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Adeno-Associated Virus–Mediated Gene Therapy for Metabolic Myopathy

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Abstract

Metabolic myopathies are a diverse group of rare diseases in which impaired breakdown of stored energy leads to profound muscle dysfunction ranging from exercise intolerance to severe muscle wasting. Metabolic myopathies are largely caused by functional deficiency of a single gene and are generally subcategorized into three major types of metabolic disease: mitochondrial, lipid, or glycogen. Treatment varies greatly depending on the biochemical nature of the disease, and unfortunately no definitive treatments exist for metabolic myopathy. Since this group of diseases is inherited, gene therapy is being explored as an approach to personalized medical treatment. Adeno-associated virus–based vectors in particular have shown to be promising in the treatment of several forms of metabolic myopathy. This review will discuss the most recent advances in gene therapy efforts for the treatment of metabolic myopathies.

Introduction

Metabolic myopathies are a heterogeneous group of rare genetic diseases in which a deficiency in cellular metabolism ultimately results in muscle dysfunction ranging from exercise intolerance to progressive muscle wasting to cardiorespiratory impairment or failure. Most metabolic myopathies are categorized as inborn errors in glycogen, lipid metabolism, or mitochondrial function, and the primary disease manifestations are usually attributed to mutations in a single gene. Unfortunately, to date, no effective treatment exists for any inherited metabolic myopathy and therapy is primarily palliative and in some instances may include modifications in diet and activity. No small-molecule drugs have been approved for a specific indication. Like all myopathies, even small improvements in muscular function can result in substantial improvement in daily living and quality of life. Over the past decade, gene transfer technology has progressed to become one of the most promising forms of therapy for the treatment of metabolic myopathies. In this review, we will discuss the current status of adeno-associated virus (AAV)–based gene therapy for the treatment of the more prevalent metabolic myopathies. Dr. Sonia Skarlatos was an unwavering advocate of these programs, first via the National Heart, Lung, and Blood Institute (NHLBI)–sponsored Programs of Excellence in Gene Therapy and then further via the Gene Therapy Resource Program.

Gene Therapy Vectors

Although earlier gene transfer studies for metabolic myopathies had been performed using adenoviral or retroviral vectors, by far the most significant advances have been made using AAV vector systems. AAVs are members of the *Dependovirus* genus of the family Parvoviridae. One of the most attractive features of AAV as a gene therapy vector system is the lack of any known association with human disease. While the original recombinant vectors were based on AAV serotype 2 (AAV2), identification of other naturally occurring serotypes have led to the development of alternate pseudotype vectors, all of which have distinct pharmacologic profiles with different cell-type tropisms and transduction efficiency levels. In the pseudotyped vectors, the vector genome retains the AAV2 inverted terminal repeats that flank both ends of the vector genome (Gao *et al.*, 2005; Byrne *et al.*, 2012). In addition, systematic analysis of the viral capsid protein has identified capsid regions and nucleotides that can be modified by either mutation or peptide insertion to generate novel vectors with even greater specificity and/or efficiency (Muzyczka and Warrington, 2005; Zhong *et al.*, 2008; Asokan *et al.*, 2012). Furthermore, modifications in the vector genome itself, such as the generation of double-stranded vector genomes, have also significantly changed the pharmacokinetics of AAV vectors (McCarty, 2008; Wang *et al.*, 2011; Zhong *et al.*, 2012). Recombinant AAV vectors

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have been shown to stably transduce both dividing and nondividing cells with both high efficiency and limited toxicity and, as a result, are currently the most actively pursued gene therapy vectors. In 2012, Glybera became the first AAV-based treatment option after receiving approval from the European Commission for lipoprotein lipase deficiency. To date, more than 40 AAV gene therapy clinical trials have been approved in the United States for myriad disease conditions.

Mitochondrial Myopathies

Mitochondrial myopathies encompass a group of disorders that share the inability to produce ATP efficiently. These myopathies become apparent in infancy and result in cardiovascular, skeletal muscle, and neurological manifestations. Although hundreds of pathological prtochopdcial mitochondrial DNA (mtDNA) mutations have been described, there are either no treatment options or merely only supportive treatments available. With many mitochondrial proteins being nuclear encoded genes, gene therapy is a promising tool for future treatment.

Adenine nucleotide translocator (*ANT-1*) mutations in humans can cause cases of progressive external ophthalmoplegia, which is caused by paralysis of the extraocular eye muscles. In addition, recent analysis clearly demonstrated progressive myopathy, exercise intolerance, and severe dilated cardiomyopathy in ANT-1-deficient patients (Strauss *et al*., 2013). The mouse model of ANT-1 deficiency results in ragged red fibers, hyperinduction of mitochondrial enzymes, and mtDNA rearrangements. Flierl *et al.* (2005) successfully treated the mitochondrial myopathy observed in ANT-1-deficient mice with AAV2 encoding mouse *ANT-1*. After direct intramuscular administration, detectable levels of the heart–skeletal muscle isoform were observed and accompanied by reversal of histopathological damage in ANT-1-deficient mice. The *ANT-1* protein was localized in the mitochondria, resulting in 25–45% of normal ATP levels. Further experiments will determine if the initial success observed in the murine model with an AAV vector can be expanded and become a viable treatment option to correct a mitochondrial metabolic defect and associated pathology.

High heteroplasmic mtDNA mutation loads are often required to reach a disease-causing threshold. In one recent study, Bacman *et al.* (2012) expressed a mitochondria-targeted restriction endonuclease packaged in AAV2/9 to manipulate mtDNA heteroplasmy systemically in all muscles. The mitochondrial restriction endonuclease, ApaLI, contained a mitochondrial targeting sequence at the N-terminus, which had been previously shown to allow for protein transport into the mitochondria (Owen *et al*., 2000). A similar study was also performed to obtain organ-specific expression in which ApaLI was packaged in AAV2/6 and adenovirus type 5 to obtain preferential cardiac transduction and liver transduction, respectively (Bacman *et al*., 2010). The viral vectors were tested in a heteroplasmic mouse model containing two polymorphic mtDNA sequence variants, NZB and BALB/c, with only the BALB/c variant containing an ApaLI site. In the systemic study with AAV2/9-mito-*APALI-HA*, delivery resulted in robust transduction of heart and skeletal muscle. APALI restriction fragment polymorphism was performed to investigate shifts in mtDNA heteroplasmy. Cardiac and skeletal muscle from treated mice had a significantly higher percentage of NZB mtDNA compared with control tissue from mice administered an AAV2/9-alkaline phosphatase reporter. Using quantitative polymerase chain reaction to measure mitochondrial and nuclear DNA ratios in skeletal muscle, heart, and liver, no changes in mtDNA levels were observed, demonstrating that endonuclease expression did not deplete overall mitochondrial content. Although this study did not directly investigate a therapeutic benefit from manipulating mitochondrial heteroplasmy, it successfully demonstrates that delivery of a mitochondrial restriction endonuclease can induce significant changes that may result in below threshold levels of mutant mtDNA. Such gene therapy strategies, once optimized, could be directed toward mitochondrial myopathies with heteroplasmy such as myoclonic epilepsy with ragged red fibers (MERRF) or mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS).

One of the many obstacles of using gene therapy to treat mitochondrial diseases is the barrier of getting the therapeutic DNA into the mitochondria. Two unique approaches have been evaluated for treatment of NADH ubiquinone oxidoreductase subunit 4 (ND4) deficiency. ND4 is a mitochondrial encoded protein and is a subunit of complex I. Mutations within *ND4* are known to cause Leber hereditary optic neuropathy. In one study, Bai *et al.* (2001) revealed that expression of the NADH–quinone oxidoreductase of *Saccharomyces cerevisiae* (*NDI1*) could restore the NADH dehydrogenase activity in human *ND4* mutant cells. NDl1 expressed by the Rous sarcoma viral promoter was packaged in an AAV2/2 capsid and was used to transduce ND4-deficient cells. Confocal fluorescence microscopy showed co-localization of vector and mitochondria. Furthermore, complex I substrate-specific oxidative phosphorylation demonstrated that respiration of treated cells was approximately two-thirds of wild-type levels and had restored capacity to grow in galactose medium. Although NDl1 is not proton translocating, is rotenone-insensitive, and requires a sequence for import into the mitochondria as opposed to ND4, the authors demonstrated that NDl1 could restore function while circumventing the dilemma of translating the therapeutic transgene in the mitochondria.

More recently, Yu *et al.* (2013) demonstrated the feasibility of directly targeting AAV to the mitochondria by fusing a mitochondrial targeting sequence to the AAV2 VP2 capsid protein to achieve ND4 expression in the mitochondria. The authors altered the AAV2 VP2 capsid protein by adding a presequence of cytochrome oxidase subunit 8 fused at the N-terminus of GFP into capsid open reading frame (ORF). Human *ND4* cDNA driven by the heavy-strand mitochondrial promoter was packaged into the mutant AAV2 capsid. Despite altering the AAV2 capsid, the authors were able to obtain high titers and maintain efficient infectivity. The vector was used to transduce hybrid cell lines containing mitochondria with a human *ND4* mutation. Agarose gel electrophoresis, quantitative reverse transcription–polymerase chain reaction, fluorescent microscopy, and immunoblotting all demonstrated that not only was the mutant AAV capsid localized within the mitochondria, but also that the delivered ND4 protein was properly translated in cells. Oxidative phosphorylation was measured using complex I substrates to investigate a therapeutic correction, and compared with controls, ATP synthesis was increased 48% in treated cells.

Because of the small size of mitochondrial genes, including *ND4*, both single-stranded AAV vectors and self-complementary AAV vectors contain an appropriate capacity for packaging. Therefore, the authors chose to reclone the *ND4* construct in an scAAV construct in order to improve rescue and overall expression for *in vitro* and *in vivo* transduction. Intraocular injections performed in mice confirmed mitochondrial localization and ND4 expression. Furthermore, they demonstrated that AAV delivery of *ND4* rescues visual dysfunction caused by a previous injection of an allotropic human *ND4* mutant in mouse eyes. This study demonstrates that mitochondrial gene delivery by AAV is achievable. Furthermore, this work paves the way for treating many mitochondrial myopathies that until now has remained an unachievable goal.

Lipid Metabolism Disorders

Glycolysis, the breakdown of glucose to pyruvate for use in the citric acid cycle, results in production of ATP. This process is traditionally the mode of energy generation in most cells. During metabolic stress, fasting, or extensive exercise, cells utilize fatty acid oxidation to break down fat stores for energy production (Keeler and Flotte, 2012). This is particularly prominent in highly oxidative tissues (Goetzman, 2011; Keeler and Flotte, 2012). Fatty acid oxidation occurs via β-oxidation within the mitochondria using precursors generated from the esterification of fatty acids by coenzyme-A to produce acyl-CoA. The catalysis of acyl-CoA and free carnitine to Acyl-carnitine by carnitine palmitoyltransferase-1 permits passage across the mitochondrial membrane where carnitine palmitoyltransferase-2 reverts acyl-carnitine to acyl-CoA and free carnitine. Inside the inner mitochondrial membrane, β-oxidation occurs via the breakdown of straight chain fatty acids by a number of acyl-CoA dehydrogenases named after the fatty acid chain lengths acting as their substrates (very long-, long-, medium-, and short-chain acyl-CoA dehydrogenase [VLCAD, LCAD, MCAD, and SCAD, respectively]) (Keeler and Flotte, 2012). Ultimately, mitochondrial membrane transfer is critical to fatty acid oxidation and in the case of β-oxidation defects; the transport system works in reverse except that the fatty acids accumulate as acyl-carnitine in the cytosol and subsequently leach out of the cell and into the blood (Keeler and Flotte, 2012).

VLCAD deficiency is one of the most common lipid metabolism myopathies with an incidence of 1:30,000 in the United States. VLCAD is responsible for the initial breakdown of fatty acids with chain lengths greater than 14 carbons in mitochondrial β-oxidation and is highly expressed in heart, liver, and skeletal muscle tissues. VLCAD deficiency results in improper metabolism of fatty acids resulting in progressive accumulation of very-long-chain fatty acids and partially metabolized fatty acids in cells. There is a range of disease phenotypes dependent on the degree of residual enzyme activity. Early-onset disease usually presents with severe hypertrophic or dilated cardiomyopathy, hypotonia, hepatomegaly, and hypoglycemia and is often fatal. A less severe hepatic phenotype typically emerges in early childhood with hepatomegaly and hypoglycemia, without cardiomyopathy. Late-onset VLCAD deficiency is the most common phenotype and presents with exercise intolerance, muscle pain, and rhabdomyolysis. VLCAD deficiency is largely managed by a low-fat, high-carbohydrate diet and monitored exercise and activity (Leslie, 2009). VLCAD deficiency provides an attractive candidate for gene therapy because of the severity of disease, lack of treatment options, and a single genetic determinant. The pathological mechanisms of VLCAD deficiency have only recently become clearer, in part because of the development of a functional animal model. In conjunction, preclinical evaluation of gene therapy for the treatment of VLCAD has so far been encouraging.

In 2009, Merritt *et al.* described the first *in vivo* demonstration of biochemical correction of VLCAD deficiency in the VLCAD-deficient mouse model using gene therapy (Merritt *et al.*, 2009). In this study, a recombinant rAAV2/8 vector encoding human *VLCAD (hVLCAD)* driven by the cytomegalovirus (CMV) promoter was administered via tail vein injection to adult VLCAD-deficient mice and analyzed between 14 and 104 days postinjection. Treated mice did not develop fasting hypoglycemia throughout the duration of the study. Acylcarnitine analysis using tandem mass spectrometry of dried blood spots showed an initial drop in C16, C18, and C18:1 acylcarnitine species at 11 days postinjection. Slight re-accumulation of acylcarnitines by day 14 (potentially because of reduced hVLCAD expression in hepatocytes) was observed, but these levels did not approach those observed pretherapy. VLCAD expression was initially evident in heart and liver tissue at 14 days postdosing; however, heart expression appeared more robust with expression still reported 104 days after vector administration. Minimal expression was seen in skeletal muscle. This study demonstrated the feasibility of gene therapy to correct VLCAD deficiency.

Recently, Keeler *et al.* (2012) further developed the potential for gene therapy to correct VLCAD deficiency using a recombinant AAV2/9 vector to express hVLCAD under control of the CMV enhancer/chicken beta-actin promoter delivered via tail vein injection to adult VLCAD-deficient mice. AAV2/9 vectors have been widely demonstrated as one of the most efficient AAV vectors to transduce heart, skeletal muscle, and liver tissue *in vivo* (Pacak *et al.*, 2006, 2008b; Bish *et al.*, 2008; Zincarelli *et al.*, 2008). As production of body heat requires fatty acid oxidation, VLCAD-deficient mice are unable to maintain body temperature in response to cold challenge. This is further exacerbated when combined with fasting, which is fatal in this animal model. All AAV2/9-*hVLCAD*-treated mice survived a cold fast challenge with lethargy, hypotonia, and blood glucose levels comparable with wild-type levels postchallenge. Using proton magnetic resonance spectroscopy (^{1}H-MRS), AAV2/9-treated mice exhibited decreased lipid content in skeletal muscle to levels seen in wild-type mice as well as reduction in accumulated acylcarnitine. Widespread hVLCAD expression was sustained throughout the duration of the study to 22–26 weeks postinjection, evident by hVLCAD expression in liver, heart, tibialis anterior, and brown adipose tissue. Together, these studies suggest that gene therapy has the potential to be a viable option for the treatment of VLCAD deficiency.

A study by Kelly *et al.* (1997) reported gene modulation as a viable therapeutic option in SCAD deficiency. The authors developed a transgenic mouse with liver-specific expression of SCAD on an otherwise systemic knockout background. The study highlights a number of important factors and

demonstrates that tissue-specific SCAD expression is sufficient to correct the organic aciduria and fatty acid changes of the liver and that overexpression of SCAD is not detrimental to homeostatic physiology. Developing these observations, Holm *et al.* (2003) reported that injection of plasmid DNA encoding *SCAD* under control of a ubiquitin promoter into the tail vein of SCAD-deficient mice induced liver-specific expression in a subset of liver cells. Although initially therapeutic as demonstrated by reduction of serum butyrylcarnitine levels, SCAD activity was undetectable 3 weeks postinjection likely because of the low transduction efficiency and insufficient cell-to-cell substrate channeling.

An alternative approach incorporating AAV2/1-*SCAD* using skeletal muscle as a depot for enzyme production has been reported by Conlon *et al.* (2006). Direct injection of the tibialis anterior muscle with AAV2/1-*SCAD* increased SCAD enzyme activity and decreased serum butyrylcarnitine levels 10 weeks postinjection with fasting to induce fatty acid oxidation. Similar systemic correction in SCAD deficiency resulting from single-tissue expression has also been demonstrated by AAV2/8-*SCAD* transduction of the liver after portal vein injection (Beattie *et al.*, 2008).

Relatively few studies have described the use of gene therapy in MCAD deficiency. Treatment of MCAD-deficient primary human fibroblasts with a first-generation adenoviral vector or AAV2 encoding human *MCAD* resulted in significant MCAD expression that was sufficient to improve acylcarnitine profile (Ii *et al.*, 2004; Schowalter *et al.*, 2005). These initial studies provide important preliminary information for future studies using AAV-based therapy in the murine model of MCAD deficiency (Tolwani *et al.*, 2005).

Collectively, these studies demonstrate great advances in the development of treatments for fatty acid oxidation disorders. Tissue-specific and systemic targeting strategies have shown benefit and should be considered as potential candidates to systemically correct β-oxidation deficiency. Specific requirements of therapeutic transgenes in affected tissues are yet to be determined, and alternative serotypes may provide enhanced therapeutic outcomes. Nonetheless, gene therapy promises to be a viable therapeutic candidate for lipid metabolism disorders.

Metabolic Myopathy

X-linked myotubular myopathy (XLMTM) is considered a phosphatase disorder with an incidence of 1 in 50,000 live male births. XLMTM is caused by mutations in the *MTM1* gene located on the X chromosome. Characteristic features include the presence of small myofibers with abnormal intracellular organization and most notably centralized nuclei and mitochondria. *MTM1* encodes a phosphoinositide phosphatase, myotubularin, which is ubiquitously expressed and has been associated with intracellular trafficking and autophagy, lipid metabolism, and cellular proliferation; however, the exact nature and function of myotubularin has yet to be elucidated (Laporte *et al.*, 2000; Grange *et al.*, 2012). Disease pathology begins *in utero*, and affected males exhibit profound muscle weakness, hypotonia, and severe respiratory insufficiency and many require assisted ventilation or do not survive past the first year of life.

Although the mechanism of disease progression is not well understood, the loss of myotubularin in XLMTM has lead to the consideration of gene therapy as a rational therapeutic approach. Work by Buj-Bello *et al.* (2008) investigated the consequences of intramuscular delivery of AAV2/1-*Mtm1* into 4-week-old Mtm1-deficient mice that already exhibited disease-related pathology. At 4 weeks postdosing, a significant increase in myofiber volume and muscle mass was noted in the treated muscle. In addition, subcellular organization and morphology was restored. Furthermore, contractile strength of treated muscle was similar to wild-type levels and treated hind limbs showed marked clinical improvement. This study demonstrated the potential for gene therapy to treat XLMTM; however, it should be noted that overexpression of Mtm1 led to plasma membrane pathology and as such, future success of gene therapy for XLMTM may require strict control of transgene expression.

Murine, canine, and zebrafish models of XLMTM exist and provide a valuable resource in understanding the process of disease pathology (Laporte *et al.*, 2000; Beggs *et al.*, 2010). Recent work by Joubert *et al.* (2013) utilized an AAV2/1 vector expressing Cre recombinase, not as a therapeutic, but as an analytic tool to further explore the consequences of MTM1 deficiency in skeletal muscle of an Mtm1 conditional knockout mouse model. Knockout of Mtm1 in adult skeletal muscle resulted in the characteristic histologic abnormalities and myopathy associated with XLMTM, indicating that MTM1 is essential not only in the early stages of life but also in maintaining adult skeletal muscle function. Interestingly, co-delivery of a therapeutic AAV2/9 vector encoding *Mtm1* with the Cre recombinase-carrying vector prevented disease pathology. This showed that the XLMTM phenotype is attributed primarily to the loss of MTM1 and again suggests the potential of AAV-mediated gene therapy to correct disease.

Glycogen Metabolism Disorders

Glycogen storage disorders are characterized by defects in glycogen metabolism manifesting in a spectrum of phenotypic severity dependent upon the disease-causing deficient component. Approximately seven disorders affect skeletal muscle, with phenotypes ranging from exercise intolerance to muscle weakness.

McArdle disease (glycogen storage disease type V, myophosphorylase deficiency) results from a deficiency in the muscle-specific isozyme of glycogen phosphorylase. Myophosphorylase allows for the release of glucose from stored glycogen in muscle, which can then enter the glycolytic pathway ultimately producing ATP. With an incidence of approximately 1:100,000 live births, McArdle disease onset occurs during childhood with patients suffering from exercise intolerance, cramping, weakness, fatigue, and myoglobinuria (Dimaur *et al.*, 2002; Nogales-Gadea *et al.*, 2007). Typically, McArdle is not diagnosed until adulthood. As no therapy has been established for McArdle disease, treatment consists of diet (high protein, low carbohydrate) and activity (submaximal aerobic) modification (Phoenix *et al.*, 1998; Haller, 2000; Vorgerd *et al.*, 2000).

Limited gene therapy studies have been conducted for McArdle disease, and to date there are only initial proof-of-concept studies that demonstrated the ability of adenoviral vectors to express myophosphorylase in myoblast cell lines increasing glycogenolytic capacity (catalysis of glycogen to

glucose) (Baque *et al*., 1994; Pari *et al*., 1999). Gene therapy approaches have been employed to restore myophosphorylase *in vivo.* Using AAV2 to mediate delivery of human myophosphorylase in a sheep model of McArdle disease, Howell *et al.* (2008) demonstrated localized glycogen metabolism was feasible with gene therapy. Future studies with optimized vectors and delivery routes may further establish the potential for gene therapy for McArdle disease.

Glycogen storage disease type VII (phosphofructokinase deficiency/Tarui disease) is clinically indistinguishable from GSDV/McArdles disease although pathologically displays polyglucosan accumulation in muscle (DiMauro and Spiegel, 2011). Polyglucosan deposits appear to result from an imbalance of glycogen synthase and glycogen branching enzyme activity (Raben *et al.*, 2002). GSDVII therefore provides an attractive candidate for substrate reduction therapy (SRT) using gene therapy approaches. In principle, SRT acts to restore the equilibrium in enzyme activity by inhibiting expression of the more active enzymes within a pathway (using short hairpin RNA [shRNA]) in patients with sufficient residual activity in the disease-causing deficient enzyme. SRT efficiency has been demonstrated in GSDII/Pompe disease both *in vitro* and *in vivo* targeting glycogenin and glycogen synthase. *In vitro* lentiviral-mediated shRNA delivery into C_2C_{12} myoblasts and primary myoblasts from Pompe mice inhibited glycogen synthase and glycogenin resulting in a decrease in accumulation of glycogen in the lysosomes. Intramuscular injection of AAV2/1-*shGYS2* reduced levels of glycogen synthase and subsequently resulted in a 62% decrease in glycogen accumulation 4 weeks postinjection in Pompe mice (Douillard-Guilloux, 2008). Although uncertainties remain surrounding the persistence and long-term effects of knockdown, these studies demonstrate AAV-mediated SRT as a potentially viable option for the treatment of GSDs where no single genetic determinant has been identified or no current alternative therapies appear prominent.

Pompe disease (glycogen storage disease type II, acid-maltase deficiency) represents a metabolic myopathy displaying the most significant therapeutic advances and development toward a gene therapy treatment. Pompe disease is caused by a deficiency in acid alpha-glucosidase (GAA), the enzyme responsible for the breakdown of lysosomal glycogen (Baudhuin, 1964; Hers, 1963; Hirschhorn and Reuser, 2001). Affecting 1:40,000 individuals, GAA deficiency results in extensive glycogen accumulation within lysosomes, resulting in cellular dysfunction. Biweekly enzyme replacement therapy (ERT) with a recombinant human GAA (Myozyme) is the only available treatment. Since the inception of ERT, a new natural history of disease has begun to emerge in which patients with Pompe disease are surviving longer but 2/3 of patients on current therapy eventually require assisted ventilation for respiratory dysfunction (Yang *et al*., 2011). Although ERT improves patient survival and disease progression, incomplete rescue warrants further investigation into alternative treatment strategies (Kishnani *et al*., 2007; Byrne *et al*., 2011a,b).

Gene therapy offers several potential treatment advantages over current ERT therapy, including the ability to directly target correction of cardiac and striated muscle in conjunction with the potential for a single treatment to provide long-term pathologic attenuation. Cell autonomous correction using gene therapy has the potential to promote natural cellular mechanisms of trafficking of GAA, reducing circulating enzyme concentrations that could trigger formation of anti-GAA antibodies. Initial gene transfer studies for Pompe disease were performed using retrovirus or adenovirus vectors; however, AAV vectors rapidly became a frontrunning candidate (Zaretsky *et al*., 1997; Pauly *et al*., 1998, 2001; Tsujino *et al*., 1998; Amalfitano *et al*., 1999; Ding *et al*., 2001).

Disruption of skeletal muscle architecture and function is well established as a primary cause of this myopathy in Pompe disease. However, an emerging potential mechanism of neuromuscular junction maladaptation can account for respiratory insufficiency in the Pompe disease mouse model. Supporting this concept, several patient case reports document glycogen accumulation in the central nervous system (CNS) and spinal neurons (Gambetti *et al*., 1971; DeRuisseau *et al*., 2009). This is also true in animals models where motoneurons dysfunction has been observed in both hypoglossal and phrenic motoneurons (DeRuisseau *et al*., 2009; Mah *et al*., 2010; Lee *et al*., 2011; Falk *et al*., 2013). Although the exact mechanism by which glycogen accumulation in motoneurons elicits dysfunction remains unknown, others have noted that motorneurons are particularly vulnerable to excessive glycogen accumulation through apoptosis (Vilchez *et al*., 2007). The inability of ERT to traverse the blood–brain barrier to the CNS suggests that a continued myopathic progression in ERT patients may result from progressive deterioration of lower motor neuron function.

As with SCAD, a gene therapy strategy for Pompe is attractive, taking advantage of the cross-correction phenomenon utilizing skeletal muscle or liver as a depot for GAA enzyme production systemically treating affected tissues. Liver-directed delivery of AAV2/5 or AAV2/8 into a Pompe mouse model significantly increased enzyme levels in the diaphragm and hind-limb muscles, reducing glycogen content in those injected tissues (Fraites *et al*., 2002; Cresawn *et al*., 2005; Sun *et al*., 2005). The level of correction, however, is partially dependent on whether a humoral immune response against the expressed and secreted rAAV-encoded GAA is present.

Systemic delivery of AAV has been investigated using alternative serotypes displaying broader cellular tropism to assess simultaneous correction of multiple muscle systems. Age of administration and viral serotype affect efficacy of treatment.

Intravenous delivery of AAV2/1 to neonatal Pompe mice resulted in sustained GAA expression and glycogen clearance in skeletal muscle. Furthermore, soleus and diaphragm contractile strength were improved, corresponding with improved ventilatory capabilities up to 1 year posttreatment (Mah *et al*., 2007; Byrne *et al*., 2011a). Systemic delivery of AAV2/9 to neonates improved cardiac pathology 3 months postinjection (Pacak *et al*., 2006). Intravenous delivery of muscle-restricted AAV2/8 or AAV2/9 vectors also lead to reduced glycogen content in striated muscle and diaphragm at 4.5 months posttreatment when administered to young adult Pompe mice (Sun *et al*., 2008).

In preclinical murine and nonhuman primate studies, recombinant AAV2/9 vectors have recently demonstrated a higher capacity to transduce heart tissue and myofibers (Pacak *et al*., 2006, 2008a; Sun *et al*., 2008; Tarantal and Lee, 2010). This makes AAV2/9 an ideal candidate vector to

correct Pompe disease-related myopathy and cardiomyopathy. As ERT can only partially attenuate Pompe pathology, our group sought to develop therapeutics targeting the respiratory system, ultimately the end point for disease progression. AAV2/9 is able to undergo retrograde transport, thereby transducing motoneurons after intramuscular injection, providing an alternative avenue of delivery route to correct the CNS (ElMallah *et al.*, 2012; Fuller *et al.*, 2013). Recently, Falk and coworkers demonstrated that a single intrapleural injection of AAV2/9-*hGAA* in adult Pompe mice could lead to simultaneous improved cardiac and respiratory function (Byrne *et al.*, 2012; Falk *et al.*, 2013). At 6 months postinjection, improved phrenic nerve burst amplitude and diaphragm EMG during a hypercapnic challenge was noted in the AAV2/9-treated mice, indicating correction of CNS deficits as well.

Direct administration of AAV has yielded the highest degree of biochemical and functional correction of the diaphragm in the Pompe mouse model (Mah *et al.*, 2004; Rucker *et al.*, 2004). AAV2/1-CMV-*hGAA* administered directly to the diaphragm of Pompe mice results in therapeutic levels of GAA activity, clearance of accumulated glycogen, and improved *in vivo* ventilatory capabilities, and provides strong preclinical evidence warranting further investigation (Byrne *et al.*, 2011b).

An open-label phase I/II study to administer AAV2/1-CMV-*hGAA* by direct intramuscular injection to the diaphragm of ventilator-dependent Pompe children has been initiated at the University of Florida (clinicaltrials.gov, identifier NCT00976352) (Stroes *et al.*, 2008; Brantly *et al.*, 2009; Mendell *et al.*, 2009). Participants received a dose of either 1×10^{12} vector genomes or 5×10^{12} vector genomes of AAV1-*hGAA* injected directly into the diaphragm muscle. Long-term follow-up of five subjects showed significantly improved unassisted tidal volume (median [interquartile range]: 28.8% increase [15.2–35.2], $p<0.05$), with most patients tolerating longer periods of unassisted breathing (425% increase [103–851%], $p=0.08$). Functional changes in unassisted ventilation were noted as early as 90 days after administration, and continued through day 365. No serious adverse events related to the vector or the therapeutic transgene product were observed. Importantly, caregivers reported that increased off-ventilator endurance resulted in meaningful and significant improvements in the daily care of their children. These observations have demonstrated that AAV gene therapy is well tolerated and can lead to clinical improvement for patients with Pompe disease (Smith *et al.*, 2013).

Strategies increasing GAA activity in the CNS and PNS could play a pivotal role in the treatment of respiratory insufficiency of Pompe patients. Together, these results provide a rational basis for the continued development of gene therapy for the treatment of Pompe disease.

Future Directions

Although heterogeneous in nature, the genetic basis and current lack of curative treatments support the rationale of gene therapy as a candidate therapeutic for metabolic myopathies. AAV gene transfer is proving to be an exceptional technology for conferring long-term expression of biologically active proteins in metabolic myopathies. While AAV-based strategies are not completely void of the pitfalls observed with recurrent administration of recombinant protein (e.g., potential for neutralizing antibody formation), numerous advantages exist.

Although many gene transfer studies for metabolic myopathies are in the initial proof-of-concept stages, the data so far indicate the potential promise of gene therapy as an effective treatment modality. Continued refinement of gene transfer strategies should yield preclinical therapeutic outcomes that will warrant entering the next phase of clinical assessment. The clinical study of AAV gene therapy for the treatment of Pompe disease was a critical first step in the establishment of gene therapy as a treatment for metabolic myopathies. The study was based on the culmination of two decades of work, and the leap from basic bench research to the clinic was immeasurably facilitated by Dr. Sonia Skarlatos and her initiatives in implementing gene therapy resource programs through the NIH and extramural centers (Lauer and Skarlatos, 2010; Tarantal and Skarlatos, 2012; McDonald *et al.*, 2013). For many, especially those in the rare disease arena such as metabolic myopathies, the NHLBI gene therapy resource programs have been and hopefully will continue to fulfill a critical need to establish a viable therapeutic option for inherited myopathies affecting heart and skeletal muscle.

Author Disclosure Statement

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