Poster Abstracts
Session I

Odd Numbered Poster Boards

Presentation Time: Wednesday, April 3rd 6:00 – 9:00 pm

Demonbreun - #1
Evesson - #3
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Kerr - #11
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Viswanathan - #21
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#1

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EHD1 Mediates Vesicle Trafficking Required for Normal Muscle Growth and Development

Avery D Posey Jr.[2], H Kieran Deveaux[1], Manuel G Alvarez[3], Judy U Earley[1], Manju George[4], Hamid Band[4], Peter Pytel[3], Elizabeth M McNally[1,2,]

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EHD proteins have also been implicated in intracellular trafficking, especially endocytic recycling, where they may mediate cytoskeletal reorganization. Ferlins are C2-domain containing proteins that display calcium-sensitive phospholipid binding. Phospholipid binding is thought to be critical for mediating vesicle fusion and trafficking and membrane repair. We previously showed that the C2 domains in myoferlin, Fer1L5, and to a lesser extent, dysferlin bind directly to EHD proteins *in vitro*. Loss of myoferlin impairs normal myoblast fusion leading to smaller muscles *in vivo,* and mutations in dysferlin cause muscular dystrophy. We now characterized muscle development in EHD1-null mice and found that loss of EHD1 leads to smaller muscles and myofibers. EHD1 mice have a substantial increase in serum creatine kinase levels, indicative of defective sarcolemmal function. Cultured EHD1-null myoblasts display reduced myoblast fusion accompanied by mislocalization of caveolin-3 and Fer1L5. EHD1-null muscle also had malformed T-tubules. These data, taken together with the interaction with ferlin proteins, suggests that the EHD proteins coordinate important events in muscle growth and maintenance likely through their interaction with ferlin proteins and the ability to reorganize the cytoskeleton.

Notes:

#3

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Membrane Repair in Living Skeletal Muscle Cells

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Background: Laser damage has been extensively employed to study membrane repair in cultured muscle cells and isolated muscle fibers, using a variety of different protocols. We are developing a damage assay using an ablation laser coupled with real time spinning disk confocal fluorescence microscopy to study repair in live cells. Our goal is to track the intracellular movements of wild type and mutant dysferlin in response to an acute damage event. **Methods:** We are using a 532nm pulse laser to damage differentiated human and mouse myotubes, with the use of lipophilic FM dyes to monitor damage sites and resealing outcomes. We use a series of short (200ms) laser pulses to make a small focused cellular lesion. We have compared the repair capacity of control and dysferlin deficient muscle cell lines, utilizing a combination of the lines currently studied in different laboratories. **Results:** We have established a reproducible laser damage assay for cultured muscle cells. As in previous studies, we have demonstrated that calcium is required for efficient membrane resealing, and shown that dysferlin deficient cells have an impaired repair outcome compared to wild type control cells. This assay allows high temporal and spatial resolution imaging at sites of precise membrane damage. **Conclusions:** Our assay provides a controlled, adaptable system to model muscle repair in live cells. We are now using this protocol to study the exact role dysferlin plays in membrane repair.

Work supported by the Jain Foundation

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#5

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Purification and Biophysical Characterization of Human Dysferlin C2 domains

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Dysferlin is composed of seven C2 domains, separated by long linker sequences of approximately 60 amino acids. The overall goal of this project is to isolate, purify, and analyze the 3D structure of each domain and to understand its role in membrane repair. Our lab is currently focused on characterization of the individual C2 domains. Isolation and purification of these domains has been challenging and time consuming. The linkers between the domains make it difficult to determine the precise amino acids that begin each C2 domain; further, the biochemical properties of the isolated domains make them difficult to manipulate. In general, C2 domains tend to co-purify to other proteins; thus, adding to the difficulty of purification. Using a variety of techniques, we have made significant progress in defining the limits and the purification of several C2 domains. Specifically, we have made significant progress purifying C2D, C2E, C2F and C2G. We have sufficiently purified each domain to the extent that we can begin performing biophysical analysis. We are in the beginning stages of collecting circular dichroism and thermal stability data on each. This data will help us understand the inner workings of the protein and how it functions in membrane repair.

Work supported by the Jain Foundation

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#7

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Quantification of Transverse Tubule Dynamics in Dysferlin-Deficient Muscle

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Mutations in the membrane protein dysferlin cause muscle disease in patients with Limb-girdle muscular dystrophy 2B and Miyoshi myopathy, and in several mouse strains. Although dysferlin has been reported to be a sarcolemmal protein, studies by our laboratory and others have localized it to the transverse tubules (TT), where it interacts with multiple proteins critical for TT function. To identify critical domains essential for function and to understand the pathological basis for patient mutations in dysferlin, we have developed a method for quantifying spatiotemporal differences in TT organization and structure using an *in vitro* myofiber injury model in which injury is induced by a brief osmotic shock. Confocal image analysis of single flexor digitorum brevis fibers from dysferlin-null A/J mice electroporated with mutant dysferlin demonstrates that mislocalization of dysferlin causes significant disruption of TT organization. Moreover, following osmotic shock injury, transient vacuole-like structures are detected in dysferlin-deficient myofibers, which can be quantified to assess differences between strains. These methods will provide a useful high-throughput platform for screening pharmacological compounds and small molecules aimed at restoring dysferlin function and identifying new pharmacotherapies for patients with dysferlinopathy. Supported by grants to RJB from the Jain Foundation and the Muscular Dystrophy Association, and by a stipend to JDG from the Muscle Biology Training Grant (T32 AR 07592, Dr. M. Schneider, P.I.)

Work supported by the Jain Foundation

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#9

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Long-term Quantitative Comparison of Muscle-Specific Promoters using AAV vectors

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Muscular dystrophies are a group of genetic disorders characterized by muscle degeneration. Currently, no effective treatments exist for this disease group. Gene therapy approaches have demonstrated promise in several dystrophic models. A challenge for the treatment of MDs is the systemic disease manifestation, requiring global gene delivery to skeletal muscle and muscle restricted transgene expression. We have demonstrated the ability to generate muscle tropic capsids that are de-targeted from other tissues using adeno-associated viral vectors and evaluated recently reported muscle specific promoters (MSPs) *in vitro* and *in vivo* using AAV mediated gene delivery. Specifically, we analyzed the transduction efficiency of three promoters; an abbreviated form of muscle creative kinase containing a triple tandem enhancer, a construct with alterations of the small upstream enhancer (USE), the promoter MS-100 which is a truncated version of the C5-12 promoter, and the cytomegalovirus promoter. AAV vectors containing an identical reporter expressed from the aforementioned promoters were generated for *in vitro*, intramuscular and intravenous applications. *In vitro*, our MSPs demonstrated complete restriction to muscle cells with tMCK demonstrating 2-3 fold enhanced activity. This observation is consistent with the results obtained following IM vector administration. To determine the restriction of our MSP panel to muscle tissue, AAV8 vectors were given to mice via IV administration and monitored for reporter activity over a year. As indicated by live imaging at several time points, apparent muscle restricted expression was noted for tMCK followed by MS-100, ΔUSE3 and CMV. Finally, a biodistribution analysis of reporter activity was performed on the systemically treated animals. Although the reporter activity determined *in vitro* was generally consistent with the live imaging data, inconsistencies were observed and suggest that the promoters affected transgene persistence. These incongruences could be explained by immunological interferences and/or inherent promoter stability and are currently being explored.

Work supported by the Jain Foundation

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#11

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Amplified X-ROS Signaling in Dysferlinopathy

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Although the genetic basis for many of the muscular dystrophies is known, there are currently no cures. Although the absence of dysferlin is the molecular “cause” of both Limb Girdle Muscular Dystrophy Type 2B and Miyoshi Myopathy, the latency of disease presentation and spectrum of disease severity suggest that additional factors contribute to the progressive muscle weakness, damage and wasting seen in these diseases. It is these factors that may provide novel therapeutic targets for intervention. We recently revealed a novel pathway in dystrophic skeletal muscle (mdx) in which a dense microtubule (MT) cytoskeleton acts as a mechano-transduction element to activate NADPH Oxidase 2 (NoX2) dependent ROS generation during stretch - a pathway we term X-ROS signaling. Excessive X-ROS sensitizes mechano-sensitive channels (MSCs), increasing sarcolemmal Ca^{2+} influx during stretch, contributing significantly to the injury susceptibility of mdx muscle *in vivo*. As dysferlin-deficient muscle exhibits an increase in contraction-induced injury in which disregulation of calcium and reactive oxygen species (ROS) play a critical role, we probed a murine model of dysferlinopathy for evidence of enhanced X-ROS signaling. In dysferlin-null muscle we revealed an increase in X-ROS components (α-tubulin, glu-tubulin, and gp91phox) and MT network density. Using our model of minimal stretch, we further revealed a dramatic increase in X-ROS amplitude in dysferlin-deficient muscle fibers. We hypothesize that targeting the X-ROS pathway may reduce acute susceptibility to muscle injury as well as the developmental trajectory of muscle degeneration in dysferlinopathy.

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#13

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Identifying the Calcium-dependent Roles of Dysferlin in Muscle Membrane Repair

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Background: Despite being implicated in muscle membrane repair a decade ago, the molecular role of dysferlin in this process has remained elusive. The precise calcium-binding role of dysferlin's C2 domains, and the nature of its interaction with MG53 in the muscle membrane repair complex, remain uncharacterized. Using a novel ballistics assay of membrane injury, we studied the calcium sensitivity of dysferlin's activation, recruitment, and nucleation at sites of injury with MG53, to coordinate muscle membrane repair. Our microscopy results reveal no consistent evidence for recruitment of EGFP-dysferlin to injury sites. In contrast, through studies of endogenously expressed dysferlin in primary human myotubes, we demonstrate that dysferlin is rapidly cleaved by calpains following membrane injury, releasing a C-terminal fragment with a specialized role in membrane repair. **Methods:** We devised a ballistics assay of membrane injury to study the injury-activated recruitment of heterologously, and endogenously, expressed dysferlin. Ballistics injury creates widespread and readily identifiable sites of membrane injury. We employed confocal and 3D structured illumination super-resolution microscopy to visualize the recruitment of dysferlin and MG53 into a membrane repair scaffold at injury sites. **Results:** Our studies reveal three important calcium-dependent stages of membrane repair. Firstly, dysferlin undergoes injury-activated, calcium-dependent cleavage by calpains to release a C-terminal fragment, mini-dysferlinC72. Mini-dysferlinC72 and MG53 are then rapidly recruited to sites of injury in response to calcium entry through L-type calcium channels. At concentrations of 200uM calcium or greater, mini-dysferlinC72 and MG53 form an intricate lattice that intensely labels exposed phospholipids of injury sites, which then infiltrates and stabilizes the membrane lesion to achieve repair. **Conclusion:** Our results demonstrate that muscle cells employ a mechanism similar to synaptic exocytosis for membrane repair. The calcium signals we identified explain the collective interplay between activated calpains, dysferlin and L-type channels, to sense a membrane injury and mount a repair response.

Work supported by the Jain Foundation

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#15

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Assessment of Disease Activity in Muscular Dystrophies by Non-invasive Imaging

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Muscular dystrophies are a class of muscle disorders that cause progressive muscle wasting. A major hurdle for discovering treatments for the muscular dystrophies is a lack of reliable assays to monitor disease progression in animal models. We have developed a novel mouse model to assess disease activity non-invasively in mice with muscular dystrophies. These mice express an inducible luciferase reporter gene in muscle stem cells. In dystrophic mice, muscle stem cells activate and proliferate in response to muscle degeneration, resulting in an increase in the level of luciferase expression which can be monitored by non-invasive, bioluminescence imaging. We have applied this non-invasive imaging to assess disease activity in a mouse model of the human disease, Limb Girdle Muscular Dystrophy 2B (LGMD2B), caused by a mutation in the dysferlin gene. We monitored the natural history and disease progression in these dysferlin-deficient mice up to 18 months of age. We were able to detect disease activity prior to the appearance of any overt disease manifestation by histopathological analyses. Disease activity was reflected by changes in luciferase activity over time, and disease burden was reflected by cumulative luciferase activity, which paralleled disease progression as determined by histopathological analysis. The ability to monitor disease activity non-invasively in mouse models of muscular dystrophy will be invaluable for the assessment of disease progression and the effectiveness of therapeutic interventions.

Work supported by the Jain Foundation

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#17

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The Use of RIPA Buffer and Sonication Improves Immunoblot Detection of Dysferlin in Skeletal Muscle Homogenates

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Background: Mutations in the DYSF gene that encodes the protein, dysferlin, lead to human muscular dystrophies known as dysferlinopathies. The purpose of our study was to compare several different methods of extracting protein from adult skeletal muscle to identify the most optimal conditions for immunoblot detection of dysferlin and its binding partners. Hypothesis: Homogenizing muscle in radioimmunoprecipitation assay (RIPA) buffer provides better extraction of dysferlin and reduces background noise in immunoblots compared to our previous standard of homogenizing muscle in Nonidet-P40 (NP40). **Methods and Results:** We prepared homogenates of the *tibialis anterior* (TA) muscle from control A/WySnJ mice, and dysferlinopathic A/J and B10.SJL mice, under the following conditions: 1) 1% NP40 in phosphate buffered saline (PBS). 2) 1% NP40 in PBS followed by sonication. 3) RIPA buffer (50mM Tris-HCl, pH8, with 150mM NaCl, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS). 4) RIPA buffer followed by sonication. Protease inhibitors were used at the same concentration in all conditions. The use of RIPA buffer and sonication yielded the best results. Using our optimized methods (n = 3 mice per strain), we were able to demonstrate the following: 1) Low levels of dysferlin can be detected in B10.SJL muscle, but not A/J muscle. 2) There is no change in dysferlin's binding partners caveolin-3, MG53 and annexin-A2 in either A/J or B10.SJL muscle. 3) The endoplasmic reticulum stress marker CHOP is elevated in A/J and B10.SJL muscle compared to controls, with higher levels seen in A/J. **Conclusion:** Our results indicate that the use of RIPA buffer and sonication for preparing skeletal muscle homogenates significantly improves the detection of dysferlin in immunoblots. Using our optimized methods, we find that endoplasmic reticulum stress is elevated in dysferlinopathies and may contribute to the phenotypic differences between the A/J and B10.SJL murine dysferlinopathy models. Funded by a grant to JAR from the Jain Foundation, Inc.

Work supported by the Jain Foundation

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#19

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Deletion of C2 Domains Inhibits the Localization of Dysferlin to T-tubules

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One approach to treating LGMD2B/MM is to transduce skeletal muscles with AAV encoding a functional form of dysferlin into skeletal muscles. To do so, we must reduce the ORF of dysferlin to ~3.4 kb without losing significant activity. We have used standard recombinant DNA technology to generate smaller versions of dysferlin, linked at their N-termini to Venus, and have introduced them into control and dysferlin null A/J FDB myofibers by electroporation. Dysferlin's cytoplasmic domain is organized from N to C terminus into 3 C2 domains (A-C), with a fer1 domain flanking C2B, 2 pairs of fer (A,B) and dysf domains, and 4 more C2 domains (D-G). We deleted each C2 domain individually, and in combination, with minimal disruption of the remaining structures. All constructs were grown in COS7 cells and protein extracts were analyzed in western blots to confirm expression of fusion proteins for the predicted sizes. Only deletion of C2A domain (DC2A) shows a localization pattern similar to the full length Venus-dysferlin. DC2B is similar to DC2A, but with more aggregation. DC2BC is partially localized to the triad junction but is highly aggregated. All the other deletion constructs we examined concentrate in short, narrow linear membrane compartments that cross the Z-disk. These results are distinct from those obtained in other laboratories, which have used mononucleate cells and myotubes to study the role of dysferlin's C2 domains in its subcellular localization. We are currently working on deletions of the gap and ferlin domains in the middle of the molecule, with the aim of determining their role in dysferlin's ability to concentrate at or near triad junctions, and, ultimately applying our results to the creation of a minidysferlin. Supported by grants to RJB from the Jain Foundation and the Muscular Dystrophy Association.

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#21

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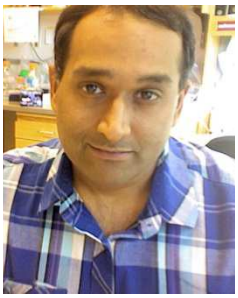

Screen for Genetic Suppressors of fer-1 Loss-of-Function in *C. elegans*

Mohan Viswanathan[1], Jennifer Fong[1], Leonard Guarente[1]

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Background: Mutation of *C. elegans* fer-1, a homolog of human dysferlin, causes infertility. This infertility is the result of defective fusion of intracellular vesicles to spermatocyte plasma membrane, an obligate event in normal sperm maturation. The identification of mutations in human dysferlin and *C. elegans* fer-1 that confer similar vesicle fusion defects strongly suggests a conserved mechanism of function and provides the rational basis for utilizing *C. elegans* fer-1 mutants as a biological model for human dysferlinopathy. **Methods:** The *C. elegans* fer-1(hc1) allele contains a single point mutation in a conserved C2 domain rendering animals temperature sensitive for fertility. We previously performed a non-independent EMS-based mutagenic screen isolating genetic suppressors of the *C. elegans* fer-1(hc1) fertility defect. More recently, we have genetically characterized a new fer-1 allele, fer-1(ok580), and have designed a genetic screen with this mutant to help isolate suppressors in this non-conditional background containing a deletion in fer-1. **Results**: We identified six fer-1(hc1) suppressor mutants: four are second-site mutations within one of the fer-1 C2 domains, while two appear to be extragenic to fer-1. We are using whole-genome SNP-based sequencing approaches to identify the nature of the extragenic mutations. As a deletion allele, fer-1(ok580) is superior to fer-1 point mutant alleles as the basis with which to find extragenic suppressor mutations as it cannot be reverted by secondary mutations within fer-1. We have developed a new screen to isolate genetic suppressor mutations of fer-1(ok580). **Conclusion:** We will describe the methods that we have developed to screen for suppressors of fer-1 loss-of-function and report on the progress of our screens to identify possible therapeutic targets for LGMD2B.

Work supported by the Jain Foundation

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#23

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Calpain Cleavage of Dysferlin is a Fundamental Response to Membrane Injury

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Background: Using a ballistics assay of membrane injury, we have recently shown that dysferlin is detected at sites of myotube membrane damage using only Hamlet-1, that recognizes a C-terminal epitope, and not by Romeo-1 or Hamlet-2 that recognize N-terminal epitopes. We have also shown that when myotubes are injured in the presence of calcium (using ballistics or scraping injury), a C-terminal dysferlin fragment (minidysferlinC72) is identifiable by only Hamlet-1 on Western blot. We show formation of minidysferlinC72 occurs at neutral pH and is sensitive to calpain inhibitors. Thus, we hypothesize that dysferlin is cleaved by calpains to release minidysferlinC72. We are currently focusing on the mechanism and regulation of this cleavage event in an effort to understand how dysferlin regulates membrane repair. **Methods:** Cells were damaged by scraping with a rubber scraper in PBS with or without calcium, lysed in SDS lysis buffer and analyzed by Western blot. **Results:** MinidysferlinC72 formation is a ubiquitous response to membrane injury in both muscle and non-muscle cells. Dysferlin cleavage is injury-dependent, calcium-dependent and sensitive to calpain inhibitors. Dysferlin undergoes injury-activated cleavage in cell types that do not express MG53, indicating minidysferlinC72 formation is MG53-independent. Cleavage of dysferlin requires >150 µM extracellular calcium, and with differential inhibition of µ- and m-calpain by Al3+, collectively implicate a role for m-calpain. **Conclusions:** Calpain cleavage of dysferlin is fundamental step in effecting calcium-dependent membrane repair. We hypothesize that minidysferlinC72 rapidly recruits to sites of membrane injury, and interdigitates with MG53 forming a membrane repair scaffold that encircles then infiltrates the plasma membrane during membrane resealing. Current studies are refining the exact calpain cleavage site and confirming the specific calpain responsible. Future studies will evaluate the role and locale of the dysferlin N-terminus following membrane injury.

Work supported by the Jain Foundation

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#25

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***In vivo* Imaging Indicates Delayed Myofiber Damage After Lengthening Contractions in Dysferlin-null Muscle**

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Background: Histological data suggest that dysferlin-null A/J mice sustain delayed myofiber damage over many hours to several days following *in vivo* injury by repeated lengthening contractions (large-strain injury, LSI). Recovery is myogenesis-dependent and takes several weeks. Control mice recover rapidly from LSI with minimal signs of damage and myogenesis. Here, we study the usefulness of live-animal, T2-weighted, magnetic resonance (MR) imaging, for non-invasively monitoring the progression and resolution of muscle damage in longitudinal studies of dysferlin-null and control mice. **Methods:** The ankle dorsiflexors of A/J and A/WySnJ mice (male, 12-16 wks) were subjected to LSI. The left hind limb served as the experimental side, while the right hind limb of each animal served as an uninjured control. Simultaneous MR imaging of both hind limbs were performed at 1 hour, 6 hours, 24 hours, 48 hours, 7 days and 14 days post-LSI. High-resolution, dual-echo proton density, and T2-weighted rapid acquisition relaxation-enhanced (RARE) MR images (195 lm inplane at 1.25-mm slice thickness; TE1/TE2/TR/ETL/NEX ¼ 17.4 ms/52.1ms/5000 ms/4/8) were acquired on a 7-Tesla MR system (Biospec 7T/30; Bruker Biospin scanner). The T2 signal in the *tibialis anterior* muscle (TA), which is the primary dorsiflexor, was analyzed by NIH's Image-J software. **Results:** The increase in T2 signal intensity, which reflects muscle edema, increases over 48 hours post-LSI in A/J muscle (~2-fold increase from baseline). In contrast, A/WySnJ muscle shows minimal changes in the T2 signal through 48 hours post-LSI (~1.3-fold increase from baseline). **Conclusion:** Similar to our histological data, our MR data show clear differences between dysferlin-null muscle and control muscle that evolve over the first 48 hours after LSI. T2-weighted MR imaging is a useful non-invasive method of monitoring changes in dysferlin-null muscle after injury.

Work supported by the Jain Foundation

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#27

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The Effects of MyD88 Deficiency on Disease Phenotype in Dysferlin-deficient A/J Mice: Role of Endogenous TLR Ligands

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Background: An absence of dysferlin leads to activation of innate immune receptors such as Toll-like receptors (TLRs) and skeletal muscle inflammation. Myeloid differentiation primary response gene 88 (MyD88) is a key mediator of TLR-dependent innate immune signaling. We hypothesized that endogenous TLR ligands released from the leaking dysferlin-deficient muscle fibers engage TLRs on muscle and immune cells and contribute to disease progression. **Methods:** To test this hypothesis, we generated and characterized dysferlin- and MyD88- double-deficient mice and studied effect of exogenous administration of TLR-7/8 agonist on muscle disease in pre-symptomatic AJ mice. **Results:** Double-deficient mice exhibited improved body weight, grip strength, and maximum muscle contractile and specific force at 8 months of age when compared to MyD88-sufficient, dysferlin-deficient A/J mice. Double-deficient mice showed a significant increase in total and centrally nucleated fibers, indicating increased regeneration. We next tested the hypothesis that endogenous ligands such as single-stranded ribonucleic acids (ssRNA) released from damaged muscle cells bind to TLR-7/8 and perpetuate the disease progression. We found that injection of ssRNA into the skeletal muscle of pre-symptomatic mice (2 months old) resulted in a significant increase in degenerative fibers, inflammation, and regenerating fibers in A/J mice. In contrast, characteristic histological features were significantly decreased in double-deficient mice. **Conclusion:** These data point to a clear role for the TLR pathway in the pathogenesis of dysferlin deficiency and suggest that TLR-7/8 antagonists may have therapeutic value in this disease.

Notes:

Poster Abstracts
Session II

Even Numbered Poster Boards

Presentation time: Thursday April 4th, 3:00 – 6:00 pm

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#2

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Validation of Dysferlin Blood Monocyte Assay as a Reliable Diagnostic Tool for Primary Dysferlinopathies

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Background: Limb girdle muscular dystrophy type 2B (LGMD2B) and Miyoshi myopathy (MM) are two clinically distinct autosomal recessive muscular diseases (dysferlinopathies) caused by mutations in human dysferlin gene, DYSF. DYSF encodes for a 230 kDa membrane repair protein dysferlin, believed to be involved in vesicle trafficking and muscle fiber membrane repair. Absence of functional dysferlin impairs muscle membrane repair especially after exercise induced damage. Disease diagnosis involves a combined evaluation of clinical phenotype and molecular diagnosis at both protein (immunoblotting or immunohistochemical analysis) and gene (sequencing or deletion/duplication analysis) level. Often, protein analysis is performed prior to molecular analysis as it is more indicative of the disease phenotype (more specifically proteotype) or mutation effect. Recently, dysferlin has been shown to be expressed in blood monocytes, allowing for a less invasive protein analysis method compared to traditional muscle biopsy. **Method:** We used a density gradient centrifugation method with Dulbecco's Phosphate Buffered Saline and Ficoll-Paque to isolate PBMCs from whole patient blood. Parallel immunoblotting was performed for both dysferlin and housekeeping protein α-GAPDH for each patient sample. Dysferlin signal intensity was quantified using Image-J software and normalized with that of α-GAPDH. Dysferlin gene sequencing and deletion/duplication analysis was performed on DNA isolated from the same blood sample. Mutation analysis and protein expression data were compared to determine correlation and specificity of the test. **Results:** We found at least 1 mutation in 82% (23/28) of cases that had relative blood dysferlin levels below 10% compared to control. Moreover, we did not find any mutations in cases that had relative blood dysferlin levels above 35% compared to control. **Conclusion:** Blood monocyte assay for dysferlin protein expression can be used as a reliable clinical tool for diagnosing dysferlinopathies. Relative blood dysferlin protein levels of 10% or less compared to control are indicative of primary dysferlin deficiency.

Work supported by the Jain Foundation

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#4

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The 5F7 ANO5 Antibody: Immunodiagnostic Applications and Functional Studies

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We have generated the 5F7 monoclonal antibody to ANO5 specific peptides. Antibody specificity has been examined by expression of YFP ANO5 fusion protein, peptide blocking and analysis of ANO5 knockdown cells. 5F7 is a C-terminal ANO5 antibody which predominantly detects a 37 kDa ANO5 isoform in control muscle. Analysis of anoctaminopathy muscle shows that the common c.191duplA mutation expressed as homozygous or heterozygous is associated with upregulation of 5F7 signal intensity in 7 out of 8 muscle samples examined. No differences in expression of the 37kDa signal are detected in R758C ANO5 muscle compared to controls. Proteomic analysis of c.191duplA and R758C muscle identifies differential expression of proteins associated with the actomyosin cytoskeleton. By immunolabelling C2C12 and fibroblast cells we show that 5F7 detects a tubular filamentous network. 5F7 positive tubular filaments can be detected at regions of membrane growth in EGF stimulated muscle cells where dysferlin accumulates. In R758C fibroblasts previously shown to be defective in membrane repair the 5F7 stained tubular filaments are disorganized. Collectively our studies indicate that the 5F7 antibody is looking promising for ANO5 immunodiagnostics. Further characterization of the tubular filaments will allow insights into the function of ANO5 and its relationship with dysferlin.

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#6

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Study of Molecular and Cellular Mechanisms of Muscular Dystrophy Related to Mutations of Dysferlin

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Neuromuscular disorders account for a broad range of ailments, where the pathological mechanisms are largely unknown. Among these, muscular dystrophies represent a sizable amount, with a worldwide distribution and an estimated incidence of 1/2000 live births. Dysferlinopathy, caused by mutations of dysferlin, a protein involved in membrane repair, is associated to Miyoshi myopathy (MM) or Limb girdle muscular dystrophy 2B (LGMD2B). In the last three years, we have identified in Chile 29 patients with dysferlinopathy bearing the MM or LGMD2B phenotypes. Preliminary data suggests that the prevalence of dysferlinopathy is higher than suspected. Interestingly, some mutations consistently repeated amongst the Chilean cohort of patients yield distinct phenotypes in either severity and/or onset of symptoms. Thus, it appears essential to explore the effects of different dysferlin mutations in skeletal muscle cell models, to evaluate the chain of events. Dysferlin is a 230 kDa protein which seems to play an important role in muscle repair. The C2A domain of dysferlin binds phospholipid in a calcium-dependent fashion, a feature common with synaptotagmin, a calcium sensor protein involved in fast exocytosis. This property of dysferlin, together with its association to the dihydropyridine receptor, suggests its possible function in calcium-dependent vesicle fusion with the plasma membrane. However, these mechanisms, critical for membrane repair, remain unclear. We therefore propose to evaluate the effect of various mutated dysferlins in vesicle trafficking and cell membrane fusion events, using *in vitro* cell models such as the RCHMH human muscle cell line, muscle primary cultures and myogenic cell lines from patients. We will pay special consideration to prevalent Chilean mutations. Regarding the association of dysferlin with the dihydropyridine receptor and proteins such as annexins and AHNAK that are involved in actin organization, we will also evaluate the role of dysferlin in calcium signals and cortical actin organization. Funding: Rings grant #ACT1121 (Conicyt, Chile)

Notes:

#8

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Gene Therapy by AAV Vector for ANO5-linked Diseases

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Dominant mutations in the ANO5 gene have been identified associated with gnathodiaphyseal dysplasia (GDD), a disorder of the bones. Recently, other loss-of-function mutations in ANO5 were identified as the cause of an autosomal recessive form of LGMD (LGMD2B) and a distal non-dysferlin Miyoshi muscular dystrophy (MMD3). As a first step towards testing therapeutic approaches for these diseases, we constructed an Ano5 knockout mouse model. This model was validated and characterized at the molecular and protein level. Histological and phenotypic characterization of the muscle level is ongoing. In the meantime, we defined the most prevalent ANO5 isoform present in human skeletal muscle and constructed an AAV vector encoding this form. This AAV vector was injected intramuscularly and intravenously into WT mice at different doses during different period of time to initially determine whether the overexpression of ANO5 in mouse muscle could be deleterious. No adverse effects were seen histologically neither in muscles nor in heart with each doses tested to 3 months of expression. This AAV vector will now be injected in our KO mouse model to thereby determine if ANO5 could be a therapeutic tool for ANO5-linked diseases in human.

Work supported by the Jain Foundation

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#10

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Detection of Dysferlin Gene Mutations in Patients with Limb-Girdle Muscular Dystrophy, in the Indian Population.

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Background: Limb- Girdle Muscular Dystrophy (LGMD) is the most common adult onset muscular dystrophy in India. The exact prevalence of dyferlinopathy in India is unknown as majority of suspected LGMD in India remain unclassified. A definitive diagnosis of dysferlinopathy can only be achieved by gene sequencing, which is expensive, hence screening of patients with high probability of LGMD2B is essential before sequencing. Aim: This JF funded project includes (i) blood-based screening (PBMCs) for defective dysferlin protein expression by monocyte assay/Western blot (ii) Detection of dysferlin gene mutations by sequencing in cases confirmed as dysferlinopathies on monocyte assay. **Methods:** (i) Blood samples collected of the patients diagnosed as LGMD2B on clinical, histopathological and immunohistochemical evaluation after running them on the Jain Foundation LGMD phenotype subtyping prediction tool. (ii) Monocyte assay/Western blot was run on all samples to ascertain the status of dysferlin protein expression. (iii) Sequencing was carried out on samples showing absence of dysferlin protein on Western blot. **Results:** A total of 146 blood samples of suspected cases of dysferlinopathy from three major Neurology Centers of India were collected and analyzed from August 2011 to date. LGMD subtyping tool based on clinical, histopathology and immunohistochemical details of the patients were run on 37 patients for prediction of probability of different LGMDs prior to collection of samples. Absence of dysferlin was identified in 75 cases on monocyte assay/Western blot. DNA mutation analysis was done on 15 samples for confirmation of dysferlinopathy. **Conclusions:** With the availability of this test through Jain Foundation, diagnosis of patients with LGMD2B in India is now possible. Given the diversity of ethnicity and genetic background of the Indian population, identifying population specific and founder mutations in Indian patients will be of immense relevance.

Work supported by the Jain Foundation

Notes:

#12

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Therapeutic Potential of the Proteasomal Inhibitor, Velcade, for Dysferlin Missense Mutations

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Background: Mutations in dysferlin cause the progressive muscular dystrophies Miyoshi Myopathy, Limb Girdle Muscular Dystrophy 2B, and distal anterior compartment myopathy. Many patients with this disease harbor missense mutations in at least one of their two pathogenic DYSF alleles. These patients have significantly reduced or absent dysferlin levels in skeletal muscle, suggesting that dysferlin encoded by missense alleles is rapidly degraded by the cell's quality-control system. We reasoned that missense-mutated dysferlin, if salvaged from degradation, might be biologically functional. **Methods**: We used a dysferlin-deficient human myoblast culture harboring the common Arg555Trp missense allele and a DYSF null allele, as well as control human myoblast cultures harboring either two wild-type or two null alleles. We measured dysferlin protein and mRNA levels, resealing kinetics of laser-induced plasmalemmal wounds, myotube formation, and cellular viability after treatment of the human myoblast cultures with the proteasome inhibitor Velcade. **Results:** We show that endogenous Arg555Trp missense mutated dysferlin is degraded by the proteasomal system. Inhibition of the proteasome by Velcade increases the levels of Arg555Trp missense mutated dysferlin. This salvaged protein is functional as it restores plasma membrane resealing in patient-derived myoblasts, and reverses their deficit in myotube formation. Velcade did not cause cellular toxicity at the regimen used. **Conclusions**: Our results raise the possibility that inhibition of the degradation pathway of missense-mutated dysferlin could be used as a therapeutic strategy for patients harboring certain dysferlin missense mutations. We are currently testing additional missense mutations to see if they retain their biological activity following Velcade treatment. Studies are being performed in recombinantly-generated missense-mutated dysferlin constructs and in patient-derived myoblast cultures.

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#14

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Multiple Antibody Colocalization Imaging of Skeletal Muscle

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Anatomical distribution of multiple proteins within complex tissue is predicted to aid in determination of musculodystrophy pathophysiology. Dysferlin is a large (~230 kDa), membrane-anchored, calcium-binding protein localized to plasmalemma and t-tubules of muscle fibers. In fibers lacking dysferlin, these structures exhibit ultrastructural abnormalities. As a means to identify the possible role(s) of dysferlin in maintenance of fiber ultrastructure and calcium homeostasis, we are using array tomography (ATomo) to detect differences in protein co-localization in dysferlin-negative vs. dysferlin-normal muscle fibers. We have screened and optimized antibodies for specific labeling of major structural components: contractile apparatus (actin, myosin, desmin), plasmalemma (dystrophin, caveolin), t-tubules (dysferlin, DHPR-α1, DHPR-α2, ryanodine receptor) and basement membrane (collagen A1, elastin) and calnexin and α-smooth muscle actin observe the fiber anatomy. Testing different temperatures for labeling incubation, we found better labeling and deeper penetration at 37℃. This labeling procedure was not associated with increased noise from non-specific labeling. We optimized labels in ATomo by comparing their specificity for anatomical structures over a range of antibody concentrations, from the minimum required for observable signal to very high concentrations demonstrating maximum signal and high noise levels. We have observed a consistent pattern between appropriate antibody concentrations for ATomo relative to other immunolabeling methods such as western blotting and immunohistochemistry that roughly encapsulates a high S:N ratio plateau, forming good first approximations for optimized labeling. We have combined up to four antibodies against epitopes colocalized within the same structure to label the complete anatomy of the T-tubule and triad architecture across the volume of a muscle fiber. We will use ATomo to colocalize dysferlin with other proteins, to assess the number, distribution and localization of L-type Ca^{2+}-channels, SERCA-type re-uptake Ca^{2+}-pumps, Ca^{2+}-leak channels and Ca^{2+} buffering proteins located in the t-tubules in normal and dysferlin-null myofibers.

Work supported by the Jain Foundation

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#16

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Dietary Intervention with Omega-3 Fatty Acid for Dysferlin Deficiency in Mice

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Background: Membrane biophysics teaches us that resistance to damage will be a function of the lipid composition of the sarcolemma. A diet rich in the polyunsaturated fatty acid (PUFA) ω-3 a-linolenic acid (ALNA) prevents the progression of muscular dystrophy in a d-sarcoglycan-null hamster model (Am. J. Pathol., 177:2176). We designed a trial in dysferlin-null A/J mice to examine whether an ALNA-rich diet can ameliorate the symptoms of dysferlinopathy. **Methods**: Mammals, lacking biosynthetic ability, require both ω-3 and ω-6 PUFAs in their diet. The standard laboratory rodent diets, containing either corn or soybean oil, have a low ratio of ω-3 ALNA to ω-6 linoleic acid (LA). To create an ALNA-rich diet, we substituted flaxseed oil for soybean oil in a standard defined diet, AIN-93G, increasing the ω-3:ω-6 ratio from 0.13 to 3.3. Mice were maintained on either the control (soy) or test (flax) diets for five months, and then evaluated for their response to muscle injury caused by a series of large strain lengthening contractions (LSI). **Results:** The two diet groups had similar weight gains for the first three months; but after five months the test (flax) group had greater weight and muscle gain. Due to within group variance, we did not observe a statically significant difference in LSI response between the two diet groups. A lipidomics analysis of muscle tissue from the two diet groups is in progress to learn if there are correlations between animal response to LSI and lipid profile. **Conclusion:** Although this study did not demonstrate a beneficial effect of dietary ALNA in a mouse model of dysferlinopathy, we did observe variability between individual animals in their response to LSI. The lipidomics analysis may identify specific muscle tissue lipids which are correlated with better recovery from LSI, and provide a basis for further dietary intervention studies.

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#18

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Dysferlinopathy in Iran: Clinical and Genetic Results

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Background: We describe the clinical course and mutational analyses of 15 patients with dysferlinopathy from 9 different families for the first time from Iran. **Methods:** Clinical and laboratory data of patients were collected. Genomic DNA was extracted from peripheral blood and 55 exons and flanking intronic boundaries of the dysferlin gene were screened for mutations and analyzed. **Results:** Five patients were male. 7 families had consanguineous marriage. Mean age of onset was 16.8 (14-24) and mean age of diagnosis was 26.6 years (delay 1-23 years). The onset was clearly distal in 7 and proximal in 6 and was asymmetric in 4 patients. Two patients had muscle swelling as their onset symptoms, one started with pain, and 13 with lower limb weakness. 3 patients had partial atrophy of the distal part of the biceps and 13 showed prominent calf muscle wasting. Foot plantar flexors, deep finger flexors and hip adductors were disproportionately involved. Genetic testing showed homozygous mutation of dysferlin gene in 8 probands, 6 of which were not previously reported. Mutational analyses of patient III-a identified a heterozygous missense mutation in exon 6 [c.509C>A (p.Ala170Glu)], which has been previously reported and two not previously reported heterozygous intronic variants in the second allele.

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#20

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Pilot Study to Assess Biomarkers of Changes in Barrier Function of Skeletal Muscle in Patients with a Fragile Sarcolemmal Muscular Dystrophy

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Background: One of the major problems in comparing the benefit of different therapeutic interventions in the muscular dystrophies is to find common outcome measurements, and thus, more reliable biomarkers have long been desired. To begin to understand the variation in potential biomarker sensitivity and the optimal time of testing, the major aim of this study is to identify biomarker and clinical correlates of changes in the barrier function of skeletal muscle membrane before and after routine motor function testing in patients with one of the Fragile Sarcolemmal Muscular Dystrophies (FSMD): LGMD2B-F, I, L, MM, BMD, and MMD3. Patients: Pilot study of 10 patients (male and female of all ethnic backgrounds) with one of the FSMDs, assessed over a 1-year period, who fulfill a set of pre-established criteria. **Methods**: We will obtain a series of patient blood draws to 1) establish a baseline of serum biomarkers and 2) to measure changes in baseline serum biomarker levels before and after patient physical therapy assessment and patient daily life routine activities. Patient sera will be screened against a panel of candidate biomarkers, including creatine kinase, lactate dehydrogenase, myoglobin, cytokines and microRNAs. **Results/Conclusions**: We have designed a clinical protocol to identify and validate biomarkers of changes in cell membrane permeability in patients with an FSMD for use in future intervention studies. The known problems associated with inter-patient variability of serum biomarkers will be addressed by analyzing the statistical properties of individualized changes in biomarkers.

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#22

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Restoring Dysferlin to Dysferlin-null Myofibers by Adenoviral Infection in Culture

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In addition to a dysferlinopathy, A/J mice exhibit an unusual set of phenotypes and an appropriate control with the same genetic background, but expressing dysferlin in muscle, is not available to determine if the muscular dystrophy in A/J mice is due solely to the absence of dysferlin. We must therefore find a way of reintroducing dysferlin into A/J muscle to ensure that the phenotypes we have been studying *in vitro* and *in vivo* are due solely to their lack of dysferlin. Although electroporation can be used with A/J muscle, it can cause considerable damage to dystrophic fibers, many of which fail to survive in culture. An alternative is to introduce exogenous dysferlin *in vitro* by adenoviral infection. Adenovirus is very efficient in infecting and transducing FDB myofibers in tissue culture. Our challenge is to ”fit” the open reading frame (ORF) of human dysferlin linked to Venus, flanked by effective promoter and polyadenylation sequences, into a construct of no more than 7.5 kb, the maximum insert size for adenovirus. We have therefore designed an adenoviral vector with the Venus-dysferlin ORF driven by a truncated version of the CMV promoter, and linked to the polyadenylation sequence of the human growth hormone gene. Combined, these sequences are ~7.4 kb, small enough to be expressed by adenovirus. Although the virus carrying this large insert grows very slowly, preliminary results indicate that it infects COS7 cells in culture and expresses full length Venus-dysferlin, confirmed by Western blot. Supported by a grant from the Jain Foundation to RJB.

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#24

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Concatemerization Versus Homologous Recombination Strategies for Full-length Dysferlin *in vivo* Expression.

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Adeno-associated virus (AAV) is currently the most advantageous vector for gene transfer in muscle. However, the packaging capacity of the AAV vector is limited to 4.7 kb, preventing encapsidation of large genes. To overcome this limitation, several gene transfer methods have been developed over the years for the *in vivo* expression of full length large transgenes: i) recombination between overlapping AAV vectors, ii) ITR-ITR mediated concatemerization of two AAV genomes, and iii) homologous recombination of partially packaged products using AAV5. In the case of dysferlin (cDNA size of 6.2 kb), the latter two techniques have been successfully tested. We present a comparative study to evaluate the relative efficiency of the different methods. We produced rAAV8 for each strategies plus rAAV5 for the "fragment" AAV homologous recombination. To compare the efficiency of muscle transduction and level of expression of the full-length protein, we performed intramuscular injections into the tibialis anterior muscle of 4 week old dysferlin deficient mice BLAJ of all the vectors. From the comparison, the vectors driving the best expression of dysferlin are currently being used for a dose-effect analysis after systemic administration in 4 weeks-old deficient mice BLAJ.

Work supported by the Jain Foundation

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#26

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Normal Migration in Dysferlin-null and Myoferlin-null Myoblasts Points to a Primary Fusion Defect

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The fusion of myoblasts during development is a complex process that is influenced by many individual components including cell signaling, migration, cytoskeletal rearrangements, and membrane fusion. We have shown previously that both myoferlin-null and dysferlin-null myoblasts display a reduced capacity to form normal myotubes. Furthermore even young dysferlin-null and myoferlin-null mice lack the largest myofibers. Our lab has also shown that both myoferlin-null and dysferlin-null myoblasts do not respond to Insulin-like Growth Factor-1 (IGF-1) stimulation as efficiently as wild type myoblasts. These findings have been interpreted as reflective of defects in membrane fusion during myotube formation and of impaired cell signaling related to disrupted receptor recycling. Defects in myoblast migration could also affect their ability to form myotubes. In this study we therefore wanted to determine if myoblasts deficient in myoferlin, dysferlin or both exhibit changes in their migrational properties compared to wild type myoblasts isolated from litter mate controls. Time-lapse microscopy was performed to track at least 60 myoblasts of each genotype and determine their average velocity. We show that there is no decrease in migration velocity in myoblast deficient in myoferlin, dysferlin or both compared to wild type controls. This suggests that loss of dysferlin and/or myoferlin does not lead to any significant defect in baseline migrational abilities and instead points to the complex process of membrane fusion as important.

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#28

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Modular Dispensability of Dysferlin's C2 Domains Reveals Rational Design for Mini-Dysferlin Molecules

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Background: Dysferlin is a large transmembrane protein composed of a C-terminal transmembrane domain, two DysF domains and seven C2 domains that mediate lipid and protein binding interactions. Recessive loss of function mutations in dysferlin lead to muscular dystrophies, for which no treatment is currently available. Dysferlin's large size precludes its encapsidation into an adeno-associated virus (AAV), the vector of choice for gene delivery to muscle. **Methods:** To design mini-dysferlin molecules suitable for AAV mediated gene transfer, we tested internally truncated dysferlin constructs, each lacking one of the seven C2 domains, for their ability to localize to the plasma membrane and to repair laser induced plasmalemmal wounds in dysferlin deficient human myoblasts. **Results:** We demonstrate that dysferlin's C2B, C2C, C2D and C2E domains were dispensable for correct plasmalemmal localization. Furthermore, we show that the C2B, C2C, C2E domains, and to a lesser extent the C2D domain are dispensable for its membrane repair function. Based on these results, we designed small dysferlin molecules, which can localize to the plasma membrane, can reseal laser-induced plasmalemmal injuries and are small enough to be incorporated into AAV. **Conclusion:** These results lay ground for AAV mediated gene therapy experiments in dysferlin deficient mouse models.

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