype) although these both diseases are connected with the same 4q35 chromosomal deletion.

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17.

Cell and animal models for DUX4 expression and FSHD

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To study the regulation of the D4Z4 repeats and gene they encode (DUX4) we are taking a multifaceted approach, incorporating human FSHD DNA into C2C12 cells, murine ES cells, and mice. We have also generated iPS cells from FSHD-affected individuals, differentiated these into mesoderm, myogenic progenitors, and myotues in vitro and evaluated D4Z4 methylation and DUX4 expression. We present an update of these models and our efforts to inhibit DUX4 expression and activity.

18.

Morpholino knock-down of PITX1 improves muscle function of the Pitx1 transgenic mice

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Paired-like homeodomain transcription factor 1 (PITX1) was specifically up-regulated in patients with facioscapulohumeral muscular dystrophy (FSHD) by comparing the genome-wide mRNA expression profiles of 12 neuromuscular disorders. In addition, it is a direct transcriptional target of the double homeobox protein 4 (DUX4). To test the hypothesis that up-regulation of PITX1 contributes to the skeletal muscle atrophy seen in patients with FSHD, we generated a tet-repressible muscle-specific Pitx1 transgenic mouse model in which expression of PITX1 in skeletal muscle can be controlled by oral administration of doxycycline. In this study, we attempted to block PITX1 expression using a morpholino molecule designed to block the translation of the mRNA. The Pitx1 mice received morpholino treatment either through intramuscular or intravenous injection. The mice received the first injection when the oral doxycycline was discontinued to induce Pitx1 expression. They then received weekly injections of morpholinos until the muscle samples were collected for pathological examinations. The results showed that the PITX1 protein expression in the muscle was reduced by the treatment. The muscle function of the mice was improved measuring by grip strength tests. Although overall health was not affected, we did observe unexpected effects when treating the mice using vivo morpholino. The results suggest that morpholino can be an option for treatment development.

19. [P]

Exome sequencing in phenotypic facioscapulohumeral muscular dystrophy (FSHD2)

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Most cases of facioscapulohumeral muscular dystrophy (FSHD1) are caused by the contraction of the 3.3kb macrosatellite repeat array D4Z4 on the chromosome 4 subtelomere. The current model of the underlying molecular disease mechanism is that the dystrophic defect results from contraction-dependent epigenetic changes at D4Z4. In turn, these allow aberrant transcription of the DUX4 retrogene, which is located within each repeat unit. DUX4 transcripts have recently been shown to be stabilized by a polymorphic polyadenylation signal at the distal end of D4Z4. This poly-A site is in linkage disequilibrium with markers proximal to the array, variants of which segregate with disease status.

Between 5% and 10% of patients are diagnosed with the FSHD phenotype but do not have a contracted D4Z4 array (phenotypic FSHD or FSHD2). However, they do show the same epigenetic changes of histones and DNA methylation at D4Z4 as FSHD1 patients. Specifically, this includes the loss of histone 3 lysine 9 tri-methylation and hypomethylation at certain restriction enzyme sites (FseI, BsaAI and CpoI). The defect that triggers these epigenetic changes and the muscle phenotype in these patients is still unknown, and we intend to use exome sequencing to identify it.

We have characterized the methylation status of a panel of FSHD2 patients by methylation-sensitive restriction digest assays combined with non-radioactive Southern blotting. For this, we developed a modification of the published protocol for D4Z4 methylation analysis for use with digoxigenin labeled probes. This analysis showed the proximal D4Z4 repeat to be hypomethylated in FSHD2 patients, confirming previous studies. For the exome sequencing we are using Agilent SureSelect 50Mb human exome capture combined with high-throughput analysis on the Illumina HiSeq2000.

What makes phenotypic FSHD interesting is the fact that the molecular disease mechanisms of FSHD1 are still only poorly understood. While the case for a DUX4 involvement is convincing, we still do not understand how it affects downstream targets to cause a muscle phenotype. If we can identify the genetic mutation in FSHD2, pathway analysis could help explain the molecular link between these two related but distinct disorders. A better understanding of FSHD2 will help us develop therapeutic strategies for the majority of FSHD cases.

We would like to thank The Muscular Dystrophy Campaign UK for funding.

20. [P]

Conservation of high-copy tandem-array organisation in the DUX-gene family

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DUX4 is causally involved in the human disorder facioscapulohumeral muscular dystrophy. It exists at high copy-number as part of the macrosatellite repeat-array D4Z4, which is composed of dozens of repeats arranged in tandem. Here, we show that DUX4's most closely related homologue, DUXC, is present in a similar genomic organisation in the cow genome. We uniquely combine read-depth analysis of publicly available next-generation sequencing data with traditional gel electrophoresis and Southern blots.

The combined results show that the DUXC macrosatellite contains hundreds of repeat units that are arranged in a head-to-tail fashion. Because DUX4 arose by retrotransposition from an intron-containing DUXC-related gene, both genes must have independently acquired this unusual genomic arrangement. This suggests a common selective pressure on maintaining a high copy-number macrosatellite of DUX-genes, the normal function of which is still unknown. What drives this expansion of DUX genes is an interesting open question.

We conclude that DUXC is probably a functional homologue of DUX4, and that the evolutionary conservation includes not just the retrogene itself but also its tandem-array organisation.

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21.

On the prevalence and inheritance of FSHD2 and its relationship with FSHD1

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In the majority of patients, FSHD is caused by contraction of the D4Z4 repeat in the subtelomere of chromosome 4q. Contraction of D4Z4 is accompanied by a local chromatin relaxation making DUX4 poised for expression. Genetic studies revealed the necessity of the distal end of the array and flanking pLAM sequence for the development of FSHD. Only on specific genetic backgrounds with a stabilizing DUX4 poly(A) signal, contraction leads to substantial levels of DUX4 mRNA in skeletal muscle and bursts of DUX4 protein in cultured myonuclei.

A small but significant group of patients show D4Z4 chromatin relaxation in the absence of D4Z4 contraction. These so-called FSHD2 patients carry at least one FSHD-permissive chromosome and show D4Z4 hypomethylation on chromosomes 4q and 10q and characteristic bursts of DUX4 protein expression in cultured myotubes. The cause for FSHD2 is currently unknown.

Until now, the genetic diagnosis for FSHD2 is only performed in a research setting and the analysis is expensive, laborious and results are sometimes difficult to interpret. To get more insight into the prevalence and genetic basis of FSHD2, and its relation to FSHD1 we modified the current methylation tests to enable more accurately the diagnosis of FSHD2. To validate the test we have analyzed >300 controls and 300 patients with FSHD1 and 2. We showed that the modified test is faster, cheaper and more robust and concluded that it can be readily implemented in the diagnostic service.

We identified 28 new FSHD2 families in addition to the 27 previously reported families and show that some FSHD2 families carry FSHD1-sized D4Z4 repeats. In total we identified 70 FSHD2 patients but also unaffected family members with D4Z4 hypomethylation. We studied the mode of inheritance in these 55 FSHD2 families and found evidence that, at least in some families, D4Z4 hypomethylation segregates as a dominant trait. In addition we studied gender-based differences in the severity of the disease. This study might explain some FSHD1 families with multiple asymptomatic carriers of FSHD1-sized D4Z4 repeats and enable future studies to identify the genetic factor(s) that determines the chromatin relaxation in FSHD2.

22. [P]

In vivo Characterization of DUX4c

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The contraction that deregulates D4Z4 transcription, leading to DUX4 expression, also alters regulation of an inverted orphaned D4Z4 repeat approximately 40 kb upstream of the main tandem repeat array, resulting in transcription of DUX4c, a gene identical to DUX4 but lacking the C-terminus due to a frameshift mutation. While over-expression of DUX4 in vitro is associated with cytotoxicity, impaired differentiation, and cell death; over-expression of DUX4c in vitro appeared to be non-toxic, but blocked differentiation of C2C12 cells into myoblasts. We have generated an inducible mouse model that expresses DUX4c upon administration of doxycycline. We find unexpectedly that although DUX4c is not cytotoxic in vitro, it is organismically toxic: upon doxycycline induction, DUX4c animals present facial swelling, ichthyosis, hair loss, hunching, and ultimately succumb. This response is dependent on the dose of doxycycline delivered. These mice also show a delay to repair following cardiotoxin-mediated muscle damage. Further, Pax7+ satellite cells isolated from DUX4c+ mice subjected to a single cell cloning assay demonstrated a lower plating efficiency, fusion index, and differentiation potential. Together, these results suggest that misregulation of DUX4c in myogenic progenitors is profoundly disruptive, and might therefore contribute to FSHD pathology.

23. [P]

Transcriptional profiling and biomarker discovery in facioscapulohumeral muscular dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is a progressive neuromuscular disorder caused by contractions of repetitive elements within the macrosatellite D4Z4 on chromosome 4q35. There is currently no treatment available for FSHD and successful future clinical trials will require sensitive biomarkers to assess the efficacy of therapeutic intervention. In order to develop mRNA-based biomarkers of affected muscles, we used GeneChip Gene 1.0 ST arrays for global analysis of gene expression in muscle biopsy specimens obtained from two distinct muscles from FSHD subjects and their unaffected first degree relatives. Gene expression from a muscle with early and severe disease involvement (biceps) was compared to a relatively uninvolved muscle (deltoid). The expression differences were mild: using relaxed cutoffs for differential expression (fold-change ≥ 1.2 and nominal P-value <0.01), we identified 191 and 110 genes differentially expressed between affected and control samples of biceps and deltoid muscle tissues, respectively, with 29 genes in common. Controlling for a false-discovery rate < 0.25 reduced the number of differentially expressed genes in biceps to 188 and in deltoid to 7. Large numbers of genes are differentially expressed between biceps and deltoid, demonstrating fundamental differences between two different muscle types. Moreover, the expression levels of 199 genes in biceps and 146 genes in deltoid were associated with the residual D4Z4 repeat length. The most significantly altered genes identified in this study can be developed into muscle biomarkers for FSHD. Furthermore, unraveling the molecular pathways disrupted in FSHD will elucidate the underlying mechanisms of FSHD pathogenesis and facilitate development of novel therapies.

24.

Expression of mu-crystallin in myoblasts inhibits myogenesis

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We report studies of the expression levels of μ-crystallin in cells cultured from biceps and deltoid muscles of families in which at least one member has facioscapulohumeral muscular dystrophy (FSHD). We show that the levels of μ-crystallin expressed in myoblasts derived from FSHD patients and their unaffected, first degree relatives, as well as in the myotubes they form, vary by family, but not with disease state. Higher levels of μ-crystallin in myoblasts are associated with a lower fusion index and reduced expression of muscle-specific genes in myotubes. These changes are not due to family-to-family variability in myogenic precursor cells, as the percentage of CD56+ cells in our cultures is constant across families. We tested the potential regulatory role of μ-crystallin in differentiation by over-expressing it in C2C12 cells by adenoviral transduction, and in cultures prepared from neonatal transgenic mice heterozygotic for a μ-crystallin transgene. Both experiments revealed a significant reduction in the formation of myotubes in cultures over-expressing μ-crystallin. We conclude that the expression of μ-crystallin in developing muscle cells varies by family, and that higher levels of expression in myoblasts reduce their ability to differentiate in culture.

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25. [P]

Two-dimensional gel proteomics of muscle

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We describe improved methods for large format, 2-dimensional gel electrophoresis (2-DE) that improve protein solubility and recovery, minimize proteolysis, and reduce the loss of resolution due to contaminants and manipulations of the gels, and thus enhance quantitative analysis of protein spots. Key modifications are: (i) the use of 7M urea + 2 M thiourea, instead of 9M urea, in sample preparation and in the tops of the gel tubes; (ii) standardized deionization of all solutions containing urea with a mixed bed ion exchange resin and removal of urea from the electrode solutions; and (iii) use of a new gel tank and cooling device that eliminate the need to run two separating gels in the SDS dimension. These changes make 2D-GE analysis more reproducible and sensitive, with minimal artifacts, than published methods. Application of this method to the soluble fractions of muscle tissues reliably resolves up to 1800 protein spots in adult human skeletal muscle and 3000 spots in myotubes.

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26.

Altered Troponin T splicing in fast fibers drives muscle weakness in facioscapulohumeral muscular dystrophy

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Facioscapulohumeral muscular dystrophy (FSHD) is characterized by weakness of selective muscle groups, and a wide range of clinical variability. Mice over-expressing FRG1 (FSHD region gene 1) develop a muscular dystrophy with features characteristic of the human disease such as abnormal curvature of the spine, reduced endurance to fatigue, selective muscle impairment. In addition we found that specific pre-mRNAs are aberrantly spliced in affected muscles of FRG1 transgenics as well as in affected muscles of FSHD patients. Here, we demonstrate that aberrant splicing mRNA encoding fast skeletal troponin T (TNNT3) observed in diseased muscles from both FSHD patients and FRG1 transgenics, translates into aberrant protein isoforms that characterize the dystrophic muscle. By contrast, muscles not affected by the dystrophic process display normal fast troponin T (fTnT) isoforms in both FSHD patients and FRG1 transgenic mice. We show that fast-twitch fibers, isolated from muscles of FRG1 over-expressing mice change their features with an anomalous myosin heavy chain/actin ratio and altered expression of myofibrillar proteins. We demonstrate that these fibers display a reduced sensitivity to calcium during tension development and produce less strength. Remarkably, replacement anomalous troponin complex with the wild-type proteins restore to normal sensitivity to calcium in tension development in dystrophic fibers. Conversely, substitution of the wild-type troponin complex with complexes isolated from FRG1-overexpressing muscle diminishes fast fiber tension. On the basis of these findings we propose that aberrant splicing of the fast skeletal troponin T mRNA is a major determinant of the contraction defects of dystrophic muscle fibers in FSHD. As a result aberrant fast skeletal troponin T represents a biological marker of muscle impairment and a target for future therapeutic interventions.

27.

Facioscapulohumeral muscular dystrophy: new insights from compound heterozygotes and implication for prenatal genetic counseling

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Facioscapulohumeral muscular dystrophy (FSHD) is considered an autosomal dominant disease with prevalence of 1 in 20,000. Almost all FSHD patients carry deletions of integral copies of tandem 3.3 kilobase repeats (D4Z4) located on chromosome 4q35. However, FSHD families have been reported in which individuals carrying a D4Z4-reduced allele remain asymptomatic. Recently it has been proposed that the D4Z4-reduced allele is pathogenic only in association with the permissive haplotype 4A161-PAS.

Through the Italian National Registry for FSHD (INRF), genotype-phenotype correlations were extensively studied in 11 non-consanguineous families in which two D4Z4-reduced alleles segregate. Overall, 68 subjects carrying D4Z4-reduced alleles were examined including 15 compound heterozygotes. Our study shows that compound heterozygotes display a more severe clinical outcome than subjects carrying a single D4Z4-reduced allele evoking the presence of a dosage effect of D4Z4-reduced alleles. Remarkably, the high frequency of compound heterozygotes and the presence of D4Z4-reduced alleles with the 4A161PAS pathogenic haplotype in 50% of non-penetrant subjects suggest that carriers of FSHD-sized alleles are more common in the general population than expected on the basis of FSHD prevalence.

28.

Telomere length as a modifying factor of FSHD

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It is becoming increasingly clear that D4Z4 contraction at chromosome 4q is not sufficient to explain FSHD, and that other factors are crucial for disease manifestation. We present experimental evidence that one of these modifying factors is telomere length, showing how replicative aging could contribute to the age-related and progressive component of FSHD. We prepared cultures of purified myogenic cells from normal and FSHD patients and manipulated telomere length through the introduction and subsequent removal of telomerase. We demonstrate that a much higher expression of 4q genes including DUX4, depends on both the presence of the D4Z4 contraction and short telomeres. Therefore, we propose a model in which D4Z4 deletions render 4q genes susceptible to be transcriptionally controlled by telomere length (Telomere Position Effect). Studies to mechanistically dissect how telomeres mediate transcriptional regulation are under way. Understanding the mechanism how modifying factors contribute to FSHD pathology may open new possibilities for targeted treatment.

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29.

Reevaluating disease progression in facioscapulohumeral dystrophy

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Background: Recent breakthroughs in the molecular pathophysiology of Facioscapulohumeral dystrophy (FSHD) have identified potential therapeutic targets. Consequently, an accurate understanding of disease progression in FSHD is crucial for the design of future clinical trials.

Methods: Data from 228 subjects in 3 clinical trials and 1 natural history study were compared to examine disease progression in FSHD. All studies utilized the same techniques for manual muscle testing (MMT) and quantitative myometry (QMT). Both techniques yield a total strength score that can be followed over time as an indicator of disease progression.

Results: Whereas, natural history data showed a linear decrease in strength in both QMT and MMT significant at 1 year, data from the clinical trials were less consistent. A surprising increase in strength versus the natural history could be seen at 6 months in clinical trials regardless of treatment assignment that persisted up to 1 year for QMT. Variability estimates for clinical trial planning support values reported in the natural history study.

Conclusions: Caution must be used when basing estimates of disease progression on prior Natural History data – an effect related to being in a clinical trial can be seen, in particular at 6 months.

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30. [P]

Patient-reported disease burden in patients with genetically confirmed FSHD

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Background: A precise understanding of patient-reported disease burden and progression of disease is important when planning clinical trials in FSHD.

Methods: De-identified data from 343 genetically confirmed FSHD patients with between 3-8 years of follow up was analyzed from the National Registry of Myotonic Dystrophy and Facioscapulohumeral Muscular Dystrophy Patients and Family Members. Measures of disease included patient-reported symptoms, extra-muscular manifestations, use of assisted devices, pain, and a patient reported functional rating scale.

Results: All patients were genetically confirmed with a median D4Z4 fragment size of 25 kb (11 to 35 kb). 51% were female. Initial age of symptom onset was 22 years, and mean time until diagnosis 10 years. At baseline 91% had facial weakness, 70% arm involvement, and 67% leg involvement. 41% use an ankle brace, leg brace, cane, or walker. 24% use a wheelchair at some point during the day, with a mean age at first use of 42 years. 4% of patients use CPAP or BiPAP, 18% report hearing loss, 7% use hearing aids, and 1% report a retinal hemorrhage or detachment, but none report history of Coat's disease. 80% experience muscle pain, most commonly in the shoulder, back and hips. We expect to include data for 3-8 years of follow up in the following measures: use of assistive devices and a patient reported functional rating scale.

Conclusions: A precise understanding of disease burden and progression of disease is important for understanding the natural history of FSHD, and planning future clinical trials.

J.S. work on this project supported by NIH Experimental Therapeutics in Neurological Disorders grant #T32 NS07338-20 (PI, R. Griggs)

31. [P]

Detection of endogenous DUX4 protein in FSHD muscles: could the spreading of a rare protein cause FSHD?

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Facioscapulohumeral muscular dystrophy (FSHD) is linked to contractions of the D4Z4 repeat array in 4q35. We identified the double homeobox 4 (DUX4) gene in D4Z4 and found that the only stable DUX4 mRNAs were derived from the distal unit and extended to a polyadenylation signal within the flanking pLAM region. The DUX4 protein is expressed in FSHD but not in control primary myoblasts. DUX4 is a transcription factor that initiates a deregulation cascade, which leads to muscle atrophy and oxidative stress, both key features of FSHD. The only known target gene of DUX4 is PITX1, which encodes a transcription factor involved in hindlimb specification during embryogenesis.

In this study, we focused on the expression kinetics of DUX4 and PITX1 during the differentiation of primary FSHD myoblasts. DUX4 was detected by immunofluorescence in a few scattered proliferating cells and in a larger number of nuclei during their differentiation into myotubes. Intriguingly, DUX4 and PITX1 staining revealed an intensity gradient between consecutive myonuclei, suggesting a protein dispersion from one nucleus to its neighbors. DUX4 and PITX1 were detected either in the same nucleus with a different subnuclear staining pattern or in different nuclei. We further demonstrated that both proteins were regulated by the ubiquitin-proteasome pathway. Finally, we could immuno-detect the DUX4 protein in FSHD muscle extracts.

In conclusion, we propose a new dynamic dispersion model of DUX4 and PITX1 proteins from one nucleus, where the gene is activated, to the adjacent nuclei within the same myotube. This model, together with the transcriptional amplification cascade initiated by DUX4, could explain how a protein that is expressed in scattered myonuclei causes the muscle defects observed in FSHD.
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32.

Efficient knockdown of DUX4 and FRG1 RNAs with viral vector delivery of sequence-targeted RNAi hairpins

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Our goal is to develop a therapy aimed at specific elimination of RNAs that play a critical role in FSHD pathogenesis. Toward this goal we have established AAV6-mediated delivery of RNAi hairpins that target FRG1 mRNA to demonstrate the potential of RNAi therapeutics for muscle disease in vivo. We have shown that an shRNA targeting FRG1 mRNA in the FRG1 over-expressing mouse prevents muscle weakness associated with muscular dystrophy and ameliorates histological and molecular effects of FRG1 expression (Bortolanza et al., 2011).

Recent identification of DUX4 mRNA expression in FSHD vs. normal muscle cells shifted the focus for our RNAi-based therapy toward targeting DUX4 RNA (Lemmers et al., 2010; Snider et al., 2010). In parallel with our AAV6-mediated RNAi knockdown approach in the FRG1 mouse, we were developing and evaluating RNAi expression cassettes for the reduction of DUX4 mRNA. DUX4 RNAi expression cassettes were cloned into plasmids for preparation of both lentiviral and AAV6 stocks. Several plasmids and viral stocks were tested in C2C12 cells expressing DUX4. Dose-dependent knockdown was evident and reached >90% for all DUX4 sequences targeted with the virus-delivered RNAi hairpins. Tests of RNAi hairpin activity are underway in FSHD immortalized myoblast cell lines to measure levels of expression of DUX4-induced biomarkers detected in the FSHD myoblasts that are not found in myoblasts from normal individuals. With our RNAi-based tools we hope to further define the best in vivo target for therapy as well as provide a potential treatment for FSHD.

We are grateful to the Pacific NW Friends of FSH Research, the Muscular Dystrophy Association, and NIH (NIAMS) for their support.

33.

RNA interference inhibits DUX4-induced muscle toxicity in vivo: Implications for a targeted FSHD therapy

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The clinical features of FSHD were described over a century ago, and 4q35-associated D4Z4 repeat contractions were linked to the disease in the early 1990's. Unfortunately, because D4Z4 contractions do not completely remove or structurally mutate any obvious genes, the pathogenic mechanisms underlying FSHD were difficult to define. As a result, FSHD research for the last two decades has been primarily focused on understanding pathogenesis, while translational studies have been largely underrepresented. Today no targeted treatment for FSHD exists, but recent findings suggest the barrier to translation may be lowering.

Several studies support an FSHD pathogenesis model involving over-expression of the myopathic DUX4 gene. DUX4 inhibition may therefore be a promising therapeutic strategy for FSHD. RNA interference (RNAi) has emerged as an important tool for silencing genes associated with dominant disorders. Indeed, we recently used an RNAi-mediated approach to knockdown over-expressed FRG1 and improve myopathy in FRG1-high mice. In this study, we tested a similar RNAi-based treatment strategy to reduce toxic DUX4 levels. We used adeno-associated viral (AAV) vector-delivered therapeutic microRNAs to correct DUX4-associated myopathy in vivo. We found that our DUX4-targeted microRNAs (miDUX4) significantly reduced DUX4 RNA and protein and protected mouse muscles from DUX4-induced damage. Specifically, miDUX4-treated muscles showed minimal to no myofiber degeneration, regeneration or activation of cell death pathways, while control-treated muscles had widespread damage associated with elevated DUX4 levels. Our results provide proof–of–principle for RNAi therapy of FSHD through DUX4 inhibition, and importantly, this strategy can be modified to target other genes that may be mis-expressed in FSHD.

34.

A Novel Xenograft Mouse Model of FSHD

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Despite being one of the most prevalent neuromuscular disorders worldwide, there have been very few clinical trials in Facioscapulohumeral muscular dystrophy (FSHD). Currently there are no clinical trials and no good therapeutic options for this progressively disabling disease. One of the main impediments to the development of novel drugs for FSHD is the lack of an accepted animal model stemming from an incomplete understanding of the pathogenesis of the disease. We are therefore developing a novel animal model which is independent of any hypothesis of pathogenesis. In this model, thin strips of biceps muscle from subjects with FSHD are transplanted into the hindlimb anterior compartment of immunodeficient, NOD-Rag1null IL2rγnull mice. Our data to date indicate that grafts are quickly vascularized and that the original myofibers degenerate over weeks. Human myoblasts fully regenerate the tissue with new myofibers and survive through 20 weeks post transplantation. Innervation can be demonstrated by colocalization of pre and postsynaptic proteins. Histological and ultrastructural analysis suggest normal architectural of the human myofibers within the host. Further optimization and characterization of this model is ongoing. However, our preliminary results are encouraging that this human xenograft model of FSHD will be a valuable tool for preclinical drug screening.