

FSH SOCIETY RESEARCH FELLOWS AND GRANTEES

Active project numbers are in bold letters.

In addition to the grants listed below, the FSH Society has made many small awards for travel expenses to attend conferences; printing and dissemination of dissertations; production of biomaterials; purchase of laboratory equipment; and honoraria for speaking at conferences.

BRONFMAN CLASS

Grant: FSHS-MB-001
Researcher: **Silvere M. van der Maarel, Ph.D.**
Institution: Leiden University Medical Center
Leiden, THE NETHERLANDS
Project Title: **“Generation of Transgenic Mouse Models for FSHD.”**
\$90,000 3 years. 7/1/1998 - 2/28/2002

Goal: To initiate groundbreaking research to create FSHD animal models.

Grant: FSHS-MB-002
Researcher: **Sara T. Winokur, Ph.D.**
Institution: Department of Biological Chemistry
University of California, Irvine
Project Title: **“Analysis of Chromatin Structure and Skeletal Muscle-Specific Gene Expression in FSHD.”**
\$90,000 3 years. 6/1/1998 - 5/31/2001

Goal: To initiate novel research using Genechip and gene expression technologies to gain insight into FSHD. Prior to this project, gene expression studies had never been done in FSHD.

Grant: FSHS-MB-003
Researcher: **Denise Figlewicz, Ph.D.**
Institution: University of Rochester School of Medicine
Rochester, New York
Project Title: **“Expression of genes proximal to the D4Z4 deletions: a quantitative study in FSHD patients and controls.”**
\$60,000 2 years. 1/1/1999 - 12/31/2000

Goal: To initiate novel research on gene expression and models to study gene expression in FSHD.

Grant: FSHS-MB-004
Researcher: **David J. Picketts, Ph.D.**
Institution: Ottawa General Hospital
Ottawa, Ontario, Canada
Project Title: **“Utilizing an epigenetic approach to identify the FSHD gene.”**
\$60,000 2 years. 5/1/1999 - 4/30/2001

Goal: To initiate novel research on epigenetic features and hyper sensitive sites to understand chromosomal aspects and models of gene expression on FSHD. Prior to this project, no work had been done internationally on elucidating the epigenetic aspects of FSHD.

Grant: FSHS-MB-005
Researcher: **Davide Gabellini, Ph.D.**
Institution: University of Massachusetts Medical Center
Howard Hughes Medical Institute
Worcester, Massachusetts
Project Title: **“Identification and characterization of a protein interacting with the DNA repetitive element causally related to FSHD.”**
\$90,000 3 years. 1/1/2000 - 12/31/2002

Goal: To initiate novel research on gene expression/repression, disease models and to gain insight into D4Z4 functionality and role in FSHD. This work has led to several landmark publications on gene mis-regulation, gene silencing and repression complexes.

Grant: FSHS-MB-006
Researcher: **Fern Tsien, Ph.D./Melanie Ehrlich, Ph.D.**
Institution: Tulane Cancer Center
Tulane Medical School
New Orleans, Louisiana
Project Title: **“DNA Methylation and Chromatin Structure of FSHD-linked Sequences in FSHD Cells, Normal Cells, and Cells from Patients with the ICF Syndrome.”**
\$70,000 2 years. 5/1/2001 - 4/30/2003

Goal: To initiate novel research on the role of methylation, chromatin structure and other epigenetic features in FSHD.

Grant: FSHS-MB-007
Researcher: **Tonnie Rijkers, Ph.D.**
Institution: Leiden University Medical Center

Leiden, THE NETHERLANDS
Project Title: “Mouse models to study candidate genes and epigenetic causes of FSHD.”

\$60,000 2 years. 2/1/2003 – 1/31/2005

Goal: To initiate research on genotype/phenotype correlations in successfully created new lines of animal models of FSHD.

Grant: FSHS-MB-008
Researcher: Cecilia Ostlund, Ph.D./Howard Worman, Ph.D.
Institution: Columbia University

New York, NY

Project Title: “The role of DUX4 in FSHD.”

\$60,000 2 years. 2/1/2003 – 1/31/2005

Goal: To initiate research on the role of DUX4, DUX4C and to examine the role of the nuclear envelope, nuclear lamina and nuclear organization in FSHD.

Grant: FSHS-MB-009
Researcher: Alberto Luis Rosa, M.D., Ph.D.
Institution(1): Washington State University -- Spokane
Spokane, Washington

Institution(2): Laboratory of Neurogenetics
Institute for Medical Research “Mercedes y Martín Ferreyra”
INIMEC-CONICET, National Research Council of ARGENTINA
Córdoba, ARGENTINA

Project Title: “Role of nuclear localization signal (NLS) and H1/H2 motifs in DUX4-mediated cell death.”

\$58,440 2 years. 8/1/2004 – 7/31/2006

Goal: To gain understanding of the molecular and cellular mechanism underlying the pathogenesis of human FSHD. To study DUX4, a putative double homeobox-containing protein encoded by a 3.3 kb polymorphic tandem repeat(D4Z4), at the locus FSHD1A on the human chromosomal region 4Q35. It is hypothesized that abnormal temporal or spatial expression of DUX4 has a toxic effect for muscle cells causing FSHD. The study will help identify the mechanism(s) by which DUX4 causes cell death.

Grant: FSHS-MB-010
Researcher: Richard Lemmers, MSc., Ph.D.
Institution: Leiden University Medical Center
Leiden, THE NETHERLANDS

Project Title: : “Refinement of the FSHD critical region on 4qA chromosomes.”

\$35,000	6/15/2005 – 6/14/2006	Year 1
\$35,000	6/15/2006 – 6/14/2007	Year 2
\$35,000	2/15/2008 – 2/14/2009	Year 3

Goal: FSHD is the third most common myopathy, with an autosomal dominant mode of inheritance. FSHD is caused by contraction of the polymorphic D4Z4 repeat in the subtelomere of chromosome 4q and the exact pathogenic mechanism is still unclear. An identical and equally polymorphic D4Z4 repeat is localized on chromosome 10, but this has never been associated with FSHD. Our approach of detailed characterization of FSHD alleles and translating these observations to disease mechanisms has provided robust mechanistic insight in FSHD pathogenesis over the past years, including the mechanism of mitotic D4Z4 instability (Lemmers et al. 2004a) and the recognition of a bi-allelic 4qter variation (designated 4qA and 4qB) of which only the 4qA allele is associated with FSHD (Lemmers et al. 2002). Moreover, our laboratory provided direct evidence for a chromatin modification associated with the contraction of D4Z4 repeats by demonstrating hypomethylation of D4Z4 in FSHD alleles (van Overveld et al. 2003).

Through our expertise in pulsed-field gel electrophoresis (PFGE)-based FSHD allele characterization, we have become the international reference center for FSHD diagnosis with on average 50 referrals of atypical FSHD patients each year and culminating in a database of >1000 patient and control genotypes for D4Z4 alleles on chromosomes 4 and 10. Our PFGE-based D4Z4 examination has led to further refinement of minimal requirement to develop FSHD in several ways including exclusion of a region of 55 kb proximal to D4Z4 by identification of proximally extended deletions in typical FSHD patients (Lemmers et al. 2003). Moreover, and novel to this field, our analysis provides evidence that within an FSHD repeat, not all units are equal, suggesting that intrinsic differences between individual D4Z4 units within one array may be important for PSEID pathogenesis (Lemmers et al. 2004a).

In the current application I propose to further refine the minimal region necessary and sufficient to cause FSHD in two ways. First, I will precisely characterize three novel patients with an unusual FSHD allele. Two of these alleles carry, analogous to proximally extended deletions, deletions of sequences distal to D4Z4. The third pathogenic allele is highly unusual, because preliminary data suggest that it is located on chromosome 10. The analysis of these alleles will be combined by the full characterization of FSHD and control alleles that display repeat exchanges between chromosome 4 and 10. Moreover, I will focus on intrinsic sequence differences between 4qA-, 4qB and 10q-derived D4Z4 units, most notably that of the most proximal unit, as we provided evidence for a linkage disequilibrium (LD) between this D4Z4 unit and the distal polymorphism 4qA or 4qB (Lemmers et al. 2004a).

I expect that this proposal will generate new and essential information on the minimal region that is required to develop FSHD. Considering the complexity of the disease mechanism, further refinement of these elements is essential for a better understanding of the primary pathogenic pathway and will assist future research

strategies based on candidate gene approaches and development of appropriate cellular and animal model systems.

Grant: FSHS-MB-011
Researcher: **Yi-Wen Chen, D.V.M., Ph.D.**
Institution: Center for Genetic Medicine Research
Children's National Medical Center
Washington, D.C.
Project Title: **"Molecular Mechanisms of Muscle Atrophy in FSHD."**
\$30,000 1 year. 1/1/2006 – 12/31/2006

Goal: In preliminary studies, we studied 125 whole genome profiles of muscle biopsies from patients with FSHD, Duchenne muscular dystrophy, Juvenile dermatomyositis, dysferlin deficiency, Emery-Dreifuss muscular dystrophy and 8 other disorders, and showed that one gene, paired-like homeodomain transcription factor 1 (Pitx1), was significantly and specifically up-regulated in patients with FSHD. Meanwhile, using data from an independent human study, we showed that the gene was up-regulated in atrophic muscles of patients with spinal cord injury. To identify molecular pathways regulated by Pitx1 in mature skeletal muscles, we over-expressed Pitx1 in mouse muscles, and showed that up-regulation of Pitx1 lead to induction of the ubiquitin-proteasome pathways, including atrogin 1 which plays a key role in muscle atrophy.

Based on the preliminary data, we hypothesize that transcriptional pathways perturbed by the contraction of D4Z4 arrays lead to up-regulation of Pitx1, and induction of atrophy pathway, which plays critical role in the patho-physiology of FSHD. In this application, we propose to generate and evaluate a conditional muscle-specific Pitx1 transgenic mouse as a potential animal model of human FSHD. The data generated in the proposed study will provide valuable findings on molecular mechanisms of muscle atrophy in general, and a potential animal model for studying FSHD.

Grant: FSHS-MB-012
Researcher: **Davide Gabellini, Ph.D.**
Institution: Howard Hughes Medical Institute
University of Massachusetts Medical School
Worcester, Massachusetts
Project Title: **"Development of an Animal Model of FSHD."**
\$37,500 1 year. 1/1/2006 – 10/31/2006

Goal: FSHD, the third most common myopathy, is an autosomal dominant neuromuscular disorder characterized by progressive weakness and atrophy affecting selective skeletal muscles. The disease has not been linked to a classical mutation within a protein-coding gene. Instead, FSHD patients carry deletions of tandem 3.3 kb repeats,

termed D4Z4, located on chromosome 4q35. An incomplete knowledge of the biochemical pathogenesis of FSHD has hampered the development of effective therapies.

D4Z4 is a repetitive element with heterochromatic features. Recently, we reported that FRG1, FRG2, and ANT1, three 4q35 genes located upstream of D4Z4, are inappropriately over-expressed, specifically in FSHD muscle. We found that an element within D4Z4 behaves as a silencer providing a binding site for a transcriptional repressing complex. These results suggest a model in which deletion of D4Z4 leads to the inappropriate transcriptional de-repression of 4q35 genes, resulting in disease.

To identify the gene(s) responsible for FSHD, we generated transgenic mice over-expressing FRG1, FRG2 or ANT1 selectively in the skeletal. FRG1 transgenic mice develop a pathology with physiological, histological, ultra-structural and molecular features analogous to those observed in FSHD patients. These include abnormal spinal curvature, progressive muscular dystrophy, skeletal muscle atrophy, and differential involvement of muscle types. Moreover, in both FSHD patients and FRG1 transgenic mice there is no evidence for mitochondrial involvement or alteration of sarcolemmal integrity. This latter feature distinguishes FSHD from other muscular dystrophies in which sarcolemmal disruption is the primary pathogenetic mechanism. By contrast, mice over-expressing two other putative FSHD-candidate genes, FRG2 and ANT1, are normal with regard to both phenotype and muscle histology.

FRG1 is a nuclear protein and several lines of evidence suggest it is involved in pre-mRNA splicing. We found that in muscle of FRG1 mice and FSHD patients, specific pre-mRNAs undergo aberrant alternative splicing. Collectively, our results suggest that FSHD results from inappropriate over-expression of FRG1 in skeletal muscle, which leads to abnormal alternative splicing of specific pre-mRNAs.

Here we propose a detailed study of FRG1 mice to provide novel insights into the molecular pathogenesis of FSHD by addressing the following questions:

1) What is the biological role of FRG1? The precise mechanism of action of FRG1 is unknown. FRG1 might bind FRG1 directly, and change splicing dynamic, or it might regulate the activity of splicing factors. We plan to identify FRG1 interaction partners as a starting point for understanding its biological role.

2) How does FRG1 over-expression trigger muscular dystrophy? Understanding the role FRG1 plays in normal and diseased muscle requires methods to identify the set of RNAs it regulates in vivo and the use of a mouse model of FSHD for RNA target validation. To address this aim systematically, we will undertake a genome-wide screen to identify and validate FRG1 dependent alternatively spliced transcripts in muscle.

These studies will provide relevant information to understand the molecular basis of FSHD that will help in the development of effective therapeutic strategies. FRG1 mice may be used as a preclinical model to test new therapies for FSHD.

Grant: FSHS-MB-013
Researcher: **Melanie Ehrlich, Ph.D.**
Institution: Tulane Medical School
New Orleans, Louisiana
Project Title: **“Finding the 4q35 FSHD Gene.”**
\$70,000 2 years. 7/24/2006 – 7/23/2008

Goal: A major obstacle in research on FSHD is the uncertainty about the identity of the 4q35 gene whose activity is directly controlled by a short D4Z4 array on the same chromosome (in cis). Circumstantial evidence strongly indicates that inappropriate expression of this gene (the FSHD gene) in certain skeletal muscle cells is caused by having a short D4Z4 array in its vicinity. Apparently, the inappropriate expression of the FSHD gene causes the painful and debilitating symptoms of FSHD by altering expression of other genes indirectly.

I propose to use a novel approach for screening for the FSHD gene in the 1-Mb region proximal to the D4Z4 array on 4q. There are now well proven examples of long-distance control of human gene expression by DNA elements that have to be on the same chromosome as the gene they regulate (cis control). My lab will identify by computer analysis about 100 sequences that might contain the elusive FSHD gene, including many sequences that would not be identified by current gene search programs as potential genes. My research group will design ~100 primer-pairs corresponding to 100-200 bp sequences in these regions and check by in silico analysis and PCR on human-rodent somatic cell hybrids and human DNA that these DNA primer-pairs work well in PCR and are unique to human chromosome 4. This broad search will compensate for the major inadequacies of available gene prediction programs and allow discovery of either a conventional or an unconventional gene, such as a gene that encodes a regulatory RNA, but not a protein. My lab will prepare and characterize myoblasts from FSHD and control patients and fix these cells. They will then be analyzed by quantitative RNA polymerase II chromatin immunoprecipitation (ChIP) assays, a DNA-based assay for engagement of the transcription machinery on specific DNA sequences. Our lab will interpret the resulting ChIP data and then, on FSHD and control myoblasts, my lab will do RT-PCR analyses to test sequences that are positive for transcription in the ChIP analysis. These RNA-based assays will be quantitative real-time RT-PCR analyses to compare FSHD and control samples and end-point RT-PCR analyses that give another level of verification by visualization of the size of the RT-PCR product. We will do these RNA-based assays to verify that the regions are transcribed from myoblasts, to determine if we can detect increased RNA amounts for one or more of these regions in FSHD vs. control myoblasts, and to test whether candidate FSHD gene sequences are transcribed from various other cell types, including FSHD and control fibroblasts and lymphoblastoid cell lines.

The method that we will employ to screen for the FSHD gene is the best one for direct identification of transcription of genes, whatever their nature. It is independent of secondary factors that can greatly impact standard RNA analyses. These complicating factors are RNA degradation in vitro despite the use of RNase inhibitors and RNase-free

reagents, RNA processing in vivo, and RNA stability in vivo. If the RNA polymerase II ChIP assays indicate differential transcription of one or more 4q35 genes in a comparison of FSHD to control myoblasts but the RNA assays do not, it could be because of one of these complications associated with RNA analysis. In that case, we will use a different type of ChIP assay to confirm the RNA polymerase II ChIP results, namely ChIP with an antibody to the general transcription factor TBP and PCR primers in the region of the putative promoter. This study holds the promise of greatly facilitating research on FSHD by elucidating the nature of the critical gene initially impacted by a short 4q D4Z4 array but unresponsive to a short, virtually identical D4Z4 array at 10q.

Grant: FSHS-MB-014
Researcher: Patrick Reed, Ph.D.
Institution: University of Maryland School of Medicine
Baltimore, Maryland
Project Title: “Analysis of Changes in the Proteome in FSHD.”
\$30,000 1/24/2007 – 1/23/2008 Year 1
\$30,000 2/15/2008 – 2/14/2009 Year 2

Goal: One of the largest hurdles to understanding the pathogenic basis of FSHD is identifying the molecular mechanisms that trigger the onset of muscular dystrophy. Although FSHD is linked to deletions of 3.3 kb non-coding repeats (D4Z4) repeats near the telomere of chromosomal position 4q35, the mechanisms linking these deletions to changes in gene expression, altered protein expression, and consequent changes in muscle structure and function, are still poorly understood. Many muscular dystrophies are linked to changes in the stability of the sarcolemma of skeletal muscle.

My previous work suggested that the sarcolemma of FSHD muscle was also affected in ways that might lead to muscle weakness, but the proteins that were altered could not be readily identified. I have now adapted a proteomic approach to identify proteins that are altered in FSHD, and to test their possible roles in pathogenicity.

The method I have chosen is high resolution, large format, two-dimensional electrophoresis (2D-GE). With the improvements I have introduced into the method, I can now detect more than 3000 distinct protein spots in normal and FSHD muscle samples. Remarkably, my preliminary results indicate that very few proteins show changes in expression levels in FSHD muscle compared to controls. One, a spot that showed strong expression in the soluble fraction from FSHD muscle but no detectable expression in controls, has an isoelectric point of 5.07 and a molecular mass of approximately 34 kDa. I used LC/MS/MS techniques to show that this protein is mu-crystallin (CRYM; also called “thyroid hormone binding protein” THBP). Western blots confirmed that this protein is highly up-regulated in deltoid muscle from FSHD patients compared to controls. Although my analysis is still incomplete, this protein is of considerable interest because it is expressed in the retina and is responsible for high frequency hearing loss, both of which are compromised in patients with FSHD.

Furthermore, its role as a thyroid hormone binding protein places it at a potentially crucial point in the regulation of myoblast cell division and differentiation, which have recently been implicated as defective in FSHD through gene array studies. It may also be linked to sarcolemmal and sarcomeric changes, as crystallins are likely to play important roles in the assembly of intermediate filaments at these structures in developing muscle.

Finally, the autosomal dominant nature of FSHD suggests a “gain-of-function” mutation, consistent with the over-expression of a protein in FSHD that is expressed at much lower levels in healthy muscle. My novel findings therefore suggest that FSHD may be caused by up-regulation of CRYM, with consequent changes in the structural organization and thyroid hormone signaling pathways.

My general aim is to test the idea that up-regulation of CRYM is an important pathogenic mechanism that leads to FSHD.

My specific aims are: (i) to learn if increased levels of CRYM are indeed specific for FSHD by applying my improved methods for 2D-GE to complete my analysis of the proteomes of FSHD and control muscle, as well as muscles from other dystrophic samples; (ii) to use cellular transfection methods to study the biology of CRYM in myoblasts and myotubes in culture; and (iii) to use transgenic techniques to try to reproduce key features of FSHD in mice. If successful, my experiments should lead to a new understanding of the molecular mechanisms underlying FSHD, and provide an animal model to use in developing therapies for it.

Grant:	FSHS-MB-015		
Researcher:	Yvonne Meijer-Krom, Ph.D.		
Institution:	Leiden University Medical Center Leiden, THE NETHERLANDS		
Project Title:	“Towards the Discovery of Early Developmental Defects in FSHD.”		
	\$35,000	1/24/2007 – 1/23/2008	Year 1
	\$35,000	1/24/2008 – 1/23/2009	Year 2

Goal: Autosomal dominant FSHD is the third most common myopathy. FSHD is mainly characterized by an often asymmetric progressive weakness and wasting of the facial, shoulder and upper arm muscles, typically starting in the second decade of life. FSHD is caused by contraction of the polymorphic D4Z4 repeat in the subtelomere of chromosome 4q (van Deutekom et al. 1993; Wijmenga et al. 1992). Contraction of D4Z4 is associated with DNA hypomethylation (van Overveld et al. 2003) and loss of a D4Z4 repressor complex containing the polycomb protein YY1 implying a complex epigenetic disease mechanism.

There is strong clinical evidence that FSHD should be regarded as a congenital disease with progressive character. Clinical and genetic features suggest an embryonic

involvement in FSSHD. These include the marked asymmetry of muscle involvement, the 1,000-fold increased occurrence of pectus excavatum unrelated to the muscle weakness and the early onset FSHD cases with complete absence of some muscle groups (Padberg 1982; Padberg 2004). In addition, two interesting candidate genes, FSHD region gene 1 (FRG1) and FRG2, located on chromosome 4, are transcriptionally deregulated in FSHD muscle culture, but not in adult muscle. Involvement of an early myogenic defect in FSHD is further supported by the observation that many of the deregulated genes in FSHD muscle are direct targets of MyoD, a key regulator of myogenesis (Figlewicz et al. 2004; Winokur, et al. 2003). Loss of YY1-Ezh2 has been demonstrated to recruit MyoD, leading to the transcriptional induction of genes involved in myogenic differentiation (Caretto et al. 2004). Therefore, we hypothesize that an unbalanced YY1 availability during early embryogenesis disturbs the myogenic program, which may render specific muscle groups more susceptible to disease later in life.

To obtain better insight in the direct targets of MyoD that are deregulated in FSHD, we will perform a transcriptome analysis of 4q-linked FSHD, phenotypic FSHD with hypomethylation of D4Z4 and control fibroblast undergoing forced myogenesis. To determine their dependency on YY1, YY1 levels will be reduced during myogenesis. In parallel, the fusion and differentiation rate of the fibroblast cell cultures will be evaluated to assess their morphologic characteristics. In advantage over the assessed gene expression profile in mature muscle, the current application mimics the early myogenic program. Furthermore, the forced myogenic cell population will be much more homogeneous compared to primary myoblast cell cultures (Bergstrom et al. 2002; Berkes et al. 2004; Padberg 1982). We expect this study to provide new and essential information on the early (embryonic) component of the FSHD phenotype.

Grant:	FSHS-MB-016
Researcher:	Darko Bosnakovski, D.V.M., Ph.D.
Institution:	Center for Developmental Biology UT Southwestern Medical Center Dallas, Texas
Project Title:	“Molecular Analyses of DUX4 and Interaction with Myogenic Regulators in FSHD.”
	\$21,488 12/01/2007 – 11/30/2008 Year 1

Goal: The prevailing model for FSHD is that deletion of D4Z4 repeats at 4q35.2 causes local modification of heterochromatin resulting in deregulation of nearby genes. Which gene(s) may be directly responsible for FSHD is controversial. In preliminary studies in which I used an inducible gene expression system to screen FSHD candidate genes, I found that only DUX4, a candidate located within each D4Z4 repeat, has structural and toxic effects at a variety of expression levels in C2C12 mouse myoblasts. Furthermore, C2C12 cells induced to express DUX4 showed striking gene expression similarities to myoblasts from FSHD patients. Both display deregulation of MyoD and oxidative stress related genes. In addition, I show that DUX4 protein is

expressed in cultured myoblasts from FSHD patients. We hypothesize that DUX4 plays a role in the pathogenesis of FSHD.

The goals of this proposal are to understand the mechanism of the toxicity of DUX4 and to explore possible therapeutic interventions. Because DUX4 is extremely toxic to myoblasts a conditional gene expression system needs to be used to study its effects (Aim 1). In the preliminary study, by using a doxycycline-inducible DUX4 expression system, I found that several crucial myogenic genes (MyoD, Myf5) are targets of DUX4 (Aim 1). I propose to study the underlying mechanism of the toxicity of DUX4 in myoblasts as well as in other cell types. I postulate that appropriate intervention of the deregulated genes (by over-expression or RNAi knockdown) in DUX4 affected cells should rescue the toxic phenotype (Aim 1). Furthermore I will test the hypothesis that DUX4 interferes with the function of myogenic regulators by competitive binding to the same target sites (Aim 2). In support of this, over-expression of Pax3, a crucial gene in myogenesis and whose homeodomain is most similar to DUX4, renders C2C12 cells resistant to DUX4-mediated toxicity. The aims of this proposed study target the most crucial and unknown aspects (both mechanism and therapy) of FSHD.

Aim 1. To understand the mechanism of action of DUX4.

Aim 2. To test the hypothesis that DUX4 interferes with the function of myogenic regulators.

Significance: The pathogenic mechanism of FSHD is controversial and largely unknown, which is the major hurdle in developing a rational therapy. Therefore it is extremely important to find the gene(s) involved in FSHD, and to understand their action, from which therapeutic strategies will arise. My proposed study is designed to answer these crucial questions. Using uniform gain of function approach, I will look closely on the effects of the FSHD candidate gene, DUX4 on myoblast phenotype, analyze the underlying mechanism in detail (Aim 1) and identify potential targets in the cascade of pathogenesis (Aim 2). Thus this study is directly relevant to progress towards a therapy for FSHD.

Grant:	FSHS-MB-017
Researcher:	Paola Picozzi, Ph.D.
Institution:	Stem Cell Research Institute Milano, ITALY
Project Title:	“Functional characterization of D4Z4 in FSHD.”
	\$35,000 3/1/2008 – 2/28/2009 Year 1

Goal: The long-term goal of our research is to identify and characterize the molecular pathways that become subverted in FSHD in order to develop therapeutic strategies. FSHD, the third most common myopathy, is an autosomal dominant neuromuscular disorder characterized by progressive weakness and atrophy affecting selective skeletal muscles. Unlike the majority of genetic diseases, FSHD is not caused by a classical mutation within a protein-coding gene but rather involves a complex cascade of

epigenetic events following contraction of a 3.3 kb subtelomeric non-coding repeat (D4Z4) located on chromosome 4q35.

At present no treatment is available for FSHD. This has been also hindered by an incomplete knowledge of the disease pathogenesis and, until recently, by the lack of an animal model. Based upon recent experimental results, it has been proposed that deletion of D4Z4 leads to the inappropriate transcriptional de-repression of the 4q35 gene FRG1 resulting in disease. Understanding how deletion of D4Z4 causes up-regulation of 4q35 genes is important to develop therapeutic approaches aimed at preventing transcriptional de-regulation in FSHD.

Our specific aims are:

1. To characterize protein/DNA interactions at D4Z4. It has been shown that a transcriptional repressor complex composed of YY1, HMGB2 and nucleolin is associated with D4Z4 (Gabellini et al, 2002). In mammalian cells, transcriptional repression is the result of the cooperation between sequence specific repressors and general co-repressors such as histone deacetylases (HDACs) and DNA methylases. Notably, the activity of YY1 is regulated at the posttranslational level, possibly through interactions with other proteins. YY1 represses transcription by interacting with HDAC-1 and 2, and this interaction is regulated by HDAC phosphorylation. (Galasinski et al, 2002). Collectively, these observations suggest that other proteins may be associated with and regulate the activity of the DRC.

2. To elucidate the mechanism underlying control of gene expression at 4q35. Our preliminary results suggest that non-coding RNAs and microRNAs generated by D4Z4 regulate chromatin structure and 4q35 genes expression. Our analysis will generate novel insights into the biological role of repetitive DNA sequences in higher eukaryotes.

The results of these studies will be very useful to identify effective therapeutic approaches for FSHD.

DELTA RAILROAD CONSTRUCTION CLASS

Grant: FSHS-DR-001
Researcher: **Alexandra Belayew, Ph.D./Stephane Plaisance, Ph.D.**
Institution: Lab. Biologie Moléculaire
Université de Mons-Hainaut
Mons, BELGIUM
Project Title: **“Characterization of a protein expressed from a 3.3 kb element not linked to FSHD.”**
\$15,000 1 year. 6/1/1998 - 12/31/1998
“Small laboratory equipment for research on FSHD.”

\$15,000

2/15/2001 - open

Year 1

Goal: To initiate research on the role of DUX, DUX1, DUX4, DUX4C and to elucidate the role of DUX in FSHD and within the D4Z4 region.

Grant: FSHS-DR-002
Researcher: **Rossella Tupler, M.D., Ph.D.**
Institution: Howard Hughes Medical Institute
University of Massachusetts Medical Center
Worcester, Massachusetts
Project Title: **“Characterization of differentially expressed genes in FSHD affected muscles.”**
\$30,000 1 year. 6/1/1998 - 5/31/1999

Goal: To initiate research into differentially expressed genes involved in FSHD. This groundbreaking research has led to major advances in our understanding of FSHD.

Grant: FSHS-DR-003
Researcher: **Robert Bloch, Ph.D.**
Institution: University of Maryland School of Medicine
Baltimore, Maryland
Project Title: **“Sarcolemmal organization in FSHD and the MYD mouse.”**
\$30,000 1 year. 7/1/1999 - 4/30/2001

Goal: To gain insight into structural aspects and patho-physiology of FSHD using the latest techniques as well as revisiting standard methodologies. To examine the structure of FSHD muscle and the sarcolemma for insights into the disease.

Grant: FSHS-DR-004
Researcher: **Jane Hewitt, Ph.D.**
Institution: Nottingham University
Division of Genetics
Queen’s Medical Centre
Nottingham, England
Project Title: **“Fugu rubripes as a model organism for FSHD gene identification.”**
\$30,000 1 year. 7/1/2000 - 6/30/2001

Goal: To sequence the analogous 4q35 region in puffer fish for insight into genomic organization of FSHD. To use data to help with mapping, assembly and finishing of the 4q35 human region. Based on this research, we were able to assist the Human Genome

Project at Washington University to complete the map and sequence this very difficult and recalcitrant region of 4q35.

Grant: FSHS-DR-005
Researcher: **Marcy Speer, Ph.D.**
Institution: Duke University Medical Center
Durham, North Carolina
Project Title: **“Genetic Linkage Studies in Non-chromosome 4 FSHD.”**
\$30,000 2/1/2002 - 1/31/2003 Year 2 (See Year 1
under Tides)

Goal: To examine and find the genetic locus of the non-chromosome 4 families through genome wide search/scan. This project is a high priority for the research community. It aims to register non-chromosome 4 pedigrees with the researchers and clinicians at Duke.

The FSH Society plays an important role in identifying such families for this project.

Grant: FSHS-DR-006A
Researcher: **Emma Ciafaloni, M.D**
Institution: University of Rochester School of Medicine
Rochester, New York
Project Title: **“The Course and Outcome of Pregnancy and Delivery in Women with FSH Muscular Dystrophy.”**

\$13,074	1/1/2004 - 12/31/2004	Year 1
(interrupt/extend)		
\$1,926	1/1/2005 - 12/31/2005	Year 2 (\$12,973 total
see under Lewis)		
\$0	1/1/2006 - 12/31/2006	Year 3 (\$13,363 total
see under Lewis)		

Goal: Very little is known about the course and outcome of pregnancy and delivery in women with muscular dystrophies. Our current ability to efficiently counsel women with muscular dystrophies when pregnant or planning a pregnancy is very limited due to the lack of studies addressing the issue of pregnancy and delivery outcome in this group. No specific attention has been paid to the possible interaction between gestation and progression of the myopathy. Objectives are: to increase our knowledge about the course and outcome of pregnancy and delivery in women with FSHD; to assess the effect of pregnancy, delivery and post-partum on the progression of muscle weakness and muscle pain and on quality of life in women with FSHD; and, to ultimately improve counseling, family planning and obstetric management of women with FSHD.

Grant: FSHS-DR-006B Honoraria
Researcher1: **Wendy M. King, PT**
Institution1: Ohio State University
Columbus, Ohio
Researcher2: **Shree Pandya, MS, PT**
Institution2: University of Rochester School of Medicine
Rochester, NY
Project Title: **“FSHD Physical Therapy Booklet/Brochure and
Article for Physical Therapy Journal.”**
\$15,000 1 year. 5/1/2004 – 4/30/2005

Goal: Gather and review of literature/information related to FSHD natural history, surgical options, orthotics, rehabilitation, physical therapy interventions, role of exercise, hydrotherapy, pain, etc. Review scientific literature, brochures and web sites of various organizations from English speaking countries to assess the type and format of information already available. Draft, peer-review and publish booklet/brochure on FSHD and Physical Therapy and submit journal article to Physical Therapy journal on P.T. and FSHD.

Grant: FSHS-DR-007
Researcher: **Sara Winokur, Ph.D./Ulla Bengtsson, Ph.D.**
Institution: Biological Chemistry
University of California, Irvine
Irvine, California
Project Title: **“Coding and non-coding RNA expression in FSHD.”**
\$35,000 1 year. 7/1/2005 – 6/30/2006

Goal: More than a decade after the position effect hypothesis was first proposed, the fundamental question of whether altered chromatin structure in FSHD affects RNA expression at 4q35 has not been answered. Several independent laboratories have addressed this question, yielding disparate and contradictory results. In part, this is due to the variability in tissues and cultures utilized by various laboratories, which are provided by different sources and often obtained and preserved using different methods. In addition, all of the experimental techniques used to examine RNA expression thus far have relied on pooled sources of RNA from tissues or cell cultures. These techniques include non-quantitative RT-PCR, real-time RT-PCR, and expression profiling. These studies assayed differential RNA expression between FSHD and control muscle, and, by nature of the experimental design, detected average RNA levels emanating from both alleles and multiple cell types.

In contrast, examination of RNA expression in a single cell context is more suited to address the question of whether an altered chromatin structure on the contracted D4Z4 allele influences RNA expression. RNA-FISH (fluorescence in situ hybridization) utilizes antisense RNA or dsDNA as hybridization probes to nascent nuclear RNA

transcripts followed by fluorescence detection of conjugated haptens or antibodies. Transcription of both coding and non-coding RNAs from each of the alleles (normal and D4Z4 contracted) can be readily identified by RNA-FISH followed by hybridization with D4Z4 and 4q specific DNA probes. In addition, the specific cell type expressing the RNA can be readily identified using this technique, either in culture or within tissue sections.

We propose to utilize RNA-FISH to answer to following questions: 1) Which 4q35 genes are transcribed in proliferating myoblasts and differentiated myotubes? 2) Are the levels of transcription different between normal and FSHD myoblasts/myotubes? 3) Is there an allele specific transcription in FSHD myoblasts/myotubes? That is, do the contracted and normal alleles display different levels of RNA transcription within single cells? For these studies, 3' hyper-biotinylated antisense oligos corresponding to 4q35 genes will be used as probes for coding RNA expression in myoblasts and differentiated myotubes. If chromatin structure is altered in FSHD, leading to aberrant RNA expression, then we should not assume that such a mechanism would affect coding RNA exclusively. Non-coding RNA has increasingly come to light as a significant player in the regulation of both transcription and translation. Although several approaches to the detection of non-coding RNAs exist, we propose to use the same technique (RNA-FISH) to examine non-coding RNA within a defined region proximal to the D4Z4 repeat. Genomic clones (cosmids) will be used to hybridize to these RNAs as the specific non-coding transcripts cannot be identified a priori.

Lastly, RNA transcription of genes affected in FSHD (as identified by expression profiling) will be examined in FSHD and control myoblasts/myotubes. A recent finding in FSHD research within the past year has been the unique and consistent localization of the 4q telomeric region to the nuclear periphery. While the biological significance of this localization is not yet known, the existence of nuclear domains either permissive or repressive of transcription is well documented. Therefore, genes affected in FSHD will be examined by RNA-FISH to determine whether co-localization with the FSHD region at the nuclear periphery might affect RNA transcription from these genes.

Grant: FSHS-DR-008
Researcher: Jane Hewitt, Ph.D.
Institution: Institute of Genetics
Queen's Medical Centre
University of Nottingham
Nottingham, UK
Project Title: "Development of Genomic Resources for Functional Studies of the Mouse DUX4 Array in Vivo."
\$44,658 2 years. 7/24/2006 – 5/31/2008

Goal: We have recently demonstrated conservation of the open reading frame and the tandem array organization of Dux4 homologues in a wide range of mammalian species, suggesting a protein-encoding function for the array and a requirement for a high copy

number. We hypothesize that the conservation of the open reading frame and the tandem array organization of DUX4 homologues in a wide range of mammalian species indicates a protein-encoding function for the array and a requirement for a high copy number. This function may be disrupted by the FSHD deletion and hence play a role in the disease mechanism. The identification of the mouse homologue (Dux4) provides a model organism in which to genetically manipulate the Dux4 array in vivo. In the work proposed in this application we plan to generate a set of resources that will then enable us to generate of mouse lines that either a) have reduced repeat numbers within the Dux4 arrays or b) in which the entire Dux4 array is deleted. In specific aims 1 and 2 we will complete the physical and the sequence map of this locus.

In specific aim 3, using information from this region obtained in aims 1 and 2, we will generate gene targeting constructs using the Mutagenic Insertion and Chromosome Engineering Resource (MICER), developed in the UK at the Sanger Genome Centre.

FSH SOCIETY, INC. GRANTS (NAMED)

FSH SOCIETY LANDSMAN CHARITABLE TRUST FELLOWSHIP

Grant: FSHS-LCT-001
Researcher: Meredith Hanel, Ph.D.
Institution: Department of Cell and Developmental Biology
University of Illinois at Urbana-Champaign
Urbana, Illinois

Project Title: “An in vivo *Xenopus* System for Studying D4Z4 Mediated Chromatin and Gene Expression.”
\$30,000 **03/01/2008 – 2/28/2009** **Year 1**

Goal: We have created a novel animal model exhibiting an FSHD phenotype using *Xenopus laevis* frogs, supporting the assertion that FSHD pathology is due to the over-expression specific genes. This provides two therapeutic targets; 1) the misregulation of the gene by the mutated D4Z4 array and 2) the activity of the over-expressed gene product. This proposal addresses the former, seeking to understand the regulation mediated by the D4Z4 array and 4q subtelomere by systematically recapitulating the human FSHD region of chromosome 4q35 in the frog.

Introducing these human sequences, and particularly the D4Z4 repeats, under controlled circumstances will allow us to determine the regulatory requirements lacking in the FSHD deletion. *Xenopus* transgenesis enables monitoring integrated genomic copies of engineered sequences in a developing animal in a high through-put method. The telomeric nature of the environment of the D4Z4 repeats will even be recapitulated in our system as we have recently engineered telomeres in *Xenopus* (Wuebbles and Jones, 2007). The *Xenopus* genome contains many of the same epigenetic characteristics as

humans, making potential findings applicable to humans. The goal of this proposal is to determine the cis and trans regulatory requirements for normal gene repression in the 4q35 region and how this is disrupted in FSHD.

Our specific aims are:

Aim 1: The impact of D4Z4 repeat number on the expression of FRG1 and neighbouring genes. D4Z4 repeats will be placed in cis with human and *Xenopus* FRG1 promoters driving the reporter GFP. Cis effects on gene expression will be visualized in a developing vertebrate and correlated with number of repeats. Subsequent analysis of DNA methylation and chromatin structure will determine the nature of repression or activation. Experiments will test the major hypotheses in the field that (1) D4Z4 repeat mediated gene repression is due to a local repressive effect of heterochromatin spreading, (2) A repressor bound to D4Z4 repeats associates with promoters, (3) An activator associated with the D4Z4 repeats activates transcription.

Aim 2: Regulatory roles of the subtelomere region. The repetitive nature of CpG rich D4Z4 repeats and their subtelomeric location suggest a structural role or maintenance of chromatin conformation. D4Z4 repeats have been proposed as an insulator from or propagator of telomere position effects. Since FSHD is strictly associated with the 4qA allele (containing a distal Beta satellite repeat), but not the 4qB allele (without beta satellites) telomeric transgenes containing D4Z4 arrays between the telomere and a reporter gene will test the influence of Beta-satellite DNA on gene expression as well as the insulator activity of the D4Z4. The number of D4Z4 repeats required to overcome telomeric position effect and the effect of the Beta-satellite DNA will be assayed. To test whether D4Z4 propagate heterochromatin we will assess the ability of D4Z4 repeats to override the effect of the Beta-globin HS4 insulators and result in gene repression.

Aim 3: D4Z4 repeats as transcriptional regulators with genome wide effects. If D4Z4 repeats regulate genes in trans, adding D4Z4 repeats to the *Xenopus laevis* genome may sequester proteins that bind D4Z4, and may ultimately manifest as a developmental phenotype. Since *Xenopus* development occurs externally, embryos at any stage of development are easily visualized and alterations in candidate myogenic markers and vasculature will be assayed.

Xenopus provides a unique opportunity to observe developmental stage and tissue specific differences in epigenetic and gene regulation. Combined with the intense research by numerous groups into pharmaceuticals targeting epigenetic regulators, elucidating the factors involved in gene regulation by the 4q35 region may make these treatments applicable to FSHD.

Grant: FSHS-LCT-002
Researcher: Scott Q. Harper, Ph.D.
Institution: Center for Gene Therapy

Columbus Children's Research Institute and Department of
Pediatrics
The Ohio State University
Columbus, Ohio

Project Title: "In Vivo Investigation of DUX4 as a Candidate FSHD Gene."
\$10,000 **03/01/2008 – 2/28/2009** **Year 1**

Goal: FSHD is an autosomal dominant disorder characterized by progressive and asymmetric weakness of facial, shoulder, and limb muscles. It is the third most common muscular dystrophy and no effective treatment exists. FSHD is caused by contraction of D4Z4 repeats on human chromosome 4q35. Though the causative mutation has been known for nearly 15 years, the underlying pathogenic mechanism for the disease remains unresolved. Current models suggest that normal chromatin structure at 4q35 is altered by pathogenic D4Z4 arrays (1-10 repeats) leading to aberrant up-regulation of nearby genes. To date, FRG1 is arguably the best candidate FSHD candidate gene; FRG1 over-expression in mice recapitulates some dystrophic changes associated with FSHD but the gene is not uniformly elevated in all patient biopsies. Thus, its uncertain role in FSHD pathogenesis justifies the search for other candidates. Recent evidence suggests DUX4 may play a role in FSHD development. DUX4 is the translated product of a transcript arising from D4Z4 which induces apoptosis of cultured myoblasts upon over-expression. However, the in vivo effects of DUX4 over-expression in muscle are unknown. The goal of this project is to determine whether viral vector-mediated DUX4 over-expression in mouse muscle causes histological changes associated with FSHD. This work will be an important step toward understanding the pathobiology of FSHD, which is necessary for ultimately developing effective therapies.

Specific Aim: To investigate the in vivo effects of DUX4 over-expression in muscle. DUX4 is candidate FSHD gene due to its chromosomal location (as a product of D4Z4 repeats) and because its over-expression induces apoptosis in cultured myoblasts. In vivo DUX4 over-expression in muscle is a logical next step toward investigating its potential role in FSHD pathogenesis. Adeno-associated viral (AAV) vectors are ideally suited for in vivo muscle gene delivery because they efficiently transduce muscle at high levels, produce no adverse effects on muscle histology/physiology, and allow cheap and rapid analysis compared to transgenic mouse methods. Here, we will use AAV serotype 8 (AAV8) vectors to deliver DUX4 or control genes to muscles preferentially or minimally affected in FSHD. We hypothesize that DUX4 over-expression will induce histological changes associated with muscular dystrophy in transduced animals.

This study will be an important first step toward understanding the potential role of DUX4 in FSHD pathogenesis and may have future implications for developing FSHD therapies.

FSH SOCIETY TIDES FOUNDATION

Grant: FSHS-TF-001

Researcher: Marcy Speer, Ph.D.
Institution: Duke University Medical Center
Durham, North Carolina
Project Title: **“Genetic Linkage Studies in Non-chromosome 4 FSHD.”**
\$30,000 2/1/2001 - 1/31/2002 Year 1 (See Year 2
under Delta RR)

Goal: To ensure that the work to examine and find the genetic locus of the non-chromosome 4 families continues. This project is of very high priority to the FSH Scientific Advisory Board.

FSH SOCIETY VICKI AND MARK RAY

Grant: FSHS-VR-001
Researcher: Robert Bloch, Ph.D.
Institution: University of Maryland School of Medicine
Baltimore, Maryland

Project 1 Title: **“Sarcolemmal organization in FSHD and the MYD mouse.”**
\$15,000 1 year. 5/1//2001 - 11/30/2001

Project 2 Title: **“To investigate the “proteome” in FSHD and to compare it to the “proteome” in control muscles and in other common myopathies and muscular dystrophies using two-dimensional gel electrophoresis”**
\$15,000 1 year. 2/15//2002 - 8/15/2002

Goal: To gain insight into structural aspects and patho-physiology of FSHD using the latest techniques as well as revisiting standard methodologies. To newly examine the structure of FSHD muscle and the sarcolemma and to examine more closely the proteins involved in FSHD using proteomic approaches.

FSH SOCIETY THELMA GREEN MEMORIAL

Grant: FSHS-TG-001
Researcher: Jeanne Lawrence, Ph.D./Y. Polly Xing, M.D., Ph.D.
Institution: University of Massachusetts Medical Center
Worcester, Massachusetts
Project Title: **“Higher level chromatin packaging and nuclear organization of FSHD cell with an emphasis on its 3.3 kb deletion involving high resolution transcript mapping by mRNA in situ and direct visualization of this region of the chromosome via in situ hybridization with loop halo DNA preparations.”**
\$30,000 1/1/2002 - 12/31/2002 Year 1
(interrupt/extend)

Goal: To gain insight into nuclear organization, scaffolding, structure and chromatin packaging involved in FSHD. To examine epigenetic features, nuclear compartmentalization and aspects of D4Z4 and telomere organization.

FSH SOCIETY SAM E. AND MARY F. ROBERTS FOUNDATION GRANT FOR NUTRITION RESEARCH

Grant: FSHS-SMRF-001
Researcher: **Graham J Kemp, M.D.**
Institution: Faculty of Medicine
University of Liverpool
Liverpool, UK
Project Title: **“Muscle damage by reactive oxygen species, muscle atrophy and effects of creatine supplementation in FSHD.”**
\$35,000 1/1/2003 - 5/01/2005 Year 1.5
(interrupt/extend)
(\$48,650 total see balance under Lewis)

Goal: This a pilot study designed to test the following hypotheses: 1) that muscle in FSHD shows evidence of damage by ROS in vivo; 2) that this is at least partly due to reduced anti-ROS protection; 3) that this is ameliorated by 6 months creatine treatment; 4) that this also partially alleviates muscle atrophy, even in the absence of training, and; 5) that this results in an increase in muscle strength and clinical indices. This is an open label pre-post protocol examining the effects of 6 months creatine supplementation in 10 patients with proven FSHD. ROS protection and damage will be studied in conchotome biopsies of deltoid. Muscle atrophy and its effect on body composition will be measured by whole-body quantitative magnetic resonance imaging (MRI). Muscle strength and effects on symptomatology will be quantified. We will compare pre-creatine results with those of control subjects, and examine differences between post- and pre-creatine values.

This study has several possible benefits: it will contribute evidence of the therapeutic usefulness of creatine over a longer time span than earlier studies; it will throw light on mechanisms of muscle damage in FSHD; if ROS are indeed important then other compounds that reduce oxidative stress in muscle may be useful; lastly, the results will help in the design and interpretation of future placebo-control trials.

Grant: FSHS-SMRF-002
Researcher: **Sara Winokur, Ph.D./Ulla Bengtsson, Ph.D.**
Institution: Biological Chemistry
University of California, Irvine
Irvine, California
Project Title: **“Restoration of normal myogenic pattern in FSHD: A nutritional approach.”**

\$60,000 2 years. 3/1/2003 – 2/28/2005

Goal: A clinically oriented project to study patterns of FSHD myogenesis in cell systems using compounds and nutritional agents that affect methylation, oxidative stress, chromatin structure and muscle cell differentiation. A major goal of this project is to build an effective model system to assay target compounds effectively. The objective of this study is to identify therapeutic compounds to treat FSHD that can be taken as part of a nutritional regimen. Nutritional compounds are selected based on functional impact on myogenesis, availability as nutritional supplement and expediency for clinical trials.

Grant: **FSHS-SMRF-003**

Researcher: **Hermien Kan, Ph.D./Arend Heerschap, Ph.D.**

Institution: Head Biomedical Magnetic Resonance group
Radboud University Nijmegen Medical Center
Nijmegen, THE NETHERLANDS

Project Title: **“Assessment of the metabolic inter-muscular heterogeneity,
and muscular creatine uptake and turnover in FSH patients vivo.”**

\$45,000 2 years. 8/14/2006 – 2/14/2008

Goal: Although substantial progress has been made in the molecular biology of FSHD, little is still known about its pathophysiology such as possible defects in skeletal energy metabolism. Asymmetric dys-functioning of muscles is a typical feature of FSHD but characteristic metabolic profiles of the affected muscles are lacking, and objective biomarkers to assess therapies, e.g. creatine supplementation, which possibly has beneficial effects, are not available. MR spectroscopy (MRS) is an ideal tool to study metabolism in muscle in a non-invasive way.

Hypothesis: The application of MRS to FSHD patients will uncover metabolic abnormalities that can serve as non-invasive biomarkers to assess, and better understand the severity of disease in specific muscles. The signals of creatine can serve as non-invasive biomarkers to assess creatine uptake, phosphorylation and turnover in skeletal muscle of patients in creatine supplementation treatment.

Study objectives:

(1). To discover metabolic abnormalities in skeletal muscle of FSHD patients by MRS as biomarkers for the severity of the disease in specific muscles.

(2). To determine if the level of Cr and PCr, by quantitative MRS is decreased in muscle of FSHD patients

(3). To determine creatine uptake, phosphorylation and turnover in different skeletal muscles in healthy volunteers (which is not known), and,

(4). in the muscles of FSHD patients.

Study design will consist of two parts.

(I). A metabolic profile of muscles will be assessed using phosphorous 31 (³¹P) and tritium (³H) MRS and possible differences between affected and non-Affected skeletal muscles will be studied (objective 1). Specifically, differences in Cr and PCr concentrations will be monitored (objective 2). Simultaneously, Cr turnover and PCr/Cr ratios after Cr supplementation will be studied in healthy volunteers (objective 3).

(II). Depending on the results of the volunteer studies Cr uptake, phosphorylation and turnover will be assessed in a single location or in several skeletal muscles of FSHD patients to meet objective 4.

Expected results A metabolic profile will be established to serve as non-invasive biomarker for the severity of disease in specific muscles and to monitor therapy in FSHD. Differences in Cr uptake and turnover between skeletal muscles in healthy persons and FSHD will guide the optimization of Cr supplementation strategies in FSHD patients.

FSH SOCIETY NEW YORK CITY SYMPHONY AND SONG BENEFIT CONCERT

Grant: FSHS-NYSS-001
Researcher: **Daniela M. Oliveira, Ph.D.**
Institution: Ottawa Health Research Institute
Ottawa, Ontario, Canada
Project Title: **“Identification of the mechanism regulating the Wnt-dependent activation of muscle progenitor cells.”**
\$30,000 1 year. 1/1/2005 – 12/31/2005

Goal: The overall goal of the project is to identify genes regulated by the Wnt signaling pathway that are responsible for the myogenic differentiation and proliferation of CD45+/Sca-1+ muscle cells. In addition muscle satellite cells, another stem cell population within muscle (CD45+/Sca-1+ muscle cells), plays a physiological role in muscle regeneration. Identification of new therapeutic targets can be used to help stimulate the Wnt-target genes that might be used to enhance stem cell transplant in FSHD.

Grant: FSHS-NYSS-002
Researcher: **York Marahrens, Ph.D./Nieves Embade, Ph.D.**
Institution: Department of Human Genetics
David Geffen School of Medicine
University of California, Los Angeles
Los Angeles, California
Project Title: **“Testing whether D4Z4 Perform Long Distance Gene Silencing via the Chromosome 4 Inactivation Network.”**
\$22,652 1 year. 11/1/2004 – 10/31/2005

Goal: A high risk and novel approach to understanding chromosome interactions, epigenetics. To test the hypothesis that long repetitive sequence on a chromosome, regardless of sequence, is tied into the network of long repeats responsible for chromosome inactivation and particular with FSHD the case of non-random mono-allelic autosomal inactivation. To test the hypothesis that the tract of D4Z4 repeats at 4q35 is tied into the chromosome 4 inactivation network and that D4Z4 deletions disturb chromosome 4 inactivation resulting in abnormal gene expression.

FSH SOCIETY RESEARCH & EDUCATION FUND

Grant: FSHS-FS-001
Researcher: Nieves Embade, Ph.D./York Marahrens, Ph.D.
Institution: Department of Human Genetics
David Geffen School of Medicine
University of California, Los Angeles
Los Angeles, California
Project Title: “Tethering Adenine (Dam) Methylase to the 3.3-kb FSHD Repeats to Identify Distant Genes that Physically Come in Contact with the Repeats.”
\$30,000 3/1/2003 – 9/30/2004 Year 1
(interrupt/extend)

Goal: A high risk and novel approach to understanding chromosome interactions, epigenetics, gene expression in FSHD and with which other parts of the chromosome(s) the FSHD chromosome 4 D4Z4 repeats are coming into contact. To locate the FSHD gene(s) that interact with the D4Z4 repeats by tethering bacterial adenine methylase to sequences in or near the 3.3 kb repeats and then identifying adenine-methylation at distant sites on the same chromosome and/or other chromosomes.

Grant: FSHS-FS-003
Researcher: Sara T. Winokur, Ph.D.
Institution: Biological Chemistry
University of California, Irvine
Irvine, California
Project Title: “FSHD nuclear organization and RNA expression in early development.”
\$38,000 1 year. 1/24/2007 – 1/23/2008

Goal: The precise mechanism responsible FSHD continues to evade elucidation using current approaches to the disease. New resources and avenues of research are necessary to provide fresh insight and perspective into this most challenging disease. To this end, we propose to examine gene expression and nuclear organization in FSHD at the earliest stages of development. Even though typical age of phenotypic onset in FSHD is during adolescence, there are many indications that the disease is influenced by early

developmental processes. Resources currently utilized for FSHD research are generated from adult tissue (skin, muscle, blood), as well as stem cell populations within these tissues (myoblasts, lymphoblasts). However, even these adult stem cells are committed to specific lineages, and may well not reveal information regarding the FSHD genome at the early in development. We propose to investigate FSHD region molecular and cell biology in embryonic stem cells (ESC) as a resource for the FSHD research community. This study will focus on FSPID region organization and gene expression in normal development, and will provide a basis for comparison to altered expression and localization in FSHD cells once they become available.

Grant: FSHS-FS-004
Researcher: **Alexandra Belayew, Ph.D.**
Institution: Lab. Biologie Moléculaire
University Academy Wallonia-Brussels
Université de Mons-Hainaut
Mons, BELGIUM
Project Title: **“Study of DUX4 mRNA and Protein Expression in FSHD.”**
\$30,000 1 year. 1/24/2007 – 1/23/2008

Goal: In this research proposal, we want to focus on expression of the DUX4 gene we mapped in each unit of the D4Z4 repeat array that is contracted in FSHD. The gene was identified several years ago, but demonstration of its expression in patient muscles proved technically very challenging because of its low level, its toxicity, and its homology to hundreds of DUX genes unlinked to FSHD. We could demonstrate expression in myoblasts and biopsies of the homologous non-toxic DUX4c protein encoded by an isolated D4Z4 element 42 kb centromeric of the repeat array. We have recently been able to develop very sensitive and specific tools and procedures to detect DUX4 expression at the mRNA and protein level. In our mRNA studies we detected 2 introns downstream from the D4Z4 stop codon: their occurrence allowed unambiguous identification of RT-PCR products as bona fide mRNA (not genomic DNA) copies in 4 FSHD myoblast lines but not 3 controls. We raised a monoclonal antibody against the DUX4 carboxyl-terminal domain that specifically detects the DUX4 (52 kDa) and homologous DUX4c (47 kDa) proteins on Western blots performed with extracts of cells transfected with p-CI-neo-DUX expression vectors. The Western blot sensitivity was recently increased about 20-fold by use of a new peroxidase substrate (Pierce) and allowed DUX4 detection in 4 additional FSHD myoblast lines provided by Dr. D. Figlewicz. (University of Michigan, Ann Arbor, MI) and Drs. D. Laoudj-Chenivresse and J. Mercier (INSERM. University of Montpellier, France)

(1) With these tools, our first aim is to evaluate DUX4 mRNA and protein expression in additional myoblast lines and in muscle biopsies of patients with FSHD and different D4Z4 copy numbers, or FSHD not linked to 4q365. as well as in controls and other neuromuscular disorders. Biopsies will be provided by Drs. D. Laoudj-Chenivresse and J. Mercier as well as by Dr. P. Lunt (United Bristol Healthcare NHS Trust, Bristol, UK) and Y.W. Chen (Children’s National Medical Center, Washington D.C). Primary

myoblast lines established from muscle biopsies have been provided by Dr. D. Figlewicz and additional ones will be by Drs. D. Laoudj-Chenivresse and J. Mercier.

(2) Our second aim is based on the observation that the DUX4 mRNA 3' ends we detected mapped outside of the D4Z4 repeat array. This region differs between the chromosome 4qA allele and the 4qB one that was never found associated with FSHD. We want to evaluate whether such DUX4 mRNA's might also be produced from the chromosome 4qB allele.

In conclusion, we expect these studies to demonstrate whether there is a correlation between DUX4 gene or protein expression and the presence of the FSHD phenotype or not.

Grant: FSHS-FS-005
Researcher: **Patricia Arashiro, B.Sc./Mayana Zatz, MSc., Ph.D.**
Institution: Universidade de São Paulo
Instituto de Biociências
Centro de Estudos do Genoma Humano
Departamento de Genética e Biologia Evolutiva
São Paulo, BRAZIL

Project Title: **“Clinical Variability in Patients Affected by FSHD.”**
\$15,000 1 year. 3/1/2007 – 3/1/2008

Goal: Previous studies from our and other groups have shown that usually males are on average more often and more severely affected than females, with approximately 20% of patients becoming wheelchair-bound (Padberg et al, 1991; Zatz et al, 1998). We have previously observed in Brazilian FSHD families that asymptomatic carriers are present in about 30% of the families and some genealogies seem to concentrate more non-penetrant cases (Tonini et al, 2004). This observation is in accordance with van der Maarel et al (2000) who have also observed a female predominance of mosaic asymptomatic carriers. A remarkable but often neglected observation in many families and populations is the occurrence of elderly individuals who inherit disease genes but who nevertheless remain healthy (Nadeau, 2006). The tendency for health to persist despite the presence of susceptibility genes has several explanations, including modifier genes and protective alleles that confer genetic resistance to disease (Nadeau, 2001).

The purpose of the present proposal is to look for modifying genes or mechanisms involved in protecting some individuals against the deleterious effect of the FSHD deletion. Understanding this mechanism may help us to develop new tools for prognosis of the disease and also for future treatment.

In order to compare the gene expression in patients with discordant phenotypes we are currently collecting muscle and skin samples from families with clinically affected and asymptomatic carriers. Total RNA will be isolated from muscle (biceps/ deltoid)

tissue using TRIzol (Invitrogen) method and their quality verified using gel electrophoresis and spectrophotometry. Sample handling and microarray hybridizations will be done in collaboration with Dr. Louis Kunkel (Biological and Biomedical Science, Harvard University). The gene expression datasets will be performed on the Affymetrix GeneChip platform. Affymetrix MAS 5.0 software and custom software (<http://db.chip.org>) will be used for initial data processing, noise analysis, and quality control. Data analysis will also be done in collaboration with Dr. Louis Kunkel at the Children's Hospital in Boston, Massachusetts.

Transcript analysis offers many technical advantages over protein analysis in that the mRNA molecules possess high affinity and specificity binding partners. Additionally, mRNA molecules exhibit equivalent biochemical properties and can be amplified. Moreover, proteomics deal with unavoidable problems of limited and variable sample material, sample degradation, vast dynamic range (more than 10⁶- fold for protein abundance alone), developmental and temporal specificity, and disease and drug perturbations (Tyers, 2003). Other aspects that must be considered are that many signaling and regulatory proteins are present in the cell at very low levels; only a small percentage of proteins are soluble and are expressed at a level compatible with structural analysis (Thornton, 2001), and more than a third of all gene products are poorly soluble membrane proteins of considerable functional importance (van Regenmortel, 2001).

The collection of informative FSHD families, in whom we have identified symptomatic and unaffected members (asymptomatic carriers and non-carriers) who are willing to be submitted to a muscle biopsy for research purposes (after informed consent), is extremely difficult in practice. It has been possible due to many years of research from our group. In addition, the comparison of gene expression from asymptomatic carriers and affected patients is a novel approach that might bring important results.

FSH SOCIETY LEWIS FAMILY RESEARCH & EDUCATION FUND

Grant: FSHS-LEWI-001
Researcher: **Graham J Kemp, M.D.**
Institution: Faculty of Medicine
University of Liverpool
Liverpool, UK
Project Title: **“Muscle damage by reactive oxygen species, muscle atrophy and effects of creatine supplementation in FSHD.”**
\$13,650 1/1/2003 - 5/01/2005 Year 1.5
(interrupt/extend)
(\$48,650 total see balance under Roberts)

Goal: This a pilot study designed to test the following hypotheses: (1 that muscle in FSHD shows evidence of damage by ROS in vivo; 2) that this is at least partly due to reduced anti-ROS protection; 3) that this is ameliorated by 6 months creatine treatment; and; 4) that this also partially alleviates muscle atrophy, even in the absence of training,

and; 5) that this results in an increase in muscle strength and clinical indices. This is an open label pre-post protocol examining the effects of 6 months creatine supplementation in 10 patients with proven FSHD. ROS protection and damage will be studied in conchotome biopsies of deltoid. Muscle atrophy and its effect on body composition will be measured by whole-body quantitative magnetic resonance imaging (MRI). Muscle strength and effects on symptomatology will be be quantified. We will compare pre-creatine results with those of control subjects, and examine differences between post- and pre-creatine values.

This study has several possible benefits: it will contribute evidence of the therapeutic usefulness of creatine over a longer time span than earlier studies; it will throw light on mechanisms of muscle damage in FSHD; if ROS are indeed important then other compounds that reduce oxidative stress in muscle may be useful; lastly, the results will help in the design and interpretation of future placebo-control trials.

Grant: FSHS-LEWI-002
Researcher: Emma Ciafaloni, M.D.
Institution: University of Rochester School of Medicine
Rochester, New York
Project Title: “The Course and Outcome of Pregnancy and Delivery in Women with FSHD.”

\$0	1/1/2004 - 12/31/2004	Year 1 (\$13,074 total see
Delta Railroad)		
\$11,047	1/1/2005 - 12/31/2005	Year 2 (\$12,973 total
see Delta Railroad)		
\$13,363	1/1/2006 - 12/31/2006	Year 3

Goal: Very little is known about the course and outcome of pregnancy and delivery in women with muscular dystrophies. Our current ability to efficiently counsel women with muscular dystrophies when pregnant or planning a pregnancy is very limited due to the lack of studies addressing the issue of pregnancy and delivery outcome in this group. No specific attention has been paid to the possible interaction between gestation and progression of the myopathy. Objectives are: to increase our knowledge about the course and outcome of pregnancy and delivery in women with FSHD; to assess the effect of pregnancy, delivery and post-partum on the progression of muscle weakness and muscle pain and on quality of life in women with FSHD; and, to ultimately improve counseling, family planning and obstetric management of women with FSHD.

Grant: FSHS-LEWI-003
Researcher: Sara Winokur, Ph.D./Ulla Bengtsson, Ph.D.
Institution: Biological Chemistry
University of California, Irvine
Irvine, California

Project Title: "Coding and non-coding RNA expression in FSHD."
\$30,000 1 year. 3/1/2006 - Bridge Fund

Goal: More than a decade after the position effect hypothesis was first proposed, the fundamental question of whether altered chromatin structure in FSHD affects RNA expression at 4q35 has not been answered. Several independent laboratories have addressed this question, yielding disparate and contradictory results. In part, this is due to the variability in tissues and cultures utilized by various laboratories, which are provided by different sources and often obtained and preserved using different methods. In addition, all of the experimental techniques used to examine RNA expression thus far have relied on pooled sources of RNA from tissues or cell cultures. These techniques include non-quantitative RT-PCR, real-time RT-PCR, and expression profiling. These studies assayed differential RNA expression between FSHD and control muscle, and, by nature of the experimental design, detected average RNA levels emanating from both alleles and multiple cell types.

In contrast, examination of RNA expression in a single cell context is more suited to address the question of whether an altered chromatin structure on the contracted D4Z4 allele influences RNA expression. RNA-FISH (fluorescence in situ hybridization) utilizes antisense RNA or dsDNA as hybridization probes to nascent nuclear RNA transcripts followed by fluorescence detection of conjugated haptens or antibodies. Transcription of both coding and non-coding RNAs from each of the alleles (normal and D4Z4 contracted) can be readily identified by RNA-FISH followed by hybridization with D4Z4 and 4q specific DNA probes. In addition, the specific cell type expressing the RNA can be readily identified using this technique, either in culture or within tissue sections.

We propose to utilize RNA-FISH to answer the following questions: 1) Which 4q35 genes are transcribed in proliferating myoblasts and differentiated myotubes? 2) Are the levels of transcription different between normal and FSHD myoblasts/myotubes? 3) Is there an allele specific transcription in FSHD myoblasts/myotubes? That is, do the contracted and normal alleles display different levels of RNA transcription within single cells? For these studies, 3' hyper-biotinylated antisense oligos corresponding to 4q35 genes will be used as probes for coding RNA expression in myoblasts and differentiated myotubes.

If chromatin structure is altered in FSHD, leading to aberrant RNA expression, then we should not assume that such a mechanism would affect coding RNA exclusively. Non-coding RNA has increasingly come to light as a significant player in the regulation of both transcription and translation. Although several approaches to the detection of non-coding RNAs exist, we propose to use the same technique (RNA-FISH) to examine non-coding RNA within a defined region proximal to the D4Z4 repeat. Genomic clones (cosmids) will be used to hybridize to these RNAs as the specific non-coding transcripts cannot be identified a priori.

Lastly, RNA transcription of genes affected in FSHD (as identified by expression profiling) will be examined in FSHD and control myoblasts/myotubes. A

recent finding in FSHD research within the past year has been the unique and consistent localization of the 4q telomeric region to the nuclear periphery. While the biological significance of this localization is not yet known, the existence of nuclear domains either permissive or repressive of transcription is well documented. Therefore, genes affected in FSHD will be examined by RNA-FISH to determine whether co-localization with the FSHD region at the nuclear periphery might affect RNA transcription from these genes.

FSH SOCIETY HELEN AND DAVID YOUNGER RESEARCH FELLOWSHIP

Grant: FSHS-HDY-001
Researcher: Kyoko Yokomori, Ph.D.
Institution: University of California, Irvine
Department of Biological Chemistry, College of Medicine,
Irvine, California

Project Title: “The molecular characterization of the chromatin structure of the D4Z4 repeat associated with FSHD.”

\$30,000	6/1/2005 – 5/31/2006	Year 1
\$30,000	6/1/2006 – 5/31/2007	Year 2
\$30,000	3/1/2008 – 2/28/2009	Year 3

Goal: FSHD is an autosomal dominant hereditary neuromuscular disorder characterized by progressive degeneration of the upper body muscles. The majority of disease cases is linked to the deletion of the D4Z4 repeat array in the subtelomeric region of chromosome 4q (4qter). Since there appears to be no functional open reading frame in this region, it was hypothesized that the D4Z4 repeat plays a structural role in governing epigenetic regulation of gene expression critical for proper muscle cell differentiation and functions, and that the disease is caused by the inability of the shortened D4Z4 to form its specialized chromatin structure leading to dysregulation of critical gene expression. However, the exact nature of this chromatin structure, factors required for the regulation, and the target genes whose dysregulation may directly evoke disease pathogenicity remain obscure. Therefore, it is vital to understand D4Z4 function in order to address the etiology and pathogenesis of FSHD.

We found using chromatin crosslinking and immunoprecipitation (ChIP) analysis that the heterochromatin binding protein HP1, and an essential protein complex required for chromatid cohesion termed “cohesin”, specifically bind to overlapping regions within the D4Z4 repeat in human muscle cells. HP1 was shown to associate with centromeric heterochromatin through interaction with the methylated lysine 9 residue of histone H3, the hallmark of silenced chromatin, and recruit cohesin to centromeres in *S. pombe* and chicken cells. Consistent with this notion, we detected H3K9 methylation in D4Z4. Intriguingly, both HP1/cohesin binding and H3K9 methylation at this region are lost in FSHD mutant cells, in which the 4qter D4Z4 is deleted. These results provide the first direct evidence that 4qter D4Z4 is heterochromatic, and that this special organization is lost in FSHD. Thus, our results provide further insight into the molecular nature and pathogenic contribution of this unique repeat sequence in FSHD.

We hypothesize that human HP1 targets cohesin to D4Z4, and together they mediate proper heterochromatin structure organization required for normal D4Z4 function, which is abrogated in FSHD. To address this, we plan to carry out biochemical and cytological analyses of the mechanism and function of cohesin and HP1 binding to D4Z4.

Specific aims are: 1) analysis of HP1/cohesin binding to D4Z4 in normal and FSHD cells; 2) characterization of the underlying mechanism and factor requirement for HP1/cohesin binding to D4Z4; and 3) analysis of the effect of cohesin and HP1 depletion on chromatin structure organization and function of D4Z4 at 4qter.

I believe that the proposed project will make unique contributions to further understanding of the chromatin structure of D4Z4 and its role in the development of FSHD and may lead to possible identification of new therapeutic targets.

TOTAL: \$ (includes small awards described at beginning of this document)