New Developments and Novel Therapies

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Therapeutic Approaches for DMD

Exon-Skipping

Gene Therapy

Utrophin

Stop-Codon Readthrough

Anti-Fibrotics

Steroid Replacement

Inflammation & Fibrosis

Stem Cells

Traditional Cardiac Drugs

Calcium Regulation

nNOS Steroid Replacement

Ryanodine Receptors

Calcium Homeostasis

Mitochondria

Mitochondrial Biogenesis

Follistatin Upregulation via Myostatin Enhancers

Follistatin Upregulation

Selective Androgen Receptor Modulators

Myostatin Inhibition

Ryanodine Receptors

Muscle Growth and Protection

Utophin Upregulation

Utophin

Inflammation & Fibrosis

Treating Duchenne

Blood Flow

Ryanodine Receptors

Calcium Homeostasis

Muscle Prc

PARENT PROJECT MUSCULAR DYSTROPHY | ENDDUCHELLEN.ORG
Gene therapy relies on modified viruses to get genes into cells.

AAV (adeno-associated virus) is the most effective gene delivery vehicle currently in the clinic.
AAV Gene Therapy for DMD

1) Express a micro-dystrophin
   - Express a truncated piece of dystrophin designed to be highly functional and stable

2) Use CRISPR/Cas9 gene editing to create a BMD gene (exon removal)
   - Use gene editing to remove DNA causing an exon to be removed from the RNA (similar to oligo-induced skipping, but a permanent change in the DNA)
Micro-Dystrophins for AAV Trials

Full-Length
Xiao Xiao
Nationwide (Chamberlain)
Solid (Chamberlain)
Limitations for Dystrophin Gene Therapy (Common to Micro-Dystrophin and CRISPR/Cas9)

- Immune response against AAV (capsid).
  - This can be overcome if immune suppression is given prior to virus administration, but to date re-administration of AAV is difficult and may require development of new approaches or alternative AAV serotypes.
  - Prior exposure to AAV may prevent this therapy from being administered. Pre-existing immunity (~20% of older boys) means that virus uptake by muscle will be blocked by circulating antibodies. We may be able to remove them by plasmaphoresis and then re-administer AAV.
Limitations for Dystrophin Gene Therapy (Common to Micro-Dystrophin and CRISPR/Cas9)

• Immune response to transgene.
  – For micro-dystrophin, if a region is deleted in the patient that is present in the micro-dystrophin, there may be an immune response to the µ-dystrophin. There may be approaches that lead to tolerance, but this has not been explored.
  – For CRISPR/Cas9, Cas9 is a bacterial protein and will provoke an immune response if expressed continuously. Strategies need to be put in place to inactivate its expression after it has excised DNA. As with exon skipping in general, there is the possibility of expressing dystrophin regions that will cause an immune response, but it may not
Micro-dystrophin for Nationwide Trial

• Micro-dys can potentially treat all DMD boys, but boys with mutations between exons 18 and 58 are not likely to have an immune response against the Nationwide micro-dystrophin.

• MHCK7 promoter enables expression in heart and skeletal muscle
CRISPR/Cas9

• Can permanently remove 1 or more exons. Will be able to be applied to a large number of mutations.
• Multiple strategies can be used to approach editing of the DMD gene.
• Further from trials.
• Issues are delivery and potential off target effects.
• Very new technology and safety needs to be established.
Gene Therapy – Impact of Age of Children

• Why dose younger?
  – Muscles are more intact.
  – Fibrosis is a barrier to virus delivery.
  – Likelihood of an immune response is lower
  – The downside is that the muscles are rapidly growing and may dilute out the dystrophin requiring another administration of virus.

• Is there a difference between CRISPR and micro-dystrophin gene therapy with regards to the age of the child?
  – No. The issues are all about AAV delivery, not the payload.
<table>
<thead>
<tr>
<th></th>
<th>Microdystrophin</th>
<th>CRISPR/Cas9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Delivery</td>
<td>AAV</td>
<td>AAV</td>
</tr>
<tr>
<td>Protein Produced</td>
<td>Micro/mini dystrophin</td>
<td>Permanent cut to Dystrophin Gene to produce exon skipping</td>
</tr>
<tr>
<td>Route</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td>Expected Result wrt % dystrophin expression</td>
<td>Stable in cell nucleus, small/stable version of dystrophin expressed at high levels</td>
<td>Modifies some % of cellular DNA allowing exon skipping; BMD protein expressed</td>
</tr>
<tr>
<td>Immune response</td>
<td>Potential immune response to viral vector</td>
<td>Potential immune response to viral vector</td>
</tr>
<tr>
<td>Unknowns</td>
<td>Potential immune response to microdystrophin in some patients</td>
<td>Likely immune response to Cas9 (bacterial protein) unless expression is inactivated</td>
</tr>
<tr>
<td>Safety</td>
<td>Immune response</td>
<td>Immune response and off target effects</td>
</tr>
<tr>
<td>Persistence</td>
<td>10+ years????</td>
<td>If satellite cells are not modified, then persistence will depend on the dystrophin that is created.</td>
</tr>
<tr>
<td>Re-treatment?</td>
<td>Eventually necessary and will need new technology</td>
<td>Will be necessary if satellite cells are not infected and will need new technology.</td>
</tr>
</tbody>
</table>
Therapeutic Approaches for DMD

- Exon-Skipping
- Gene Therapy
- Utrophin
- Stop-Codon Readthrough
- Anti-Fibrotics
- Inflammation & Fibrosis
- Stem Cells
- Traditional Cardiac Drugs
- Calcium Regulation
- Ryanodine Receptors
- Calcium Homeostasis
- Mitochondria
- Mitochondrial Biogenesis
- Fibrotics
- Follistatin Upregulation
- Myostatin Inhibition
- Selective Androgen Receptor Modulators
- Utrophin Upregulation
- Ryanodine Receptors
- Muscle Growth and Protection
- Inflammation & Fibrosis
- Exon-Skipping
Functional Roles of Dystrophin:

- **Mechanical** - transmits force from the contractile apparatus to connective tissue/tendon

- **Organizer** - positions a number of proteins at the muscle membrane (NOS, ion channels, etc.)

- **Signaling** - likely plays a number of signaling roles, including a key role in calcium homeostasis

Contraction causes **rupture of the muscle membrane**, which allows calcium inflow. There also may be increased flux through ion (TRPC) channels and leakiness of the internal calcium storage compartment (SR) via the ryanadine receptor (SR-calcium release channel).

Excessive calcium activates breakdown of muscle (via calpain and other proteases), calcium overloads mitochondria causing ROS production, and may trigger cell death program.

Cell death triggers an **inflammatory response**. Activation of fibroblasts can lead to fibrosis, which inhibits muscle regeneration by satellite cells (modulated by IGF-I and myostatin).
Therapeutic Targets: Disease progression can be altered by either expressing dystrophin or overexpressing utrophin and/or targeting the downstream pathological cascade.

Current Duchenne muscular dystrophy therapeutic targets can be grouped into six categories. Only the first addresses the primary genetic defect (resulting in the loss of dystrophin protein). The rest address downstream aspects of the pathogenesis.

1) Replacement of dystrophin/utrophin
2) Decreasing inflammation and fibrosis
3) Increasing muscle mass and regeneration
4) Correcting blood flow regulation
5) Correcting perturbations in calcium handing
6) Mitochondria dysfunction and ROS generation
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Current standard of care: early use of corticosteroids (prednisone or deflazacort)
Repair and Remodeling of Skeletal Muscle in Response to Injury

1. Degeneration
   - Necrosis of myofibers

2. Inflammation
   - Neutrophils
   - Macrophages
   - M1: Debris Removal, Pro-inflammatory cytokines production
   - M2: Satellite cell activation

3. Regeneration
   - Satellite cell activation
   - Stem cell recruitment
   - Regenerating fibers

4. Remodeling/Repair
   - Extracellular matrix remodeling
   - Angiogenesis
   - Functional recovery
NF-κB Activation is a Pathology Observed in DMD

**A.** Muscle biopsies from infants with DMD compared to age matched controls

- NF-κB p65 staining in DMD muscle biopsy
- NF-κB p65 staining in control muscle biopsy

**B.** Muscle biopsies from infants and young boys with matched controls demonstrate activation of the TGF-β pathway occurs by 5 years of age

Chen, 2005
Inhibition of NF-κB Activation Reduces Muscle Necrosis in *mdx* Mice

- IgG positive fibers (degenerative) are reduced with genetic reduction of NF-κB activation (compare red stained fibers on left to right)

Acharyya et al., JCI 2007
Heterozygous Deletion of p65/NF-κB in \textit{mdx} Mice Activates Muscle Satellite Stem Cell Growth

A. Gastrocnemius cryosections from 8-week-old \textit{mdx}/SCID mice in which \textit{p65\textsuperscript{+/-}} and wild-type (wt) MDSCs were implanted. Engraftment was determined by immunostaining for dystrophin (red)

B. Quantitation of regenerated dystrophin-positive myofibers

Huard et al., Molecular Therapy 2012
NF-κB Activation is Seen in All Key Muscles

**Working Hypothesis**

- Loss of Dystrophin (DMD)
- Sarcolemmal instability/injury
- NF-κB (myofibers and immune cells)
- MMP-9
- Inflammation
- Fibrosis
- ECM degradation
- Regeneration

**NF-κB Activation in diaphragm muscle in mdx mice**

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Control</th>
<th>mdx</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>

*Kumar and Boriek (2003) FASEB J, 17:386-396*

- First report showing that NF-κB is highly activated in diaphragm of mdx mice compared with age-matched wild-type mice
NFκB system is elevated in dystrophic muscle

Components of both pathways elevated in \textit{mdx} muscle

Activation of inflammatory genes
CAT-1004 and CAT-1041 are novel NFκB inhibitors

Increased intracellular accumulation

Potent NFκB inhibition in LPS treated cells

CAT compounds are possible therapeutics for DMD
CAT-1041 improves exacerbated *mdx* phenotype

**Exacerbated mouse model**
Male *mdx* mice provided *ad libitum* access to running wheel

- Increased activity
- Limb muscle hypertrophy
- Ex vivo functional improvements
- Reduced muscle fibrosis
Decreased NFkB, Inflammation, and Fibrosis

Multiple measures indicate less damage/degeneration with CAT-1041

No change in utrophin

How does NFkB inhibition protect from mechanical damage?
Membrane repair protein, dysferlin, increased by CAT-1041

Dysferlin is a disease modifier.
Therapeutic Targets: Disease progression can be altered by either expressing dystrophin or overexpressing utrophin and/or targeting the downstream pathological cascade.

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Pathways Controlling Skeletal Muscle Growth and Atrophy
Myostatin Null Boy
Signaling via the Activin Receptors

Activin
Myostatin
GDF11

Type IIR
(ActRIIA, IIB)

Type IR
(ALK4,5,7)

ALK kinase inhibitors

Follistatin for activin and myostatin
Myostatin propeptide for myostatin
Receptor ectodomain (ActRIIA-Fc, ActRIIB-Fc)

MAPK
(ERK1/2, p38MAPK, JNK)

Smad2, 3

Smad4

Transcription cofactors

Smad2/3/4 complex

Smad7,6

off
gene expression

on
Approaches to Achieve Post-natal Myostatin Inhibition

• Neutralizing agents against myostatin (Pfizer, Regeneron, Lilly, BMS)
  – MYO-29 clinical trial by Wyeth failed because of the antibody
  – New generation antibody developed by Pfizer in trial for DMD
  – BMS/Roche DMD trial with anti-myostatin adnectin

• Delivery of N-terminal myostatin propeptide

• Delivery of follistatin or FLRG (myostatin binding proteins)

• Antibody against Activin IIB receptor

• Delivery of soluble Activin IIB receptor (Acceleron clinical trial was halted due to bleeding)
Summary of Mouse Experiments

The amount of hypertrophy resulting from myostatin inhibition in mice differed with age and disease:

- Old Adult C57 (5-10%)
- > Young Adult C57 (10-25%)
- > C57 Neonates (25-40%)
- > Mdx (30-50%)

The hypertrophy resulting from myostatin inhibition in mice was restricted to the fast fiber types, with the most hypertrophy in the fastest (IIb) fibers.
Multiple benefits of increasing or inhibiting myostatin in dystrophic muscle

IGF-I↑

MSTN↓

Regenerative Capacity
Hypertrophy
Muscle Survival
Fibrosis
PPMD Sponsored Preclinical Efforts at UF: Feeding the Pipeline

Preclinical Assessment of Therapeutics Lab
PPMD Sponsored Preclinical Efforts at UF: Feeding the Pipeline

Preclinical Assessment of Therapeutics Lab
Translational Pathway

**Disease Mechanism**
- Cause of pathology
  - ex. Mutation of a gene

**Target Identification**
- Identification of disease modifiers
- Molecules capable of manipulating target
- Usually begins \textit{in vitro} as proof of concept
- Rigorous testing in animal models

**Therapeutic Development**
- Safety and efficacy evaluations in humans

**Preclinical Trials**

**Clinical Trials**
Preclinical Assessment of Therapeutics Lab

Overview:

**Purpose**: To evaluate the therapeutic potential of novel interventions and/or to validate the potential of published interventions in mouse models of DMD to help companies and PPMD prioritize therapeutic interventions that can potentially move into the clinic.

**Mouse models of DMD**: mdx, mdx/ \( C_{mah}^{-/-} \), mdx/DBA, and mdx/Utrophin\(^{+/−}\)

**Rationale**: To see if there is long-term benefit of interventions in mouse models of DMD to identify best candidates for clinical studies. The assumption is that if an intervention is unable to show sustained benefit in the severe mouse model, then it is unlikely to provide any long-term benefit in DMD boys.
$T_1$-weighted images of the thigh with age and disease progression

Age 5

Age 6 -

Age 8 - 9

Age 10 - 11

Age 12 - 13

Age 14
New mdx models show accelerated disease progression with increased fibrosis and fatty replacement, as evidenced in muscles from 3 month old mice shown below.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisolone</td>
<td>Inflammation</td>
<td>N/A</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>Estrogen Receptor</td>
<td>N/A</td>
</tr>
<tr>
<td>Tadalafil</td>
<td>Phosphodiesterase 5</td>
<td>Lily</td>
</tr>
<tr>
<td>PF-943</td>
<td>Phosphodiesterase 9</td>
<td>Pfizer</td>
</tr>
<tr>
<td>BGP-15</td>
<td>HSP70</td>
<td>N-Gene</td>
</tr>
<tr>
<td>Givinostat</td>
<td>Histone deacytelases</td>
<td>Italfarmaco</td>
</tr>
<tr>
<td>Mstn Propeptide</td>
<td>Myostatin</td>
<td>N/A</td>
</tr>
<tr>
<td>Nitrates</td>
<td>Nitric oxide synthase</td>
<td>N/A</td>
</tr>
<tr>
<td>GKT831</td>
<td>NOX4</td>
<td>Genkyotex</td>
</tr>
<tr>
<td>GTX-026/27</td>
<td>Androgen Receptor</td>
<td>GTX</td>
</tr>
<tr>
<td>Plioglitazone</td>
<td>PPARγ</td>
<td>N/A</td>
</tr>
<tr>
<td>MP-101</td>
<td>Mitochondria</td>
<td>Mitochon</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>Mitochondria</td>
<td>Cardero</td>
</tr>
<tr>
<td>Nicotinamide riboside (NR)</td>
<td>Mitochondria</td>
<td>Supplement</td>
</tr>
<tr>
<td>PSC5</td>
<td>Fibrosis (Ras inhib)</td>
<td>N-Gene</td>
</tr>
<tr>
<td>Ang (1-7)</td>
<td>Fibrosis</td>
<td>N/A</td>
</tr>
</tbody>
</table>
DBA/mdx (D2.mdx) mouse model shows accelerated disease progression with increased fibrosis and fatty replacement.
Daily prednisolone causes atrophy but slows disease progression in DBA/mdx (D2.mdx) mouse model.
Daily prednisolone causes atrophy but slows disease progression in DBA/mdx (D2.mdx) mouse model.
Combination Therapies

1) Does prednisone enhance or interfere with other drug actions?
   - Combining Pred (daily or weekly) with:
     a) MSTN inhibition

2) What are the most synergistic drug combinations?
   - Combining CAT-1004/1041 with:
     a) MSTN inhibition
     b) Once a week prednisolone
     c) Mitochondrial drugs
     d) ACE inhib / ARBs
Acknowledgements

Parent Project Muscular Dystrophy
LEADING THE FIGHT TO END DUCHENNE