2009 Research Activities Supported by PPMD  
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Project 1:  
**Novel small molecules for the treatment of DMD: PROJECT CATALYST**  
Duchenne muscular dystrophy is a devastating, X-linked disease that affects approximately 1 in 3000 boys and is characterized by muscle weakness, loss of ambulation and eventual respiratory and cardiac complications that result in the death of these young men in their late teens or early twenties. There is no cure for this rare muscle wasting disorder. In an ongoing effort to identify new treatments for DMD patients, we are using a proprietary drug discovery platform technology (developed by PTC Therapeutics) called GEMS (Gene Expression Modulation by Small-molecules) to identify small molecules that up- or down-regulate the production of proteins. GEMS has proven to be a very robust technology that can address difficult drug targets. We have performed high throughput screens against four targets believed to be medically relevant in DMD. Compounds have emerged from the screen that demonstrate sufficient activity, selectivity and potency in cell-based assays to merit further characterization and chemical optimization. Lead optimization is a complex and iterative process of refining the structure of the chemical scaffolds identified in HTS in order to improve the activity, selectivity and specificity of the compound as well as their drug characteristics. The aims of this work are to explore the chemical space surrounding these molecules to evaluate the structure-activity relationships (SAR) for these molecules. SAR is the relationship between chemical structure and biological (in vitro and in vivo) and pharmacological activity for a given series of compounds. Four groups consisting of Biology, Chemistry, Pharmacology and Efficacy Assessment Cores have been assembled to work in a concerted effort to rigorously characterize and optimize the biological, chemical, pharmacological and in vivo efficacy of the compounds that modulate production of each of our four targets. This we seek to develop small molecule therapeutics that: 1) increased expression of utrophin; 2) increased expression of α7-integrin; 3) decreased expression of myostatin; and 4) increased expression of IGF-I in muscle. Our plan is to move small molecules that modulate either utrophin or α7-integrin and either myostatin or IGF-I into clinical trials.

Project 2:  
**Systemic myostatin inhibition via liver targeted AAV expression in mice and dogs**  
Myostatin inhibition is an attractive therapeutic target to maintain muscle mass in a variety of disorders, including the muscular dystrophies, cachexia and sarcopenia. Previously described approaches to blocking myostatin signaling include injection of neutralizing antibodies and the inhibitory propeptide domain. Failure of anti-myostatin antibodies to attenuate muscular dystrophy in clinical
trials indicates more efficacious inhibitors should be pursued. To ascertain the
degree of benefit in skeletal muscle, and possible positive and/or negative effects
in the heart, we are studying a novel approach for inhibitor of myostatin that
consists of mutant myostatin propeptide resistant to proteolytic activation that is
systemically expressed from the liver following AAV injection. This leads to
increased skeletal muscle mass throughout the body in mice. The inhibitor was
tested in C57 and mdx animals. We also demonstrate persistent expression of
the inhibitor in the serum of dogs following hepatic artery or intravenous AAV
injection, including GRMD dogs (dog model of DMD). These experiments are
ongoing and should give a realistic view of the pros and cons of myostatin
inhibition as a potential treatment for DMD

**Project 3:**
**Influence of long-term myostatin inhibition on dystrophic cardiomyopathy**
An unexpected finding in mdx mice following long term myostatin inhibition is an
inhibitor dose dependent increase in heart weight and decline in systolic function.
To investigate the effect on heart function further, we have initiated experiments
with mdx and gsg mice. The virus used in these experiments is the same virus
used in the C57 and mdx studies described above. The promoter is liver specific
and leads to persistently high levels of circulating myostatin inhibitor, especially
when injected at one month of age or older. Mdx mice exhibit dilated
cardiomyopathy beginning at 11 months of age, follow a mild clinical course in
comparison to human disease and expire prematurely at 21-24 months of age.
Gsg mice have a less well characterized cardiomyopathy that includes a
hypertrophic phase and results in death at approximately 12 months of age in our
line. Mdx mice have been injected at 1 month (n=9 treated) and 6 months of age
(n=12 treated) and will be followed to heart failure. Gsg mice were injected at 1
month of age (n=15 treated) and will also be followed to heart failure. Currently
we plan to perform serial echocardiograms on these animals to precisely track
alterations in heart function.

**Project 4:**
**Increasing calcium buffering in mdx mice with overexpression of SERCA1a**
Sarcoplasmic or endoplasmic reticulum ATPase proteins (SERCA) are
responsible for removing calcium from the cytosol following muscle contraction.
The two major forms of SERCA are the “fast” SERCA1 and “slow” SERCA2a.
These proteins demonstrate differential tissue expression as SERCA1
predominates in fast muscle fibers and SERCA2a is found mainly in slow muscle
fibers and cardiac muscle. It has been well documented that dystrophin
deficiency in muscle results in excess calcium influx following contraction that
ultimately leads to muscle damage through multiple maladaptive mechanisms.
Additionally, experimental evidence indicates there may be a calcium pumping
deficit due to the loss of expression of SERCA1a in fast fibers (Divet A 2005).
We have hypothesized that excess calcium influx due to membrane
destabilization and concurrent calcium pumping defects may be compensated for
by overexpression of SERCA1a in cardiac and skeletal muscle. Toward this aim,
neonatal mdx mice have been injected via a subxyphoid approach with AAV2/6 CB.SERCA1a to target the diaphragm and heart. The diaphragm will be functionally analyzed at six months of age and heart function will be assessed as per the myostatin inhibition/mdx study. Shorter term limb skeletal muscle studies will also be performed to examine the effect of enhanced calcium pumping on the function and histopathology of EDL and soleus muscle. The animals for the long term studies were injected 11/07 to 2/08. These experiments could validate altering calcium handling in skeletal muscle in DMD patients as a therapeutic approach.


Project 5: Increasing calpastatin in mdx mice
Calpain activation following pathological calcium influx and the resulting cleavage of cytoskeletal proteins have been proposed to be a component of progressive muscular dystrophy in the mdx mouse model. Recent evidence explicitly demonstrates that calcium influx and the activation of calpains are critical for the development of muscle damage due to eccentric contractions (Zhang BT 2008). Damage to myofibers due to repeated eccentric contractions has been implicated in the initial loss of myofibers early in the lifespan of the mdx mouse and then the accumulation of damage throughout life. Previous studies investigating the efficacy of calpain inhibition in mdx animals include leupeptin treatment and a cross with a calpastatin overexpressing transgenic line. While the calpastatin/mdx cross appears to partially protect from muscle damage early in life, it is unclear if part of the therapeutic benefit is due to altered prenatal muscle development. Inhibition of calpain with the small molecule leupeptin has been reported to be successful in ameliorating disease in the mdx animal, however in house studies have been unable to replicate the previously published findings and indicate leupeptin treatment does not improve disease in longer term experiments (4 months). We also found that calpain inhibition due to leupeptin administration led to an upregulation of micro- but not milli- calpain. This finding suggests the inhibition of calpain by leupeptin is inducing compensatory upregulation and/or increased activation of calpains. The endogenous inhibitor of calpains, known as calpastatin, may provide protection from calpain proteolysis, is most likely a more efficient inhibitor of calpains than leupeptin and its inhibitory action is less likely to be overwhelmed by compensatory calpain upregulation. We have produced AAV2/6 and paired mouse calpastatin with a strong ubiquitous promoter (CB7) in order to see if upregulation of calpastatin might provide a means to counter calpain activation in dystrophic muscles that cannot be approached by standard calpain inhibitors.

Project 6:
Investigation of PGC-1α as a target and resveratrol as an activator
Increased utrophin expression has repeatedly been shown to reduce pathology in dystrophic skeletal muscle. In addition, dystrophic muscle demonstrates mitochondrial abnormalities (likely due to oxidative free radical damage). Recently, PGC-1α activation has been shown to increase oxidative genes, and transgenic, mdx mice over-expressing PGC-1α had increased utrophin mRNA and improved histology. While it was not shown in previous studies, if over-expression of PGC-1α in post-natal mdx mice increases utrophin, then the dystrophic muscles should be more resistant to damage caused by lengthening contractions. Additionally, there should be increased oxidative gene expression, making the muscle more fatigue resistant. However, it is possible that these changes are simply due to a fast to slow fiber type conversion mediated by PGC-1α, then there may also be a loss of muscle mass and force which could somewhat offset the increased protection. This is of considerable interest, since SIRT-1 activators, such as resveratrol, could be used to drive PGC-1α in DMD patients. To test these possibilities, we are examining neonatal mdx mice that were injected in the right hind limb with $1 \times 10^{11}$ gc of an AAV expressing PGC-1α while the left hind limb serves as a control. We are also conducting parallel studies involving the feeding of resveratrol to mdx mice in order to activate the PGC-1α pathway.

Project 7:
Continuing examination of BBI in dystrophic mice
Current treatments for DMD have evolved little over the last 30 years and include only steroids as a standard of care. We have been studying a known serine protease inhibitor, the Bowman Birk inhibitor (in the form of an impure concentrate; BBIC), which potentially could attenuate a number of extracellular proteases that are activated in DMD muscle as a consequence of the disease process. To date we have found that, BBIC improves the overall phenotype of the muscles of the mdx mouse, including increasing mass and strength and decreasing necrosis, but, as expected, did not treat the underlying increased sensitivity damage that is due to loss of dystrophin. Nevertheless, it is likely the increased force production and decreased necrosis will lead to improved quality of life, as well as slow disease, for those afflicted with DMD. We are now in the process of evaluating if the effects can be seen with the administration of pure BBI. If so, then we will perform the necessary preclinical efficacy and toxicology to move this into clinical trials in DMD.

Project 8:
Developing High-Throughput Phenotypic Screens for DMD
Most drug screening for DMD relies on known targets that can be modulated in cultured cells. However, there may be many unknown pathways that could provide benefit. The key to being able to screen for such targets is to be able to generate membrane damage in a repeatable manner in either mouse or human dystrophic muscle cells in culture and then perform high throughput screens for
compounds that lower the damage readout. We are in the process of developing such an assay system with the goal of performing high throughput screens for novel targets and drugs in DMD.

**Project 9: Preconditioning stem cells in culture**
The delivery of muscle adult stem cells may provide a means to correct the skeletal muscle of patients with a number of different forms of muscular dystrophy. However, a number of problems remain to be solved including an optimal adult stem cell source, culture conditions to expand and prepare the cells for delivery, and preconditioning of the cells that will provide optimal engraftment and repair of skeletal muscle. The goal of this work is to develop technologies that will promote and reinforce stem cell commitment to the myogenic lineage prior to implantation in a diseased tissue. This project is a collaboration between the Sweeney and Butler-Browne labs, and will use engineered substrates of muscle-like elasticity (~11kPa) to direct the lineage specification of naive stem cells. Two types of easily attainable human adult stem cells will be used; mesenchymal stem cells (MSCs), which can be isolated from a variety of tissues, and AC133+ stem cells, which are isolated from blood. We will follow the fate of the cells after matrix preconditioning when transplanted into mice in which muscle is either acutely damaged, or has an ongoing dystrophic process. An important objective of the overall project is not only to understand to what extent external signals associated with disease can be overridden in stem cells leading to the desired specification, but also to determine to what extent the physical properties of the diseased matrix will interfere with specification and ultimately differentiation into muscle.

**Project 10: AAV-mediated cardiac gene therapy in the dog model of DMD**
Preclinical cardiac involvement in DMD is present in one-fourth of affected individuals by 6 years of age and the mean age of death is 19 years, most often the result of pulmonary or cardiac failure. The disease is a progressive disorder with respiratory or cardiac death common by age 20 to 25 years. A primary cardiac etiology for death occurs in about one-fourth of patients, with an equal distribution of death from progressive heart failure and sudden death. Gene replacement therapy is a promising technique that may result in direct myocardial and skeletal muscle improvement. Although there are strategies currently under investigation using AAV vectors to provide dystrophin expression in skeletal muscle in DMD patients, it is unknown whether the myocardial response to therapy will mirror the skeletal muscle response. In fact, there are documented cases of Becker muscular dystrophy (BMD) with mild skeletal muscle disease, but severe dilated cardiomyopathy. Thus we are comparing AAV-directed exon skipping (U7) in the GRMD dog, which creates a nearly full-length dystrophin protein, to the delivery of a micro-dystrophin, which will fail to rescue a number of components of the cardiac DGC, including NOS. We are also evaluating transgenes designed to improve calcium handling in the heart.