CORRECTION OF AUTOPHAGIC ACCUMULATION IN SKELETAL MUSCLE OF A POMPE DISEASE MOUSE MODEL FOLLOWING GENE THERAPY ADMINISTRATION

By

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To my Nana and Grandma, my biggest fans in heaven
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<td>Adenovirus</td>
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<td>AP</td>
<td>Autophagosome</td>
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<td>CI-MPR</td>
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<td>Chaperone Mediated Autophagy</td>
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<td>Cytomegalovirus Promoter</td>
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<td>CNM</td>
<td>Central Nuclear Myopathy</td>
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<td>CRIM</td>
<td>Cross-Reactive Immunologic Material</td>
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<td>Desmin Promoter</td>
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<td>DMD</td>
<td>Duchenne Muscular Dystrophy</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>Eagle’s Minimal Essential Medium</td>
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<td>ERAD</td>
<td>Endoplasmic Reticulum Associated Degradation</td>
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<td>ERT</td>
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<td>GAA</td>
<td>Acid α-Glucosidase</td>
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<td>Gastroc</td>
<td>Gastrocnemius</td>
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<td>GFP</td>
<td>Green Flourescent Protein</td>
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<td>GFR</td>
<td>Growth Factor Receptor</td>
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<td>H&amp;E</td>
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<td>hGAA</td>
<td>Human Acid α-Glucosidase</td>
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<td>Herpes Simplex Virus</td>
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<td>IF</td>
<td>Immunofluorescence</td>
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<td>IP</td>
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<td>IPD</td>
<td>Infantile-onset Pompe disease</td>
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<td>ITR</td>
<td>Inverted Terminal Repeat</td>
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<td>JPD</td>
<td>Juvenile-onset Pompe disease</td>
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<td>kb</td>
<td>kilobases</td>
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<td>kDa</td>
<td>kiloDalton</td>
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<td>LAMP</td>
<td>Lysosomal Associated Membrane Protein</td>
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<td>LGMD</td>
<td>Limb-Girdle Muscular Dystrophy</td>
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<td>LOPD</td>
<td>Late-Onset Pompe disease</td>
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<td>LSD</td>
<td>Lysosomal Storage Disease</td>
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<td>LSP</td>
<td>Liver Specific Promoter</td>
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<td>MCK</td>
<td>Muscle Creatine Kinase Promoter</td>
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<td>mGaa</td>
<td>Murine Acid α-Glucosidase</td>
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<td>MTM1</td>
<td>Myotubularin 1</td>
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<td>MTMPro</td>
<td>Myotubularin 1 Promoter</td>
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<tr>
<td>mTROC1</td>
<td>Mammalian Target of Rapamycin Complex 1</td>
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<tr>
<td>NGLY1</td>
<td>N-glycanase 1</td>
</tr>
<tr>
<td>NMJ</td>
<td>Neuromuscular Junction</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<tr>
<td>PAS</td>
<td>Periodic Acid Schiff</td>
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PBS  Phosphate-Buffered Saline
PD    Pompe disease
PI3K-III Phosphoinoside-3-Kinase Class-III
PI3P  Phosphotidylinositol-3-Phosphate
PI3,5P₂ Phosphotidylinositol-3,5-Diphosphate
rAAV recombinant Adeno-Associated Virus
rER   rough Endoplasmic Reticulum
rhGAA Recombinant Human Acid α-Glucosidase
TA    Tibialis Anterior
TFEB  Transcription Factor EB
TBS   Tris-Buffered Saline
UF-PGTC-VC University of Florida – Powell Gene Therapy Center – Vector Core
UTR   Untranslated Region
vg    vector genomes
wt    Wildtype
XMEA  X-Linked Myopathy with Excessive Autophagy
XLMTM X-Linked Myotubular Myopathy
CORRECTION OF AUTOPHAGIC ACCUMULATION IN SKELETAL MUSCLE OF A POMPE DISEASE MOUSE MODEL FOLLOWING GENE THERAPY ADMINISTRATION

By

Angela Layne McCall

May 2017

Chair: Barry John Byrne
Major: Medical Sciences - Genetics

Pompe Disease (PD) is a fatal metabolic disorder caused by mutations in the GAA gene leading to a deficiency in acid α-glucosidase (GAA) and affects 1 in 40,000 births. Clinically, PD presents with cardiomegaly and skeletal muscle weakness, leading to cardiorespiratory failure. GAA is responsible for the breakdown of glycogen in the lysosome, an important energy source for striated muscle and neurons.

Currently, the only approved treatment for PD is enzyme replacement therapy (ERT). While ERT has increased patient survival, there are several limitations to this strategy, chiefly that it is dependent upon receptor-mediated endocytosis. A low abundance of receptor at the plasma membrane in skeletal muscle and physical barriers by accumulated vesicles, results in much of the enzyme captured in amphisomes never reaching the lysosome. This issue illustrates the need for an alternative treatment. This project proposes that endogenous production of GAA enzyme mediated by recombinant adeno-associated virus (rAAV)-delivery of GAA will improve lysosomal delivery and reduce the overall dysregulation of vesicular systems.

In this study, AAV serotype 9 vectors carrying the GAA gene driven by a tissue restrictive promoter (rAAV9-DES-coGAA) at three doses (1x10^{11} vg/kg, 1x10^{13} vg/kg,
and $1 \times 10^{14}$ vg/kg) were intravenously delivered to 12-week old $Gaa^{-/-}$ mice. One month after AAV or sham injection skeletal muscles, soleus, diaphragm, gastrocnemius, and tibialis anterior, were harvested for biochemical and histological analyses. GAA activity assays demonstrate that therapeutic levels of enzyme in skeletal muscle were attained only with a dose of $1 \times 10^{14}$ vg/kg. Vacuolization of fibers was lower in high dose treated mice compared to untreated controls. Immunofluorescence and western blot analysis of autophagy-associated proteins, LAMP1, LC3-I, LC3-II, Beclin1, and p62 showed a decrease in protein levels indicating an improvement in autophagic regulation in diaphragm, gastrocnemius, and tibialis anterior. Cross-sectional area, a measure of muscle growth was improved compared to hypotrophic fibers in untreated mice. Preliminary evaluation of tibialis anterior of mice treated with the high dose at birth or at 3 months of age and analyzed 6 months later, demonstrated the persistence of GAA expression and resolution of lysosomes and autophagosomes. Overall, this treatment is promising for PD patients and this study provides evidence of the amelioration of vacuolar accumulation.
CHAPTER 1
LITERATURE REVIEW

Pompe Disease

Clinical Perspective

In 1932 Dutch pathologist J.C. Pompe observed a deceased infant, whose cause of death was idiopathic cardiac hypertrophy\(^1\). This is the first report of a patient who had generalized muscle weakness and vacuolar glycogen accumulation in several tissues, but predominantly in the heart. This phenotype would later be coined “Pompe disease,” (PD) and was differentiated from “von Gierke’s disease,” in which a similar glycogen storage primarily affects the liver\(^2\). Upon the discovery of the lysosome, an acidic compartment within cells, by Christian de Duve in 1955\(^3\), Henri-Gery Hers demonstrated that the accumulation of glycogen described by Pompe was due to a lack of maltase, the enzyme responsible for hydrolyzing glycogen into glucose within lysosomes\(^4\). In this seminal article, which would pioneer the identification of a group of disorders known as lysosomal storage diseases (LSDs), it was shown that cells of children with PD were unable to metabolize either maltase substrate, maltose or glycogen, at an acidic pH. Maltase, now known as acid $\alpha$-glucosidase (GAA), is the gene product of the GAA gene, located on Chromosome 17\(^5,6\) at position q23\(^7-9\). The gene spans 28 kilobases (kb), and is composed of one non-coding exon followed by nineteen coding exons resulting in an mRNA of 3.4 kb\(^10,11\). Mutations within GAA produce genetic heterogeneity\(^12\). Deficiency of the enzyme is caused by at least one of the nearly 200 mutations compiled by Kroos, et al.\(^13\). Some of the most commonly detected DNA changes include: c.-32-13T>G\(^14-18\), c.525ΔT\(^19\), c.1076-1C>T\(^20\), c.1935C>A\(^21\), c.2560C>T\(^22\), and Ex18Δ\(^23\) (Table 1-1). While there has been some correlation
between mutation location, amount of functional enzyme, and degree of severity, it is
difficult to predict disease progression based solely on mutation analysis\textsuperscript{24}. Some even
assert that no correlation exists\textsuperscript{25}. Additionally, modifying genes appear to exist; for
example, variations in levels of angiotensin-converting enzyme and α-actinin 3 have
been shown to affect severity of PD\textsuperscript{26,27}. Founder effects have delineated genotypes
and corresponding phenotypes to specific regions of the globe\textsuperscript{20,28–30}. Global
differences in the heritance of specific mutations have led to a more severe phenotype
being observed at a greater proportion in Southern China, while the Netherlands have a
higher proportion of patients with a mild to moderate phenotype.

Due to of the vast number of mutation combinations and modifying genes that
may be present, there exists a gradient of phenotypes. In the forty years since Hers’
discovery, many of these patients’ phenotypes have been described alongside their
genetic and biochemical analysis. Generally, they have been separated into three
levels of clinical severity and age at onset\textsuperscript{31}: infantile (IPD)\textsuperscript{32,33}, juvenile (JPD)\textsuperscript{34,35}, late-
onset (LOPD)\textsuperscript{36,37}. Those with the most severe phenotype, IPD, typically have
mutations that result in <1% of normal levels of functional GAA produced\textsuperscript{38}. They begin
to experience symptoms anytime from birth through the first few months of life\textsuperscript{38,39}.
These symptoms include rapid and progressive muscle weakness particularly in the
facial and distal limb muscles\textsuperscript{32,36,40}. Although it is not lethal, hepatomegaly is
observed\textsuperscript{2}. By one year of age, glycogen accumulation in the heart causes hypertrophy
of the ventricles and obstructs outflow of blood from the left ventricle, leading to cardiac
failure, the primary cause of death in this subset of patients. Additionally, patients
display a weakened diaphragm, macroglossia, and delayed motor skills. IPD patients
that live beyond infancy may also experience osteopenia\textsuperscript{41}. Juvenile-onset and late-onset are characterized by the presence of 1-10\% and 2-40\% of normal GAA levels, respectively\textsuperscript{39}. LOPD patients do not typically have cardiac related symptoms but rather experience significantly greater skeletal muscle hypotonia. For most, symptoms manifest in the second to fourth decade of life, but LOPD has been described in patients as late as their sixties\textsuperscript{42}. Those with JPD have symptoms and life-expectancy in between those of IPD and LOPD, with a greater skeletal muscle dysfunction leading to loss of ambulation, and variable cardiomegaly\textsuperscript{34}. Respiratory insufficiency is a common symptom among all three subsets of the disease, with three-quarters of children and one-third of adults requiring mechanical ventilation\textsuperscript{43,44}. Another result of diaphragm weakness is that patients are at an increased risk of complications when undergoing anesthesia as well as pulmonary infections due to an inability to produce a full-force cough \textsuperscript{45,46}.

While cardiac and skeletal muscle dysfunction are the organ systems impacted the most in PD, the central nervous system (CNS) including motorneurons show significant glycogen accumulation and functional disturbance. When this was first reported, patients' electromyographs and histopathology of the CNS were deemed abnormal\textsuperscript{47}. In recent years, evaluation of the $Gaa^\text{-/-}$ mouse model has revealed the mechanisms of CNS pathology. Pro-inflammatory signaling and pro-apoptotic pathways are significantly upregulated at both the mRNA and protein levels\textsuperscript{48}. Downstream effects of these pathways, such as DNA fragmentation, have also been noted\textsuperscript{49}. Additionally, the neuromuscular junction (NMJ) is susceptible to damaging pathology. Alterations on both sides of the junction such as a reduction in neurofilament heavy
protein and myelin sheath, and an increase in endplate fragmentation lead to decreased endplate innervation. These CNS-related pathologies, particularly within the phrenic and sciatic nerves, are becoming more important as it is understood that it is not just the glycogen accumulation within the diaphragm and lower limb skeletal muscles that leads to a loss of motor function. Despite the presence of neural cell pathologies and the large energy demand in photoreceptors, no retina-related pathologies have been identified. All ocular studies have attributed blurred vision to the glycogen accumulation in extraocular muscles and surrounding ciliary bodies.

In the 1960s many patients with LOPD were incorrectly diagnosed with “progressive muscular dystrophy” but upon an improved understanding of glycogen presence in vacuoles and assays for enzyme activity, they were later correctly characterized. Upon initial onset of symptoms, a broad-spectrum of muscle disorder tests are performed. Diagnosis has previously relied on the collection of a muscle biopsy to evaluate the presence of Periodic-Acid-Schiff (PAS) positive material and identify glycogen accumulation in membrane-bound vesicles by electromicroscopy. Now, early signs include elevated creatine kinase levels, as well as raised liver enzymes: asparagine aminotransferase, alanine aminotransferase, and lactate dehydrogenase. Differential diagnoses come from an echocardiogram, revealing diminished ventricular space and force, and an electrocardiogram, in which a classic sign is a shortened PR interval and tall, elongated QRS complexes. The diagnosis is confirmed with a GAA activity assay from a dried blot spot, skin fibroblasts, or muscle biopsy, and finally GAA mutation sequencing. Biopsies of striated muscle reveal a plethora of histological findings including a “lacework” appearance of muscle fibers.
where glycogen filled lysosomes have taken over the majority of a fiber, central vacuolization, and displacement of myofibrils where vacuolar accumulation-induced mechanical stress has impinged on the muscle ultrastructure.  

Molecular Mechanism

GAA, the enzyme, is synthesized in the rough endoplasmic reticulum (rER) as a 110 kiloDalton (kDa) precursor and is N-glycosylated with a combination of seven high mannose oligosaccharides and complex-type glycans, which are required for efficient folding and rER exportation. Once in the Golgi apparatus, the glycans are modified and mannose moieties are phosphorylated. This addition is crucial to the proper trafficking of nascent polypeptides from the trans-Golgi network to lysosomes, and also facilitates uptake of exocytosed enzyme by other cells. Approximately 90% of the enzyme is transported to the lysosome via endosomes. As the vesicle matures by increasing H⁺ concentration, the enzyme also matures through a specific series of self-cleavages that result in proteins of sizes 95-, 76-, and 70-kDa. All forms of the enzyme have some catalytic function, but become more active with maturation, with the 76kDa form being the most abundant and the most active. Inside lysosomes, GAA has the ability to cleave glycogen fully at both α-1,4 and α-1,6 linkages (Figure 1).

Mutations in GAA may have a variety of effects. They can impact transcript stability, cause an insertion, deletion, or change of amino acids, or generate a premature stop codon and thus produce a truncated protein. The location and type of mutation has a great effect on the amount of functional enzyme produced. Those that yield an unstable mRNA transcript or severely truncated protein result in <1% of functional protein produced (Figure 1-2). Similarly, mutations that affect a cleavage site or glycosylation residue can prevent the enzyme from reaching the compartment in
which its substrate is available\textsuperscript{59}. Milder mutations such as IVS1 and Ex18Δ reduce the total amount of enzyme that is synthesized at the translational level, rather than hindering the catalytic site. Currently, no known mutations result in a decrease in a single molecule’s enzymatic efficiency, rather those that generate some protein, present symptomatically because of a reduction in the total number of active enzyme molecules. This is reflected in the similarity of Km values among PD patients and unaffected samples.\textsuperscript{39}

**Animal Models**

Generalized glycogen storage disorders have been reported in Japanese quail, Finnish and Swedish Lapphunds, and Brahman and Shorthorn cattle species\textsuperscript{60–64}. All exhibit signs and symptoms similar to those in human patients, specifically, myocardial hypertrophy and progressive muscular weakness, with additional weakness observed in smooth muscle. Their histopathological and biochemical analyses display glycogen accumulation. Mutations in the gene for acid α-glucosidase leading to 0 – 20% of normal GAA activity has been attributed to the phenotype produced in the animals. Each has been explored for its usefulness as an animal model for pathology mechanism elucidation and treatment evaluation\textsuperscript{65}.

Despite the natural occurrence of these animals, a more manageable species was needed to study the response to protein replacement. In the late 1990s, two mouse models were generated. The first disrupted Gaa in Exon 13, and while histologically skeletal muscle, cardiac muscle, and hepatocytes appeared to have classic Pompe disease signs, the mice did not exhibit global physiological symptoms\textsuperscript{66}. Then, Raben et al. generated the mouse model that has since been used throughout the Pompe disease research community\textsuperscript{67}. Exon 6 of Gaa was interrupted with a neo
gene to result in a lack of GAA production. This model experiences classic symptoms, glycogen accumulation, cardiac hypertrophy, and skeletal muscle weakness that affect mobility by nine months of age.

**Autophagy**

**General Overview**

Autophagy is the cellular process by which proteins are selectively and non-selectively sequestered and transported to the lysosome for degradation. There are three types of autophagy: chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy. CMA and microautophagy involve direct incorporation of a protein into the lysosomal lumen, through chaperone unfolding via lysosomal-associated membrane protein 2 (LAMP2), or invagination of the lysosomal membrane, respectively. Macroautophagy, colloquially referred to as “autophagy,” is the process of a phagophore developing around a group of proteins and/or organelles, and fusion with a lysosome to deliver its contents (Figure 1-3). This pathway is activated by a number of physiological and chemical stimuli, most of which act through the mammalian target of rapamycin complex 1 (mTORC1). TOR activation and suppression has a centralized role in many pathways that control responses to cell stress such as cell cycle, transcription, translation, and actin organization. Physiological stimuli include nutrient starvation, growth factors and hormones, as well as cellular stresses like DNA damage. mTORC1 activity is regulated by nutrient sensors for amino acids, monosaccharides, nucleotides, divalent metals, and lipids that reside in the cytosol near the lysosomal membrane where the complex is tethered.

Skeletal muscle autophagy is most robustly activated under conditions of nutrient deprivation through both transcriptional-dependent mechanisms and signaling pathways.
controlled by protein-protein interactions and post-translational modification\textsuperscript{78}. Transcriptionally, FoxO3 (Forkhead box O3)\textsuperscript{79,80} and TFEB (transcription factor EB)\textsuperscript{81} are crucial factors that coordinate the expression of the majority of autophagy-related proteins. The autophagic pathway is regulated by a multi-step process which includes over twenty proteins\textsuperscript{82}. Most of what is known about autophagy comes from a gross number of genetic manipulation studies completed in yeast, but homologs of most identified autophagy related genes (Atgs) have been found in higher level eukaryotes from \textit{Drosophila melanogaster} and \textit{Caenorhabditis elegans} to mouse and man\textsuperscript{73}. Upon suppression of mTORC1, an unphosphorylated ATG13 is activated to complex with ULK1 and FIP200\textsuperscript{83}, then recruits PI3K-III (composed of VPS34-VPS15-ATG14-Beclin1) to the site of nucleation. These two complexes, along with ATG9 and WIPI proteins\textsuperscript{84}, activate two ubiquitin-like conjugation systems to control nucleation and elongation of the phagophore, or sequestering membrane. In the first system, E1-like ATG activates ATG12 at the C-terminus. Then it is transferred to E2-like ATG10; the process is completed by the conjugation to Lysine130 of ATG5. This multiprotein molecule interacts with ATG16L on the growing phagophore and dissociates once the autophagosome (AP) fully fuses\textsuperscript{85–88}. A similar process occurs to attach phosphatidylethanolamine to LC3-I, creating LC3-II. The activator ATG7 is also used here, this time transferring to ATG3, an E3-like conjugator. Unlike ATG12-ATG5, LC3-II remains within the AP membrane throughout the lifespan of the vesicle, from initiation to full maturation\textsuperscript{89–92}. The phagophore is generated from one of a number of existing membranes, ER, trans Golgi network, plasma membrane, or synthesized \textit{de novo}\textsuperscript{93}. Proteins, fatty acids, organelles, and other cytosolic materials are gathered within the
growing membrane until the two ends fuse together, facilitated by SNARE proteins\textsuperscript{94}, forming an AP. Characteristic of APs are double and multi-membranes, making them readily identifiable by electron microscopy\textsuperscript{93}. APs are then able to fuse with endosomes, forming amphisomes\textsuperscript{95}, which allows for the delivery of lysosomal enzymes and acidification through H\textsuperscript{+} vacuolar-ATPases; followed by fusion with lysosomes, forming an autolysosome\textsuperscript{96,97}. Once the latter event occurs, material delivered has reached a terminal stage and is thus degraded by hydrolases active within lysosomes\textsuperscript{98}.

**Autophagic Dysregulation in Pompe Disease**

For the autophagy pathway to be completed, contents within APs must be delivered to lysosomes\textsuperscript{98}. The flow through this system resulting in the degradation of APs is characterized as autophagic flux. In a study of cultured muscle cell precursors from the Gaa knockout mouse model, 40\% of lysosomes were not within the normal pH range\textsuperscript{99}. As glycogen accumulates within lysosomes and their luminal volume expands, v-ATPases, which acidify the lumen, are unable to maintain the low pH environment. When the lumen alkalizes autophagosome-lysosome fusion is diminished. Thus, a secondary vesicular accumulation of APs occurs\textsuperscript{100}. A model proposed by Fukuda and colleagues suggests that there are multiple factors feeding into the dysregulation of this pathway. At first the cell enters a nutrient deprivation status because glycogen cannot be degraded, and activates autophagy in response\textsuperscript{99}. Simultaneously, when glycogen amasses within lysosomes they lose the ability to fuse with endosomes, amphisomes, and APs, thus halting the reduction of vesicles. As material within lysosomes accumulates mechanical stress is placed on their limiting membrane and causes them to burst. The accumulation of APs creates a similar stress on the sarcolemma membrane until the contractile unit becomes unstable\textsuperscript{101,33}. This phenomenon has been
well documented in both Pompe disease patients and the Gaa<sup>−/−</sup> mouse model, with some distinct characteristics<sup>99,102–104</sup>. In the mouse model, build-up occurs most predominantly in type II muscle fibers, which is accompanied by a decrease in the diameter of each fiber, although an increase in total autophagy is observed in type I fibers<sup>99</sup>. As analyzed by electron microscopy, APs are present in more than 60% of distal skeletal muscle fibers<sup>105</sup>. mRNA and protein levels of Beclin1, Atg12, LC3, and GABARAP indicate an increase in the expression of autophagy-associated proteins and the persistence of this pathology in skeletal muscle<sup>106</sup>. In patients, the dysregulation is equally seen in type I and type II fibers<sup>100,103,104</sup>. The earliest reports precede elucidation of the pathway’s mechanism and describe structures as secondary lysosomes<sup>107</sup>. In biopsies taken from IPD and LOPD patients, accumulation of p62 protein aggregates correlated with prolific vacuolization and myofiber atrophy<sup>104</sup>. In addition to the disruption of myofibrillar structure, lipofuscinosis, defined as irregularly shaped autofluorescent inclusions composed of abnormal mitochondria, undegradable proteins, and lipids result from oxidative damage<sup>100,108</sup>.

**Treatments**

**Enzyme Replacement Therapy**

From the premise that cells secrete ~10% of GAA into the blood stream, which is then able to be taken up by neighboring or distal cells<sup>57</sup> arose the concept of enzyme replacement therapy (ERT) for Pompe disease<sup>109</sup>. Early attempts at developing ERT for PD administered a purified extract from the fungus *Aspergillus niger*, which contained a mixture of α-amylase, amyloglucosidase, and maltase<sup>109–112</sup>. Original studies delivered the enzyme extract in a number of methods, including direct intramuscular or intravenous injections, some within liposomes, in an attempt to find a favorable route.
However, efficacy of the mixture was dismal, having a small effect on glycogen-containing lysosomes only within the liver\textsuperscript{110,111}. Patients with Gaucher disease, a lysosomal storage disease similar to PD, demonstrated encouraging success following administration of recombinant cerebrosidase (Ceredase\textsuperscript{®}) which in 1995 became the first FDA approved ERT and led the way for the development of ERT for PD\textsuperscript{113–115}. One of the most notable influences recombinant cerebrosidase had was a shift from the theory that lysosomal hydrolases were taken up by pinocytosis\textsuperscript{110} to the fact that they enter through receptor-mediated endocytosis, specifically via the cation-independent mannose-6-phosphate receptor (CI-MPR)\textsuperscript{56,116,117} (Figure 1–4). Enzymes purified from the human placenta and bovine testes were shown to have the mannose-6-phosphate moiety. When they were applied to the medium of cultured fibroblasts and skeletal muscle cells, they were endocytosed efficiently through the CI-MPR and confirmed to be active in the lysosome, clearing glycogen\textsuperscript{118,119}. Upon administration of bovine testes-derived GAA to mice, activity was 43\% and 70\% of \textit{wildtype} levels in the cardiac and skeletal muscle, respectively\textsuperscript{120}. Another hurdle that needed to be overcome was the ability to mass-produce the enzyme. Results of \textit{in vivo} mouse studies yielded an expected dose of 200-400 milligrams (mg) delivered weekly to human patients, an amount unable to be provided from bovine testes purification methods.

Two alternative production methods were developed and tested in parallel. Chinese Hamster Ovary (CHO) cells had been used by many others for the production of recombinant mammalian proteins\textsuperscript{121–123}. Recombinant human acid α-glucosidase (rhGAA) was expressed in CHO cells where the 110kDa form of the enzyme that is properly glycosylated and phosphorylated is secreted into the media and can be
collected for purification\textsuperscript{123,124}. Functionality of this product was tested in patient fibroblasts and a guinea pig model. Glycogen accumulation in patient cells was 46% higher than that in unaffected control cells\textsuperscript{123}. This accumulation was completely mitigated in affected cells treated with CHO-derived GAA\textsuperscript{123}. With the same product, an increase in activity was also observed within the heart and liver of the guinea pig\textsuperscript{123}. The second method of protein production was through the milk of transgenic rabbits\textsuperscript{125}. Intravenous delivery of purified GAA produced in transgenic rabbits to Gaa knockout mice resulted in an increase of 67500%, 375%, and 590% of wt GAA activity in the liver, cardiac muscle, and skeletal muscle, respectively, measured twelve hours after final dose\textsuperscript{125}. The higher enzyme amounts corresponded with a decrease in glycogen content. Two clinical trials comparing the products of these methods were completed at the turn of the millenium\textsuperscript{126,127}. In the first, rabbit milk-derived rhGAA, was administered to both healthy volunteers, to demonstrate safety, and to four patients with IPD\textsuperscript{126}. Activity in muscle by twenty-four weeks of dosing at 40 mg/kg, infused one time per week, was within the normal range (8-40nmol/mg/hour), and resulted in improved strength. An obvious effect was seen in the heart, where the left ventricular wall thickness decreased in all patients. In the second trial, which utilized CHO-derived rhGAA at a lower dose than the previous, three IPD patients began ERT between 2.5 and 4 months of age, and were treated for an average of 16 months at time of publication\textsuperscript{127}. Similar improvements in cardiac muscle were observed, but no improvement was measured in skeletal muscle. Importantly, all patients survived through 12 months of age, which served as the critical primary endpoint in both studies. Success, specifically by survival past one year of age, paved the way for FDA approval.
in 2006 of rhGAA as an enzyme replacement therapy known as alglucosidase alfa, or Myozyme®, which has since been replaced in the United States with an identical product, Lumizyme®. A crucial finding in the latter study was the influence of the immune system on the receptiveness to the drug. Patients whose mutations result in no GAA produced, are considered cross-reactive immunological material- (CRIM) negative, as compared to those who do have GAA produced, regardless of its functionality, and are labeled CRIM-positive. For CRIM-negative patients, delivery of rhGAA is recognized by the immune system as foreign and antibodies are formed against it, reducing efficacy\textsuperscript{128}. The mechanism has been explored more in-depth in the Gaa\textsuperscript{-/-} mouse model and a CRIM+ PD model\textsuperscript{129}. In the trial described above, Patient 3 was CRIM-positive, and had significantly better outcomes, including remaining ventilator independent, than Patients 1 and 2 who were CRIM-negative. CRIM status has become a strong predictor of how responsive a patient will be to ERT. In a retrospective study of patients over a twelve-year period, none of the six CRIM-negative patients survived to five years of age, even those who were started on ERT before six months of age\textsuperscript{130}.

While ERT has demonstrated efficacy in cardiac pathology correction leading to an increased average life expectancy, many other organ systems remain unaffected by the therapy and continue to deteriorate. The central nervous system and skeletal musculature are the two greatest organ systems that are unable to be fully penetrated by infused rhGAA. Neural cells in the brain and motoneurons in the spinal cord that control proximal and distal skeletal muscle function are unable to be treated due to the inability of proteins to cross the blood-brain barrier\textsuperscript{105}. In addition, skeletal muscle, particularly Type II muscle fibers, are resistant to ERT, due to a low abundance of CI-
MPR present along the sarcolemma (Figure 1-5)\textsuperscript{59,99}. Better outcomes for patients on ERT have been associated with those who have a higher percentage of Type I fibers, which may be influenced by transcriptional modifiers and activity ability\textsuperscript{131}.

Lack of uptake in skeletal muscles, specifically the diaphragm and lower limb muscles have resulted in a poor quality of life for patients who rely on mechanical ventilation for many years and are wheelchair bound\textsuperscript{44}. A cyclical cascade then ensues, since physical inactivity from being non-ambulatory causes a transition of fibers to type II, perpetuating the problem\textsuperscript{132}. Accumulated amphisomes and autophagosomes that are unable to fuse with lysosomes create a physical barrier preventing endosome movement. Without endosome movement and acidification, rhGAA is inefficiently trafficked to the lysosome. Because of this, the enzyme is trapped within a vesicle separate from its substrate, glycogen, and is not able to undergo necessary self-cleavages without proper maturation and acidification of the vesicle. This is one of the largest contributors to rhGAA efficacy in skeletal muscle\textsuperscript{59,133}. This blockage also reduces the already paltry number of CI-MPRs present at the sarcolemma membrane, preventing the uptake of enzyme. In the mouse model, ERT reduces glycogen in skeletal muscle by a mere 30\%\textsuperscript{134}. The accumulation, marked by the presence of ubiquitin aggregates, persists despite treatment\textsuperscript{135}.

**Alternative Therapies**

Following the failure of early ERT studies, alternative approaches such as cell and bone marrow transplantations were attempted\textsuperscript{136–138}. These were unfortunately also met with obstacles preventing their success. Dietary regimes have also been implemented for PD patients. A high-fat, low carbohydrate diet reduced total protein turnover, and thus reduced muscle mass loss, in IPD and LOPD patients. However,
only a few subjects made functional clinical improvements\textsuperscript{139–142}. Studies in the mouse model of PD and PD patients have demonstrated that exercise therapy is somewhat effective in reducing glycogen storage, and improving functional outcomes, but only when used in combination with another therapy\textsuperscript{143–145}. Successful studies completed \textit{in vitro} and \textit{in vivo}, target mRNA transcripts of glycogen synthase for degradation by RNAi\textsuperscript{146,147}. In this, the glycogen is not produced and the need for GAA is not as pertinent.

\textbf{Adeno-Associated Virus-Mediated Gene Therapy}

In 1965, a virus-like contaminant was discovered in a preparation of simian adenovirus stocks. Small, replication deficient viral particles were isolated and thus named “adeno-associated viruses” (AAVs)\textsuperscript{148,149}. Thirteen serotypes have since been identified from human and non-human primate tissue samples, along with over 100 isolates\textsuperscript{150–152}. AAVs are small (~260Å), single stranded DNA viruses that have been classified within the Dependovirus genus of the Paroviridae family\textsuperscript{153}. Their lifecycle can be divided into two phases, latent and lytic, which is controlled by the presence of a helper virus that is required for replication. Adenovirus (AdV) and Herpes Simplex Virus (HSV) are among the two most well-known helper viruses for AAV\textsuperscript{154,155}. Without a helper virus, AAV genomes exist in two forms. A small percentage of genomes become integrated into the AAVS1 locus of the Chromosome 19 q arm \textit{in vitro}, while the majority persist as an episome \textit{in vitro} and \textit{in vivo}\textsuperscript{156}. Even though the replication of AAV relies on cellular and helper virus factors, its small genome contains a sequence that contains several diverse factors\textsuperscript{157}. The \textit{wildtype (wt)} AAV genome is \textasciitilde 4.7 kb\textsuperscript{158} in length and contains three open reading frames (ORFs) that are flanked by 145 base-pair inverted terminal repeats (ITRs) (Figure 1-6a). ORF transcription is initiated at three promoters,
p5, p19, and p40. The first ORF, \( Rep \), codes for four replication proteins, Rep78, Rep68, Rep52, Rep40. The second ORF, \( Cap \), encodes three viral proteins, VP1, VP2, and VP3 that make up the capsid. The AAV genome makes use of an alternative start codon at VP2 and a cryptic splice site such that the VPs are produced in a 1:1:10 ratio, respectively, with a total of 60 monomers generating a \( T=1 \) icosahedral capsid.

Characteristics of AAV capsid symmetry include the two-fold depression, three-fold protrusions, and five-fold pore\(^{159}\). Just upstream of the VP3 translation start site, is a third ORF which encodes the assembly-activating protein, required for transport of VPs from the rER into the nucleolus and capsid formation\(^{160}\). Once the capsid is assembled, both positive and negative DNA genomes (“flip” and “flop”) are thread through the five-fold pore\(^{161,162}\). The ITRs, contain palindromes and thus form hairpin structures at the 3’ and 5’ ends of the AAV genome and have many roles crucial to the life cycle of these viruses\(^{162,163}\). The 3’ end serves as a primer for second strand synthesis. Lending to their persistence in cells, the ITRs are resolved by recombination to form episomes and concatamers\(^{164}\). These multifunctional sequences also contain a packaging signal for DNA encapsidation.

The capsids of the thirteen known serotypes have demonstrated a broad tissue tropism, an asset that has been exploited toward their development as effective gene therapy vectors. Tropism is dictated by the properties of the capsid surface and its interaction with glycans expressed on the plasma membrane in a cell-type specific manner. Nine of these AAV serotypes have been structurally characterized by cryo-electron microscopy 3D reconstruction or x-ray crystallography\(^{165–172}\). Understanding the capsids at this level provides a wealth of functional information spanning from their
receptor binding domain to their antibody footprint. Glycan receptors for eight serotypes have been identified, and the AAVs are delineated into three categories, sialic acid-binding, heparan sulfate proteoglycan-binding, and galactose-binding, based on the glycan utilized for cell binding. Meanwhile, the binding moieties for AAV-7, 8, 10, 11, and 12 are still unknown. Co-receptors for several serotypes have also been characterized and include a number of growth factor receptors (GFR) (fibroblast GFR, hepatocyte GFR, platelet-derived GFR, epidermal GFR), integrin, and the 67kDa laminin receptor. Following binding to a receptor, AAVs enter the cell in an endosome. As the pH decreases in the endosome, the capsid stability changes and a phospholipase A2 (PLA2) domain at the N-terminus of VP1, which is typically buried on the inside of the capsid, becomes externalized. The PLA2 domain functions to degrade the endosomal membrane, allowing the capsid to escape into the perinuclear region. The mechanism by which AAVs enters the nucleus is still uncertain, but it is known that the capsid does not uncoat until inside.

Due to their lack of pathogenicity and weak immunogenicity, AAVs were investigated as a potential gene therapy vector. In 1984, Hermonat and Muzuczka, explored this potential by demonstrating its ability to package and express a foreign gene exchanged for part of the wt genome. In this pinnacle study, packaging limits of the capsid were revealed, as well as the ability to provide the cap gene in trans, which paved the way for vector production. Early pre-clinical and clinical studies were all conducted using recombinant AAV2 (rAAV2) vectors, until cross-dressing, the ability to package DNA vectors flanked with wt AAV2 ITRs, into each of the various serotype’s capsids, was known to be possible. This represented a major breakthrough,
providing the necessary foundation for targeting rAAV vectors toward specific tissues, utilizing their natural evolution in a productive manner. rAAV1 and rAAV6 vectors demonstrate high transduction in skeletal muscle, while rAAV2 and rAAV5 have been used in the treatment of retinal diseases, and rAAV3b and rAAV8 are liver-tropic. Because of its unique glycan receptor, rAAV9 has come to the forefront in the treatment of systemic diseases for its ability to transduce cardiac muscle, skeletal muscle, and the CNS\textsuperscript{192,193}.

rAAV vectors cannot only be cross-packaged using AAV2 ITRs, but these are the only \textit{wt} DNA elements required for packaging; no coding sequence of the \textit{wt} AAV genome is necessary for rAAV vector transduction. Even though genome packaging size limits exist, only a mere 300 base-pairs of the crucial ITRs is conserved across rAAV vectors, leaving nearly 4.4\textit{kb} of space to be filled with a promoter, transgene cDNA, and regulatory elements of interest (Figure 1-6b)\textsuperscript{194}.

**Muscle AAV gene therapy**

Skeletal muscle is an attractive AAV-mediated gene therapy target since it makes up nearly 40\% of lean body mass and plays a large role in the production of proteins that can be secreted into the plasma and taken up by proximal or distal organs\textsuperscript{195}. In 1996, AAV transduction of muscle was initially demonstrated; when it was previously believed that only dividing cells could be transduced\textsuperscript{196}. In this study the expression of a reporter gene, was present throughout the length of transduced fibers for eight months\textsuperscript{196}. Early studies of intramuscularly delivered AAV-FIX, pioneered the way for AAV-mediated treatment of hemophilia B\textsuperscript{197}. Similarly, efficient and long-term secretion of α-1 antitrypsin into plasma was achieved\textsuperscript{198}. Toward the treatment of myopathies, localized gene transfer to animal models of Duchenne muscular dystrophy
(DMD)\textsuperscript{199}, Pompe disease\textsuperscript{200}, and limb-girdle muscular dystrophy (LGMD)\textsuperscript{201} were among the first. Once the potential of cross-packaging was realized and the tropisms of AAV1 and AAV6 began to be characterized, these quickly arose as the preeminent serotypes for muscle directed therapies\textsuperscript{202}. Longevity of vector genome presence and persistence of their expression following intramuscular administration is one of its most attractive features. This phenomenon has been observed in low and high order mammals from one and half years of \textit{lacZ} expression in mice\textsuperscript{203} to eight years and five years of \textit{FIX} expression in dogs and non-human primates, respectively\textsuperscript{204,205}. Ongoing and completed clinical trials have demonstrated the safety and efficacy of rAAV vectors in muscle\textsuperscript{206–210}. The gene-transfer drug approved globally, is alipogene tiparvovec (Glybera®), an rAAV1 capsid carrying a transgene for lipoprotein lipase that is administered via intramuscular injections\textsuperscript{211}. Its approval represents a groundbreaking decision toward the approval of subsequent therapies\textsuperscript{211}.

**Pompe disease gene therapy**

In 2002, Fraites et al, revealed the ability of a rAAV2 vector to provide active GAA enzyme to knockout mice and efficiently reduce lysosomal glycogen in skeletal muscle\textsuperscript{200}. In follow-up studies, delivery of \textit{hGAA} by rAAV1 and rAAV8 vectors was explored and contractile strength in the diaphragm and lower limb muscles was increased. Glycogen can be cleared and provide minor respiratory benefits when treatment is provided even as late as 21 months of age\textsuperscript{212}. This is an important finding for translation to patients, who are not diagnosed until cellular dysregulation has greatly amassed. Direct injections of rAAV1-\textit{hGAA} to the diaphragms of rabbits provided necessary toxicology evidence and feasibility for a novel route of administration. A Phase I/II clinical trial, which included both safety and efficacy measures, utilized this
vector and method to treat patients five to nine years old, whose enzyme replacement therapy was insufficient at preventing mechanical ventilation\textsuperscript{210}. Respiratory function improved and length of time off mechanical ventilation was elongated\textsuperscript{210}.

As the involvement of damaged motor neurons began to be fully comprehended, it became apparent that for systemic correction of Pompe disease pathologies, AAV serotype 9 would be a more suitable vector\textsuperscript{45,213,214}. Its ability to transduce the CNS has been explored as a potential vehicle in the treatment of spinal muscular atrophy\textsuperscript{215}, mucopolysaccharidosis VII\textsuperscript{216}, and Parkinson’s disease\textsuperscript{217}. AAV9 is also capable of retrograde travel from muscle through the NMJ and into motor neurons\textsuperscript{218–220}. Administration of rAAV9-\textit{hGAA} has proven to nearly eliminate lysosomal glycogen and provide corresponding clinical benefits to the phrenic nerve, responsible for innervating the diaphragm, skeletal muscle contractile force, left ventricular mass, and increased ejection fraction in the PD mouse model\textsuperscript{221,222}. Success with this vector has led to its use in a clinical trial enrolling early this year (ClinicalTrials Identifier: NCT02240407).

\textbf{Rationale}

The overall goal of this project is to evaluate a gene therapy-based approach to correct and prevent autophagic dysregulation in PD (Figure 3-1). Autophagic dysregulation, characterized by abnormal flux, increases the rate of disease progression by stimulating muscle dystrophy and debilitating muscle function. Enzyme replacement therapy, the currently the only FDA-approved therapy for the treatment of PD, is unable to clear enough glycogen from lysosomes for fusion of accumulated autophagosomes to fuse properly and clear the vacuolar pathology. Much research has aimed to increase autophagic flux to improve ERT delivery to lysosomes. However, few therapeutics tested have been successful at achieving this and there are potentially serious negative
consequences of those that have. As such, there still exists a need for an effective
treatment for patients with PD that can correct skeletal muscle pathology and yield
improved respiratory outcomes as well as ambulation, both of which will lead to an
enhanced quality-of-life. Several preclinical studies and clinical trials have
demonstrated the effects of rAAV-mediated gene transfer of a GAA gene, wherein
increased GAA enzyme activity led to lysosomal glycogen clearance, and correlated
with muscle function improvement. The significance of this study is that it employs
correction-of-autophagy as an endpoint measurement for musculoskeletal disorders.
The outcomes of this study will provide insight into the effectiveness of AAV-mediated
gene therapy for the treatment of diseases where autophagic dysregulation persists.
Figure 1-1. GAA is Produced in Healthy Mammals. GAA is produced in the rough endoplasmic reticulum then transported to the Golgi apparatus for mannosylation (a). It is trafficked in endosomes to either lysosomes (b) or secreted (c) from the cell. Secreted enzyme is taken up through receptor-mediated endocytosis via the cation-independent mannose-6-phosphate receptor (CI-MPR) and trafficked to the lysosome (d). Once in the lysosome, GAA degrades glycogen, which has been delivered by autophagosomes.
Figure 1-2. GAA is Not Produced in Pompe Patients. In Pompe patients with no GAA production the first consequence is glycogen accumulation in lysosomes.
Figure 1-3. The Autophagy Pathway Delivers Cellular Contents to the Lysosome for Degradation. Autophagy is stimulated chiefly by signals of nutrient deprivation. Following propagation of this signal through a cascade, a phagophore or initiating membrane is brought to the site of nucleation and is decorated with lipidated LC3 (LC3-II). The membrane closes around cytoplasm in selective and non-selective processes, and eventually the two growing ends fuse to form the full autophagosome (AP). APs, identified by their double membrane, may fuse with endosomes first, but the pathway always concludes with fusion with lysosomes to generate an autolysosome (AL). In ALs cellular contents are degraded by hydrolases and nutrients are released.
Figure 1-4. Enzyme Replacement Therapy (ERT) is Delivered to the Lysosome. Recombinant human GAA, can be taken up through CI-MPR receptor-mediated endocytosis, as is done with endogenously produced and secreted enzyme. It is then trafficked to the lysosome where it degrades glycogen.
ERT is Ineffective in Skeletal Muscle. In skeletal muscle, there is a lower abundance of CI-MPR, which takes up rhGAA, than is found in cardiac muscle. Additionally, as glycogen accumulates in lysosomes they become unable to fuse with endosomes, autophagosomes, or amphisomes thus these vesicles also begin to accumulate. All together there is a deficiency in trafficking rhGAA to lysosomes were it can be effective.
Figure 1-6. wt and Vector AAV Genomes Have ITRs in Common. (a) The wt AAV genome is flanked by 145bp inverted terminal repeats. Three promoters direct the expression of eight proteins. Rep78, Rep68, Rep52, and Rep40, replication proteins, which are expressed from the p5 and p19 promoter with a splice site at the 3’ end. VP1, VP2, and VP3, capsid proteins are expressed from the p40 promoter through the use of alternate start codons and a splice site. AAP is also expressed from p40. (b) The recombinant AAV genome is made up of four crucial elements: wt AAV ITRs, most commonly AAV2 sequence, a promoter, cDNA or transgene of interest, and polyadenylation signal.
Figure 1-7. rAAV mediated GAA Delivery is Hypothesized to be Therapeutic in Skeletal Muscle. rAAV vectors transduce cells by binding to proteoglycan receptors and activating receptor-mediated endocytosis (a). As pH decreases in late endosomes, the capsid’s lipase is exposed and the virus escapes the endosome. It then enters the nucleus through a nuclear pore, upon which the capsid uncoats from the genome (b). GAA is subsequently produced, as it normally would be from the delivered genome. It is mannosylated in the Golgi apparatus (c), and traffics to the lysosome where degrades glycogen (d), that has been delivered by autophagosomes. When GAA is able to reach lysosomes and degrade accumulated glycogen, lysosomes will be able to fuse to endosomes, amphisomes, and autophagosomes, resolving these vesicles and restoring normal functions to the muscle fiber. As in healthy cells, a portion of GAA is secreted (e) from the cell and can be taken up by CI-MPR (f).
Table 1-1. Common GAA Mutations

<table>
<thead>
<tr>
<th>Mutation (cDNA)</th>
<th>Disease Severity</th>
<th>Demographic</th>
</tr>
</thead>
<tbody>
<tr>
<td>c.-32-13T&gt;G</td>
<td>LOPD</td>
<td>Global&lt;sup&gt;14-16&lt;/sup&gt;</td>
</tr>
<tr>
<td>c.525ΔT</td>
<td>IPD</td>
<td>Northern Europeans&lt;sup&gt;19&lt;/sup&gt;</td>
</tr>
<tr>
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<td>IPD</td>
<td>Spanish&lt;sup&gt;20&lt;/sup&gt;</td>
</tr>
<tr>
<td>c.1935C&gt;A</td>
<td>IPD</td>
<td>Taiwanese with Chinese ancestors&lt;sup&gt;21&lt;/sup&gt;</td>
</tr>
<tr>
<td>c.2560C&gt;T</td>
<td>IPD</td>
<td>African Americans&lt;sup&gt;22&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ex18</td>
<td>IDP</td>
<td>Caucasians&lt;sup&gt;23&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
CHAPTER 2
METHODS

Vector Production

rAAV vectors were produced by the University of Pennsylvania Vector Core and purified by the University of Florida Powell Gene Therapy Center Vector Core (UF-PGTC-VC) by traditional double transfection methods described previously\textsuperscript{223}. HEK293 cells were maintained in DMEM supplemented with 5% FBS and 1% antibiotic at 37°C with 5% CO\textsubscript{2}. 1x10\textsuperscript{9} cells were seeded in a 10-layer CellSTACK (Corning, #3270) and allowed to grow to 75-80% confluency. A calcium phosphate precipitate was made by mixing, pXZY9\textsuperscript{223} (a kind gift from Dr. James Wilson, University of Pennsylvania, Philadelphia, PA) and rAAV plasmid, here pTR-DES-coGAA at a 1:1 molar ratio, calcium chloride, and 2x HBS, then incubated at room temperature for 1-2 minutes. Complete DMEM was added to stop the precipitate, and this solution was added to the cells, after they were washed with PBS. Following a 60-hour incubation the media was removed, and cells were washed with PBS then harvested with EDTA-PBS. Cells were pelleted by centrifugation at 1000 xg for ten minutes. The pellet was resuspended in lysis buffer (150nM NaCl, 50mM Tris pH 8.4). A series of three freeze/thaws and incubation with Benzonase (50U/mL) and magnesium chloride (1mM) for 30 minutes at 37°C lysed the cells. Cell debris was cleared by centrifugation at 3700rpm for 20 minutes at 4°C. The supernatant was kept and overlayed onto a step gradient of 15%, 25%, 40%, and 60% Optiprep Iodixanol (Sigma-Aldrich: D1556) in an ultracentrifuge tube (Beckman: #341625). The tubes were capped and centrifuged at 69000 rpm for one hour at 18°C in a 70Ti rotor. Virus was collected from the 40%-60% interface through half of the 40% fraction, 3-4mL, using an 18G needle and syringe. The purified
sample was loaded into a prepared Apollo 100K centrifugal concentrator with citrate buffer and spun at 3000rpm in a Beckman JS-4.2 rotor, for six minutes intervals, until the final volume was 500μL.

**Animals**

The University of Florida Institutional Animal Care and Use Committee (IACUC) approved all animal use under the protocol 201408522. *Wildtype (wt) 129SVE* and *Gaa*−/−/129SVE mice were obtained (Taconic, Hudson, NY, USA) and housed at the University of Florida Animal Care Services. The *Gaa*−/− mouse generated by Raben et al. was previously bred onto a 129SVE background67,220.

**Correction Aim**

Equal numbers of twelve-week old male and female mice were utilized in this study. Experimental mice were randomly assigned to one of five groups: untreated *Gaa*−/−, untreated *wt* 129SVE, and *Gaa*−/− treated with 1×10^{11} or 1×10^{13} or 1×10^{14} vector genomes (vg) per kilogram (kg) rAAV9-DES-coGAA. Mice were placed under a heat lamp to allow the lateral tail veins to become pronounced. Then each was moved into a moderately confined restraining device. The tail was palpated and wiped with 70% ethanol. rAAV vector diluted to 100 microliters (μL) was injected into the tail vein using a 28-gauge insulin syringe. At 16 and 36 weeks of age (4 and 24 weeks after injection, respectively), animals were anesthetized using 2-4% isofluorane inhaled through a nose cone and euthanasia was completed by thoracotomy, after which the diaphragm, tibialis anterior (TA), gastrocnemius, and soleus, from both legs were collected for molecular, biochemical, and histological analysis. Muscles were split and preserved in a number of methods as described below.
Prevention Aim

Another set of animals consisting of three groups: untreated Gaa\(^{+/+}\), untreated \(wt\), and \(Gaa^{+/+}\) treated with \(1 \times 10^{14}\) vg/kg dosed within the first forty-eight hours of life, as previously described\(^{225}\), were evaluated. Briefly, newborn pups were placed on ice for thirty to sixty seconds to anesthetize them. Then using a 30 Gauge needle and syringe, vector was delivered to the temporal vein. Following injection, they were returned to the dam back in a cage, placed on a heating pad warmed to 37°C. Twenty-four weeks after injection animals were sacrificed and muscled were collected as above.

GAA Enzyme Activity Assay

Fifty milligrams of each muscle, flash frozen in liquid nitrogen, was homogenized in deionized water with protease inhibitor cocktail (Roche Life Science: 04-693-124-001) using a FastPrep24 (MP Biomedicals) followed by a series of three freeze-thaw cycles. Lysates were clarified by 4°C centrifugation at 16,000 \(xg\) for ten minutes. GAA enzyme activity was evaluated with modifications to methods previously described\(^{226}\). Samples were incubated for one hour at 37°C with 3mM 4-methylumbelliferyl-\(\alpha\)-D-glucoside (Sigma-Aldrich: M9766) in 200mM sodium acetate buffer pH 4.3. The reaction was stopped with the addition of 500mM sodium carbonate pH 10.7. A standard curve was generated using 4-methylumbelliferone (Sigma M1381) for comparison. Fluorescence produced by substrate cleavage was detected using a Biotek Synergy HTX Multimode Reader. These measurements were normalized to protein levels determined by DC Protein Assay (Bio-Rad: 500-0111) performed according to manufacturer’s instructions.
Histology

Formalin Fixation

Upon collection, muscles were cut such that a 5mm cross-section from the middle of the muscle was obtained. These were fixed in formalin for 24 hours at 4°C, then exchanged into PBS. They were processed on an automated Sakura Tissue Tek VIP tissue processor. Following dehydration through a gradient of alcohols and xylene they were infiltrated with wax. The wax-filled tissues were oriented in embedding molds, then wax was added to surround the tissues and the blocks were allowed to harden.

Muscles were serially sectioned in two sets using a microtome (Microm HM 330) to six microns, mounted to Superfrost Plus Microscope Slides, and air-dried overnight at room-temp. The first set was stained with Periodic Acid Schiff (Sigma-Aldrich: 395B-1KT) according to manufacturer’s instructions. The second set was stained with a rabbit polyclonal anti-hGAA antibody. The muscles were blocked for biotin, avidin, and peroxidase prior to incubation with the primary antibody. DAB was used to detect the peroxidase-conjugated secondary antibody.

Fresh-Frozen

Whole muscles were flash-frozen as previously described\textsuperscript{227}. They were mounted on cork with a small dot of Tissue-Tek® O.C.T. Compound (VWR: 25608-930) at the distal end of the muscle. Then dipped for fifteen seconds in isopentane chamber submerged in liquid nitrogen so that the isopentane is cooled to -150°C. The cork was immediately placed in a Styrofoam box with dry ice, wrapped in aluminum foil, and stored at -80°C. These muscles were transversely sectioned to six microns on a
cryostat (Microm HM 505) set to -21°C, mounted to Superfrost Plus Microscope Slides (Fisher Scientific: 12-550-15), and air-dried overnight at room-temp.

**Hematoxalin and Eosin**

One set of slides containing fresh-frozen sections were fixed in cold 4% paraformaldehyde for five minutes and stained with Hematoxalin and Eosin (H&E) according to manufacturer's instructions (Leica). Coverslips were mounted with cytoseal.

**Immunofluorescence**

A second set of slides was probed with anti-Lamp1 (Developmental Studies Hybridoma Bank: 1D4B) and anti-LC3 (Novus Biologicals: NB100-2331) antibodies detected by immunofluorescence. Slides were first permeabilized with acetone, and then blocked with 2% normal horse serum in PBS for one hour at room temperature. Next, the primary antibodies diluted to 1:100 and 1:300, respectively, in Antibody Diluent (Invitrogen: 00-3118) were applied overnight at 4°C. Secondary antibodies, donkey anti-Rat IgG AlexaFluor488 (Molecular Probes, Invitrogen: A21208) and donkey anti-Rabbit IgG AlexaFluor594 (Molecular Probes, Invitrogen: A21207), were diluted 1:500 in 1.5% horse serum in PBS and incubated for one hour at room temperature. Finally, slides were fixed in normal buffered formalin and coverslips were mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories: H-1200).

**Imaging and Analysis**

Images were taken using an upright light microscope (Olympus BX43), fluorescent light source (Olympus U-HGLGPS) and cellSens software (Olympus Standard).
H&E stained images were analyzed for degree of vacuolization. Each muscle fiber was characterized as (a) having no vacuoles, (b) having only small vacuoles, and (c) having at least one large vacuole. Based on the total percentage of (a) and (b) characterized fibers an overall vacuolization score was assigned on a scale of one to nine for each sample.

Immunofluorescence images were analyzed for the number of fibers containing (a) no LC3-positive puncta (b) only small LC3-positive puncta, or (c) at least one large LC3-positive vacuole. A similar method was used to evaluate LAMP1 stained images where level of staining was determined.

Zizhao Zhang, a graduate student in the laboratory of Lin Yang, PhD, determined the cross-sectional area (CSA) of fibers from H&E stained images as previously described\textsuperscript{228}. Briefly, each muscle fiber was segmented to determine the pixel membership (cell or non-cell) within a particular image. The method adopted deep convolutional neural networks (CNN), a powerful deep learning technology, to achieve highly accurate and fast muscle image segmentation. Next, morphological operations of image processing techniques quantified the pixels within each fiber to calculate CSA.

**Western Blot Analysis**

The represents the water-insoluble fraction of muscle homogenate was homogenized in a 0.5% SDS in phosphate buffered saline (PBS) solution containing protease inhibitor cocktail using the same FastPrep, freeze and thaw method as for the water soluble fraction. Fifty micrograms of total protein, determined by Bio-Rad DC Protein Assay was separated by SDS-PAGE on 4-15% gradient Criterion\textsuperscript{TM} TGX\textsuperscript{TM} Precast Midi Protein Gels (BioRad: 597-1084) for an average of seventy-five minutes at ninety Volts. The proteins were then transferred to PVDF membrane (EMD Millipore:}
IPFL00010) for ninety minutes at 250mAmps. These blots were probed with the following antibodies, anti-LAMP1 (1:250) (Developmental Studies Hybridoma Bank: 1D4B), anti-p62 (1:1000) (Progen: GP62-C), anti-Beclin1 (1:1000) (Cell Signaling Technology: #3738), anti-GAPDH (1:2500) (Cell Signaling Technology: #2118), and LC3A (1:1000) (Cell Signaling Technology: #4599). Membranes probed with Cell Signaling Technology (CST) antibodies were blocked in accordance to manufacturing suggestions for fluorescent western blot detection with modifications. Briefly, they were blocked in 5% non-fat dry milk in Tris buffered saline (TBS). Primary antibodies were diluted in 5% bovine serum albumin (BSA) in TBS with 0.1% Tween-20 (TBS-T) (Gentrox: 40-059), applied for one hour at room temperature or overnight at 4°C. LI-COR secondary antibodies were diluted 1:10,000 in 5% non-fat milk in TBS-T, and applied for one hour at room temperature with three, five-minute washes of TBS-T between each antibody solution. For membranes probed with all other antibodies, LI-COR Odyssey Blocking Buffer in PBS was used as the blocking buffer and antibody diluent for all steps. These blots were washed only after primary and secondary antibody incubation with PBS supplemented with 0.1% Tween-20 (Gentrox: 40-028). All blots were imaged using the LI-COR Odyssey infrared detection system. Band densitometry, measured as Integrated Intensity, was determined with coordinating software.

**Statistical Analysis**

All numerical data was evaluated using GraphPad Prism software. Statistical analysis was performed on all quantitative data collected, using an Ordinary One-Way ANOVA and Dunnett’s Multiple Comparison’s test to determine the differences between treated and untreated groups.
CHAPTER 3
RESULTS

Expression of Vector

Cellular pathology including glycogen accumulation and dysregulation of the autophagy pathway has been noted in Gaa-deficient mice as early as one month of age, and by five months, the majority of muscle fibers contain an accumulation of autophagosomes\textsuperscript{59}. Mice used in this study were treated at three months of age, because cellular pathology was at a stage representative of that in human children at six months of age, even if enzyme replacement therapy has been initiated\textsuperscript{103}. Based on effective doses from previous studies\textsuperscript{212,229}, two dose levels of rAAV9-DES-coGAA were chosen, 1x10\textsuperscript{11} (low) and 1x10\textsuperscript{13} (mid), delivered systemically through the tail vein to Gaa\textsuperscript{-/-} mice. While expression of a transgene administered via rAAV vectors can occur relatively immediately, it takes approximately three weeks for protein expression to reach a maximum\textsuperscript{230}. Here, biochemical and histological changes resulting from the introduction of endogenously produced GAA were analyzed four weeks after injection to evaluate the relatively short-term effect (Figure 3-1). Treated mice were compared to untreated Gaa\textsuperscript{-/-} mice and untreated wt mice.

Four skeletal muscles, soleus, diaphragm, gastrocnemius (gastroc), and tibialis anterior (TA), were chosen for analysis in this study because of their dissimilar proportions of the three most common muscle fiber types, I, IIa, and IIb\textsuperscript{231,232}. The soleus is composed of nearly 60\% type I, slow oxidative, fibers, \textasciitilde 40\% type IIa, fast oxidative glycolytic fibers, and 0\% IIb, fast glycolytic, fibers\textsuperscript{231}. In contrast to the gastrocnemius has a nearly opposite composition\textsuperscript{231}. The diaphragm and tibialis anterior have higher proportions of type IIa fibers. Diaphragm muscle is made up of
nearly exclusively IIa fibers\textsuperscript{232}, while TAs have 65% IIa fibers and 35% IIb fibers\textsuperscript{231}.

These muscle fiber types are distinguished by myosin heavy chain expression, material stored for energy production, and method of ATP production, which correlates to the abundance of mitochondria\textsuperscript{233,234}.

Expression of the transgene, \textit{coGAA}, was first determined by a GAA enzyme activity assay (Figure 3-2). In all muscles analyzed, activity from the animals dosed with the low- and mid-doses was not significantly above knockout levels. Upon this finding, a group of mice treated with a higher dose of 1x10^{14} vg/kg (high-dose) was added. In soleus and diaphragm, supra-physiological levels of activity were achieved with the high-dose, 267% and 185% of \textit{wt}, respectively. In gastroc and tibialis anterior, near \textit{wt} levels of activity were reached with the high-dose, 67% and 66% of \textit{wt}, respectively. From these data, it was determined that only the animals receiving the high-dose would experience any therapeutic benefit and thus only these muscles were further analyzed. Accordingly, hereinafter all references to “treated mice” refer to those that received the high-dose.

Formalin-fixed muscle was embedded in paraffin, sectioned, and stained with a polyclonal anti-GAA antibody specific for the human GAA, as observed by the lack of murine GAA staining in the \textit{wt} samples (Figure 3-3). Soleus and diaphragm sections showed much more variegated distribution of GAA expression, where some fibers had strong expression and others had none. Gastroc had a relatively homogenous staining pattern with a few cells having a greater concentration of GAA. Comparable patterns were seen in the TA, with few fibers lacking any staining, but also few fibers with a dense concentration.
Similarly prepared muscle sections were stained with Periodic Acid-Schiff (PAS), which detected polysaccharides, such as glycogen (Figure 3-4). In both soleus and diaphragm of Gaa<sup>-/-</sup> mice, lysosomal glycogen was observed in discrete puncta. In treated mice, clearance of lysosomal glycogen was prevalent, where some fibers were completely devoid of any puncta. PAS staining of gastroc and TA from Gaa<sup>-/-</sup> mice was more diffuse, while treated mice had a generalized decrease in PAS intensity, with a somewhat inverse pattern to that observed in GAA stained sections. Visually, gastroc muscle had the greatest decline in overall glycogen.

**Correction of Vacuolization**

Vacuolization, as observed by H&E staining of fresh-frozen muscle cross-sections, was an indicator of the presence of vesicles such as lysosomes, endosomes, amphisomes, and autophagosomes. Increased vacuolization, corresponding to fiber damage, has been correlated with diminished clinical outcomes. Images were collected from a single, stained section of each muscle (Figures 3-5a, 3-6a, 3-7a, 3-8a). Fibers from the muscles studied here generally had three phenotypes - having no vacuoles, only small vacuoles, or at least one large vacuole with several small vacuoles. Percentages of total fibers for each phenotype within a section were used to assign a vacuolization score from one to nine (i.e. 1 = little to no degree of pathology, 9 = high degree of pathology) in each section (Figures 3-5b, 3-6b, 3-7b, 3-8b). Fibers with large, centrally located vacuoles represent an aggregation of vesicles and sections with a high proportion of these fibers were considered to have an advanced stage of cellular disorganization. In all muscles studied, a decrease in vacuolization score was observed. Soleus and diaphragm from untreated Gaa<sup>-/-</sup> mice had few large vacuoles, leading to a smaller change with treatment, with a decrease in average score of 0.9.
units and 0.75 degree units, respectively. In gastroc and TA, average scores decreased by 2.7 and 2.2 degree units respectively, both statistically significant compared to untreated Gaa<sup>-/-</sup> mice. While muscles were not fully cleared of vacuoles, each one demonstrated at least partial resolution.

**Correction Autophagic Flux**

Studies that elucidated the autophagic accumulation phenotype in Gaa<sup>-/-</sup> mouse muscle, described the alignment of autophagosomes along the median of fibers<sup>99</sup>. Based on prior observations it was surmised that the large vacuoles seen in fibers of H&E stained sections were likely aggregated autophagosomes and the small vacuoles were likely lysosomes<sup>105</sup>. To elucidate this, antibodies for LAMP1 (Lysosome Associated Membrane Protein 1) and LC3, an autophagosomal membrane protein, were used to identify these structures detected by immunofluorescence (IF) on fresh-frozen, transverse sections of muscles (Figures 3-9a, 3-10a, 3-11a, 3-12a). For LAMP1 quantification, fibers were triaged as having nearly no LAMP1 (“no puncta”), some generally small LAMP1 puncta (“some puncta”), or fibers that were mostly filled with LAMP1 puncta (“puncta filled”). A similar characterization method was applied to LC3 fibers, where degrees of pathology were distinguished as no LC3 puncta, several small puncta, or having one large punctum. While 64% and 44% of soleus and diaphragm fibers of Gaa<sup>-/-</sup> mice were classified as LAMP1 puncta filled, less than 25% were noted to have a large LC3 punctum in soleus, while there were none in diaphragm. In all Gaa<sup>-/-</sup> sections of gastroc, gross numbers of LAMP1 puncta and at least one large LC3 punctum was observed within 44% and 62% of muscle fibers, respectively, while wt mice had low levels of LAMP1 puncta fibers and undetectable puncta of LC3 (Figures 3-11b,c). 76% and 50% of fibers within Gaa<sup>-/-</sup> TA were classified as LAMP1 puncta-filled
and large LC3 punctum positive (Figures 3-12b,c). The findings of this study were similar to those in previous reports that identified large autophagosome aggregates within fibers and larger-than-normal lysosomes filling the surrounding space\textsuperscript{59,99}.

In each muscle type a global reduction in total LAMP1 puncta and thusly lysosomes resulted following administration of rAAV9-\textit{DES-coGAA} vectors. The percentage of fibers classified as LAMP1 puncta filled was minimized by 29%, 20%, 7%, 42%, in soleus (Figure 3-9b), diaphragm (Figure 3-10b) gastrocnemius (Figure 3-11b), and tibialis anterior (Figure 3-12b), respectively. This was in conjunction with an increase in “no LAMP1 puncta” fibers by 21%, 28%, 8% and 19%, respectively.

In soleus, there was a reduction of fibers with large puncta and small puncta fibers by 2% and 17% each, which resulted in a 19% increase of fibers with no LC3 puncta. (Figure 3-9c). Discrete LC3 puncta were not observed in diaphragm sections from mice in any of the three groups, and for this reason, fibers were not triaged. A diffuse staining pattern was noted and from the images, there does appear to be more fibers with less staining in the \textit{wt} and treated mice compared to \textit{Gaa}\textsuperscript{-} mice (Figure 3-10c). In gastroc, fibers containing a large LC3 punctum were reduced by 20% and those having none were increased by 1% (Figure 3-11c). Statistically significant changes were observed in TA sections, in which treated mice had a 25% decrease in fibers with large LC3 puncta and a 15% decrease in fibers with no puncta (Figure 3-12c).

To more specifically quantify these proteins and delineate the species of LC3, LC3-I (non-lipidated, cytosolic form) and LC3-II (lipidated, autophagosomal form), muscle lysates were analyzed by western blot. Additionally, two other autophagy-
associated proteins, Beclin1 and p62, were quantified by this method. Beclin1 is involved in the PI3K-III complex, responsible for autophagosome initiation. p62 links ubiquitinated proteins and organelles, signaled for degradation, to the LC3-II that is within the membranes of autophagosomes\(^{235}\) (Figure 1-3). The water-insoluble fraction of each muscle was homogenized in an SDS-containing buffer to release the membrane-bound proteins. These were separated on a gradient gel, transferred to PVDF membrane, and probed. Blots were scanned and intensity of the bands was determined. \(\text{wt}\) levels of LAMP1 and LC3-II were lower than in untreated \(\text{Gaa}^{-/-}\) mice in all muscles (Figures 3-13, 3-14, 3-15, 3-16). Surprisingly, in soleus, levels of LAMP1, LC3-I, LC3-II, Beclin1, and p62 all increased in treated mice compared to untreated knockouts, some reaching statistical significance (Figures 3-13). In diaphragm, LAMP1, LC3-II, and p62 all significantly decreased in treated mice compared to untreated \(\text{Gaa}^{-/-}\) mice in accordance with the hypothesis (Figures 3-14). A mitigation of LAMP1, LC3-II, LC3-I, Beclin1, and p62 was observed in gastroc of treated mice with statistically significant changes in both forms of LC3 (Figures 3-15). No change in LAMP1 occurred in TA, but a decrease in LC3-II, LC3-I, and Beclin1 did (Figures 3-16). While levels by western blot of key proteins in soleus of treated mice are not as expected, levels in diaphragm, gastroc, and TA, indicate there has been resolution of lysosomes and autophagosomes.

**Growth of Healthy Muscle Fibers**

Following correction of molecular cell pathology specifically accumulated glycogen-filled lysosomes and autophagosomes, muscle fiber growth was evaluated. This was done by measuring fiber cross-sectional area (CSA)\(^{236}\). Weakened fibers of \(\text{Gaa}^{-/-}\) mice are, on average, smaller in diameter, compared to \(\text{wt}\) muscle fibers\(^{104}\). A
small, 2.5%, decrease in average CSA was measured in soleus of treated mice (Figure 3-5c) as opposed to the increase in average CSA by 27%, 4%, and 25% in diaphragm (Figure 3-6c), gastroc (Figure 3-7c), and TA (Figure 3-8c), respectively. The average CSA in TA reached near normal sizes at 93% of wt. These results demonstrated that when dysregulated vesicular pathways are corrected, the muscle fibers become healthier and are able to grow.

**Long-Term Correction**

After establishing that endogenously expressed active GAA from rAAV9-*DES-coGAA* vectors correlated to a reduction in glycogen, lysosomes, and autophagosomes, and resulted in trends toward muscle growth preliminary analysis was performed in mice treated with the same dose for six months. Activity in TA of treated mice was 51% of wt (Figure 3-17). Vacuolization and CSA analysis could not accurately be determined as many of the borders fibers in untreated *Gaa^-/-* mice were broken down and ill defined. Visually, ~25% of fibers in the treated group have few vacuoles and a wt-like appearance (Figure 3-18). While there were more fibers with fewer vacuoles in the treated mice, levels of membrane proteins, LAMP1 and LC3-II, measured by western blot only mildly decreased and associated proteins, LC3-I, Beclin1, and p62 were elevated (Figure 3-19). The fibers lacking vacuoles appear larger in size indicating their growth (Figure 3-18).

**Long-Term Prevention**

Examining correction of the cellular phenotype is relevant in the current treatment of PD patients due to delay in onset of symptoms and time required for diagnosis confirmation. However, studying prevention of pathology is also vital for the widespread acceptance of newborn screening practices. Preliminary evidence provided here in TA
of mice treated at the highest dose of vector shortly after birth and analyzed at six months of age, was promising. GAA activity was 51% of wt (Figure 3-20), and led to a complete improvement in average vacuolization score $Gaa^{-/-}$ mice had a score of 7.66 degree units, and the score for treated mice was 2.5 degree units, close to the 1.0 degree units score of wt mice (Figure 3-21). All proteins quantified by western blot from treated mice were less than in untreated mice, and all but Beclin1 were statistically significant (Figure 3-22).
Figure 3-1. Study Design. Mice were treated at two time points to elucidate the effects of correcting pathology and the effects of preventing pathology. Animals in the correction aim of the study were treated at 3 months of age, and analyzed one and six months after administration. Animals in the prevention aim of the study were treated within the first forty-eight hours of life and analyzed six months later.
Figure 3-2. GAA Enzyme Activity is Restored with a High Dose of rAAV9-DES-coGAA One Month After Corrective Treatment. GAA enzyme activity levels are representative of the level of expression produced after effective transduction by rAAV9-DES-coGAA administered at three months of age. Activity was measured in four muscles, soleus, diaphragm, gastrocnemius, and tibialis anterior, of five groups of mice: untreated Gaa<sup>−/−</sup>, untreated wt, Gaa<sup>−/−</sup> treated with low-dose (1x10<sup>11</sup> vg/kg), Gaa<sup>−/−</sup> treated with mid-dose (1x10<sup>13</sup> vg/kg), and Gaa<sup>−/−</sup> treated with high-dose (1x10<sup>14</sup> vg/kg) of vector. Data are represented as mean ± SEM. Levels of activity above background are not achieved with low- and mid-doses, but near- and supra-physiological levels are reached with the high-dose. n = 3-6 mice for each muscle per group. Significance is relative to Gaa<sup>−/−</sup>, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Figure 3-3. GAA Staining is Present in Skeletal Muscle of Treated Mice.
Representative images of formalin-fixed muscles from untreated Gaa<sup>−/−</sup>, untreated wt, and treated Gaa<sup>−/−</sup> mice, and hGAA was detected in sections using immunohistochemistry. Hematoxalin was used as a counter stain to define nuclei. Positive staining is present in fibers of treated muscles only. Scale bar = 40μm
Figure 3-4. PAS staining of Skeletal Muscle from Treated Mice Depicts Reduced Glycogen. Representative images of formalin-fixed muscles from untreated Gaa−/−, untreated wt, and treated Gaa−/− mice, sectioned and stained with PAS to detect polysaccharides, specifically glycogen. A reduction of lysosomal and total glycogen is observed in all treated muscles. Scale bar = 40μm.
Figure 3-5. Treated mice had Reduced Vacuolization in Soleus. (a) Representative images of H&E stained, transverse sections of soleus muscle from fresh frozen untreated Gaa−/−, untreated wt, and treated Gaa−/− mice. Scale bar = 40μm. (b) Fibers were characterized based on degree of pathology and each sample was assigned a vacuolization score, where 1 = no pathology and 9 = highest degree of pathology. Treated mice had an average score that was 0.9 units better than untreated Gaa−/− mice. (c) Cross-sectional area of each fiber was measured as a determinant of muscle growth. Treated mice had a 2% smaller average CSA compared to untreated Gaa−/− mice. Data are represented as mean ± SEM. n = 3-5 mice per group with a minimum of 65 fibers per mouse counted.
Figure 3-6. Treated mice had Reduced Vacuolization and Increased Cross-Sectional Area in Diaphragm. (a) Representative images of H&E stained, transverse sections of soleus muscle from fresh frozen untreated Gaa<sup>−/−</sup>, untreated wt, and treated Gaa<sup>−/−</sup> mice. Scale bar = 40μm. (b) Fibers were characterized based on degree of pathology and each sample was assigned a vacuolization score, where 1 = no pathology and 9 = highest degree of pathology. Treated mice had an average score that was 0.75 units better than untreated Gaa<sup>−/−</sup> mice. (c) Cross-sectional area of each fiber was measured as a determinant of muscle growth. Average CSA increased 27% compared to untreated Gaa<sup>−/−</sup> mice. Data are represented as mean ± SEM. n = 2-4 mice per group with a minimum of 100 fibers per mouse counted.
Figure 3-7. Treated mice had Reduced Vacuolization and Improved Average Cross-Sectional Area in Gastrocnemius. (a) Representative images of H&E stained, transverse sections of soleus muscle from fresh frozen untreated Gaa<sup>−/−</sup>, untreated wt, and treated Gaa<sup>−/−</sup> mice. Scale bar = 40μm. (b) Fibers were characterized based on degree of pathology and each sample was assigned a vacuolization score, where 1 = no pathology and 9 = highest degree of pathology. Treated mice had an average score that was 2.7 units better than untreated Gaa<sup>−/−</sup> mice. (c) Cross-sectional area of each fiber was measured as a determinant of muscle growth. Average CSA improved by 4% compared to Gaa<sup>−/−</sup>. Data are represented as mean ± SEM. n = 3-5 mice per group with a minimum of 100 fibers per mouse counted. Significance is relative to Gaa<sup>−/−</sup>, * p ≤ 0.05, **** p ≤ 0.0001.
Figure 3-8  Treated mice had Reduced Vacuolization and Improved Average Cross-Sectional Area in Tibialis Anterior. (a) Representative images of H&E stained, transverse sections of soleus muscle from fresh frozen untreated Gaa\textsuperscript{−/−}, untreated wt, and treated Gaa\textsuperscript{−/−} mice. Scale bar = 40μm. (b) Fibers were characterized based on degree of pathology and each sample was assigned a vacuolization score, where 1 = no pathology and 9 = highest degree of pathology. Treated mice had an average score that was 2.2 units better than untreated Gaa\textsuperscript{−/−} mice. (c) Cross-sectional area of each fiber was measured as a determinant of muscle growth. Average CSA improved by 25% compared to Gaa\textsuperscript{−/−}. Data are represented as mean ± SEM. n = 3-6 mice per group with a minimum of 100 fibers per mouse counted. Significance is relative to Gaa\textsuperscript{−/−}, * p ≤ 0.05, *** p ≤ 0.001, **** p ≤ 0.0001.
Figure 3-9. Treated Mice Had Fewer Fibers with Lysosomes and Autophagosome Aggregates in Soleus. (a) Representative images of IF stained, transverse sections. The top panels detect LAMP1, the middle panels detect LC3, and the bottom panels are a merged image with LAMP1 in red, LC3 in green, and DAPI, a DNA stain, in blue. Scale bar = 40μm. (b) Quantification of fibers with each LAMP1 phenotype. Treated mice had 29% fewer puncta-filled fibers and 21% more fibers with no puncta. (c) Quantification of fibers with each LC3 phenotype. Treated mice had 2% less fibers with large puncta, 17% fewer fibers with small puncta, and 21% more with no puncta compared to untreated Gaa−/− mice. Data are represented as mean ± SEM. n = 3-4 mice per group with a minimum of 50 fibers per mouse counted. Significance is relative to Gaa−/− mice. *No Puncta: *p < 0.05, **p < 0.01, ^Some/Small Puncta: ^p < 0.05, +Filled/Large Puncta: ++p < 0.01
Figure 3-10. Treated Mice Had Fewer Fibers with Lysosomes and Autophagosome Aggregates in Diaphragm. (a) Representative images of IF stained, transverse sections. The top panels detect LAMP1, the middle panels detect LC3, and the bottom panels are a merged image with LAMP1 in red, LC3 in green, and DAPI, a DNA stain, in blue. Scale bar = 40μm. (b) Quantification of fibers with each LAMP1 phenotype. Treated mice had 20% fewer puncta-filled fibers and 28% more fibers with no puncta. (c) Discrete LC3 Puncta were not observed, and fibers were thus not triaged. Data are represented as mean ± SEM. n = 2-4 mice per group with a minimum of 60 fibers per mouse counted. Significance is relative to Gaa-/mice. *No Puncta: *p < 0.05, **p < 0.01, +Filled/Large Puncta: ++p < 0.01
Figure 3-11. Treated Mice Had Fewer Fibers with Lysosomes and Autophagosome Aggregates in Gastrocnemius. (a) Representative images of IF stained, transverse sections. The top panels detect LAMP1, the middle panels detect LC3, and the bottom panels are a merged image with LAMP1 in red, LC3 in green, and DAPI, a DNA stain, in blue. Scale bar = 40μm. (b) Quantification of fibers with each LAMP1 phenotype. Treated mice had 7% fewer puncta-filled fibers and 8% more fibers with no puncta. (c) Quantification of fibers with each LC3 phenotype. Treated mice had 20% fewer fibers with large LC3 Puncta and 1% more fibers with no puncta. Data are represented as mean ± SEM. n = 3-4 mice per group with a minimum of 55 fibers per mouse counted. Significance is relative to Gaa<sup>−/−</sup> mice. *No Puncta: **p < 0.01, ****p < 0.0001, +Filled/Large Puncta: ++p < 0.01
Figure 3-12. Treated Mice Had Fewer Fibers with Lysosomes and Autophagosome Aggregates in Tibialis Anterior. (a) Representative images of IF stained, transverse sections. The top panels detect LAMP1, the middle panels detect LC3, and the bottom panels are a merged image with LAMP1 in red, LC3 in green, and DAPI, a DNA stain, in blue. (b) Quantification of fibers with each LAMP1 phenotype. Treated mice had 42% fewer puncta-filled fibers and 19% more fibers with no puncta. (c) Quantification of fibers with each LC3 phenotype. Treated mice had a 25% fewer fibers with large LC3 Puncta, 10% fewer fibers with small puncta, and 13% more fibers with no puncta. Data are represented as mean ± SEM. n = 3-4 mice per group with a minimum of 90 fibers per mouse counted. Significance is relative to Gaa/− mice. *No Puncta: **p < 0.01, ****p < 0.0001, ^Some/Small Puncta: ^p < 0.05, ^^^^^p < 0.0001, ++Filled/Large Puncta: ++p < 0.01, ++++p < 0.0001
Figure 3-13. Treated Mice Had an Increase in Lysosome and Autophagosome Proteins in Soleus. Data are mean ± SEM of integrated intensity (artificial units) of bands observed on western blot detecting lysosome (LAMP1) and autophagosome (LC3-II) specific proteins and autophagy associated proteins (LC3-I, Beclin1, and p62) in soleus of untreated Gaa<sup>−/−</sup>, untreated wt, and treated Gaa<sup>−/−</sup> mice. Levels for each protein analyzed increased in treated mice compared to untreated Gaa<sup>−/−</sup> mice. LC3-II / LC3-I ratio, is decreased indicative of flux restoration through the system in treated mice. n = 5-6 mice per group. Significance is relative to Gaa<sup>−/−</sup>, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Figure 3-14. Treated Mice Had a Decrease in Lysosome and Autophagosome Proteins in Diaphragm. Data are mean ± SEM of integrated intensity (artificial units) of bands observed on western blot detecting lysosome (LAMP1) and autophagosome (LC3-II) specific proteins and autophagy associated proteins (LC3-I, Beclin1, and p62) in untreated Gaa<sup>−/−</sup>, untreated wt, and treated Gaa<sup>−/−</sup> mice. Levels for each protein analyzed decreased in treated mice compared to untreated Gaa<sup>−/−</sup> mice. LC3-II / LC3-I ratio, is decreased indicative of flux restoration through the system in treated mice. n = 4-6 mice per group. Significance is relative to Gaa<sup>−/−</sup>, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Figure 3-15. Treated Mice Had a Decrease in Lysosome and Autophagosome Proteins in Gastrocnemius. Data are mean ± SEM of integrated intensity (artificial units) of bands observed on western blot detecting lysosome (LAMP1) and autophagosome (LC3-II) specific proteins and autophagy associated proteins (LC3-I, Beclin1, and p62) in untreated Gaa⁻/⁻, untreated wt, and treated Gaa⁻/⁻ mice. Levels for each protein analyzed decreased in treated mice compared to untreated Gaa⁻/⁻ mice. n = 3-6 mice per group. Significance is relative to Gaa⁻/⁻; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Figure 3-16. Treated Mice Had a Decrease in Autophagosome Proteins in Tibialis Anterior. Data are mean ± SEM of integrated intensity (artificial units) of bands observed on western blot detecting lysosome (LAMP1) and autophagosome (LC3-II) specific proteins and autophagy associated proteins (LC3-I and Beclin1) in untreated Gaa<sup>−/−</sup>, untreated wt, and treated Gaa<sup>−/−</sup> mice. Levels for LC3-II, LC3-I, and Beclin1 were decreased in treated mice compared to untreated Gaa<sup>−/−</sup> mice. LC3-II / LC3-I ratio, is decreased indicative of flux restoration through the system in treated mice. n = 3-6 mice per group. Significance is relative to Gaa<sup>−/−</sup>, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Figure 3-17. GAA Enzyme Activity is Restored Long Term with a Corrective High Dose of rAAV9-DES-coGAA. GAA enzyme activity levels are representative of the level of expression produced six months after effective transduction by rAAV9-DES-coGAA administered at three months of age. Activity measured in tibialis anterior, of three groups of mice: untreated Gaa<sup>−/−</sup>, untreated wt, and treated Gaa<sup>−/−</sup>. Data are represented as mean ± SEM. Treated mice had an average activity level 51% of wt. n = 4-6 mice per group. Significance is relative to Gaa<sup>−/−</sup>, **p ≤ 0.01.
Corrective Treatment Improved Vacuolization Long Term in Tibialis Anterior. Representative images of H&E stained transverse sections of TA muscle from fresh frozen untreated Gaa<sup>−/−</sup>, untreated wt, and treated Gaa<sup>−/−</sup> mice. Scale bar = 40μm.
Figure 3-19. Reduced Lysosome and Autophagosome Build-Up Does Not Continue to Be Prevented in Tibialis Anterior in Long Term Following Corrective Treatment. Data are mean ± SEM of integrated intensity (artificial units) of bands observed on western blot detecting lysosome (LAMP1) and autophagosome (LC3-II) specific proteins and autophagy associated proteins (LC3-I, Beclin1, and p62) in untreated Gaa<sup>−/−</sup>, untreated wt, and treated Gaa<sup>−/−</sup> mice. Levels of proteins had variable responses, LAMP1 and LC3-I were slightly lower in treated animals, while LC3-II, Beclin1, and p62 were increased. LC3-II / LC3-I ratio, is increased indicative of flux through the system in treated mice has stalled. n = 5-6 mice per group. Significance is relative to Gaa<sup>−/−</sup>, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Figure 3-20. GAA Enzyme Activity is Restored with a Preventative, High Dose of rAAV9-DES-coGAA. GAA enzyme activity levels are representative of the level of expression produced six months after effective transduction by rAAV9-DES-coGAA administration at birth. Activity measured in tibialis anterior, of three groups of mice, untreated Gaa−/−, untreated wt, and treated Gaa−/−. Data are represented as mean ± SEM. Treated mice had an average activity level 51% of wt. n = 6-10 mice for each muscle per group. Significance is relative to Gaa−/−, ****p ≤ 0.0001.
Figure 3-21. Preventative Treatment Reduced Vacuolization and Improve Average Cross-Sectional Area Long Term in Tibialis Anterior. (a) Representative images of H&E stained, transverse sections of TA muscle from fresh frozen untreated Gaa^+/−, untreated wt, and treated Gaa^+/− mice. Scale bar = 40μm. (b) Fibers were characterized based on degree of pathology and each sample was assigned a vacuolization score. Treated mice had a 5.2 unit improvement in average score compared to untreated Gaa^+/− mice. (c) Cross-sectional area of each fiber was determined at a determinant of muscle health. Average CSA improved by 21% compared to Gaa^+/− mice. n = 2-3 mice per group with a minimum of 70 fibers per mouse counted. Significance is relative to Gaa^+/−, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Figure 3-22. Preventative Treatment Reduced Lysosome and Autophagosome proteins Long Term in Tibialis Anterior. Data are mean ± SEM of integrated intensity (artificial units) of bands observed on western blot detecting lysosome (LAMP1) and autophagosome (LC3-II) specific proteins and autophagy associated proteins (LC3-I, Beclin1, and p62) in untreated Gaa<sup>−/−</sup>, untreated wt, and treated Gaa<sup>−/−</sup> mice. Levels for each protein analyzed decreased in treated mice compared to untreated Gaa<sup>−/−</sup> mice. LC3-II / LC3-I ratio, is decreased indicative of flux restoration through the system in treated mice. n = 4-6 mice per group. Significance is relative to Gaa<sup>−/−</sup>, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
CHAPTER 4
DISCUSSION

Correction of the autophagic accumulation present in skeletal muscle of PD patients is currently the goal to achieve. ERT, while quite successful in mediating glycogen clearance in cardiac muscle, reducing left ventricular mass, and restoring physiological function, has proven inefficient for skeletal muscle. However, there are several promising outcomes from the delivery of rAAV9-DES-coGAA vectors in skeletal muscle, as well as in cardiac muscle and motorneurons, as described by others\textsuperscript{221,237}. This study described the systemic resolution of total vacuolization to the skeletal musculature from the diaphragm to multiple muscles of the lower-hind limbs with rAAV-GAA gene therapy. Specifically demonstrated is the reduction of autophagic dysregulation in ERT-resistant, type IIb-rich gastrocnemius muscle (Figure 3-15). Preliminary evidence indicates that AAV-mediated GAA expression has the ability to persist long term and maintain reduced vacuolization in fibers in the tibialis anterior (Figures 3-17, 3-20). Lastly, while correction of pathology appears to be possible, preventative treatment may lead to even better outcomes in patients (Figure 3-21).

Each of the major components of an rAAV vector, the capsid, ITRs, promoter, and cDNA transgene, can all be manipulated to improve transduction and gene expression\textsuperscript{194}. While the thirteen naturally occurring AAV serotypes have broad tissue tropism based on capsid-cell receptor chemistry, researchers have developed novel serotypes through library screening and rational design to have a more specific tropism\textsuperscript{181,238}. These mutant capsids have also been generated to evade humoral and cellular immunity, by modifying the antibody recognition site\textsuperscript{176}, removing surface tyrosine residues that lead to degradation by the ubiquitin-proteasome pathway\textsuperscript{239,240}.  

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and those that are detargeted from the liver\textsuperscript{241,242}. In the present study, a wt AAV9 capsid was chosen because of its broad biodistribution profile with high transduction in cardiac muscle, skeletal muscle, and motorneurons\textsuperscript{192,219}, three cell types that store glycogen to be degraded for energy and need to be targeted by potential PD therapeutics\textsuperscript{46}. The choice of promoter and other regulatory elements, microRNAs and untranslated regions, provide an additional level of regulating tissue-specific expression following capsid usage. Ubiquitous promoters, \textit{CMV} (cytomegalovirus) and \textit{CBA} (chicken β-actin), are frequently used for expression in both systemic and local administrations because of their high activity in many cell types\textsuperscript{200,229,243,244}. Although, a negative consequence of ubiquitous promoters is their expression in immune related cells at high levels that provoke a humoral response against the gene product, particularly in CRIM-negative patients in which the protein is considered foreign. Cell specific promoters are gaining popularity because of their ability to combat this issue by reducing off-target expression and increasing expression in target tissues\textsuperscript{245–247}. For example, use of a skeletal muscle specific promoter, \textit{MCK} (muscle creatine kinase) driving GAA expression in the PD mouse model, elicited a limited immune responses which correlated to greater activity and to decreased glycogen when compared to \textit{CMV}\textsuperscript{248}. Another method of reducing immune responses is to induce tolerance\textsuperscript{249}. One way this has been achieved, using PD as a model, was through delivery of rAAV9 vectors carrying a liver specific promoter (\textit{LSP})-driven \textit{GAA} and a tissue restrictive, desmin (DES) promoter-driven \textit{GAA} at a 1:9 ratio\textsuperscript{250}. The transgene was expressed at a low level in hepatocytes, and therapeutic levels were expressed in relevant tissues. Codon optimization is yet another method of improving transgene expression by
increasing translation. Algorithms modify the nucleotide in the third position based on the wobble theory to account for abundance of tRNAs and those that enhance mRNA stability\textsuperscript{251}.

All together, the development of an rAAV vector is dictated by multiple factors including disease pathology, target tissue desired, and most importantly safety\textsuperscript{252}. Efficient transduction that produced therapeutic effects from the vector used in the current study came from careful selection of each of these elements, which were evaluated through a series of studies over the last twenty years. Original vectors proving gene delivery and phenotypic correction in the mouse model utilized rAAV2-CMV-mGaa\textsuperscript{200}. Following the identification of crossdressing\textsuperscript{191} and a greater understanding of AAV1 efficacy to transduce skeletal muscle, it replaced AAV2, and was the capsid serotype chosen for the clinical candidate delivered during the first clinical trial\textsuperscript{210,229,237}. Then, as the neurological phenotype became more prominent in patients surviving on ERT, use of an AAV9 capsid came to the forefront. It has been used in recent preclinical studies, such as this one, and will be employed in the upcoming PD clinical trial\textsuperscript{218,250}. Many promoters including CMV, LSP, DES, MCK, and even a combination have conferred GAA expression with varying benefits\textsuperscript{221,250}. Here, a modified DES was built into the construct. It’s small size, (~300 base pairs) accommodates the large GAA transgene (~3000 base pairs). Expression is greatest in cardiac muscle and is relatively high in skeletal muscle and motorneurons as well, but limited in hepatocytes\textsuperscript{220,221,245}. The human GAA (hGAA), quickly replaced the murine cDNA, mGAA, used in early vectors in the pursuit of a viable clinical candidate. The construct was then exchanged for one that is codon optimized (coGAA), which is the
cDNA utilized in this study following demonstration of its ability to be efficiently produced\textsuperscript{250}. Route of administration plays a crucial role in therapeutic outcomes. The effects of local delivery to specific target tissues has been elucidated\textsuperscript{200,212,218}. Nevertheless, these organs do not exist individually and systemic correction as shown here, has the potential to provide phenotypic correction to the entire body.

Last year, the first study to examine an autophagic marker in a PD model following \textit{GAA} gene transfer was conducted\textsuperscript{243}. A dose of $1 \times 10^{11}$ viral particles of \textit{rAAV9-CBA-hGAA} was systemically delivered to four month-old mice. GAA activity was slightly increased in cardiac muscle, but not elevated in skeletal muscles (diaphragm and quadriceps). LC3-II abundance in the heart and quadriceps of treated was not appreciably different to that found in untreated mice. Based on the work presented herein, potential causes for the lack of response in this study include low dose and the choice of promoter. Here, a dose of $1 \times 10^{14}$ vg/kg, or $2.5 \times 10^{12}$ total vg on average, was required to create near to supra-physiological levels of GAA activity in lower limb skeletal muscles (Figure 3-2) whereas doses in that study were nearly two logs lower.

Based on the hypothesis that clearance of autophagosome accumulation is secondary in the cascade following clearance of glycogen in lysosomes, it is a fitting theory that without an increase in active GAA to clear the glycogen, downstream pathologies cannot be corrected. As such, the present study is the first to demonstrate a reduction in autophagosome accumulation in any skeletal muscle of PD mice following administration of AAV-mediated \textit{GAA}.

Diaphragm and TA had the best overall responses to \textit{rAAV9-coGAA} administration (Figures 4-2, 4-4). Activity in the diaphragm was over 250% of \textit{wt} levels,
which cleared lysosomal glycogen (Figure 3-2). Large vacuolar aggregates were not noted in many fibers of Gaa\(^{-/-}\) mice, consistent with previous reports\(^{105}\), but total vacuolization was less in treated mice (Figure 3-6a,b). This matches lysosome and autophagosome detection by IF (Figure 3-10). Total protein, quantified by western blot, revealed that LAMP1, LC3-II, and p62, the key proteins analyzed, were statistically lower in treated mice compared to Gaa\(^{-/-}\) mice (Figure 3-14). In conjunction with vacuole clearance, fiber growth trended positively, evidenced by the increase in average CSA by more than 25% (Figure 3-6c). The amelioration of pathology at the cellular level may account for the observed improvements to contractile force observed previously\(^{237}\).

Expression of GAA in TA of treated mice led to an increase in activity that was 66% of wt levels, and reduced total glycogen (Figures 3-2, 3-4). A statistically significant 2.2 unit lower vacuolization score marks the correction of damage in a high percentage of fibers (Figure 3-8b). Specifically, the proportion of fibers characterized as LAMP1 puncta filled decreased by a statistically significant 32%, allowing considerable resolution of autophagosomes, measured by LC3, through IF and western blot (Figures 3-12, 3-16). With a lower LC3-II/LC3-I ratio, an indicator of increased flux, and decreased initiation by Beclin1, correction of the pathway’s dysregulation is apparent, leading to healthy fibers, which were able to show growth trends to almost wt sizes with average CSA at 92% of wt (Figure 3-8c).

As widely reported, type IIb fibers are resistant to ERT and extensive research has focused on increasing their tolerance\(^{99,103,106,253}\). Other groups have considered
several methods to improve the effectiveness of ERT in the PD mouse model using genetic manipulations and pharmacologic agents\textsuperscript{134,253,254}.

Two double knockout (DKO) transgenic mouse models were generated to evaluate autophagy suppression in this system by crossing the Gaa\textsuperscript{+/−} mouse to a muscle specific Atg5\textsuperscript{+/−} mouse\textsuperscript{254} or to a Type II muscle fiber specific Atg7\textsuperscript{+/−} mouse\textsuperscript{134}. Both DKO models resulted in an ablation of autophagosome production. However, in the Atg5\textsuperscript{+/−}:GAA DKO, lysosomal glycogen still accumulated, ubiquitinated proteins amassed, and mice had an exaggerated clinical phenotype\textsuperscript{254}. On the other hand, when Atg7\textsuperscript{+/−}:GAA DKO mice were treated with rhGAA, glycogen and total ubiquitinated proteins were reduced in the targeted fast muscles\textsuperscript{134}. Nevertheless, translation of this finding to a therapeutic has not been proposed in the literature yet.

Another strategy attempted to convert Type II to Type I fibers, by creating a novel transgenic mouse model\textsuperscript{253}. This was achieved by crossing the Gaa\textsuperscript{+/−} mouse to one that constitutively overexpresses Pgc-1α, a member of the PPARγ cotranscription factor family, which enhances expression of genes responsible for mitochondria production. Muscles typically predominant in Type II fibers had fibers with an intermediate phenotype in the transgenic model. While there was an increase in myoglobin and a decrease in autophagosome accumulation, the fibers still stained positive for MHC type II, and expression of several autophagy-associated proteins, LC3-II, Beclin1, Bnip3 was induced. An increased fusion between autophagosomes and lysosomes did not enhance the ability of ERT to resolve glycogen in lysosomes when administered to this model, and lysosomal rupture still occurred.
With a greater understanding of Transcription Factor EB (TFEB) in recent years, it has emerged as a potential therapeutic for PD and other lysosomal storage diseases\textsuperscript{255,256}. TFEB binds and activates the expression of CLEAR (coordinated lysosomal expression and regulation) network proteins\textsuperscript{81}. Overexpression of TFEB in vitro resulted in increased autophagosome and lysosome biogenesis and sped up their motility, followed by lysosomal exocytosis\textsuperscript{255}. When Gaa\textsuperscript{-/-} mice were administered TFEB in an rAAV1 vector delivered intramuscularly to the gastroc, lysosomal glycogen was reduced which was attributed to increased exocytosis\textsuperscript{256}.

Others explored already FDA-approved selective β2 receptor agonists as a potential combination treatment for ERT\textsuperscript{243}. Water spiked with Clenbuterol and Salmeterol was provided to Gaa\textsuperscript{-/-} mice. These drugs enhanced the expression of CI-MPR and led to a lightly reduced glycogen load, and strengthened skeletal muscle function tested by wire hang test latency.

Autophagy induction and flux is tightly regulated through several signaling pathways that compete to turn the pathway on and off\textsuperscript{76,78,257,258}. A number of neurological and muscular disorders are caused by mutations in any one of the proteins within these pathways resulting in dysregulation of autophagy by increased induction, decreased induction, or decreased autophagosome-lysosome fusion\textsuperscript{259}. Each of the methods described previously to enhance autophagy at a specific step may alter the balance too far in the opposing direction, causing a subsequent problem\textsuperscript{260}. Despite the fact that the some methods were successful at clearing lysosomal glycogen, there are potentially detrimental outcomes that result from turning off autophagy, some of which were observed at low levels in the Atg DKO study. These consequences include
accumulation of dysfunctional mitochondria, increased oxidized cytosolic material, and muscle atrophy which may outweigh the benefits observed in the long run\textsuperscript{134,261}. Additionally, altering the end of the pathway with excessive lysosomal exocytosis, may introduce a pH imbalance in the interstitial space, along with a higher abundance of degradative hydrolases. Moreover, if macromolecules are being non-selectively removed from the cell, they will not being catabolized efficiently to release nutrients back into the cytosol.

By directly addressing the primary cause of PD, lack of endogenous GAA production, therapeutic levels of activity were achieved even in type IIb-rich muscle (Figure 3-2). With this, a decrease in glycogen was observed (Figure 3-4). Total vacuolization in the gastroc of treated muscle was significantly mitigated compared to untreated controls (Figure 3-7b). The reduced presence of vacuoles corresponds to a resolution of lysosomes and autophagosomes, observed by IF (Figure 3-11). Lysosome and autophagosome markers as well autophagy-associated proteins were lower in abundance in treated mice than in untreated controls (Figure 3-15). Molecular changes led to a slight increase in CSA, which did not reach statistical significance potentially due to the quick evaluation time point (Figure 3-7c). Taken together, this method of GAA delivery has the ability to correct autophagic dysregulation in a muscle type previously resistant to therapies (Figure 4-3).

One of the most anomalous findings of this study was the response produced in soleus muscle (Figure 4-1). It had the second highest level of therapeutic transgene expression, indirectly measured by activity, at 185\% of \textit{wt} (Figure 3-1), as well as a correlative clearance of lysosomal glycogen determined by PAS staining (Figure 3-3).
and a visual improvement in fiber vacuolization is noted (Figure 3-5a,b). The quantification of IF sections indicates a decrease in lysosomes with treated mice having 26% fewer LAMP1 filled fibers compared to untreated controls, and a 21% more fibers containing no detectable LAMP1 puncta (Figure 3-9b). By this method there was also an improvement in the proportion of fibers with small LC3 puncta by 15% (Figure 3-9). However, quantification of proteins by western blot revealed increases in LC3-II, LC3-I and p62, the latter of which had statistical significance (Figure 3-13). CSA in treated mice also had not just a neutral response, but a negative one, with a non-statistical decrease in size by 2.5% (Figure 3-6c). Interestingly, there was a lower LC3-II to LC3-I ratio (Figure 3-13), often a measure of the fusion of autophagosomes and lysosomes, where lower ratios correlate to an increase in flux262. A somewhat similar outcome was seen previously in type I-rich soleus, in which despite a reduction in autophagosome accumulation, associated proteins were upregulated253. Taken together, it is postulated that a multi-step cascade is occurring. (1) During the time prior to treatment, as vesicles accumulated, aberrant organelles such as mitochondria, which are naturally greater in abundance in type I fibers, have also accumulated234. (2) As GAA is produced and able to resolve glycogen in lysosomes, they are able to fuse with autophagosomes. (3) It is possible that, with the restoration in function of the pathway, the cell is upregulating it in order to resolve the remaining nonfunctional proteins and organelles. With this theory, total protein levels would be increased but vesicles would be smaller and less detectable than the enlarged ones found in untreated muscle by H&E and IF. Since at this time the fibers are still in the process of restoring homeostasis, they are not yet in a growth period, leading to a smaller CSA.
Currently, all patients who are enrolled in gene therapy clinical trials continue to receive ERT because it would be unethical to withdraw them from a treatment that improves survival. Evidence shows ERT is more effective at clearing autophagic accumulation in type I muscle fibers in Gaa<sup>−/−</sup> mice and PD patients. It is possible that, when administered together, these two treatments will result in complementary correction.

The half life of rhGAA is approximately seven days<sup>118,220,263</sup> and the current recommendation for its administration is a biweekly infusion of 20-40 mg/kg, delivered over the course of four hours<sup>264</sup>. The frequency of delivery creates a burden on the patients’ lives. It is also accompanied by a hefty price tag, at $120,000 - $300,000 per year<sup>265</sup>. The only AAV-mediated gene therapy on the market has thus far been deemed “the world’s most expensive drug,” at ~$1,000,000<sup>266</sup>. If therapeutic levels of gene expression last for several years, the cost may be comparable, and will only have burdened the patient once, rather than approximately one hundred times, over the same duration. Preclinical and clinical studies demonstrated the potential longevity of rAAV-mediated gene expression<sup>205,229,267</sup>. Many Gaa<sup>−/−</sup> mice live to twenty-one to twenty-four months of age despite lower survival rates<sup>220</sup>. Here we demonstrate the ability of vector derived GAA to persist and provide some pathological phenotypic relief for six months when delivered after vesicular dysfunction amasses.

By six months after delivery, GAA activity had diminished by 25%, compared to the one month post-injection evaluation (Figures 3-2, 3-17), but because carriers display no symptoms, the remaining 50% of wt levels of activity should provide enough to yield improvements. Although an exact vacuolization score was not determined, repaired
fibers lacking vacuoles are apparent. In contrast to the short term evaluation, lysosome and autophagosome membrane proteins have risen again to levels just below untreated mice. A higher LC3 ratio and greater abundance of Beclin1 and p62 indicate that autophagy dysregulation continues to persist, despite the presence of active GAA. This is a perplexing finding considering the functional improvements of the whole muscle, measured by others at the same time point or longer. Based on the heterogenous transduction pattern, it is possible that although fibers expressing GAA have vacuole clearance, that untransduced fibers continue to amass lysosomes and autophagosomes resulting in increased amounts of associated proteins. This finding and other recent data collected from a study in non-human primates (unpublished) shed light on the need for vector readministration. Elucidating the link between molecular pathology and clinical symptoms even further may provide insight into redosing strategies.

Timing of treatment is of critical importance as demonstrated in previous studies as well as this one. Taiwan has implemented newborn screening through dried blood spot GAA activity assays and organizations are recommending that it be added to the list of metabolic disorders evaluated similarly in the United States. In Taiwan, the average age of diagnosis has significantly dropped, from three to four months of age to a mere twenty-two days. This difference followed by earlier treatment initiation has led to greatly improved clinical outcomes including increased ventilator free-survival and overall survival.

Studies of neonatal early gene delivery have correlated with similar positive outcomes in mouse models of various lysosomal storage diseases (LSDs), such as Mucopolysaccharidosis. Mah and colleagues observed restored GAA activity,
glycogen degradation, and functional improvements in lower limb and respiratory skeletal muscles following gene transfer to PD mice within the first 48 hours of life\textsuperscript{229,237}. Others have shown that, as time passes, the disease progresses to a point where interventions are only weakly efficient\textsuperscript{222}. \textit{Gaa}\textsuperscript{-/-} mice treated at three, nine, and twenty-one months of age and analyzed three months after, all had respiratory improvements, but those treated later had reduced functionality compared with those that were dosed at three months\textsuperscript{222}.

An impactful finding here is the long-term ability of rAAV-derived GAA to prevent autophagic dysregulation when administered prior to gross vesicular accumulation, particularly compared to the six-month evaluation time point following corrective treatment. GAA activity was about the same, at 51% of \textit{wt}, but vacuolization was considerably ameliorated in contrast to the six month old untreated \textit{Gaa}\textsuperscript{-/-} mice, and mice treated at three months of age evaluated at both one month and six months later. Lysosome and autophagosome membrane proteins, as well as associated proteins, were nearly all significantly improved.

Dysregulation of the autophagy pathway is a noted secondary pathology in a number of muscle diseases, including Duchenne Muscular Dystrophy\textsuperscript{273} and Limb-Girdle Muscular Dystrophy\textsuperscript{274}, but their pitfall is inefficient initiation. Danon disease and X-Linked Myopathy with Excessive Autophagy (XMEA) along with PD constitute a new classification of disorders known as autophagic vacuolar myopathies, where accumulation is observed rather than an absence. Danon disease is caused by mutations in \textit{LAMP2}, which has a role in the fusion of autophagosomes and lysosomes\textsuperscript{275}. Unlike other X-Linked protein deficiencies\textsuperscript{276–279}, female carriers
experience significant symptoms, but onset is delayed to adulthood, illuminating the importance of this protein\textsuperscript{275}. The clinical profile is similar to that of PD, with hypertrophic cardiomyopathy, skeletal muscle weakness, and neurological manifestations. XMEA is caused by mutations in \textit{VMA21} and manifests predominantly as a skeletal myopathy. \textit{VMA21} encodes the v-ATPase, a crucial protein that resides in the membranes of vacuolar membranes and pumps protons into their lumens to maintain an acidic pH. As mentioned previously, the acidic environment is necessary for effective fusion of autophagosomes and lysosomes\textsuperscript{280}. While neither of these diseases have an abundance of lysosomes, autophagosome accumulation is apparent\textsuperscript{281}. Currently, there are no therapies for Danon disease or XMEA, but the work here provides a rationale for using gene therapy, rather than a chemical drug that would enhance or inhibit an autophagy pathway step.

In this study, the underlying mechanism of vacuolar accumulation is examined following the delivery of rAAV-mediated GAA at multiple time points. The central hypothesis identified the lack of endogenously produced GAA to all subsequent pathologies, beginning with glycogen accumulation which led to lysosomal dysfunction and finally lysosome and autophagosome build-up. Evidence supporting this hypothesis has been provided through this study. Additionally, several important findings were noted, chiefly the ability of a systemic administration to treat the lysosomal and autophagosomal pathologies in skeletal muscles located in the thoracic cavity as well as the lower leg. Also demonstrated was the reduction of autophagosome accumulation in ERT-resistant, type II-rich muscles. Lastly, timing and longevity effects from vector derived GAA was explored, providing support for early intervention. Overall
this therapy is effective at preventing and correcting the secondary pathology of autophagic dysregulation that occurs in skeletal muscle of the PD mouse model.
Figure 4-1. Summary of Results for Soleus. Green Arrows indicate that treatment yielded a result that was concordant with the hypothesis for a particular parameter. Red Arrows indicate that treated animals yielded a results opposing to the hypothesis. Asterisks below arrows indicated where treated mice were significantly different than Gaa<sup>−/−</sup> mice. An increase in GAA activity was accompanied by a decrease in glycogen and vacuolization. Presence of lysosomes and autophagosomes was variable depending on method used to evaluate them. Autophagy associated proteins and CSA were worse in treated animals than in untreated controls.
Figure 4-2. Summary of Results for Diaphragm. Green Arrows indicate that treatment yielded a result that was concordant with the hypothesis for a particular parameter. N.D. = not determined. Asterisks below arrows indicated where treated mice were significantly different than \( \text{Gaa}^{+/−} \) mice. An increase in GAA activity was accompanied by a decrease in glycogen and vacuolization. Presence of lysosomes and autophagosomes were reduced, as detected by IF and western blot. Autophagy-associated protein, p62 and CSA were improved.
Figure 4-3. Summary of Results Gastrocnemius. Green Arrows indicate that treatment yielded a result that was concordant with the hypothesis for a particular parameter. N.C. = no change. Asterisks below arrows indicated where treated mice were significantly different than Gaa-/- mice. An increase in GAA activity was accompanied by a decrease in glycogen and vacuolization. Presence of lysosomes and autophagosomes were reduced, as detected by IF and western blot. Autophagy-associated proteins, Beclin1 and p62 and CSA were improved.
Figure 4-4. Summary of Results TA. Green Arrows indicate that treatment yielded a result that was concordant with the hypothesis for a particular parameter. N.C. = No Change. Asterisks below arrows indicated where treated mice were significantly different than Gaa−/− mice. An increase in GAA activity was accompanied by a decrease in glycogen and vacuolization. Presence of lysosomes and autophagosomes were reduced, as detected by IF and western blot. Autophagy-associated protein, Beclin1 and CSA were improved.
APPENDIX A
DEVELOPING A GENE THERAPY VECTOR FOR X-LINKED MYOTUBULAR MYOPATHY

Introduction

Clinical Manifestation

X-Linked Myotubular Myopathy (XLMTM), the most well described Central Nuclear Myopathy (CNM) is a fatal metabolic disorder arising annually in 1 in 50,000 male births. Spiro et al. fundamentally identified the pathology in 1966, and named the disease following the observation of a muscle biopsy in which fibers resembled fetal myotubes. In the thirty years following this report, a multitude of cases have been described in the literature. Clinically, patients are phenotypically diagnosed at birth or within the weeks after, by failure to spontaneously breathe and profound skeletal muscle hypotonia also known as floppy baby syndrome. Some mothers report decreased fetal movement during their pregnancy and polyhydraminos is often noted. Many of these patients require mechanical ventilation within the early weeks of life due to weakness of the diaphragm causing respiratory insufficiency. Other symptoms and signs at birth include a low APGAR score (a measure of appearance, pulse, grimace, activity, and respiration) at both one minute and five minutes, length and head circumference in the 90th percentile or above, and areflexia. Cardiac muscle-related pathologies are not typically observed and creatine kinase levels are normal to slightly elevated. Since there is no treatment available, boys with the most severe disease pathology have a life expectancy of one year due to respiratory failure. Those who live beyond one year with a less severe form of the disease have a higher incidence of developing peliosis hepatis, a liver disorder in which cystic cavities filled with blood accumulate. Scoliosis and myopia
have also been noted in teenage boys with XLMTM\(^{292}\). While female carriers do not
typically display muscle related symptoms biopsies reveal centralized nuclei in a small
percentage of fibers\(^{293,294}\).

Beyond the outward clinical phenotype, the disease is diagnosed based on
histological findings from a muscle biopsy, identifying the distinguishing characteristic of
centralized nuclei, followed by DNA sequencing\(^{295–297}\). XLMTM is caused by mutations
in the \(MTM1\) gene, which was independently and simultaneously localized the \(MTM1\)
gene to the q arm of the X chromosome at locus 28 by linkage analysis and known
polymorphic DNA markers by three labs\(^{298–300}\) \(MTM1\) encodes the lipid phosphatase,
myotubularin 1 (MTM1), which acts on phosphatidyl-inositol-3-phosphate (PI3P) and
phosphatidyl-inositol-3,5-bisphosphate (PI3,5P\(_2\))\(^{301–303}\). \(MTM1\) is comprised of fifteen
exons that span a range of 100kb\(^{304}\); the coding sequence begins in Exon 2, and
generates an mRNA transcript 3.4kb in length\(^{301}\). The gene is ubiquitously expressed
but a muscle-specific alternative transcript is predominant due to the use of a different
poly-adenylation signal\(^{301}\). The nearly 200 pathogenic mutations are found evenly
distributed throughout the gene with missense mutation hotspots clustered in exons 8
and 12, which surround the active site\(^{284}\). A genotype-phenotype correlation has been
made, wherein truncating mutations and large deletion mutations are found in patients
with a more severe disease progression, and non-truncating mutations, missense, in-
frame insertion/deletions, lead to a milder phenotype\(^{286}\). One common genetic
alteration is the c.205C>T transition which results in p.R69C amino acid
change\(^{284,295,296}\). This residue lies within the PH-GRAM domain of the protein, the
domain responsible for substrate binding\(^{305–307}\). Because the mutation is predicted only
to reduce the specificity of lipid binding, specifically for PI3,5P_2 and not ablate the 
enzymatic activity, a less severe disease is observed. Approximately 17% of boys with 
XLMTM have mutations that arose \textit{de novo} demonstrating the instability of the genome 
within this region^{284}.

Prior to identification of the gene responsible for XLMTM, observations made 
from muscle biopsies were, and to a large extent still are, the gold standard for a 
diagnosis^{294}. The most prominent histopathological feature is the presence of 
centralized nuclei with a halo of mitochondria in 50 – 100% of the muscle fibers^{308}. In 
fibers where a centralized nucleus is not found, the center of the fiber appears pale or 
devoid of any cellular materials^{309}. Many reports describe a preponderance of round, 
hypotrophied Type I muscle fibers, although others have noted a similar phenotype in 
Type II fibers with equal abundance^{310,311}. Muscle fibers in XLMTM patients have an 
appearance more like fetal fibers at early developmental stages like that originally 
described by Spiro and stain positively for early development cytoskeletal protein, 
vimentin^{283,312}. Both an autosomal recessive and autosomal dominant disorder with 
similar clinical and histological symptoms and signs have been reported. However, they 
are associated with juvenile and adult onset, respectively, and have milder 
phenotypes^{294}.

\textbf{Molecular Mechanism}

Myotubularin 1 is the founding member of a family of Myotubularin-related 
proteins (MTMRs), all of which have ubiquitous promoters, but each is predominant in a 
specific tissue that leads to pathogenically different diseases when the gene is 
modified^{301,313}. Each of the enzymes has two functional domains, a protein tyrosine 
phosphatase (PTP) domain and a PH-GRAM domain. Surprisingly nearly half of the
enzymes in this family have an inactive PTP domain, for currently unidentified reasons\textsuperscript{314}. MTM1 was originally studied as a protein tyrosine phosphatase, until it was discovered to have little activity toward phosphorylated proteins and that this domain along with PH-GRAM domain has much greater affinity for lipids\textsuperscript{301}. Its role in lipid dephosphorylation lies within the PI3P pathway regulating intracellular membrane trafficking and transport vesicles\textsuperscript{315}. PI3P and PI3,5P\textsubscript{2} are important in the signaling of cytoskeletal filaments and movement of organelles, specifically they control the fate of early and late endosomes\textsuperscript{316,317}. In addition to its enzymatic functions, MTM1 provides structural support to the cell, interacting with desmin to regulate filament assembly and cytoskeletal architecture\textsuperscript{318}. This interaction is theorized to be involved in the positioning of mitochondria and nuclei.

Animal Models

A zebrafish model was created using antisense morpholinos to knock down mtm\textsuperscript{1}\textsuperscript{319}. Myofibers are hypertrophied, leading to a thinned muscle compartment and dorsal curvatures. The neuromuscular junction also displays abnormalities. An important finding from this model is the 60-120\% increase in PI3P, the primary substrate for mtm1.

Two mouse models have been generated in an effort to study the mechanism of pathology and its correction with therapies. The Mtm1\textsuperscript{δ4} mouse was created using a Cre-loxP system, which resulted in the removal of Exon 4, a frameshift mutation, causing a premature stop codon\textsuperscript{320}. In this model, Mtm1 is absent systemically. This is not a completely embryonic lethal mutation but knockout males are only produced \~17\% of the time rather than 25\%, expected by Mendelian genetics. They have an early onset of symptoms, with hindlimb weakness at four weeks post-natal, and rapid progression of
disease with a life expectancy of fourteen weeks of age. As in the human disease, nuclei are found in the central region of the fibers, and mitochondria accumulate in the perinuclear region. The contractile unit is disrupted and atrophy of myofibers is observed. The second mouse model, the p.R69C mouse, was created by introducing a single base pair mutation, c.205C>T, commonly found in humans. This line produces progeny closer to Mendelian expectations, at 22% and that have a longer survival time, living until just over one year of age. With their slower disease progression, they also have a later onset of symptoms, not showing hypotonia until three months. Their fibers display the typically small, rounded phenotype with centralized nuclei appearing by one month of age. Ultrastructural analysis reveals abnormal T-tubules and sarcoplasmic reticulum.

A naturally occurring mutation, c.465C>A, was found in Labrador Retrievers. This mutation lies within Exon 7, and results in a single amino acid change, p.N155K, located between the substrate binding and enzymatic domains. These dogs experience similar signs as in human patients such as centralized nuclei, mislocalization of dihydrolyradine receptor α1 and ryanodine receptor 1, a predominance of Type I muscle fibers. Progressive lower limb weakness and muscle atrophy becomes evident around 13 weeks of age and within the following five weeks they become non-ambulatory.

**Therapies**

While there are preclinical studies evaluating the efficacy of ERT and gene therapy for XLMTM, no treatment has been approved by the FDA. It is believed that uptake of exogenous myotubularin 1 for the treatment of XLMTM will face many pitfalls observed in other myopathic patients receiving ERT. Lawlor, et al. demonstrated that intravenous delivery of myotubularin 1 is inefficiently targeted to the skeletal muscle...
of mice without an antibody tag, an aspect that may hinder the enzyme being taken up by other affected organs\textsuperscript{322}. Additional pitfalls include high cost (\textasciitilde $300,000 per year)\textsuperscript{265,276}, due to the repetitive infusions, and a strong immunogenic response in CRIM negative patients\textsuperscript{128,325,326}. These issues illustrate the need for an alternate treatment.

AAV mediated gene therapy is an attractive option for the treatment of XLMTM. The thirteen identified serotypes have been isolated from humans and non-human primates and their capsids have the potential to transduce a variety of tissues. AAVs are not known to cause any disease and recombinant vectors are weakly immunogenic, compared to other gene transfer vectors\textsuperscript{327}. XLMTM is a monogenic disease with a relatively small cDNA sequence that adequately fits into an rAAV capsid, two characteristics that make it ideal for AAV gene therapy\textsuperscript{194}. Additionally, because MTM1 is involved in a signaling cascade, very low levels of protein are predicted to be required, an attainable goal demonstrated in preclinical and clinical studies\textsuperscript{328}. Clinical trials and the first approved gene therapy in western medicine, provides evidence of safety for muscle directed treatment.

Preliminary studies utilizing AAV-mediated gene therapy, completed with a murine cDNA and canine cDNA delivered to their respective models have had promising results. Following administration of rAAV8-\textit{DES-mMtm1} vectors to the \textit{Mtm1Ex\textdegree4} mice, cellular and physiological functions were improved, and life expectancy was elongated. However, the hearts of treated knockout mice displayed focal lesions at the time of necropsy likely due to cardiac infarctions. Diseased canines treated with a similar vector administered intramuscularly also exhibited prolonged ambulation and survival\textsuperscript{324}.
Rationale

While rAAV-mediated gene therapy vectors are currently in development by others, an ideal vector has not yet been generated. Expression regulation by a specific promoter, microRNA, or untranslated regions needs to be evaluated. Additionally, the biodistribution profile of rAAV9 vectors is likely give better systemic skeletal muscle transduction than rAAV8 vectors\textsuperscript{192}. Currently, the major method of evaluating a therapy is through survival and histological analysis, but better biochemical assays including an activity assay coupled with a western blot will help advance the research in this field.

Methods

Plasmid Transfection into C2C12 Cells

C2C12 cells were maintained at 37°C with 5% CO\textsubscript{2} in Dulbecco’s Modified Eagle’s Medium (DMEM) (Cellgro: 10-013) supplemented with 10% fetal bovine serum (FBS) (Cellgro: 35-010-CV) and 1% antibiotic-antimycotic (Gibco: 15240062). They were transfected with plasmids containing either DESPro-Luciferase or MTMPPro-Luciferase, using the Amaza\textsuperscript{®} Cell Line Nucleofector\textsuperscript{®} Kit V (Lonza: VCA-1003). Cells were washed with PBS, then trypsin (Gibco: 25200056) was added and they were incubated at 37°C until the majority of cells were detached, approximately three minutes. The trypsin was neutralized with an equal volume of DMEM. Cells were collected and counted using an Invitrogen™ Countess™. Cells were pelleted, by centrifugation for five minutes, 1500 rpm, at 4°C. They were resuspended in Nucleofector\textsuperscript{®} Solution for a final concentration of 1\times10^6 cells per 100μL. 100μL of cells and plasmid DNA were added to a cuvette. The cuvette was inserted into the
Nucleofector® Device, and transfection was performed using the B-032 Program. The cells were then plated in a 6-well plate and 2mL of supplemented DMEM was added.

**Plasmid Transfection into HepG2 Cells**

HepG2 cells were maintained at 37°C with 5% CO₂ in Eagle’s Minimum Essential Medium (EMEM) (ATCC: 30-2003) supplemented with 10% FBS and 1% antibiotic-antimycotic. Cells were plated in a 6-well culture plate at 60-70% confluency and allowed to grow overnight. Lipofectamine® 2000 Reagent (Invitrogen: 11668027) and Opti-MEM® (Gibco: 11058021) was mixed together with Opti-MEM diluted plasmid DNA, so that Lipofectamine® to DNA ratio was 2:1. The mixture was incubated at room temperature for ten minutes, and then added to cells with fresh EMEM.

**Plasmid Transfection into HEK293 Cells**

HEK293 cells were obtained from the UF-PGTC-VC and maintained at 37°C with 5% CO₂ in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic. Cells were seeded in a T-225cm² flask at 60-70% confluency and allowed to grow overnight. HEK293s were transfected using a standard Calcium Phosphate method. Briefly, 50μg of pTarget-\textit{hMTM1} was added to Calcium Chloride and water. An equal volume of 2x HEPES Buffered Saline (HBS) was added and the mixture was incubated at room temperature for 1-2 minutes, and then applied to the cells with a fresh layer of DMEM. Cells were collected 48 hours after transfection, split into 1x10⁶ cell aliquots for analysis by activity assay and western blot.

**Vector Production**

The University of Florida Powell Gene Therapy Center Vector Core (UF-PGTC-VC) produced rAAV by traditional double transfection methods described previously. HEK293 cells were maintained in DMEM supplemented with 5% FBS and 1% antibiotic.
1x10^9 cells were seeded in a 10-layer CellSTACK (Corning: #3270) and allowed to grow to 75-80% confluency. A calcium phosphate precipitate was made by mixing, pXYZ9223 (a kind gift from Dr. James Wilson, University of Pennsylvania, Philadelphia, PA) and rAAV plasmid at a 1:1 molar ratio, calcium chloride, and 2x HBS, then incubated at room temperature for 1-2 minutes. Complete DMEM was added to stop the precipitate, and this solution was added to the cells, after they were washed with PBS. Following a 60 hour incubation at 37°C with 5% CO_2, the media was removed, and cells were washed with PBS then harvested with EDTA-PBS. Cells were pelleted by centrifugation at 1000g for ten minutes. The pellet was resuspended in lysis buffer (150nM NaCl, 50mM Tris pH 8.4). A series of three freeze/thaws and incubation with Benzonase (50U/mL) and magnesium chloride (1mM) for 30 minutes at 37°C lysed the cells. Cell debris was cleared by centrifugation at 3700rpm for 20 minutes at 4°C. The supernatant was kept and overlayed onto a step gradient of 15%, 25%, 40%, and 60% Optiprep Iodixanol (Sigma-Aldrich: D1556) in an ultracentrifuge tube (Beckman: #341625). The tubes were capped and centrifuged at 69000 rpm for one hour at 18°C in a 70Ti rotor. Virus was collected from the 40%-60% interface through half of the 40% fraction, 3-4mL, using an 18G needle and syringe. The purified sample was loaded into a prepared Apollo 100K centrifugal concentrator with Lactated Ringer’s Solution and spun at 3000rpm, for six minutes, until the final volume was 500μL.

**rAAV Vector Infection into C12 Cells**

C12329 (a kind gift from Dr. Paul Johnson) cells were obtained from UF-PGTC-VC and maintained at 37°C with 5% CO_2 in DMEM supplemented with 5% FBS and 50μg/ML Geneticin (Sigma: A1720). Cells were infected with rAAV9-DESPro-Luc or rAAV9-MTMPro-Luc, at a multiplicity of infection (MOI) of 10⁴. The cells were
superinfected with rAdV5 at and MOI of 20. 48 hours after infection, cells were collected as above. Cells infected with a Luc-containing vector were prepared and enzyme activity was measured as described below.

**Luciferase Detection**

48 hours after transfection or infection, cells were harvested by using a cell scraper to lift them into the medium. They were collected and spun at 1500 rpm at 4°C for 5 minutes. The pellets were washed twice with phosphate buffered saline (PBS). The cells were lysed in Reporter Lysis Buffer (Promega: E3971). Homogenates were clarified by 4°C centrifugation at 13.2k rpm for 10 minutes. 20μL of lysate was plated into a white, opaque 96-well plate. Using an ElmerPerkin Enspire, a single ten-second luminescence read was taken to measure background levels. Then 100μL of Luciferase Assay Reagent (Promega: E1483) was injected and 5 subsequent ten-second luminescence reads. The 5 reads were averaged and the background levels were subtracted for a final luminescence measurement. These values were normalized to protein levels determined by DC Protein Assay (Bio-Rad: 500-0111) performed according to manufacturer’s instructions.

**Animals**

The University of Florida Institutional Animal Care and Use Committee (IACUC) approved all animal use under the protocol 201308155. Wildtype 129SVE mice were obtained (Taconic, Hudson, NY, USA) and housed at the University of Florida Animal Care Services. Experimental mice were randomly assigned to one of 11 groups: Lactated Ringer’s control, 2.3x10⁸ vg (low-dose), 1.15x10¹⁰ vg (mid-dose), or 2.3x10¹⁰ vg (high-dose) of rAAV8-DESPro-hMTM1-3’UTR, rAAV9-DESPro-hMTM1-3’UTR, and rAAV9-MTMPRO-hMTM1. Animals were anesthetized using 2-4% isofluorane inhaled
through a nose cone. A heating pad was placed under the surgical board to maintain appropriate body temperature. The hair of the lower limbs was shaved and scrubbed three times with Povidone/Iodine scrub alternating with 70% alcohol. An insulin syringe (29.5 gauge) was used to draw up vector diluted in Lactated Ringer’s to 20μL and then inserted into the midbelly region of the TA. Single injections of rAAV were administered bilaterally. One month after vector delivery, mice were anesthetized as above and euthanasia was completed by thoracotomy, after which TAs were harvested. Both TAs from each animal were flash-frozen together in liquid nitrogen, then stored at -80°C until further use.

**Immunoprecipitation of MTM1**

Anti-MTM1 antibody (Santa Crux Biotechnology: sc-14781) was conjugated to magnetic Dynabeads® M-270 Epoxy beads (Invitrogen: 14311D) according to manufacturer’s instructions. For cell culture samples, cells were lysed in buffer with or without protease inhibitor, followed by a series of 3 freeze-thaw cycles. Buffers included water, Pierce™ IP Lysis Buffer (ThermoFisher: 87787), and RIPA Buffer (Sigma-Aldrich: R0278). For mouse muscle samples, approximately 10ng of muscle, was homogenized in deionized water with protease inhibitor cocktail (Roche Life Science: 04-693-124-001) using a FastPrep24 (MP Biomedicals) followed by a series of 3 freeze-thaw cycles. Lysates were clarified by 4°C centrifugation at 13.2k rpm for 10 minutes. For cell culture samples, 40μg total protein and for mouse muscle samples, 600μg total protein was immunoprecipitated. The lysates were incubated with the anti-MTM1 antibody conjugated Dynabeads® for 1 hour while shaking at 4°C. Beads were washed with TBS-T 3 times before utilizing further.
**MTM1 Phosphatase Activity Assay**

The immunoprecipitated MTM1, still attached to the antibody-coupled beads, was incubated with 0.1mM of PI3,5P$_2$ (Echelon: Q-0125) in TBS pH 6.5 for 10 minutes at 37°C. The reaction was stopped by adding supernatant to N-ethylmaleimide. 20μL of the stopped solution is added to 80μL of Malachite Green in a clear 96-well plate, and then incubated at room temperature for 20 minutes as described by manufacturer’s instructions (Echelon: K-1500). Absorbance is read at 620nm and compared to a standard curve of phosphate.

**Western Blot**

Upon completion of the activity assay, the beads were placed on a magnet and all liquid was removed. Beads were resuspended in 25μL of Laemeli Buffer, then heated to 100°C for ten minutes. The beads were placed on the magnet and the buffer was pipetted off and loaded into 7.5% Criterion™ TGX™ Precast Midi Protein Gels (BioRad: 597-1024). The gel was electrophoresed for 30 minutes at 90 Volts followed by 60 minutes at 120 Volts. The proteins were then transferred to PVDF membrane (EMD Millipore: IPFL00010) for 90 minutes at 250mAmps. Blots were blocked in LI-COR Odyssey Blocking Buffer in PBS, which was also used as the antibody diluent for all steps. These blots were probed with an anti-MTM1 antibody and they were washed after primary and secondary antibody incubation with PBS supplemented with 0.1% Tween-20 (Gentrox: 40-028). All blots were imaged using the LI-COR Odyssey infrared detection system. Band densitometry was determined with coordinating software.

**Statistical Analysis**

All numerical data was evaluated using GraphPad Prism software. Statistical analysis was performed on all quantitative data collected, using an Ordinary
One-Way ANOVA and Dunnett’s Multiple Comparison’s test to determine the differences between treated and untreated groups.

Results & Discussion

Verification of Expression And Function Of Mtm1 cDNA / Development of Coordinating Mtm1-Specific Phosphatase Activity Assay & Western Blot

The transgene was designed by acquiring a codon-optimized human MTM1 cDNA (hMTM1) sequence. As a control, this transgene was first analyzed for expression and function through a CMV-driven plasmid (pTargetT™-hMTM1) transfected into HEK293 cells. There was not previously an MTM1-specific activity assay, which was needed by the field because measuring phosphatase activity through phosphate levels in the cell is highly non-specific due to the plethora of phosphatases present in the cytoplasm. Aliquots of transfected 293s were lysed in one of three buffers with and without protease inhibitor. Cleared and uncleared lysates were immunoprecipitated (IP’d) using anti-MTM1-conjugated Dynabeads®. Using a Malachite Green Phosphatase Assay and PI3,5P₂ as a substrate, activity of MTM1 was determined for each sample (Figure A1.3a). Western Blots were performed to detect the sequestered MTM1 (Figure A1.3b-c). While the IP Lysis buffer yielded the largest amount of MTM1 IP’d, the activity level was diminished due to the strength of detergent present. Lysis in RIPA buffer gave poor yields of activity and western blot detection. It was clear that non-specific antibody binding was occurring, based on the presence of increased activity in the sample lysed with water and was not spun down, as compared to the activity in the sample lysed with water but was spun down. However, due to the presence of membranous particulates in the water-lysed, unspun sample, MTM1 was unable to be resolved properly on the western blot. It was determined that lysis buffers
containing protease inhibitor had no measurable effect on the level of activity measured. We found that cell lysis in water with particulate clearance gave the best results (Figure A1.3d-f) for measuring MTM1 presence through an activity assay and western blot and were consistent. The activity from transfected cells is measured at three times greater than both controls, mock cells transfected with GFP and untransfected cells.

**Comparison of DESPro And MTMPro in vitro**

In vectors previously used in XLMTM preclinical studies, a human desmin gene promoter (DESPro) was used to direct Mtm1 transgene expression\(^{324}\). However, some data suggest that this promoter expresses Mtm1 at an excessive level in cardiac muscle, resulting in lesions noted upon necropsy\(^{324,330}\). Additionally, for patients surviving longer than one year of life peliosis hepatis, a fatal liver condition is observed\(^{292}\). This indicates that MTM1 may be more prominently expressed in liver, an organ in which DESPro does not express\(^{245}\). For this reason, we examined the MTM1 promoter for expression that may be more analogous to wt levels. I have analyzed a 1.3kb region immediately upstream of the MTM1 gene (MTMPro) was analyzed (GeneCopoeia: HPRM15185), and the presence of endogenous promoter elements was confirmed. The DNA sequence was examined using TFSEARCH and results indicate presence of both ubiquitous and muscle-specific transcription factors (data not shown). Preliminary experimental data confirms MTMPro is able to drive expression of a downstream gene using a Gaussia Luciferase reporter construct (data not shown). To compare the activity of DESPro and MTMPro, AAV plasmid vectors were generated; one each with MTMPro and DESPro driving Firefly Luciferase. The two plasmids were transfected into C2C12 murine myoblast cells and hepatocellular carcinoma, HepG2, cells. Expression activity of the promoters was measured by quantifying luciferase.
activity (Figure A1.2). Presence of luciferase showed MTMPro is able to drive expression in muscle progenitor cells. C2C12 myoblast and HepG2 cell lines were chosen based on their relatedness to tissue types that are most greatly affected in XLMTM. Results of this assay are as expected, where expression of MTMPro was lower than DESPro in myoblasts, but higher in the hepatocyte-derived cells. These promoters were then cloned in front of the hMTM1 transgene. rAAV vectors were produced and used to infect C12 cells, superinfected with rAdV5. An MTM1 activity assay reveals MTMPro expression is greater than that of DESPro in this cell type, a cervical adenocarcinoma-derived line.

Following the comparison of plasmid and rAAV vectors in vitro, expression was examined in skeletal muscle of wt animals. Vector was bilaterally injected into the TA muscle of 129SVE mice at three dose levels, 2x10⁹, 1x10¹⁰, 2x10¹⁰ total vgs. Animals injected with Lactated Ringer’s solution were used as controls. After one month muscles were harvested, and the two TAs from each mouse were combined. Muscle tissue was homogenized and immunoprecipitated to sequester MTM1. An MTM1 Phosphatase Activity Assay and Western Blot were completed (Figure A1.4a,b). Samples from rAAV8-DESPro-hMTM1-3’UTR and rAAV9-DESPro-hMTM1-3’UTR injected animals have detectable levels of MTM1 in a dose-dependent manner by western blot, as opposed to those samples from animals administered rAAV9-MTMPro-hMTM1. Concordant with WB results, activity of DESPro injected mice is also observed to be dose-dependent, and there is no increase in activity observed in MTMPro injected mice.
Here, a 3’ untranslated region (UTR) of the 1.0 kb that follows the end of the MTM1 coding sequence was added to the DESPro-hMTM1 construct. This addition had two purposes. The first was to fill out the rest of the AAV DNA vector to achieve the optimal 4.7kb size, since DESPro is a much smaller promoter than MTMPro. Specifically, this sequence was chosen because of its potential regulatory capabilities. 3'UTRs can function in a variety of ways with silencer and enhancer binding sites as well as having an effect on mRNA stability\(^{331}\).

The group that observed cardiac toxicity with DESPro-driven mMtm1 added a microRNA that regulated expression in cardiac muscle. Here, an extension of the MTM1 gene was chosen as it may confer more endogenous regulatory elements.

Since these preliminary studies were completed, the DESPro-hMTM1-3’UTR DNA vector was packaged in AAV2i8 capsids. This rationally designed capsid mutant demonstrates efficient systemic biodistribution and is detargeted from the liver to reduce immune responses\(^{242}\). This vector has successfully been administered to Mtm1p.R69C mice and canine model with longer survival and improved clinical outcomes (data not shown).
Figure A-1. Vector Diagram. Four rAAV DNA vectors were generated for this study. Previously studied DESPro, was compared to MTMPro, 1.3kb of DNA upstream of the MTM1 transcription start site. These promoters were cloned in front of a Luc (firefly luciferase) reporter gene. The promoters were also cloned in front of hMTM1, for more in depth comparison of their expression of the gene of interest. (a) MTMPro-Luc, (b) DESPro-Luc, (c) MTMPro-hMTM1, (d) DESPro-hMTM1-3’UTR
Figure A-2. Harsh lysis buffers ablate MTM1 activity. (a) Free phosphate as a measure of MTM1 activity from transfected and non-transfected cells lysed in water, IP Lysis Buffer, or RIPA Buffer. The addition of protease inhibitors was evaluated, as well as lysate clearance. (b) Samples from (a) run on western blot to detect total levels of expression. MTM1 is found at ~66kDa, while bands from the IgG heavy chain and light chain from the antibody used in IP, are detected at ~50kDa and ~25kDa, respectively. (c) MTM1 activity of transfected cells with final lysis and activity assay protocol. Mock transfected cells, are transfected with a GFP cassette, while untreated cells are exposed to no DNA or transfection reagent. (d) Western blot of samples used to confirm final lysis and activity assay plus western blot protocol.
Figure A-3. *MTMP* and *DESP* have cell specific expression *in vitro*. (a) Luciferase activity, representative of expression levels produced from transfection of *MTMP-Luc* and *DESP-LUC* into C2C12 Myoblasts and HepG2 Cells. n=3. (b) Luciferase activity, representative of expression levels produced from infection of rAAV9-*MTMP-Luc* in C12 HeLa cells. n = 3
Figure A-4. Comparison of DESPro and MTMPRO in vivo. *WT* 129SVE mice were injected bilaterally into the tibialis anterior with either a high, mid, or low dose of rAAV-hMTM1. Muscle was analyzed one month after administration. (a) Western blots of muscle homogenate after Immunoprecipitation from animals in each dose and vector group. Negative (-) control is homogenate from an animal sham injected with Lactated Ringer’s Solution. Positive Control (+) is immunoprecipitation of transfected cells as in Figure A-2. n = 2-3 mice per group (b) Quantification of band densitometry on western blot. n = 2-3 mice per group. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (c) MTM1 Activity measure by release of free phosphate when immunoprecipitated homogenate from injected muscles is incubated with substrate. n = 1-2 animals per group. Dashed line represents background endogenous activity in *WT* mice.
APPENDIX B
INTRAVENOUS DELIVERY OF rAAV9 VECTORS TO GRAVID MOUSE TRANDUCES
HEART OF PUPS

Introduction

Patients with N-glycanase 1 (NGLY1)-deficiency have only just been diagnosed, using whole exome sequencing that found mutations within the NGLY1 gene within the last 5 years\textsuperscript{332,333}. Parents of these infants became alarmed when their child had generalized lethargy and most notably never produced tears when crying\textsuperscript{334,335}. Other symptoms include developmental delay and seizures, while clinical tests recognize elevated liver enzymes, alanine aminotransferase and aspartate aminotransferase, and peripheral neuropathy\textsuperscript{334}. Due to the heterogeneity in the clinical phenotype, and a lack of identification of NGLY1 mutation pathogenicity, DNA sequencing has become the gold standard for diagnosis\textsuperscript{332}. It has now been reported that p.R401X is the most common mutation observed. This truncation diminishes NGLY1 activity, while other less common mutations reduce the translation of NGLY1 from mRNA, producing fewer total protein molecules\textsuperscript{335}.

N-glycanase 1 is a deglycosylation enzyme involved in endoplasmic reticulum associated degradation (ERAD)\textsuperscript{335–337}. When proteins are synthesized, before they can exit the ER and enter the Golgi apparatus they undergo extensive quality control to ensure proper folding and glycosylation\textsuperscript{338}. When abnormal proteins are identified, they are targeted for degradation\textsuperscript{339}. This process is beneficial for the cell because it frees the ER from aberrant proteins and limits the formation of toxic protein aggregates. The first step in this process is for the protein to retrotranslocate out of the ER, then is deglycosylated, prior to ubiquitination, and finally is broken down by the proteasome. While the exact mechanism of this process is not known, NGLY1 has been deemed a
vital component in the removal of N-linked glycans\textsuperscript{335,336}. During the degradation process, N-linked glycans are removed to generate free oligosaccharides in the cytosol. Specifically, NGLY1 catalyzes the deglycosylation of misfolded N-glycosylated proteins by cleaving the $\beta$-aspartyl glycosylamine linkage of the glycan and the amide side chain of asparagine\textsuperscript{340,341}. This converts the asparagine to aspartic acid.

One of the greatest hindrances to studying the mechanism and potential therapeutics for this disease is the lack of a good mouse model. Attempts by the Lutz research group at the Jackson Laboratory have struggled in the creation of a mouse model by conventional methods, due to embryonic lethality (personal correspondence). Necropsies of the developing pups reveals that a disorder within the cardiovascular system may be culprit to their early death. This detrimental phenomenon is also observed in the attempts at generating rodent models for other human diseases. We propose a model here that delivers a corrected copy of the mutant gene via an Adeno-associated virus (AAV) mediated vector that will provide the missing enzyme to overcome developmental issues. \textit{In utero} studies examining the delivery and expression of AAV vectors have been studied\textsuperscript{342,343}. These studies utilize direct injections to the uterus or fetus, both of which require potentially harmful, invasive procedures. In this study, we evaluate the ability of recombinant AAV serotype 9 (rAAV9) vectors delivered intravenously to a pregnant mouse to be transported in the blood across through the umbilical vein and transduce fetal tissues. In order to provide evidence that this route of administration would be possible we introduced \textit{GFP} under the control of a \textit{CMV} promoter in AAV9 to pregnant mice.
Methods

Vector Production

rAAV9-CMV-GFP vectors were produced and purified as previously described\textsuperscript{344}. rHSV vectors containing either the AAV9 helper plasmid or the transgene plasmid were coinfectected into adherent HEK293s. Forty-eight hours after incubation, cells were collected, washed with PBS, and pelleted. Pellets were lysed and purified by column chromatography followed by tangential flow filtration as described previously\textsuperscript{345}.

Animals

All animal work was completed at the Jackson Laboratory. Equal numbers of female and male FVB/NJ mice, 8 weeks or older, were set up in pair-wise timed matings. The mice were housed in individually and positively ventilated polysulfonate cages with HEPA filtered air at a density of one paired mating per cage. The females were removed to their own cage after the appearance of a plug. The animal room was lighted entirely with artificial fluorescent lighting, with a controlled twelve hour light/dark cycle (6 am to 6 pm light). The normal temperature and relative humidity ranges in the animal rooms were 22 ± 4°C and 50 ± 15%, respectively. The animal rooms were set to have 15 air exchanges per hour. Filtered tap water, acidified to a pH of 2.5 to 3.0, and normal rodent chow was provided ad libitum.

Three females were weighed and injected with a high dose of AAV9-CMV-GFP seven days prior to mating (Group 1) (Table 1). Females were checked daily for plugs first thing in the morning after matings were initiated. Females with plugs were moved to their own cage. Females without plugs were moved to the other side of a duplex and were returned to the male at the end of each day. Pregnancy was confirmed at day E8 via ultrasound. Twelve females, not already assigned to Group 1, that were confirmed to
be pregnant were randomly grouped into Groups 2 - 5 (Table 1). Groups 2, 3, and 4 were weighed and dosed with either rAAV9-CMV-GFP at a low dose and a high dose or Excipient, the vector solvent. Group 5 was weighed and dosed with a high dose of rAAV9-CMV-GFP at day E14.

All dams were allowed to give birth. Three to four pups per dam, depending on litter size, were sacrificed on post-natal days 1 and 30. On pup post-natal day 30, the dams were also sacrificed. At time of sacrifice, the liver, heart, brain, and quadriceps were collected. Tissues were cut in half and rinsed with PBS then snap frozen in liquid nitrogen.

**Vector Genome Quantification**

Vector genome (vg) quantification was completed by the University of Florida, Powell Gene Therapy Center, Toxicology Core (UF-PGTC-TC) as previously described\textsuperscript{346}. Genomic DNA (gDNA) was extracted from the hearts of the pups and dams using a DNeasy blood and tissue kit (Qiagen: 69504) according to manufacturer's instructions. DNA Concentration was determined by spectrometry using a NanoDrop\textsuperscript{®} ND-1000 (ThermoFisher Scientific). An ABI PRISM 7900HT Taqman PCR Machine (Applied Biosystems), was used to quantify rAAV genome copies within one microgram (μg) of gDNA from each sample, using Real-Time Polymerase Chain Reaction (qRT-PCR). Fluorescently labeled primers and probes contain the SV40 polyA target sequence found downstream of the transgene in the rAAV vectors. Reactions were run under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, and 45 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Samples were run in triplicate. To determine PCR inhibition the third replicate was spiked with plasmid DNA at a ratio.
of 100 copies / μg gDNA. Only when this was measured at least 40 genome copies / μg gDNA, were results considered viable.

**Western Blot**

Approximately ten (10) nanograms (ng) of tissue, was homogenized in deionized water with protease inhibitor cocktail (Roche Life Science: 04-693-124-001) using a FastPrep24 (MP Biomedicals) followed by a series of three (3) freeze thaw cycles. Lysates were clarified by 4°C centrifugation at 13.2k rpm for ten minutes. Protein concentrations were determined by DC Protein Assay. 50μg of total protein was loaded into 15% Criterion™ TGX™ Precast Midi Protein Gels (BioRad: 597-1024). The gel was electrophoresed for 30 minutes at 90 Volts followed by 60 minutes at 120 Volts. The proteins were then transferred to PVDF membrane (EMD Millipore: IPFL00010) for 90 minutes at 250mAmps. These blots were probed with an anti-GFP antibody. Blots were blocked in LI-COR Odyssey Blocking Buffer in PBS, which was also used as the antibody diluent for all steps. These blots were washed only after primary and secondary antibody incubation with PBS supplemented with 0.1% Tween-20 (Gentrox: 40-028). All blots were imaged using the LI-COR Odyssey infrared detection system. Band densitometry was determined with coordinating software.
Table B-1. Animal Study Outline

<table>
<thead>
<tr>
<th>Group No.</th>
<th>No. Of Mice Per Arm</th>
<th>Compound Used</th>
<th>Dosage</th>
<th>Dose Timing</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Excipient</td>
<td>200μL</td>
<td>E8</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>rAAV9-CMV-GFP</td>
<td>1x10^{14} vg/kg</td>
<td>-E8</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>rAAV9-CMV-GFP</td>
<td>1x10^{13} vg/kg</td>
<td>E8</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>rAAV9-CMV-GFP</td>
<td>1x10^{14} vg/kg</td>
<td>E8</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>rAAV9-CMV-GFP</td>
<td>1x10^{14} vg/kg</td>
<td>E14</td>
</tr>
</tbody>
</table>
Results & Discussion

While many biodistribution studies have demonstrated a lack of transduction in the germ line cells, one question pertinent to this study was whether the persistence of vector in the bloodstream would be enough to transduce fetal mice. To evaluate this possibility female mice were administered an intravenous dose of $1 \times 10^{14}$ vg/kg rAAV9-CMV-GFP, then mated eight days later (negative embryonic day 8, -E8). Pups were allowed to develop and be delivered naturally at full term. Two pups from each litter were sacrificed at post-natal day 1 (P1) and day 30 (P30). The dams were sacrificed when the pups reached P30, or eight weeks post-injection. Hearts from these mice were evaluated for vector genome copies and protein expression. qPCR and Western Blot analysis for the moms confirmed presence of rAAV DNA and protein expression. Two of the twelve (16.6%) pups evaluated in this group had positive levels of vector genomes. These were not enough to produce enough protein for western blot detection.

The next question to be asked in this study was whether fetal transduction could be achieved following intravenous dosing of a pregnant female. Six females were mated with six males. Eight days after the presence of a plug (embryonic day 8, E8) and positive ultrasound, the females received the vector at two doses, $1 \times 10^{13}$ vg/kg and $1 \times 10^{14}$ vg/kg, through a tail vein injection. As with the previous females, two pups from each litter were sacrificed at P1 and P30 for analysis. Similarly, rAAV DNA was confirmed in the in the hearts of the dams in a dose dependent manner. Two and three pups from each group were positive for vector genomes, yet protein was not detected by western blot.
A final time point of E14, determined by dosing was evaluated. As to be expected and observed in the prior to dosing times, all dams were positive for vector genomes and GFP expression in their hearts. An average of 186 rAAV genomes were present in the three of six positive pups sacrificed at P1, while none of those sacrificed at P30 had detectable copies.

Of the 10 total pups that had positive levels of rAAV DNA, the average of the bottom 9 was 224±38 vg. The highest number of genomes detected in a pup was 1050. While these numbers are significantly lower than what is found in the dams, and protein was unable to be seen by western blot, it is possible that there is enough to rescue an embryonic lethal phenotype.

While rAAV vectors transduced 16.7% of the pups’ cardiac muscle, there was grave heterogeneity in the distribution of the vector. As a proof-of-concept for a method of correcting an embryonic lethal phenotype, the success of transducing the predicted one-quarter homozygous recessive progeny would be low, although not necessarily impossible. The data from this study contribute to the knowledge about how rAAV vectors function in a biological system during fetal development. Despite the large ethical debate regarding vertical transmission of recombinant vector, evidence here demonstrates the low potential for transmission to a female that becomes pregnant shortly after administration, or who receives an rAAV injection while gestating.
Figure B-1. Vector Genome Copy Number in Hearts of Dams, P1 pups, and P30 pups by Quantitative Polymerase Chain Reaction. The left-most column contains the timing of dosing as well as the dose level. Excipient is the vector vehicle delivered to mice as a negative control. The second column contains the dam number for each group. The next column is the vector copy number, obtained by qPCR, present in the heart of dams 5 – 8 weeks after injection. Columns labeled P0 and P30 list the vector copy number found in the heart for 2 pups per dam, collected at age either P0 or P30. Vector copy numbers over 100 are considered positive. Heterogeneity between pups of the same litter reveal the unequal distribution of vector from dam to pups.
Figure B-2. GFP is expressed in hearts of injected dams. (a) Western Blotting was used to detect GFP in the cardiac muscle of injected dams, to confirm expression in a dose dependent manner. Loading control Gapdh was used. (b) Quantification of band densitometry of GFP western blot bands normalized to the densitometry of Gapdh. Group 1: Excipient, Group 2: -E8, 1x10^{14} \text{vg/kg}, Group 3: E8, 1x10^{13} \text{vg/kg}, Group 4: E8, 1x10^{14} \text{vg/kg}, Group 5: E14, 1x10^{14} \text{vg/kg}.
LIST OF REFERENCES


264. Lumizyme | Dosing & Administrationat


BIOGRAPHICAL SKETCH

Angela Layne McCall is the daughter of Mr. and Mrs. William R. McCall, Jr. and grew up in Okeechobee, Florida. In the summer of 2007, Angela attended the University of Florida, Center for Precollegiate Education and Training, Student Science Training Program, where she worked under the direction of Mavis Agbandje-McKenna, PhD. It was during this time that she originally developed an interest in Adeno-associated viruses. After graduating from Okeechobee High School in 2008, she chose to attend the University of Florida and pursue a bachelor’s of science degree. Angela majored in Interdisciplinary Studies in Biochemistry and Molecular Biology, continuing her research on AAV capsid and host cell interaction with Dr. Agbandje-McKenna. She worked for UF-CPET in the summers and minored in Extension Education, each of which sparked a passion for teaching. Upon graduating magna cum laude in 2012, Angela entered the Interdisciplinary Program in the Biosciences at UF. She wanted to turn her focus from structural basic biology of AAVs to the translational side of gene therapy, and thus began her research with gene therapy pioneer, Barry J. Byrne, MD, PhD, in the Genetics Concentration. She received her Ph.D. in the spring of 2017.