4th Annual Dysferlin Conference
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1. Introduction

The annual Dysferlin Conference [1] brings together scientists and clinicians working to understand and develop a therapy for the dysferlinopathies, Limb Girdle Muscular Dystrophy 2B (LGMD2B) and Miyoshi Myopathy. These diseases, caused by mutations in dysferlin, are characterized by progressive muscle wasting beginning in the late teenage years [2,3]. Although dysferlin has been implicated in repair of muscle fiber membrane tears [4,5], a more complete understanding of dysferlin's function and role in pathology is required for effective development of therapies. The objective of the 4th Annual Dysferlin Conference, held from September 11-14, 2010 in Bellevue, WA, USA, was to speed research on dysferlinopathy by promoting the sharing of ideas and unpublished data between scientists actively working on dysferlin and other relevant therapeutic approaches. The meeting implemented a novel conference format with an informal lab-meeting atmosphere to encourage the open exchange of information that typically exists within individual laboratories, as well as a unique agenda in which speakers presented individual scientific results, rather than full-length presentations, in relevant topic-oriented sessions to ensure a more logical flow of information and discussions. The conference was sponsored and organized by the Jain Foundation, and participants included 36 invited speakers, 16 additional

scientists and clinicians, 26 poster presenters, 4 dysferlinopathy patients, 2 representatives of other muscular dystrophy foundations, and 7 members of the Jain Foundation scientific team. Research was presented from 10 countries around the world.

2. Session I: What Does Dysferlin Look Like?

This session focused on understanding the structure and organization of the dysferlin protein.

R. Bryan Sutton (USA) presented the newly determined atomic-resolution crystal structure of the C2A domain of human dysferlin, which has a beta-sandwich fold similar to that of the human myoferlin C2A domain [6]. The asymmetric unit contains two dysferlin C2A trimers, each arranged as an equilateral triangle, although there was no indication of oligomerization by light scattering at low concentration in solution. The electrostatic surface of each C2A trimer is strikingly polarized, with the two opposing faces of the triangular structure almost entirely oppositely charged, which could promote interaction with lipid membranes. In addition, molecular dynamics analysis of the C2A domain suggests that it binds two Ca^{2+} ions and contains two putative lipid-binding residues. Dr. Sutton also discussed ongoing efforts in Thomas Walz's laboratory (USA) to visualize the structure of full-length dysferlin protein by electron microscopy. Preliminary micrographs of purified mouse dysferlin reveal that the protein most commonly adopts a folded U-shape structure.

Sandra Cooper (Australia) presented a phylogenetic analysis of ferlin genes, which revealed that ferlins are present in almost all eukaryotes, but not in prokaryotes [7]. This phylogenetic correlation with the existence of intracellular membranes is consistent with the presumed role of ferlins in intracellular membrane trafficking. Structurally, all ferlins have at least two C2 domains immediately preceding the transmembrane domain at the C-terminus, and a more N-terminal FerI motif sandwiched between two other C2 domains (C2B and C2C in human dysferlin). Almost all ferlins also contain a FerB motif following the C2-FerI-C2 sandwich, and some ferlins (including human dysferlin) also contain a FerA motif and DysF domain surrounding the FerB motif. Most organisms have at least one DysF-containing ferlin and one non-DysF-containing ferlin.

3. Session II: Where is dysferlin located?

This session addressed the localization and trafficking of dysferlin in muscle and other cell types, as well as changes in subcellular localization in response to injury and other stimuli.

Robert Bloch (USA) presented immunohistochemical studies of the intracellular localization of dysferlin in rat myofibers, using antigen retrieval at elevated temperature to enhance dysferlin staining in interior compartments. In longitudinally-sliced myofibers from an uninjured rat tibialis anterior muscle, dysferlin co-localizes with desmin on either side of the Z-lines. Dysferlin partially co-localizes with dystrophin on the plasma membrane, and also partially co-localizes with the dihydropyridine receptor (DHPR) in the cell interior, suggesting T-tubule localization. High co-localization of venus-dysferlin with the membrane dye Di-8-Anepps in

cultured FDB myofibers is also consistent with dysferlin localization in the T-tubules. Dysferlin appears to be localized in unique domains near the A-I junctions between the T-tubules and the sarcoplasmic reticulum.

Steve Laval (UK) reviewed studies of dysferlin localization in various cell types [8,9]. In C2C12 myotubes, GFP-dysferlin localizes to the developing T-tubule system and is trafficked to the cell membrane in response to wounding [8]. Dysferlin co-localizes with Caveolin-3, and Caveolin-3 mutants can cause retention of dysferlin in the Golgi apparatus [10,11]. Expression of dysferlin in myeloid cells was also investigated. Dysferlin is expressed specifically in non-classical monocytes in mice (the Ly6-Clo/F4-80hi subset, equivalent to the CD14+/CD16+subset in humans). Dysferlin may play a role in the transformation of monocytes/macrophages from pro-inflammatory to anti-inflammatory following muscle injury, accounting for the abnormal inflammatory response observed in dysferlin deficiency.

Sandra Cooper (Australia) presented data on the localization and trafficking of normal dysferlin and several dysferlin deletion constructs. Dysferlin co-localizes with mitsugumin 53 (MG53), and both localize to longitudinal T-tubules along with annexin A1 (but not caveolin-3 or spectrin). Full-length dysferlin has a half-life of only about five hours before degradation, and slightly less than three hours on the plasma membrane before being endocytosed [12]. Many mutant dysferlin proteins, for instance the L344P patient mutation and constructs lacking one or more C2 domains, have enhanced rates of endocytosis. Comparing many dysferlin deletion constructs revealed that the C2B-FerI-C2C domain sandwich regulates membrane localization

and endocytosis. Finally, Dr. Cooper showed that mutant dysferlin follows the same endocytosis and degradation pathway as syntaxin-4.

Gillian Butler-Browne (France) has studied the "secretome" of muscle satellite cells as they fuse into myotubes; the secretome contains secreted soluble proteins/peptides as well as microvesicles exocytosed from the cells. About 72% of the proteins detected in the secretome are non-soluble, vesicle-associated proteins. Proteins of the secretome may play a role in intercellular signaling during myogenesis and muscle regeneration, and Dr. Butler-Browne's group has shown that secreted microvesicles can fuse with adjacent cells. Dysferlin, as well as MG53, is present in the microvesicles; thus it may have a role in intercellular signaling to coordinate muscle formation and maintenance.

4. Session III: What Happens When Dysferlin is Missing?

This session focused on characterizing the cellular and muscle-level phenotypic changes that occur in the absence of dysferlin in patients and animals.

Miranda Grounds (Australia) presented the hypothesis that the cell membrane of growing muscle fibers is more dynamic and/or fluid (to accommodate the large increase in surface area during growth) than the membrane of mature muscle fibers, which could make growing muscle better able to prevent or repair membrane tears and thus explain the onset of dysferlinopathy symptoms around the end of puberty. Many factors differentially affect growing and mature muscle fibers: for example, IGF-1 causes myofiber hypertrophy only in growing muscle [13] and

absence of dysferlin causes degeneration only in mature muscle. Dr. Grounds emphasized that cultured myotubes (a growing muscle cell) may not reflect the situation in adult myofibres *in vivo,* as indicated by differences in subsarcolemmal localization of Ca^{2+} [14]; thus careful selection of models is required to study mature post-growth myofibres.

Bradley Williams (USA) presented an analysis of the clinical history of 420 dysferlinopathy patients. About 77% of patients experienced their first symptoms between ages 15 and 30, confirming that the typical age of onset is around the end of puberty. There were no statistically significant differences in the average onset age or average time between onset and wheelchair use between male and female patients, but the age-of-onset distribution for females was broader than that for males; in other words, females were more likely than males to have particularly early or late onset. As a function of age, the number of female patients with symptoms begins to rise sharply about two years earlier than the number of male patients with symptoms, consistent with the hypothesis that onset is correlated with cessation of muscle growth, since females on average stop growing two years earlier than males.

Joseph Roche (USA) characterized the recovery and response of muscles after injury induced by 15 large-strain lengthening contractions (described in [15]) in 3-month old dysferlin-deficient mice. He tested several dysferlin-deficient mouse models and found that all models have significantly increased necrosis and immune cell infiltration in their injured muscles 3 days after injury when compared to control mice [16]. Despite the fact that dysferlin-deficient mice are not more susceptible to the initial injury than control mice, recovery of contractile torque after injury is significantly slower in dysferlin-deficient mice [17].

Katie Maguire (USA) discussed two new mouse models that can be used to study the rates of muscle degeneration and regeneration in live dysferlin-deficient mice non-invasively over time. The first strain of dysferlin-deficient mice expresses luciferase only in Pax7-expressing cells (muscle satellite cells and their progeny) and only after tamoxifen injection (as described in [18]), so that total fluorescence intensity in muscles of these mice is correlated to the amount of regeneration they have undergone. The second strain of dysferlin-deficient mice expresses luciferase in MRF4-expressing cells, so that fluorescence intensity is correlated to the amount of remaining muscle. Preliminary data using the first mouse strain suggest an increased rate of regeneration in dysferlin-deficient versus wild-type mice, detectable as early as 2.5 months of age.

Simone Spuler (Germany) characterized the response of pre-symptomatic dysferlin-deficient mice to exercise training, motivated by the fact that dysferlinopathy patients are often particularly athletic prior to onset of symptoms [19]. Dr. Spuler tested the performance of 4 week-old dysferlin-deficient BLA/J mice during one week of endurance exercise training on an ascending treadmill, and found that the dysferlin-deficient mice could run slightly farther and did not become exhausted as rapidly as wild-type mice. Despite this slightly better physical performance, exercise training had a significant negative effect on muscle histology in dysferlin-deficient mice compared to wild-type mice, suggesting that exercise prior to onset of muscle weakness may speed the progression of dysferlinopathy.

5. Session IV: What Does Dysferlin Do?

This session focused on dysferlin's cellular function(s) and how dysferlin's absence changes the response of a muscle cell to use or injury.

Robert Bloch (USA) followed up on his dysferlin localization studies by presenting evidence that labeling for dysferlin in T-tubules increases dramatically following muscle injury induced by large strain eccentric contractions. This increase in T-tubule labeling occurs within hours, and there is no change in the total amount of dysferlin in the muscle by western blot, suggesting that the observed increase is either due to re-localization of existing dysferlin to the T-tubules, or due to a conformational change in dysferlin leading to increased exposure of the Hamlet-1 antibody antigenic site. Dysferlin's distribution along T-tubules also changes, increasing preferentially at the vertices of the T-tubules.

Elisabeth Barton (USA) studied the response of dysferlin-deficient EDL, soleus and diaphragm muscles to eccentric contractions. Procion orange dye uptake (an indicator of membrane disruption) varied widely between the three muscles, and only the diaphragm muscle showed the expected increase in dye uptake for dysferlin-deficient muscles versus controls [20]. In the soleus muscle, on the other hand, absence of dysferlin seemed to reduce dye uptake following eccentric contractions. When subjected to low Ca^{2+} conditions in an effort to inhibit membrane repair, the muscles that produced the least stable force profiles were those that had shown the most dye uptake, suggesting that there may be a connection between Ca^{2+} handling and susceptibility to damage.

Sandra Cooper (Australia) has been testing the role of dysferlin in membrane repair in C2C12 myotubes using two different wounding assays. The first assay used small projectiles to make well-defined holes in cells. Surprisingly, Dr. Cooper found that GFP-dysferlin does not accumulate at the site of the membrane wound, in contrast to other proteins known to play a role in membrane repair, including MG53 and annexin A1. A second set of experiments used detergent to wound cells, followed by flow cytometry to monitor dye entry. Cells in which dysferlin was knocked down using siRNAs were slightly more susceptible to detergent injury than wild-type cells; however, overexpression of full length dysferlin (or several truncated dysferlin forms of dysferlin) had a detrimental effect on membrane resealing and cell survival.

Simone Spuler (Germany) presented a wounding assay in which an atomic force microscope (AFM) probe tip is used to scrape a cell membrane in a controlled manner, resulting in a white spot in transmission light images that may represent a hole or deep depression in the membrane. The nature of the wound is now being characterized by both dye entry and AFM surface analysis. Additional experiments using a laser to wound primary human myotubes showed that dysferlin-deficient myotubes have a reduced rate of membrane repair compared to normal myotubes. This result is notable because many groups have had difficulty recapitulating the dysferlin-dependent membrane repair defect (originally shown in primary muscle fibers, [4]) in cultured myotubes. Other variables, such as immortalization, time in culture, and species differences, may explain the lack of repair defect observed by these other investigators.

Isabelle Richard (France) used genetic manipulation of mouse models to demonstrate that expression of either myoferlin or a mini-dysferlin construct [21] in the muscles of dysferlin-

deficient mice can partially restore the muscle fiber membrane resealing defect that is observed in response to laser wounding in untreated dysferlin-deficient mice (as described in [4]). However, this partial restoration does not have a measurable impact on histological signs of muscle pathology, such as the presence of central nuclei, in contrast to expression of full-length dysferlin, which both restores the membrane resealing defect and corrects pathology [22]. These results suggest that either the membrane repair defect is not the primary disease-causing effect of the absence of dysferlin, or that the partial restoration of membrane repair was not sufficient to impact the overall course of the disease.

Steven Vogel (USA) reviewed his work on the function of a sea urchin homolog of dysferlin [23]. Wounding a single cell in a sea urchin embryo causes a spike of high intracellular Ca^{2+} that is subsequently observed in neighboring unwounded cells, both in the same embryo and in nearby embryos in the culture. The intracellular signal for this response was shown to be ATP, and the release of ATP from the wounded cell depends on the action of voltage-gated Ca^{2+} channels. Morpholino knockdown of the sea urchin dysferlin homolog also inhibits release of this ATP signal, a phenotype that can be rescued by microinjection of mRNA encoding human dysferlin. This result is consistent with a role for dysferlin in exocytosis of ATP-containing vesicles. Dr. Vogel speculated that an analogous intercellular signaling mechanism might coordinate remodeling responses following muscle use in humans.

Paul Blank (USA) described a method to monitor membrane wounding and repair in cultured myotubes and myofibers by monitoring intracellular Ca^{2+} levels. The timing and quality of the Ca^{2+} influx depend on the severity of the wound, and by this assay dysferlin-deficient

myotubes and myofibers do not show a membrane resealing defect except under the most extreme wounding conditions. Inspired by Dr. Vogel's results in sea urchins [23], Dr. Blank found with C2C12 myotubes that neighboring myotubes in the culture show an increase in intracellular Ca^{2+} levels shortly after one myotube is wounded (depending on the severity of the wound). This bystander response was absent in an immortalized dysferlin-deficient myoblast line derived from the A/J mouse ("GREG" cells). Analysis of the timing of the bystander response versus the distance between the bystander cell and the wounded cell reveals slower kinetics than would be expected from simple diffusion of ATP, suggesting that an additional step or alternative signaling mechanism is required.

Elizabeth McNally (USA) presented her studies of the role of dysferlin in muscle growth [24]. She showed that dysferlin-null myoblasts have a defect in myoblast-to-myotube fusion, resulting in smaller myotubes in culture, and also have an accumulation of different types of vesicles. In studies of whole animals treated with IGF1, a growth stimulant, dysferlin-null muscle did not respond while control muscle showed a 30% increase in myofiber diameter. These data indicate that dysferlin may be involved in multiple pathways that regulate muscle growth, likely including vesicle trafficking events.

6. Session V: What do dysferlin partners do?

This session addressed the identity and function of proteins that interact with dysferlin.

Silvère van der Maarel (The Netherlands) discussed the isolation of dysferlin protein partners by immunoprecipitation (IP) of myogenic protein sources (myoblasts, myotubes, and muscle tissue) with dysferlin. Hundreds of potential partners were identified, including seven out of the nine known dysferlin partners (AHNAK, CAPN3, MG53, ANXA2, PARVB, DHPR, Tubulin), as well as additional cell adhesion, endocytosis, mitochondrial, ER chaperone, and ATP signaling proteins [25]. Many of the partners varied between the different myogenic sources, suggesting spatiotemporal regulation of dysferlin interactions during myogenic differentiation. Dr. van der Maarel followed up on two candidate partners, myoferlin (a close ferlin family member) and vinculin (a cell adhesion protein). Specific co-IPs of each protein with dysferlin were confirmed by western blot, the possibility that the dysferlin antibody was cross reacting with myoferlin was ruled out, and vinculin and dysferlin were shown to co-localize at the sarcolemma [25].

Robert Bloch (USA) compared the expression dynamics of dysferlin and some of its known partners in mouse muscle after injury in order to test the hypothesis that dysferlin and its partners are co-regulated. Dysferlin protein levels do not change just after injury, but increase by ~1.5-fold from day 3 to day 7 post-injury, and return to normal by day 14. Annexin II levels increase from day 3-7 in parallel with dysferlin; AHNAK levels decrease from day 3-7 and return to normal by day 14; and caveolin 3 levels do not change through day 14. Evaluation of other proteins, including calpain 3, is ongoing. Since it is known that AHNAK levels decrease when calpain 3 is active [26], these findings may indicate that calpain 3 levels increase from day 3-7 in parallel with dysferlin.

Rumaisa Bashir (UK) discussed the possibility of an interaction between ANO5 and dysferlin. Patients with *ANO5* mutations manifest a limb girdle muscular dystrophy or Miyoshi myopathy that is similar to dysferlinopathy [27] and cells isolated from these patients have a membrane repair defect [28]. ANO5 is a member of a family of Ca^{2+}-activated chloride channels [29] but does not appear to have a Ca^{2+} binding site by sequence analysis, leading to the hypothesis that dysferlin may act as the Ca^{2+} sensor for ANO5. To support an interaction between dysferlin and ANO5, Dr. Bashir showed that ANO5 is expressed in human muscle and that its expression increases upon differentiation in culture, a pattern similar to dysferlin. In addition, ANO5 and dysferlin have overlapping co-sedimentation profiles and ANO5 levels are substantially reduced in dysferlin-deficient cells. Experiments are ongoing to see whether dysferlin levels are also reduced when ANO5 is absent.

7. Session VI: What is the role of inflammation in dysferlinopathy?

This session focused on the reasons for and role of the unusually high immune and inflammatory responses in dysferlin-deficient dystrophic muscle.

Eric Hoffman (USA) focused on the role of muscle as a pro-inflammatory organ. Several components of the inflammasome complex are upregulated in skeletal muscle in the dysferlin-deficient SJL mouse model compared to wild-type muscle, and some of these factors, including NALP-3 and vinculin, are specific to dysferlin deficiency versus other muscular dystrophies [30]. Primary SJL myotubes as well as shRNA-mediated dysferlin knockdown C2C12 and RAW (macrophage) cells have increased IL-1 secretion in response to treatment with

lipopolysaccharide and benzylated ATP compared to control cells, possibly because of the NALP-3 upregulation. Dr. Hoffman proposed that increased IL-1 secretion from both muscle cells and macrophages could result in a positive-feedback loop. He also presented microarray data showing that the timing of the activation of proteins involved in muscle regeneration is perturbed in muscular dystrophies, suggesting that out-of-sync activation of these pathways may cause detrimental cross-talk between them that contributes to pathology.

Joseph Roche (USA) presented data suggesting that macrophage infiltration following large-strain injury in dysferlin-null A/J mouse muscle is a consequence and not the cause of myofiber death. Three hours after injury, A/J muscle and control A/WySnJ muscle recover equally well; however, histological studies indicate that over the next several hours, necrotic fibers begin to appear in dysferlin-deficient muscle, peaking at 72 hours post-injury, whereas no significant necrosis is seen in control muscle. MRI scans indicate a difference in recovery between control and A/J muscle beginning around 6 hours after injury. Both immunofluorescence and flow cytometry suggest that large numbers of macrophages invade injured A/J muscle later, around 72 hours after injury, suggesting that macrophage infiltration follows myofiber death. In addition, depletion of macrophages by clodronate does not significantly decrease myofiber damage in A/J muscle.

Eduard Gallardo (Spain) discussed his latest findings on a chemotactic factor released by dysferlin-deficient cells that promotes monocyte recruitment [31]. A chemotaxis assay was designed to measure the extent of monocyte migration towards cell culture supernatants derived from normal or dysferlin-deficient human myotubes. Increased chemotaxis was induced by the

dysferlin-deficient supernatants versus normal supernatants. A microarray screen was performed on the cultured myotubes to identify candidate proteins that could be responsible for this increased chemotaxis, and it revealed an upregulation of thrombospondin-1 (TSP-1) that was confirmed with subsequent analyses. Blocking TSP-1 activity decreased monocyte chemotaxis. TSP-1 expression was found to be higher in muscle biopsies from LGMD2B patients, but not other muscular dystrophies, and TSP-1 was also found to be upregulated in siRNA-mediated dysferlin knockdown cells.

Steve Laval (UK) discussed the finding that muscles in dysferlin-deficient BL10-SJL mice have delayed regeneration following notexin injury compared to control mice [32]. This delay was found in both the removal of necrotic fibers and in functional recovery, and there was also an extended inflammatory phase associated with early impairment of neutrophil recruitment to the injured muscle. Dr. Laval proposed that the absence of dysferlin may perturb inflammatory cell recruitment due to improper signals sent out by the damaged muscle, likely due to a role for dysferlin in the fusion of vesicles carrying signal transduction molecules to the plasma membrane, in addition to its role in sarcolemmal repair.

Simone Spuler (Germany) discussed the involvement of the complement system in dysferlinopathy. She has shown that dysferlin deficiency is associated with down-regulation of the complement protection factor DAF1/CD55 in the skeletal muscles of dysferlin-deficient mouse models as well as LGMD2B/MM patients [33]. The absence of DAF1/CD55 on the surface of skeletal muscle fibers increases their susceptibility to membrane attack via the complement cascade, as evidenced by deposition of the membrane attack complex (MAC) on

dysferlin-deficient muscle fibers and the susceptibility of dysferlin-deficient myotubes to complement-mediated lysis. Administration of anti-C5 antibodies to antagonize complement activity reduced muscle fiber damage in mice, and IVIG therapy in three LGMD2B/MM patients decreased MAC levels and improved muscle strength.

Renzhi Han (USA) described his studies on the role of complement in dysferlinopathy. Complement factor C5b-9 was found to uniformly label muscle fibers in dysferlin knockout mice, but to label only necrotic fibers in dystrophin-deficient mdx mice. Similarly, immunofluorescence analysis of patient muscle biopsies showed uniform labeling of fibers in LGMD2B patients but labeling of only necrotic fibers in LGMD2I patients. Knockdown of complement factor C3 in the dysferlin knockout mice improved muscle pathology, reduced the number of centrally nucleated fibers, and increased fiber diameters [34]. There was no effect on membrane repair in response to laser wounding. Dr. Han proposed a model in which a primary membrane repair defect causes downstream complement activation, resulting in positive feedback and additional muscle damage.

8. Session VII: Is There a Regeneration Defect in Dysferlinopathy?

This session focused on whether the absence of dysferlin directly affects the ability of skeletal muscle to regenerate after normal use or injury.

Tatiana Cohen (USA) presented studies of the ability of myoblasts to fuse and form myotubes in the presence and absence of dysferlin. Dysferlin-deficient myoblasts cultured from

SJL and A/J mice form myotubes with smaller diameters than those formed by wild type myoblasts, but with the same diameter per nucleus, suggesting that the defect is in fusion rather than growth. The dysferlin-deficient myotubes were also delayed in fusing with neighboring myotubes in the culture, and showed downregulation of muscle-specific markers by gene expression profiling. While this fusion defect was only shown in cultured cells, in vivo it could slow the recovery of dysferlin-deficient muscle from injury.

Steve Laval (UK) also studied the ability of dysferlin-deficient myoblasts to fuse into multinucleated myotubes, and found that early passage primary myoblasts from dysferlin-deficient BL10-SJL mice have no apparent deficit in fusion, despite the fact that these mice have a regeneration defect in whole muscle in response to notexin injury [32]. This led to speculation that a number of factors, such as how long the cells have grown in culture or the extent of disease progression in the muscle from which the original sample was taken, may affect whether dysferlin deficiency causes a fusion defect.

Joseph Roche (USA) assessed the ability of the mouse tibialis anterior muscle to recover from two rounds of large-strain muscle injury in 1-year-old dysferlin-deficient A/J and control A/WySnJ mice. In A/J mice, recovery from acute muscle injury requires myogenesis, but the rate of recovery was the same after a second round of injury (7 weeks after the first injury) as after one round of injury. This result suggests that the regenerative capacity of dysferlin-deficient muscle is not depleted in 1-year-old mice.

Isabelle Richard (France) used myoferlin-overexpressing mice to demonstrate a role for myoferlin (a close dysferlin family member) in muscle regeneration in both wild type and dysferlin-deficient (Bla/J) genetic backgrounds. Recovery from notexin injury was monitored histologically by Alizarin Red uptake 7, 10, and 14 days after injury. In both dysferlin-positive and dysferlin-negative background strains, myoferlin overexpression resulted in faster recovery from the muscle injury.

9. Session VIII: How do we put dysferlin back?

This session covered approaches that would introduce dysferlin back into dysferlin-deficient muscle, including gene therapy and cell therapy.

Nicolas Lévy (France) discussed an approach to dysferlin gene therapy using just a portion of the dysferlin gene (a mini-dysferlin gene), motivated by the fact that the packaging limit (~5 kb) of the AAV vectors used to deliver genes to muscle is too small to contain the full-length dysferlin cDNA (6.2 kb). The specific mini-dysferlin tested by Dr. Levy was based on a patient with a large deletion in dysferlin (exons 1-40, leaving only the two C2 domains and the transmembrane domain at the C-terminus of the protein) but only a mild phenotype, suggesting that the patient's shortened dysferlin protein is partially functional. AAV-mediated transfer of an equivalent mini-dysferlin construct into dysferlin-null mice significantly improved membrane repair following laser wounding of muscle fibers, but did not make muscle histology significantly better or worse [21].

Marc Bartoli (France) discussed a proof-of-principle study of exon skipping in dysferlinopathy. The challenge for exon skipping is to determine which exons of dysferlin can be skipped while maintaining function of the protein. Based on a mild phenotype observed in a patient with a mutation causing skipping of exon 32 of dysferlin [35], Dr. Bartoli and colleagues designed oligonucleotides to skip exon 32, and then applied the technique to myoblasts derived from a patient with a frame-shifting mutation in this exon. The myoblasts were found to express dysferlin after skipping was induced [36]. They also had improved myogenesis and improved membrane repair following laser wounding relative to dysferlin-negative controls.

Isabelle Richard (France) discussed three methods for assessing the efficacy of gene therapy treatments in mice: eccentric treadmill exercise, the large strain injury protocol developed by the Bloch lab [15], and use of a mSeAP reporter gene. Using the reporter gene approach, they found that transduction in dysferlin-deficient Bla/J muscle was reduced relative to wild type, which may complicate interpretation of results. Dr. Richard also compared the results of using mini-gene and dual AAV vector approaches to deliver the dysferlin gene to mice. Delivering the mini-dysferlin gene with a single AAV vector [21] produced more dysferlin expression in mice, but the dual vector approach for delivering full-length dysferlin [22] led to less Evans Blue dye uptake following the large strain injury protocol, indicating better functional recovery.

Matt Hirsch (USA) gave a quantitative comparison of large gene (e.g. dysferlin) delivery strategies based on homologous recombination using dual AAV vectors. Two approaches were tested using luciferase as a reporter gene. In one approach ("split AAV"), an intron was

introduced in the middle of the luc gene, and the construct was split between two AAV vectors with the split and recombination sites in the middle of the intron. Since the recombination event occurs in the intron, any misalignment should not affect the final protein product. The other approach ("fragment AAV") uses a region of overlap between the split portions of the gene, which is thought to recombine by non-canonical homologous recombination. Fragment AAV appears to give better efficiency in producing the recombined gene product in mouse muscle. A dysferlin cDNA codon optimized to enhance protein expression and remove alternative reading frames in the coding sequence was also developed for use in these dual-vector approaches.

Jordi Díaz-Manera (Spain) discussed cell therapy in a mouse model of dysferlinopathy using transplantation of adult mesoangioblasts (MABs). MABs are progenitor cells associated with blood vessels [37], which can be obtained in large quantities from many tissues, including adipose, and can colonize muscle under stimulation by inflammatory molecules. MABs from C57/Bl6 mice were found to differentiate into muscle tissue spontaneously in vitro. When these C57/Bl6 MABs were injected into dysferlin-deficient A/J-SCID mice, all tested muscles were efficiently transfected and expressed dysferlin at levels between 6% and 35% of wild type, depending on the transfection method used. Muscle fibers expressing dysferlin were isolated from these transfected mice and had recovered their ability to repair their membranes using the laser wounding assay.

Michele Calos (USA) discussed cell therapy combined with ex vivo gene correction of autologous cells using phiC31 integrase, which integrates the transgene into specific locations in the human genome, minimizing insertional mutagenesis [38]. Use of a patient's own cells with

gene correction would be ideal for histocompatibility, but may be problematic due to the expansion potential of the cells following correction. Dr. Calos is working with adipose-derived mesenchymal stem cells (MSCs), but noted that induced pluripotent stem cells (IPS cells) could overcome the expansion potential limitation and could themselves be generated using phiC31 integrase to add the four genes required to convert them from adult stem cells.

10. Session IX: How Do We Compensate for Dysferlin's Absence?

This session discussed a variety of ways to intervene in the dystrophic processes that result from the absence of dysferlin.

Jeffery Molkentin (USA) hypothesizes that unregulated entry of Ca^{2+} into muscle fibers through sarcolemmal breaches is the trigger for muscle fiber death through apoptosis and necrosis. By generating transgenic mice expressing a variety of cation exchangers and channels and crossing them with mouse models of muscular dystrophy (including dystrophin-null and sarcoglycan-null), he demonstrated that excess Ca^{2+} influx is sufficient to cause extensive muscle pathology and that reducing cytosolic Ca^{2+} levels can ameliorate muscle degeneration [39]—including reduction by overexpression of SERCA1. Various transgenic crosses to dysferlin-deficient A/J mice are underway to determine if these approaches are also effective for dysferlinopathy.

Eric Hoffman (USA) presented preliminary results from preclinical trials of two different compounds in dysferlin-deficient mice. The first compound, fasudil, is a Rho kinase inhibitor

that is used as a vasodilator and also affects the immune system. In dysferlin-deficient mice, fasudil reduced the number of inflammatory cells in the muscles, but negatively impacted several measures of muscle function, including grip strength and rotorod performance. The second drug is a modification of prednisolone, the glucocorticoid used to treat patients suffering from Duchenne muscular dystrophy, that can no longer activate the glucocorticoid receptor (which causes detrimental side effects) but still activates other beneficial prednisolone signaling pathways. In dystrophin deficiency, this modified prednisolone eliminated many side effects without decreasing the positive effects of the drug. In dysferlin-deficient mice, Dr. Hoffman found beneficial effects of this drug on grip strength, number of centrally-nucleated fibers, and Cy5.5 dextran uptake.

Noah Weisleder (USA) discussed the therapeutic potential of recombinant mitsugumin 53 (MG53), motivated by the role of endogenously-expressed MG53 in membrane repair [40,41]. Externally applied recombinant MG53 purified from *E. coli* concentrates at the site of a membrane wound and facilitates the in vitro repair of damaged muscle fibers after laser wounding in wild type, dysferlin-null, and MG53-null backgrounds. Recombinant MG53 also prevents cardiotoxin-induced damage to skeletal muscle in mice, as assayed by dye uptake and creatine kinase (CK) levels, and can reduce pathological hallmarks of dystrophic muscle both in vitro and in vivo.

Joshua Zimmerberg (USA) is testing whether changes in the lipid composition of myoblast membranes can alter their ability to tolerate tears and reseal in the absence of dysferlin, motivated by the theoretical prediction that negative curvature lipids will increase the line

tension along open tears, promoting their closure. Altering lipid composition in vivo through the introduction of specific dietary lipids could therefore provide a means of mitigating the effects of dysferlin deficiency. Dr. Zimmerberg found that dysferlin-deficient myotubes treated with the polyunsaturated fatty acid DHA reseal more quickly than untreated cells as measured by a conductance assay. He hopes to start a human trial to measure exercise-induced CK levels in normal individuals, either taking DHA supplements or not, to see if the dietary lipid has an effect on spontaneous closure of membrane disruptions in vivo.

H. Lee Sweeney (USA) discussed the therapeutic potential of PTC124 (Ataluren), a small molecule that promotes ribosomal read-through of premature stop codons [42]. In mdx mice, it successfully restores dystrophin expression, improves force generation, and lowers CK levels. In cultured myoblasts derived from a dysferlinopathy patient heterozygous for the dysferlin R1905X mutation, exposure to PTC124 restores dysferlin expression to 20% of normal levels, as measured by a novel assay for dysferlin function based on membrane blebbing [43]. Unlike wild-type myotubes, dysferlin-deficient myotubes do not exhibit membrane blebbing when exposed to hypotonic conditions, but this blebbing response was restored after treatment with PTC124.

Peter Serafini (USA) presented preliminary evidence from Louis Kunkel's laboratory that knocking down dysferlin with morpholinos in zebrafish results in a developmental muscle phenotype that includes bent tails and disorganized contractile filaments that can be easily assayed using birefringence. These phenotypes, if confirmed in a dysferlin-mutant zebrafish, can be used to screen for compounds that compensate for dysferlin's absence in the zebrafish model

system. The same approach is currently being used to screen for compounds that compensate for the absence of dystrophin.

Kevin Sonnemann (USA) presented preliminary results from a medium-throughput screen for small molecules that promote membrane resealing after wounding in Xenopus oocytes. So far a handful of candidate molecules have shown positive effect, and will be validated in additional assays. Dr. Sonnemann is also producing recombinant full-length human dysferlin protein with a TAT tag to help facilitate uptake by cells. Because dysferlin is a large protein with a transmembrane domain, protein production has been a major challenge, but has recently been achieved. Dr. Sonnemann is now testing the uptake and localization of TAT-tagged dysferlin applied externally to cultured cells, in preparation for future studies in which the recombinant dysferlin will be injected into dysferlin-deficient mice to test delivery to muscle fibers and ability to alleviate the symptoms of muscular dystrophy.

Tina Duong (USA) presented data on low intensity submaximal exercise interventions in dysferlin-deficient A/J mice. The study tested three different exercise regimens (voluntary wheel, horizontal and downhill treadmill) and an unexercised control group. The results demonstrate that all three exercise regimens had a beneficial effect on a number of behavioral, physiological, and histological tests, including reduced inflammatory markers and increased functional strength. The implication that low intensity exercise may be beneficial for dysferlinopathy will be tested by the CINRG network in a pilot study of body weight supported treadmill exercise in patients with LGMD.

François-Jérôme Authier (France) analyzed of the level of pathology and response to exercise regimens in different muscle groups of dysferlin-deficient Bla/J mice. Analysis of sedentary animals showed much more involvement of the hindlimb muscles as compared to the forelimbs. Because different types of exercise can have different effects on muscle [44], Bla/J mice were subjected either to forced downhill running, which requires eccentric muscle activity, or to forced swimming exercises with minimal eccentric muscle activity as described in [45]. Analyses of grip strength and histology demonstrated that downhill running had a detrimental effect on muscles, while the swimming regimen seemed to improve muscle function and histology relative to unexercised Bla/J mice.

11. Session X: What's new in diagnostic tools for dysferlinopathy?

This session covered various methods for improving the diagnosis of dysferlinopathy in patients.

Eduard Gallardo (Spain) described the accuracy of the dysferlin blood monocyte test as a diagnostic tool for the diagnosis of dysferlinopathy. Dysferlin is highly and consistently expressed in human peripheral blood monocytes (PBMs) in normal individuals, and Dr. Gallardo determined an optimal threshold dysferlin protein level for diagnosis of dysferlinopathy by testing the PBM dysferlin levels in known dysferlinopathy patients, carriers, and normal controls. Analysis of additional undiagnosed muscular dystrophy patients confirmed the accuracy of this threshold in predicting dysferlin mutations. Dr. Gallardo advised using the PBM

test, as opposed to the more invasive and less predictive muscle biopsy test, as an initial screen for dysferlinopathy prior to full dysferlin genetic analysis.

Martin Krahn (France) discussed new tools to reduce the expense and effort and increase the accuracy and interpretation of dysferlin mutational analysis. Chip-based comparative genomic hybridization (CGH) arrays are being developed to identity duplications and deletions in the dysferlin gene that are missed by classical sequencing methods. Sequence capture arrays are also being developed for exhaustive analysis of the entire dysferlin locus (i.e. introns, exons, regulatory regions) and other candidate genes (e.g. dysferlin protein partners). Finally, the new UMD-DYSF database contains over 772 individual dysferlin mutation entries as well as bioinformatics programs (e.g. UMD predictor, Human Splice Finder) to predict whether a given sequence variant is pathogenic or not. The goal is for the UMD-DYSF database to develop into an international dysferlinopathy patient registry.

Ibrahim Mahjneh (Finland) described clinical features that can be used to distinguish Miyoshi Myopathy caused by mutations in dysferlin (MMD1) from those caused by mutations in ANO5 (MMD3). MMD3 patients often have an uncomfortable burning sensation prior to the onset of muscle weakness, and the distal muscles of the forearms and anterior muscles of the legs are spared. By MRI, MMD3 patients have muscle fat degeneration of the gluteus minimus (as in MMD1), but no involvement of the gluteus maximus, medium muscles, shoulder and paravertebral muscles. Histologically, muscle biopsies from MMD3 patients lack the inflammation and vesicle accumulation under the sarcolemma that are observed in MMD1 patient biopsies.

12. Conclusions

The unique lab meeting format of the 4th Annual Dysferlin Conference received extremely positive feedback from participants, who felt that it created a more collegial atmosphere and that the grouping of individual results by topic led to more focused discussions of both synergistic and conflicting data. We hope that this meeting has provided the catalyst for participants to resolve any apparent conflicts and to build on their synergies, in an effort to conclusively address the open questions in the dysferlin field that were highlighted at each session. Tangible therapeutic progress will require collective, rather than individual, effort, and the Jain Foundation continues to encourage all researchers to welcome collaborations and share resources and ideas.

Note: Please visit the Jain Foundation website (www.jain-foundation.org) for more details on the 4th Annual Dysferlin Conference proceedings.

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