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Continuing to make progress in understanding and treating FSHD

Grant awards for February 2016 cycle

Since 1998, the FSH Society has transformed FSHD research by providing grants for vital start-up funding for investigators in FSHD and research projects on FSHD. The FSH Society has two rounds of grant applications each year, with deadlines in February and August. Grant applications are thoroughly analyzed and vetted by the SAB. An initial letter of intent is submitted, which is reviewed by Professor David Housman, Chair of the SAB. If a letter of intent is accepted, the applicant submits a full application. The main section where researchers describe the proposed work and workflow is around 12 pages long.

Upon receipt of all full grant applications for a particular round, Professor Housman assigns teams of two or more members of the SAB to critique each proposal. Any potential conflicts of interests are noted, and SAB members who may have a conflict are not assigned to review, and do not vote on, the particular proposal. The two reviewers review the application in depth and provide a detailed written description and recommendation to the other members. Initial critiques are due within three weeks of the assignment and a full meeting of the SAB is held around two weeks thereafter. Grant applications are reviewed and voted upon by the entire SAB, with discussion led by the two primary reviewers. SAB recommendations for approved applications are then sent to the Society's Board of Directors for a vote. When the SAB disapproves an application, it provides the applicant with a detailed description of the reasons for disapproval, and the applicant may resubmit the application for consideration in a later round. SAB members and the chair serve without pay.

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

The milestones and insights gained are significant. The fellowship program allows innovative and entrepreneurial research to develop, prove successful, and ultimately to attract funding from large funding sources such as the US National Institutes of Health (NIH) and large private sources. We are very pleased to list the grantees funded in the February 2016 cycle.

Awards from February 2016 cycle

The SAB reviewed grant applications and progress reports for the February 2016 round on June 28 and July 5, 2016. The SAB had two meetings (sub-reviews) under Prof. Housman's chairmanship and on July 8, 2016, the SAB reached a final ranking and majority consensus. On July 29, 2016, the FSH Society Board of Directors reviewed and approved the SAB recommendations for funding. Below is a list of the funded projects, including project descriptions as submitted by the grant applicants. For the February 2016 round of grant applications nine grant applications were received; five were funded in the amount of \$648,774.

1. Sujatha Jagannathan (Robert Bradley and Stephen Tapscott, mentors). Fred Hutchinson Cancer Research Center, Seattle, Washington. Novel role for reduced RNA quality control in FSHD pathogenesis. \$ 59,225 for 1 year.

Specific Aims: Facioscapulohumeral muscular dystrophy (FSHD) is caused by the misexpression of the germline transcription factor DUX4 in muscle cells. Several mechanisms have been proposed to explain DUX4-induced myotoxicity, including activation of apoptotic pathways, perturbed proteostasis and protein aggregation, among others (Wallace et al., 2011; Wallace et al., 2012; Tassin et al., 2013; Homma et al., 2015). We recently observed profound inhibition of an essential RNA quality control mechanism – nonsense-mediated RNA decay (NMD) – following DUX4 expression that could potentially account for several aspects of FSHD biology (Feng et al., 2015). Temporal analysis of DUX4 expression in human myoblasts revealed proteolytic degradation of core NMD factors upon DUX4 expression and concomitant inhibition of NMD. Following NMD inhibition, the DUX4-expressing cells upregulated various protein folding stress response pathways, leading us to hypothesize that DUX4-induced NMD inhibition could allow synthesis of aberrant protein products and cause proteotoxicity and cell death. In support of this hypothesis, pilot quantitative mass spectrometry studies detected truncated proteins as well as a small number of novel peptides derived from NMD targets in the DUX4-expressing cells. To rigorously test whether NMD inhibition drives proteotoxicity and contributes to DUX4-induced cell death, we sought to identify the mechanism of NMD inhibition by DUX4 (Specific Aim 1) and to determine the contribution of inefficient NMD to DUX4 toxicity (Specific Aim 2).

2. Peter L. Jones/ Robert Bloch. University of Massachusetts Medical School, Worcester. CRISPR approaches targeting DUX4 in vivo. \$ 240,014 for 2 years.

Specific Aims: We recently published the first, and still only, report utilizing the CRISPR/Cas system for reducing or eliminating DUX4-fl expression as an avenue to an FSHD treatment [Himeda et al. "CRISPR/dCas9-mediated transcriptional inhibition ameliorates the epigenetic dysregulation at D4Z4 and represses DUX4-fl in FSH muscular dystrophy. 2015. Molecular Therapy, (In Press)]. Importantly, in this work we showed that it is in fact feasible to design sgRNAs that target Cas9 to the 4q35 D4Z4 in primary human myogenic cells. The next phase is to move to an in vivo system and ask if we can target the D4Z4/DUX4 in an animal model; however, due to off target concerns, it is also vital to be targeting mature human muscle fibers. Fortunately, we have been collaborating with Dr. Bob Bloch and his colleagues at the University of Maryland School of Medicine on their humanized mouse models for FSHD. Recently, they

published the first study on these mice and show that their technique produces spectacular engraftment and development of mature human muscle fibers, with minimal contamination by mouse myonuclei [Sakellariou et al. “Neuromuscular electrical stimulation promotes development in mice of mature human muscle from immortalized human myoblasts” 2016. Skeletal Muscle (In Press)]. Thus, the timing works out perfectly for us to continue our collaboration and merge our CRISPR/Cas studies with their mouse model. In this collaborative grant the Jones lab at UMMS will develop new sgRNAs targeting the D4Z4/DUX4 region that are compatible with the smaller Cas9s which fit into AAV. The Bloch lab at UMSOM will generate humanized FSHD mice, inject these CRISPR AAVs, and assess their effects on muscle physiology and morphology. The Jones lab will then analyze the expression of DUX4, downstream targets, and off targets. This project will test if AAV-mediated CRISPR technology is indeed a viable approach to treating FSHD.

3. Eugénie Anseau (Frédérique Coppée and Alexandra Belayew, mentors). University of Mons, Belgium. Study of the unexpected cytoplasmic functions of double homeodomain proteins DUX4 and DUX4c during differentiation: focus on healthy and pathological muscle cells. \$ 124,807 for 1 year.

Specific Aims: The DUX proteins are known as transcription factors and until now functional studies from several laboratories only focused on that perspective. Intriguingly, the transcriptional target genes of DUX4 identified to this date cannot explain the cytoplasmic alterations observed in FSHD muscle sections. In a search for DUX4/4c protein partners we have unexpectedly identified several classes of cytoplasmic or nucleo-cytoplasmic proteins. The functionality of these interactions was underscored by the observation of DUX4/4c cytoplasmic localization upon myoblast fusion. Moreover, our recently published (Anseau et al 2016) and new preliminary data indicate that DUX proteins are associated to specific IGF2BP1-dependent ribonucleoparticles (mRNP). In these granules, IGF2BP1 is associated a.o. with ACTB mRNA and regulates the synthesis of non-muscle cytoskeletal actins that is essential in the first steps of myotube formation (elongation, fusion, nuclear dynamics). Later these actins will be replaced by the muscle forms in myofibrils. The DUX4 and DUX4c inductions observed in FSHD muscle cells could therefore interfere with this precise dynamics and contribute to the muscle pathology. Moreover, as DUX4c is normally expressed in almost all myoblasts and as many identified partners are identical for DUX4 and DUX4c, the pathological increase of DUX4/DUX4c proteins in FSHD muscle cells could titrate out some partners and interfere with the normal DUX4c function in muscle and would contribute to explain why this tissue is particularly sensitive to pathological DUX4 expression (one of the FSH Society research priorities for 2016).

In this project which is with the follow up of our on-going FSH Society grant (see Annex for a progress report) we therefore want to define the DUX4/4c functions in the cytoplasm with the following specific aims :

- (1) to monitor DUX4/4c trafficking, cytoskeletal dynamics and nuclear movements during muscle cell differentiation in culture,
- (2) to identify the RNAs associated with DUX4/4c
- (3) to study the impact of DUX4/4c gain or loss of function on IGF2BP1-associated mRNP granules

(4) to determine the pathological interactions caused by excess DUX4 or DUX4c in muscle cell cultures and in a FSHD mouse model in collaboration with the group of Yi-Wen Chen (George Washington University).

The identification of the DUX4/4c peptidic domains involved in the interactions with partners will later be used to select either peptides (using a phage display library), or aptamers that suppress or decrease the interaction with partners involved in toxic pathways. The biological significance of the DUX4/4c-partner interaction will then be evaluated by introducing these agents in FSHD muscle cell cultures and analysing the resulting phenotype. This approach might present a therapeutic interest in FSHD by blocking some DUX4/DUX4c toxic functions.

We expect this project will help (i) to define new functions for DUX4 and the poorly studied DUX4c, (ii) to discover their putative interactions through shared partners (iii) to bring new light on the mechanisms of DUX4 toxicity in FSHD muscle and (iv) to propose new therapeutic strategies preventing protein/protein or protein/RNA interactions.

4. Amber Mueller (Robert Bloch, mentor). University of Maryland, Baltimore. Assessing the pathologic role of DUX4 in a humanized mouse model of FSHD. \$ 134,728 for 3 years.

Specific Aims: Facioscapulohumeral Muscular Dystrophy (FSHD) is a muscle wasting disease caused by a genetic mutation in the subtelomeric region of chromosome 4. Healthy individuals have 11-100 repeated D4Z4 microsatellite units, whereas individuals with FSHD have 1-10 D4Z4 units. This genetic contraction makes the 4q35 chromosomal region more accessible to transcriptional machinery and permits transcription of nearby genes including DUX4, the proposed pathological agent in FSHD. DUX4 is a transcription factor that when aberrantly expressed induces transcription of many genes implicated in muscle development, the actin cytoskeleton, regulation, apoptotic signaling pathways, and germline development. While many potential biomarkers of FSHD, including TRIM43, MBD3L2, ZSCAN4, and PITX1, all of which are downstream of DUX4, have been identified from studies of muscle biopsy and of muscle cells in vitro, the pathophysiological role of DUX4 and these target genes has yet to be studied in a humanized in vivo model. The D4Z4 contraction is not replicable in animal models, nor do the effects of DUX4 expression in murine muscle cells replicate those seen in human cells. To overcome these problems, the laboratory has developed a method of xenografting human derived muscle precursor cells, isolated from patients with FSHD and healthy controls, into the tibialis anterior compartment of immune-deficient mice. These cells grow, fuse, and mature into force-producing human muscle fibers within the mouse hind limb. These human muscle fibers can be studied in intact grafts or following dissociation and isolation in culture. This novel in vivo FSHD model will be used to study DUX4 and its potential downstream gene targets, TRIM43, MBD3L2, ZSCAN4, and PITX1. The ultimate goal is to determine how their patterns of expression and localization in relation to DUX4 within individual fibers lead to dystrophy - experiments that have never been performed in an FSHD model system before. Further analyses will attempt to reveal the primary defect in FSHD muscle, leading to the creation of targeted FSHD therapies. This study hypothesizes that DUX4 expression in a small percentage of the nuclei of mature human muscle fibers is responsible for activating DUX4 gene targets in the same or nearby myonuclei and that together these induce local muscle wasting, leading to the phenotype seen in FSHD. The hypothesis will be tested by i) comparing the molecular phenotype of intact muscle as well as fibers isolated and cultured from FSHD-derived xenografts to controls and by ii) examining the functional phenotype of FSHD derived

xenografts compared to controls. Molecular methods such as qRT-PCR, immunofluorescent labeling, and confocal microscopy combined with electrophysiology-based functional measures will be employed to explore the phenotype in living muscle from FSHD xenografts. This study overcomes the limitations of current models of FSHD associated with viral vectors or incomplete development in culture, as it examines the pathologic consequences of endogenous DUX4 expression and its downstream targets in mature human muscle fibers grown within the mouse hind limb.

5. Nizar Saad (Scott Q. Harper, mentor). Nationwide Children's Hospital, Columbus, Ohio. MicroRNAs as potential modifiers of Facioscapulohumeral Muscular Dystrophy (FSHD). \$ 90,000 for 1 year.

Specific Aims: My doctoral and postdoctoral studies aimed at investigating the implication of non-coding RNAs in the regulation of gene expression in bacteria. Therefore, I am quite familiar with gene expression processes as well as their implication in the control of cell homeostasis. One of my objectives for joining Dr. Harper's lab is my interest in understanding dysregulation processes of gene expression in human diseases (e.g., FSHD). In fact, FSHD arises from complicated pathogenic mechanisms, with epigenetic origins, that ultimately cause overexpression of the transcription factor DUX4, which is toxic to muscle and numerous non-muscle cell types. FSHD presentation is non-uniform, and there may be extreme variability in severity of symptoms, rate of progression and age at onset, even in families with several affected relatives. Similarly, asymmetrical weakness is common. It has been hypothesized that this non-uniformity of presentation might be due to the regulation of DUX4 expression that becomes initiated upon the presence of various but yet undetermined factors. So far, the regulation of DUX4 gene expression is still relatively unexplored, and except SMCHD1, genes that directly modify DUX4 expression have not been identified. We think that DUX4 gene expression modifiers might influence DUX4 toxicity and FSHD disease penetrance. In this proposal, our central hypothesis is that endogenous DUX4-targeted microRNAs (miRNAs) are modifiers of DUX4 expression and toxicity. Specific Aim 1: To define miR-675 regulation of DUX4 gene expression. Specific Aim 2: To define the DUX4-targeted miRNome among all human microRNAs.