Speaker Abstracts
Sessions I – VII

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***In vitro* and *in vivo* Assays of Dysferlin's Role in T-Tubules**

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We transfected fluorescent dysferlin constructs into murine FDB muscles and localized the fluorescent fusion protein to transverse (t-) tubules (TT).To test the role of dysferlin in maintaining TT integrity, we subjected hind limb muscles in A/J and control mice to large strain eccentric injury (LSI), and FDB myofibers in culture to osmotic shock (OSI). Following LSI, we assayed torque loss and recovery, TT disruption, necrosis and inflammation. Following OSI, we assayed TT disruption, changes in Ca^{2+} transients and baseline $Ca^{2+,}$ and changes in efflux of the impermeant dye, Sulforhodamine B (SulfB), from the TT lumen. We find that after either LSI or OSI, TT are more disrupted structurally in A/J than controls. Disruption occurs soon after LSI; necrosis and inflammation occur later. OSI rapidly causes disregulation of Ca^{2+} homeostasis and slowing of SulfB efflux from TT. Expression of full length dysferlin or reducing extracellular [Ca^{2+}] protects against the changes after OSI. We conclude that dysferlin is present in TT and that in its absence TT are more susceptible to OSI *in vitro* and LSI *in vivo*. Our results establish significant parallels between the effects of LSI and OSI that should be useful in future pre-translational studies of dysferlinopathies.

Work supported by the Jain Foundation

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Cellular Models for Dysferlinopathy Identifies a Role of Dysferlin in Myoblast Repair and Injury-Triggered Lysosomal Exocytosis

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Use of cell-based models for dysferlinopathy and understanding the subcellular defects in these cells would help in developing therapies for this disease. Here we have tested the suitability of two different dysferlin deficient mouse muscle cell lines - C2C12shRNA dys and H2K-A/J as cellular models for dysferlinopathy. Lack of dysferlin does not affect the ability of these cells to proliferate or differentiate. However, using two independent methods for cell membrane injury, we find that dysferlin deficient myoblasts repair slowly and poorly, establishing the suitability of these cells as models for dysferlinopathy. As lysosomal exocytosis is associated with repair of injured cell membrane, we tested if dysferlin deficit affects injury-triggered lysosomal exocytosis. Dysferlin deficient cells have several lysosomal deficits including: a) fewer lysosomes docked at the cell membrane, b) delay in injury-triggered lysosome exocytosis and c) reduced injury-triggered lysosomal exocytosis. These findings demonstrate a role of dysferlin in cell membrane repair even in myoblasts and demonstrate that dysferlin is involved in docking and calcium triggered fusion of lysosomes. Based on these findings we propose that defective lysosomal exocytosis contributes to poor healing of dysferlin deficient muscle cells.

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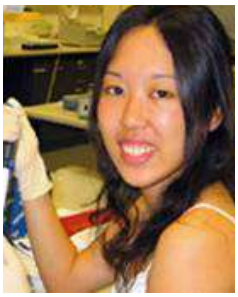

Mini-dysferlinC72: Conjuring a Synaptotagmin Out of the Biological Hat?

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We have studied the recruitment of dysferlin and MG53 to sites of ballistics injury using super-resolution 3D structured illumination microscopy (3D-SIM). I will present evidence suggesting it is the synaptotagmin-like cleavage product of dysferlin, minidysferlinC72 that specifically binds exposed phospholipids at injury sites, forming a highly organized repair lattice with MG53. Injury-activated cleavage of dysferlin is calcium dependent, sensitive to calpain inhibitors, and occurs most efficiently at neutral pH. Together, our results collectively point to calpain-cleavage of dysferlin as a fundamental response to membrane injury in multiple cell lineages. I will present new data refining the calpain cleavage site within dysferlin, adding another layer of complexity to the current paradigm of defective membrane repair in dysferlinopathy.

Work supported by the Jain Foundation

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Dysferlin Stimulates Muscle SNARE-Mediated Vesicle Fusion

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We have utilized recombinant dysferlin proteins in several *in vitro* assays to better understand how dysferlin modulates membrane fusion events *in vivo*. Importantly, recombinant full-length dysferlin stimulated fusion between muscle v-SNARE- and muscle t-SNARE-containing vesicles in a dose-dependent and calcium-dependent manner. Dysferlin likely regulates fusion in part by acting on muscle SNARE proteins: recombinant dysferlin directly bound the t-SNARE protein heterodimer SNAP23/syntaxin 4 in a pull-down assay and preferentially aggregated t-SNARE-containing vesicles, all in a calcium-dependent manner. Dysferlin may also facilitate fusion by directly modulating membrane lipids: purified recombinant GFP-dysferlin mixed with fluorescent (protein-free) vesicles localized to areas of membrane tubulation upon the addition of calcium, suggesting dysferlin can induce or stabilize extreme membrane curvature. Together, these data indicate that dysferlin performs a primary role in muscle membrane fusion and offer mechanistic insight into the membrane repair deficit in dysferlinopathies.

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Evaluating Cellular Repair Potential in Skeletal Muscle

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The ability to repair disruptions in the plasma membrane is critical for maintaining cellular integrity and health; this is especially true for cells that routinely experience membrane damage as a result of activity such as skeletal muscle. We are developing techniques to evaluate the repair potential of dysferlin null skeletal muscle fibers and myotubes using fluorescence assays of dye-leakage, dye-uptake, and calcium activity. In isolated muscle fibers or myotubes containing the indicator calcein red-orange and subjected to IR laser wounding, the amount of dye-leakage is measured using confocal microscopy. Upon wounding repair-competent fibers, a "puff" of dye is released at the wound site and dye leakage ceases within 60 seconds. Under conditions where resealing is inhibited (i.e. low extracellular calcium) a large amount of dye is lost; leakage continues for >10 minutes. Problems associated with the use of FM-143 have been overcome using the nuclei-staining indicator Sytox Green for measuring dye-uptake. Changes in intracellular calcium are measured using a combination of the calcium indicator dyes fluo-4 and Fura Red. Upon wounding, a rise in calcium is observed at the wound site. Calcium either returns to the fiber or myotube pre-wound level following successful resealing, or in the absence of resealing remains high and spreads through the fiber or myotube. Dysferlin null muscle is repair competent but differs in the developed calcium load and wounding threshold compared to dysferlin positive muscle. Since our overall goal is to develop interventions that boost cellular repair potential, quantifiable metrics of this complicated cellular process are required when testing the efficacy of repair-targeted treatments.

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Actin Cytoskeleton Regulates Membrane Remodeling During Cell Membrane Repair

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To understand the sub-cellular processes involved in repairing injured muscle cells we have compared the cell surface proteome of injured and uninjured cells. This identified various organelles that can participate in the repair of injured cell membrane. Additionally, it suggested a preferential involvement of actin in the process of cell membrane repair. Use of real time imaging of cytoskeleton during cell membrane repair confirmed that injury dynamically regulates local polymerization and depolymerization of cortical F-actin. This is not the case for microtubules. Remodeling of actin is associated with annexin and dysferlin trafficking and is critical for the repair of both, large and small injuries. Additionally, during repair of large injuries, cortical actin remodeling enables cleavage of the damaged cell membrane from the rest of the cell. Blocking actin remodeling blocks cleavage of the damaged cell membrane and prevents repair of the injured cell. Our findings suggest that local control of actin remodeling and membrane trafficking regulates cell membrane repair.

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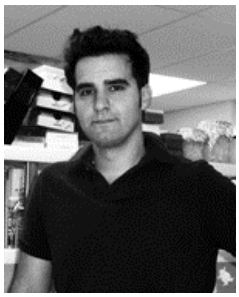

Dysferlin and Myoferlin in Hyperproliferative, Non-Mechanically Active Tissues: Do They Really Regulate Sarcomere Repair?

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The muscle weakness observed in dysferlinopathies is generally considered to be the result of blunted dysferlin-dependent application of patches to a damage sarcomere. Many reports have shown robust dysferlin expression at the sarcomere, although these data are a likely the result of 'fixation artifacts', casting doubts about the role of dysferlin as a direct membrane repair protein. Moreover, our laboratory has shown that the blood vessels that perfuse skeletal muscles show little to no dysferlin or myoferlin presence at the plasma membrane, but rather that they regulate transmembrane protein translocation to the plasma membrane. Providing further evidence about the confusing actions of ferlins in mammalian cells, our most recent report (AmJPathology 2013) documents the unexpected expression of dysferlin and myoferlin in human and animal breast cancer cells and tumors, characterized by exceptionally high rates of cellular proliferation and complete lack of mechanical properties. However, profound differences between the two ferlins were found: while dysferlin knock-down had no effect on tumorogenesis, myoferlin knock-down decreased cancer cell proliferation *in vitro* and tumor burden in mice. Hence, cancer epithelial cell lines might prove to be valuable tools to study ferlins, and that dysferlin and myoferlin are profoundly heterogeneous in their capacity to regulate proliferation.

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NCX1 and Reverse Mode Ca^{2+} Entry Promotes Muscular Dystrophy in Mice

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Muscular dystrophy typically arises from a diverse series of mutations in genes that comprise or assist the dystrophin-glycoprotein complex to provide stability to the muscle cell membrane (sarcolemma). Destabilization or inefficient repair of the sarcolemma is thought to promote unregulated calcium entry that directly induces myofiber necrosis and muscle degeneration. However, the calcium hypothesis of disease in muscular dystrophy remains controversial. Accordingly, here we investigated the role of sodium-calcium exchanger 1 (NCX1) and observed that its expression was dramatically increased in dystrophic skeletal muscle from d-sarcoglycan (Sgcd-/-) deficient mice (and mdx and dysf-/-). Hence, we generated skeletal-muscle specific NCX1 transgenic mice to model this increase in NCX1 expression, which induced a dystrophic-like disease state in all hind limb musculature, but not the diaphragm. The NCX1 transgene also dramatically exacerbated the muscular dystrophy phenotypes in the hind limb musculature of Dysf-/-, mdx, and Sgcd-/- mouse models. In contrast, muscle-specific deletion of the Slc8a1 (NCX1) gene diminished hind limb pathology. In contrast to the effects observed in the hind limb with NCX1 overexpression and worsening disease, NCX1 expression in the diaphragm was protective and rescued MD disease. Mechanistically, we show that NCX1 operates in reverse mode in hind limb musculature to promote greater calcium entry, while in diaphragm NCX1 maintains forward mode operation to efflux calcium and protect from disease. Indeed, Na+/K+ ATPase a2 heterozygous mice, which have reduced sodium clearance rates, showed exacerbated disease in the hind limbs of NCX1 TG mice given even greater predicted reverse mode activity of the exchanger. Hence, NCX1 activity promotes muscular dystrophy in hind limb musculature through reverse mode operation. Inhibitors of reverse mode operation, or enhanced sodium clearance strategies, would represent novel therapeutic avenues for this disease.

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Glycerol Sensitivity from Loss of Dysferlin and Myoferlin

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Dysferlin is a membrane-associated protein found in vesicles and also at the transverse (T-) tubules of muscle. The precise role of dysferlin has been debated, in part because of the mild phenotype in dysferlin null mice. To address this, Dysf129 mice were bred to mice lacking myoferlin on the similar 129SvJ background to generate mice lacking both myoferlin and dysferlin, referred to as FER. Comparison of muscle morphology shows that FER animals display progressive muscle damage with myofiber necrosis, internalized nuclei and, at older ages, chronic remodeling and increasing creatine kinase levels. These changes were most prominent in proximal muscles of the hind limb, paraspinal, and abdominal muscles and were more severe than Dysf129 mice. Ultrastructural studies uncovered progressive sarcoplasmic reticulum dilation as well as ectopic and misaligned T-tubules FER skeletal muscle. FER muscle, as well as Dysf and Myof, exuded lipid, and serum glycerol levels were elevated in FER and Dysf mice. Glycerol injection into muscle is known to induce myopathy, and glycerol exposure promotes "pinching-off" of T-tubules, referred to as de-tubulation. FER muscle was highly susceptible to glycerol exposure *in vivo* and *in vitro*, demonstrating a defective sarcotubule system and a role in muscular dystrophy.

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Skeletal Muscle Glucose Utilization Depends on Dysferlin

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Mutations in the dysferlin gene cause adult onset muscular dystrophy. Children are unaffected and often athletic. During adolescence, skeletal muscle metabolism changes from a primarily oxidative to a more glycolytic type. We wondered about a role of dysferlin in muscle metabolism. Glucose metabolism, glucose tolerance and glucose uptake were studied in dysferlin-deficient B6.A/J-Dysf^{prmd} mice and in primary human myotubes. Our data reveal that skeletal muscle glucose utilization depends on dysferlin. Loss of dysferlin leads to an age dependent increase in lipid oxidation as indicated by reduced respiratory quotient and increase in α-ketoglutarate. Control experiments on dystrophin deficient mdx mice demonstrated that our findings were specific for dysferlin.

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Dysferlin-Deficient Myoblasts Show an Attenuated Response to Pro-Inflammatory Macrophages Due to Upregulated TGFβ1 and IL-1β Signaling

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Limb-girdle muscular dystrophy 2B results from mutations in dysferlin and is characterized by inflammatory foci and macrophage infiltration. During an acute injury, classically activated (M1) and alternatively activated (M2a) macrophages are sequentially recruited to remove necrotic cells, and facilitate regeneration of muscle fibers, respectively. This process is modified in dysferlin-deficient muscle, in which there are ongoing rounds of necrosis and regeneration. The aim of these studies is to understand the interactions between macrophages and myoblasts, in order to improve the targeting of treatments that potentiate regeneration by shifting the balance away from the pro-inflammatory M1 macrophage and towards the M2a macrophage phenotype. We hypothesized that the interactions between muscle and macrophages will be perturbed in dysferlin-deficient muscle. Thus, we tested the effect of these macrophage phenotypes on muscle regeneration in dysferlin-deficiency using an *in vitro* co-culture approach. WT or A/J myoblasts were differentiated in the presence of M1 and M2 macrophages. Myogenesis was assayed by calculating the myogenic index. Gene expression profiles were obtained using Illumina gene arrays. Supernatants were assayed for secreted cytokines and chemokines. Gene arrays show that co-culture with M1 macrophages in WT myoblasts activates pro-inflammatory networks and down-regulates MyoD-activated muscle specific genes. The robust response to M1 is attenuated in A/J myotubes. In contrast, the response to M2 co-culture in WT myotubes does not activate pro-inflammatory networks. In A/J myotubes, the response to M2 co-culture includes up-regulation of muscle-specific genes. Additionally, WT and A/J cells differ in their response to IL-1β, a key mediator of macrophage activation. Upregulated pro-inflammatory networks in dysferlin-deficient myoblasts decrease their regenerative capacity and lessen their response to external signals from macrophages that promote myogenesis. Our studies suggest potential therapeutic approaches in modifying the responses to these macrophages and promoting regeneration.

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The Absence of Dysferlin Results in ER Stress, RyR Redistribution, and Mitochondrially-Mediated Myofiber Death

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We hypothesized that ER/SR stress is elevated in dysferlin-null muscle, and that mitochondrially-mediated myofiber death occurs following injurious lengthening contractions. We found by immunoblots that the ER stress marker CHOP was elevated in dysferlin-null A/J muscle compared to control A/WySnJ muscle. We subjected the *tibialis anterior* muscle of A/J and A/WySnJ mice to *in vivo* dynamometry and injurious lengthening contractions, and found no difference in tetanic torque before injury or at 10 minutes and 3 hours thereafter. At 6 hours and beyond, torque declined in A/J muscle, whereas A/WySnJ muscle continued to recover. In A/J muscle, organization of RyR was altered in >75% myofibers 10 minutes after injury; disruption to DHPR and desmin increased 3 hours later. Immunolabeling of cytochrome-c and complex IV showed that the intermyofibrillar mitochondrial network in A/J muscle becomes condensed 3 hours after injury; and that at 6 hours after injury, cytochrome-c is released into the myoplasm. Between 6 hours and 12 hours after injury, TUNEL+ apoptotic nuclei increased from 72±21 nuclei/mm^2 to 247±48 nuclei/mm^2 in A/J muscle, but only from 12±7 nuclei/mm^2 to 77±28 nuclei/mm^2 in A/WySnJ muscle. Injurious lengthening-contractions cause changes in mitochondria associated with cell death in dysferlin-null myofibers, which may be linked to ER/SR stress and RyR instability.

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Elevated Protein Thiol Oxidation and Metabolic Disturbance in Dysferlin-Deficient Myofibers of Mice and Humans

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Dysferlin is a Ca^{2+} binding protein that plays an important role in membrane vesicle trafficking in skeletal muscle. Lack of dysferlin can lead to disregulation of cellular Ca^{2+} that can drive an increase in reactive oxygen species (ROS) production by mitochondria. ROS can cause cellular damage by directly and irreversibly damaging macromolecules such as proteins, membrane lipids and DNA. Another major cellular consequence of ROS is the reversible modification of protein thiol side chains that can affect many aspects of molecular function. In dysferlin-deficient muscles of A/J mice at various ages, we show high levels of lipofuscin (a measure of cumulative oxidative damage) plus significantly increased protein thiol oxidation within myofibers, yet minimal myofiber necrosis [1]: This contrasts with the situation for dystrophin-deficient mdx mice where necrosis is a major feature of the disease. We also identify striking changes in histopathology, indicating severe metabolic disturbance, of dysferlin-deficient muscles for mice (A/J, BlaJ, SJL/J) and humans. [1]. Terrill JR, Radley-Crabb HG, Iwasaki T, Lemckert FA, Arthur PG, Grounds MD. (2013) Oxidative stress and pathology in muscular dystrophies: focus on protein thiol oxidation and dysferlinopathies FEBS J. Jan 19. doi: 10.1111/febs.12142.

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Protein Expression Changes with Disease Status in the BlaJ Mouse

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We have used mass spectroscopy to compare the proteome of a WT mouse labeled with C14 lysine – a SILAM mouse – with that of a non-labeled BlaJ mouse. This permits analysis of the relative amounts of signature peptides and thereby of the proteins from which these peptides were derived in the two strains. Six month old BlaJ mice with active pathology expressed, as expected, an excess of inflammatory proteins, but also an excess of structural proteins, which was not expected. This is reinforced by preliminary data from a separate investigation, showing that nuclear and fibrous actin content of muscle isolated from pre-symptomatic 4 month BlaJ mice contained fewer myonuclei per fiber and lower amounts of fibrous actin per fiber than age-matched C57Bl6 controls implying defects in muscle growth. Surprisingly, fibers of the 6 month old BlaJ mice contained more myonuclei and more fibrous actin per nucleus than WT. This accords with the proteomic investigation: signifying a hypertrophic response in association with the onset of disease. The nature of the stimulus to this hypertrophic component is of interest for future investigations.

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***In Vivo* Imaging of Molecular Interactions at Damaged Sarcolemma Reveals Unexpected Complexity in Repair Patch Formation**

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Muscle cells have a remarkable capability to repair plasma membrane lesions. Mutations in dysferlin (Dysf) are known to elicit a progressive myopathy in humans, probably due to impaired sarcolemmal repair. We show here that loss of Dysf and annexin A6 (Anxa6) function lead to myopathy in zebrafish. By use of high resolution imaging of myofibers in intact animals, we reveal sequential phases in sarcolemmal repair. Initially, membrane vesicles enriched in Dysf together with cytoplasmic Anxa6 form a tight patch at the lesion independently from one another. In the subsequent steps, annexin A2a (Anxa2a) followed by annexin A1a (Anxa1a) accumulate at the patch - the recruitment of these annexins depends on Dysf and Anxa6. Thus, sarcolemmal repair relies on the ordered assembly of a protein-membrane scaffold. Moreover, we provide several lines of evidence that the membrane for sarcolemmal repair is derived from a specialized plasma membrane compartment.

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Long-term Enzymatic, Histological and Behavior Study of the BlaJ Dysferlin Deficient Mouse Model

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The development of a treatment for dysferlinopathy requires validation in animal models and the BlaJ dysferlin deficient mouse model generated by Dr. I. Richard and colleagues provides an invaluable resource. To date no comprehensive report exists thoroughly characterizing the BlaJ mouse model which is prerequisite to therapeutic studies in order to define consistent and universal disease parameters. In this work, we have performed enzymatic, histological, and behavioral analyses on a large cohort of BlaJ and wild type (WT) mice for over 1.3 years. At all time points tested, elevated levels of circulating creatine kinase and central nucleated myofibers were detected, along with compromised sarcolemmal integrity in the BlaJ mice in a gender-independent manner. Regarding our behavioral analyses, in general, the emergence of significant deficits became apparent at approximately 1 year old for challenges including the roto-rod, a marble bury test, grip strength, and swimming speed. Earlier observations of decreased performance for the BlaJ mice were evident during extended monitoring of overall exploration and of rearing activity, as well as in alterations in stride length. Phenotypically, the older BlaJ mice developed symetrical large fat deposits creating a distinct “saddlebag” appearance in the hip region often accompanied by tumors associated with leg muscle. However, at 18 months of age there remains no significant difference in the life expectancy of the BlaJ mice compared to the WT parents. Collectively, this long-term analysis of a large cohort of WT and BlaJ mice identifies consistent disease parameters that can be indicators of efficacy/toxicity in studies developing emerging technologies for the treatment of human dysferlin deficiency.

Work supported by the Jain Foundation

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Generating Targeted Mutations of Dysferlin in the Zebrafish
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The ability to manipulate the genome is critical to develop and test hypotheses of gene function. A new gene targeting approach, utilizes engineered zinc finger nuclease (ZFN) technology to directly modify specific loci in zebrafish. Our lab is using CompoZr® Zinc Finger Nucleases (Sigma-Aldrich) to generate mutants of many of the human muscular dystrophy related proteins. By injecting zebrafish embryos with RNA encoding ZFN's we hope to generate mutations at high frequency in a locus-specific manner. Importantly, our first verified target is dysferlin, a transmembrane protein implicated in surface membrane repair of muscle cells in human. Mutations in dysferlin cause the progressive muscular dystrophies Miyoshi myopathy, limb girdle muscular dystrophy 2B, and distal anterior compartment myopathy. We have been able to generate multiple stable mutants with both deletions and insertions of the dysferlin gene in less than 9 months. The mutants transmit to the second generation allowing for phenotypic analysis and we will present our preliminary data on the phenotype of the dysferlin null phenotype. We hope our findings will allow us to study the function of dysferlin in living muscle tissue *in vivo* and screen for molecules that may improve the muscle structure.

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Mutagenesis of a Zebrafish Dysferlin Variant Isoform by TALEN Proteins

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Zebrafish are an ideal vertebrate model to develop therapies for disorders such as LGMD2B. We have identified and cloned a variant of fish dysferlin (6.3kb) whose n-terminal region is different. This transcript has high homology (68%) with the human dysferlin variants numbered 8-15. We have found this variant is more highly expressed in the brain, eye, heart, and skeletal muscle than other fish dysferlin isoforms. Using transcription activator-like effector nucleases (TALENs), we have mutated the variant transcript of fish dysferlin. Injection of TALEN encoding mRNA directed against dysferlin resulted in several different somatic mutations in 3-day-old zebrafish embryos. Zebrafish injected with TALENs were cultured to adulthood and tail-clipped. Fish that showed dysferlin mutations in the tail were bred and their offspring were screened to detect germ line transmission. We now have a mutant line with a 5bp deletion and one with an 8bp deletion. We have begun to analyze the RNA stability of the mutant dysferlin variant by doing RT-PCR and Western blotting. We have noticed no distinct muscle phenotype associated with the homozygous mutant zebrafish of both lines early in development and intend to see if any brain or muscle phenotype occurs as the fish grow into adulthood.

Work supported by the Jain Foundation

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Homologous Recombination Mediates Functional Recovery of Dysferlin Deficiency Following AAV5 Gene Transfer

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As with other forms of muscular dystrophy, adeno-associated virus (AAV) gene transfer is a particularly auspicious treatment strategy, however the size of the dysferlin (DYSF) cDNA (6.5 kb) negates packaging into traditional AAV serotypes known to express well in muscle (i.e. rAAV1, 2, 6, 8, 9). AAV5 has demonstrated unique plasticity with regards to packaging capacity and recombination of virions containing homologous regions of cDNA inserts has been implicated in the generation of full-length transcripts. We showed for the first time *in vivo* that homologous recombination following AAV5.DYSF gene transfer leads to the production of full length transcript and protein. Moreover, gene transfer of full-length dysferlin protein in dysferlin deficient mice resulted in expression levels sufficient to correct functional deficits in the diaphragm and importantly in skeletal muscle membrane repair. Intravascular regional gene transfer through the femoral artery produced high levels of transduction and enabled targeting of specific muscle groups affected by the dysferlinopathies setting the stage for potential translation to clinical trials. We are currently finishing long-term pre-clinical studies in mice and non-human primates and will present our strategy for bringing AAV5.DYSF gene transfer to the clinic.

Work supported by the Jain Foundation

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Overview of Compensation Strategies for Therapeutic Approach in Dysferlinopathies

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Mutations in the dysferlin gene are the cause of Limb-girdle Muscular Dystrophy type 2B and Miyoshi Myopathy. The dysferlin protein has been implicated in sarcolemmal resealing, leading to the idea that the pathophysiology of dysferlin deficiencies is due to a deficit in membrane repair. In addition, to test various strategies to transfer the full length dysferlin using AAV vectors (see abstract by Marina Pryadkina), we are investigating the possibility to compensate dysferlin deficiencies using different proteins. First, we generated a transgenic mouse overexpressing myoferlin, the member of the ferlin family closest to dysferlin. The myoferlin overexpressors show no skeletal muscle abnormalities, and crossing them with a dysferlin-deficient model rescues the membrane fusion defect present in dysferlin-deficient mice *in vitro*. However, myoferlin overexpression does not correct muscle histology in vivo. In addition, our data suggest that the pathogenicity of dysferlin deficiency is not solely related to impairment in sarcolemmal repair and highlight the care needed in selecting assays to assess potential therapies for dysferlinopathies. Second, we tested the hypothesis that the Anoctamin 5 protein (ANO5) could compensate for dysferlin absence (see abstract by Karine Charton). As for dysferlin, ANO5 when mutated can lead both to limb-girdle muscular dystrophy or Miyoshi-like myopathy and has been implicated in sarcolemmal resealing. We demonstrated that AAV-mediated transfer of human ANO5 (hANO5) was not able to compensate for dysferlin deficiency in the Dysf^{prmd} mouse model and that hANO5 overexpression does not improve the membrane repair defect seen in the absence of dysferlin. Third, we want to assess the consequences of modulating the expression level of calpain 3, another protein involved in limb-girdle muscular dystrophy, on the myopathology of dysferlin deficiencies. For this purpose, a double knock-out mutant is under generation in our laboratory. In conclusion, our attempts to compensate dysferlin deficiencies have not been successful so far. These observations, together with data generated with minidysferlins, suggest the importance of restoring the totality of dysferlin functions.

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AAV-Mediated Dysferlin Delivery; Observation of a Goldilocks Effect

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The popularity of adeno-associated viral vectors (AAV) for human gene delivery has been demonstrated in over 70 human trials for diverse genetic diseases. In the case of dysferlinopathy, reports demonstrate that AAV gene addition strategies based on the delivery of dysferlin cDNA to skeletal muscles have proven successful in relieving disease phenotypes in a mouse model. Following designation of fAAV as the most efficient AAV large gene delivery strategy, we demonstrated restoration of dysferlin protein in the BlaJ dysferlin deficient mouse model. To quantitatively gauge any potential therapeutic effects of fAAV-Opt-dysferlin transduction, a unique muscle cell labeling technique was validated that allows visualization of muscle degeneration by live imaging. The results demonstrated that dysferlin over-production in WT muscle results in muscle fiber loss, whereas in BlaJ muscle an initial therapeutic effect of dysferlin was observed followed by treated muscle fiber loss in some animals. Currently, dose escalation studies are underway to determine what level of dysferlin abundance is therapeutic/toxic in transduced muscle fibers. In addition, we will present data regarding dysferlin overexpression throughout wild type and dysferlin deficient mice to determine the importance of targeted muscle transduction, muscle-specific expression and the possible requirement of transgene regulation following systemic fAAV administrations.

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Applicability of Exon-Skipping as a Therapeutic Strategy for Dysferlinopathies

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Dysferlinopathies are disabling muscular dystrophies including LGMD2B and Miyoshi myopathy as the main phenotypes. They are associated with molecular defects in DYSF, encoding dysferlin, a key player of sarcolemmal homeostasis. Previous investigations suggest that exon-skipping is a promising therapy for a subset of patients with dysferlinopathies. Such an approach aims to rescue almost functional proteins, when targeting modular proteins and specific tissues. This has been well documented in another myopathy, Duchenne Muscular Dystrophy, for which exon-skipping recently led to encouraging results in phase II/III clinical trials. We intend to translate into pre-clinics our proof of principle of exon-skipping feasibility for dysferlinopathies, and evaluated the dysferlin function recovery following exon 32 skipping in mutated human cells. We will present the preliminary characterization of a newly generated KI mouse model carrying an exon 32 nonsense mutation (as identified in a human patient), which will be used to further evaluate the functionality of the mouse dysferlin deleted of exon 32 in living animals after AON treatment.

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The Phenotype of Dysferlin-Deficient Mice is not Rescued by AAV-Mediated Transfer of Anoctamin 5

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Mutations in dysferlin and anoctamin 5 (ANO5) have been shown to lead to limb-girdle muscular dystrophy types 2B and 2L respectively and both also lead to Miyoshi myopathy. These two proteins have both been implicated in sarcolemmal resealing. On the basis of similarities in associated phenotypes and protein functions, we tested the hypothesis that ANO5 protein could compensate for dysferlin deficiency. We first defined that the main transcript of ANO5 expressed in human skeletal muscle is the 22-exon full-length isoform and we demonstrated that dysferlin deficient mice ($Dysf^{prmd}$) have lower ANO5 expression levels, observation that further enhanced the rational of the tested hypothesis. We constructed an adeno-associated virus (AAV) vector that encoded for human ANO5 and performed intramuscular injection in wild-type (WT) and dysferlin deficient mice. We showed that AAV-mediated transfer of ANO5 did not lead to apparent toxicity in 4 week-old WT mice after 1 month of expression. We then demonstrated that AAV-hANO5 injection does not improve the membrane repair defect as tested by laser-wounding nor muscle histology and does not prevented damage to muscle following eccentric exercise seen in the absence of dysferlin. Consequently, overexpressing hANO5 does not seem to provide a valuable therapeutic strategy for dysferlin deficiency.

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Crystal Structure of Human Dysferlin Inner DysF Domain

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Dysferlin and the other type I ferlins contain a nested DysF double domain, where the inner domain is surrounded by two sections of the outer domain. Our group solved the first three dimensional structure of this family, the inner DysF domain of myoferlin, by NMR (Patel et al, 2008 J.Mol. Biol. 379, 981–990). We have now confirmed the unique nature of this fold by solving the crystal structure of the dysferlin inner DysF domain at a resolution of 1.9 Å. The DysF forms a long two-stranded beta sheet held together by tryptophan and arginine stacks. Most of the dysferlinopathy causing mutations found in the DysF domains fall in these residues, indicating that the integrity of this domain is needed for the function of dysferlin or at least to prevent its degradation. The loop between the two strands is structurally well conserved between dysferlin and myoferlin and the combination of crystal and NMR data gives an idea of the flexibility of regions of this domain. We have also looked at the wider occurrence of the DysF domain in sequences genomes.

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Alternate Splicing of Dysferlin C2A Confers Ca^{2+}-Dependent and Ca^{2+}-Independent Binding for Membrane Repair

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Dysferlin plays a critical role in the Ca^{2+}-dependent repair of micro-lesions that occur in the muscle sarcolemma. Of the seven C2 domains in dysferlin, only C2A is reported to bind both Ca^{2+} and phospholipid thus, acting as a key sensor in membrane repair. Dysferlin C2A exists as two isoforms, the "canonical" C2A, and C2A variant 1 (C2Av1). Interestingly, these isoforms have markedly different responses to Ca^{2+} and phospholipid. Structural and thermodynamic analyses are consistent with the canonical C2A domain as a Ca^{2+}-dependent, phospholipid-binding domain, whereas C2Av1 functions as a Ca^{2+}-independent, membrane-sensing domain. Additionally, both isoforms display remarkably low free energies of stability, indicative of a highly flexible structure. The inverted ligand preference and flexibility for both C2A isoform suggest a complexity in effector interactions ranging from constitutive to Ca^{2+}-regulated interaction, likely relevant to its role as mediator of membrane repair.

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Evaluating Exogenous Lipids for Slowing Progression of Dysferlinopathy

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We are investigating the hypothesis that altering the physical parameters of the lipid bilayer component of skeletal muscle membranes will play a role in controlling disease progression for patients suffering from a dysferlinopathy. Whole fibers are evaluated for membrane resealing using laser wounding and the calcein red-orange leakage assay. In addition to replicating our previous results with DHA strengthening the membrane, we have tried a new class of lipids that are derived from plant material. Chlorophyll itself has a lipid moiety that is cleaved by enzymes in ruminants to give rise to the branched chain fatty acid, phytanic acid. Ingestion leads to partial breakdown of this compound and partial incorporation into phospholipids. In planar bilayers, membrane stability is exceptional. Even the addition of only 5% of diphytanoylPC to a mixture of lipids derived from soybeans leads to significant stabilization of planar phospholipid bilayers. Of all the lipids we have tested on either skeletal myotubes or isolated FDB fibers; the largest effect was obtained with phytanic acid. The results confirm a profound effect of lipid addition to the stability of muscle membranes. We are currently determining the toxicity of this new lipid as a dietary supplement for mice, coupled with lipidomics to determine both the degree of penetration of the dietary lipid and the compensatory changes in the other lipids of skeletal muscle. We are also developing array tomography for determining the degree to which these lipids reverse the disorganization of muscle structure seen in the A/J mouse. In order to move from preclinical to clinical studies, we wish to have a clinical correlate of a therapeutic response to dietary intervention. We have initiated a pilot project to evaluate different biomarker for this purpose, based on intra-patient variations with activity.

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Myostatin: A Molecular Rheostat for Muscle Mass

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Myostatin is a secreted protein that plays a critical role in regulating muscle growth. Myostatin mutations in multiple species have been shown to cause dramatic increases in muscle mass and inhibition of myostatin activity in adult mice has been shown to induce muscle fiber growth. As a result, there has been considerable interest in the possibility that agents capable of targeting this pathway may have clinical applications in disease states characterized by muscle loss. Indeed, numerous studies have now documented the potential benefits of targeting this pathway in preclinical models of muscle diseases and at least five companies have initiated clinical trials testing myostatin inhibitors. Although the potential benefits of targeting this pathway seem to be clear, there are two general concerns about using this therapeutic approach in the setting of muscle degeneration. First, there is the concern that targeting myostatin signaling may lead to premature exhaustion of satellite cells and thereby accelerate the rate of disease progression. Second, there is the concern that inducing muscle hypertrophy may cause additional stress on myofibers that are already suboptimal and thereby cause additional myofiber damage. In my talk, I will summarize our recent studies addressing these issues.

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Membrane Stabilizing Properties of Δ-9,11 Glucocorticoid Analogues (VBP Compounds) are Beneficial to Dystrophic Skeletal Muscle

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We have recently shown that VBP compounds are effective in reducing inflammation in two mouse models of muscular dystrophy; mdx (dystrophin-deficiency) and SJL (dysferlin-deficiency). Daily oral administration of VBP-3 compound showed reduction of muscle inflammation and improvements in multiple muscle function assays without the side effects on body weight or spleen size, that are typically seen with glucocorticoids (Baudy et al., 2012). ReveraGen's lead compound VBP-15 is developed to have better plasma membrane stabilizing properties via compression of the head groups in the lipid bilayer by decreasing permeability while disordering the hydrophobic core. *In vitro* experiments demonstrate that VBP-15 not only shows resistance to laser-induced injury but also enhances membrane repair after injury. Therefore, we have hypothesized that membrane stabilization of dystrophic muscle with VBP-15 before the spontaneous necrotic peak would reduce inflammation and improve muscle function in mdx mice. To test this we have treated mice starting from the pre-necrotic stage for 6 weeks and found significant improvements in muscle strength and inflammatory disease phenotypes. *Ex vivo* procion orange uptake by EDL muscle in response to contraction-induced injury during lengthening contraction protocol indicates a dose-dependent decrease in the number of necrotic muscle fibers in VBP treated mice. This data suggests that the membrane stabilization properties of these compounds provide beneficial effects in vivo in dystrophic mice. ReveraGen, in collaboration with MDA and NIH/NCATS is pursuing clinical development of VBP-15 for the treatment of Duchenne muscular dystrophy.

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Diltiazem Protects Dysferlin-Null Muscle from Injury *in vivo* and *in vitro*

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Treatments for dysferlinopathies in man are not yet available. Our studies have shown that dysferlin is present in t-tubules (TT), which are extensively damaged when muscles lacking dysferlin are injured. Our results also indicate that the damage caused by injury *in vitro* requires extracellular Ca^{2+}. We tested the possibility that injury of dysferlin-null muscle is mediated by Ca^{2+} influx through the L-type Ca^{2+} channel (LTCC), concentrated in TT at triad junctions. We cultured FDB myofibers from A/J and control mice and subjected them to injury by osmotic shock (OSI) in the presence or absence of the LTCC blocker, diltiazem (10µM). We assayed TT disruption, and changes in Ca^{2+} transients and baseline Ca^{2+}. Diltiazem protected A/J fibers from OSI-induced changes in TT structure and Ca^{2+} regulation. We also assayed diltiazem's effect on A/J muscle subjected to large strain eccentric injury (LSI) *in vivo*. Diltiazem injected daily for 7d prior to LSI and immediately before and after LSI protected TT from LSI-induced disruption. Diltiazem also improved torque recovery and reduced necrosis and inflammation at 3 d post-LSI. Our results suggest diltiazem treatment as a potential therapeutic option for LGMD2B and MM patients.

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Bone Marrow Transplantation in Dysferlin Deficient Mice Results in a Mild Functional Improvement

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Dysferlinopathies are caused by mutations in the DYSF gene. Dysferlin is a protein mainly expressed in skeletal muscle and monocytes. Cell therapy constitutes a promising tool for the treatment of muscular dystrophies. The aim of our study was to evaluate the effect of bone marrow transplantation (BMT) using the A/J Dysf^{prmd} mouse model of dysferlinopathy after 12 weeks of treatment. We studied dysferlin expression by Western blot (WB) and/or immunohistochemistry in transplanted mice and controls. Computerized analysis of locomotion and electrophysiological techniques were also performed to test functional improvement. We observed dysferlin expression in splenocytes but not in the skeletal muscle of the transplanted mice. However, locomotion test, electromyography studies and muscle histology showed an improvement in all transplanted mice that was more significant in the animals transplanted with dysferlin+/+ cells. In conclusion, although BMT restores dysferlin expression in monocytes but not in skeletal muscle, muscle function was partially recovered. We propose that the slight improvement observed in the functional studies could be related with changes in the inflammatory response due to dysferlin expression in monocytes and/or factors released after BMT that enhanced muscle regeneration. Future experiments will unravel whether the effect of BMT persists six months after treatment.

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Repeated Administration of Polyvalent Immunoglobulins Improves Muscle Strength in Dysferlin-Deficient SJL/J Mouse

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Muscle pathology in dysferlinopathy is characterized by the presence of widespread complement activation and conspicuous inflammation, suggesting that immunomodulatory drugs could be effective. We pre-clinically investigated the potential benefit of polyvalent immunoglobulins (po-Igs) in dysferlinopathy by subjecting dysferlin-deficient SJL/J mice to 5 consecutive weekly infusions of 2mg/g human po-Igs. Evaluation included (i) traction strength measurement; (ii) spontaneous activity quantification; and (iii) quantification of histopathogical injuries. Po-Igs administration induced a significant increase of forelimbs strength. Hind limb strength, spontaneous activity and muscle pathology remained unchanged. In conclusion, these results provide the first evidence for a potential therapeutic efficiency of po-Igs in dysferlinopathy. Further studies are needed to determine the actual place of po-Igs and other immunomodulatory drugs in Miyoshi/LGMD2B patient care.

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Clinical Evaluation of Patients with Dysferlinopathy from a LGMD Natural History Study

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Dysferlin deficiency results in the clinical phenotypes of limb-girdle muscular dystrophy (LGMD) 2B and Myoshi myopathy. Participants in this study were part of a larger natural history study with the Cooperative International Neuromuscular Research Group (CINRG) of LGMD, which included participants with LGMD2A (Calpain-3 deficiency), LGMD2I (FKRP deficiency We also had age matched disease controls (Becker Muscular Dystrophy) and disease free controls. We analyzed clinical and serum biomarkers in 11 participants with LGMD2B. Evaluations included strength testing, functional testing, 6 Minute Walk Test (6MWT), timed tests (climb 4 stairs, sit to stand), and pulmonary function testing (sitting and supine). Cardiac function, serum creatine kinase levels, serum growth factors and cytokine levels, and self-reported questionnaires of their genetic understanding of their disease, quality of life and activity limitations were assessed after the clinical evaluations. A total of 11 LGMD2B participants (6 male, 5 females) ranged in age from 26.69 years to 47.43 years (mean age 36.8 +/- 7.4 years) with 9/11 being ambulatory. The 6MWT ranged from 59.5-620 meters (328.4 +/- 183.6 meters). Greater variability existed in the velocity to climb 4 stairs (0.88 steps/sec +/-0.45) compared to normal controls, 2.03 steps/sec +/- 0.3). Grip strength of controls was nearly twice that of LGMD2b of 87.94 lb +/-21.14) compared to 48.52 lb+/- 25.46. Overall, lower extremity strength was more impaired than upper extremity strength. Preliminary analysis of pulmonary and cardiac function showed 3 who had pulmonary forced vital capacity <75% and only one who had a cardiac ejection fraction <50. This study provides baseline measures of strength and function differences in LGMD2B compared to age matched normal and disease with less impact on cardiac and pulmonary function. Future study aims will follow these measures longitudinally to evaluate disease progression of dysferlin deficient muscular dystrophy.

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Dysferlin Carriers Frequency in the General Population

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Dysferlin is expressed in skeletal muscle and also in peripheral blood CD14+ monocytes. We have reported a good correlation between the expression in skeletal muscle and monocytes. Due to its minimal invasivity, this method gives us the chance to study dysferlin protein expression in the general population. The aim of this work was to study the frequency of carriers of a mutation in DYSF in the general population. For this purpose, 100 blood samples have been collected from volunteers. In blood, dysferlin is expressed only in monocytes, which represent 3-7% of total blood cells. CD14+ cells were isolated to study the expression of dysferlin in this subpopulation of cells at the protein level. The results obtained so far show that 6/80 subjects display levels of dysferlin compatible with those of confirmed dysferlinopathy carriers (< 70%). The presence of mutations in DYSF in these subjects is under study. In conclusion, the frequency of carriers of dysferlinopathy in the general population is higher than expected. These results indicate that: 1) the number of asymptomatic patients would be higher than expected or 2) Low levels of dysferlin may be reduced in some patients with no mutations in DYSF due to unknown factors.

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Clinical and Genetic Characterization of Dysferlinopathy in the Chilean Population: Preliminary Results

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Mutations in the dysferlin gene lead to limb girdle muscular dystrophy 2B, Miyoshi myopathy and several other clinical myopathic phenotypes. A cohort of Chilean patients affected by dysferlinopathy is described. Based on clinical findings or absence of dysferlin in muscle biopsy, 39 patients suspected to have dysferlinopathy were selected. Assessment workup consisted of clinical evaluation, Motor Function Measure (MFM) scale, CPK levels, electrodiagnostic testing, whole body MRI, echocardiogram, basal spirometry and direct sequencing of the DYSF gene. We identified 29 patients from 22 non-related Chilean families harboring point mutations in DYSF. Eight mutations were consistently found in the cohort, four of which (c.5979dupA; c.2858dupT; c.2779delG and c.4390G>T) accounted for 78% of the total, these are considered recurrent mutations. In three patients only one mutation was found after complete DYSF gene sequencing. Symptoms at onset ranged from 10 to 33 years, invariably as weakness in lower legs, either distally (21/29) or in the pelvic girdle (8/29), progressing later to upper limbs. Electrodiagnostic assessment showed consistently normal NCV and repetitive stimulation testing, with distinct degrees and distribution of myopathic changes on needle EMG. Single fiber EMG was normal in four confirmed dysferlinopathy patients. Muscle impairment observed through MRI in 27/29 was proportional to disease duration, but distribution of affected muscles was similar, despite of clinical phenotype. MFM scale score in 26/29 patients at different disease's stages showed major impairment for standing and transfers and axial and proximal motor function, with relative sparing of distal motor function. Cardiac and respiratory evaluation was normal in all patients assessed (50%). The clinical spectrum of dysferlinopathy in the series is in agreement with similar cohorts reported. The relative high frequency of some mutations suggests a founder effect for such mutations in the Chilean population. Funding: FONDECYT grant #1110159 (Conicyt, Chile).

Notes: