

In some FSHD1 patient with quite severe clinical phenotype, FSHD1 contracted permissive allele has been found in combination with *SMCHD1* pathogenic mutation (FSHD1+FSHD2 patients). Interestingly these patients carry a borderline repeat of 8-10 RU. On the other hand, most of the FSHD2 patients identified up to now carry a relatively low number of repeat raising the possibility of a *continuum* between these two diseases that may represent a confounding issue in genetic diagnosis and counseling and need to be better clarified. This possibility may have important consequences in understanding FSHD physiopathology and in developing future therapeutic strategies.

Epigenetic derepression of DUX4 in FSHD

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Facioscapulohumeral dystrophy (FSHD) is one of the most common types of muscular dystrophy typically involving the muscles of the face and upper extremities. With disease progression also other muscles may become affected. In most cases, this autosomal dominantly inherited muscle disorder can be molecularly recognised by discrete chromatin changes of the D4Z4 macrosatellite repeat array on chromosome 4 in somatic cells. The polymorphic D4Z4 repeat array normally varies between 8-100 units, and adopts a repressive chromatin structure in somatic cells. Because of repeat array contractions to a size of 1-10 units (FSHD1), or mutations in the chromatin repressor SMCHD1 (FSHD2), this epigenetic silencing is incomplete leading to aberrant expression of the DUX4 retrogene in skeletal muscle of patients.

The DUX4 retrogene is encoded within each unit of the D4Z4 repeat array but lacks a stabilizing polyadenylation signal. Therefore, only in combination with a disease-permissive genetic background of chromosome 4, which contains a polymorphic DUX4 polyadenylation signal, these chromatin changes lead to aberrant expression of DUX4 protein in skeletal muscle. DUX4 is a double homeobox transcription factor normally expressed in the germline and its ectopic expression results in activation of several muscle damaging pathways.

FSHD is characterized by a marked inter- and intra-familial variability in disease onset and progression. For a long time, the molecular mechanisms underlying this phenomenon were largely unknown, but recent studies indicate that a combination of genetic and epigenetic factors that act on the D4Z4 repeat array determine the probability of DUX4 expression in skeletal muscle. This possibly explains the extensive clinical variation in disease onset and progression, and the frequent presence of borderline FSHD repeat arrays in the control population. In this regard, FSHD1 and FSHD2 should not be considered separate disease entities, but rather opposite extremes of a disease continuum.

Phenotypic and molecular characterization of FSHD families: a systematic approach towards trial readiness

Rossella Tupler

Facioscapulohumeral muscular dystrophy (FSHD) is characterized by vast clinical variability. The majority of FSHD patients, termed FSHD1, carry a reduced number of D4Z4 repetitive elements on chromosome 4q. There are also FSHD patients, termed FSHD2, who carry mutations in the *SMCHD1* gene but have D4Z4 alleles of normal size. Both FSHD1 and FSHD2 patients carry D4Z4 alleles associated with the 4APAS haplotype, which is considered permissive for FSHD and present D4Z4 hypomethylation, suggesting a common pathogenic mechanism altering chromatin structure at D4Z4. However, 1.3% of healthy subjects carry a D4Z4 reduced allele (DRA), associated with the 1614APAS haplotype arguing for the role of additional elements in FSHD onset and progression.

This idea is supported by clinical data, in which we have used the power of very large patient cohorts from the Italian National Registry for FSHD, combining detailed phenotypic analyses with molecular characterization. For these analyses we designed a clinical evaluation form that measures the grade of motor impairment in FSHD and describes the diverse clinical phenotypes observed in FSHD families. I will discuss our analyses of 530 subjects from 176 families, all carriers of a DRA that showed that the genetic background influences the disease development and that additional elements influence disease progression. I will also discuss the study of 68 index cases carrying a DRA with 1-3 repeats that showed the size of D4Z4 allele is not always predictive of the clinical outcome, and that additional factors contribute to the phenotype complexity. We also analyzed the D4Z4 methylation status on FSHD families, healthy controls, affected subjects by other neuromuscular diseases and tested for the presence of SMCHD1 variants. Our study revealed that D4Z4 methylation does not strictly correlate with the presence and severity of a FSHD phenotype as we detected both D4Z4 hypomethylation ($\leq 25\%$) and normal levels of methylation ($\geq 35\%$), in all analyzed subgroups. We also found FSHD patients showing D4Z4 hypomethylation and wild-type SMCHD1 sequences, suggesting the contribution of additional unidentified elements.

Overall, systematic studies of large cohorts of FSHD families suggest that a complex genetic and epigenetic network is altered in FSHD as highlighted by genotype-phenotype studies and inter- and intra-familial clinical variability. We propose standardized tools to study FSHD families on the basis of clinical phenotypes and follow diversified approaches for the interpretation of molecular results to be used in clinical practice. This approach will have important clinical implications with particular regard to genetic counseling and clinical trial readiness. It will also foster the dissection of genetics and epigenetic mechanisms involved in developing FSHD.

Plenary Session- Advances in Myology

• Helge Amthor (FRANCE) • H. Lee SWEENEY (USA) • Ana Buj-Bello (FRANCE)

The role of TGF- β signaling factors in skeletal muscle plasticity

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Signaling molecules of the Transforming Growth Factor- β (TGF- β) family, such as Bone Morphogenetic Proteins (BMPs) and Myostatin, are main regulators of skeletal muscle growth. BMP signaling induces muscle hypertrophy, whereas Myostatin signaling causes muscle atrophy. Lack of Myostatin stimulates fiber growth, however, this entirely depends on the presence of BMP signaling. Furthermore, BMP signaling is required for satellite cell dependent muscle growth and for the generation of the adult muscle satellite cell pool.

Importantly, Myostatin, BMPs and TGF- β s regulate muscle plasticity in response to diverse physiological and pathophysiological stimuli and three examples will be presented.

Example 1: BMP signaling is upregulated following denervation and this antagonizes denervation induced muscle atrophy. Abrogation of BMP signaling in denervated muscle results in exacerbated muscle atrophy and this cannot be reversed by additional suppression of Myostatin signaling.

Example 2: Myostatin endows skeletal muscle with high oxidative properties and in absence of its signaling, muscle fibers convert towards a glycolytic metabolism, which grossly diminishes the capacity for aerobic exercise. This is associated with a loss of muscle capillaries. Notably, dystrophic muscle requires Myostatin signaling for maintaining oxidative properties. Blockade of Myostatin signaling in *Mdx* mice, despite causing larger muscle, results in extreme fatigability and in a secondary metabolic myopathy. Interestingly, endurance exercise can partly overcome the metabolic deficiency due to the absence of Myostatin, however, muscle size is reduced and changes towards a wildtype phenotype.

Example 3: Myostatin and TGF- β signaling are largely required for correct adaption during muscle overload (model for resistance exercise). In absence of Myostatin, muscle fails to convert towards an oxidative phenotype. Whereas wildtype muscle strongly upregulates TGF- β signaling and increases connective tissue following overload, this response is reduced in lack of Myostatin. Moreover, lack of Myostatin yields no advantage for overload-induced force increase compared to the wildtype situation. Treatment with halofuginone, an inhibitor of the TGF- β signaling pathway, strongly mitigates the effect of overload on muscle force, muscle mass and connective tissue and this is independent of Myostatin signaling.

In summary, TGF- β signaling guides multiple cellular processes in skeletal muscle including muscle growth, metabolic phenotype, force and exercise properties, muscle connective tissue and vascularization.

Therapeutic Targets for Duchenne Muscular Dystrophy: Moving towards combinatorial therapy

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Duchenne muscular dystrophy (DMD) is caused by loss of the force transmitting and membrane complex organizing protein, dystrophin, and is characterized by progressive muscle deterioration with failed regeneration and replacement with a fatty-fibrous matrix. Dystrophin replacement therapies to date have shown only limited ability to slow the disease process, and thus therapeutics targeting other aspects of the disease, which can be used in combination, are needed. We are examining a number of potential targets, representing five aspects of the pathological cascade: (1) Decreasing inflammation and fibrosis; (2) Increasing muscle mass and regeneration; (3) Correcting blood flow regulation; (4) Correcting perturbations in calcium handling; (5) Mitochondria dysfunction. For inflammation, a major potential target is nuclear factor κ B (NF κ B), which is increased in the DMD muscles. We examined a novel class of NF- κ B inhibitors in *mdx* mouse and golden retriever muscular dystrophy (GRMD) dog models of DMD. These orally bioavailable compounds improved the phenotype of voluntarily run *mdx* mice, in terms of amount of activity, muscle mass and function, inflammation, and fibrosis. Surprisingly, the muscles were also more resistant to contraction-induced damage, which we demonstrated was significant increases in dysferlin, a protein required for membrane damage repair. We also evaluated the cardiac impact of a phosphodiesterase 5 (PDE5) inhibitor, tadalafil, which has been shown to improve blood flow in exercising skeletal muscles in *mdx* mice and human DMD patients. Cardiomyopathy is a leading cause of mortality among DMD patients and is well modeled by the golden retriever muscular dystrophy (GRMD) dog model of DMD. Prophylactic use of the PDE5 inhibitor, tadalafil, improved GRMD histopathological features of the hearts, decreased levels of the pathogenic cation channel TRPC6, increased phosphorylation of TRPC6, decreased m-calpain levels and indicators of calpain target proteolysis, and elevated levels of the dystrophin ortholog, utrophin. The progressive loss of cardiac function was significantly slowed by these effects. These data demonstrate that prophylactic use of tadalafil delays the onset of dystrophic cardiomyopathy in a severe animal model of DMD. The benefit is likely attributed to modulation of TRPC6 levels and permeability and inhibition of protease content and activity, which results in higher levels of the protective protein, utrophin. Thus PDE5 inhibition and NF- κ B inhibition are potential therapeutics to consider in developing a combinatorial approach to the treatment of DMD.

Gene replacement therapy for myotubular myopathy.

Ana Buj-Bello

Loss-of-function mutations in the myotubularin gene (*MTM1*) result in X-linked myotubular myopathy (XLMTM), a fatal pediatric disease of skeletal muscle characterized by small centrally nucleated myofibers containing abnormal mitochondrial accumulations. Patients typically present with severe hypotonia and respiratory failure. We have performed gene replacement studies in mouse and dog models of the disease and shown that intravenous administration of a single dose of a recombinant serotype 8 adeno-associated virus (AAV8) vector expressing myotubularin under the muscle-specific desmin promoter restores muscle function, corrects pathology and leads to long-term survival in the absence of a humoral or cell-mediated immune response against *MTM1*. These results demonstrate the therapeutic efficacy of AAV-mediated gene therapy for myotubular myopathy in small- and large-animal models, and provide proof of concept for a clinical trial in XLMTM patients.