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Continuing to make progress in understanding and treating FSHD

Grant awards for August 2012; also includes February 2012, August 2011 and February 2011 Cycles

Since 1998, the FSH Society has transformed FSHD research by providing grants for vital start-up funding for investigators in FSHD and research projects on FSHD. The FSH Society has two rounds of grant applications each year, with deadlines in February and August. Grant applications are thoroughly analyzed and vetted by the SAB. An initial letter of intent is submitted, which is reviewed by Professor David Housman, Chair of the SAB. If a letter of intent is accepted, the applicant submits a full application. The main section where researchers describe the proposed work and workflow is around 12 pages long. Upon receipt of all full grant applications for a particular round, Professor Housman assigns teams of two or more members of the SAB to critique each proposal. Any potential conflicts of interests are noted, and SAB members who may have a conflict are not assigned to review, and do not vote on, the particular proposal. The two reviewers review the application in depth and provide a detailed written description and recommendation to the other members. Initial critiques are due within three weeks of the assignment and a full meeting of the SAB is held around two weeks thereafter. Grant applications are reviewed and voted upon by the entire SAB, with discussion led by the two primary reviewers. SAB recommendations for approved applications are then sent to the Society's Board of Directors for a vote. When the SAB disapproves an application, it provides the applicant with a detailed description of the reasons for disapproval, and the applicant may resubmit the application for consideration in a later round. SAB members and the chair serve without pay.

Upon acceptance by the Society's board, the grantee receives a letter of acceptance and a grants policies and procedures document. The grantee is then asked for written confirmation indicating their intention of accepting or declining the fellowship knowing that the grant is administered in accordance with the FSH Society's policies document. It is understood that the funds awarded have not been provided for any other purpose than research on FSHD. The grantee is asked to reply within two weeks where upon a check is issued in advance for the first six months with equal installments to follow at subsequent six month intervals based on review of requested progress reports.

The milestones and insights gained are significant. The fellowship program allows innovative and entrepreneurial research to develop, prove successful, and ultimately to attract funding from large funding sources such as the US National Institutes of Health (NIH) and

large private sources. We are very pleased to list the grantees funded in the August 2012, February 2012, August 2011 and February 2011 cycles.

Awards for August 2012 Cycle

The FSH Society Scientific Advisory Board (SAB) met in December 2012 to review grant applications received for the August 2012 round of FSH Society grants funding. Below is a list of the funded projects, including project descriptions as submitted by grant applicant(s).

1. Role of Polycomb Group Proteins in Facioscapulohumeral dystrophy

Valentina Casà, M.S.

Davide Gabellini, Ph.D.

Division of Regenerative Medicine, Fondazione Centro San Raffaele, Milan, Italy

\$45,000 over 18 months

Summary (Provided by Applicant): Facioscapulohumeral muscular dystrophy (FSHD), the third most common myopathy, is an autosomal dominant neuromuscular disorder characterized by progressive weakness and atrophy affecting specific muscle groups. FSHD is not due to a mutation within a protein-coding gene, but is caused by contraction of the 3.3 kb macrosatellite repeat D4Z4 in the subtelomeric region of chromosome 4q35. While there is general agreement that D4Z4 deletion leads to over-expression of 4q35 genes, the molecular mechanism through which D4Z4 regulates chromatin structure and gene expression is poorly understood. Consequently, no therapeutic tool to control the aberrant 4q35 gene expression in FSHD is currently available.

Polycomb (PcG) and Trithorax (TrxG) group proteins act antagonistically in the epigenetic regulation of gene expression and they play crucial roles in many biological aspects such as development, cell proliferation and cancer. In *Drosophila*, PcG and TrxG proteins bind to specific DNA regions termed Polycomb/Trithorax Response Elements (PREs/TREs), constituting a regulated switchable element that influences chromatin architecture and expression of nearby genes.

D4Z4 shares several features with PREs/TREs. Indeed, my previous results (*Cell* 2012 149:819-31). showed that Polycomb group of epigenetic repressors targets D4Z4 in healthy subjects. Furthermore, I found that Polycomb proteins are required to maintain 4q35 genes repressed and that D4Z4 deletion is associated with reduced Polycomb silencing in FSHD patients (*Cell* 2012 149:819-31).

My preliminary results strongly suggest that D4Z4 could be the first PRE involved in a human genetic disease. An attractive hypothesis would be that a D4Z4 copy number above the threshold of 11 repeats is able to stably sustain a Polycomb-mediated repression of 4q35 genes, while few copies of the repeat fail to do this efficiently. Here, I propose to rigorously investigate the PRE activity of D4Z4. These studies will allow a deep understanding of the D4Z4 mechanism of action and will lay the basis to develop therapeutic approaches aimed at normalizing aberrant 4q35 gene expression in FSHD.

My specific aims are:

- 1.) To understand the mechanism through which the deletion of D4Z4 repeats below a threshold copy number is affecting 4q35 gene expression in FSHD.
- 2.) To identify potential therapeutic targets.

2. Derivation of human induced pluripotent stem cells from FSH patient fibroblasts

Gabsang Lee, Ph.D.

Johns Hopkins University, Baltimore, Maryland

\$49,705 over 1 year

Summary (Provided by Applicant): The genetic and biological events that result in Facioscapulohumeral muscular dystrophy (FSHD) pathogenesis are complex and the link between the genetic aberration and manifestation of symptoms is still elusive. We hypothesize that there might be cellular and genetic alteration in the early stage of myogenesis in FSHD patients. The establishment of human induced pluripotent stem cells (hiPSCs) ushered a new era in biomedicine and can be useful for modeling pathogenesis of human genetic diseases, autologous cell therapy after gene correction, and personalized drug screening. Our lab has been studying human genetic disorders by using induced pluripotent stem cells (hiPSCs) that is a new type of stem cells without destruction of any embryonic tissues or embryos. In addition, we already built a novel methodology in highly innovative manner to directly derive and prospectively isolate skeletal muscle from the hiPSCs. Here we propose to establish hiPSC lines from FSHD patient's somatic cells. Our proposed study will enable us to isolate FSHD-specific skeletal muscle cells for better understanding of FSHD pathogenesis in human system as well as potential autologous cellular therapy accompanying with genetic correction in near future.

3. Autophagy defects in FSHD

Sachchida Nand Pandey, Ph.D.

Children's Research Institute, Washington, DC

\$99,599 over 2 years

Summary (Provided by Applicant): Our previous study showed that DUX4 was up-regulated in patient's muscles of FSHD and transcriptionally regulated paired-like homeodomain transcription factor 1 (PITX1). The muscle-specific expression of Pitx1 in transgenic mouse model showed muscular dystrophy phenotype similar to FSHD [Pandey et al., 2012]. Expression profiling data of Pitx1 transgenic mice showed that 16 major autophagy genes, including damage-regulated autophagy modulator (Dram1) were mis-regulated in the muscle over-expressing PITX1. To determine whether the autophagy pathways were also affected in FSHD, we investigated the autophagy state in FSHD myoblasts as well as patients' muscle biopsies. Our data showed disease-specific up-regulation of a master autophagy regulator, DRAM, in FSHD muscle biopsies but not DMD or controls.

To further characterize the autophagy state in FSHD myoblasts we cultured the myoblast in differentiation media and we found that DRAM was up-regulated in FSHD myoblasts compared to the control myoblasts. We then examined two proteins critical to autophagy activities, p62 and LC3B. The p62 protein binds both ubiquitinated substrates and

LC3B [Pankiv et al., 2007], and has been used as an indicator of autophagic flux. In addition, the accumulating of p62 has been used as an indicator of defective autophagy [Settembre et al., 2008; Ju et al., 2010]. In our study, instead of down regulation when autophagy is activated, p62 showed up-regulation in FSHD myoblasts suggesting a defect in autophagy activation. We further checked the LC3B-II to LC3B-I ratio (LC3B-II/I) which is a commonly used marker for autophagy activation. Because LC3B-II is formed only when autophagosomes are generated, the LC3B-II/LC3B-I ratio represents the density of autophagosomes in cells. The significantly lower LC3B-II/LC3B-I ratio in the FSHD myoblasts indicated again a suppression of autophagy in the myoblast. The suppression of autophagy is also supported by accumulation of ubiquitinated protein in the FSHD cells. While the activation of DRAM should activate the downstream autophagy pathways, we observed a defect in autophagosome formation. Interestingly, the up-regulation of LAMP1 and 2 at mRNA level in muscle biopsy of patients with FSHD suggests that the lysosomal system is activated and ready for the later steps of forming autophagolysosomes. However, the autophagy process is somehow disrupted in FSHD myoblast. In addition, the aberrant expression of DUX4 is a cause of FSHD so we would like determine whether defect in autophagy process is directly linked with expression of DUX4. On the basis of our preliminary result we hypothesize that defect in autophagy causes differentiation defect in myotubes formation in FSHD. In addition, autophagy defect is directly induced by aberrant expression of DUX4.

In proposed study, we will examine the expression changes of the key regulators of autophagy and further investigate the mechanisms involved in autophagy defects in FSHD. In addition, we will knock-down the DUX4 expression in the FSHD myoblasts to determine whether the autophagy defects are directly induced by the aberrant expression of DUX4 in the cells. The goal of this current proposal is to understand the mechanism and to identify molecular pathways for treatment development. The aim for this study as follows:
Aim 1: To determine the expression of DRAM, p62, Autophagy related gene 5 (ATG5), Autophagy related gene 4B (ATG4B), LC3B, and LAMP1 in patients with FSHD. We anticipate that DRAM, p62 and LAMP1 will show higher expression whereas LC3B-II/LC3B-I ratio will be low in FSHD. Aim 1A: To determine the expression of DRAM, p62, ATG5, ATG4B, LC3B, and LAMP1 in FSHD myoblasts with and without autophagy induction. Aim 1B: To determine the expression of DRAM, p62, ATG5, ATG4B, LC3B, and LAMP1 in muscle biopsies of patients with FSHD. Aim 2: To determine whether the defects in autophagy are due to inhibition of fusion between the autophagosomes and lysosomes. We anticipate a reduction in fusion of lysosome to autophagosomes will be observed in FSHD myoblasts but not in the control myoblasts. Aim 2A: To determine the inhibition of fusion efficiency between the autophagosomes and lysosomes in immortalized FSHD myoblasts. Aim 2B: To determine the expression of ATG4B, a key regulator of LC3B conversion, in immortalized FSHD myoblasts. Aim 3: To determine whether the defects are induced by DUX4 by knocking down DUX4 in the immortalized human myoblasts using antisense oligonucleotides against DUX4. Aim 3A: To determine the expression of DRAM, p62, ATG5, ATG4B, LC3B in proliferating and differentiating myoblasts after knocking-down DUX4. Aim 3B: To determine whether DUX4 knock-down can correct the inhibition of fusion between the autophagosomes and lysosomes in FSHD myoblasts.

4. Evaluation of an FSHD-specific patient reported outcome measure and a disease specific functional rating scale

Jeffrey Statland, M.D.

University of Rochester, Rochester, New York

\$59,185 over 2 years; \$43,666 year 1, \$11,931 year 2

Summary (Provided by Applicant): Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common forms of muscular dystrophy with an estimated prevalence between 1:15,000 and 1:20,000. The clinical spectrum of disease severity is wide, and the regional distribution of muscle weakness, as well as the pattern of progression, is unique. The molecular defect in FSHD on chromosome 4q35 was described in 1992 but the molecular pathophysiology remained unknown until recently. A unifying model has now emerged proposing the aberrant reactivation of the DUX4 gene - resulting in a toxic gain of function - in the pathophysiology of FSHD. This FSHD model has provided, for the first time, therapeutic targets for FSHD, and it is expected that several potential therapeutic interventions will emerge in the coming years. Because of these recent discoveries, there is an urgent need to develop the tools necessary to effectively and efficiently conduct therapeutic trials in FSHD. Existing validated outcome measures in FSHD are neither sensitive to change nor intuitively patient-relevant. More sensitive outcome measures are needed for a more efficient drug development process.

The need for patient-relevant outcome measures was emphasized in the proceedings of the 2010 FSHD European Neuromuscular Centre meeting. Moreover, there is increasing emphasis by the FDA on the development of outcome measures that are clinically meaningful and based on the patient's perspective. There are currently two validated, commonly utilized outcome measures in FSHD (manual muscle testing [MMT] and quantitative myometry) both of which are based on direct strength testing. Although direct measurement of muscle strength makes intuitive sense in a myopathy, what minimum change in such a measure can be considered clinically relevant is not clear. There are, additionally, two FSHD-specific clinical severity scales that have been validated in cross-sectional studies; however, neither the responsiveness to change over time nor the direct relevance to patients has been demonstrated. Moreover, as 10 and 15 point ordinal scales, they are not likely to be highly sensitive to change. Here we plan to test the reliability, validity and responsiveness to change of two FSHD-specific outcomes: the FSHD Health Inventory (FSHDHI) and the FSHD Functional Outcome (FSHD-FO). Both of these instruments were developed based on direct patient input to reflect the most prevalent and important physical limitations of FSHD. We will recruit 35 participants with FSHD for 4 visits over 1 year of follow up. Outcomes will be compared at baseline and longitudinally to traditional measurements such as the composite MMT score, existing FSHD clinical rating scales, and SF-36 health survey. Additionally an anchoring technique will be used to determine the minimally clinically important change. We expect that this proposal will provide preliminary data on the utility, ease of administration, reliability and validity, and responsiveness to change over 1 year of two novel and clinically relevant FSHD-specific outcome measures. We have designed our budget so that reliability

and convergent validity are tested in year 1; and responsiveness in year 2, months 12-18. It is of vital importance for the FSHD research community that development of outcome measures parallels advancements in molecular pathophysiology and drug development. The scales presented here both represent valuable, patient-relevant tools for the FSHD clinical trial toolkit.

5. Specific Silencing of FAT1: Role in Pathogenesis of FSHD

Angela K. Zimmermann, Ph.D.

Centre National de la Recherche Scientifique, IBDML – Development Biology Institute of Marseille, Campus de Luminy, Marseille, France

\$140,000 over 2 years

Summary (Provided by Applicant): This proposal outlines a postdoctoral fellowship project, which I (AK Zimmermann) am planning to conduct in the laboratory of Françoise Helmbacher at IBDML in Marseille, France over the next 2 years. I arrived in the lab 5 months ago and have already initiated the research project described below (see preliminary data). The Helmbacher laboratory studies mechanisms that contribute to assembly of neuromuscular circuits during development and associated pathologies. Recently the lab has begun work on facioscapulohumeral muscular dystrophy (FSHD), a devastating human disease characterized by degeneration of muscles in the face and shoulder area. Mechanisms contributing to FSHD remain elusive although pathogenic deletions within a D4Z4 macrosatellite array on chromosome 4 have been identified in the majority of clinical cases. Several studies investigating how this genetic alteration exerts its pathogenic effect have led to propose a contribution of deregulated expression of genes proximal to the deleted region (FRG1 & 2, ANT1), as well as a toxic effect of DUX4, a transcription factor whose transcription is enabled in the pathogenic context. However, a role for these genes in the selective muscle deficits associated with FSHD has not been irrevocably established and therefore additional candidates remain to be identified. The Helmbacher laboratory has shown that FAT1, a protocadherin gene also located near the D4Z4 locus, regulates muscle development and may play a role in physiology of mature skeletal muscles as well. In two mouse mutant models, FAT1-deficient mice were found to develop an FSHD-like phenotype, including both muscle and non-muscle defects. Notably, the developmental abnormalities of muscle shape appear to prefigure the map of muscles affected in FSHD patients. In addition, analyses of human foetal biopsies suggested that tissue-specific silencing of FAT1 might be a causal mechanism in FSHD. This was supported by the finding that several FSHD patients without the classical D4Z4 abnormality carried a deletion of a cis-regulatory enhancer of the FAT1 gene. Here, I propose to study the role of FAT1 in FSHD pathogenesis, and specifically to answer the following questions: 1. Owing to a conditional allele of FAT1 developed in the Helmbacher lab, I will use a tissue-specific ablation approach to ask in which tissues FAT1 expression is relevant for proper muscle migration (candidates include muscle, nerve, vascular and connective tissues) 2. Using primary culture assays on myoblasts and myotubes isolated from Fat1 mutant embryos, I will ask whether FAT1 also play a role in muscle function, as suggested by its subcellular association to the t-tubule excitation-contraction coupling system. Ultimately, alterations of these functions in FSHD patients might be accessible to preventive

therapies. 3. Finally, I will ask how FAT1 silencing contributes to dysregulation of retina vasculature, a symptom of FSHD that may provide clues about the molecular mechanisms associated with both muscle and non-muscle phenotypes of disease? Ultimately we hope these strategies will contribute to the development of therapeutic targets aimed at bypassing FAT1 silencing in FSHD and maintaining functional Fat1 levels in muscle prior to worsening of the muscle degeneration symptom.

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Awards for February 2012 Cycle

The FSH Society Scientific Advisory Board (SAB) met in June 2012 to review grant applications received for the February 2012 round of FSH Society grants funding. Below is a list of the funded projects, including project descriptions as submitted by grant applicant(s).

1. A transgenic model of DUX4-mediated FSHD

Peter Jones, Ph.D.

Boston Biomedical Research Institute, Watertown, Massachusetts

\$105,000 over 2 years, \$60,000 year 1, \$45,000 year 2

Summary (Provided by Applicant): The most critical need in the FSHD field is a reliable and faithful mouse model of FSHD. This has been inhibited in the past by lack of a consistent and consensus understanding of the gene misregulation in the human condition that leads to FSHD pathology. Now that there is wide spread agreement about the involvement of DUX4-fl in FSHD pathology there are different barriers; the severe cytotoxicity of DUX4 and its lack of conservation in mammals. As such, the field has so far failed to generate a genetic mouse model based on DUX4 expression that recapitulates the DUX4-fl expression profile and FSHD-like pathophysiology. This project proposes to generate a regulable and tunable strain of D4Z4/DUX4 transgenic mice using the Cre/lox system and targeted transgenesis into the Rosa26 locus. Importantly, this model incorporates the downstream cis regulatory elements and DUX4 splicing and polyadenylation of the FSHD-associated 4q35 locus. This is different from any of the mouse models discussed at meetings (none are published) that fail to show any phenotype. The targeting construct has already been generated and shown to function properly in human and mouse myogenic cell culture and myotubes. With this construct we believe we can manipulate DUX4-expression in mice 1) to a range of cells in a population (1:50 down to 1:5000) in the developmental profile of DUX4 expression and/or 2) in any select tissue or spacio-temporal pattern desired. These mice will prove invaluable for therapeutic screening and understanding DUX4 function. As such, once generated and initially characterized we will make these mice available to the FSH community at large in a timely manner for those with therapeutic approaches.

2. Expression of Human DUX4 in Zebrafish Development

Hiroaki Mitsuhashi, Ph.D. / Louis Kunkel, Ph.D.

Children's Hospital Boston, Boston, Massachusetts
\$60,000 over 1 year

Summary (Provided by Applicant): FSHD is characterized by an asymmetric progressive weakness and wasting of the facial, shoulder and upper arm muscles. Hearing loss and retinal vasculopathy are frequently accompanied. Accumulating evidence supports the hypothesis that derepression of DUX4, a double homeobox gene located within D4Z4 unit in chromosome 4q35, play a role in the pathogenesis of FSHD. However, a major problem with this hypothesis is the extremely low abundance of DUX4 expression in FSHD muscle. It has been shown that approximately one cell per 1000 expresses DUX4 protein in cultured FSHD muscle cells. How this sporadic burst of DUX4 expression can cause a chronic and progressive myopathy is largely unknown. To address this question, generation of DUX4 animal model is essential. However, there is currently no good animal model due to the toxic nature of DUX4 where overexpression of DUX4 induces apoptosis to many types of cells, resulting in embryonic lethality. This has hampered the further understanding of FSHD pathogenesis and the development of therapeutic approaches. To generate DUX4 animal model, we injected extremely small amount of human DUX4 mRNA into zebrafish embryos. Microinjection of 0.2 or 0.1 pg of DUX4 mRNA ($\approx 1 \times 10^5$ copies) caused asymmetric abnormalities on their eyes and fins. Injected embryos also showed affected muscle birefringence and slow swimming, suggesting muscle degeneration and weakness. These phenotypes are very similar to those observed in FSHD patients. We believe that DUX4 injected in small amounts into zebrafish is a good animal model for investigating the pathogenesis of FSHD and the impact of DUX4 on development. In this proposal, we plan to define the phenotype of the DUX4 mRNA injected zebrafish model to determine how much and where in development expression of DUX4 can cause the human FSHD-like phenotype in zebrafish. Furthermore, we plan to develop a conditional DUX4-transgenic zebrafish to create stable model. These zebrafish models will help us to understand DUX4-mediated pathogenesis in vivo, and provide us a platform to screen a number of small molecules for therapeutic approach.

3. FAT1 roles in muscular physiology and FSHD onset

Virginie Mariot, Ph.D. / Julie Dumonceaux, Ph.D. and Gillian Butler-Browne, Ph.D.

Thérapie maladies du muscle strié / Institut de myologie, Paris, France

\$68,000 over 1 year

Summary (Provided by Applicant): FSHD is an autosomal dominant pathology recently ranked to the most prevalent muscular dystrophy. The genetic locus of the FSHD pathology has been identified 20 years ago, but the molecular mechanisms leading from this genotype to FSHD are still not clearly understood. Indeed, despite recent findings which highlighted the notion of permissive chromosome for FSHD, and the fact that Dux4 is always expressed in FSHD biopsies from these permissive chromosomes, the consequence of this expression on muscle development and function is not well established and the link between Dux4 expression and the development of FSHD pathology is not clearly understood. This

reinforces the complexity of FSHD and emphasizes the need to identify other genetic elements putatively involved.

Recently, in a collaborative effort involving 3 French laboratories (Francoise Helmbacher, Nicolas Levy and ours), we have identified a new gene named FAT1 which, when down-regulated in mice, recapitulates FSHD muscular phenotypes: at early post natal stages, shoulder and face muscles present an asymmetric atrophy, whereas at later stages, a widespread muscular dystrophy is observed. Moreover, the FAT1 mutant mice also present some non muscular FSHD characteristics such as a retinal vasculopathy. In human, FAT1 is located in 4q35 and belongs to the Planar Cell Polarity (PCP) family which is involved in coordinating tissue polarity, morphogenetic movements, and polarized cell flow. Interestingly, we have also observed that FAT1 mRNA is systematically down-regulated in human FSHD fetal muscle biopsies (but not in brain) as compared to age matched control fetuses. Moreover, in several FSHD2 human samples which do not present the typical FSHD contraction, deletions in the FAT1 gene have been found.

All together, these observations strongly suggest that FAT1 may play a major role in the FSHD pathology. Since FAT1 has never been described to play a role in muscle physiology in mammals, our goal is now to understand its biological function and to analyze how it may underlie the onset and progression of FSHD disease. We have already demonstrated that different FAT1 isoforms co exist in human muscle cells and that they are differentially expressed in proliferating and differentiating conditions. Moreover we have also observed that the expression of some isoforms can be coregulating each other, thus highlighting the complex regulation of FAT1 expression and the urgent need to decipher its specific expression. Our aim is to specifically up regulate or down regulate each isoform in normal or FSHD fetal myoblasts and to analyse the effects on myotube formation, on the expression of the other isoforms, on the localization of the protein using FAT1 specific antibodies and on the modulation of expression of other factors we have recently identified in biopsies and in cell cultures (proteins, miRNA), which represent putative new biomarkers for FSHD. Finally, different miniFAT1 will be cloned in viral vectors which will be used to transduce the FAT1 mutant mice in order to try to rescue the FSHD phenotype.

4. A humanized mouse model for investigations of FSHD pathology and therapeutic development

James Windleborn, Ph.D. / Charles Emerson, Ph.D.

Boston Biomedical Research Institute, Watertown, Massachusetts

\$60,000 over 1 year

Summary (Provided by Applicant): 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. Because of the unique nature of human D4Z4 repeats, there is currently no mouse disease model. To provide such a valuable tool, we have developed a humanized mouse model for FSHD. This model was obtained by the engraftment of FSHD patient derived myoblasts into mouse muscle. Because of the dominant nature of the disorder, we hypothesized that the FSHD 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 work has established the feasibility of this project. Our findings demonstrate the successful, high efficiency engraftment of myogenic cells from FSHD and control subjects into injured, regenerating tibialis anterior (TA) muscle of immune-compromised mice. Early passage myoblast cells from cohorts of FSHD probands and their appropriate controls (i.e., a first degree relative) for these studies were provided by the unique cell repository of the Boston Biomedical Research Institute (BBRI) Wellstone Cooperative Research Center for FSHD Research. We have grafted these standardized cultured cells into mouse muscle to obtain the FSHD humanized mouse model, thereby generating a well-controlled in vivo model for the study of FSHD. We here propose to investigate FSHD disease progression in the humanized mouse model, through studies of the expression of DUX4, an FSHD candidate disease gene, and a larger set of 143 putative FSHD disease biomarkers, which includes a number of DUX4 target genes, identified by the BBRI Wellstone Center by microarray analysis of differentiated FSHD and control myogenic cells. Furthermore, in vivo animal imaging technologies will be utilized to investigate the survival, regenerative capacity and maintenance of FSHD myogenic cells engrafted into injured mouse TA muscle as a pathological mechanism for FSHD. In summary, this work will contribute to the understanding of the FSHD pathogenesis in vivo by defining the cellular and molecular disease pathology of FSHD using the unique humanized mouse model of FSHD. Research findings will enable identification of new drug targets for FSHD treatment and provide an animal model for preclinical studies of RNA silencing and small molecule FSHD drugs.

5. Tri-dimensional organization of the FSHD locus during proliferation and differentiation of muscle Cells in FSHD patients and controls

Marie Gaillard, M.S. / Frederique Magdinier, Ph.D.

INSERM UMR_S 910, Epigenetics, chromatin & diseases team, Faculté de Médecine de Marseille, FRANCE

\$30,000 over 1 year

Summary (Provided by Applicant): FSHD is an autosomal dominant neuromuscular disorder, with an incidence of 7: 100,000 recently ranked as the most common and prevalent rare muscular dystrophy of the adult. The number of D4Z4 is a critical determinant of the age of onset and clinical severity of the disease. A region distal to D4Z4 generates two allelic forms, 4qA and 4qB both equally common in the general population but FSHD is mainly associated with the 4qA allele. In addition, other polymorphisms, qualified as permissive alleles, exist; the pathology being often associated with the 4qA161 allele. In 5-10 % of families with a typical FSHD phenotype, there is no linkage to 4q35 and this type is referred to as type 2. Over the last decade, major advances have occurred in the understanding of the genetics of this disorder however the exact patho-mechanisms secondary to the genetic defect are still not understood. The DUX4 ORF is localized within D4Z4 and since at least one repeat is necessary to generate a pathogenic phenotype it has been hypothesized that DUX4 overexpression contributes to the pathology by leading to the production of a toxic protein in 1 in 1000 muscle nucleus. While several groups are actively seeking targets of the DUX4

protein, a technically challenging approach, our goal is to understand what leads to DUX4 expression. The deletion of repetitive elements and changes in epigenetic marks across the D4Z4 array such as DNA hypomethylation or decrease in H3K9 trimethylation also indicates that FSHD involves chromatin changes and epigenetic alterations. Interestingly, a localization of the 4q telomere at the nuclear periphery has been reported including by our group (Ottaviani et al., 2009; Arnoult et al., 2010), in close proximity to heterochromatin suggesting that subnuclear positioning contributes to this peculiar pathology. However, the links between epigenetic changes, nature of the 4q35 sequences, DUX4 expression and muscle phenotype have never been fully demonstrated.

Therefore, based on our current knowledge and expertise, we are deciphering the link between the subnuclear positioning of the D4Z4 array, epigenetic changes and DUX4 expression in the pathology. Thus, we focus on the regulation of the 4q35 region as a whole through the constitution and exploration of a unique cohort of patients' samples including atypical cases and cellular models. The strategies proposed here, would lay new grounds for the deciphering of this complex disorder.

- Within the frame of this project, a cohort of samples from patients is available including accompanying detailed patient histories and genotyping.
- Primary myoblasts and fibroblasts for FSHD pathogenesis,
- Induced pluripotent cells (iPSCs) from FSHD1 and 2 patients. This cellular model will be a very valuable tool to investigate the role of epigenetic changes in the tri-dimensional organization of the FSHD locus and the regulation of a putative "FSHD gene" during differentiation and investigate how DNA methylation contributes to the pathogenesis.

The epigenetic mechanisms regulating the 4q35 locus remain poorly understood, including how DNA methylation is controlled in the pathology and whether hypomethylation is a cause (increased instability) or a consequence of D4Z4 array shortening. We aim at understanding whether D4Z4 hypomethylation is an early event that precedes shortening of the repeated array or simply the consequence of the loss of a certain number of repeats by comparing methylation profiles in normal and diseased cells, and investigate the intergenerational transmission of DNA methylation in FSHD1 and 2 patients and correlate this methylation level with disease penetrance, D4Z4 array compaction and DUX4 expression. We have already shown that it is possible to visualize methylated regions through the detection of methylcytosines on combed DNA molecules using anti-m5C antibodies. We wish to use MC combined with DNA methylation detection with the probes designed for genomic analysis in order to evaluate the DNA methylation pattern of the D4Z4 array and flanking sequences for each individual allele on the slide and all D4Z4 copies. The sensitivity and stringency of the method requires further development and tests of different experimental conditions are in progress.

Hypomethylation occurs in FSHD2 suggesting that DNA methylation metabolism is globally altered in FSHD. The de novo methyltransferase (DNMT3b) contributes to D4Z4 methylation suggesting that epigenetic programming of the repeat occurs at early developmental steps. We investigated the expression of catalytical DNMTases and co-regulatory splicing variants in myoblasts. Demethylation and chromatin changes might also

be subsequent to differentiation. iPSCs provide a unique platform to dissect the mechanisms of epigenetic reprogramming during differentiation for typical FSHD cases and FSHD2. Using this type of model, we are investigating the timing of production of DUX4, and testing whether repeat's transcription initiates epigenetic regulation in different genomic contexts and vice versa.

Gene localization in the nucleus is not arbitrary, it's a dynamic process and we have previously identified D4Z4 as the first human sequence able to control the localization of its abutting telomere by tethering this chromosome end toward the nuclear periphery. We hypothesize that the tri-dimensional organization of the 4q35 locus is altered in patients, being responsible for deregulation of disease's gene expression. Using a large collection of patients' biopsies, we have shown that the expression of several genes located in the 4q35 region is modulated in patients and we are now trying to describe how the tri-dimensional organization of the D4Z4 array might affect gene expression by comparing 4q35 chromatin conformation in patients and unaffected individuals in order to understand the interactions between sequences, either distant or in close proximity during proliferation of muscle precursors and muscle differentiation. To study the tridimensional distribution of DNA sequences within the nuclear volume, we have developed a FISH technique coupled with immunofluorescence in 3D. Since FSHD is a progressive pathology, we have chosen to focus on three different cell types, fetal and adult primary myoblasts and fibroblasts from patients or controls, as well as induced pluripotent stem cells (iPSc) derived from primary fibroblasts. Our preliminary results show that hybridization patterns differ in patients and controls cells. Signal hybridization volumes display a significant difference between patients and controls which might reflect the degree of chromatin relaxation in the pathology. Moreover, signals corresponding to 4q35 probes are more colocalized in patient cells, suggesting an interaction between sequences undergoing the same regulatory mechanisms. Hence, we will consolidate our data by using iPSCs as a model of the pathology. These approaches should bring further insights into the underlying mechanisms of FSHD in order to identify target genes and to understand by which mechanism, a decrease in the number of D4Z4 leads to this muscular dystrophy.

This project is expected to lead to a better understanding of the regulation of the 4q35 at the genomic and epigenomic levels. We believe that the different aspects proposed here are required to get further insights into the patho-mechanism of FSHD, a key step for the future development of specific therapeutic targets.

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Awards for August 2011 Cycle

The FSH Society Scientific Advisory Board (SAB) met in October 2011 to review grant applications received for the August 2011 round of FSH Society grants funding. Below is a list of the funded projects, including project descriptions as submitted by grant applicant(s).

1. Identification of the epigenetic mechanisms that regulate DUX4 activity in skeletal muscle

Richard J.L.F. Lemmers, Ph.D. / Silvere van der Maarel, Ph.D.

Leiden University Medical Center (LUMC)

Department of Human Genetics, Leiden, Netherlands

\$80,000 over 2 years

Summary (Provided by Applicant): D4Z4 repeat array chromatin relaxation and transcriptional de-repression of the non-polyadenylated double homeobox 4 (*DUX4*) gene unifies D4Z4 contraction-dependent FSHD1 and contraction-independent FSHD2. Only from FSHD-permissive genetic backgrounds *the DUX4 transcript* originating from the most telomeric unit of the array can be stabilized by a polyadenylation (polyA) signal outside the array. Non-permissive chromosomes fail to stabilize *DUX4* in the absence of this polyA signal. Somatic *DUX4* derepression in FSHD1 and FSHD2 leads to bursts of *DUX4* protein in sporadic nuclei of cultured FSHD myotubes.

DUX4 is highly expressed in the germ line. It is low expressed in embryonic stem cells and it subsequently gets silenced during differentiation. FSHD iPS cells fail to silence *DUX4* during differentiation. The regulatory mechanisms that act upon *DUX4* in muscle are largely unknown and currently we do not know how a protein that is expressed in minute amounts causes a chronic and progressive muscle wasting.

While others have used conventional over-expression vectors to study the effect of *DUX4*, we have consistently observed that using constructs in which the genomic organization of *DUX4* is retained, i.e. within the context of D4Z4, the locus creates sporadic bursts of *DUX4* expression: not only in FSHD1 and FSHD2 cultured muscle cells, but also in muscle cells cultured from our transgenic L42 mice and in C2C12 cells stably transfected with a genomic D4Z4 construct. These bursts already occur at low frequency in proliferating cells and increase in frequency during differentiation.

I aim to identify the epigenetic mechanisms that regulate the bursts of *DUX4* activity. I will develop reporter constructs in which the *DUX4* ORF in D4Z4 is replaced by a reporter gene but in which otherwise the genomic integrity of the distal *DUX4* gene is preserved. These reporter constructs will be used in the following set of experiments:

1. Fluorescent reporter constructs will be used in life cell imaging studies to precisely characterize the bursts of expression. Although the highest somatic expression of *DUX4* is observed in differentiated myotubes, occasional nuclei expressing *DUX4* can also be observed during proliferation. Life cell experiments will establish whether bursts of *DUX4* are cell-cycle dependent or whether other factors regulate *DUX4* expression. It will also establish whether a single nucleus can repeatedly express *DUX4*, or whether this is a one-time event.
2. Inserting a fluorescent reporter in the construct allows for the separation of expressing muscle cells by FACS sorting and comparison of the chromatin structure of expressing and non-expressing cells by ChIP with a panel of histone modifications that allows for the recognition of the major chromatin states in mammalian cells. These chromatin studies will be validated in our extensive panel of primary muscle cells of FSHD patients and controls. I expect this study yield a

comprehensive epigenetic map of the FSHD locus in DUX4 expressing and non-expressing cells.

3. The reporter construct will also be used in dedicated and in large-scale screens for compounds that activate or repress DUX4. I will use an established RNA-interference (RNAi) screen (collaboration with Dr. Agami, NKI, Amsterdam) to identify chromatin modifiers that affect the D4Z4 chromatin structure. I will validate these studies in primary muscle cells of FSHD patients and controls. I expect this study to provide mechanistic insight in the chromatin structuring of the FSHD locus in patients and controls.

Currently we have identified a uniform molecular mechanism for FSHD. I expect this study to contribute to the current gap in our model of how a protein that is expressed in minute amounts causes a progressive muscle disease.

A FSH Society Marjorie Bronfman research grant FSHS-MGBF-019 FSHS-82011-01

2. Resonance Imaging and Spectroscopy Biomarkers in FSHD

Doris G. Leung, M.D. / Kathryn R. Wagner, M.D., Ph.D.

Hugo W. Moser Research Institute at Kennedy Krieger, Baltimore, Maryland

\$43,650 over 1 year

Summary (Provided by Applicant): Facioscapulohumeral muscular dystrophy (FSHD) is the most prevalent hereditary progressive muscle disorder in humans. It is an autosomal dominant disease that causes wasting and weakness in multiple muscle groups (face, shoulders, and upper arms initially, and legs later in the disease) as well as significant disability in affected individuals. Recent advances in understanding the patho-physiology of FSHD have led to the identification of therapeutic targets. However, we lack appropriate biomarkers that reflect the degree of muscle degeneration and regeneration in these patients. Such biomarkers will be necessary for the successful completion of clinical trials. The proposed study will test the hypothesis that proton magnetic resonance spectroscopy (MRS) and quantitative magnetic resonance imaging (MRI) can be used to define unique metabolic profiles in the skeletal muscle of patients with FSHD and controls with normal muscles. These profiles can then be used as biomarkers of disease severity and surrogate outcome measures in therapeutic clinical trials in FSHD.

The initial aim of the project will be to establish quantitative MRS patterns in the skeletal muscles of patients with FSHD. We will accomplish this by performing a cross-sectional imaging study of 30 subjects with genetically-confirmed FSHD and measurable biceps weakness. Each subject will undergo MRI/MRS imaging of the upper extremity, and we will correlate the metabolic profiles of the biceps muscles to disease severity (as measured with quantitative muscle testing). Further specific aims will be developed to: 1.) compare magnetic resonance spectroscopy profiles of subjects with FSHD to groups of healthy and diseased controls, 2.) identify correlations between MRS biomarkers and molecular biomarkers collected from muscle biopsy samples, and 3.) observe longitudinal changes in muscle spectroscopy on repeat studies. The combined output of the proposed project will be an imaging protocol that can be used in future clinical trials in FSHD.

A FSH Society Irene Lai research grant FSHS-SLMM-002 FSHS-82011-02

3. Additional Support for Medicinal Chemistry Developing anti-DUX4 therapeutics for FSHD

Michael Kyba, Ph.D.

Lillehei Heart Institute, University of Minnesota, Minneapolis, Minnesota

\$25,000 over 2 years

Summary (Provided by Applicant): This \$12,500 per year grant is supplementary support linked to an NIH R21 1R21NS076671-01 (2-year) application directed towards identifying chemical inhibitors of DUX4. We have previously screened 200,000 compounds and identified approximately 600 inhibitors of cell death induced by DUX4, and current work is directed towards identifying the most promising leads within this set of 600. The NIH application was recently selected for funding and the grant was initiated. This support from the FSH Society will be used to supplement the NIH project, principally by providing additional medicinal chemistry support (allowing us to increase the number of compounds that we follow-up by purchase/synthesis. These compounds help us to understand pathways that can indirectly inhibit toxicity associated with DUX4. See 1R21NS076671-01 [http://projectreporter.nih.gov/project_info_description.cfm?aid=8225734&icde=10980193] for more information on the R21.

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Awards for February 2011 Cycle

The FSH Society Scientific Advisory Board (SAB) met in June 2011 to review grant applications received for the February 2011 round of FSH Society grants funding. Below is a list of the funded projects, including project descriptions as submitted by grant applicants.

1. Antisense strategies against DUX4 as therapeutic approaches for FSHD

Eugénie Anseau, Ph.D./Alexandra Belayew, Ph.D.

Universite de Mons, Mons, Belgium

\$70,500 over 2 years, \$25,000 year 1, \$45,500 year 2

Summary (Provided by Applicant): FSHD is a muscle degeneration disease genetically linked to contractions of the D4Z4 repeat array on the 4q35 subtelomeric region. Our group has identified the double homeobox 4 (DUX4) gene within each unit of the D4Z4 repeat array and shown that the encoded protein was expressed in primary myoblasts and biopsies of patients with FSHD but not in non-affected individuals. We found that the only stable DUX4 messenger RNAs derive from the last unit and extend to the flanking pLAM sequence that provides a polyA addition signal. This signal is required to develop FSHD as independently confirmed by an eight-laboratory consortium which studied genetic polymorphisms in hundreds of patients and thousands of healthy individuals. In aggregate our collaborative studies with four different groups have shown that the DUX4 protein is a

transcription factor that targets a large set of genes, some of which encode other transcription factors that in turn target additional genes. Globally, DUX4 activation at the FSHD locus initiates a transcription cascade leading to muscle atrophy, inflammation, decreased differentiation potential and oxidative stress, the key features of the disease. By differential protein, RNA and gene studies we keep identifying additional FSHD biomarkers and define whether they are direct or indirect DUX4 targets.

Strikingly, we found that DUX4 expression in human myoblast induces an atrophic myotube phenotype and atrophy markers. The rationale of our on-going project is that inhibition of DUX4 expression should prevent the global gene deregulation process and allow muscle regeneration in patients. We have first developed small inhibitory RNAs (siRNAs) and conditions to suppress DUX4 protein expression either in primary myoblast cultures transfected with a DUX4 expression vector, or in primary FSHD myoblasts. Addition of DUX4 siRNA to FSHD myoblasts allowed recovery of a normal myotube phenotype with a decrease of atrophy markers. We have started a collaboration with Prof. Steve Wilton (ANRI, Australia) because of his expertise in the exon skipping therapeutic approach with antisense oligonucleotides (AOs) in Duchenne muscular dystrophy. Prof. Wilton provided us with 7 AOs directed against various parts of the DUX4 mRNA characterized in our group: the aim was to either block translation or induce mRNA degradation to prevent DUX4 protein expression. We were able to identify conditions for selective DUX4 inhibition by 3 AOs as done for the siRNAs above in human primary myoblast cultures. Moreover DUX4 mRNA inhibition also affects the expression of several FSHD markers such as μ -crystallin, β -catenin and TP53. These results constitute a proof of concept in myoblast cultures that DUX4 inhibition might reverse the FSHD phenotype. In the present project we want to validate these results by other techniques (RNA and protein expression profiling) and to test the effect of these AOs and siRNA in mouse models *in vivo*.

A FSH Society New York Festive Evening of Music and Song fellowship grant

2. Humanized mouse model for the study of Facioscapulohumeral Dystrophy

Marietta Barro, Ph.D./Charles P. Emerson Jr., Ph.D.

Boston Biomedical Research Institute, Watertown, Massachusetts

\$40,000 over 1 year

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 will develop 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 hypothesize 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 studies have already established 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 will graft these standardized cultured cells into mouse muscle to obtain the FSHD humanized mouse model, thereby generating a well-controlled in vivo model for the study of FSHD. 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 will contribute to the understanding of the role of DUX4 in vivo, thus providing a better understanding of FSHD pathogenesis.

The proposed project will be completed following 2 specific aims:

Specific Aim 1: Optimization of the FSHD humanized mouse model. We will improve results obtained in preliminary experiments by designing more efficient transplantation strategies. In order to fully interpret the disease model, we will seek to increase the amount of muscle formed from implanted human cells, by devising more efficient transplantation strategies. The cell repository of the BBRI Wellstone Center provides access to freshly isolated FSHD and their appropriate control muscle cells sorted for CD56 expression, which are expected to have particularly high engraftment potential. However, the timing between the toxin injection and the cell injection, as well as depletion of endogenous satellite cells by irradiation of the mouse legs, may affect the ability of implanted cells to regenerate the murine muscle and will be optimized during this aim. Upon establishment of an effective mouse model, we will look for disease characteristics, as described in Specific Aim 2.

Specific Aim 2: Characterization of the FSHD humanized mouse model to evaluate the role of DUX4 during in vivo muscle regeneration. The model obtained in Specific Aim 1 will be characterized by establishing differences between the fibers generated from FSHD cells and fibers from their appropriate control cells in injected muscles. Recent breakthroughs in the field suggest that DUX4, a gene identified inside D4Z4 repeats, expresses a toxic protein in the muscles of patients with FSHD, thus causing the disease. DUX4 may have a normal role during development and the FSHD pathology might involve incomplete developmental silencing of DUX4. However, the precise molecular and cellular mechanisms involving DUX4 remain to be uncovered. The BBRI Wellstone Center, currently investigating DUX4 expression in muscle samples from its cohort collection, has been able to detect DUX4 transcripts in FSHD samples, and these cohorts will be selected for the generation of the humanized FSHD mouse model. Initially, the expression of DUX4 at the mRNA and/or protein levels will be assessed in FSHD- and control transplanted muscles. This will be followed with experiments designed to compare the biological characteristics of the resulting muscle fibers. Finally, we will develop a dynamic approach to investigate the current DUX4 model in following the evolution of the engrafted fiber over time using in vivo bioluminescence live imaging. Murine models surpass in vitro limitations due to their ability to reproduce complex in vivo environment thereby providing a deeper understanding of disease mechanisms. Our model for creating humanized FSHD fibers in murine muscle will recapitulate the mechanisms of pathological fiber formation in vivo, allowing us to fully characterize the disease progression and test potential therapeutic agents.

A FSH Society New York Festive Evening of Music and Song fellowship grant

3. Testing a therapeutic approach for FSHD: evaluation of the efficacy of AOs blocking DUX4 in a mouse model of isolated myofibres

Alexandra Tassin, Ph.D./Alexandra Belayew, Ph.D.

Universite de Mons, Mons, Belgium

\$15,000 over 1 year

FSHD is considered the most frequent hereditary muscle disorder in adults, affecting one individual in 20,000. It is associated with contractions of the D4Z4 repeat array in the 4q35 subtelomeric region. In non-affected individuals, this array comprises 11-100 tandem copies of the 3.3-kb D4Z4 element while in patients, only 1-10 D4Z4 copies are left (Wijmenga et al., 1992). Our group has identified the double homeobox 4 (DUX4) gene within each unit of the D4Z4 repeat array (Gabriels et al., 1999) and several studies have now demonstrated the causative role of DUX4 in FSHD. We have demonstrate that the stable full-length DUX4 messenger RNA (mRNA) is produced from the last D4Z4 unit in FSHD, using a polyadenylation signal in the flanking pLAM region, located telomeric to the distal repeat (Dixit et al., 2007) as recently confirmed by a study of genetic polymorphisms in hundreds of patients and thousands of non-affected individuals (Lemmers et al., 2010). This polyadenylation site is necessary to develop FSHD on a contracted allele therefore called “permissive chromosome” (Lemmers et al., 2010). The mRNA from this distal D4Z4 unit contain the entire DUX4 open reading frame (ORF) and 1 or 2 alternatively spliced introns in the 3’UTR (DUX4-fl). In addition, a short DUX4 mRNA terminates at the previously described polyadenylation site in the pLAM region but uses a cryptic splice donor site within the DUX4 ORF (DUX4-s). DUX4-fl was only detected in FSHD muscle cells and biopsies, whereas DUX4-s is detected both in control and some FSHD samples (Snider et al., 2010). A long DUX4 mRNA was detected in induced pluripotent stem cells (iPS cells) and human testis where the gene contains 4 additional exons and a more distal polyadenylation signal. Expression of this DUX4 mRNA was suppressed during differentiation of control iPS cells to embryoid bodies whereas expression of full length DUX4 mRNA persisted in differentiated FSHD iPS cells (Snider et al., 2010). These data, together with the conservation of the DUX4 ORF through evolution (Clapp et al., 2007) suggests a possible role of DUX4 in human development.

Dr. Tassin intends to undertake a post-doc for 3 months in 2011 at King’s College London, to initiate a collaborative research project between our lab and that of Dr. P. Zammit. In agreement with Dr. Zammit, our collaborative project will consist of testing antisense oligonucleotides (AOs) directed against the 3’UTR of the DUX4 gene that we have developed in our laboratory, in collaboration with Prof. S. Wilton (ANRI, University of Western Australia). These AOs have undergone preliminary screening in cell culture, but require more extensive testing. Dr. Zammit has developed mouse myofibre models that provide an ideal system to further test our AOs. The satellite cells associated with the isolated myofibres will be infected with retroviral vectors encoding DUX4, and the effects on myogenic progression and apoptosis of AO administration analysed. We want specially to focus on the pLAM region responsible for the stabilisation of the DUX4 mRNA leading to FSHD. This system will allow better understanding of the action AOs, for evaluating their potential suitability as a

human therapy. We believe that this collaboration will give us new insights into a potential therapy for FSHD.

A FSH Society California Walk and Roll fellowship grant

4. Investigating mouse models of FSHD

Paraskevi Sakellariou, Ph.D./Robert J. Bloch, Ph.D.

University of Maryland School of Medicine, Baltimore, Maryland

\$40,000 over 1 year

There is a great need for a valid mouse model for FSHD. Such an animal model would provide a valuable tool for exploring the effects of newly cloned genes and novel proteins on the pathophysiology of this disease. It would also greatly facilitate research towards the development and testing of new therapeutic approaches to FSHD. We propose to examine two possible mouse models of FSHD, the FRG1 over-expressor, from Drs. Davide Gabellini and Rossella Tupler, and mu-crystallin over-expressor, developed by Drs. Patrick Reed and Robert Bloch. I will breed these mice and test them for their physiological and morphological characteristics, and their susceptibility to injury and ability to recover from injury. I will also initiate xenografting studies to create mice with humanized normal and FSHD ankle dorsiflexor muscles, combining methods that are routine in the Bloch laboratory with unique reagents provided by collaborators in the Wellstone Muscular Dystrophy Cooperative Research Center (MDCRC), "Biomarkers for Therapy of FSHD." These experiments should reveal the usefulness of available transgenic models for the study of FSHD, and promote the development of humanized mouse muscles for the study of the pathophysiology of FSHD in situ.

A FSH Society New York Festive Evening of Music and Song fellowship grant

5. Epigenetic abnormality in FSHD

Weihua Zeng, Ph.D./Kyoko Yokomori Ph.D.

University of California, Irvine, California

\$8,875 for 3 month extension

Our preliminary findings indicate that D4Z4 repeat regions indeed interact with other genome regions, and that these interactions are indeed disrupted in FSHD. With a three-month extension of my fellowship, I plan to perform a high-throughput identification of potential target genes that interact with D4Z4 using the recently developed "Chromatin Interaction Analysis using a Paired-End Tag" (ChIA-PET) technique. This strategy enables the genome-wide detection of chromatin interactions mediated by specific factors that are normally assembled at D4Z4. Identification of additional FSHD pathogenic genes other than FRG1 and DUX4 is important to explore future therapeutic targets to improve or prevent the clinical symptoms of FSHD.

Previously, with the support from the FSH Society in 2010, we found that a set of factors that normally assemble at D4Z4 repeats do not bind to these repeats in FSHD cells. Interestingly, these factors are known to function in gene silencing and long-distance genomic

interactions, which appear to be particularly important for coordinated developmental gene regulation in human cells. Two candidate genes, FRG1 in a neighboring region and DUX4 encoded within D4Z4, have been identified whose artificial over expression did cause muscular dystrophy in vivo or a myoblast differentiation defect in vitro, respectively. The loss of chromatin structure associated with gene silencing at D4Z4 may explain the abnormal expression of these genes in the disorder. However, FSHD patient muscle cells do not always over express these genes. Thus, there are likely to be additional unidentified genes and signaling pathways involved in the pathogenesis of FSHD. Our hypothesis is that D4Z4 normally spreads a silencing effect to target genes through genomic interactions mediated by D4Z4-bound factors. This function is lost in FSHD, resulting in the abnormal over expression of a set of target genes that leads to clinical manifestations of the disorder. I am taking two strategies to test this model; (1) screen for any genes that might have lost factors similar to those that are lost from D4Z4 in FSHD by high-throughput genome-wide chromatin immunoprecipitation (ChIP)-sequencing, and (2) directly search for genomic regions that interact with D4Z4 using biochemical chromatin conformation capture (3C)-related methods. Any candidate genes identified by these assays will be tested for their effect on cell viability, proliferation/differentiation, and muscle-related downstream gene expression. I will try to re-create the expression change detected in FSHD cells in normal human myoblasts (by over expression or repression) and compare it to the phenotypes of FSHD myoblasts to determine whether the candidate gene contributes to the FSHD cellular phenotype. My research aims to decipher the epigenetic abnormality mechanism in FSHD, which should provide novel insight into the disease mechanism and thus potentially present new therapeutic strategies.

A FSH Society Sanford Batkin & Helen Younger and David Younger research fellowship grant

6. Analysis of DUX4-fl expression

Peter L. Jones, Ph.D.

Boston Biomedical Research Institute, Watertown, Massachusetts

\$7,500 for 1 year

We request support from the FSH Society for our pilot project investigating DUX4 expression in unaffected and FSHD subjects. The DUX4-fl expression model for FSHD has not been independently validated, likely due to the lack of quality clinical resources in the field. At this point in FSHD research, validating and expanding upon the DUX4-cytotoxicity model for pathogenesis is vital to the entire field and we are best positioned to do the necessary experiments with the unique set of highly controlled reagents being generated by the NIH Wellstone Muscular Dystrophy CRC for FSHD at BBRI. Each Wellstone cohort consists of an FSHD affected subject and an unaffected first-degree relative. Each subject donated two biopsies, one from the biceps and one from the deltoid. A portion of each biopsy was used to derive myogenic cell cultures. Quite surprisingly, in our initial preliminary results using 4 cohorts we found some inconsistencies with the published DUX4 expression results that have warranted further investigation. Therefore we have begun a much larger effort to analyze DUX4-fl mRNA and protein expression in a larger set of Wellstone cohorts using RT-PCR and immunostaining (ICC). However, this project is not funded at all in my lab or in

the original Wellstone budget and my lab receives no financial support from the Wellstone Center. The Wellstone has supported us by providing us with cells, which we culture, and RNA which the Louis Kunkel lab purified from biopsies (we do not actually work with the biopsies) and we have been fortunate to receive these Wellstone samples. At this point, to ensure that our results are statistically meaningful, we need to analyze many more cells and biopsy RNAs and it has become cost prohibitive. Therefore I am requesting financial support for consumables and services (DNA sequencing) to conduct these experiments.

A FSH Society Cape Cod Walk and Roll fellowship grant