3rd Annual Dysferlin Conference
2-5 June 2009, Boston, Massachusetts, USA

1. Introduction

The 3rd Annual Dysferlin Conference, held from June 2-5, 2009 in Boston, MA, USA, brought together leading scientists and clinicians to discuss recent progress towards understanding and developing a therapy for the dysferlinopathies, Limb Girdle Muscular Dystrophy 2B (LGMD2B) and Miyoshi Myopathy. These diseases involve progressive muscle wasting, typically beginning in the late teenage years, and are caused by mutations in the protein dysferlin. Dysferlin has been shown to be required for repair of muscle fiber membrane tears, but little is known about how this defect contributes to disease pathology or whether dysferlin is also involved in other cellular processes. The conference was sponsored and organized by the Jain Foundation, and participants included 36 invited speakers, 9 additional scientists and clinicians, 40 poster presenters, 5 dysferlinopathy patients, 3 representatives of other muscular dystrophy foundations, and 7 members of the Jain Foundation scientific team. Research was presented from 11 countries around the world. In addition, a satellite meeting on the first morning of the conference brought together 17 scientific participants, including 7 speakers, to discuss the design of a natural history study for the dysferlinopathies.

2. Natural History Satellite Meeting

Prior to the start of the main conference, the Jain Foundation conducted a natural history satellite meeting with the goal of determining the most effective strategy for a natural history study of dysferlinopathy. The satellite meeting consisted of 7 short talks and discussion that centered on lessons learned from other natural history studies and addressed issues specific to a study on dysferlinopathy, such as clinical variability, small patient numbers, and slow disease progression.

The satellite meeting reached the following conclusions about the design of a dysferlinopathy natural history study. *Purpose/goal:* To determine the best clinical and/or laboratory parameters to use as outcome measures for future clinical trials and to identify the optimal period over which to assess the progression of the disease. *Parameters to test:* As many as possible, including muscle MRI because of its sensitivity and ability to detect muscle damage prior to the onset of clinical symptoms. *Inclusion criteria:* Dysferlinopathy patients with two identified pathogenic dysferlin mutations regardless of their clinical diagnosis, gender, age of onset, or stage of disease. *Number of patients:* Should be calculated based on the objectives and comparisons that the study is designed to test. *Duration and frequency:* The study will likely take 3 or more years. The frequency of evaluation should be determined based on the slow progression of the disease, the likelihood that a patient will return for subsequent testing, and cost.

3. Session I: Dysferlinopathy – Mechanisms of Pathology I

The talks in this session focused on changes that occur in muscle as a consequence of the loss of dysferlin function, and how these

changes lead to muscle fiber degeneration and pathology.

Robert Bloch (USA) presented research on the role of dysferlin in the repair of damaged muscle fibers *in vivo*. Dysferlin's role in muscle plasma membrane repair *in vitro* is widely accepted, yet it is not clear what happens to muscle fibers *in vivo* following injury in dysferlin-deficient animal models. His laboratory employs acute large strain eccentric contractions (Lovering et al. 2007) to induce muscle injury in control and dysferlin-deficient A/J mice. They then monitor the extent of contractile function loss and recovery, membrane disruption and resealing, and markers of muscle regeneration and inflammation over a time course of several weeks. The results demonstrate that A/J mice are no more susceptible to large strain muscle injury than control mice, but that they take longer to recover from muscle damage and require myogenesis to replace the fibers that were damaged directly or indirectly by injury (Roche et al. 2008). Remarkably, however, the muscle fibers in A/J mice that were initially damaged are impermeant to fluorescent dextrans three days after the injury, suggesting that their plasma membranes reseal the initial wound. Examination of injured muscles during the recovery period reveals a massive inflammatory response in A/J but not control mice, which Dr. Bloch suggests may be responsible not only for triggering myogenesis, but also for causing additional damage to fibers that had not been injured during the initial large strain contractions.

Jeffery Molkentin (USA) discussed the role of Ca^{2+} in muscle degeneration. Ca^{2+} can enter cells through sarcolemmal breaches, which are common in muscular dystrophies due to increased susceptibility to contraction-induced membrane tears or, in the case of dysferlin deficiency, due to reduced ability to repair tears. Dr. Molkentin hypothesized that in both cases, unregulated entry of Ca^{2+} into the muscle fiber is the key event that triggers muscle fiber death through apoptosis and necrosis. By generating transgenic mice expressing a variety of cation exchangers and channels, he demonstrated that excess Ca^{2+} influx is sufficient to cause extensive muscle pathology, and that Ca^{2+} efflux can ameliorate muscle degeneration in mouse models of muscular dystrophy. To determine whether this is also the case in dysferlin deficiency, he plans to cross the Ca^{2+} channel and exchanger overexpressing mice with dysferlin-deficient mice, which have indicators of intercellular Ca^{2+} overload such as swollen mitochondria. Dr. Molkentin has also generated transgenic mice that expresses dysferlin from a muscle specific promoter. Crossing these mice to dysferlin-deficient A/J mice corrects the dysferlin-deficient pathology, demonstrating that dysferlin only needs to be expressed in skeletal muscle (and not other cell types, such as immune cells) to prevent disease.

4. Session II: Dysferlinopathy – Mechanisms of Pathology II

The talks in this session focused on new evidence that intercellular signaling pathways, in addition to membrane resealing, may be disrupted in the absence of dysferlin.

Steven Vogel (USA) discussed recent experiments on the function of a sea urchin homolog of dysferlin. He has previously shown that morpholino-mediated knockdown of the sea urchin ferlin in one cell of a 2-cell stage embryo inhibits the cytokinesis step of cell division and decreases the rate of uptake of FM1-43 dye into cells, suggesting reduced endocytosis.

Now he has also found that wounding a cell in an embryo causes a flash 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. ATP added to the medium can also trigger such Ca^{2+} flashes, while apyrase (which degrades ATP and ADP), cadmium (a voltage-gated Ca^{2+} channel blocker), and omega-agatoxin IVA (a P-type voltage-gated Ca^{2+} channel blocker) all block the Ca^{2+} pulses in nearby cells but not in the initially wounded cell. Dr. Vogel hypothesizes that wounding results in depolarization of the wounded cell and thus opens voltage-gated Ca^{2+} channels, which allows an influx of Ca^{2+} into the cell that triggers exocytotic release of ATP. Knockdown of the sea urchin ferlin also inhibits release of this signal from wounded cells, a phenotype that can be rescued by microinjection of the human dysferlin mRNA. This result is consistent with a role for dysferlin in exocytosis of ATP-containing vesicles. Dr. Vogel mentioned that there is evidence in the literature that extracellular ATP stimulates muscle satellite cell differentiation, suggesting a potential physiological role for wounding-induced ATP release in mammalian muscle.

Todd Lamitina (USA) discussed his research on the role of the *C. elegans* homolog of dysferlin, fer-1, in *C. elegans* muscle. Fer-1 was originally identified for its role in sperm motility and fertility (Argon and Ward 1980; Achanzar and Ward 1997). Dr. Lamitina has now shown that fer-1 is expressed in *C. elegans* body wall muscle, and that the expression of genes involved in locomotion and muscle sarcolemma stabilization is altered in two different fer-1 mutants. Based on the recent finding that fer-1 mutations confer resistance to normally fatal doses of aldicarb and levamisole, drugs that increase cholinergic signaling, Dr. Lamitina hypothesized that fer-1 plays a post-synaptic role in muscle cells at the neuromuscular junction (NMJ). He found that post-synaptic acetylcholine receptors (AchRs) in fer-l mutant worms did not have the normal punctate staining associated with their proper localization to synaptic varicosities. He also found reduced muscle power and force generation in fer-1 mutant worms, which was improved by increasing acetylcholine signaling using a mild dose of aldicarb. These results suggest that dysferlin is required for proper localization of AchRs at the muscle cell membrane and point to a role for dysferlin in membrane cycling.

Tejvir Khurana (USA) hypothesized that dysferlin-deficient A/J mice may have a defect in response to cholinergic signaling at the NMJ, as do *C. elegans* fer-1 mutants (see above), and that their pathology might be improved by treatment with drugs that increase the efficiency of cholinergic signaling. He found that although the NMJs in A/J mice appear normal by histology, the mice have reduced muscle force and electromyogram intensity after repetitive low frequency muscle stimulation (a typical test for NMJ signaling defects). Acetylcholinesterase inhibitors are used to treat a similar cholinergic signaling defect in myasthenia gravis patients, and oral administration of the acetylcholinesterase inhibitor pyridostigmine bromide to post-symptomatic A/J mice eliminated the force generation and electromyogram defects. Although A/J mice typically have a normal lifespan, 3 out of 7 untreated A/J mice died by 14.5 months of age in this trial, compared to none of the treated A/J mice. The results of this pilot study suggest that acetylcholinesterase inhibitors may improve pathology in dysferlinopathy patients.

5. Session III: Dysferlin Structure and Function

This session focused on dysferlin's function at the molecular level; specifically its structure, interactions with other proteins, lipids and ions, and how these interactions influence its localization and movement within muscle fibers.

Silvère van der Maarel (The Netherlands) described his ongoing efforts to identify new interaction partners of dysferlin. He has developed anti-dysferlin antibodies using a phage display system of the heavy chain antibody repertoire, taking advantage of the fact that llama antibodies consist of only a heavy chain that can be cloned, tagged, and produced in large quantities in bacteria. These antibodies were used to immunoprecipitate dysferlin and its associated proteins from different tissues and cell lines. A number of known and novel dysferlin interacting proteins were identified by mass spectrometry, including calpain 3, caveolin 3, annexin-1 and -2, AHNAK, and MG-53, as well as many novel interactors that still need to be verified. Bioinformatic analysis of the identified proteins supports the hypothesis that dysferlin acts in a dynamic protein complex depending on the tissue or cell line analyzed. Dr. van der Maarel has also continued to study the interaction of dysferlin with AHNAK, and how calpain-3 regulates this interaction through cleavage of AHNAK.

Sandra Cooper (Australia) discussed the trafficking and membrane biology of dysferlin and dysferlin truncation mutants. The constructs were tagged with EGFP and His/Myc at the N- and C-termini, respectively, allowing for differential visualization of the intracellular and extracellular domains. Her studies demonstrate that dysferlin requires the Golgi secretory pathway for trafficking to the plasma membrane and that dysferlin remains at the cell surface for several hours. This trafficking pattern may help explain the abnormal localization of dysferlin in many patients with muscular dystrophy. Dr. Cooper also found that dysferlin, and some of the truncation mutants, appear to cluster by light and electron microscopy, and preliminary experiments using FRET suggest that individual dysferlin molecules may associate with one another and form oligomers.

Michael Sinnreich (Canada) described three different projects that his lab is pursuing on dysferlinopathy. The first is to characterize the lipid binding properties of the C2 domains of dysferlin (Therrien et al. 2009). C2 domains from other proteins can bind to specific phospholipids, often in a manner that is regulated by Ca^{2+}. Dr. Sinnreich demonstrated that the C2A domain of dysferlin does bind phospholipids in a Ca^{2+}-dependent manner but that the other C2 domains do not. Dr. Sinnreich also described his efforts to identify new dysferlin binding partners by combining immunoprecipitation with mass spectroscopy. These studies identified a number of proteins known to interact with dysferlin, including calpain-3, caveolin-3, AHNAK, annexin-1 and -2, and MG-53, as well as a novel interaction with alpha tubulin that is supported by additional biochemical evidence. Finally, Dr. Sinnreich described his efforts to design a functional mini-dysferlin protein for use in gene therapy. The design is based on his finding of a particularly mild phenotype in a patient with a lariat branch point mutation that produces a partially functional mini-dysferlin protein (Sinnreich et al. 2006).

Bryan Sutton (USA) described his approach to determining the structure of the dysferlin protein, including electron microscopy imaging of the full cytoplasmic portion of dysferlin and X-ray crystallography to determine the atomic resolution structure of the individual C2 domains. Dr. Sutton has re-evaluated the boundaries of each C2 domain using bioinformatics analysis and is generating individual C2 domain expression constructs. The domains that have been expressed so far, C2A and C2F, can be purified from bacteria and are amenable to growing protein crystals for structural analysis. Crystals of C2A are now available and diffract to a very high resolution. Dr. Sutton has not had success using the myoferlin C2A domain NMR structure for molecular replacement of the dysferlin C2A data, suggesting that there are substantial differences between the C2A domains of these two ferlins. The electron microscopy analysis is being done in collaboration with Dr. Tom Walz and is still in the development phase. Finally, Dr. Sutton has found by bioinformatics that the portion of dysferlin between C2C and C2D (containing the ferlin domains) has substantial homology to glutamine synthetase, which is known to form pentamers.

6. Session IV: Membrane Fusion and Repair

This session focused on the molecular mechanisms underlying membrane fusion events—in which dysferlin is hypothesized to be involved—during plasma membrane repair and myoblast fusion.

Jianjie Ma (USA) discussed his studies on the muscle-specific mitsugumin 53 (MG53) protein and its involvement in sarcolemmal repair. MG53-/- cells cannot effectively reseal their plasma membranes following laser wounding or mechanical damage, and MG53 in wild-type cells rapidly concentrates to the plasma membrane wound site. MG53 does not contain a transmembrane domain, but is likely associated with membranes through its interaction with phosphatidyl serine. Studies performed under Ca^{2+}-free conditions revealed that the local oxidative environment near the injury site is required for recruitment of vesicles, which can subsequently fuse in a Ca^{2+}-dependent manner to seal the membrane wound. Oligomerization of MG53 under oxidative conditions appears to be the signal for vesicle recruitment. Unlike MG53-/- cells, dysferlin-/- cells have normal vesicle accumulation at the wound site, implying that their defect is in a subsequent step of the repair process, such as Ca^{2+}-dependent membrane fusion. Finally, MG53 was found to interact with dysferlin and caveolin-3, suggesting that these three proteins may function as a part of a molecular complex.

Norma Andrews (USA) presented her studies on the sequence of membrane cycling events during membrane repair, which is known to involve Ca^{2+}-mediated exocytosis of intracellular vesicles. Previous work from her lab has shown that exocytosis of lysosomal vesicles is involved in the repair process and that the lysosomal marker LAMP-1 can be readily detected on the surface of repaired cells. In addition, formation of endosomes can also be observed in multiple cell types, including C2C12 myoblasts, shortly after membrane wounding by either scraping or treatment with streptolysin O (a toxin that generates stable, protein-lined pores in the membrane). Enhancement of the endocytic process (by disruption of the actin cytoskeleton) accelerates repair, while its inhibition (by cholesterol depletion) blocks the repair process. Dr. Andrews proposed a model in

which lysosomal exocytosis adds additional membrane to the plasma membrane and brings about the release of hydrolases that participate in endosome formation. These studies highlight the interplay between exo- and endocytosis in the plasma membrane repair process.

Elizabeth McNally (USA) presented her research on the protein myoferlin, the closest ferlin homolog of dysferlin, and its involvement in myoblast fusion. Expression studies and characterization of the myoferlin promoter revealed that myoferlin expression is upregulated in response to cardiotoxin-induced muscle damage (Davis et al. 2000; Doherty et al. 2005). Examining the upstream regulatory regions suggests a role for the NFAT and calcineurin pathways in regulating myoferlin expression, presumably in response to Ca^{2+} influx caused by membrane wounding. Dr. McNally also found that myoferlin interacts with the endocytic recycling protein EHD2 via its C2B domain (Doherty et al. 2008). Myoferlin-null myoblasts are defective in endocytic recycling, and expression of an EHD2 point mutation previously associated with defective endocytic recycling results in myoferlin aggregation and impaired myoblast fusion. Dr. McNally proposed that recycling of plasma membrane receptors involved in signaling events associated with myoblast fusion is mediated by myoferlin and is critical for maintaining the fusion process.

7. Session V: Muscle Satellite Cells

Talks in this session focused on methods to study disease pathology and the muscle regenerative process in dysferlinopathy by making use of muscle progenitor cells.

Gillian Butler-Browne (France) described the generation of immortalized cell lines from primary cells of dysferlinopathy patients. These cell lines will help overcome the bottlenecks posed by limited availability of primary patient cells, and provide an inexhaustible source of cells harboring different pathogenic dysferlin mutations. Expression of telomerase is sufficient to immortalize fibroblasts but not myoblasts, indicating fundamental differences in how senescence is achieved in these two cell types. Senescent myoblasts have high p16 and cyclin D1 activity, and low levels of phospho-Rb. Further studies showed that myoblast immortalization can be achieved by the combined expression of telomerase and inhibition of the p16 pathway through expression of cdk4 (Zhu et al. 2007). Importantly, the immortalized myoblasts retain the ability to differentiate and form normal myotubes in culture. Dr. Butler-Browne is currently working to immortalize fibroblasts and cells from muscle biopsies from 3 dysferlinopathy patients.

Terence Partridge (USA) described the use of thymidine analogues, BrdU and EdU, for tracking and quantifying loss and regeneration of muscle fibers in mouse models of muscular dystrophies. The technique involves administering the thymidine analog to the animals twice daily during the first post-natal week so that it is incorporated into many myonuclei. BrdU-labeled nuclei will be diluted by addition of non-labeled nuclei during growth and will be lost from fibers that degenerate and regenerate. The frequency of BrdU labeled myonuclei therefore gives an estimate of muscle fiber growth and degeneration. Labeling levels in pathological versus wild-type muscle can be used to monitor muscle degeneration during disease progression *in vivo* and to test the effects of therapeutic interventions. Another potential application is administering the label prior to an acute muscle injury to measure the robustness of

the regenerative process. Dr. Partridge has shown that *mdx* (dystrophin-deficient) mice almost entirely lose the BrdU signal by 50 days of age, in contrast to wild type controls, suggesting rapid myonuclear turnover and massive muscle degeneration in these dystrophic mice. Similar studies are planned with dysferlin-deficient mice.

Thomas Rando (USA) described the generation of reporter mice that can be used to follow disease progression through non-invasive whole animal imaging and serum-enzyme monitoring. Luciferase and Alkaline Phosphatase are expressed using a constitutive promoter and an IRES sequence, respectively, in one mouse strain (LUSEAP mice) (Nishijo et al. 2009). The promoter is immediately followed by a transcriptional stop signal that can be floxed out in a regulated manner upon conditional expression of Cre. Another set of animals express Cre under the control of either the Pax7 promoter (satellite cell-specific; Pax7-Cre-ER mice) or the Myf6 promoter (mature muscle fiber-specific; Myf6-Cre-ER mice) (Brack et al. 2007). Both the Pax7 and Myf6 promoters are tamoxifen-inducible. Crossing the LUSEAP and Cre mice generates animals in which the luciferase enzyme can be expressed in satellite cells or mature muscle fibers upon administration of tamoxifen. This enables tracking of muscle regeneration (as luciferase-expressing satellite cells fuse with muscle fibers) or degeneration (as luciferase-expressing mature fibers are lost) using whole animal imaging. These reporter strains are being crossed to dysferlin-deficient SJL mice in order to study muscle loss and regeneration in dysferlin deficiency.

8. Session VI: Stem Cell Therapies

This session discussed the characterization and use of muscle- and blood-derived stem cells as a therapeutic tool for the treatment of dysferlinopathy.

Amy Wagers (USA) first discussed her attempt to identify systemic blood-circulating factors that ameliorate dysferlinopathy by surgically joining the circulatory systems (parabiosis) of dysferlin-deficient mice to wild type partners. Dr. Wagers also discussed SMP cells, a subpopulation of satellite cells that express satellite cell and early myogenic markers, but lack differentiation markers: $CD45^-$, $Sca\text{-}1^-$, $Mac\text{-}1^-$, $CXCR4^+$, $\beta1\text{-integrin}^+$ (Sherwood et al. 2004; Cerletti et al. 2008). These cells exhibit robust, lineage-selective myogenic differentiation, long-term engraftment capability, and self-renewal capacity (Cerletti et al. 2008). Similar to her previous observations in dystrophin-deficient mdx mice (Cerletti et al. 2008), Dr. Wagers presented data showing significant changes in the SMP number in dysferlin-deficient mice (carrying the SJL mutation on a BL10 background) that correlate with disease severity. She believes that SMPs are relevant targets for cell-based therapy in muscle based on her finding that SMPs intramuscularly injected into dystrophic mice can achieve high levels of muscle chimerism and enhance muscle function. Dr. Wagers ended her talk by discussing her recent efforts to find a method of expanding SMPs using Notch activation and iPS technology.

Mayana Zatz (Brazil) described her approach to stem cell therapy for dysferlinopathy using mesenchymal stem cells (Vieira et al. 2008(1); Vieira et al. 2008(2)). Dr. Zatz has performed intravenous injections of human adipose stromal cells (hASCs) into three groups of 7 dysferlin-deficient SJL mice weekly and then once a month for 6 months (Vieira et al. 2008(2)). The three groups received either

no injections (control), undifferentiated hASCs, or hASCs pre-committed to the myogenic phenotype. She found unexpectedly that undifferentiated hASCs were not rejected after systemic injection into the SJL mouse, even without immunosuppression. Furthermore, the injected undifferentiated hASCs cells (but not the pre-committed cells) were able to fuse with the host muscle, express a significant amount of human muscle proteins, and improve motor ability.

Morayma Reyes (USA) has isolated wild type skeletal muscle perivascular cells (SMPCs) by FACS ($CD45^-$, $Sca\text{-}1^+$, $CD31^-$, $CD34^+$) and found that they express dysferlin perinuclearly when undifferniated and at the plasma membrane upon differentiation into myogenic cells. SMPCs from dysferlin-deficient mice appear to have impaired cell-cell fusion. Dr. Reyes transduced the dysferlin-deficient SMPCs with lentiviral vectors expressing full-length dysferlin containing one of the two exon 1 variants and found that both variants independently correct the fusion defect, suggesting that they have redundant functions. Dr. Reyes also showed that autologous and allogenic transplants of SMPCs result in high levels of engraftment over 2 weeks. She performed allogenic transplant experiments into dysferlin-deficient mice preceded by a bone marrow (BM) transplant with cells from the same donor to induce immune tolerance, and found that while BM irradiation initially resulted in the death of satellite cells and a worsening of the dystrophic phenotype, injected mice showed increased muscle mass in the injected leg compared to the contralateral leg.

Jacques Tremblay (Canada) discussed his clinical trials using myoblast transplantation as a treatment for muscular dystrophies. Myoblasts are muscle precursor cells responsible for the regeneration of muscle fibers *in vivo* and can be obtained by proliferation of satellite cells *ex vivo*. Dr. Tremblay showed that human allogenically-derived myoblasts transplanted into mice maintain their myogenic capacity and do not cause tumor formation. He then performed a Phase 1A clinical trial with 9 Duchene muscular dystrophy (DMD) patients who received 25-100 injections of allogenically-derived myoblasts into a 1 cm square section of one leg. One month post transplantation, 8 out of 9 patients showed some level of dystrophin positive fibers (Skuk et al. 2004; Skuk et al. 2006). Dr. Tremblay also conducted a trial in the thenar muscle of the hand in one DMD patient and found increased strength 1-14 months post transplantation and dystrophin expression for up to 18 months (Skuk et al. 2007). Finally, in a preliminary study he has demonstrated that transplantation of human myoblasts into mouse muscle also results in human dysferlin expression.

Michele Calos (USA) described her efforts to combine autologous stem cell therapy with phiC31 integrase-based gene therapy. PhiC31 integrase is a site-specific recombinase that can catalyze the integration of a transgene into a small number of sequence-specific integration sites in the host genome (Calos 2006). This system has no size limit for the transgene and achieves robust lifetime expression even through differentiation and cell division. Dr. Calos is attempting to implement *ex vivo* integrase-mediated integration of dysferlin into two different stem cell types: mesoangioblasts and mesenchymal stem cells (MSC). Mesoangioblasts are multipotent stem cells derived from blood vessels (Minasi et al. 2002), while MSC cells are myogenic stem cells that can be isolated from a number of tissues, including

white adipose tissue (Crisan et al. 2008). Preliminary injections of phiC31 integrase-modified mesoangioblasts into both mdx and dysferlin-deficient mice show good engraftment into both the injected and contralateral TA muscles.

Yvan Torrente (Italy) discussed autologous transplantation of CD133+ stem cells that have been modified to skip mutation-containing exons of dysferlin. CD133+ cells are adult muscle- or blood-derived progenitor cells that express muscle cell markers and display clonogenic, self-renewal, and multi-potency properties (Torrente et al. 2004; Benchaouir et al. 2007). CD133+ stem cells isolated from both muscle and blood express dysferlin. Using human myoblasts from an LGMD2B patient carrying mutations in exons 27 and 22, Dr. Torrente tested anti-sense oligonucleotides (AONs) designed to skip the mutation-containing exons. While he has observed the desired skipped product, the results are not always reproducible and the efficiency is low. He plans to use bifunctional AONs cloned into a lentiviral backbone to improve efficiency. Dr. Torrente is also studying the functionality of the shortened dysferlin proteins that would result from skipping exons 22-24, 24-29, and 22-29.

9. Session VII: Emerging therapies for dysferlinopathy

This session discussed an array of approaches for treating dysferlinopathy, including mutation-specific therapies, dysferlin gene therapy, enhancing muscle regeneration, and boosting the stability of the plasma membrane.

Lee Sweeney (USA) discussed an ongoing clinical trial of PTC124 (Ataluren), a small molecule that promotes ribosomal read-through of premature stop codons. PTC124 was identified after a high-throughput *in vitro* chemical screen for read-through activity. In mdx mice, it successfully restores dystrophin expression, improves force generation, and lowers CK levels. Dr. Sweeney has now shown that exposure to PTC124 restores dysferlin expression to 20% of normal levels in cultured myoblasts derived from a muscle biopsy from a patient heterozygous for the dysferlin R1905X mutation. Dr. Sweeney also presented a novel assay system to test functional rescue of dysferlin-deficient myotubes: in hypotonic conditions wild type myotubes exhibit membrane blebbing, whereas dysferlin-deficient myotubes do not. Blebbing can be restored in the latter by transferring full-length dysferlin into these myotubes via AAV. Dr. Sweeney found that treating the human myotubes harboring the dysferlin R1905X mutation with PTC124 also restores blebbing, establishing the therapeutic potential of PTC124 for treating dysferlinopathy patients with nonsense mutations.

Joshua Zimmerberg (USA) discussed the relationship between membrane lipid curvature and the ability of a membrane to repair or resist the formation of tears and pores. Surface tension tends to make membrane pores larger, while line tension tends to make pores smaller. In response to these forces, pores smaller than a critical size will spontaneously close. Increasing the proportion of negative curvature lipids in the membrane increases line tension and favors closure. Dr. Zimmerberg is directly assessing whether changes in the lipid composition of myoblast membranes can alter their ability to tolerate tears and reseal in the absence of dysferlin. Altering lipid composition *in vivo* through the introduction of specific dietary lipids may 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 creatine kinase (CK) levels in normal people taking DHA versus controls to see if the dietary lipid has an effect on spontaneous closure of membrane disruptions.

Isabelle Richard (France) discussed the use of adeno-associated viruses (AAV) for delivering the dysferlin gene to muscle. To get around the 4.5-kb upper limit to the capacity of the virus, Dr. Richard is using a concatamerization strategy in which the two halves of the dysferlin gene (a total of close to 7-kb) are delivered by two different AAV vectors with specific sequences that allow recombination inside a cell. She has administered the two AAV vectors (using either AAV1 or AAV9) either intramuscularly or intravenously into dysferlin-deficient mice (BLA/J mice, containing the A/J mutation on a C57BL/6 background). Injection into the tibialis anterior results in dysferlin expression detectable by quantitative RT-PCR, in situ hybridization, and western blotting up to 12 months post injection. The transferred dysferlin shows sarcolemmal localization by immunohistochemistry, and a membrane repair assay based on FM 1-43 entry into fibers after laser wounding demonstrates improved repair in AAV-injected dysferlin-deficient mice compared to uninjected mice. Systemic injection also results in detectable dysferlin protein and significantly improves activity level and distance traveled in an assay for locomotor activity one month post-injection. Dr. Richard has also created myoferlin overexpressing transgenic mice,and found that crossing them to dysferlin-deficient mice does not rescue the dysferlin-deficient phenotype.

Simone Spuler (Germany) discussed a strategy to re-target missense mutant dysferlin that has become trapped in the endoplasmic reticulum (ER) to the sarcolemma. Misfolded proteins are rapidly degraded in the ER via the ubiquitine proteasome system. She used TAT-labeled short 15-mer peptides corresponding to the dysferlin amino acid sequence around the location of each of 3 different missense mutations, versus control peptides with random sequence. She delivered the peptides to C2C12 myoblasts transfected with wild type or mutant GFP-dysferlin. She showed by immunoflouresence (IF) that a small amount of GFP protein found its way to the sarcolemma when she used peptides from the mutated dysferlin sequence or wild type dysferlin sequence but not when she used control nonsense peptides. Dr. Spuler also showed similar results using primary human myoblasts from patients.

Se-Jin Lee (USA) has previously demonstrated that inhibiting the protein myostatin can increase the muscle mass of both normal and dystrophic mice (Lee 2004). Anti-myostatin antibodies are already being tested in clinical trials as a potential therapy for muscular dystrophy; however, it is unclear whether directly targeting myostatin will be an effective approach in patients. Dr. Lee's recent work has focused on how myostatin's interacting protein partners regulate its activity. In particular, Dr. Lee presented genetic studies supporting a role for the BMP-1/tolloid family of metalloproteases in regulating myostatin activity by cleaving the propeptide, which normally maintains myostatin in a latent, inactive state. Dr. Lee is also using genetic studies to try to identify the other ligands that cooperate with myostatin to regulate muscle growth and is attempting to develop

novel biologics capable of targeting these ligands.

10. Session VIII: Lessons from the Clinic – Clues to a Cure

The talks in this session covered aspects of the natural history and pathology of dysferlinopathy, genetic diagnostics, and lessons on organizing multicenter trials from other forms of muscular dystrophy.

Isabel Illa (Spain) discussed recent studies on disease progression in dysferlinopathy patients. Dysferlin deficiency has been associated with a wide variety of clinical diagnoses, such as Miyoshi myopathy (MM), LGMD2B, distal anterior compartment myopathy, hyperCKemia, and others, based on the patient's initial pattern of muscle weakness at presentation. Dr. Illa's lab analyzed muscle MRIs in a group of 27 (14 MM, 12 LGMD2B, 1 hyperCK) dysferlinopathy patients and assessed the images by the degree of fat replacement on a four-level scale. Dr. Illa found that dysferlinopathy patients have similar muscle involvement in their upper and lower legs, regardless of their clinical diagnosis, but that the pattern differs from other muscular dystrophies. Dr. Illa also performed a natural history pilot study of 5 patients diagnosed with LGMD2B and 4 with MM over an 18-month period, including manual muscle testing (MMT), quantitative muscle testing (QMT), MRI imaging, and functional testing. A significant decline in muscle strength was noted over the 18-month period for pooled data from LGMD2B and MM patients, and the two clinical groups had similar evolution of symptoms.

Kate Bushby (UK) recently analyzed UK patients (Klinge et al. 2009) and showed that out of 36 patients from the UK diagnosed with dysferlin deficiency, 25% had onset prior to the age of 13. Also, 53% reported performing well in sports prior to onset, although this athletic activity did not appear to influence the later course of the disease (in contrast to some evidence presented by Dr. Corrado Angelini at the 2008 Dysferlin conference). These data indicate that the generally accepted age of onset range (15-30) may need to be modified and that there may be a "pre-symptomatic" period in dysferlinopathy. Dr. Bushby has also found that dysferlin-deficient mice (with the SJL strain mutation backcrossed onto the BL/10 background) are not more susceptible to damage by contraction than wild type mice, but that the repair process after damage is abnormal. Following needle and saponin wounding, the dysferlin-deficient mice have a reduction in cytokine release and in neutrophil recruitment. Regeneration is also abnormal (Chiu et al. 2009), including slower recovery of contractile force, fibers at an arrested state of regeneration, and permanent damage resulting from repeated bouts of injury. These findings suggest that dysferlin may play a role in secretion of chemokines responsible for attracting neutrophils to damaged muscle.

Nicolas Lévy (France) is developing a chip-based approach to detect mutations, including deep intronic mutations and copy number variations (duplications and deletions), in dysferlin, calpain-3, caveolin-3, and 45 other candidate genes. This approach should aid in identifying mutations in patients with confirmed dysferlin protein deficiency but whose mutations have been difficult to detect by standard diagnostic techniques. Dr. Lévy also discussed how molecular diagnosis of patients can provide valuable clues for new therapeutic approaches. He described a patient with a mild phenotype who has a naturally-occurring mini-dysferlin missing 40 exons. This fragment of dysferlin appears to be at

least partially functional, based on localization (monitored by a GFP tag) and membrane wound repair assays. Dr. Lévy is refining the construct by adding additional C2 domains to allow for increased "physiological" interactions with known partner proteins, while keeping the construct small enough to fit into an AAV vector for gene therapy. Dr. Lévy also discussed a "natural" proof-of-concept for exon skipping strategies: a patient with an in-frame deletion of exon 32 of dysferlin and only a moderate phenotype. Based on this clinical finding, Dr. Lévy is testing an exon skipping strategy for patients with mutations in exon 32.

Eric Hoffman (USA) discussed a recent study to identify genetic polymorphisms that modify the phenotype of Duchenne muscular dystrophy (DMD) through the CINRG network in collaboration with Dr. Elena Pegoraro in Padova, Italy. Candidate SNPs were identified through studies of muscle strength in normal populations (FAMUSS cohort) (Orkunoglu-Suer et al. 2008) and in severe versus mild DMD boys by mRNA profiling. The investigators have identified a genetic polymorphism that influences the severity of the DMD phenotype, and it is possible that this polymorphism may also have an effect on dysferlinopathy. Dr. Hoffman also described a study of the effect of exercise on dysferlin-deficient A/J mice, done by Dr. Kanneboyina Nagaraju's laboratory. There was a relatively mild yet statistically significant improvement of behavioral and physiological endpoints (including forced treadmill running and voluntary wheel running) in A/J mice with training, and the greatest improvement coincided with the known histological onset of the disease in A/J mice.

11. Session IX: Clues from Other Ferlins and Myopathies

This session included talks on the function of ferlin family members in non-muscle tissues and on the identification of a gene responsible for a form of non-dysferlin Miyoshi Myopathy.

Dennis Drescher (USA) discussed the role of otoferlin in the ear. Otoferlin and dysferlin are both members of the ferlin protein family, which also includes myoferlin, Fer1L4, Fer1L5, and Fer1L6. Patients with otoferlin mutations have profound deafness (DFNB9) but have normal otoacoustic emissions (Yasunaga et al. 1999, Tekin et al. 2005). Otoferlin is strongly expressed in inner hair cells of the ear and plays a role in exocytosis in the synaptic region between the hair cells and the acoustic nerve. Dr. Drescher's lab has studied the interaction of otoferlin with SNARE proteins through two of its C2 domains (C2F and C2D) (Ramakrishnan et al. 2009; Drescher et al. 2009). The C2F domain of wild-type otoferlin exhibits a calcium-dependent interaction with Syntaxin-1 and SNAP-25, and the C2D domain exhibits calcium-dependent binding to the calcium channel $Ca_v1.3$. Dr. Drescher's lab engineered otoferlin with known pathological mutations in the C2D and C2F domains (Leu1011Pro and Pro1825Ala, respectively) and demonstrated that the calcium-dependent interactions were inhibited by these mutations.

John Robinson (USA) presented studies of dysferlin and myoferlin in the human placenta. Dysferlin and myoferlin are both highly expressed in purified tissue from the syncytiotrophoblast, a large polynuclear cell structure at the boundary between maternal blood and the fetus that is formed by fusion of fetal cytotrophoblasts (mononuclear

precursor cells). Trophoblastic cell lines express myoferlin, but do not express dysferlin until they are induced to fuse. Knockdown of either dysferlin or myoferlin in one of the trophoblastic cell lines (BeWo cells) did not disrupt fusion. In the syncytiotrophoblast, there is a turnover of nuclei in which dead nuclei are removed though exocytosis. Dr. Robinson hypothesizes that high levels of dysferlin and myoferlin may be needed for extensive membrane remodeling due to the high rate of exocytosis. In collaboration with Dr. Paul McNeil, he is testing whether trophoblastic cell lines show a membrane repair defect in the absence of dysferlin. Dr. Robinson also found that expression of dysferlin was reduced by about 50% in placentas associated with pre-eclampsia, a disease of pregnancy in which the placenta causes health problems to the mother.

Rumaisa Bashir (UK) discussed two other genetic loci, in addition to dysferlin, that give rise to the clinical diagnosis of Miyoshi myopathy (MM). One, designated MMD2, is located on Chromosome 10 and was identified in a study of patients in the Netherlands (Linssen et al. 1998). The gene responsible for MMD2 has not yet been identified. The second is the MMD3 locus, located on Chromosome 11, which overlaps a genetic locus for an autosomal recessive form of LGMD (LGMD2L) (Jarry et al. 2007). Dr. Bashir demonstrated that cells from MMD3 patients have defective membrane repair (Jaiswal et al. 2007). In two MMD3 families, homozygous pathogenic mutations were found in TMEM16E, a member of the TMEM16 (Anoctamin) gene family. Mutations in TMEM16E are also responsible for LGMD2L and autosomal dominant gnathodiaphyseal dysplasia (GDD1), which causes bone fragility (Tsutsum et al. 2004). From studies of other TMEM16 proteins (Hartzell et al. 2009; Pifferi et al. 2009), TMEM16E is predicted to function as a calcium-activated chloride channel. Dr. Bashir hypothesizes that during repair of membrane tears, TMEM16E may function as a counter-ion channel following calcium influx to maintain charge neutrality for efficient exocytosis.

12. Session X: New Tools for Studying Dysferlin

This session highlighted technological advances that may improve the study of dysferlin or muscular dystrophies.

Robert Brown (USA) presented his development of a C2C12 cell line that stably expresses shRNAs that suppress dysferlin expression. However, injuring these cells using detergents or osmotic shock and monitoring release of creatine kinase or luciferase has not demonstrated a significant difference in membrane repair compared to wild type C2C12 myotubes. Gene expression analysis of these dysferlin-deficient C2C12 cells indicates that a number of genes involved in myogenesis and regeneration are upregulated. Dr. Brown also discussed a collaboration with Dr. Jerry Mendel to develop mini-dysferlins that can be used in AAV gene therapy. They have also recently begun to explore delivery of full-length dysferlin to the muscles of mice, based on a recent report that AAV5 can package larger genomes than other AAVs.

Paul McNeil (USA) discussed his progress on developing a high-throughput assay to monitor repair of membrane tears. He developed a tool to damage cells in a 96-well plate by simultaneously scraping cells off the bottom of each well and transferring the damaged cells in suspension to a new 96-well plate. The cells are stained with two different dyes, one retained by live cells that

successfully repair their membranes and one retained only in dead cells. The live/dead cell ratio is a reproducible measure of the cell population's ability to reseal. The entire process for each plate takes about 10 minutes by hand. This cell scraping technique works well on a variety of mononuclear cell types, but applying it to myotubes has proven difficult and may require further modifications to the protocol, such as application of drugs that inhibit muscle contraction. Dr. McNeil has surprisingly not found the same membrane repair defect in cultured dysferlin-deficient myotubes (derived from two different dysferlin-deficient cell lines) that he observes in isolated myofibers from dysferlin-deficient mice. In contrast, myotubes derived from primary dysferlin-deficient human myoblasts have been shown to have defective repair, suggesting that membrane repair may be influenced by the immortalization process or by differences between species.

Daniel Stockholm (France) is attempting to non-invasively monitor the membrane permeability of muscle fibers *in vivo* by labeling albumin with the radioactive tracer Technetium-99m. However, he found that the background levels of circulating Technetium-99m in the blood were too high to reliably detect an increase in the amount of Technetium-99m in the skeletal muscle of dystrophic mice. Audience members suggested that he try Technetium-99m alone rather than bound to albumin, as this may change its clearance properties. Dr. Stockholm is also using live photonic imaging of mouse skeletal muscle to follow the pathological events that occur after large strain injury, in collaboration with Dr. Robert Bloch. He monitors the entry and distribution of fluorescently labeled dextrans of various sizes into muscle fibers during and after injury. Preliminary results reveal patterns of dextran infiltration that suggest a segmental model of muscle fiber degeneration.

13. Special Session: NIH Funding for Neuromuscular Diseases

John Porter, Program Director at the National Institute of Neurological Disorders and Stroke at the NIH, talked about the current state of funding for neuromuscular disease and muscular dystrophy, and new opportunities for funding in these fields. In fiscal year 2008 the NIH spent $239 million on neuromuscular disease research, with $56 million of that going toward muscular dystrophy. The total amount of research dollars available to the muscular dystrophy research community has remained fairly flat in recent years, but there has been an increase in translational research program awards, which are designed to help move promising ideas for therapies closer to clinical implementation. For additional information about these programs please visit the office of translational research on the NINDS website.

14. Conclusions

The Dysferlin Conference has continued to grow rapidly in both number of participants and breadth of research presented since the first conference in 2007. It is particularly noteworthy that unpublished data presented at the 3rd Annual Dysferlin Conference point to a variety of cellular processes in addition to membrane repair that may be disrupted in the absence of dysferlin and contribute to pathology. It is clear that many assumptions about dysferlin and the pathogenesis of dysferlinopathy need to be revisited. At the Jain Foundation, we are very encouraged by reports of new collaborations that were formed at this conference and hope that all researchers will

take to heart the closing comments of our founder, Ajit Jain, and endeavor to accelerate the pace of dysferlin research by welcoming collaborations and sharing all of the resources and ideas that are available in the field. We look forward with great expectations to the next Dysferlin Conference in 2010.

Participants:

Norma Andrews (College Park, MD)
Corrado Angelini (Padova, Italy)
Rumaisa Bashir (Durham, UK)
Robert Bloch (Baltimore, MD)
Robert Brown (Worcester, MA)
Kate Bushby (Newcastle, UK)
Gillian Butler-Browne (Paris, France)
Michele Calos (Palo Alto, CA)
Pierre Cau (Marseille, France)
Avital Cnaan (Washington, DC)
Sandra Cooper (Sydney, Australia)
Dennis Drescher (Detroit, MI)
Kevin Flanigan (Salt Lake City, UT)
Julaine Florence (St. Louis, MO)
Matthew Harms (St. Louis, MO)
Eric Hoffman (Washington, DC)
Isabel Illa (Barcelona, Spain)
Tejvir Khurana (Philadelphia, PA)
Todd Lamitina (Philadelphia, PA)
Se-Jin Lee (Baltimore, MD)
Robert Leshner (Washington, DC)
Nicolas Lévy (Marseille, France)
Jianjie Ma (Piscataway, NJ)
Elizabeth McNally (Chicago, IL)
Paul McNeil (Augusta, GA)
Jerry Mendell (Columbus, OH)
Jeffery Molkentin (Cincinnati, OH)
Steven Moore (Iowa City, Iowa)
A. Nalini (Bangalore, India)
Terence Partridge (Washington, DC)
Alan Pestronk (St. Louis, MO)
John Porter (Washington, DC)
Thomas Rando (Palo Alto, CA)
Morayma Reyes (Seattle, WA)
Isabelle Richard (Evry, France)
John Robinson (Columbus, OH)
Michael Sinnreich (Montreal, Canada)
Simone Spuler (Berlin, Germany)
Daniel Stockholm (Evry, France)
Bryan Sutton (Lubbock, TX)
H. Lee Sweeney (Philadelphia, PA)
Yvan Torrente (Milan, Italy)
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Andoni Urtizberea (Paris, France)
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