

Facioscapulohumeral Muscular Dystrophy FSHD

International Research Consortium Research Workshop Meeting 2005

**Tuesday, October 25, 2005
10 a.m. – 6:30/7:00 p.m.**

**The Sheraton City Centre Hotel
150 West 500 South
Salt Lake City, UT 84101 USA**

Smokehouse through Harvest Room(s)

**Chair: Silvère van der Maarel, Ph.D.
Organizers: Daniel Paul Perez
Silvère van der Maarel, Ph.D.
William R. Lewis, M.D.**

Sponsored by:

**FSH Society, Inc.
Facioscapulohumeral Muscular Dystrophy Society, Inc.
Muscular Dystrophy Association (MDA USA)
Association Française Contre les Myopathies (AFM)**

FSHD INTERNATIONAL RESEARCH CONSORTIUM RESEARCH WORKSHOP MEETING 2005

PROGRAM SCHEDULE AND OUTLINE

9:55-10:00 a.m. OPENING REMARKS & CHARGE FOR THE MEETING

10:00-11:00 a.m. PLATFORM PRESENTATION(S) I

DUX and DUXC

10:00-10:15 a.m. Cellular Toxicity of DUX4 Depends on Nuclear Localization.

10:15-10:30 a.m. Over-expression of the Double Homeodomain Protein DUX4c in FSHD Muscle.

MODEL SYSTEMS

10:30-10:45 a.m. Studying the Over-expression of FRG1 in Transgenic Mice.

10:45-11:00 a.m. The Facioscapulohumeral Dystrophy (FSHD) as an Example of Position-effect Pathology?

11:00-12:15 p.m. PLATFORM PRESENTATION(S) II

MECHANISM(S)

11:00-11:15 a.m. RNA-FISH: An Approach to Single Cell Gene Expression in FSHD.

11:15-11:30 a.m. Chromatin Accessibility in D4Z4 Arrays by In Vivo DNaseI-Sensitivity Assay.

11:30-11:45 a.m. D4Z4 DNA Methylation Studies in FSHD.

11:45-12:00 noon HP1/Cohesin-containing Heterochromatin Structure at Chromosome 4q is Disrupted in FSHD.

12:00-12:15 p.m. FSHD and Retinal Vascular Disease. A Signal from Wnt?

12:15-1:45 p.m. POSTER PRESENTATION SESSION & LUNCH (Buffet Lunch Served)

POSTERS

1. Altered Differentiation of Myoblasts Derived from Affected and Unaffected Muscles of FSHD Patients.

2. Large Deletion Encompassing D4F104S1 in an Extended Pedigree with Facioscapulohumeral Muscular Dystrophy.

3. Functional Analysis of the Mouse D4Z4 Homologue.

4. The Autosomal Dominant Facioscapuloperoneal Muscular Dystrophy with 4q35 Chromosomal Deletion is Probably an Independent Form but is not a Variant of Facioscapulohumeral Muscular Dystrophy.

5. Paired-Like Homeodomain Transcription Factor 1 (Pitx1) Over-expression Regulates Genes Involved in Muscle Atrophy.

6. Can a Normally Asymptomatic D4Z4 Allele Exacerbate Another Muscular Dystrophy?

7. Tethering Adenine (Dam) Methylase to the 3.3-Kb FSHD Repeats to Identify Distant Genes that Physically Come in Contact with the Repeats.

8. Identification of the Mechanism Regulating the Wnt-Dependent Activation of Muscle Progenitor Cells.

9. A Retrospective One Year Audit Study of FSHD Testing in Bristol, United Kingdom.

10. Molecular Analysis of FSHD: a) Further p13E11 (D4F104S1) Deletions; b) Indication for the Presence of Third Allele Variant Distal to D4Z4.

11. Studies on the Expression of the DUX4 and DUX4c Proteins.

1:45-2:15 p.m. PLATFORM PRESENTATION(S) III

U.S. NIH INITIATIVES AND GRANT REVIEW PROCESS

1:45-2:00 p.m. National Institutes of Health (NIH) Planning Process in Muscular Dystrophy.

2:00-2:15 p.m. Strategies for Writing a Good NIH Grant.

2:15-6:15 p.m. WORKSHOP AND DISCUSSION IV

2:15-2:25 p.m. THE OBJECTIVE(S) OF THE PANEL DISCUSSION

2:25-4:40 p.m. PANEL DISCUSSION(S)

2:25-2:45 p.m. Non Chromosome 4q-Linked Families will give Direction to FSHD Molecular Pathways.

2:45-3:05 p.m. Systems Biology is a Bridge too Far in FSHD Research.

3:05-3:25 p.m. The Nuclear Localization of the Chromosome 4q35 Region explains the FSHD Pathogen

3:25-3:45 p.m. There is no FSHD Gene.

3:45-4:00 p.m. BREAK

4:00-4:20 p.m. Bio-banking is Instrumental to Progress.

4:20-4:40 p.m. Non-humanoid/Non-homonoid Animal Models are not Useful.

4:40-5:10 p.m. GENERAL DISCUSSION

5:10-5:30 p.m. 20 MINUTE RECESS

5:30-6:15 p.m. ROADMAP TO SOLUTIONS

6:15 p.m.- CONCLUDING REMARKS

October 18, 2005

PREFACE

Dear Colleagues,

We have very timely and important work to accomplish!

As we begin the Facioscapulohumeral Muscular Dystrophy (FSHD) International Research Consortium (IRC) Workshop of 2005 we ask ourselves -- how can we best accelerate the rate of discovery in FSHD research and how best to approach the task? In order for us to bring more funding, capital and resources to the research community, we need to develop more deliverables, research plans, business plans that philanthropists, foundations, businessmen, private and government funding agencies and volunteer health agencies can use to discretely break the problem down into fundable projects.

The FSH Society is often asked to help define and to assemble business plans and consensus on research questions that need funding. Funding agencies tell us they are more inclined to issue request for applications and to fund research projects if they knew exactly what needed to be funded and addressed. The research community needs to provide this input and documents to help garner this financial support.

Given the complexity of FSHD we are always seeking a consensus and continued input on the following questions. What are the important unanswered questions in FSHD? What is the current status of the research? How can we best focus the basic science? What is the data? What are new and important research directions?

A major initiative is underway at the U.S. National Institutes of Health (DHHS NIH) to develop a national research plan under the MD CARE Act 2001 law. The U.S. federal advisory committee is called the Muscular Dystrophy Coordinating Committee (MDCC) and has been working on the dystrophy research plan for several years. The committee has developed a backbone for a national research plan. The MDCC research plan covers five broad sections -- 1. Disease mechanisms, 2. Screening, surveillance and epidemiology, 3. Treatments, 4. Quality of life issues, and, 5 Research infrastructure needs. Over the summer of 2005, Dr. Porter, Program Director, Neuromuscular Disease and the Executive Secretary, Muscular Dystrophy Coordinating Committee (MDCC) at the National Institutes of Neurological Disorders and Stroke (NINDS), National Institutes of Health (NIH) brought together five work groups to define specifically what is needed to be done in each of the five areas of the national research plan.

Today we have the honor of having two important speakers from the NIH to speak on U.S. NIH initiatives and grant review process. John D. Porter, Ph.D. the Executive Secretary, Muscular Dystrophy Coordinating Committee (MDCC) at the NIH will talk on the "NIH Planning Process in Muscular Dystrophy." Richard Bartlett, Ph.D. is the Scientific Review Administrator for SMEP (Skeletal Muscle and Exercise Physiology) and Orthopaedics SBIR, at the Center for Scientific Review (CSR), National Institutes of Health (DHHS NIH). Dr. Bartlett will speak on "Strategies for Writing a Good NIH Grant."

Two weeks from now, on November 9, 2005, there is a meeting and hearing at the NIH on facioscapulohumeral muscular dystrophy (FSHD) research. The 2005 MDCC meeting agenda is dealing with several important issues, including an examination of the state of research in FSHD, potential shared mechanisms between FSHD and Emery-Dreifuss (EDMD), and has asked Daniel Paul Perez, FSH Society, to report on the Society's strategies to aid research in the field through a grants program and an annual FSHD meeting at the ASHG meeting. The MDCC has also asked Rune R. Frants, Ph.D. to speak on FSHD research and its uniqueness and its unique research needs. Together, we have been asked to present our scientific and strategic views in our field with the ultimate goal of improving the funding opportunities for FSHD research.

To optimize the input, we hope to get feedback from the work floor. We kindly ask your collaboration on the following two ideas.

1. During this FSHD workshop 2005, we propose a session to discuss a number of relevant issues. To stimulate the discussion, we suggest a format with two introductions; one in favour and one against a certain thesis. The two speakers do not actually need to sell their own favorite opinion. They only need to analyze the issue in a purely scientific manner. Our intent is not to create the feeling of having to defend personal and professional differences, but rather to openly discuss the differences and how to rule in, rule out or corroborate data and research findings. For the purpose of this discussion today we ask that previous conflicts and past judgment(s) be left at the door on the way into this meeting. Enclosed, please find the program.

2. We ask that we make a SWOT analysis of FSHD research and resources. A SWOT examines the current environment, both internal and external to the FSHD research community. Areas internal to FSHD research labs usually can be classified as strengths (S) or weaknesses (W), and those external to the labs can be classified as opportunities (O) or threats (T). The main issues during the meeting are: Disease mechanism, screening, treatment, quality of life and infrastructure. Please consider the whole field, not only your own field of expertise. Your comments and suggestions, in particular on future research efforts, do not necessarily need to be extensive; just some key words may help organize our thoughts. Think big; also dreams should be described. We need your input to help define a collegial and collaborative proposal to present to the NIH that clearly outlines the specific direction and needs of the FSHD research community in each of the five areas of the national muscular dystrophy research plan. This will help corroborate the work of the five NIH working groups recommendations on FSHD as well as provide a check and balance to the process on record.

Hopefully you will find the motivation to participate in these two tasks!

This meeting of the FSHD IRC could not have been organized without the support of our sponsors. We express my gratitude for the generous support of the FSH Society, the Muscular Dystrophy Association of the USA and the Association Française Contre les Myopathies (AFM France). We are honored to have several directors, program directors and senior staff from numerous institutes of the United States National Institutes of Health (DHHS NIH).

Please be sure to stop by the FSH Society at booth 1209 in the exhibit hall of the ASHG meeting.

It is truly a pleasure to be meeting with the entire group and know that we will have a fast moving and interesting workshop!

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

Rune R. Frants, Ph.D.
Leiden University Medical Center, Leiden, The Netherlands

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

William R. Lewis, M.D.
FSH Society, Inc., Monterey, California, USA

WELCOME

9:55-10:00 a.m.

OPENING REMARKS & CHARGE FOR THE MEETING

William R. Lewis, M.D., Vice-Chairman, FSH Society, Inc.

10:00-11:00 a.m.

PLATFORM PRESENTATION(S) I

Silvère van der Maarel, Ph.D., Moderator

Leiden University Medical Center, Leiden, The Netherlands

DUX and DUXC

10:00-10:15 a.m.

Alberto Luis Rosa, M.D., Ph.D., and, C. B. Conde, Ph.D.

Instituto de Investigación Médica “Mercedes y Martín Ferreyra” (INIMEC-CONICET), Córdoba, Argentina

Cellular Toxicity of DUX4 Depends on Nuclear Localization.

C.B. Conde, C. Arias and A.L. Rosa

Instituto de Investigación Médica “Mercedes y Martín Ferreyra” (INIMEC-CONICET), Córdoba, Argentina. e-mail: cconde@immf.uncor.edu

10:15-10:30 a.m.

Dalila Laoudj-Chenivesse, Ph.D.

Centre de Recherche en Biochimie Macromoléculaire, CNRS, 1919 route de Mende, 34293, Montpellier, France

Over-expression of the Double Homeodomain Protein DUX4c in FSHD Muscle.

Dalila Laoudj-Chenivesse², Marietta Barro², Sébastien Sauvage¹, Alexandra Tassin¹, Eugénie Anseau¹, Aline Marcowycz¹, Denise Figlewicz³, Annette Van Acker⁴, Oberdan Leo⁴, Alexandra Belayew¹, Frédérique Coppée¹.

¹Laboratory of Molecular Biology, University of Mons-Hainaut, Mons, Belgium

²C.R.B.M. (Research Center of Macromolecular Biochemistry), FRE2593 CNRS, Montpellier, France

³Department of Neurology, University of Michigan, Ann Arbor, MI, USA

⁴Laboratory of Animal Physiology, I.B.M.M., Free University of Brussels, Gosselies, Belgium.

MODEL SYSTEMS

10:30-10:45 a.m.

Rossella Tupler, M.D., Ph.D., and, Davide Gabellini, Ph.D.

University of Massachusetts Medical School, Worcester, Massachusetts, USA

Biologia Generale e Genetica Medica, Pavia, Italy

Studying the Over-expression of FRG1 in Transgenic Mice.

Davide Gabellini¹, Chiara Zecca², Maurizio Moggio², Robin Fitzsimons³, Phil Luthert⁴, Barbara Angeletti⁵, Simona De Gregori⁵, Marie McConkey¹, Michael R.Green¹, Rossella Tupler^{1,5}

¹Program in Gene Function and Expression, University of Massachusetts Medical School, Worcester, MA, USA

²Dipartimento di Scienze Neurologiche, Università di Milano, I.R.C.C.S Ospedale Maggiore, Milano, Italy

³University of Sidney, Australia

⁴Institute of Ophthalmology, London, UK

⁵Biologia Generale e Genetica Medica, Pavia, Italy

PLATFORM PRESENTATION(S) I (Continued)

MODEL SYSTEMS (Continued)

10:45-11:00 a.m.

Alexandre Ottaviani, Ph.D.

Laboratory of Cell Molecular Biology, CNRS UMR 5161, Telomeric & Epigenetic Regulations Group, Ecole Normale Supérieure de Lyon, 69007 Lyon, France

The Facioscapulohumeral Dystrophy (FSHD) as an Example of Position-effect Pathology?

Frédérique Magdinier, Alexandre Ottaviani, Andrea Förster, Eric Gilson

Laboratory of Cell Molecular Biology, CNRS UMR 5161. Telomeric & Epigenetic Regulations Group.

Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, 69007 Lyon FRANCE.

11:00-12:15 p.m.

PLATFORM PRESENTATION(S) II

York Marharens, Ph.D., Moderator

University of California, Los Angeles, California USA

MECHANISM(S)

11:00-11:15 a.m.

Sara T. Winokur, Ph.D.

Department of Biological Chemistry, University of California, Irvine, California USA

RNA-FISH: An Approach to Single Cell Gene Expression in FSHD.

Ulla Bengtsson¹, Jorge Martin¹, Peter Masny², Jane Hewitt³, Rabi Tawil⁴, Sara Winokur¹

¹Department of Biological Chemistry, University of California, Irvine, California, USA

²University of Colorado Health Science Center, Denver, Colorado, USA

³Institute of Genetics, University of Nottingham, Nottingham, UK

⁴Department of Neurology, University of Rochester, Rochester, New York, USA

11:15-11:30 a.m.

Melanie Ehrlich, Ph.D.

Human Genetics, Tulane Medical School, New Orleans, Louisiana, USA

Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland USA

Chromatin Accessibility in D4Z4 Arrays by In Vivo DNaseI-Sensitivity Assay.

Melanie Ehrlich^{1,2} and Koji Tsumagari^{1,2}

¹Human Genetics, Tulane Medical School, New Orleans, Louisiana, USA

²c/o Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland USA

11:30-11:45 a.m.

Silvere M. van der Maarel, Ph.D.

Leiden University Medical Center, Center for Human and Clinical Genetics, Department of Human Genetics, Leiden, The Netherlands

D4Z4 DNA Methylation Studies in FSHD.

EL van der Kooij¹, JC de Greef², P. van Overveld², M Wohlgemuth¹, E. Ricci³, L Felicetti³, M Jeanpierre⁴, BGM van Engelen¹, RR Frants², GW Padberg¹, SM van der Maarel²

¹Dept. of Neurology, University Medical Center Nijmegen, Nijmegen, Netherlands

²Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, Netherlands

³Center for Neuromuscular Diseases, UILDM-Rome Section, Rome, Italy and Institute of Neurology, Università Cattolica, Rome, Italy

⁴U129 INSERM and Service de Biochimie Génétique, ICGM, CHU Cochin, Paris, France

PLATFORM PRESENTATION(S) II (Continued)
MECHANISM(S) (Continued)

11:45-12:00 noon

Kyoko Yokomori, Ph.D.

Department of Biological Chemistry, School of Medicine, University of California, Irvine, California, USA

HP1/Cohesin-containing Heterochromatin Structure at Chromosome 4q is Disrupted in FSHD.

Weihua Zeng¹, Heather C. Gregson¹, Sara Winokur¹, Silvere van der Maarel², Kyoko Yokomori¹

¹Department of Biological Chemistry, School of Medicine, University of California, Irvine, California 92697-1700, USA

²Leiden University Medical Center, Center for Human and Clinical Genetics, P.O. Box 9502, 2300 RA Leiden, The Netherlands

12:00 noon-12:15 p.m.

Robin B. Fitzsimons, M.D., Ph.D.

University of Sydney, Sydney NSW 2000 Australia

FSHD and Retinal Vascular Disease. A Signal from Wnt?

Robin B. Fitzsimons, M.D., Ph.D.,

University of Sydney, Sydney NSW 2000 Australia

12:15 p.m.-1:45 p.m.

POSTER PRESENTATION SESSION & LUNCH

(Buffet Lunch Served)

Poster 1. Dalila Laoudj-Chenivresse, Ph.D.

Centre de Recherche en Biochimie Macromoléculaire, CNRS, route de Mende, 34293, Montpellier, France

Altered Differentiation of Myoblasts Derived from Affected and Unaffected Muscles of FSHD Patients.

M. Barro¹, M. Kitzmann¹, J. Mercier², D., G Carnac¹, Laoudj-Chenivresse¹

¹“Adult stem cells and Facioscapulohumeral dystrophy,” Centre de Recherche en Biochimie Macromoléculaire, CNRS FRE2593, 1919 route de Mende, 34293 Montpellier cedex5, France

² Muscle et pathologies chroniques EA 701, Université Montpellier I, Montpellier, France

Poster 2. Kristen L. Bastress Deak, Ph.D.

Marcy Speer, Ph.D.

Center for Human Genetics, Duke University Medical Center, Durham, North Carolina USA

Large Deletion Encompassing D4F104S1 in an Extended Pedigree with Facioscapulohumeral Muscular Dystrophy.

K. L. Bastress Deak¹, S.M. van der Maarel², R.J.L.F Lemmers², J.M. Staijch¹, M.C. Speer¹, J.R. Gilbert¹

¹Center for Human Genetics, Duke University Medical Center, Durham, NC

²Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands

Poster 3. Jane E. Hewitt, Ph.D.

Institute of Genetics, Queens Medical Centre, University of Nottingham, Nottingham, NG7 2UH, UK

Functional Analysis of the Mouse D4Z4 Homologue.

Jannine Clapp, Laura Mitchell, Paul Scotting, Jane Hewitt

Institute of Genetics, Queen's Medical Centre, University of Nottingham, Nottingham, UK

Poster 4. Valery M. Kazakov, M.D., Ph.D., D.Sc. [presented in absentia]

Department of Neurology, Pavlov State Medical University, St. Petersburg, Russia

The Autosomal Dominant Facioscapulo-peroneal Muscular Dystrophy with 4q35 Chromosomal Deletion is Probably an Independent Form but is not a Variant of Facioscapulohumeral Muscular Dystrophy.

Valery M. Kazakov, M.D., Ph.D., D.Sc.¹, Dmitry I. Rudenko, M.D., Ph.D.^{1,2}, Vladislav O. Kolynin, M.D.²

¹Department of Neurology, Pavlov State Medical University of St. Petersburg, Russia

²Second Neurological Department, City Hospital 2 of St. Petersburg, Russia

POSTER PRESENTATION SESSION & LUNCH (Continued)

*Poster 5. **Yi-Wen Chen, D.V.M., Ph.D.***

Center for Genetic Medicine Research, Children's National Medical Center, Washington, D.C. USA

Department of Pediatrics, George Washington University, Washington, D.C. USA

Paired-Like Homeodomain Transcription Factor 1 (Pitx1) Over-expression Regulates Genes Involved in Muscle Atrophy.

Matthew Kostek, Ling Li, Rongye Shi, Yi-Wen Chen

Center for Genetic Medicine Research, Children's National Medical Center, Washington, D.C. and Department of Pediatrics, George Washington University, Washington, D.C.

*Poster 6. **Peter W. Lunt***

Department Clinical Genetics, Institute of Child Health, Bristol Children's Hospital, Bristol, England

Can a Normally Asymptomatic D4Z4 Allele Exacerbate Another Muscular Dystrophy?

Peter Lunt¹, Suzanne O'Shea², Maggie Williams², Claire Willoughby³, Steve Abbs³, Tim Moss⁴, Caroline Sewry⁵, Philip Jardine⁶

¹Clinical Genetics, St Michaels Hospital, Bristol BS2 8EG, UK

²Molecular Genetics, Lewis Laboratory, Southmead Hospital, Bristol BS10 5NB, UK

³Molecular Genetics, Guys Hospital, London SE1 9RT, UK

⁴Neuropathology, Frenchay Hospital, Bristol BS16 1LE, UK

⁵Dubowitz Neuromuscular Centre, Hammersmith Hospital, London W12 0HS, UK

⁶Paediatric Neurology, Frenchay Hospital, Bristol BS16 1LE, UK

*Poster 7. **York Marahrens Ph.D.***

UCLA, Department of Human Genetics, Gonda Center, Los Angeles, California, USA

Tethering Adenine (Dam) Methylase to the 3.3-Kb FSHD Repeats to Identify Distant Genes that Physically Come in Contact with the Repeats.

York Marahrens¹, Fyodor D. Urnov², Nieves Embade¹

¹UCLA Department of Human Genetics, Gonda Center, 695 Charles E. Young Drive, Los Angeles, California 90095, USA

²Sangamo BioSciences, Inc., Pt. Richmond Tech Center 501, Canal Blvd, Suite A100 Richmond, California 94804, USA

*Poster 8. **Daniela Mara de Oliveira, Ph.D.** [presented in absentia]*

Ottawa Health Research Institute, Molecular Medicine Program, Ottawa, Ontario, Canada K1H 8L6

Identification of the Mechanism Regulating the Wnt-Dependent Activation of Muscle Progenitor Cells.

Daniela Mara de Oliveira, Michael A Rudnicki

Ottawa Health Research Institute, Molecular Medicine Program, 501 Smyth Road, Ottawa, Ontario, Canada K1H 8L6

POSTER PRESENTATION SESSION & LUNCH (Continued)

Poster 9. Suzanne O'Shea, Ph.D.

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

A Retrospective One Year Audit Study of FSHD Testing in Bristol, United Kingdom.

Suzanne O'Shea¹, Maggie Williams¹, Sarah Burton-Jones¹, Peter Lunt²

¹Molecular Genetics, Lewis Laboratory, Southmead Hospital, Bristol BS10 5NB, UK

²Clinical Genetics, St Michaels Hospital, Bristol BS2 8EG, UK

Poster 10. Meena Upadhyaya, Ph.D.

Institute of Medical Genetics, Cardiff University, Heath Park, Cardiff CF14 4XN, United Kingdom

Molecular Analysis of FSHD: a) Further p13E11 (D4F104S1) Deletions; b) Indication for the Presence of Third Allele Variant Distal to D4Z4.

Gill Spurlock, Meena Upadhyaya

Institute of Medical Genetics, Cardiff University, Heath Park, Cardiff CF14 4XN, UK

Poster 11. Alexandra Belayew, Ph.D.

University of Mons-Hainaut, Mons, Belgium

Studies on the Expression of the DUX4 and DUX4c Proteins.

Alexandra Tassin¹, Sébastien Sauvage¹, Christel Mattéotti¹, Annette Van Acker², Denise Figlewicz³, George Padberg⁴, Oberdan Leo², Frédérique Coppée¹, Alexandra Belayew¹

¹Laboratory of Molecular Biology, University of Mons-Hainaut, 7000-Mons, Belgium

²Laboratory of Animal Physiology, IBMM, Free University of Brussels, Gosselies, Belgium

³Department of Neurology, University of Michigan, Ann Arbor, Michigan, USA

⁴Department of Neurology, University Medical Center, Nijmegen, The Netherlands

1:45 p.m.-2:15 p.m.

PLATFORM PRESENTATION(S) III

U.S. NIH INITIATIVES AND GRANT REVIEW PROCESS

William R. Lewis, M.D., Moderator

Vice-Chairman, FSH Society, Inc., Monterey, California and Boston, Massachusetts, USA

1:45-2:00 p.m.

John D. Porter, Ph.D.

Program Director, Neuromuscular Disease

Executive Secretary, Muscular Dystrophy Coordinating Committee (MDCC)

National Institutes of Neurological Disorders and Stroke (NINDS), National Institutes of Health (DHHS NIH), Bethesda, Maryland USA

National Institutes of Health (NIH) Planning Process in Muscular Dystrophy.

John D. Porter, Ph.D.

Extramural Programs, Muscle Biology Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), National Institutes of Health (DHHS NIH), Bethesda, Maryland USA

2:00-2:15 p.m.

Richard Bartlett, Ph.D.

Scientific Review Administrator for SMEP (Skeletal Muscle and Exercise Physiology) and

Orthopaedics SBIR, the Center for Scientific Review (CSR), National Institutes of Health (DHHS NIH), Bethesda, Maryland USA

Strategies for Writing a Good NIH Grant.

Richard Bartlett, Ph.D.

Scientific Review Administrator for SMEP (Skeletal Muscle and Exercise Physiology) and Orthopaedics SBIR, Center for Scientific Review (CSR), National Institutes of Health (DHHS NIH), Bethesda, Maryland USA

2:15-6:15 p.m.

WORKSHOP AND DISCUSSION IV

2:15-2:25 p.m.

THE OBJECTIVE(S) OF THE PANEL DISCUSSION

Silvère van der Maarel, Ph.D., Moderator

Leiden University Medical Center, Leiden, The Netherlands

&

William R. Lewis, M.D., Moderator

Vice-Chairman, FSH Society, Inc., Boston, Massachusetts, USA

Principle: The two opponents get 5 minutes each to defend the ALLOCATED opinion. The audience can discuss for 10 minutes. General discussion of 30 minutes will be held after all six discussions. After 20 minute break a proposal for November 9, 2005 will be presented based on discussion.

2:25 p.m.-4:40 p.m.

PANEL DISCUSSION(S)

2:25 p.m.-2:45 p.m.

DISCUSSION ONE

Non Chromosome 4q-Linked Families will give Direction to FSHD Molecular Pathways.

Arguing for the Pro position: Peter W. Lunt

Department Clinical Genetics, Institute of Child Health, Bristol Children's Hospital, Bristol, England

Arguing for the Contra position: Robin B. Fitzsimons, M.D., Ph.D.

University of Sydney, Sydney NSW 2000 Australia

2:45 p.m.-3:05 p.m.

DISCUSSION TWO

Systems Biology is a Bridge too Far in FSHD Research.

Arguing for the Pro position: To Be Assigned (TBA)

Arguing for the Contra position: Rune R. Frants, Ph.D.

Leiden University Medical Center, Leiden, The Netherlands

3:05 p.m.-3:25 p.m.

DISCUSSION THREE

The Nuclear Localization of the Chromosome 4q35 Region explains the FSHD Pathogenesis.

Arguing for the Pro position: Sara T. Winokur, Ph.D.

Department of Biological Chemistry, University of California, Irvine, California USA

Arguing for the Contra position: Melanie Ehrlich, Ph.D.

Human Genetics, Tulane Medical School, New Orleans, Louisiana, USA

Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland USA

3:25 p.m.-3:45 p.m.

DISCUSSION FOUR

There is no FSHD Gene.

Arguing for the Pro position: Rossella Tupler, M.D., Ph.D.
University of Massachusetts Medical School, Worcester, Massachusetts, USA, and,
Biologia Generale e Genetica Medica, Pavia, Italy

Arguing for the Contra position: Alexandra Belayew, Ph.D.
University of Mons-Hainaut, Mons, Belgium

3:45 p.m.-4:00 p.m.

BREAK

(Tea, Refreshments, and Desserts)

4:00 p.m.-4:20 p.m.

DISCUSSION FIVE

Bio-banking is Instrumental to Progress.

Arguing for the Pro position: Denise A. Figlewicz, Ph.D.
Department of Neurology, University of Michigan, Ann Arbor, Michigan USA

Arguing for the Contra position: Kevin Flanigan, M.D.
Eccles Institute of Genetics, University of Utah School of Medicine, Salt Lake City, Utah USA

4:20 p.m.-4:40 p.m.

DISCUSSION SIX

Non-humanoid/Non-homonoid Animal Models are not Useful.

Arguing for the Pro position: Silvère van der Maarel, Ph.D.
Leiden University Medical Center, Leiden, The Netherlands

Arguing for the Contra position: Jane E. Hewitt, Ph.D.
Institute of Genetics, Queens Medical Centre, University of Nottingham, Nottingham, UK

4:40 p.m.-5:10 p.m.

GENERAL DISCUSSION

Rune R. Frants, Ph.D., Moderator
Leiden University Medical Center, Leiden, The Netherlands

5:10 p.m.-5:30 p.m.

20 MINUTE RECESS

5:30 p.m.-6:15 p.m.

ROADMAP TO SOLUTIONS

Rune R. Frants, Ph.D., Moderator
Leiden University Medical Center, Leiden, The Netherlands

6:15 p.m.-

CONCLUDING REMARKS

Silvère van der Maarel, Ph.D.

ABSTRACTS SECTION

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1. Altered Differentiation of Myoblasts Derived from Affected and Unaffected Muscles of FSHD Patients.

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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant neuromuscular disease characterized by a progressive weakness of the facial, shoulder girdle and upper arm muscles. FSHD has been causally related to deletions of 3.3kb subtelomeric repeats, namely D4Z4, on chromosome 4q35, but the molecular mechanism of this disease remains unknown.

Although increased expression of several 4q35 genes has been reported, two following studies disputed this, finding no significant change in the transcriptional level of any 4q35 gene, among which is the heart and muscle specific isoform of the adenine nucleotide translocator ANT1. We have previously shown increased levels of ANT1 protein in both affected and unaffected FSHD muscles, suggesting that ANT1 could be implicated in FSHD pathogenesis.

We have purified muscle precursor cells from clinically unaffected and affected muscles from FSHD patients, and explored their ability to form muscle tissue in vitro and to recapitulate the pathological process. Compared to control donor cells, FSHD myoblasts displayed no defect under proliferative conditions, whereas they showed abnormal differentiation with some heterogeneity consistent with the phenotypic variability observed in FSHD patients. Furthermore, we also reported increased levels of ANT1 protein in differentiated FSHD myoblasts, further supporting the implication of ANT1 in critical event of the FSHD pathogenesis.

As the level of alterations is not correlated with the length of repeat units, our results suggest that contraction of D4Z4 is necessary to cause abnormal myoblasts differentiation but is not sufficient to explain the phenotypic variability.

2. Large Deletion Encompassing D4F104S1 in an Extended Pedigree with Facioscapulohumeral Muscular Dystrophy.

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Facioscapulohumeral muscular dystrophy (FSHD) is a disease of skeletal muscle, with symptoms including both facial and shoulder girdle weakness. In the majority of FSHD cases, the molecular basis is a contraction of the D4Z4 repeat on the end of the long arm of chromosome 4. In a small number of cases, there is an apparent absence of the contracted D4Z4 repeat that is associated with FSHD. This is due to the presence of a deletion in the region proximal to the D4Z4 repeat array, which encompasses the p13E-11 (D4F104S1) probe-binding site. The frequency of such extended deletions is unknown, due to the difficulties in the molecular identification of such cases. To date, only a few patients with an extended deletion have been described, with no more than 2 members of the same family.

Here we describe a family (DUK 2531) with 20 sampled individuals, where 10 affected individuals that segregate a contracted D4Z4 allele in addition to large proximal deletion of approximately 70 kb. This is the largest deletion to be described and the first extended pedigree showing such a deletion. This family was originally characterized as being unlinked to the 4q35 region (FSHD1B) and was thought to have normal D4Z4 fragment size. Because such deletions can lead to misinterpretation in the diagnostic setting, this suggests the need for the use of additional diagnostic tests in FSHD such as the 4qA probe. MseI digestion and probing with the D4Z4 specific probe 9B6A reveals the presence of ten D4Z4 repeat units (33 kb), and the 4qA probe also reveals a fragment of this length. Subsequent MseI/BlnI digestion confirms that the deletion allele is of chromosome 4 origin and NotI fragment analysis for chromosome assignment suggests that the allele resides on chromosome 4. It appears that the extended deletion includes the p13E-11 binding site, the B31 probe site, the inverted repeat D4S2463, and several genes including FRG2 and TUBB4Q. Mapping of the exact breakpoint is currently underway.

3. RNA-FISH: An Approach to Single Cell Gene Expression in FSHD.

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²University of Colorado Health Science Center, Denver, Colorado, USA

³Institute of Genetics, University of Nottingham, Nottingham, UK

⁴Department of Neurology, University of Rochester, Rochester, New York, USA

In order to directly address the position effect hypothesis in FSHD, we have implemented the use of RNA-FISH for allele-specific gene expression studies. RNA-FISH is a technique that allows for examination of RNA expression within single cells, rather than the pooled sources of RNA used for RT-PCR and expression profiling. Thus, RNA expression from the deleted and normal 4q alleles can be independently assayed, rather than detecting average RNA levels emanating from both alleles and multiple cell types within cell cultures or muscle tissue.

Initial studies have focused on optimizing hybridization with a variety of probes, including antisense biotin- or digoxigenin-labeled oligonucleotides, nick translated genomic clones and fluorochrome-incorporated PCR fragments generated from cDNA clone templates. FSHD and control myoblasts were cultured in chamber slides, and assayed for RNA expression from multinucleated myotubes at seven days post-differentiation. The first two 4q35 genes chosen for these studies were adenine nucleotide translocator 1 (ANT1, SLC25A4) and PDZ and LIM domain 3 (PDLIM3, ALP). ANT1 and ALP expression in FSHD have been analyzed previously by other techniques, and are both expressed in mature skeletal muscle. In order to verify expression in our differentiated myoblasts, we performed co-IF with anti-PDLIM3 and anti-actin, as well as anti-ANT1 in parallel with the mitochondrial stain mito-tracker. Preliminary results of ANT1 and ALP RNA expression in normal and FSHD differentiated myoblasts will be discussed in light of the position effect hypothesis.

In addition, ongoing nuclear localization studies indicate that both lamin A/C and emerin are required for proper localization of the FSHD genomic region to the nuclear periphery. Both lamin A/C & emerin null fibroblasts exhibit a slight shift of the 4q telomere away from the nuclear envelope. In the emerin deficient fibroblasts, the 4q telomere often appears unraveled, with multiple hybridization signals relative to the discrete hybridization signal seen with the 1q telomere probe. Thus, RNA-FISH studies will be conducted on these cell cultures as well, to determine whether differential localization affects RNA expression of 4q35 genes.

We are grateful to the Facioscapulohumeral Society, Inc., The Muscular Dystrophy Association, and the Fischer-Shaw Foundation for their generous support of our research.

4. Functional Analysis of the Mouse D4Z4 Homologue.

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Institute of Genetics, Queen's Medical Centre, University of Nottingham, Nottingham, UK

Facioscapulohumeral muscular dystrophy (FSHD) is caused by a unique genetic rearrangement close to the telomere of human chromosome 4q. The disease is causally associated with deletions within a tandem DNA repeat (D4Z4). Initially, because of homeobox sequences within D4Z4, it was thought that the mutation might alter a protein product encoded by the repeat. However, although cDNAs and RT-PCR products containing closely related sequences have been identified, none of these originate from D4Z4. An alternative mechanism is that the deletion alters the local chromatin environment, resulting in a position effect on one or more nearby genes. Although changes in 4q35 gene expression levels in FSHD muscle have been reported, these findings remain controversial. It is apparent that the function of D4Z4 and its role in FSHD remains to be defined.

In light of this, we have returned to study the evolution of D4Z4 as this may shed further light on its function. Although DNA hybridization techniques have previously identified D4Z4 homologues only in primates, here we show that the mouse genome also contains sequences homologous to D4Z4. These are arranged in a large (100-200kb) tandem array, with each 5kb repeat unit having an open reading frame containing two homeobox sequences. The rat genome contains an orthologous array at the equivalent genome location. Phylogenetic analysis indicates that human and mouse D4Z4 homeodomains are more closely related to each other than to other homeodomain proteins. We have used RT-PCR and in situ hybridisation to show that the mouse array (mD4Z4) is transcribed in a wide range of embryonic and adult tissues, including skeletal muscle. Transfection of epitope-tagged mD4Z4 into C2C12 cells shows the protein to localise to the nucleus.

This is the first time that any D4Z4 homologous sequence, apart from very closely related primate species, has been identified and suggests that the hypothesis that the human D4Z4 repeat encodes a protein should be revisited.

5. Cellular Toxicity of DUX4 Depends on Nuclear Localization.

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DUX4 is a putative double-homeobox protein encoded at D4Z4, the 3.3-kb tandem repeat contracted on 4q35 in patients with FSHD. A nearly identical ORF (DUX4c) is located ~40 kb centromeric to D4Z4. Anti-DUX4 antibodies prepared in our laboratory specifically recognize a DUX4-related endogenously-expressed protein in cultured human adult and fetal rhabdomyosarcoma cells. The protein is not DUX4c as indicated by lack of immunostaining using a specific anti-DUX4c antibody (provided by A. Belayew, Belgium). The endogenously expressed DUX4-related protein, like DUX4 expressed in cell transfection experiments, is a ~46 kDa protein that localizes to the cell nuclei. Transient expression of DUX4 in cultured cells leads to cell death, and no stable transfectants constitutively expressing DUX4 could be isolated. PCR-mediated mutagenesis was used to study the functionality of two putative nuclear localization signals (NLS-1 and NLS-2) present in DUX4. Selected amino acid residues at NLS-1 and NLS-2 were independently replaced by threonines and the sub-cellular distribution of the various DUX4 mutants was analyzed in transfected cells. DUX4 mutants that do not localize to the nuclei highlight amino acid residues that mediate sub-cellular trafficking of this protein.

The typical DUX4-mediated cell death phenotype observed in transfection experiments was abolished in some of these DUX4 mutants. We propose that NLS-1 and NLS-2 are functional DUX4 NLSs and that nuclear entrance is required for DUX4-mediated cell death.

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6. Chromatin Accessibility in D4Z4 Arrays by In Vivo DNaseI-Sensitivity Assay.

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Although by histone acetylation assays and transcription assays, DNA sequences proximal to the subtelomeric D4Z4 arrays are not embedded in highly condensed heterochromatin, the structure of the arrays themselves is uncertain. Therefore, we are testing D4Z4 chromatin structure by in vivo DNaseI-sensitivity assays. We designed a set of probes that allow comparison on the same Southern blot of two reference gene regions that are unexpressed in the examined cell types, two 5' regions of reference genes that are constitutively expressed; a constitutively heterochromatic satellite DNA sequence, and D4Z4 arrays. These probes were used for blot-hybridization to DNA from cultured cells permeabilized by lysolecithin, briefly incubated with various concentrations of DNaseI, purified, and digested with a restriction endonuclease before gel electrophoresis.

We determined the relative rate of decrease in the signal of the analyzed restriction fragments with increasing concentrations of DNaseI. The standard gene regions, the satellite DNA region, and the test D4Z4 sequence could be cleaved by a single restriction endonuclease (StyI) to generate DNA fragments from 1.2-3.3 kb. We determined hybridization conditions that gave no interfering cross-hybridization. The analyzed restriction fragments were as follows: D4Z4 (3.3 kb from 4q-type repeats and 2.8-kb from 10q-type repeats); genes unexpressed in the cells being studied, interleukin 2 (IL-2, intron2 to intron3; 3.1-kb) and cystatin D (CST5, intron2 to intron3; 1.5-kb); constitutively expressed genes, beta-2 microglobulin (B2M, promoter region through intron 1; 1.2-kb) and hydroxymethylbilane synthase (HMBS, promoter region through intron 1; 1.4-kb) and constitutive heterochromatin, satellite 2 DNA from chromosome 1 (Chr1 Sat2 from 1qh; 0.46, 1.3, 1.7, and 2.3 kb). The gene probes were from a sub-region of the examined restriction fragment and were obtained by PCR of genomic DNA. The D4Z4 probe was a 1-kb subfragment obtained by PCR of plasmid DNA, and the Chr1 Sat2 probe was an insert from a recombinant plasmid. We analyzed initially a control lymphoblastoid cell line (AG14953; Coriell Institute).

As expected, progressive decreases in the percent of DNA fragment remaining with increasing DNaseI concentration were observed for all tested DNA sequences. Moreover, according to prediction, Sat2 chromatin was much more resistant to DNaseI than the unexpressed genes, which, in turn, were more resistant than the constitutively expressed genes. Also, as expected, the ladder of specific bands obtained from Sat2 showed an approximately linear dependence of increasing DNaseI sensitivity and increasing DNA fragment size. With or without normalization for this fragment size relationship, D4Z4 chromatin was found to be considerably more resistant to DNaseI than all of the gene regions tested, but more sensitive than the constitutively heterochromatic Sat2.

This suggests that D4Z4 arrays are in an appreciably more condensed chromatin structure than unexpressed euchromatin. Although we have not yet examined myoblasts, we predict that FSHD-related gene expression in the 4q35 region is dependent on cell type, but that D4Z4 chromatin structure is not. A moderately condensed D4Z4 structure may play a role in the ability of D4Z4 array size to control expression of genes in cis so as to determine FSHD or non-disease status. Supported in part by NIH Grant NS 048859.

7. Studying the Over-expression of FRG1 in Transgenic Mice.

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Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common hereditary myopathies. The disease has been causally related to deletion of tandemly arrayed 3.3 kb repeat units (D4Z4) on chromosome 4q35 possibly affecting chromatin organization and nearby gene expression. Consistently, we have observed that three genes mapping at 4q35, FRG2, FRG1 and ANT1, are over-expressed in the FSHD affected muscle. An element within D4Z4 has been shown to behave as a silencer that provides a binding site for a transcriptional repressing complex formed by YY1, HMGB2, and nucleolin. These results suggest a model in which deletion of D4Z4 leads to the inappropriate transcriptional de-repression of 4q35 genes resulting in disease.

To test the proposed pathogenic model in vivo, we generated transgenic mice selectively over-expressing ANT1, FRG1, and FRG2 in skeletal muscle; these are the genes that were found over-expressed in muscle tissues affected by FSHD. To this purpose, ANT1, FRG1, and FRG2 have been cloned into an expression vector carrying the human skeletal actin (HSA) promoter that is expressed in all skeletal muscle fibers of the adult mouse, with minor fiber to fiber variation. The HSA-FRG1, HSA-FRG2, and HSA-ANT1 transgenes were expressed uniquely in skeletal muscle tissues. Transgenic mice over-expressing FRG1 develop signs of muscular dystrophy whose degree of severity is proportional to FRG1 expression level. By contrast, mice over-expressing two other putative FSHD-candidate genes, FRG2 and ANT1, are normal with regard to both phenotype and muscle histology. Notably, FRG1 transgenic mice display a variety of features found in FSHD, including abnormal spinal curvature, progressive muscle dystrophy, skeletal muscle atrophy, and a differential involvement of muscle types that is analogous to that observed in FSHD but not other muscular dystrophies. 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. Collectively, our results suggest that FSHD results from inappropriate over-expression of FRG1 in skeletal muscle.

Extra-muscular symptoms such as sensori-neural deafness, and retinovasculopathy, are also part of the clinical spectrum of FSHD. To investigate whether the over-expression of FRG1 is responsible for extra-muscular symptoms in FSHD patients, we generated transgenic mice ubiquitously over-expressing the human FRG1 gene under the control of CAG promoter, a composite promoter connecting the cytomegalovirus immediate early (CMV-IE) enhancer sequence to the chicken b-actin promoter. Genotype was verified by PCR and Southern blot analysis. RT-PCR confirmed that the transgene was expressed in all tissues analyzed.

The development of a faithful animal model of FSHD should provide further insights into the molecular basis and pathogenesis of the disease, as well as a model for evaluating therapeutic strategies.

8. The Autosomal Dominant Facioscapulooperoneal Muscular Dystrophy with 4q35 Chromosomal Deletion is Probably an Independent Form but is not a Variant of Facioscapulohumeral Muscular Dystrophy.

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We give the description of a two autosomal dominant with 4q35 linked facioscapulooperoneal muscular dystrophy (FSPD) families in which the patients were re-examined by V.K. after 24, 28 and 35 years and the typical changes of the pattern of muscle affections on the different stages of the disease were established and confirmed by muscle CT and MRI. The disease began with initial involvement of the face and shoulder girdle muscles and some time later of the peroneal group (anterior tibial) muscles. However the dystrophic process is not limited of these anatomical regions and gradually extends to the thighs (posterior group of the muscles), pelvic girdle (gluteus maximus) and not always to upper arm (biceps brachii). In this connection, the inaccuracy of the name “facioscapulooperoneal” (FSP) or “scapulooperoneal with minimal/slight affection of facial muscles” [(F)SP] muscular dystrophy becomes evident. The term “facioscapulolimb muscular dystrophy, type 2 (FSLD2), descending with a “jump” with initial (F)SP or FSP phenotypes with 4q35 deletion” would be more correct.

In observed patients we revealed very slight weakness (or atrophy) of individual mimic muscles or their parts, especially during the stage of the disease presenting the scapulooperoneal phenotype. Weakness of the biceps brachii muscles usually in slight degree appeared as a rule after weakness of the peroneal group, posterior group of the thigh and gluteus maximus muscles. The (F)SP or the FSP phenotypes predominated in the clinical picture during a many years and they constituted merely a stage in the development of FSLD2. We suppose the FSLD2 is an independent form of muscular dystrophy.

9. Paired-Like Homeodomain Transcription Factor 1 (Pitx1) Over-expression Regulates Genes Involved in Muscle Atrophy.

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Paired-like homeodomain transcription factor 1 (Pitx1) is a homeobox transcription factor, which plays a critical role in specification of hind-limb identity during embryonic development. Although the role of Pitx1 in developing limbs has been well studied, the function of the gene in postnatal skeletal muscle is unknown. By comparing expression profiles of Facioscapulohumeral muscular dystrophy (FSHD) to 128 muscle profiles of 12 neuromuscular disorders, we showed that PITX1 is specifically up-regulated in muscles of FSHD patients (4 fold, $P < 0.005$). To further study the function of PITX1 in mature skeletal muscle, we over-expressed Pitx1 in mice by in vivo electroporation, and showed that over-expression of the gene lead to activation of the ubiquitin-proteasome pathways, including up-regulation of atrogen 1 which is a key regulator of skeletal muscle atrophy. To identify the direct targets of Pitx1, we searched for a known consensus sequence of Pitx1 (CAATCC) in the promoter region of the genes differentially regulated in the mouse study. Only genes with the consensus sequence in the first 2000 bp of the promoter or the 5' UTR of exon 1 in mice and located in an evolutionary conserved region were included in the final list.

The search identified 7 of 62 genes containing the consensus binding sequence. Only Foxo1a, an upstream regulator of atrogen 1, reached the final criteria. We over-expressed Pitx1 in C2C12 cells and RNA was isolated at 0, 24 and 48 hrs post-differentiation, followed by RT_PCR of Foxo1a. Foxo1a was found to have a seven-fold increased expression at 24 hours post-differentiation. Our results suggest that Pitx1 is likely to be involved in the early skeletal muscle atrophy process through regulating Foxo1a, an upstream regulator of atrogen1 and the ubiquitin-ligase proteasome pathway.

10. Over-expression of the Double Homeodomain Protein DUX4c in FSHD Muscle.

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We have identified the putative double homeobox gene DUX4c in an isolated D4Z4 unit mapping 42 kb centromeric of the D4Z4 repeat array and encoding a 374-residue protein. DUX4c mRNA's encompassing its open reading frame were detected when the natural gene was introduced by transfection into muscle cell cultures. 3'RACE experiments showed alternative splicing downstream of the STOP codon.

A rabbit antiserum raised against a DUX4c-specific peptide detected a 47 kDa protein on a Western blot prepared with nuclear extracts of cells transfected with a DUX4c expression vector. In muscle cells transfected with the natural gene, the DUX4c protein was detected by immunoprecipitation of nuclear extracts with the rabbit antiserum, followed by a western blot with a very sensitive mouse monoclonal antibody raised against the carboxyl-terminal domain of DUX4 and cross reacting with DUX4c. This weak protein expression from the DUX4c natural gene was confirmed in the nucleus of a few transfected muscle cells by immunofluorescence. We have investigated the DUX4c expression by Western blot with this rabbit antiserum in muscle biopsies of patients with FSHD and controls, and in the primary myoblast cultures derived from them. DUX4c was detected in controls and at increased levels in all FSHD muscle biopsies analyzed. Interestingly, higher DUX4c expression was observed in samples with lower D4Z4 repeat copy number. Moreover, the strongest signal was found in a patient homozygous for the 4q35 deletion. In myoblast primary cultures (DMEM, 10% fetal calf serum and 1% Ultrosor G), DUX4c was detected both in FSHD and control samples but when the culture medium was changed (SKGM and 10% fetal calf serum), it could not be detected in controls anymore. DUX4c expression was induced upon differentiation in both myoblast types with increased levels in FSHD samples. We then performed Western blots on biopsies of patients with Duchenne muscular dystrophy and found a higher DUX4c amounts as compared to controls. In myoblasts cultures, similar increases in DUX4c expression were detected during differentiation of Duchenne or control samples. Additional analyses will be needed to evaluate whether DUX4c expression might be linked in part to muscle regeneration.

In another set of experiments, we transfected TE671 rhabdomyosarcoma cells grown in vitro with a pCINeo-DUX4c expression vector and observed induced Myf5 protein expression and DNA binding activity, but decreased MyoD and MEF2 activities.

In conclusion, our data demonstrated that the DUX4c gene was functional, expressed in myoblasts, with higher levels in FSHD samples, and could affect some myogenic factor activities. DUX4c might not be involved in FSHD since a deletion extending from the D4Z4 repeat array to DUX4c was found in two affected families (Lemmers et al, 2003, *Neurology* 22, 178-83). Nevertheless, the second DUX4c allele is still present in these families and could be activated by transvection as recently reported for the FRG2 gene (Rijkers et al, 2004, *J Med Genet.* 41, 826-36).

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11. Can a Normally Asymptomatic D4Z4 Allele Exacerbate Another Muscular Dystrophy?

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We present a manifesting female carrier of Duchenne muscular dystrophy (DMD), aged 6 years, who has an unusually more severe presentation for this than would be expected even in an affected male, and who on testing for FSHD shows a 40kb Bln-resistant D4Z4 fragment.

The girl (BG) has hypotonia and progressive scapular and pelvic girdle weakness, losing ambulation by 6 years. Serum CK is in the BMD range (7000 IU/l), and muscle biopsy shows the mosaic pattern of dystrophin immunostaining and utrophin pattern characteristic for a manifesting DMD carrier. Dystrophin DNA screening has identified heterozygosity for a maternally inherited predicted in-frame novel duplication of exons 54 & 55. The mother is asymptomatic and has normal CK (58 IU/l). BG has moderate facial weakness, but testing for FSHD showed only a 40kb (37kb) Bln-resistant fragment with p13E-11. Estimation of likelihood for being a 4q fragment by Bln2/Bgl2 dosage, and characterisation as 4qA or B is underway.

If confirmed as a 4q35 type A allele, we propose that in this girl, a mild heterozygous DMD mutation and a usually innocuous D4Z4 allele may be having a synergistically deleterious effect to give a very severe DMD presentation with some additional features of FSHD.

Since previous work has suggested 4q35 Bln-resistant fragments in the size range 32-40 kb are found in at least 2-4% of the normal population, it will be interesting to test whether these alleles may occur in higher frequency in patients with unusually severe presentations of other muscular dystrophies.

12. The Facioscapulohumeral Dystrophy (FSHD) as an Example of Position-Effect Pathology?

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In several human pathologies, disorders of different levels of the epigenetic regulations have been observed. Genetic alteration of modulators of the higher-order chromatin architecture can lead directly to developmental diseases or cancer. Also, the disorganization of the genome can disrupt the equilibrium between coding sequences, adjacent regulatory elements or heterochromatin regions and lead to inappropriate gene expression through position effect mechanisms.

A link between position effect and the 4q telomeric region rearrangement is a popular hypothesis to explain the molecular mechanism involved in the FSHD. Indeed, despite intense efforts over the past decade the gene responsible for this disease has not been identified. Therefore, key to understanding the pathogenesis leading to FSHD is elucidation of how deletion of a tandemly repeated fragment named D4Z4 in the subtelomeric region of 4q35 to a critical threshold causes this syndrome and how flanking sequences cooperate in the pathological process.

In order to investigate the molecular mechanisms leading to FSHD, we constructed different cellular models by putting D4Z4 and other subtelomeric sequences near a GFP reporter gene at a subtelomeric position. In this simple experimental system, the effect of one or several D4Z4 and flanking sequences on transcription was followed-up by flow cytometry for up to 100 days. Preliminary results show that a single D4Z4 exhibit a strong enhancer activity on gene expression whereas other elements present at 4q35 behave as repressor suggesting that 4q35 is a mosaic of regulatory sequences probably involved in long-range chromatin interaction and sub-nuclear localization. Our goal is now to understand what mediates the transcriptional regulation in term of chromatin architecture and epigenetic regulation and to identify both cis- and trans-sensing effects of the 4q subtelomeric sequences on expression. Therefore, different SiRNA directed against partners of the chromatin architecture and telomere maintenance were tested either on transgenes or endogenous 4q genes expression. Preliminary results will be discussed. However, our data suggest that FSHD might be caused by different mechanisms combining nuclear organization, telomere structure and chromatin insulation.

13. Tethering Adenine (Dam) Methylase to the 3.3-Kb FSHD Repeats to Identify Distant Genes that Physically Come in Contact with the Repeats.

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Although deletion of the 3.3-Kb D4Z4 repeats is almost certainly responsible for FSHD, the FSHD disease mechanism and FSHD disease gene(s) have not been identified. We hypothesize that physical interactions bring the 3.3-kb repeats near the FSHD gene(s) and thus influence gene expression. Our goal is to map genomic loci that come in physical contact with the D4Z4 repeats. Our approach is to create fusion proteins that contain (1) a zinc finger DNA binding domain designed to bind to the D4Z4 repeats, and (2) bacterial adenine methyltransferase. Mammalian cells do not normally contain adenine methylation. By tethering the adenine methyltransferase to the D4Z4 repeats in this manner we hope to identify distant sites that are not bound the fusion protein but are nonetheless adenine-methylated due to physical looping interactions that bring the fusion protein tethered to the D4Z4 repeats in contact with these sites.

To this end, we mapped the positions of the nucleosomes in the D4Z4 repeats on chromosome 4 and found that the nucleosomes occupied precise positions. We then turned to Sangamo Biosciences, a company that designs proteins to bind specific DNA sequences. Sangamo possesses a library of clones for zinc fingers that bind DNA. Each zinc finger binds a different 3-bp sequence and combinations of 3 or 4 zinc fingers can be used to recognize specific sequences in the genome. However, these zinc finger proteins tend to bind much better to target sequences that are in between nucleosomes than within nucleosomes. We provided Sangamo with the DNA sequences that are in between the phased nucleosomes in the D4Z4 repeats on chromosome 4 and obtained clones for two different zinc finger proteins. The ZFPs each recognize a 12 bp target and one makes specific contact with the chromosome 4-specific residues. We constructed chimeric genes that encode an adenine methylase-Zinc Finger fusion protein whose expression can be induced using the steroid hormone ecdysone analog ponasterone A and made several transgenic cell lines that express the fusion proteins. We determined that the adenine methyltransferase is functional in these cell lines and are currently using chromatin immuno-precipitation to determine whether the fusion proteins bind the D4Z4 repeats in vivo.

14. Identification of the Mechanism Regulating the Wnt-Dependent Activation of Muscle Progenitor Cells.

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Recently, our group showed that an adult stem cell population in muscle is capable of undergoing myogenic differentiation in response to canonical Wnt signaling pathway. This signaling pathway results in the transcription of genes that contain binding domains for members of the LEF/TCF family of transcription factors. Our group has also previously determined that the paired box transcription factor Pax7 is required for the specification of myogenic satellite cells in adult skeletal muscle. The expression of Pax7 is activated in response to Wnts during muscle regeneration. However, the target genes that function downstream of Wnt signaling to induce expression of Pax7 in adult myogenic stem cells have not been identified. In addition, analysis of Pax7 gene sequencing reveals putative LEF/TCFs binding sites within introns and upstream the coding sequence. We are exploring the hypothesis that Pax7 is the direct target of Wnt signaling that directs the myogenic specification of adult stem cells.

Members of the LEF/TCF family of transcription factors present in muscle progenitor cells were identified. We found that LEF-1 and TCF-1 are expressed in primary myoblasts. In addition, we also found significant levels of β -catenin in the nucleus of primary myoblasts, indicating activation of Wnt pathway. Our data together indicate that the Wnt signaling pathway is active in primary myoblast; and LEF-1 and TCF-1 are the transcription factor that work as a switcher in the regulation of this pathway in primary myoblasts.

Currently, we are proceeding to find the minimal myoblast elements in the Pax7 promoter. To do this mapping, truncation constructions encompassing 5Kb, 10 Kb sequences upstream the initiation site of Pax7 and also 10kb + first intron were made. These genomic sequences were retrieved and cloned into luciferase reporter plasmids using recombining technology. These constructions are being accessed for luciferase activity as a measure of promoter strength. Once the promoter region of Pax7 is defined, we will use primary myoblasts for ChIP analysis of bona-fide TCF-1 and LEF-1 binding sites.

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15. A Retrospective One Year Audit Study of FSHD Testing in Bristol, United Kingdom.

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The Bristol regional genetics laboratory is one of three U.K laboratories offering FSHD tests as part of the United Kingdom Genetic Testing Network (UKGTN). The current service supports diagnostic, exclusion, predictive and prenatal testing. Since 2000 the laboratory has handled over 1600 cases (averaging 320 cases/year), forwarded to us through local clinicians or other UKGTN laboratories. Patient referrals come from several clinical specialities including geneticists, neurologists and paediatricians.

The tests we currently offer as a routine diagnostic series are based on linear gel electrophoresis, run as a triple digest (EcoRI and EcoRI/BlnI and XapI) to confirm or exclude the presence of short 4q fragments <35kb, supported by BglII/BlnI dosage and linkage as appropriate.

Patients with deletions of P13E-11 probe region/locus can appear as false negatives with this first level of service. In addition there is lack of clarity for patients with more than one short fragment. To address this we are introducing 4qA/4qB polymorphism testing, but aim to target this appropriately.

We therefore have undertaken a retrospective one year audit of referrals classified according to the information provided by the requesting clinicians. We have classified the patients into five distinct groups (A-E): Group A –referring clinicians diagnosis as FSHD; Group B - clinical information compatible with FSHD but insufficient for certainty; Group C- clinically unlikely FSHD, exclusion test; Group D -patients with family history for predictive tests (including asymptomatics); Group E- no clinical information supplied.

There are 74 patients from Group A, 42 Group B and 42 from Group C.

Fragments < 35kb were present in	47%,	24%,	19%	of Group A, B, C, respectively.
Fragments >35kb were present in	16%,	7%,	7%	of Group A, B, C, respectively.
Sensitive fragments <35kb were in	8%,	14%,	7%	of Group A, B, C, respectively.
No fragment<48kb was seen in	29%,	55%,	67%	of Group A, B, C, respectively.

We propose to offer second order testing (i.e. 4qA/4qB tests) to negative patients (37%) in group A and positive patients (19%) in Group C. For group B in particular we feel that the data presented here highlights the need for a proforma for clinical data collection to enable appropriate gate keeping in such a labour intensive diagnostic test.

16. Molecular Analysis of FSHD: a) Further p13E11 (D4F104S1) Deletions; b) Indication for the Presence of Third Allele Variant Distal to D4Z4.

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Extended deletion that includes both the more proximal D4F104S1 region and D4Z4 array have been reported in 4 FSHD subjects. It is important to determine the frequency of such deletions in order to improve the interpretation of diagnostic results for FSHD. Previous studies suggest that the region distal to the D4Z4 repeat array on chromosome 4 is polymorphic. Two allelic variants 4qA and 4qB have been identified that differ by the presence of a large beta-satellite repeat (6kb) in the 4qA allele. Both the alleles are equally common in the population. FSHD-associated D4Z4 repeat contractions appear to be associated with 4qA allele.

We have studied DNA from 116 patients referred to our centre for FSHD analysis, 97 of these individuals had EcoRI/BlnI fragments smaller than 38kb using probe p13E11. DNA from the remaining 19 patients without a small EcoRI/BlnI fragment was digested with Hind III, the resulting fragment encompasses p13E11, D4Z4 repeat array, 4qA and 4qB sequences. Hind III Southern blot was hybridised with probe A. In two DNA samples, a small 20 kb Hind III fragment was detected. Sequential analysis of this Hind III Southern blot with probe p13E11 failed to reveal the small fragment, confirming the deletion of the p13E11 region in the disease chromosome. The size of the deletions and their chromosomal origin are currently being defined.

Of the 97 DNA samples analysed with probes A and B which detect alleles 4qA and 4qB, two samples with FSHD associated small EcoRI/BlnI fragments failed to generate a hybridisation signal with either probe A or B, suggesting the presence of a third allele. D4Z4 repeat array length with Hind III is 6kb larger than with EcoRI. Study of Hind III Southern blots has allowed us to detect size variability in 4qA alleles.

P13E11 Eco RI alleles of identical size have yielded variable size Hind III alleles when hybridised with probe A, further suggesting the presence of a potential polymorphic site in the beta satellite repeat region.

In summary, FSHD patients with EcoRI/BlnI fragment size larger than 38kb should be tested for p13E11 deletions. Our results suggest the presence of additional rare allelic variants of distal polymorphism. Identification of these rare variants may help in better defining the molecular pathology of FSHD.

17. Studies on the Expression of the DUX4 and DUX4c Proteins.

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The DUX4 double homeobox gene maps within each of the D4Z4 units repeated in tandem in the 4q35 chromosome region. The homologous DUX4c gene is located in a single truncated and inverted D4Z4 element 42 kb centromeric of the D4Z4 array. The encoded DUX4 and DUX4c proteins are 424- and 374- residue long, respectively, and are identical over their first 342-residues including the double homeodomain. In the carboxyl-terminal domain, the last 32 residues of DUX4c differ in 13 positions from DUX4 that presents in addition a unique 50-residue end.

The DUX4 carboxyl-terminal domain outside of the homeodomains (“DUX4 tail”, 297 residues) was expressed as a His-tag fusion in *E. coli* and purified by affinity chromatography on Zn⁺⁺-agarose beads. Mice were immunized with this protein and a hybridoma producing a specific monoclonal antibody (MAb) was selected for reactivity of its culture medium in ELISA on plates coated with the DUX4 tail, but not with another His-tagged protein. This MAb was shown to react specifically with DUX4 and DUX4c by immunoprecipitation of radioactive proteins expressed by transcription/translation *in vitro*, western blotting performed on extracts of TE671 cells transfected with different pCIneo-DUX expression vectors, and immunofluorescence on transfected cells. In a preliminary experiment, a Western blot was performed with this MAb on protein extracts of human myoblast lines and detected two proteins with the expected sizes of DUX4 (52 kDa) and DUX4c (47 kDa) in two FSHD lines, but not in one control. Additional samples are being evaluated.

Because DUX4 induces cell death upon over-expression in rhabdomyosarcoma TE671 cells grown *in vitro*, we reasoned that its expression in patients might similarly lead to cell death. The DUX4 protein present in the dead cells might thus be exposed to the patient immune system and trigger production of specific antibodies. We coated ELISA plates with the recombinant “DUX4 tail” protein (see above) to evaluate the presence of anti DUX4/4c antibodies in sera of patients: an OD value about 2-fold higher than 3 controls was found for 6 out of 10 FSHD samples. In one sample of a patient with infantile FSHD, the ELISA signal was 3.5-fold higher than for his unaffected parents and a control. This observation suggests that these patients have been exposed to either DUX4 and/or DUX4c. Additional samples will be analyzed, and for the most reactive ones, immuno-precipitation and western blot studies should define which antigen these sera recognize.

In conclusion these data underscore our hypothesis that the DUX4 and DUX4c proteins are expressed in patients with FSHD.

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18. D4Z4 DNA Methylation Studies in FSHD.

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Facioscapulohumeral muscular dystrophy (FSHD) is associated with contraction of the polymorphic D4Z4 repeat array on chromosome 4qter. In healthy individuals, D4Z4 consists of 11-100 units on both chromosomes, whereas individuals with FSHD carry one 4q array of 1-10 units. Recently, we demonstrated that this contraction is associated with significant hypomethylation of the shortened D4Z4 allele. In 'phenotypic FSHD' in which patients do not have a contraction of D4Z4 on chromosome 4q, both their D4Z4 alleles show hypomethylation. These findings suggest a central role of D4Z4 hypomethylation in the pathogenetic pathway of FSHD.

Although FSHD alleles are significantly hypomethylated compared to control alleles as a group, there is a large inter-individual variation of methylation at D4Z4. Therefore, we studied whether there are potential relationships between D4Z4 hypomethylation and both residual repeat size and clinical severity in unrelated FSHD patients.

Our study showed that correcting the clinical severity score for age at examination improves the parameter to define clinical severity and provides further support for hypomethylation of FSHD alleles. A linear relationship between repeat size and clinical severity of the disease could not be established. However, patients with residual repeat sizes of 10-20kb that are severely affected show severe D4Z4 hypomethylation. In contrast, patients with repeat sizes of 20 to 31kb show large interindividual variation in clinical severity and D4Z4 hypomethylation.

As DNA methylation and demethylation are reversible processes, DNA methylation levels and patterns can potentially be influenced. Folic acid and vitamin B12 are essential for the synthesis of methionine and S-adenosyl methionine (SAM), the common methyl donor required for the maintenance of DNA methylation. Intervention studies in humans taking folic acid and/or vitamin B12 supplements show that DNA hypomethylation, chromosome breaks, and uracil misincorporation are minimized when serum concentration of folate is higher than 34 nmol/l, and serum vitamin B12 concentration is higher than 300 pmol/l.

We therefore performed a pilot study to evaluate the effect of supplemental folic acid and methionine on the methylation level of D4Z4 alleles on chromosome 4qter in peripheral blood lymphocytes of FSHD patients (both 4q and non-4q linked) and in healthy controls in order to decide if a larger clinical trial is warranted. Preliminary results of this study will be discussed.

19. HP1/Cohesin-containing Heterochromatin Structure at Chromosome 4q is Disrupted in FSHD.

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Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant hereditary neuromuscular disorder usually resulting from reduction of the D4Z4 repeat array in the subtelomeric region of chromosome 4q (4qter). Although the mechanism of the disease is still not completely understood, accumulating evidence suggests that FSHD is caused by abnormal chromatin structure resulting in deregulation of gene expression. In normal cells, D4Z4 is thought to be heterochromatic accompanied by hypermethylation of DNA. In FSHD, the inability of the shortened D4Z4 to form its specialized chromatin structure likely leads to deregulation of expression of genes critical for disease pathogenicity.

To further understand the nature of the chromatin structure in this region, it is vital to identify proteins that function through binding to the D4Z4 repeat. We found using chromatin cross-linking and immunoprecipitation (ChIP) analysis that a protein complex essential for higher-order chromatin organization, termed “cohesin”, specifically binds to the D4Z4 repeat in primary human muscle cells. Cohesin is critical for genome integrity and is responsible for sister chromatid pairing, which is prerequisite to faithful chromosome segregation during mitosis and homologous recombination repair during interphase. Genetic studies also suggest cohesin’s role in transcriptional regulation although the underlying mechanisms are unclear. We also found that the heterochromatin binding protein HP1 binds to a site adjacent to the cohesin-binding site within D4Z4. Since HP1 recruits cohesin to heterochromatin in *S. pombe*, it is possible that HP1 targets cohesin to D4Z4. Intriguingly, cohesin and HP1 binding was lost in both 4q-linked and “phenotypic” FSHD, a less frequent form of FSHD with no D4Z4 repeat contraction.

Our results suggest that cohesin and HP1 are involved in chromatin structure organization at the D4Z4 repeat region, and that loss of cohesin and HP1 binding at 4qter D4Z4 plays a critical role in the pathogenesis of FSHD.

20. FSHD and Retinal Vascular Disease. A Signal from Wnt?

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FSHD is associated with retinal vascular abnormalities (telangiectasis), including peripheral capillary dropout and abnormal leakage. In the vast majority of cases these findings are subclinical, but occasionally exudative retinal detachment and even (usually unilateral) blindness results. The clinical picture and vascular changes are indistinguishable from those which otherwise occur in 'Coats Disease', usually a unilateral disease of young boys. FSHD can also be associated with subclinical sensorineural hearing impairment or partial deafness, which on the basis of neurophysiological studies is assumed to be cochlear.

Might these associated phenomena give clues as to FSHD pathogenesis?

Although most cases of Coats' Disease are of unknown aetiology, some cases are due to mutation of 'norrin' – formerly thought to be a member of the TGF-beta family of signalling proteins, but now known to be an extracellular ligand for wnt signalling. 'Norrin' mutations classically cause 'Norrie Disease', an X-linked and much more severe congenital retinal vascular disorder, with fibrotic pseudogliomas and failure of peripheral retinal vascularization. Norrie Disease used to be considered quite distinct from Coats' Disease, and is associated with cochlear hearing impairment, and additional features.

X-linked Familial Exudative Vitreoretinopathy (FEVR) is yet another retinal disease, of supposedly separate classification but with a similar retinal picture, which can be caused by norrin mutation. Other families with FEVR, with different (incl dominant) modes of inheritance are reported with mutations affecting 'frizzled-4' (fz-4) protein or lipoprotein receptor related protein 5/6 (LRP5/6)- which are respectively trans-membrane receptor and co-receptor for norrin. Norrin/fz-4 signalling also determines vascular development in the inner ear, as well as retina.

Wnt signalling causes activation of selected nuclear transcription factors via very complex canonical (beta-catenin) or non-canonical pathways leading to modification of chromatin. Wnt pathways are important in embryonic myogenesis (although apparently not in normal muscle regeneration, Zhao and Hoffman 2004). They are important in muscle/somite patterning and polarities; FSHD is extraordinarily muscle-pattern specific and often highly asymmetrical.

Could an abnormality of wnt signaling early in myogenesis affect both the functional viability of selected satellite/stem cells in mature muscle, and also the vascular development of retina and cochlea?

