

Proceedings of the **First Annual Dysferlin Conference**

The First Annual Dysferlin Conference, held from July 9-12, 2007 in Hamilton, Bermuda, brought together leading scientists and clinicians to discuss progress towards a therapy for dysferlin deficiency (LGMD2B / Miyoshi myopathy) and to build collaborations for future research. The conference opened with a statement from the founder of the Jain Foundation, describing his personal experience with LGMD2B and highlighting the importance of focused research on this disease.

An important goal of the conference was to 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 it 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, muscle regeneration, stop codon read-through, and exon skipping).

SESSION I: STEM CELL THERAPY

The Stem Cell Therapy session discussed in detail the potential of cellular therapy for muscle diseases such as the dysferlinopathies. If successful, a muscle stem cell approach should be applicable to any disease with an intrinsic muscle defect because the goal is to regenerate muscle from a progenitor cell type. Stem cell therapies may also be combined with ex vivo modification strategies that are specific to dysferlinopathy.

An essential question for stem cell therapy is what cell type to use for the treatment. There are many different muscle progenitor cell types being tested in different laboratories, and not much work has been done to determine how these cell populations overlap or to reproducibly test which population gives the best results in animal models.

Dr. Giulio Cossu discussed mesoangioblasts, progenitor cells associated with blood vessels that are capable of differentiating into muscle. Wild type mesoangioblasts have been able to partially restore dystrophin expression and ameliorate symptoms in a dog model of Duchenne

muscular dystrophy after allogenic transplantation. One caveat to this work is that Dr. Cossu has seen a very variable production of dystrophin in muscle fibers of the recipient dogs and also very variable production of dystrophin from post-natal mesoangioblasts isolated from non-dystrophic humans. If variability after transplantation is patient-specific, careful screening of patients may be essential at the time of a clinical trial. There has also not yet been success using autologous, ex vivo modified mesoangioblasts in the dog model, possibly because lower levels of dystrophin were produced by these genetically modified cells. Dr. Cossu is currently planning a clinical trial for Duchenne muscular dystrophy, in which HLA-matched donor mesoangioblasts will be transplanted either intra-muscularly or intra-arterially into DMD patients, who will be monitored for both safety and efficacy (improvement in muscle strength). In response to questions following his talk, Dr. Cossu mentioned that all the dogs used in his studies had been treated with steroids throughout their lives, and also that he did not observe any production of anti-dystrophin antibody in any of the treated dogs.

Dr. Morayma Reyes discussed an adult progenitor cell type (multipotent adult progenitor cells, MAPCs) that can be isolated from either bone marrow or muscle. She has focused on these cells because of their ability to multiply in culture more efficiently than other muscle and bone marrow mononuclear cells, and has now developed a method to isolate these MAPCs, as well as satellite cells, by FACS. She has also had some success improving the proliferation of these cells by culturing them in low oxygen, possibly because low oxygen conditions may mimic the in vivo environment. (Cells in the body are not exposed to atmospheric concentrations of oxygen.) The MAPCs share surface markers with vascular and perivascular cells and can be found in the perivascular region of the muscle in situ. Dr. Reyes hypothesized during her talk that many of the stem cell types used by researchers may have vascular origin. She plans to test delivery of MAPCs, derived from both muscle and bone marrow, to dysferlin-deficient mice.

Dr. Yvan Torrente discussed adult blood-derived progenitor cells, selected by their cell-surface expression of CD133. Although these cells are isolated from blood, they express a number of muscle markers and are found enriched in the muscle after exercise, suggesting that they may be recruited to muscle after damage in order to help

regenerate muscle fibers or to help replenish the muscle stem cell pool. CD133+ stem cells can also be isolated directly from muscle. Dr. Torrente has achieved expression of dystrophin in mdx/SCID mice after autologous transplantation of blood-derived CD133+ cells that had been ex vivo modified by lentiviral insertion of a U7 exon skipping cassette. Dr. Torrente is planning to repeat this exon skipping approach with the dysferlin gene, in CD133+ stem cells isolated from human patients. Exon skipping by U7 can achieve permanent skipping, but it does carry a tumorigenic risk—due to random insertion of the exon skipping cassette into the genome—that is common to viral gene therapy. During the discussion following Dr. Torrente's talk, it was suggested that ex vivo modification of stem cells might be possible by homologous recombination designed to replace just a targeted mutation-containing exon.

Dr. Amy Wagers discussed a progenitor cell type (skeletal muscle precursors, SMPs) that can be isolated from myofiber-associated mononuclear cells. She originally isolated these cells based on their high myogenicity in culture, and has found that they are a subset of muscle satellite cells. These cells may be the sub-population responsible in vivo for renewing the muscle satellite cell pool. (As muscle regenerates, the satellite cell pool must either self-renew or be replenished by another population.) Dr. Wagers has found that donor SMP cells injected intramuscularly into mdx mice successfully differentiate into muscle fibers and also replenish satellite cells. She emphasized that this latter point (incorporation into the satellite cell pool as well as into mature muscle fibers of recipient animals) is important to test in all stem cell studies, because the injected stem cells must contribute to the muscle stem cell pool in order to have a long-term effect on muscle regeneration. Dr. Wagers has also shown that there are fewer SMP cells present in the muscles of both aged mice and dystrophic (mdx) mice than in wild type mice. In the case of aged mice, it is known that factors present in the blood of young mice can improve the regenerative capacity of the aged muscle, and Dr. Wagers plans to test this effect in dysferlin-deficient muscle as well.

During the brainstorming session, an important topic of discussion was the fact that stem cell therapy has not had much success treating older animals. This could be due to an environment of the serum or muscle that does not promote stem cell regeneration or differentiation in older animals, or it could be due to the difficulty of reversing changes (such as fibrosis and fatty infiltrates) that are present in older diseased animals. One example of

successful reversal of damage in older individuals that was brought up during the discussion is in treatment for retinopathies—studying that treatment could provide insights into how to reverse damage in muscle diseases.

The hypothesis of a poor myogenic environment in older animals is supported by experiments presented by Dr. Wagers, showing that combining the circulatory systems of old and young mice (parabiosis) improved the regenerative capacity of muscle in the old mice. During the brainstorming session it was suggested that pregnancy might be a good natural analog of parabiosis, but it differs from parabiosis in not allowing any mixing of blood cells.

Another important issue that was discussed in the brainstorming session is how best to confirm that dysferlin (or another muscular dystrophy protein) is present and functioning in treated muscle. Methods such as immunofluorescence and Western blotting can be used to detect dysferlin in treated muscle, but it is also important to test for protein function, especially if autologous cells are modified ex vivo to express dysferlin. One good indicator of function could be to confirm that dysferlin's interactions with other proteins are preserved.

Other points mentioned in this brainstorming session were the fact that exercise in mice increases macrophage infiltration into muscle, and that the resistance of A/J mice to atherosclerosis could be related to lack of dysferlin in their blood monocytes.

SESSION II: DYSFERLIN GENE THERAPY

The Dysferlin Gene Therapy session discussed recent advances in methods for delivering genes to muscle. The gene therapy approach is similar for most muscle-targeted genes, but dysferlinopathy faces a few specific challenges due to the large size of the dysferlin gene and also due to the repair defect in dysferlin-deficient cells, which could increase the toxicity of some gene delivery methods.

There are two broad categories of gene delivery: viral and non-viral. Adeno-associated virus (AAV) is the most promising viral vector for gene therapy, but it still faces a number of challenges, including the limited DNA packaging capacity of the virion and the immune response to the viral capsid that is elicited in recipients. There are also many non-viral gene delivery methods, which involve transient generation of pores in the target cell membrane. These pores could be more toxic to cells with

a structural or repair defect (such as dysferlin-deficient cells) than to normal tissues. Whatever the method of delivery, all gene replacement approaches may also trigger a dangerous immune reaction in patients to the new transgene product.

Dr. Jude Samulski discussed work that has led to a clinical trial with AAV in Duchenne Muscular Dystrophy patients. Pre-clinical studies were done in mdx mice (treated with AAV carrying mini-dystrophin) and TO-2 hamsters (treated with AAV carrying delta-sarcoglycan). Treated animals had both increased functional ability and increased lifespan. Dr. Samulski also engineered an improved AAV vector (AAV2.5) by modifying 5 amino acids of the AAV2 capsid protein to match the AAV1 capsid protein—these changes were enough to convey muscle tropism to the modified vector. AAV2.5 has a distinct immune profile from either AAV1 or AAV2, so that it can be used even in patients who have received previous AAV treatments. Additional modified vectors are being developed to facilitate repeat treatment of patients. For the Phase 1a trial, 6 DMD patients were injected intramuscularly in one arm with vector containing mini-dystrophin and in the other arm with empty capsids, in order to distinguish whether any observed immune response is due to the viral capsid or to the transgene product. Data from this trial will soon be available. In preparation for a Phase 1b trial with full limb infusion, AAV carrying the canine mini-dystrophin gene was infused into a single hindlimb of GRMD dogs, resulting in restoration of the dystrophin glycoprotein complex to the membrane of muscles in the treated limb.

Dr. Isabelle Richard discussed an approach to overcome the DNA size limitation for packaging into AAV vectors. Multiple AAV genomes that infect the same cell can concatemerize using inverted terminal repeats at the ends of the genome. If the dysferlin cDNA (a total of 6.9kb) is split into two halves, each of which fits into an AAV vector, concatemerization may result in the full-length coding sequence being integrated into the recipient chromosome. When two vectors prepared along these lines were tested by IM injection into A/J mice, both full-length dysferlin and a smaller product were produced. The effect of this smaller product is not known, and Dr. Richard is currently working to eliminate its expression. She is also in the process of generating an improved mouse model for dysferlin deficiency by back-crossing the A/J mutation onto a B16 background. These back-crossed mice have a slightly more severe phenotype than the original A/J mice. In response to questions following her talk, Dr. Richard mentioned that she is using the C5-12

muscle-specific promoter, which gives a low level of expression that should not have negative effects on the muscle.

Dr. Michele Calos discussed ways to achieve stable expression of genes delivered by non-viral methods. Genome integration of a transgene is important even in post-mitotic cells, because it often increases expression and because an expression plasmid can otherwise be silenced or lost over time. Her approach is based on co-delivery of a plasmid containing Φ C31 integrase and a plasmid containing the transgene with an attB integration site. Integration facilitated by Φ C31 is site-specific (using pseudo-attP sites in the genome), gives fewer integrated copies of the transgene than does random integration, and has been successful at integrating the full-length dystrophin gene into mouse muscle after electroporation into hindlimbs. Dr. Calos is in the process of using this integration method for non-viral ex vivo modification of autologous mesoangioblasts from dystrophic mice. In response to questions following her talk, Dr. Calos mentioned that integration can be targeted to muscle cells either by expressing the integrase under a muscle-specific promoter, or by using a DNA delivery method that is highly targeted to muscle. She has not observed any immune reaction in mice to the integrase protein.

Dr. Daniel Scherman discussed the electrotransfer method of non-viral gene delivery and its applicability to dysferlin deficiency. Large cells (such as myotubes and fibers) can be permeabilized using a lower electric field strength than is necessary for small cells (such as satellite cells). Dr. Scherman hypothesized that an even lower E field might be sufficient in the case of dysferlin deficiency because the cell membrane is already somewhat permeable. In wild type mice, electrotransfer into muscle leads to robust local expression of reporter genes. There is some toxicity (nuclear centralization and infiltration from immune cells), which heals within a month. This toxicity could be more serious in the case of dysferlin deficiency, but in the discussion following Dr. Scherman's talk it was mentioned that repair of electropores is calcium-independent and probably does not require dysferlin. Electrotransfer has also been used to successfully deliver dystrophin and lamin alpha2 to diseased muscle. Dr. Scherman proposed that the localized nature of this technique may be beneficial for delivering muscle-stimulating growth factors (such as IGF-1), which normally act locally. Recently, electro-transfer has been used to deliver the follistatin gene to mdx mouse muscle, resulting in increased muscle mass and improvement in myoblast transplantation to the treated muscle.

During the brainstorming session, an important topic of discussion was how to avoid the immune response in patients both to the viral capsid (in the case of viral delivery) and to the transgene product. Reaction to the transgene product can be severe in patients whose mutations in their endogenous gene have completely prevented its expression. In that case, it may be possible to tolerize patients to the transgene product prior to administering therapy (as is done in the case of hemophilia). Tolerization might also be used as a strategy to reduce immune response to the viral capsid, in addition to making structural modifications to the capsid.

Gene therapy can be done either in vivo or as ex vivo modification of progenitor cells. If done in vivo, it is important to consider whether the delivery method targets satellite cells as well as mature muscle fibers. On the other hand, ex vivo modification of progenitor cells faces the same challenges discussed in the Stem Cell Therapy session, including what cell population to use and how to expand the population in culture. In fact, modification of an autologous population in culture often makes the cells even more difficult to expand than an unmodified, allogenic population. One participant suggested avoiding clonal populations for ex vivo modification, because they are particularly hard to expand and differentiate rapidly.

There was also some discussion of the relative merits of different non-viral delivery techniques, particularly with respect to the amount of damage that they produce in recipient cells. While electropores can generally seal on their own, it is not known whether hydro pores are similar or whether they require cellular repair mechanisms. Finally, it was mentioned that hydrodynamic delivery could potentially be improved by treatment with histamines.

SESSION III: MUTATION ANALYSIS AND DIAGNOSIS

The Mutation Analysis and Diagnosis session discussed dysferlin mutation analysis, protein testing, and other aspects of the diagnosis of dysferlinopathies. For patients to benefit from any future therapies, they will first need to be accurately diagnosed. In addition, many mutation-specific therapies (exon-skipping, premature stop codon read-through, or homologous recombination) that are currently being contemplated will only be applicable to patients with certain types of mutations, or will require knowledge of the exact location of a patient's mutations.

Unfortunately, diagnosis of dysferlinopathies has been very difficult historically, especially before this particular cause of muscular dystrophy (dysferlin deficiency) was recognized and reported in the literature. In particular, many patients have been misdiagnosed as having an inflammatory myopathy, due to the inflammatory features often present in dysferlinopathy (see Session VII). In spite of greater awareness of dysferlin deficiency in the literature and among neurologists, a definitive diagnosis remains challenging in many cases for the following reasons:

- 1) Clinical symptoms can vary widely among patients, even in the same family; symptoms of dysferlinopathy can also overlap those of other forms of muscular dystrophy. This necessitates diagnosis at the molecular level.
- 2) The dysferlin gene is very large, having approximately 6900 bp of cDNA and over 55 exons. Therefore, mutation screening without prior knowledge of a deficiency in dysferlin protein expression is not practical.
- 3) There do not seem to be any mutational "hot spots," as have been identified in the dystrophin gene, nor are there any mutations which account for a large proportion of cases, except for "founder effects" in certain specific populations.
- 4) Dysferlin protein expression can sometimes be reduced or mis-localized as a secondary result of a mutation in another gene (for example, calpain-3 and caveolin-3 have been identified in this regard). Therefore, interpreting whether dysferlin deficiency is a primary or secondary effect from protein analysis of a muscle biopsy can sometimes be difficult.

Dr. Nicolas Levy discussed his laboratory's experience sequencing dysferlin at both the DNA and RNA levels. One interesting fact is that a large proportion of identified dysferlin mutations are missense, involving the substitution of a single amino acid. Since polymorphisms (non-pathological amino acid substitutions) also occur, it is important to know whether an identified amino acid substitution is pathological. To this end, Dr. Levy's group has put together a database containing all mutations identified in his laboratory or written up in the literature, together with predictions of the result of a missense mutation and whether it is likely to be pathological, based on the particular substitution and its location in the protein. Dr. Levy thinks that some of the dysferlinopathy cases for which mutations have not been found are due to

large deletions in the dysferlin gene, which are not well detected with the techniques currently employed. He expects to begin employing a new method to address this issue in the near future.

Dr. Eduard Gallardo discussed a number of different research topics related to diagnosis. His group has identified a premature stop codon (R1905X) in a patient cluster in Spain, who may be candidates for testing a stop codon read-through strategy. His group also collaborated in a recent report of two manifesting carriers of dysferlin mutations. The two symptomatic Spanish individuals are both family members of patients in whom mutations on both dysferlin alleles were identified. Because the family's mutation(s) are known, the symptomatic carriers could be confirmed as having only one mutant allele. Either a 50% reduction in dysferlin levels is enough to sometimes cause symptoms, or else the dysferlin defect is combined with some other defect in these carriers. Dr. Gallardo has also found that dysferlin-deficient myoblasts do not form normal myotubes *in vitro*, supporting a role for dysferlin in myogenesis as well as in membrane maintenance and repair.

Dr. Steven Moore discussed correlations between protein analysis and genetic analysis based on several years of patient diagnosis at the University of Iowa. Typically, patient muscle biopsies are used for protein testing (Western Blot or immunohistochemistry), followed in some cases by genetic sequencing. The easiest way to identify a patient who is likely to have primary dysferlin deficiency is by the complete absence of dysferlin staining. This is often, but not always the case, and Dr. Moore showed a number of biopsy slides which give less clear results: e.g. splotchy staining pattern (the cell membrane does not uniformly give a dysferlin antibody signal, or only some muscle fibers show dysferlin staining) or mis-localization (the absolute quantity of dysferlin is normal, but it resides primarily in the cell cytoplasm, rather than in the cell membrane). Some of these staining patterns can result from a secondary deficiency of dysferlin due to a primary deficiency in another muscle protein; thus genetic analysis is required to determine which gene causes the primary deficiency. Dr. Moore also showed a case of an abnormal staining pattern for dysferlin in which mutation analysis found one dysferlin mutation and one calpain-3 mutation. Either the sequencing simply failed to identify an existing second mutation in either calpain or dysferlin, or else being a heterozygote for dysferlin and calpain-3 mutations can be pathological.

SESSION IV: DYSFERLIN FUNCTION

The Dysferlin Function session discussed our current understanding of the cellular function of the dysferlin protein. Although this is a basic science question, understanding dysferlin's function is essential for any therapy that might replace this function or maintain cell survival even in the absence of this function. To develop a complete therapy, it is also important to know whether dysferlin has a function in tissues other than muscle, but dysferlin's expression pattern is still incompletely characterized. Family members of dysferlin (other ferlins) are potential candidates to substitute for dysferlin in the muscle, but developing a treatment using one of these family members will also require understanding in more detail their functions and expression patterns.

Dr. Kevin Campbell discussed experiments that led to the hypothesis and confirmation that dysferlin is involved in the repair of muscle membrane tears after wounding. Two different assays of wound repair (with wounding accomplished either by laser or by passage through a syringe) have demonstrated that dysferlin is concentrated in the myocyte membrane at sites of newly-repaired damage and that dysferlin-deficient skeletal muscle fibers are unable to repair membrane tears. The laser wounding assay has recently been repeated with dysferlin-deficient cardiac myocytes, which show the same defect in Ca-dependent resealing. If cardiac muscle cells have the same defect as skeletal muscle cells in the absence of dysferlin, it is unclear why cardiomyopathy is not normally a symptom of dysferlin deficiency in patients. Dr. Campbell has recently found that cardiac dysfunction and increased Evans blue dye uptake can be induced by exercise in dysferlin knockout mice more than 1 year old, but the relevance for human patients is not yet known. It may be that dysferlin's role in a process other than membrane damage repair (for example, a role in myoblasts during regeneration) is involved in the phenotypic difference between skeletal and cardiac muscle in the absence of dysferlin.

Dr. Eric Hoffman discussed recent mRNA profiling studies comparing gene expression between different types of muscular dystrophies. In comparing muscle biopsies from dysferlin-deficient, FKRP-deficient, and normal individuals, he found that expression of genes involved in inflammation and vesicle trafficking was uniquely increased in dysferlinopathy. Dr. Hoffman suggested that the observed increase in vesicle trafficking proteins Rab27a and Slp2a (synaptotagmin-like protein a) may

indicate a compensatory shift toward alternative vesicle trafficking and membrane fusion pathways in the absence of dysferlin. This increase varied significantly between patients, suggesting that some may be more successful than others in compensating for dysferlin's loss, though the profiling results have not yet been correlated to the severity of disease in these patients. Dr. Hoffman also presented data suggesting that during muscular dystrophy, the expression of proteins involved in muscle regeneration pathways does not follow the normal pattern and allows for inappropriate crosstalk between expressed proteins. Dr. Hoffman hypothesizes that this crosstalk may lead to fibrosis instead of successful regeneration during muscular dystrophy. Glucocorticoids can affect the timing of expression of these muscle regeneration proteins and may reduce inappropriate crosstalk.

Dr. Sandra Cooper discussed studies of dysferlin missense mutants, which are degraded in patient muscle and so cannot perform partial function. She has studied both patient myoblasts and patient fibroblasts that are triggered to undergo myogenesis by lentiviral delivery of the MyoD gene. Although mutant dysferlin is not detected by Hamlet-1 in muscle biopsies from patients with missense mutations, it is detected in cultured myotubes generated from their fibroblasts. EGFP-tagged dysferlin constructs carrying one of two different patient missense mutations in the C2F domain revealed that one mutant was transported to the membrane of myotubes while the other was not. A variety of C2 domain deletion constructs (some retaining only the C2F domain) were all successfully targeted to the membrane of cultured myotubes, transported by vesicles from the Golgi. However, in at least one case the loss of C2 domains affected the persistence of dysferlin at the membrane. Dr. Cooper has also noticed that the Hamlet-1 antibody is not as good at recognizing membrane dysferlin as intracellular dysferlin, and that dysferlin may be able to adopt a conformation that is not recognized by the Hamlet-2 antibody. These concerns should be kept in mind by anyone using these antibodies.

Dr. Rita Barresi discussed recent findings that dysferlin in cultured C2C12 myotubes is localized to the T-tubule network, despite its sarcolemmal localization in mature muscle fibers. In the myotubes, GFP-tagged dysferlin constructs lacking various C2 domains all failed to localize properly to T-tubules, in contrast to the full length protein. Using a membrane wounding and repair assay based on glass bead damage, it was found that wounding of myotubes causes dysferlin to change its localization from the T-tubules to the sarcolemma and accumulate at

sites of wounding. Again, none of the tested C2 domain deletion mutants were able to accomplish this translocation. Dr. Barresi's group is also planning to study the effects of Poloxamer 188 (a membrane resealing agent) on the phenotype of dysferlin-deficient mice. In the discussion following her talk, it was mentioned that it is often difficult to distinguish between developing T-tubule membrane and ER membrane in muscle cells.

Dr. Rumaisa Bashir discussed studies of other ferlin family members and other proteins associated with dysferlin. Dysferlin and its two closest relatives, myoferlin and Fer1L5, all increase in expression during the transition from myoblasts to myotubes, with Fer1L5 showing the most dramatic increase. These three proteins are primarily found in different types of intracellular vesicles (with different densities and detergent resistances), but the origins and functional overlap of these vesicles are still unknown. Dr. Bashir has also found a membrane repair defect in patients with Miyoshi myopathy whose disease is not caused by mutations in dysferlin or in other known membrane repair-associated proteins. She is trying to identify the responsible protein defect, which may be in a protein that interacts with dysferlin or represents a possible compensatory pathway.

During the brainstorming session, one point of discussion was the apparently conflicting results from Dr. Cooper and Dr. Barresi on proper targeting and localization of dysferlin C2 domain deletion constructs. Their experiments suggested opposite conclusions about how many C2 domains are necessary for localization. The results could reflect different mechanisms of targeting to the T-tubules versus the sarcolemma—in which the full length protein is required for targeting to the T-tubules, but only the transmembrane domain (and possibly the C2F domain) is required for targeting to the sarcolemma. Alternatively, the difference in results may be due to differences between C2C12 cells and patient-derived cells. Researchers should be cautious when comparing results from different cell types or from cultured versus primary cells.

Another issue discussed in the brainstorming session is the relationship between dysferlin and dystrophin deficiencies. Dr. Campbell mentioned that crossing transgenic dysferlin-expressing mice with mdx mice does not improve the mdx phenotype. There are a number of possible explanations for this result, including that mdx muscle may already engage in a saturated level of membrane repair, that the membrane ruptures in mdx muscle are too large to be corrected by the dysferlin repair

pathway, or that dysferlin-mediated repair cannot take place without some secondary structure that is lost in the absence of the dystrophin glycoprotein complex.

There are still many important unknowns about dysferlin's function. These include: Do dysferlin-deficient muscle cells have trouble with any other types of exocytosis? What are all of the other components of the dysferlin membrane repair pathway? Does dysferlin participate in myoblast fusion? What is the source of vesicles containing dysferlin or other ferlin family members? What are the expression levels of the ferlin family members in muscle throughout an individual's lifetime? What is the function of dysferlin in monocytes and neutrophils? Is dysferlin involved in immune cell development and does this have any consequences for patients? What is the function of dysferlin in other tissues (heart, placenta, blood-brain barrier)?

SESSION V: MEMBRANE FUSION AND REPAIR

The Membrane Fusion and Repair session focused on the cellular process of membrane repair and on therapeutic prospects for improving or restoring this process even in the absence of dysferlin. It might be possible to trigger other cellular fusion proteins to substitute for the function of dysferlin in membrane repair. Some candidates include other ferlin family members, or other C2 domain proteins such as synaptotagmins. Another possibility is to identify drugs that stabilize the muscle membrane, making it less likely for tears to form, or that facilitate membrane fusion even in the absence of cellular fusion proteins.

To screen for drugs or genetic modifiers of dysferlin, it is essential to have both *in vitro* and *in vivo* assays of membrane repair. *In vitro* assays are complicated by the question of how to physiologically and reproducibly damage cells, while *in vivo* assays are complicated by the necessity of using non-human homologs of dysferlin, whose function may differ somewhat from that of human dysferlin.

Dr. Paul McNeil discussed various methods for damaging cells *in vitro* to trigger calcium-dependent repair—these include laser wounding, impaling cells with a microneedle, and scraping cells off the bottom of a dish using a pipette. Cell scraping is a good initial screen to test for cell survival after wounding, while the laser assay allows detailed monitoring of membrane tears as they are resealed. The laser wounding assay has recently been

used to test fibroblasts in which one of the calpain subunits is knocked out, and has demonstrated that calpains (like dysferlin) are required for calcium-dependent membrane resealing. Calpains may be involved in cytoskeletal remodeling at the site of injury. Similarly, HeLa cells in which the function of annexin A1 is blocked are unable to resealed after laser wounding. Annexins are known to interact with dysferlin and to concentrate at sites of membrane disruption. Further studies may clarify the roles of these three proteins in the repair process. Finally, Dr. McNeil discussed preliminary data showing that overnight incubation with glutamine enhances membrane resealing after laser wounding of wild type HeLa cells. Depending on the mechanism of this enhancement, glutamine or one of its downstream targets could potentially improve the resealing phenotype of dysferlin-deficient cells.

Dr. Joshua Zimmerberg discussed the relationship between membrane lipid curvature and the ability of a membrane to repair or resist the formation of tears (or 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. Surfactants such as Poloxamer 188 decrease surface tension, increasing the critical size and making it more likely that membrane tears will spontaneously close. Another way of making closure more likely is to increase the proportion of negative curvature lipids in the membrane, which increases line tension. A biological example of this effect is in the life cycle of malaria, a pathogen that exits red blood cells by creating pores in two membrane layers. A positive curvature lipid (LPC) promotes the replication of malaria because it makes pore formation and expansion easier. Dr. Zimmerberg also discussed the process of membrane fusion, which involves hemifusion (stalk formation) followed by expansion into a fusion pore. The hemifusion step is facilitated by lipids with negative curvature, while the fusion pore expansion step is facilitated by lipids with positive curvature that increase surface tension. C2 domain proteins or other proteins may also help increase surface tension around the pore. In response to questions following his talk, Dr. Zimmerberg mentioned that cholesterol has negative curvature but is often associated with molecules with positive curvature, so that its overall effect on membrane curvature is hard to determine. One interesting question for the future is whether it may be possible to target lipids to specific vesicles or rafts in a cell.

Dr. Steven Vogel discussed the sea urchin model system for studying calcium-dependent membrane fusion. Sea

urchin eggs have long been used to study exocytosis, and recently Dr. Vogel has developed a controlled system in fertilized sea urchin eggs by injecting one of two cells at the two cell stage with a gene expression construct or with anti-sense morpholinos to knock down expression of a target gene. As the embryo develops, the un-injected half serves as a control for the injected half, and the laser wounding and repair assay (or other assays of endo- or exocytosis) can be performed on cells from both halves of the embryo. Sea urchins have a dysferlin homolog, whose function can be tested by this method. Dr. Vogel also discussed past experiments on exocytosis in sea urchin eggs, which revealed that the fusion step of repair happens too quickly for binding partners in both membranes to meet. It may be that vesicles are already docked at the membrane prior to fusion, or that fusion is carried out by proteins present in only one of the membranes. This differs from the SNARE model of membrane fusion (requiring interaction between proteins in both membranes), which Dr. Vogel hypothesizes may be responsible for constitutive exocytosis but is too slow to give the rapid fusion response seen in repair.

Dr. Samuel Ward discussed the role of the dysferlin homolog Fer-1 in *C. elegans*, where its known function is in sperm motility. *C. elegans* sperm move by pseudopod extension, which requires fusion of membranous organelles (MOs) with the plasma membrane at the site of extension. In normal worms, the MOs maintain stable fusion pores with the plasma membrane, while in Fer-1 mutant worms, the MOs dock at the membrane but do not fuse. These docked MOs have a characteristic appearance in electron microscopy images, with the tip of the vesicle (closest to the membrane) protruding from a protein collar. A possibly important functional difference between Fer-1 and human dysferlin is that Fer-1 is initially located in the membrane of the MOs, rather than in the plasma membrane (though it is deposited in the membrane after fusion). Dr. Ward also discussed the Fer-1 C2 domains, each of which is more conserved across the different ferlin family members than it is similar to the other C2 domains, suggesting that the domains have distinct biological roles. In *C. elegans*, unlike in humans, missense mutations of Fer-1 are observed to cluster in the C2 domains. In response to questions following his talk, Dr. Ward mentioned that the identity of the protein making up the vesicle collar is unknown, but is not believed to be a dynamin homolog.

Dr. Steven L'Hernault discussed the maturation of *C. elegans* MOs and the potential for using *C. elegans* as a model system for dysferlin function. He has identified

proteins required for formation of MOs and has found that these vesicles become acidified prior to fusion with the plasma membrane, both results suggesting that the MOs are similar to lysosomes in human cells. The acidification is also interesting given that many viral fusion proteins are believed to be triggered by pH changes. As a demonstration of the potential for using *C. elegans* as a model system, Dr. L'Hernault has recently done a suppressor screen with two different Fer-1 mutants by a self-fertility assay. The identified suppressors are located in the same chromosome as Fer-1 and another ferlin-like protein (T05E8.1), of which a targeted knockout is now available. Finally, Dr. L'Hernault discussed the potential for genetic studies in *C. elegans*, an organism that has traditionally been very difficult to engineer. The laboratory of Dr. Stephen Hauschka has recently had some success engineering *C. elegans* using a low copy number integration technique. RNAi is not normally successful but can work with certain genes, and manipulating introns is possible.

During the brainstorming session, it was pointed out that of all the different methods of wounding cells, it is still unknown which type of damage is most similar to natural damage in the muscle. There may be some broad classifications of damage, since certain types of damage require calcium-dependent repair mechanisms and other types don't. (Dr. McNeil mentioned electro-poration as an example of the latter—the pores created by electroporation are small enough that they presumably reseal on their own.) If all repair based on calcium-dependent exocytosis relies on the same fusion machinery, then any type of damage that triggers calcium-dependent repair should be a possible assay for the role of dysferlin.

Another issue discussed in the brainstorming session was whether fibroblasts can be used as a cell type for membrane repair assays in the absence of dysferlin, since they are easier to collect from patients than muscle. This depends on the expression of dysferlin in fibroblasts, which turns out to be a controversial question. Dr. Rumaisa Bashir has found that dysferlin is expressed in fibroblasts, while Dr. Nicolas Levy has found the opposite. It may be that dysferlin is expressed in fibroblasts from only certain parts of the body, but this needs to be more rigorously tested.

On the question of whether dysferlin deficient cells have trouble with any other types of exocytosis, it was noted that there is no evidence of any insulin abnormality in dysferlin deficient patients. Fusion of vesicles containing

glucose transporters with the plasma membrane is a function of muscle cells that could potentially be affected by dysferlin.

Finally, there was discussion of Dr. Ward's observation that Fer-1 is cleaved into two smaller forms in *C. elegans* sperm cells. This cleavage could simply be a product of degradation, or it could occur during maturation of the protein. It was pointed out that many viral fusion proteins require cleavage into two smaller subunits before they can carry out fusion. It is possible that Fer-1 (and dysferlin) must also be primed for fusion by some cleavage reaction. More information is needed about these Fer-1 subunits, and whether the same can be observed in the case of dysferlin, to determine whether the cleavage is biologically relevant.

SESSION VI: OTHER THERAPEUTIC STRATEGIES

The Other Therapeutic Strategies session discussed a variety of different approaches to muscle disease therapy that do not fall into the categories of gene therapy or stem cell therapy. This session included therapies designed to modify the effect of specific genetic mutations in dysferlin, to replace the function of dysferlin by inducing expression of other cellular proteins, and to increase endogenous levels of muscle regeneration.

Dr. Lee Sweeney discussed an ongoing clinical trial of PTC124, a small molecule that promotes ribosomal read-through of premature stop codons. (Aminoglycoside antibiotics such as gentamicin can also cause read-through, but have significant toxicity.) PTC124 was identified after a high-throughput in vitro chemical screen for read-through activity. In mdx mice, it successfully restores some dystrophin expression, improves force generation, and lowers CK levels. Phase 1 studies in humans revealed no significant toxicity or side effects. Phase 2 studies have been carried out with Cystic Fibrosis and Duchenne muscular dystrophy patients. Dr. Sweeney suggests that these two conditions represent distinct efficacy tests because in CF the relevant protein has a short half-life but not much is needed to correct the disorder, while in DMD the relevant protein has a long half-life but much more is needed. Prior to the trial, cultured myotubes from the DMD patients were tested with PTC124 and showed dystrophin expression in vitro. During the trial, a higher dose of the drug was required for DMD patients than for CF patients, because it turned out that children clear the drug faster than adults. After 4

weeks of treatment, dystrophin could be detected in muscle biopsy from about half of the DMD patients. A Phase 3 trial will begin later this year. Dr. Sweeney is now evaluating the ability of PTC124 to read through the dysferlin R1905X mutation in an artificial in vitro system.

Dr. Kathryn Wagner discussed an ongoing clinical trial of a myostatin neutralizing antibody, MYO-029. Loss of myostatin in animals and some human cases is known to cause muscle hypertrophy, faster regeneration after injury, and reduced fibrosis. Increased amounts of myostatin, on the other hand, cause increased fibrosis. A Phase 1/2 trial of MYO-029 was carried out in patients with several different types of MD, and functional data from the trial are still being analyzed. New types of inhibitors of myostatin's production or action are now being developed and tested in animals to determine which has the greatest functional effect. In response to questions following her talk, Dr. Wagner mentioned that old mdx/myostatin null mice have normal regeneration capacity following cardiotoxin injury, suggesting that there is no negative long-term effect of myostatin inhibition, such as reduction in satellite cell number. However, she acknowledges that inhibiting myostatin in adults might cause long-term effects that do not show up in knockout animals (which could, for example, be born with increased numbers of satellite cells).

Dr. Elizabeth McNally discussed studies of myoferlin, the human ferlin family member most closely related to dysferlin. Myoferlin is highly expressed in regions of muscle damage and can be seen at sites of myoblast fusion into myotubes. Myoferlin null mice have more myoblasts but smaller muscles than wild type mice, suggesting that myoblasts in these mice can proliferate but are unable to fuse into muscle fibers. In contrast to myoferlin, dysferlin is not normally upregulated at damaged sites after cardiotoxin injury, but there is sometimes a compensatory upregulation of dysferlin in myoblasts of myoferlin null mice. Dr. McNally has found that only the C2A domain of both myoferlin and dysferlin binds calcium. Myoferlin proteins with missense mutations in this domain are able to bind calcium at low concentrations but not at higher concentrations, suggesting that changes associated with calcium binding destabilize the missense mutants. Finally, Dr. McNally has shown that myoferlin interacts with EHD2, a protein involved in vesicle trafficking and endocytic recycling. The binding site, in the C2B domain of myoferlin, does not appear to be present in dysferlin.

Dr. Terence Partridge discussed exon skipping as a possible treatment for dysferlinopathy. Oligo-nucleotides

that interfere with splicing and make the cell skip particular exons can be used to remove stop or frameshift mutation-containing exons from a processed transcript, assuming that the rest of the transcript remains in frame. U7 constructs can achieve permanent expression of these oligos; however, oligo sequences need to be optimized in vivo for each targeted exon, so Dr. Partridge advocates starting treatment with transient skipping (e.g. Morpholinos). The success of an exon skipping approach is highly dependent on the target protein's structure, and revertant muscle fibers are generally a good indicator that exon skipping may be effective. While revertants are common in dystrophin deficiency, there has only been one report of revertant fibers in dysferlin deficiency (by Dr. Jens Reimann in Germany). However, these dysferlin revertants are isolated fibers and have not expanded within the muscle, suggesting that there is no functional benefit of the restored protein. It may be that dysferlin is highly-structured and cannot tolerate loss of whole exons, a possibility supported by the fact that disease-causing missense mutations can be found throughout the protein rather than clustered in domains.

To progress towards an exon-skipping therapy for dysferlin deficiency, Dr. Partridge suggests generating a panel of dysferlin constructs, each missing an exon. These constructs can be transfected into dysferlin null cells to test for functional recovery, and then transgenic mice can be made with the promising constructs to check for improved symptoms relative to dysferlin null mice. Other participants suggested working directly with cells (either myoblasts or monocytes) from patients who have mutations in candidate exons, such as 21/22 or 32. One question raised in the discussion was whether the function of the skipped protein should be tested in other cell types (e.g. immune cells) in addition to muscle.

During the brainstorming session, an important question was whether and how to screen patient cells for efficacy before enrolling them in a trial of PTC124. One feature of PTC124 is that it inserts a substitute amino acid in place of the premature stop codon (usually a missense substitution). In the case of dysferlin, missense mutations are often detrimental to protein function, so it may be necessary to test for protein function after read-through of each patient's specific mutations. Depending on the stop codon, PTC124 has preferences for certain substitute amino acids. Dr. Sweeney is studying the R1905X dysferlin mutation because in that case the most likely substitute amino acid is arginine (R), so a significant percentage of full-length protein may be produced after read-through without any missense substitution. Dr.

Sweeney also recommended testing patient cells with PTC124 directly, without first testing the efficacy of gentamicin at promoting read-through of their mutations.

Another important question was how to monitor the effectiveness of myostatin inhibitors, other than looking for large-scale functional effects. Dr. Wagner mentioned that it is very difficult to detect even normal levels of myostatin in human serum, and detection must be done by immuno-precipitation. Furthermore, many inhibitors affect myostatin activity but not its serum concentration, so it is important to monitor downstream pathways. This may be a difficult task if myostatin targets many different pathways.

Finally, there has been some confusion about the myoferlin approach and whether myoferlin is already upregulated in dysferlin deficiency. Dr. McNally clarified that myoferlin often appears to be upregulated in many different muscle wasting diseases, but this is because it is highly expressed in myoblasts while wasted muscle is regenerating. It does not remain expressed in mature muscle fibers after myoblast fusion. The goal of this approach, therefore, is to express myoferlin (or to prevent it from being turned off) in mature fibers. Dr. McNally is now attempting to screen for compounds that will activate the myoferlin promoter.

SESSION VII: IMMUNE INVOLVEMENT AND CELL DEATH

The Immune Involvement and Cell Death session discussed the mechanisms by which damaged muscle cells die in dysferlin deficiency. As in other muscular dystrophies, the absence of dysferlin causes an accumulation of damage to the muscle cell membrane, in the form of membrane tears. The mechanism by which these tears lead to cell death is unknown, but one possibility (discussed below) is that increased calcium influx into the cell activates proteins involved in cell death pathways. Dysferlin deficiency is distinct from other dystrophies in that it is often also associated with a high level of muscle inflammation. Inflammation is a response to cellular injury and death, but it may also mediate a significant amount of death and cause additional damage to neighboring cells, in a positive-feedback loop that accelerates muscle wasting.

Reducing inflammation or preventing the death of muscle cells, even after injury, are potential therapeutic strategies because they may break this feedback loop and may also

reduce muscle fibrosis. Another possible strategy is to shift dying cells from necrotic to apoptotic pathways in order to reduce muscle inflammation.

Dr. Simone Spuler discussed methods for reducing immune-mediated cell death in dysferlin deficiency. She has found that expression of CD55 (a complement regulator) is significantly reduced in the skeletal muscle of SJL/J mice and LGMD2B patients compared to controls. Dysferlin-deficient myoblasts from LGMD2B patients were found to have increased susceptibility to complement-mediated lysis relative to normal human myoblasts, suggesting that blocking complement activity could reduce muscle cell death in this disease. In addition, A/J dysferlin-deficient mice, which lack complement factor 5 (C5), have a milder phenotype than SJL/J mice. Dr. Spuler treated SJL/J mice with anti-C5 and found significantly reduced muscle damage in the treated mice. (A control IgG1 antibody also caused some improvement, and she will follow up on that observation as well.) Dr. Spuler has already treated three LGMD2B/MM patients with IVIG to block complement activity and seen some improvement in muscle strength, but IVIG is a heterogeneous preparation with many different effects, so she suggests that a monoclonal antibody (such as anti-C5) would be a better treatment option. In the discussion following Dr. Spuler's talk, it was suggested that a good test of her hypothesis would be to provide C5 to A/J mice to see if it increases the severity of their phenotype.

Dr. Kanneboyina Nagaraju discussed characteristics of the unusual immune response seen in dysferlin deficiency, which is marked by mononuclear cell infiltration and the presence of complement/MAC even on non-necrotic muscle fibers. Although dysferlin deficiency is sometimes mis-diagnosed as polymyositis, it is not an autoimmune condition because auto-antibodies are not found in dysferlin-deficient patients. Dr. Nagaraju has found that monocytes and macrophages have increased phagocytic activity in SJL/J mice relative to controls, though there is not as much of an effect in dysferlin-deficient human patients. Suppression of dysferlin in a macrophage cell line also increases phagocytic activity. Expression levels of inflammatory mediators, including caspase-1 and NALP3, are often elevated in muscle from LGMD2B patients. Dr. Nagaraju also encouraged collaborative use of the CNMC preclinical drug testing facility, dedicated to testing muscular dystrophy models. This facility performs standardized tests of muscle function, including treadmill exercise, Rota-rod testing, grip strength testing, MRI and ultrasound imaging.

Dr. Jeffery Molkentin discussed the role of calcium in muscle cell death pathways. Both necrosis and apoptosis can be triggered by rupture of mitochondria after a rise in intracellular calcium levels and formation of a pore complex in the mitochondrial membranes. When one component of this pore complex (cyclophilin D, encoded by Ppif) is knocked out in mice, cell death after peroxide damage is reduced. Dr. Molkentin hypothesizes that damage to the sarcolemma in muscular dystrophies allows unregulated calcium influx and also triggers enhanced influx of calcium through membrane channels, leading to mitochondrial calcium overload. He has found evidence of swollen mitochondria in both delta-sarcoglycan and laminin alpha-2 knockout mice. Crossing delta-sarcoglycan knockout and Ppif knockout mice rescues the muscle loss normally associated with sarcoglycan deficiency, increases force generation, reduces inflammation, and improves histological characteristics of the muscle. Muscle membrane damage is still present, however, demonstrating that the cyclophilin D pathway acts downstream of the damage. In the discussion following Dr. Molkentin's talk, it was suggested that inhibitors (or inducible knockouts) of cyclophilin D or other pathway members should be tested to ensure that the beneficial effect is not due to absence of cyclophilin D before birth.

Dr. Christina Jamieson discussed steroid hormone receptors and their role in the immune system. Although glucocorticoids are not effective at treating dysferlin deficiency, other steroid pathways could have a beneficial effect. Activated androgen receptor, for example, enhances myogenic differentiation of stem cells and helps build muscle. Dr. Jamieson plans to screen for androgen receptor modulators that promote muscle-building even in the absence of dysferlin. She has also studied activated glucocorticoid receptor, which leads to immune suppression by repressing cytokine production and by triggering apoptosis in T lymphocytes. During the late stages of dexamethasone-induced T cell apoptosis, she found that dysferlin expression was upregulated, along with that of cytoskeletal remodeling and vesicle trafficking proteins. This timing of expression coincides with membrane blebbing and the fusion of internal vesicles with the plasma membrane. Dr. Jamieson hypothesizes that apoptosis may be defective in dysferlin deficiency. Defective apoptosis during T cell development can result in autoimmune diseases later in life, and impaired apoptosis might account for the fact that glucocorticoids are not effective in treating this disease. It is still unknown what role apoptosis plays in normal muscle.

During the brainstorming session, there was much discussion of potential complement inhibition therapies and IVIG. The three dysferlin-deficient patients who were treated with IVIG by Dr. Spuler had all been tested for the presence of MAC proteins in their muscle biopsies prior to treatment, and Dr. Spuler believes that IVIG will only be beneficial in patients who have MAC binding to non-necrotic muscle fibers. Although IVIG is commercially available, it was recommended that patients undergo this screening before trying the drug. It was also mentioned that complement inhibition may be a particularly useful strategy for this disease if dysferlin-deficient cells are more susceptible to complement because of their inability to repair membrane pores. Though the complement-generated pores are different from normal damage in that they are ringed by proteins, there is some evidence that these pores can be repaired by calcium-dependent mechanisms because complement activity is often sub-lethal in the presence of calcium.

Another point of discussion was how to clarify the many different effects of the immune response in dysferlin deficiency. Inflammation may cause a significant amount of damage to muscle cells, but the inflammatory response is also an important mediator of reconstruction and repair of muscle tissue after damage. Anti-IL-1 therapy was suggested as a way to reduce the pro-inflammatory response while retaining the muscle reconstruction phase of the immune response. Because it is complicated to predict the changes in pathology that may result from immune modulation or suppression strategies, Dr. Nagaraju suggested transplanting muscle from dysferlin-deficient mice into nude mice to test the effects of a lack of T cell responses. Another possibility is to cross dysferlin-deficient mice with immune-deficient mice and then characterize the resulting phenotype.

Questions were also asked about possible cardiac involvement in dysferlin deficiency, and Dr. Spuler recommended that LGMD2B/MM patients be screened for reduced heart rate (elevated RR interval) and rigorously treated for any abnormality.

LOOKING AHEAD

As organizers, we were very pleased with the scientific quality of the First Annual Dysferlin Conference and the amount of discussion that took place. In 2008, we intend to keep the size of the conference about the same in order to facilitate the same level of discussion and maintain the intimate feel of the meeting.

We implemented a number of unique initiatives at this conference designed to stimulate discussion and encourage collaboration. First, the conference schedule included time after each set of 4-5 research presentations for 30-50 minutes of general "brainstorming" on that topic. These brainstorming sessions turned out to be essential to airing all participants' questions and suggestions on each area of research. Second, each invited speaker was asked to provide a list of resources available in his or her laboratory that could be shared with other scientists, as well as a wish-list of resources to which he or she would like to have access. These lists were made available to all of the conference participants. Many scientists mentioned that they used these lists to make connections with others—with whom they now plan to share their resources for future collaborative research projects in the dysferlin field.

Both of these initiatives will be continued and extended at future conferences. We hope to make the brainstorming sessions even more effective next year by preparing questions ahead of time, splitting the participants into smaller brainstorming groups, and encouraging more targeted output from these sessions.

Finally, we would like future conferences to build on this first conference by including almost exclusively new and unpublished results. All speakers invited in the future will be encouraged to contribute to this effort.

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