Proceedings of the

Second Annual Dysferlin Conference

• June 14-18, 2008 •

Organized, sponsored, and moderated by the JAIN FOUNDATION INC

The Second Annual Dysferlin Conference, held from June 14-18, 2008 in Puerto Rico, brought together leading scientists to discuss recent research and progress towards a therapy for Limb Girdle Muscular Dystrophy 2B (LGMD2B) and Miyoshi Myopathy, which are caused by mutations in the protein dysferlin.

An important goal of the Dysferlin Conference is to bring together and facilitate discussion between the leaders of different fields that may contribute to the development of a therapy or cure: clinician scientists who see dysferlin patients and study their genetic mutations; researchers who study the role of the dysferlin protein and how its absence causes this disease; experts in the processes of membrane fusion and repair (in which dysferlin is now known to participate); and leaders in emerging muscle-targeted and gene-targeted therapies (including gene therapy, stem cell therapy, stop codon read-through, exon skipping, and enhanced muscle regeneration).

The Jain Foundation implemented a number of new initiatives at the conference designed to stimulate discussion and encourage collaboration around tangible, focused goals. For example:

1) The conference included two 100-minute breakout discussion sessions, during which participants were split into groups to discuss the roadblocks facing different avenues of research. The Jain Foundation team members pushed each group to reach a consensus on tangible experiments that can address these roadblocks. *Blue boxes (below) present the proposed experiments that resulted from each breakout session.* The Jain Foundation intends to find and support researchers who have the interest and expertise to conduct each of these experiments. In some cases, researchers attending the conference have already approached us to pursue one of the projects from these lists.

2) Investigators funded by the Jain Foundation were asked to develop a flow chart outlining the path their project will ideally take towards a therapy for LGMD2B/Miyoshi, including the new materials, technologies, and basic information that they will need along the way, and to highlight their progress along that path so far. These flow charts were included in the conference booklet to encourage constructive discussion of each funded project.

SESSION I:
DYSFERLINOPATHY – MECHANISMS OF PATHOLOGY

This session addressed the mechanisms that contribute to dysferlinopathy at the molecular and cellular levels. Although defective membrane repair plays a role in pathology, the details of how and why dysferlin mutations cause disease, and what happens downstream of a torn, unrepaired sarcolemma *in vivo,* remain poorly understood. Resolution of these key questions is critical for choosing and developing appropriate drugs and other therapeutic strategies.

Dr. Sandra Cooper (Children's Hospital at Westmead) discussed the trafficking and membrane biology of both wild type (WT) dysferlin and a missense mutant isolated from a patient that results in a F1871S amino acid substitution in the last C2 domain of the dysferlin protein. To study trafficking, she made tagged dysferlin constructs that code for the full-length WT and mutant proteins, as well as deletion fragments that contain only the C2F and transmembrane domains (C2F-TM, F1871S C2F-TM). These proteins were tagged with EGFP and His/Myc at the N- and C-termini, respectively, thus allowing for differential visualization of the intracellular and extracellular domains. Her studies evaluated both the protein half-life and the plasma membrane half-life of dysferlin.

She found that dysferlin requires the Golgi secretory pathway for trafficking to the plasma membrane (PM) and that it has a relatively transitory life at the PM (several hours), confirming that dysferlin indeed cycles to and from the PM. This trafficking pattern may help explain the abnormal localization of dysferlin in many patients with muscular dystrophy. In contrast, the deletion construct lacking the majority of the N-terminal cytosolic domain displays a much shorter half-life at the PM and is rapidly endocytosed and degraded, implying that element(s) within the dysferlin N-terminal domain are required for stable anchorage at the PM. The F1871S mutant is inefficiently targeted to the PM, and is largely retained intracellularly. Dr. Cooper speculates that this is probably due to a subtle folding defect and/or failure to interact with a factor that facilitates dysferlin's transport to the PM. Further investigation of additional deletion constructs and elucidation of relevant trafficking pathways are currently underway to clarify the molecular details involved.

Dr. Jeffery Molkentin (Cincinnati Children's Hospital Medical Center) discussed the role of Ca^{2+} in muscle degeneration. Ca^{2+} can enter cells through the sarcolemmal breaches that are encountered in various forms of muscular dystrophy. Mutations in a number of different sarcolemma-associated factors (such as dystrophin and the sarcoglycans) make the membrane more prone to contraction-induced damage, while mutations in dysferlin leave the membrane unable to repair itself following membrane tears. Dr. Molkentin hypothesizes that in either case, the result is unregulated entry of Ca^{2+} into the muscle fiber that in turn initiates intracellular signaling cascades culminating in muscle fiber death through apoptosis and necrosis. In order to test this hypothesis, Dr. Molkentin developed cyclophilin D null mice (a component of the mitochondrial permeability transition pore that plays a role in mitochondrial disruption and cell death) and crossed them with δ-sarcoglycan and laminin-α2 knockout mice. The resulting animals showed improvement in muscle pathology and amelioration of the dystrophic phenotype.

Further support for the Ca^{2+} hypothesis came from studies using the drug Debio-025, a cyclophilin inhibitor, which upon administration to mdx mice prevented dystrophic changes in the animals. In addition, Dr. Molkentin has generated several transgenic mice expressing different types of cation exchangers and channels to study how Ca^{2+} influx and efflux influence muscle degeneration. Recent studies with dysferlin-deficient A/J mice have revealed the presence of swollen mitochondria in muscle fibers, suggesting that elevated intracellular Ca^{2+} may indeed contribute to the muscle pathology observed in dysferlinopathy. Future work will include crossing dysferlin-deficient mice with transgenic animals that overexpress various Ca^{2+} channels, pumps and exchangers to directly test whether Ca^{2+} is involved in mediating muscle degeneration associated with dysferlin deficiency.

Dr. Robert Bloch (University of Maryland School of Medicine) presented research on the role of dysferlin in 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. Joseph Roche, in the Bloch laboratory, employed acute large strain eccentric contractions to induce muscle injury in control and dysferlin-deficient A/J mice, and monitored the extent of membrane disruption and the process of recovery from the injury by labeling muscle fibers with Fluorescein dextran and Rhodamine dextran, as well as markers of muscle regeneration and inflammation, and by monitoring contractile function.

They found that A/J mice are no more susceptible to muscle injury than control mice, but differ in how they recover after the injury protocol. In particular, the results suggested that dysferlin-deficient mice take longer than WT mice to recover from muscle damage and that recovery in dysferlin-deficient mice involves myogenesis rather than membrane resealing. Myogenesis in A/J mice eventually replaces all the fibers that were damaged directly or indirectly by injury. Remarkably, however, the muscle fibers in A/J mice that were initially damaged by injury remain impermeant to both fluorescent dextrans for 3 days after injury, suggesting that their plasma membranes do initially reseal. Examination of injured muscles during the recovery period revealed a potent inflammatory response in A/J but not control mice, which Dr. Bloch suggests is responsible not only for triggering myogenesis, but also for causing additional damage to fibers that were unharmed by the initial large strain contractions. These results highlight dysferlin's role in sarcolemmal stability and the potential role of the immune system in pathogenesis in dysferlin-deficient muscular dystrophy.

SESSION II:
DYSFERLINOPATHY AND THE IMMUNE SYSTEM

This session addressed the role of dysferlin in the immune system and highlighted studies that suggest a more direct involvement of immune system dysfunction in dysferlinopathy. While an inflammatory response to damaged muscle is expected, certain observations—such as the robust expression of dysferlin in immune cells, the unusually marked inflammation associated with the disease, and the development of autoimmunity in mouse models of dysferlinopathy—have raised the possibility that an aberrant immune response may play a more active, rather than passive, role in the progression of LGMD2B/Miyoshi.

Dr. Eric Hoffman (Children's National Medical Center) presented his recent studies on a possible mechanism behind the aggressive inflammatory response seen in many dysferlinopathy patients. Work carried out in collaboration with Dr. Kanneboyina Nagaraju has uncovered enhanced phagocytotic activity in dysferlin-deficient murine monocytes and macrophages, as well as upregulation of multiple vesicular trafficking proteins in these cells. Investigation of how these immune cells interact with and are recruited to damaged myofibers has revealed a specific induction of the Rab27A/Slp2a vesicle trafficking pathway in dysferlinopathy but not in two other dystrophies, LGMD2I and Becker's muscular dystrophy (BMD). These results suggest a possible hypothesis: that this alternative vesicle trafficking pathway is induced to compensate for the absence of dysferlin, but that this mode of compensation is also accompanied by abnormal release into the extracellular environment of vesicular contents that serve as paracrine signals for the recruitment of cytotoxic T-cells and natural killer cells, leading to inflammasome formation and fiber death. Components of the inflammasome are increased in the dysferlin-deficient SJL mouse model and LGMD2B/MM patients, and this finding appears to be specific to dysferlin deficiency. Dr. Hoffman also briefly mentioned that the reason why prednisone does not benefit dysferlinopathy patients may not have to do with its anti-inflammatory activity but rather with the fact that this steroid helps re-synchronize regeneration in muscle, a process that is already in place and not excessively affected in LGMD2B/Miyoshi patients. Overall, Dr. Hoffman's work underscores the contribution of aberrant immune responses to dysferlinopathy, and suggests that therapeutic strategies to intervene these responses should be explored.

BREAKOUT SESSION:
IMMUNE INVOLVEMENT

Proposed Project:

Dissecting the role of dysferlin in the immune system. The aim of this project is to selectively study the role of dysferlin in immune system function, and to assess the relative contribution of dysferlin deficiency in immune cells to dysferlinopathy. This may be accomplished by:

1. Selective ablation of individual immune cell types in dysferlin-deficient mice: Rag-/- for B and T lymphocytes, dichloro-methylene-bisphosphonate for macrophages; and
2. Muscle-specific knockdown of dysferlin.

Dr. Simone Spuler (Charité University Medicine Berlin) discussed the involvement of the complement system in disease pathology associated with dysferlin deficiency. Complement is a major effector of the humoral immune response and consists of a group of serum proteins that generate a cytolytic membrane attack complex (MAC). Dr. Spuler's studies have shown that dysferlin deficiency is associated with absence of the complement protection factor DAF1/CD55 from the surface of skeletal muscle fibers in mouse models as well as LGMD2B/MM patients, which may make the fibers more prone to membrane attack via the complement cascade. This hypothesis is supported by Dr. Spuler's findings of MAC deposition on dysferlin-deficient muscle fibers and the susceptibility of dysferlin-deficient myotubes to complement-mediated lysis. Furthermore, the administration of anti-C5 antibodies to antagonize complement activity in dysferlin-deficient mice led to reduction of muscle fiber damage, and IVIG therapy in three LGMD2B/MM patients led to muscle strength improvement and decreased levels of MAC. These results indicate that inhibition of complement activity should be explored as a therapy for LGMD2B/MM.

Dr. Christina Jamieson (University of California, Los Angeles School of Medicine) presented data suggesting that dysferlin may be involved in T-cell apoptosis. Gene expression profiling of murine T-cells treated with the glucocorticoid dexamethasone revealed that dysferlin mRNA, along with that of some synaptotagmins, is upregulated during apoptosis. This induction of expression was abolished by inhibiting apoptosis using TCR-mediated survival signaling. Another apoptotic stimulus, gamma irradiation, also induced expression of

dysferlin mRNA and protein in the mouse thymus, albeit with different kinetics than was observed for dexamethasone treatment. Comparison of irradiation-induced apoptosis in wild type C57BL/6 versus dysferlin-null A/J mice showed that CD8+ T-cells were more resistant to apoptosis in dysf-null mice than wild type. Overexpression of mouse dysferlin cDNA in T-cells resulted in a 50% reduction in viability even without dexamethasone treatment. Taken together, these data suggest that induction of dysferlin expression in T-cells promotes apoptosis. Dr. Jamieson hypothesized that dysferlin may be involved in the vesicle trafficking and membrane remodeling steps associated with late-stage apoptosis, such as membrane blebbing, and that the absence of dysferlin may lead to aberrant apoptosis in T-cells. She also briefly discussed the effect of glucocorticoids on muscle development, and showed that dysferlin is also induced in C2C12 cells upon dexamethasone treatment. She speculates that the inability to upregulate dysferlin may contribute to the ineffectiveness of glucocorticoid treatment for LGMD2B/MM patients, in contrast to other MD patients.

SESSION III:
MEMBRANE FUSION AND REPAIR

This session focused on research into the basic cellular mechanisms for repairing membrane tears. Dysferlin is required for membrane repair, but its specific role in the repair process—a process that is itself poorly understood—is unknown. Additional studies of the basic mechanisms of repair will help define dysferlin's function and generate new ideas for therapies. For example, identification of other proteins that participate in repair or alternate (non-dysferlin-dependent) repair pathways may suggest candidate proteins that could compensate for the absence of dysferlin. Knowledge of other cellular processes involved in or dependent on repair of membrane tears may help identify additional downstream effects of dysferlin deficiency that could contribute to pathology. Finally, a more detailed understanding of the steps of repair will also aid development of high-throughput assays to screen for membrane repair-enhancing drugs.

Dr. Paul McNeil (Medical College of Georgia) discussed his ongoing efforts to identify proteins that are required for repair of membrane tears in addition to dysferlin. He had previously shown that the dysferlin-interacting protein annexin A1 is required for repair in fibroblasts and has now extended his experiments to myotubes. He found that both annexin A1 and annexin A2 concentrate at the site of membrane damage after laser wounding. He also found that C2C12 myotubes with annexin A1 function disrupted (by expression of a dominant negative annexin A1 mutant or by siRNA knockdown) and myotubes from annexin A1 knockout mice cannot repair laser-induced wounds, similar to dysferlin-deficient myotubes. Some of the unrepaired cells in culture appear to undergo rapid contraction and membrane blebbing after wounding. It is unclear whether this happens physiologically, but it may be a sign of changes that could prevent repair-deficient cells from pursuing alternate, delayed mechanisms of repair (such as activating another repair pathway) after repair fails initially. Dr. McNeil mentioned during the question period that significant membrane blebbing sometimes occurs even when cells successfully repair, if the original damage was particularly severe.

Dr. McNeil also discussed his progress in developing a high-throughput assay to monitor repair of membrane tears. The limiting factor for such a membrane repair assay has been the method used to generate reproducible damage in a high-throughput manner. Dr. McNeil has developed a tool to damage cells in a 96-well plate by simultaneously scraping cells off the bottom of each well

BREAKOUT SESSION:
MEMBRANE REPAIR

Proposed Project:

Determining the size and site of membrane tears. This project involves using cryobiopsy to determine and compare the size and site of membrane tears in wild type (WT) and dysferlin-deficient muscle fibers. This could be done on WT and dysferlin-deficient mice immediately after exercise, using biopsy and cryofracture/EM to detail the defects in the sarcolemma. The cryobiopsy approach, for example, is described in Shimoni and Muller, J. Microscopy (1997) 192: 236-247. Care must be taken to obtain the biopsy quickly enough so as to preserve the membrane changes associated with tearing (this may be easier to do in dysferlin-deficient mice due to defective/slower membrane repair). Another approach for determining the size of membrane holes is to use dye-tagged molecules of various sizes (for example, fluorescein- or rhodamine-glycans of various molecular weights). The size of the hole can be estimated based on the molecular size limit of the glycan that can diffuse through it. Care must be taken to use diffusible molecules whose mass cut-off is determined solely by the size of the sarcolemmal hole and not by impediment from extracellular structures such as the matrix, fat deposits, etc.

and then transferring the damaged cells in suspension to a new 96-well plate. The entire process for each plate takes about 10 minutes by hand. The cells are stained with two different dyes, one retained by cells that successfully repair the damage and one retained only in dead cells, and the ratio of repaired-to-dead cells gives a reproducible measure of the cells' ability to reseal.

Dr. Steven Vogel (National Institutes of Health) discussed recent experiments on the function of a sea urchin homolog of dysferlin. Sea urchin eggs have long been used as a model system to study membrane repair. Dr. Vogel is now using early sea urchin embryos, rather than eggs, to study the role of ferlins in the sea urchin. He identified a sea urchin ferlin with similarity to dysferlin and found that morpholino-mediated knockdown of its expression (in one cell of 2-cell stage embryos) inhibits the cytokinesis step of cell division, which is thought to require endocytosis. He also found reduced uptake of FM1-43 dye into cells with sea urchin ferlin knocked down, suggesting that endocytosis is inhibited. These observations could result from a primary defect in endocytosis or from a primary defect in exocytosis that subsequently inhibits compensatory endocytosis. Dr. Vogel has now confirmed in preliminary experiments that knockdown of sea urchin ferlin in embryonic cells also inhibits membrane repair.

While monitoring Ca^{2+} levels inside wild type embryonic cells using a fluorescent reporter, Dr. Vogel observed occasional flashes of high intracellular Ca^{2+} that became much more frequent shortly before cell division. He also found that wounding a single cell in an embryo causes a flash of high intracellular Ca^{2+} that is subsequently observed in neighboring unwounded cells. These Ca^{2+} spikes observed in unwounded cells are inhibited by cadmium (a Ca^{2+} channel blocker) and occur even in cells of nearby embryos in the culture, suggesting that they are triggered by a soluble signal. Dr. Vogel has found that ATP applied onto the cells can mimic these flashes. He is continuing to study this Ca^{2+} signaling to determine the nature of the soluble signal and whether transmission of the signal is blocked by knockdown of the sea urchin ferlin.

Dr. Norma Andrews (Yale University School of Medicine) discussed her recent work on the sequence of membrane cycling events during membrane repair, which is known to involve Ca^{2+}-mediated exocytosis of intracellular vesicles. She had previously shown that lysosomes participate in exocytosis during the repair process and has now confirmed this result in muscle cells, showing that inhibition of lysosomal exocytosis in C2C12 myoblasts using bromoenol lactone interferes with repair. She also presented her recent finding that newly-formed endosomes can 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 a protein-lined pore in the membrane). Inhibition of lysosomal exocytosis prevents the formation of these endosomes, suggesting that they are the result of compensatory endocytosis after exocytosis. Enhancement of this endocytic process (by disruption of the actin cytoskeleton) accelerates repair, while inhibition (by cholesterol depletion) blocks it, which led Dr. Andrews to hypothesize that these endosomes contain the tear or pore from the plasma membrane and carry it into the cell. If compensatory endocytosis is indeed required for repair, it is possible that dysferlin's primary role could be in endocytosis rather than exocytosis.

Dr. Andrews also discussed her work on another protein, synaptotagmin VII (SytVII), that she has found to be involved in membrane repair in muscle. The synaptotagmins are a family of transmembrane proteins similar to dysferlin (with two C2 domains instead of seven), and synaptotagmin I is known to mediate Ca^{2+}-dependent exocytosis in nerve cells. Dr. Andrews found that SytVII is expressed in muscle and localizes to lysosomes. SytVII knockout mice have progressive loss of muscle strength coupled with inflammatory cell infiltration and fibrosis in skeletal muscle and skin, similar to the symptoms of dysferlinopathy, and fibroblasts from these mice have defective membrane repair. Additional work is needed to determine whether dysferlin and SytVII function in different repair pathways, both required for muscle cell maintenance, or whether they have different functions in the same repair pathway.

SESSION IV:
CLINICAL ASPECTS OF DYSFERLINOPATHY

There are many dysferlinopathy-related clinical challenges that need to be addressed and studied in order for us to better understand the disease process and to more accurately diagnose, treat, and care for dysferlinopathy patients. Some questions include: what is the best, most accurate, and cost effective way to diagnose patients; what causes the late onset of disease

BREAKOUT SESSION:
CLINICAL ISSUES IN DYSFERLINOPATHY

Proposed Projects:

Factors that cause disease variability. This project investigates the possible reasons behind the high degree of variability seen in the onset and progression of LGMD2B/Miyoshi. For example: 1) Male and female dysferlin-deficient mice will be compared to test whether gender differences play a role. 2) Different exercise protocols will be employed to test the influence of physical activity on the rate of disease progression in various mouse models.

Causes of differential muscle involvement. This project examines if differential gene expression and/or fiber-type involvement are contributing factors to differential muscle-involvement in LGMD2B/Miyoshi. Different muscle groups will be compared to correlate their gene expression profiles and the ratio of their fast and slow muscle fibers to their "affected" status.

Development of natural-history protocols for dysferlin-deficient patients. The aim of this project is to develop a standardized natural-history protocol for LGMD2B/Miyoshi patients by gathering data from a statistically significant number of patients. Such a protocol is critically needed to confidently determine the outcome measures for clinical trials.

and the highly variable phenotype; and what issues need to be addressed before recruiting dysferlinopathy patients into clinical trials. The presentations in this session focused on these three different, but equally important clinical challenges: dysferlin mutational analysis and interpretation, factors that contribute to disease onset and progression, and issues related to the planning and analysis of clinical trials.

Dr. Martin Krahn (Hopital d'enfants de la Timone) discussed mutational analysis tools currently being developed in France for the identification and characterization of dysferlin mutations. Tools such as these are urgently needed because of the size of the dysferlin gene, the high proportion of missense/intronic variants, the high number of unique variants, and the large percentage of patients without two confirmed pathogenic dysferlin mutations. Dr. Krahn first described the UMD Locus Specific Database developed for dysferlin. As of June 2008, the UMD-DYSF database contains 629 entries that describe 227 mutants and 201 protein variants. Along with information on the individual variants, the database contains bioinformatic tools to aid in predicting the pathogenicity of new variants. These tools include the splice site analysis/ splice site sequence finder for analysis of intronic variants, the UMD predictor for analysis of missense/ isosematic variants, and Global Analysis for statistical analysis of mutational events.

Dr. Krahn went on to describe two mutational analysis techniques that could help identify additional mutations in the 30% of patients that have 0-1 dysferlin mutations identified. The first technique involves sequencing the alternative exons 1, 5, and 40. In 29 patients tested, no mutations in these alternative exons were found, implying that if mutations in these alternative exons exist, they are rare. The second technique is Multiplex Ligand-dependent Probe Amplification (MLPA), developed by the MRC-Holland company, which identifies large intragenic rearrangements. Dr. Krahn showed how this technique has been used to identify single to multi-exon deletions and duplications in a number of dysferlin patients. The development and use of these important tools and techniques will be essential to help us diagnose dysferlinopathy patients, better understand the disease, and develop new therapeutic approaches.

Dr. Corrado Angenlini (University of Padova) discussed prognostic factors that may adversely affect dysferlinopathy disease progression. He began his presentation by describing the distribution and clinical course of the various clinical phenotypes that are associated with a dysferlinopathy. Distal myopathies, such as Miyoshi Myopathy, accounted for the largest phenotypic group at 80%, following by HyperCKemia at 12%, and LGMD2B at 8%. By comparing the correlation between muscle strength (using the MRC grade) and various muscle functional scores (gait, Gower sign, etc.), Dr. Angelini showed that patients who present with a distal Miyoshi phenotype have a more rapid disease progression compared to those patients who present with a more proximal or mixed proximodistal phenotype, even though onset generally occurs later for the Miyoshi subgroup. Therefore, distal weakness at disease onset is an adverse prognostic factor.

Another adverse prognostic factor appears to be strenuous physical activity prior to disease onset. Dr. Angelini evaluated 11 molecularly confirmed LGMD2B patients using a modified Garner-Medwin and Walton clinical scale. Five out of the 11 patients never performed regular physical activity, while 6 out of the 11 performed competitive physical activity prior to onset of the disease. By comparing the time it took to reach the "loss of Gowers" stage on the modified

Gardner-Medwin and Walton scale, he showed that the physically active ("sportive") patients reached this stage after only 5.75 years compared to 10 years for the non-sportive patients. Therefore, Dr. Angelini suggests that patients who are suspected to have a dysferlinopathy should limit their physical activity. The third adverse prognostic factor that he described was prominent inflammation. He showed that an increased inflammatory response in muscle biopsies correlated with faster disease progression and decreased fiber regeneration, as documented by fetal myosin histopathological studies.

Dr. Kathryn Wagner (Johns Hopkins School of Medicine) discussed the recently completed Phase I/II MYO-029 trial of myostatin inhibition in adult muscular dystrophy. Myostatin inhibits muscle growth and directly regulates fibrosis, and inhibition of myostatin has been shown to improve muscle regeneration. These properties were the rationale for developing neutralizing antibodies to myostatin, including MYO-029, as treatment for muscular dystrophies. The Phase I/II clinical trial was a multicenter, double-blind, placebo controlled, randomized trial of MYO-029 sponsored by Wyeth Pharmaceuticals and included adult patients with a number of different muscular dystrophies, including LGMD2B.

Dr. Wagner discussed a number of findings from the trial, including the following:

- Short term, partial myostatin inhibition is likely safe and well tolerated.
- Myostatin inhibition can increase muscle mass and shows positive trends in the percent change in lean body mass and muscle fiber diameter.
- At the doses used in this trial, MYO-029 does not produce substantial changes in muscle strength or function.

In addition, while the exploratory endpoints were found to be feasible for all disease populations studied, outcome measurements need to be refined based on the previously underappreciated amount of fibrosis observed.

She also discussed why the trial was unsuccessful in answering the following questions:

- Can MYO-029 reduce myostatin levels or activity? Many of the patients' myostatin levels were below the limit of quantification.
- Can MYO-029 increase muscle function? The "power" to detect change in function was low in this study.
- Are some conditions more responsive to myostatin inhibition than others? There were too few patients in each disease category.

While this trial provides some hope for the use of myostatin inhibition as a treatment for adult muscular dystrophies, Dr. Wagner believes that there are a number of issues that need to be considered before moving forward with another trial in the future. These include identifying biomarkers as a way to assess myostatin activity, determining surrogate endpoints to assess efficacy, and conducting phase 0 trials with a small number of patients to provide proof-of-principle.

SESSION V: NEW RESEARCH TOOLS AND TECHNOLOGIES

This session highlighted recent technological advances that may be helpful in the study of dysferlin or muscular dystrophies. New research tools and technologies have a profound impact on the kinds of scientific questions that can be addressed and our ability to determine the pathological mechanisms that underlie LGMD2B/ Miyoshi. This session was subdivided into two parts, one part covering new advances in imaging technologies for muscular dystrophy that will help researchers follow the progression of muscle diseases with increased accuracy and in some cases non-invasively; and the second part on progress in developing dysferlin-deficient myoblast cell lines that will help facilitate a variety of research endeavors, including high throughput screening for small molecule therapies and fundamental studies of dysferlin function.

Advances in imaging technologies

Dr. Thomas Rando (Stanford University School of Medicine) discussed the development of two genetically altered strains of mice that use a Luseap reporter gene to track skeletal muscle destruction and regeneration. This system uses two distinct reporters, serum alkaline phosphatase and luciferase (luciferase activity can be measured non-invasively). These markers are inducible

and tissue specific, and should be very useful for tracking the destruction and regeneration of muscle fibers noninvasively once crossed with dysf-null mice. One mouse strain will express the Luseap reporter only in mature muscle fibers and will serve as a marker of muscle loss; in the other mouse strain, the reporters will only be expressed in satellite cells and serve as a marker of muscle regeneration. Examples of the luciferase *in vivo* imaging technology were shown from normal mice with luciferase expression in mature skeletal muscle fibers. One limiting factor of this technology is that because it is light based, the signal intensity can be influenced by a number of factors such as the presence/absence of fur, skin pigmentation, and thickness of the skin/subcutaneous fat. Because of these factors, it was noted that the strength of the signal doesn't correlate well with the sites of greatest muscle bulk on an individual animal. However, the ability to monitor the same muscles in a single live animal over time should ameliorate these issues and give a good estimate of changes in muscle pathology or regeneration. The mice carrying the Luseap reporter gene were recently crossed with the SJL model of dysferlin deficiency, and it is anticipated that initial studies on these mice will proceed by late summer 2008.

Dr. Daniel Stockholm (Généthon) began his talk with an overview of the advantages and limitations of current imaging technologies. This discussion included photonic imaging, such as 3D confocal microscopy, Fluorescence Resonance Energy Transfer (FRET) and Fluorescence Recovery After Photobleaching (FRAP), as well as some examples of medical imaging techniques such as MRI, CT and gamma-scintigraphy, as applied to animals. The advantages of photonic imaging include the ability to examine the fine distribution of labeled proteins in living mice, and in some cases to track them in real time at the cellular and sub-cellular level. The major disadvantage is that in order to obtain high resolution images of myofibers, the skin of the animal must be opened, making it difficult to examine the same animal over time. Furthermore, photonic imaging has very few clinical applications. Medical technologies such as MRI, CT, and gamma-scintigraphy have the advantage of being non-invasive and thus able to sample the same patient or animal multiple times in a longitudinal study. However, MRI, CT and gamma-scintigraphy don't offer the fine resolution or specific labeling of a molecule of interest that can be achieved with photonic imaging.

Dr. Stockholm then presented some preliminary results from his attempts to use technicium-labeled albumin to track changes in muscle membrane integrity. This project combines the commonly used membrane permeability assay based on Evan's blue dye uptake (Evan's blue dye is known to bind to albumin in the blood) with the non-invasive imaging techniques of gamma-scintigraphy and MRI. By labeling albumin

BREAKOUT SESSION:
MODELING DISEASE IN MICE

Proposed Projects:

Comparative study of pathology in different mouse models of dysferlinopathy. This project involves comparing disease progression between the 4 mouse models of dysferlinopathy – A/J, SJL, Bla/J and the Bittner mice. The mice will be studied at different ages to compare which types of muscles are involved (and to what extent) by examination of muscle pathology and by functional measurements.

Determining the effects of physical activity on pathology in mouse models of dysferlinopathy. This project involves exposing dysferlin-deficient mice of different ages (both pre- and post-symptomatic) to a variety of physical activities (both weight-bearing and non-weight-bearing) for various lengths of time and in various combinations. Muscle pathology in these mice (versus un-exercised dysferlin-deficient mice) will be monitored both immediately following exercise and in longer-term follow-up examinations to evaluate the short-term and long-term effects of physical activity on the progress of dysferlinopathy in mice.

Gene expression analysis of affected and unaffected muscles in pre- and post-symptomatic mice. This project will look for differences in gene expression between muscles in pre-symptomatic dysferlin-deficient mice versus affected muscles in post-symptomatic mice, in order to identify any changes that may contribute to the onset of pathology. It will also look for differences between unaffected and affected muscles in the same symptomatic animals, in order to identify any genes that may contribute to the resistance of some muscle groups to pathology.

Investigating the effects of muscle fiber type on pathology. This project involves determining the muscle fiber type ratios in affected versus unaffected muscles in symptomatic dysferlin-deficient mice, in order to investigate a possible difference in susceptibility of the different fiber types to pathology.

Testing whether inflammation can be included as an outcome measure in preclinical studies. This project will rigorously test a variety of assays of muscle inflammation on dysferlin-deficient and control mice to determine the reproducibility of these assays and their statistical power in distinguishing between affected and unaffected mice. The project should only be done on dysferlin-deficient mice with appropriate controls (e.g. Bla/J vs. Bl6 mice, or Bittner vs. Bl10 mice).

with the radioactive tracer technicium, Dr. Stockholm is hoping to non-invasively monitor the membrane permeability of muscle fibers *in vivo*. The development of this technique may be useful for monitoring disease progression in patients, or during clinical trials, since technicium-labeled albumin is already commonly used in the clinic for other purposes.

Development of dysferlin-deficient myoblast cell lines

Dr. Terence Partridge (Children's National Medical Center) began by describing several complications associated with using immortalized myoblast models for studying muscle diseases such as dysferlin deficiency. The major concern with immortalized muscle cell lines is the genetic drift that occurs while they are in culture. This changes their characteristics over time, and it is difficult to compare results between different labs even when they use the same cell line. Dr. Partridge then described how the immortomouse has been used to generate immortalized myoblast cell lines that are superior to the commonly used C2C12 cell line in their ability to fuse and form myotubes, and that do not show the high level of genetic drift that has been reported to occur in C2C12 cells. Because the immortomouse takes advantage of a temperature-sensitive mutation, there is some concern that growing the cells under permissive conditions (33°C, plus 10ng/ml interferon gamma) could impact other aspects of cell function. However, the ease of use, genetic stability, and high myogenicity of these lines are significant advantages over traditional cell lines. Furthermore, crossing the immortomouse with mouse models of muscular dystrophy is a strategy that may facilitate the production of high quality muscle lines to study various forms of muscular dystrophy. Dr. Partridge has plans to do just that, and is currently crossing the dysferlin-deficient A/J mouse line with the immortomouse to create a high quality dysferlin-deficient myoblast cell line.

Dr. Robert Brown (Harvard Medical School) presented his work on the development and characterization of a C2C12 cell line that stably expresses shRNAs that suppress dysferlin expression. His laboratory began the experiments with commercially available (Sigma) siRNAs and then modified the sequences for greater efficacy. These sequences were used to create plasmids that were stably introduced into the C2C12 cells. He now has a mixed population of C2C12 cells that show an 80% knock-down of dysferlin. Selection and expansion of single clones to create a more homogeneous cell population is planned. Dr. Brown also presented gene expression data suggesting that a number of genes involved in myogenesis and regeneration are increased in his dysferlin-deficient C2C12 cells. He is interested in using this information to identify biomarkers that are specific to dysferlin deficiency. Dr. Brown also discussed preliminary experiments in which primary cultures of dysferlin-deficient cells were used in a detergent-based wounding assay (.025% SDS for 2min) that successfully demonstrated the inability of the dysferlin-deficient cells to reseal membrane wounds. The ultimate goal of the project is to develop a method for high throughput screening of small molecules that can help compensate for the loss of dysferlin. Finally, Dr. Brown discussed his attempts to over-express dysferlin in transgenic mice and the surprising discovery that too much dysferlin expression causes a severe muscle pathology.

SESSION VI: GENE THERAPY

This session focused on the different therapeutic strategies that are being explored to restore dysferlin expression in patients with LGMD2B/Miyoshi. Gene delivery strategies include intramuscular injection or systemic delivery via the blood, using different DNA delivery vehicles ranging from viral delivery to direct injection, or reimplantation of progenitor cells taken from the patient and modified by *ex vivo* gene therapy. This session included discussion of potential challenges associated with genetic therapies that specifically relate to the dysferlinopathies. These challenges include how to achieve sufficient and sustained dysferlin expression, how to express dysferlin in the right cell types (e.g. immune cells and muscle cells), how to circumvent the issue of the large size of the dysferlin gene, and whether an immune response to the delivery agent (e.g. virus) and/or to the newly-expressed dysferlin protein might be an issue.

Dr. Isabelle Richard (Généthon) discussed the use of adeno-associated viruses (AAV) for delivery of the dysferlin gene. The advantages of AAV vectors include their lack of pathogenicity, lower immunogenicity than other vectors, and efficiency of muscle cell transduction. Some issues to be resolved include the administration route, the possibility of an immune response, and the 4.5-kb upper limit to the capacity of the virus (since the dysferlin cDNA is almost 7-kb without any regulatory

elements). To address the latter problem, Dr. Richard has taken advantage of a concatamerization strategy that uses two different AAV vectors to deliver the dysferlin gene in two pieces, which contain specific sequences that allow them to reassemble. She first demonstrated effective concatemerization of 2 separate AAV vectors that produce a full length dysferlin protein in cell culture. She then administered the AAV particles either intramuscularly or intravenously into dysferlin-deficient mice. Injection into the tibialis anterior resulted in dysferlin expression, detectable by western blot, up to 12 months later. Functional restoration of dysferlin in the injected mice was tested by a membrane repair assay: FM 1-43 entry into wounded fibers was found to be decreased for fibers from AAV-injected dysferlin-deficient mice compared to fibers from uninjected dysferlin-deficient mice, demonstrating improved repair. In an assay for locomotor activity conducted one month after systemic injection of the AAV vectors, the mice expressing the concatamerized dysferlin gene showed significant improvements in their level of activity, speed, and distance travelled when compared to untreated dysferlin-deficient mice.

Dr. Richard is also backcrossing the dysferlin-deficient A/J mice onto a C57BL/6 background in order to have a dysferlin-deficient mouse model with a strain-matched control. This is very important as the A/J mice are known to have mutations in several other genes. Initial characterization of the F4 generation showed that it has a similar index of centronucleation to the parental A/J line. Dr. Richard very generously offered to distribute the mice freely to anybody interested in using them.

Dr. Michele Calos (Stanford University School of Medicine) discussed two different strategies to restore dysferlin expression in muscles. In both strategies, her laboratory takes advantage of phiC31 integrase, a site-specific recombinase, to incorporate the dysferlin gene stably into the genome of the recipient cells. Initial experiments to test gene delivery to mice by hydro-dynamic injection into the saphenous vein yielded limited expression of GFP in the muscle fibers. When naked DNA was directly injected into the hindlimb muscles and followed by electroporation, the efficiency of delivery was significantly higher as measured by luciferace imaging in the mice as well as immuno-fluorescence staining.

BREAKOUT SESSION:
GENETIC THERAPIES

Proposed Project:

Choice of dysferlin isoform for gene therapy. This project addresses the question of whether different dysferlin isoforms are equally or only partially functional by testing their therapeutic effectiveness upon administration to mice. *Dr. Isabelle Richard (Genethon, France) will test the ability of the two dysferlin exon 1 variants to restore function upon AAV-mediated administration to dysferlin-deficient mice.*

The second strategy being used by Dr. Calos is cell therapy transplantation with mesoangioblast cells, which are multipotent, self-renewing stem cells that can differentiate into skeletal muscle, smooth muscle, osteoblasts, adipocytes and cardiomyocytes in vitro and can migrate to sites of muscle degeneration within the body. Dr. Calos has shown that these mesoangioblasts can be successfully transfected and has shown stable DNA integration mediated by the phiC31 integrase. She further demonstrated the capacity of the modified mesoangioblasts to undergo differentiation into myotubes in vitro. Mdx mice injected with phiC31 integrase-modified mesoangioblasts showed expression of luciferase in the hindlimbs of the mice, and, more importantly, correctly-localized dystrophin expression in muscles. Future studies will focus on delivery of dysferlin to dysferlin-null mesoangioblasts, followed by transplantation into dysferlin-null mice.

Dr. Nicolas Lévy (Hopital d'enfants de la Timone) presented his work on the identification and characterization of dysferlin mutations. Primary dysferlinopathies have a large mutational spectrum. Analysis of the dysferlin gene has revealed no mutational hot spots, with most disease-causing mutations being missense or frame-shift mutations, and about 20-25% being nonsense mutations. Dr. Lévy described a patient with atypical and moderate primary dysferlinopathy whose cells still produce a mini-dysferlin protein. In this patient, there is a large multi-exonic genomic dysferlin deletion (exons 2 to 40). The truncated protein is 73 KDa in size, and detectable on the plasma membrane in blood monocytes (by IF, FACS and WB). This truncated dysferlin is at least partially functional in the patient as well as in a membrane repair assay. Since the size of the truncated dysferlin's cDNA is within the packaging size limit of AAV-vectors, it might provide a therapeutic alternative to full length dysferlin for gene therapy. Importantly, the partial functionality of this mini-dysferlin molecule demonstrates the partial modularity of wild type

dysferlin, which is an essential pre-requisite for exon-skipping strategies in dysferlinopathies.

Dr. Lévy also discussed the DYSFonCHIP project, in which he is using comparative genomic hybridization (CGH) and sequence capture arrays to identify mutations in dysferlin and related partners. CGH is a powerful tool that he showed can be used to detect several types of dysferlin mutations, including the previously-described heterozygous large deletion (exons 2-40), as well as a short homozygous deletion. Sequence capture arrays detect point mutations and small insertion/deletions. Currently dysferlin mutational analysis is an expensive and time consuming task that often fails to find both pathogenic mutations. The use of the CGH and sequence capture arrays could eliminate these issues by making mutational analysis cheap, accurate, and quick. The use of these arrays has the potential to revolutionize the way mutational analysis is performed for dysferlin, as well as many other genes.

SESSION VII: COMPENSATING FOR DYSFERLIN DEFICIENCY

This session focused on ways to counter the pathological process that is initiated by the absence of dysferlin. The current hypothesis in the field is that when dysferlin is absent, skeletal muscle cells are unable to efficiently repair membrane tears that occur as a consequence of normal muscle use. These unrepaired tears precipitate a cascade of events that ultimately leads to the destruction of the muscle fiber or severe damage that necessitates recovery by regenerative mechanisms. Therefore, it is possible that preventing this destructive process or increasing the regenerative response of the muscle may slow, or even stop, the progression of the disease in patients.

Dr. Se-Jin Lee (Johns Hopkins University School of Medicine) is exploring how pathways regulating the muscle growth inhibitor myostatin might be used to increase muscle regeneration. Previous work by Dr. Lee and others has demonstrated that inhibiting myostatin can increase the muscle mass of normal and dystrophic mice. While simply increasing muscle mass won't prevent the pathological process initiated by dysferlin deficiency from occurring, it may still help patients retain greater muscle function and substantially improve their quality of life. Anti-myostatin antibodies are already being tested in clinical trials as a potential therapy for muscular dystrophy (see above); however, it is unclear whether directly targeting myostatin will be an effective approach in patients. Dr. Lee's talk focused on how myostatin's interacting protein partners regulate its activity. In particular, myostatin's activity is regulated by the BMP-1/tolloid family of proteases, which cleave the inactive form of myostatin to create the active molecule, and the activin receptors that initiate a cascade of intracellular signals upon binding myostatin. Multiple members of these two protein families are involved in myostatin processing and signaling, and represent potential targets for small molecule therapies to increase muscle mass by inhibiting the negative effects of myostatin on muscle growth.

Dr. Joshua Zimmerberg (National Institutes of Health) is directly assessing whether changes in the lipid composition of myoblast membranes can alter their ability to reseal in the absence of dysferlin. The composition of a lipid bilayer determines many of its properties, including its curvature and stability. The intrinsic curvature of different lipids can influence the spontaneous resealing of membrane tears or promote specific steps in the membrane fusion process. Therefore, precisely determining the composition of the myoblast membrane and altering it through the introduction of specific dietary lipids may provide a way to mitigate the effects of dysferlin deficiency. Dr. Zimmerberg described the development of several tools designed to help him address this question, including a

BREAKOUT SESSION: DEVELOPMENT OF SMALL MOLECULE THERAPIES

Proposed Project:

Developing high throughput assays for screening small molecules. The aim of this project is to design and develop a variety of assays for screening small molecules and drugs that may compensate for dysferlin deficiency in mammalian cells. Specific examples of such assays could include:

1. Restoration of normal membrane repair in dysferlin-deficient cells after a variety of methods of wounding the cell membrane.
2. Upregulation of the rate of membrane repair in wild type cells (to identify compounds that improve membrane repair in general and are not dysferlin-specific).
3. Restoration of a normal rate of phagocytosis in dysferlin-deficient macrophages.
4. Restoration of a normal growth rate and normal cell-to-cell fusion in dysferlin-deficient skeletal muscle cell culture.

dysferlin-deficient myoblast cell line, a method for assessing lipid composition that makes use of mass spectrometry, and a patch clamp based wounding assay. Dr. Zimmerberg has shown that dysferlin-deficient myoblasts wound more easily than wild type cells using the laser wounding method, and fail to repair their wounds as quickly. In addition, treatment of the dysferlin-deficient myoblasts with a poloxamer had a beneficial effect on the rate of membrane resealing. Experiments to test the effects of specific dietary lipids on these processes are in progress.

Dr. Mohan Viswanathan (Cambria Pharmaceuticals Inc.) is taking advantage of a temperature sensitive mutation in the *fer-1* gene of *C. elegans* to do a high throughput screen for small molecules that can correct for the loss of *fer-1*. *fer-1* is the *C. elegans* orthologue of dysferlin. *fer-1* was originally identified as a protein that plays a role in membrane fusion during sperm development, a function that is mechanistically similar to dysferlin's role in wound repair. As a result, *fer-1* mutant worms are sterile. Thus it is possible to use a simple proliferation assay to screen for molecules that can compensate for the absence of *fer-1* and restore the worm's ability to proliferate. Of 85,000 compounds screened, 260 positives have been found in the primary screen and passed a preliminary retest. 189 of these compounds have been repurchased and retested, yielding 29 (15%) false positives and 160 (85%) that continue to be positive in the worm proliferation assay. These remaining 160 positive compounds have been grouped into structural subclasses to assess whether common features can be identified that correlate with activity. The compounds will then be subjected to a secondary screen in *C. elegans* using a different *fer-1* mutant, and then to a cell wounding assay in mammalian cells. The results of these experiments will be used to further narrow down the number of positive compounds and to select a small number of lead compounds that will proceed to the next stage of drug development.

Dr. Kanneboyina Nagaraju (Children's National Medical Center) tied the session together by describing assays that can be used pre-clinically to evaluate muscle pathology, and any reduction of pathology, in dysferlin-deficient mice. Any therapeutic strategy developed for dysferlinopathy will likely be tested in dysferlin-deficient mice first to assess efficacy and safety. Therefore, it is critical to develop a series of tests that are effective at evaluating the severity and progression of this disease in mice, so that mice treated with potential therapies can be accurately monitored for improvement. In behavioral tests, Dr. Nagaraju found that A/J mice are markedly different from wild type mice in their ability to stay on a rotating rod (rotarod) and that they showed a dramatic decrease in voluntary movement in an open field apparatus measuring total distance moved, movement time, number of movements, and both horizontal and vertical movements (Versamax activity monitor). *In vitro* assessment of muscle function using electrode stimulation of a muscle attached to a force monitor revealed no significant difference between normal and dysferlin-deficient mice.

SESSION VIII:
FERLIN STRUCTURE AND FUNCTION

This session addressed the comparative expression patterns, folding structure, and interaction partners of ferlin family member proteins. There are six human ferlins, which can be separated into two groups based on homology and conserved domain structure. The proteins dysferlin, myoferlin, and FER1L5 comprise one of the two homology groups, with a distinguishing feature being the presence of a DysF domain of unknown function in the central region of the protein.

Dr. Rumaisa Bashir (University of Durham) presented studies comparing the expression patterns of the three DysF-containing ferlins. Using antibodies raised against myoferlin and FER1L5, Dr. Bashir's laboratory found that all three proteins show co-localization in C2C12 myotubes, and that dysferlin and FER1L5 partially co-localize in adult muscle. To prompt discussion on whether dysferlin may also have a role in signaling, Dr. Bashir reported that she has found nuclear localization of all three DysF ferlins during early myogenesis. Other experiments, however, showed that these three proteins are *not* early response genes during myogenesis. Cell lines deficient in myoferlin and FER1L5 were created and found to have delayed myoblast fusion, which has also been reported for dysferlin. Dr. Bashir also briefly discussed another project in her laboratory, in which she is analyzing two genetic loci from families who show symptoms similar to Miyoshi myopathy but who do not have dysferlin deficiency. Identification and study of the proteins involved in these cases may identify interaction partners of dysferlin and/or dysferlin-independent alternative repair pathways.

BREAKOUT SESSION:
DYSFERLIN STRUCTURE AND FUNCTION

Proposed Project:

Determining the identity of the dysferlin doublet band in Western blots. In Western blots, a doublet is often seen for the dysferlin signal. This could be caused by different dysferlin isoforms having slightly different molecular masses or by post-translational modifications such as glycosylation, phosphorylation, etc. Determining the identity of the doublet could help clarify the roles of these different isoforms or post-translational changes. Experimental approaches for determining the identity of the doublet may involve:

1. Employing proteolytic cleavage of the full-length protein to enhance size-resolution on the gel (the doublet is not well resolved for the full-length protein).
2. Testing if glycosylation is involved by tryptic digestion followed by mass spectrometry.
3. Testing for phosphorylation by using phospho-specific antibodies or by mass spectrometry.

Dr. Silvère van der Maarel (Leiden University Medical Center) reported his work on identifying interaction partners of dysferlin. Much of his effort has been directed to developing appropriate antibodies for these proteins, and he described in detail the use of phage display-derived antibodies from llamas. Although it has been known for some time that calpain-3 (the protein that is defective in LGMD2A) can sometimes show secondary deficiency in cases of primary dysferlin deficiency, the relationship between the two proteins has been unclear. Dr. van der Maarel's laboratory has found that dysferlin binds to the large protein AHNAK, and that calpain-3 cleaves AHNAK, which disrupts the association of AHNAK and dysferlin. Dr. van der Maarel also briefly discussed the expression of dysferlin in different cell types and noted that dysferlin is highly expressed in Purkinje cells of the cerebellum. This might imply that some aspects of the phenotypes of the mouse models of dysferlin deficiency, for example, the animals' inability to balance on a rotating rod, might be an effect of coordination as well as strength.

Dr. Nicholas Keep (Birbeck University of London) discussed the folding structure of the DysF domain of dysferlin and myoferlin. The DysF domains in these proteins are nested, resulting from an insertion of one DysF domain inside another. Dr. Keep's laboratory determined the structure of the inner DysF domain of myoferlin. The amino acid sequences of this region are very similar for dysferlin and myoferlin, and for technical reasons the myoferlin structure was easier to determine experimentally. The structure was determined by first creating a soluble protein fragment of the appropriate region of the protein (just the inner DysF domain), and then using nuclear magnetic resonance (NMR) imaging of ^{1}H, ^{13}C and ^{15}N (the latter two using isotopically-labeled amino acids). The wide distribution of ^{1}H-^{15}N peaks indicated that the protein was folded. By assigning each atom to a particular chemical shift, constraints on distances between the atoms can be used to obtain a computed protein structure that is consistent with the NMR spectra.

The major feature of the structure obtained for the DysF domain of myoferlin is a beta sheet which has a "stack" of tryptophan and arginine residues (a W/R stack). This stacking occurs in some otherwise unrelated proteins, such as the cytokine receptor and the thrombospondin repeat. Various missense mutations located in this DysF region and known to be pathological were mapped onto the structure to determine their likely effect on folding structure or charge distribution, and many of them are in locations where they would disrupt the W/R packing. Analysis of the charge distribution of the DysF domain indicates there are regions that may bind metals, but not calcium or magnesium. It was found that addition of zinc, nickel, or copper caused the protein to precipitate.

SESSION IX:
THE FERLIN FAMILY – BEYOND MUSCLE

Ferlins are found in many different organisms, and are expressed in many tissues besides skeletal muscle. The expression and function of ferlins in other tissues and organisms may provide clues to their roles in muscle. This session included two presentations that highlight the role of ferlins in non-muscle tissues.

Dr. John Robinson (Ohio State University) presented studies of the expression of dysferlin and myoferlin in the human placenta. In the placenta, dysferlin and myoferlin are both highly expressed in the syncytiotrophoblast. The syncytiotrophoblast is a large polynuclear cell structure formed by fusion of cytotrophoblasts (mononuclear precursor cells), roughly analogous to the relationship between myocytes and a mature muscle fiber. The syncytiotrophoblast is the largest polynuclear structure in the human body, having roughly the area of a tennis court in a full-term placenta. It forms the boundary between maternal blood and the

fetus. Cytotrophoblasts are fetal cells, so a lack of dysferlin in the syncytiotrophoblast would be due to the genotype of the fetus, not the mother.

One of the technical challenges in studying protein expression in the syncytiotrophoblast was finding a method of isolating it, since it only forms a small portion of the placenta. Once purified, the syncytiotrophoblast tissue was found to express extremely high levels of both dysferlin and myoferlin. Trophoblastic cell lines were found to express myoferlin, but did not express dysferlin until they were induced to fuse together. In the syncytiotrophoblast, new cytotrophoblasts are continuously added to increase the syncytiotrophoblast's size. The nuclei of the newly-added cells die after a few days, and are removed from the syncytiotrophoblast though exocytosis. Therefore, an explanation for the high expression levels of dysferlin and myoferlin in the syncytiotrophoblast may be the need for extensive membrane remodeling due to the high rate of exocytosis. The fact that there does not seem to be a defect in gestation of dysferlin-deficient and myoferlin-deficient animals indicates that a deficiency of either of these proteins can be compensated in the placenta.

Dr. Barbara Wakimoto (University of Washington) discussed the Drosophila ferlin protein. Drosophila has a single ferlin gene called *misfire*, which lacks a DysF domain. Comparison with other insect species indicates that the ancestor of current insects had two ferlin genes, one of which Drosophila lost in the course of evolution. *Misfire* was originally identified in a search for genes whose mutations cause male infertility. The *misfire* gene encodes multiple mRNA transcripts, including four expressed in males and three in females. Interestingly, some of the transcripts predict isoforms lacking a transmembrane domain. In females, *misfire* regulates dorsal/ventral egg patterning during oogenesis. From an analysis of mutants, it appears that the long isoform (containing five C2 domains) is necessary for male fertility. The isoform necessary for egg patterning in females contains the last two C2 domains, C2E (mutated in the SJL mouse) and C2F, as well as the transmembrane domain.

In Drosophila, fertilization is different from that of mammals in that it does not involve a membrane fusion event. Instead, the sperm enters the egg intact and sperm plasma membrane breakdown (PMBD) must occur to allow the sperm nucleus to participate in development. *Misfire* and another highly conserved protein called *Sneaky* are essential for PMBD and both are expressed in the acrosome, a vesicle located at the tip of the sperm head. The co-localization of the proteins and similarities in their mutant phenotypes suggest a signaling role for these acrosomal membrane proteins in PMBD. Like dysferlin, these proteins are proposed to function in mediating membrane-membrane interactions. It would be interesting to know if dysferlin, like the Drosophila ferlin, is associated with a member of the Sneaky protein family in humans.

SESSION X:
MUSCLE STEM CELLS

This session highlighted the characterization and use of muscle- and blood-derived stem cells as a therapeutic tool for the treatment of dysferlinopathies. While the use of stem cells as a therapy for dysferlinopathies is promising, there are still a number of challenges that need to be addressed. These include the best stem cell type to use (which could vary depending on use), the best way to compare/contrast the different stem cell types, the best delivery method(s), and the best donor type (allogenic vs. autologous). Each presentation in this session highlighted a different stem cell type, focusing on its isolation, characterization, dysferlin expression/ function, and potential therapeutic use.

Dr. Amy Wagers (Harvard Stem Cell Institute) discussed the characteristics of skeletal muscle precursor cells (SMPs), evidence that they are muscle stem cells, and their potential use as a cell-based therapy for dystrophic muscle. SMPs were originally isolated from myofiber-associated mononuclear cells based on their high myogenic capacity in culture and can be isolated by FACS based on their expression of cell surface markers (CD45-, Sca-1-, Mac-1-, CXCR4+, β1-integrin+). SMP cells are a subpopulation of satellite cells that express satellite cell and early myogenic markers, but lack differentiation markers. These cells exhibit robust, lineage-selective myogenic differentiation, long-term engraftment capability, and self-renewal capacity. Dr. Wagers believes that SMPs are relevant targets for cell-based therapy in muscle based on her findings that SMPs can achieve a high level of muscle chimerism and enhance muscle function in intramuscularly-injected mdx mice. Similar to what she previously observed in aged and dystrophic mdx mice, dysferlin-deficient mice (from Reginald Bittner, with the SJL mutation crossed onto a BL10 background) have fewer SMP cells in their

muscle, but show no difference in SMP clonal efficiency, average proliferation, or differentiation capacity.

Dr. Wagers also believes that soluble factors and/or cellular factors (such as SMP cells) could modulate muscle function, repair, or regeneration in dystrophic muscle, and she is trying to identify these factors using parabiosis experiments in which the blood systems of two different mice are surgically combined. Using this technique, she has previously shown that factors present in the blood of young mice can improve the regenerative capacity of aged muscle. She is currently testing whether she can identify similar systemic factors that can enhance regeneration and ameliorate dystrophy in dysferlin-deficient mice by parabiosing them with wild type animals.

Dr. Morayma Reyes (University of Washington) presented the isolation and characterization of various muscle stem cell types by FACS based on the expression of cell surface markers (i.e., endothelial, perivasular, and satellite cells) and the role that dysferlin may play in these cell populations. In the satellite cell population (CD45-, Sca-1-, CD31-, α7-integrin+), she identified two different sub-populations, one that is CD34- and one that is CD34+. CD34+ satellite cells express higher levels of myogenic commitment markers and may represent the more differentiated satellite cell population compared to the CD34- satellite cell population, which appears to be less committed and more quiescent. This is supported by Dr. Reyes' cardiotoxin injury experiments, which show that CD34-/GFP+ transplanted cells can give rise to CD34+/GFP+ cells and that after cardiotoxin injury in a wild type mouse, the number of CD34- cells declines, while the number of CD34+ cells increases. When she performed similar experiments in the dysferlin-deficient A/J mouse, she showed that the A/J mouse has impaired and delayed regenerative capacity.

Dysferlin appears to be expressed in a number of adult muscle-derived stem cells, such as perivascular MAPC cells, endothelial cells, and both satellite cell subtypes (CD34- and CD34+), and the lack of dysferlin in these cells appears to affect cell fusion. In order to determine whether the two dysferlin exon 1 variants can rescue the cell fusion defect, Dr. Reyes transduced dysferlin-deficient cells (satellite and MAPC cells) with lentiviral vectors expressing the two different variants. Preliminary results indicate that both variants are independently able to correct the fusion defect, suggesting that they have redundant functions. Additional studies with the two variants and stem cell transplants of various muscle stem cells into the A/J mouse model are planned.

BREAKOUT SESSION:
STEM CELLS

Proposed Projects:

Generation of chimeric mice by muscle stem cell transplantation. This project involves transplanting muscle stem cells from control mice into the muscle of dysferlin-deficient mice (and vice versa) and monitoring the cells' engraftment potential (both with and without muscle injury), in order to determine whether the environment in dysferlin-deficient mice (rather than a defect in dysferlin-deficient stem cells themselves) contributes to inefficient regeneration. This project should be repeated for each stem cell type. *This project is being carried out by Amy Wagers, Morayma Reyes, and Yvan Torrente.*

Determining the lineages of stem cells that are naturally recruited to dysferlin-deficient muscle. This project involves looking for the presence of cells with unusual surface markers (indicating that they represent lineages not normally found in muscle) in muscle samples from dysferlin-deficient mice (both pre- and post-symptomatic) and patients. The presence (or an increase in number) of such cells in dysferlin-deficient muscle versus controls would suggest a deficiency in the regeneration capacity of dysferlin-deficient muscle stem cells, compensated for by recruitment of other stem cell lineages to muscle.

Bone marrow transplantation from normal to dysferlin-deficient mice. This project involves transplanting either the bone marrow alone or bone marrow and muscle stem cells from the same donor to dysferlin-deficient mice, in order to reduce immune rejection and to investigate the potential contribution of dysferlin-deficient immune cells to pathology. *This project will be performed by Morayma Reyes.*

Dr. Yvan Torrente (University of Milan) discussed autologous transplantation of exon-skipped CD133+ stem cells for the treatment of dysferlinopathies. CD133+ cells are adult progenitor cells derived either from muscle or blood. They display clonogenic, self-renewal, and multi-potency properties and even the blood-derived cells express muscle cell markers. CD133+ stem cells isolated from both muscle and blood show dysferlin mRNA and protein expression and are therefore ideal templates for an exon-skipping strategy. Using human myoblasts from an LGMD2B patient carrying a deletion in exon 22, Dr. Torrente tested, both individually and in combination, a number of anti-sense oligonucleotides (AONs) that targeted the donor and

acceptor splice sites, as well as the enhancer splicing elements in exons 22 and 23. In order to get an in-frame product, exons 22-23 or 22-23-24 need to be skipped. Of the five oligos tested, only one AON produced a robust, efficiently skipped in-frame product deleted for exons 22, 23, and 24. Dr. Torrente went on to show that these *in vitro* skipped CD133+ positive cells derived from blood tissue can express dysferlin and muscle markers when co-cultured with A/J murine myotubes. In addition, the exon 22-24 skipped dysferlin was able to correct the membrane repair defect of dysferlin-deficient cells. These results are proof-of-principle for the combined approach of stem cell therapy and exon-skipping and indicate that some regions of dysferlin may be removed without affecting function. On the other hand, the results of the skipping experiments highlight the unpredictable nature of exon-skipping and the need to increase the specificity and efficiency of this process before planning its use for therapy.

LOOKING AHEAD

We are very pleased with the scientific progress that was presented during the Second Annual Dysferlin Conference. It was clear that the dysferlin field is beginning to take off in a number of new directions and that some of our assumptions about dysferlin and LGMD2B need to be revisited. The presentation of such a variety of high-quality new data on dysferlin and LGMD2B made for an exciting meeting with many lively discussions, new ideas, and new collaborations.

In 2009, we plan to have another conference of similar size and format in order to facilitate the same level of discussion and maintain the intimate feel and scientific focus of the meeting. In the meantime, we hope that all researchers working on dysferlin will take to heart the closing comments of our founder, Ajit Jain, and endeavor to accelerate the pace of dysferlin research and make the most of their ideas by welcoming collaborations and sharing and using all of the resources available in the field.

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