

2021

Adeno-associated virus vectors-mediated gene therapies for inherited neuromuscular diseases

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SCHOOL OF MEDICINE

Thesis

**ADENO-ASSOCIATED VIRUS VECTORS-MEDIATED GENE THERAPIES
FOR INHERITED NEUROMUSCULAR DISEASES**

by

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B.A., Boston University, 2019

Submitted in partial fulfillment of the
requirements for the degree of
Master of Science

2021

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DEDICATION

I would like to dedicate this work to my amazing parents Amy and Colin, my readers Dr. Vandana Gupta and Dr. Simon Levy for their time and valuable feedbacks.

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QIFAN SHANG

ABSTRACT

Inherited neuromuscular diseases are a group of disorders that affect skeletal muscle or neuronal control of the skeletal muscle. Due to the incredible clinical and genetic heterogeneity of these disorders, very few curative therapies are available. Affected patients often require lifetime treatments to manage their symptoms and a great number of them succumb to premature deaths due to respiratory failures. Recent development in gene therapy, especially Adeno-Associated Virus (AAV) vectors provide exciting avenues for curing monogenic neuromuscular diseases by delivering a functional copy of the defective gene into the musculature. This review covers recent progress made to implement AAV vectors as a viable treatment option for inherited neuromuscular diseases including advancements in engineering technologies for AAV vector optimization, promising results from preclinical animal studies and clinical trials. Current obstacles to clinically translate AAV gene replacement therapy are further discussed.

In conclusion, AAV-based gene therapy serves as an ideal treatment to correct the genetic defects underlying inherited neuromuscular diseases with recent regulatory approvals as the basic and translational science proves safe and effective. Further understanding of AAV vector mechanisms and advancements in capsid engineering are needed to build a stronger foundation to establish AAV vector-mediated gene

replacement therapy as a standard treatment option clinically to treat inherited neuromuscular diseases.

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LIST OF ABBREVIATIONS

AAT	Alpha-1-Antitrypsin
AAV	Adeno-Associated Virus
AAVR	Adeno-Associated Virus Receptor
AdV	Adenovirus
AIP	Acute Intermittent Porphyria
BMD	Becker Muscular Dystrophy
CAG	CMV Enhancer-Chicken-Beta-Actin
Cas	CRSPR-associated
CB	Chicken Beta-Actin
CHOP-INTEND	Children Hospital of Philadelphia Infant Test of Neuromuscular Disorders
CMV	Cytomegalovirus
CNS	Central Nervous System
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
C-SSRS	Columbia Suicide Severity Rating Scale
DES	Desmin
dMCK	Double Muscle Creatine Kinase
DMD	Duchenne Muscular Dystrophy
ECG	Electrocardiogram
EDL	Extensor Digitorum Longus
EGFR	Epidermal Growth Factor Receptor

EMA	European Medicines Agency
FDA	Food and Administration
FGFR	Fibroblast Growth Factor Receptor
GAA.....	Acid-Alpha-Glucosidase
GFP	Green Fluorescence Protein
hAAT	Human AAT
HFIX16.....	Human FIX16
hGAA.....	Human GAA
HGFR.....	Hepatocyte Growth Factor Receptor
HSV	Herpes Simplex Virus
IDUA	Alpha-L-Iduronidase
IM	Intramuscular
ITR.....	Inverted Tandem Repeats
IV.....	Intravenous
IVIg.....	Intravenous Immunoglobulin
KO	Knockout
LamR	Laminin Receptor 1
LCA	Leber's Congenital Amaurosis
LPL	Lipoprotein Lipase
LPLD	Lipoprotein Lipase Deficiency
LTFU	Long-Term Follow-Up
LVEF	Left Ventricular Ejection Fraction

MCK	Muscle Creatine Kinase
MIP	Maximal Inspiratory Pressure
nAMD	Neovascular Age-Related Macular Degeneration
NSAA	North Star Ambulatory Assessment
PBGD	Porphobilinogen Deaminase
PCR	Polymerase Chain Reaction
PDGFR	Platelet-Derived Growth Factor Receptor
polyA	Polyadenylation
rAAV	Recombinant AAV
scAAV	Self-Complementary AAV
sIBM	Sporadic Inclusion Body Myositis
SMA1	Spinal Muscular Atrophy Type 1
TA	Tibialis Anterior
tMCK	Triple Muscle Creatine Kinase
VEGF	Vascular Endothelial Growth Factor
VG	Vector Genome
XLMTM	X-linked Myotubular Myopathy

INTRODUCTION

Neuromuscular diseases are a broadly defined group of inherited and acquired disorders that disrupt motor unit functions, and which can affect peripheral nerves, motor and sensory neurons, neuromuscular junctions, and skeletal muscles. Primarily inherited neuromuscular disorders include muscular dystrophies and non-dystrophic myopathies. These conditions are generally characterized by abnormal muscle functions including muscles weakness and impaired locomotion, are co-morbid with serious complications and can often lead to premature fatalities associated with respiratory failure. Although these conditions are individually rare, they are often debilitating and collectively take a noteworthy toll on families and the entire medical system. Neuromuscular diseases form a highly clinically, genetically and pathologically heterogeneous group of hereditary diseases that vary in severity and prevalence depending on factors such as age, ethnicity, and gender. This complex nature of neuromuscular diseases in turn is reflected in the large number of pathological pathways that are affected in the disease condition. These diseases are highly heterogeneous within a subtype, therefore, therapeutic interventions targeting specific pathways often lead to incomplete or ineffective clinical responses in the whole patient group (Dowling et al, 2018). Current standard of care for neuromuscular diseases includes pain management, assistive ventilatory and mobile devices and drugs targeting different steps in the dysregulated downstream pathways. These methods can sufficiently increase the quality of life but fail to deliver a permanent improvement in muscle function because the primary cause of the disease is not fixed.

To meet this challenge, significant progress has been made in recent decades on identifying the disease-causing mutations responsible for the pathogenesis of inherited neuromuscular diseases. As of December 2020, the journal of Neuromuscular Disorders recognized 1079 monogenic neuromuscular diseases in humans linked to primary defects residing in 608 different genes (Benarroch et al, 2020). Owing to the tremendous strides made to understand the genomic structures and functions, recent therapeutic efforts are focused on resolving the primary cause by replacing the genetic mutation with a normal copy of the disease-causing gene to restore the normal function.

Adeno-associated virus vectors serve as leading viral vehicles for gene therapy and which hold great potential to addressing these disorders as they lead to precise delivery to replace the impaired gene which can potentially permanently ameliorate the underlying pathology. Moreover, AAVs have clinical appeals such as broad tropism, non-pathogenicity and ease of production.

This review details a fundamental understanding of AAV biology highlighting recent AAV-mediated vector engineering principles and current consensus of therapeutic strategies. Existing challenges such as off-target toxicity and immunogenicity are also important to consider, as well as advances aimed at perfecting the vector development process to achieve high efficiency and tissue tropism in skeletal muscle. This review further discusses comprehensive summary of results of published preclinical animal models and clinical trials of three representative inherit neuromuscular diseases: X-linked

myotubular myopathy (XLMTM), Duchenne muscular dystrophy (DMD) and Spinal muscular atrophy type 1(SMA1). A discussion of the future outlooks of translating the technology of AAV-mediated gene replacement therapies to the clinics is written at the end to conclude this review.

Fundamentals of AAV

AAV Biology

AAV was first discovered as a contaminant in adenovirus preparations back in 1965 (Atchison et al, 1965) and has been found to exist in various species including humans and non-human primates. AAV contains a linear, single-stranded DNA genome around 4.7 kb flanked by two inverted tandem repeats (ITR) (Hastie & Samulski, 2015) and can be either the sense strand or anti-sense strand. AAV's replication and infectious activities (Geoffroy & Salvetti, 2005) require a helper virus such as adenovirus (AdV), and Herpes Simplex Virus (HSV). Only the two ITRs, each taking up 145 base pairs, are essential for AAV's survival in serving as the origins of replication and ensuring the viral transduction and vector production.

Recombinant AAV (rAVV) can therefore be constructed by replacing most of its protein-coding genome with a therapeutic transgene expression cassette for maximal therapeutic packaging that contains the single-stranded transgene DNA which can be further codon modified, a tissue-specific promoter, and a regulatory element for targeted

expression of the virus (Greiger & Samulski, 2012; Figure 1). The regulatory element can include the Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element (WPRE) which can form a stabilizing tertiary structure when transcribed to help increase transgene nuclear transport (Wilmott et al, 2019). The polyadenylation (polyA) signal, sandwiched in between WPRE and the 3' end ITR, is also a crucial part of the transgene cassette's regulatory element. This polyA site further aids the mRNA nuclear transport and translation longevity (Wilmott et al, 2019).

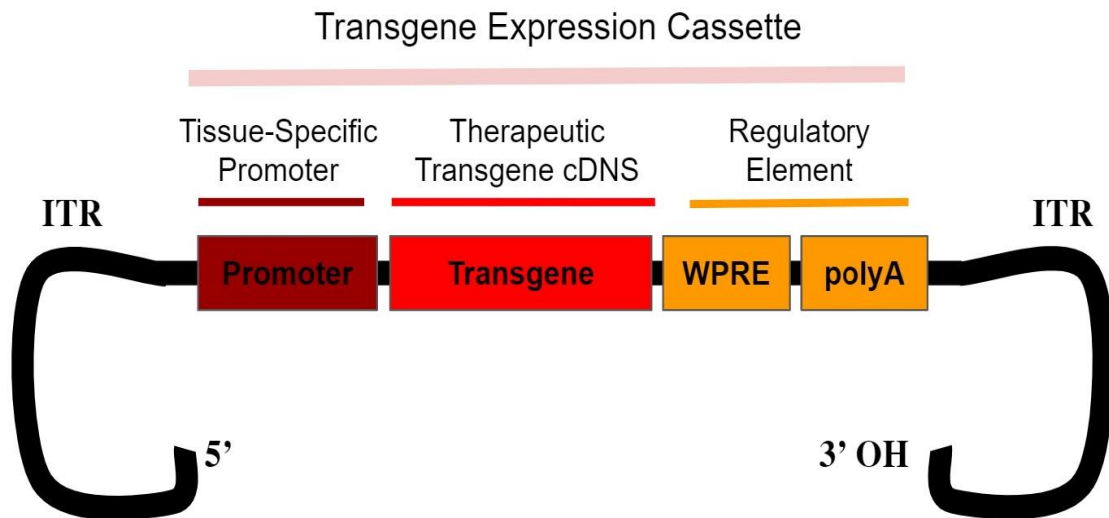


Figure 1. Basic Structure of rAAV. ITR-flanked AAV genome replaced with the transgene expression cassette for therapeutic and immunogenicity purposes. The therapeutic cassette should include transgene, which is the functional copy of the gene targeted a tissue-specific promoter and regulatory elements such as WPRE and polyadenylation signal.

AAV infection is commonly observed in a wide range of cells in humans, both dividing and non-dividing (Balakrishnan & R. Jayandharan, 2014). They are not associated with any known diseases, since rAAVs are unable to integrate into the human

genome (Calcedo et al, 2011). These advantages have made AAV one of the leading viral vehicles for clinical gene replacement therapy, RNA therapy, and gene editing when used in conjunction with CRISPR-cas9 (Wang et al, 2017). Luxturna was the first AAV-mediated gene therapy approved by the U.S Food and Administration (FDA) for treating patients with RPE65-related Leber's congenital amaurosis (LCA) (Jacobson et al) in 2017. Further success in AAV gene therapy was achieved in clinical trials treating patients with heart diseases, hemophilia, neurological disorders, and neuromuscular diseases, which is the main focus of this review (Ishikawa et al, 2018; Nathwani et al, 2011; Deverman et al, 2018; Aguti et al, 2018).

AAV Serotypes

The versatility of AAV vectors is facilitated by its large array of selection comprising at least 12 naturally occurring human serotypes and over 100 mutants (Kotterman & Schaffer, 2014) which bind to different receptors with tissue preferences. They all differ in respect to binding capacity of their capsid proteins to specific cell surface receptors which in turn would affect tropism, types of the cells susceptible to transduction and degrees of immunogenicity of different serotypes.

All AAVs interact with a primary receptor on the cell surface and require a proteinaceous co-receptor for the initial attachment to the cell surface and post attachment cellular transduction, respectively. Most of these primary receptors on cell

surface have been identified that typically contain glycan moieties, while for other serotypes these remain to be identified (Li and Samulski, 2020; Table 1).

Serotypes	Primary Receptor	Secondary Receptor
AAV1	Sialic Acid	AAVR
AAV2	Heparin	AAVR, Integrin, FGFR, HGFR, LamR
AAV3	Heparin	AAVR, FGFR HGFR, LamR
AAV4	Sialic Acid	Unknown
AAV5	Sialic Acid	AAVR, PDGFR
AAV6	Heparin, Sialic Acid	AAVR, EGFR
AAV7	Unknown	Unknown
AAV8	Unknown	AAVR, LamR
AAV9	Galactose	AAVR, LamR
AAV10	Unknown	Unknown
AAV11	Unknown	Unknown
AAV12	Unknown	Unknown

Table 1. AAV Serotypes' Interactions with Primary Receptors and Co-Receptors. AAVR, AAV receptor; FGFR, fibroblast growth factor receptor; HGFR, hepatocyte growth factor receptor; LamR, laminin receptor 1; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor.

AAV1 has been used as the delivery vehicle in clinical trials treating patients with several diseases including muscular diseases such as Pompe disease, heart failures, alpha1-antitrypsin (AAT) deficiency and was used in the first approved gene therapy to treat patients with lipoprotein lipase deficiency (LPLD) in Europe (Keeler and Flotte, 2019). A clinical phase 1A trial to treat Becker muscular dystrophy (BMD) and sporadic inclusion body myositis (sIBM) was carried out in 2012 using intramuscular (IM) injections of AAV1-cytomeglovirus (CMV)-follistatin (NCT01519349). BMD and sIBM patients exhibit weakness in the lower extremities and follistatin gene is delivered to the skeletal muscle to improve muscle strength and size. Phase 1 trials of this approach showed decreased fibrosis and improved muscle regeneration (Mendell et al, 2017). Pompe disease is another devastating neuromuscular disease resulting from mutations of the acid-alpha-glucosidase (GAA) gene which leads to disruptions of cardiac and skeletal muscles due to a pathological buildup of glycogen (Hers, 1963). In 2017, a clinical trial using AAV1 to treat early ventilatory failures in patients with Pompe disease involved delivery of AAV1- CMV-human GAA (hGAA) intramuscularly in the diaphragm. AAV1- CMV-human GAA did not result in any severe adverse events, however, anti-capsid and anti-GAA antibodies were observed in subjects (Corti et al, 2017). Another clinical trial was initiated to determine the safety and efficacy of intracoronary administration of AAV1-SERCA2a (MYDICAR) in patients with heart failure. In 2016, researchers concluded that a single infusion of MYDICAR was not efficient in improving the clinical outcomes of patients with heart failure (Greenberg et al, 2016). There are two clinical trials using AAV1 to treat patients with AAT deficiency where they both inject

rAAV1-chicken beta-actin (CB)-human AAT (hAAT) into subjects (ClinicalTrials.gov Identifier NCT00430768; NCT01054339). In 2012, the European Medicines Agency (EMA) approved alipogene tiparvovec (Glybera) which uses rAAV-lipoprotein lipase (LPL) to treat patients with LPL deficiency. This approval marked a milestone in gene therapy development. However, the drug was withdrawn from the market in 2017 due to economic concerns (Keeler and Flotte, 2019).

AAV2 is the first discovered serotype and therefore is the most well-characterized. AAV2-based therapies that have been used in several clinical trials treating eye diseases, hemophilia, CNS diseases, and AAT deficiency (Russel et al, 2017; George et al, 2020; Li and Samulski, 2020). In 2017, the FDA approved voretigene neparvovec (Luxturna) for the treatment of LCA. This was the first ever approved *in vivo* gene therapy treatment by the FDA. LCA is caused by mutations in the RPE65 gene and is characterized by progressive loss of visual acuity and nystagmus (Russel et al, 2017). Luxturna is delivered by subretinal injection of AAV2-human RPE (hRPE65v2) to affected patients to improve their vision and light sensitivity (Russel et al, 2017). An intrahepatic AAV2 delivery of the factor IX gene (rAAV2-human FIX16 (hFIX16)) to adults with severe hemophilia B was administered from 2001 to 2004. A long-term follow-up (LTFU) of the study subjects was initiated in 2007 for multiple physical examinations yearly to collect longitudinal data of the safety and tolerability of the AAV gene therapy (NCT00515710). In 2020, George and colleagues reported data of four patients after 12-15 years of follow-up post-vector injection. No major safety concerns

were observed, and a persistent increase in AAV2-neutralizing antibodies was detected (George et al, 2020). Another clinical trial was initiated in 2006 injecting AAV2-neurturin (CERE-120) in a bilateral stereotactic manner to patients' putamen region for the treatment of idiopathic Parkinson's disease (NCT00400634). Phase 2 of the trial was completed in 2008 and showed no significant improvement in treated subjects. Many serious adverse events were observed in patients who received the AAV treatment. Although the events are thought to be caused by trophic factors rather than AAV2-neurturin treatment, longer periods of follow-up is needed to determine the primary cause (Marks Jr et al, 2010). Lastly, AAV2 has also been used in a clinical trial treating patients with ATT deficiency where rAAV2-CB-hAAT was administered intramuscularly into patients (NCT00377416). Post injection, no adverse safety events were observed and a low transient increase in serum AAT was detected 30 days after. By day 90, patient muscle biopsies showed strong immunostaining of AAT, further proving the safety and feasibility of the approach (Flotte et al, 2004).

AAV3 has moderate tropism across a wide variety of tissues. Currently, there are no trials underway using AAV3. AAV4 has strong tropism towards eyes and CNS. Therefore, it has been used in a clinical trial to treat the eye disease LCA where rAAV-2/4, hRPE65 was injected subretinally into subjects (NCT01496040). Patients were monitored for 1-3.5 years post-injection. All injected subjects showed tolerance to the vector and exhibited improvement in visual functions (Le Meur et al, 2018).

AAV5 has been used in multiple clinical trials treating hemophilia, eye disease, and acute intermittent porphyria (AIP). Valoctocogene roxaparvovec (Roctavian) is an open-label, phase 3 AAV5-based gene therapy aimed at delivering the F8 gene to patients with severe hemophilia A (NCT02576795). It has previously shown great promise in a multiple year follow-up of injected patients where they showed greatly reduced spontaneous bleeding episodes, persistent transgene expression and good tolerance (Pasi et al, 2020). AAV2/5 is an rAAV engineered to have AAV5 ITRs in combination with AAV2 capsid proteins. This engineered rAAV vector was used in a clinical trial targeting LCA by delivering a functional copy of the RPE65 gene (NCT02781480). AIP is a metabolic disorder characterized by the defect in the heme biosynthesis pathway caused by deficiency of porphobilinogen deaminase (PBGD). rAAV2/5-PBGD is also delivered to patients to evaluate safety in a clinical trial (NCT02082860). No cellular response towards the transgene or AAV capsid was observed and a reduction of hospitalizations of treated patients was observed (D'Avola et al, 2016).

AAV6 uses both heparin and sialic acid as its primary receptor, therefore it has a broad tropism across multiple tissues such as muscle, central nervous system (CNS), heart, liver, and lung, and used as the primary delivery agent in clinical trials involving hemophilia and CNS diseases. Pfizer started a dose-ranging trial in 2017 using rAAV2/6 to deliver human factor VIII to subjects with severe hemophilia A (NCT03061201). Recombinant AAV2/6 has also been used in attempts to correct the metabolic and neurological complications caused by mucopolysaccharidosis type 1 (MPS1). MPS1 is an

autosomal lysosomal storage disorder resulting from the mutations in the alpha-L-iduronidase (IDUA) gene. Recombinant AAV6 carryings a correct copy of IDUA was injected in subjects intravenously to provide them with a lifelong production of the missing enzyme (NCT02702115). However, the trial was rather unsuccessful due to low transgene expression efficacy (Ou et al, 2020).

There are currently no active clinical trials using AAV7, 10, 11, and 12. AAV8 and AAV9 have been heavily used in more recent clinical trials, especially regarding treatments of neurological disorders due to their ability to cross the blood brain barrier. AAV8 and AAV9 have become the current standard delivery vehicles in animal models and clinical trials treating inherited neuromuscular diseases.

AAV8 is the delivery vehicle in multiple clinical trials ranging from eye diseases, hemophilia, to muscle diseases. RGX-314 is a recombinant AAV8 gene therapy aimed to treat neovascular age-related macular degeneration (nAMD) by delivering the cDNA of a soluble anti-vascular endothelial growth factor (VEGF) protein (NCT03066258). RGX-314 can treat it by inhibiting VEGF as excessive VEGF is one of the primary cause of the nAMD,. Recombinant AAV8 is used to deliver a functional copy of factor IX to patients suffering from severe hemophilia B via a single intravenous (IV) injection (NCT00979238). An increase of circulating factor IX is observed in patients with persistent long-term transgene expression up to at least three years post-injection (Nathwani et al, 2014). ACT132 is a gene replacement therapy using rAAV8 to deliver

one functional copy of human MTM1 gene to children affected by XLMTM (NCT03199469). Despite promising results, the trial was halted in the summer of 2020 due to the unexpected deaths of three participants. This is discussed further in a later section (page 37).

AAV9 stands out from other serotypes due to its ability to transduce virtually every tissue. It has been used to deliver treatments for neuromuscular diseases such as SMA1 and DMD in humans. Moreover, Zolgensma, a gene therapy using rAAV9 to treat SMA1, was approved by the FDA in 2019. The details of these clinical studies are discussed in further in this review (page 42).

Rationale for AAV Vector Design

AAVs are prime candidates for targeting the primary cause of monogenic neuromuscular diseases. There are several naturally occurring serotypes that have tropism towards skeletal muscles, cardiac muscles, and the CNS. Several serotypes have been applied to treat neuromuscular disorders in preclinical animal experiments and clinical trials with varying success. Furthermore, AAVs can be engineered to further refine targeting of specific cell types and maximize transgene transduction.

AAV1, 6, 7, 8, and 9 have been utilized to achieve high transduction in skeletal muscles (Arnett et al, 2014). Early studies were focused on AAV1, 6, and 7 to transduce

muscles whereas the relatively newly discovered AAV8 and 9 are becoming more popular. AAV8 and AAV9 not only can transduce striated muscle at least as high as others but AAV9 has also a strong tropism towards myocardium and can cross the blood brain barrier (Schultz and Chamberlain, 2008; Inagki et al, 2006; Zincarelli et al, 2008). Furthermore, the specific type and level of maturation of the muscle fibers targeted need to be taken into consideration before choosing the most appropriate serotype. Since inherited neuromuscular diseases usually impact the entire musculature, a serotype that can transduce all muscle types efficiently is ideal. Skeletal muscle originates from muscle progenitor cells and muscle stem cells (satellite cells) regulate post-natal growth. Therefore, in a pool of muscle fibers at different maturation stages in their life cycle, delivery of therapeutic viral vector into satellite cells is ideal for sustained growth. Damaged muscle fibers from diseases prompt muscle stem cells to proliferate and regenerate into new fibers to replenish the degeneration. Therefore, satellite cells targeted by AAV could continuously differentiate into genetically corrected fibers and thus provide a self-sufficient source of transgene.

The ultimate goal of any gene therapy is to achieve a persistent, therapeutic level of transgene expression in the organ of interest while minimizing off-target toxicities and immune reactions. To achieve this, various factors need to be considered, including but not limited to, route of administration, gene therapy development, and supplemental therapies.

To minimize off-target effects of the viral vectors, they can be administered locally via an intramuscular injection into affected muscle area. This approach is ideal in that it ensures that the virus stays in the muscles injected, rather than traveling globally throughout the body. By doing so, off-target effects can be minimized. As neuromuscular disease affect the entire skeletal musculature, this approach may prevent broad and even viral distribution over the entire organ. Therefore, the recommended route of administration for AAV gene therapy treating neuromuscular diseases would be via systemic injection. In order to target neuromuscular diseases, viral vectors that have natural tropism towards the entire motor unit are ideal candidates. Although the viral expression will be unavoidably observed in other tissues, comparatively muscles should be the main target.

To maximize the efficacy of the viral vector, optimization of all parts that make up the transgene cassette needs to be considered. The key components of gene therapy development are vector selection, promoter design, and transgene cDNA optimization. Vector selection determines the overall safety profile and transduction efficiency by targeting the tissue of interest and reducing cytotoxicity and immunogenicity. Promoter design relies on choosing a promoter that naturally drives gene expression in muscle. To expand on that, promoter enhancers can be utilized to further the targeting effectiveness of the promoter. Choosing the right promoter is crucial in ensuring high virus expression levels and specificity. Transgene cDNA sequence is key for determining the functional impact of the therapy. By optimizing the codons in the human cDNA, researchers can

ensure the most preferred codon for a gene sequence and increase the stability of the RNA sequence and secondary structures. All these extra measures can lead to more protein expression, which is useful in driving the maximum effect of the therapy although it is important to take into consideration that codon optimization is not necessarily equivalent to superiority and many different versions of cDNA need to be optimized *in vitro* or *in vivo*.

Lastly, supplemental therapies such as proteasome inhibitors can be used in conjunction with the viral gene therapy (Monahan et al, 2010) Given the fact that AAV vectors are naturally broken down by proteasomes following ubiquitination, inhibiting this cascade anywhere along the way can lead to longer expression of the same amount of viral dosage. This implicates the possibility of minimizing viral vectors and achieving the same or higher therapeutic gene expression in muscles.

Capsid Engineering

Genetic delivery to the entire musculature to treat neuromuscular diseases ultimately requires widespread transduction while maintaining a therapeutic level of expression and evading the immune system. To address this, several strategies have been developed focusing on AAV capsid design and ITR modifications. It is extremely difficult to fit a natural serotype to a disease process since it may possess traits that are both beneficial and harmful for therapeutic development. Therefore, approaches such as

directed evolution and rational design are often employed to give viruses clinically relevant properties.

Rational design is an approach where scientists utilize the knowledge of AAV transduction pathways and the relationship between capsid structure and functionality to configure an AAV mutant that fits their research purposes. Peptides can be engrafted onto the virus surface to enhance cell-to-receptor binding and target specific cell types (Li and Samulski, 2020). Efforts have been made to identify receptor-binding domains, AAV capsid sequences and crystal structures. Enhanced muscle transduction may be achieved by identifying capsid regions that are responsible for the strong muscle tropism and combining said regions to an AAV2 capsid for its established safety profile and purification ease (Li and Samulski, 2020; Bowles et al, 2012). For example, AAV 2.5, a combination of AAV2 capsid with 5 amino acid residues engrafted from AAV1 produced more efficient muscle transduction than either of them alone in a clinical trial (Bowles et al, 2012). Such approaches not only lend the benefits of increased tropism specificity, but also help the virus evade the host immune system and decrease off-target effects. Therefore, rational designing of the virus leads to prolonged virus expression and reduced side effects. AAV2i8 was generated by reengineering the heparan sulfate receptor footprint 585-RGNRQA-590 on AAV2 and replacing it with AAV8 hexapeptide sequence. This created an alternative antigenic profile in mice following tail vein injection. The synthetic AAV2 vector achieved high transduction in both cardiac and

skeletal muscles while presenting markedly decreased hepatic tropism (Asokan et al, 2010).

Moreover, mutants of a serotype can be engineered to achieve a specific goal. AAV9.HR was designed by inducing mutations His527Tyr and Arg533Ser on a chimpanzee-derived variant of AAV9. As a result, AAV9.HR retains the ability to cross the blood-brain barrier but also shares greatly reduced peripheral transduction following intravenous injections in mice (Wang et al, 2008). Rational design can also increase targeted viral transduction by inhibiting cellular degradation of the virus. Zhong and colleagues induced site-directed point mutations in surface-exposed tyrosine residues on AAV2 capsids (Zhong et al, 2008). This resulted in a 30-fold increase in hepatocyte transduction since the mutation allowed the vector to avoid phosphorylation and therefore ubiquitination induced proteasome-mediated degradation of the capsids. This approach accomplished the goal of reaching a high viral count with reduced virus dosage which is vital for avoiding off-target effects and cytotoxicity in the host.

Thanks to multidisciplinary efforts to better understand and elucidate the relationship between capsid structure and functionality of the virus, scientists are able to assemble their virus capsid by combining useful characteristics to hyper target the desired target. However, the rational design approach where new variants are added onto the vector capsid does raise some concerns regarding the stability of the virus in its 3D structure and is limited by our knowledge pertaining the inner workings of the virus.

Therefore, greater efforts need to be made to better understand the working mechanism of AAV to make rational design a viable approach to be widely adapted across labs and clinics.

Directed evolution employs methods such as gene shuffling, and error-prone polymerase chain reaction (PCR) allows simulating natural selection by screening a wide range of barcoded AAVs with different tissue tropism to construct highly diversified AAV mutant libraries with advantageous genetic properties (Grimm et al, 2008). They can then be systemically administered in animal models to investigate their tropism and transduction efficiency *in vivo*. However, species difference between mice and humans may lead to the failure of their effectiveness in humans. Although there are humanized animal models that partially mimic human cells, this option is not widely available for every tissue. This strategy is favorable because it bypasses the need to understand the biological mechanisms of the mutant, however, is also time consuming and could be nonspecific due to the random gene shuffling process.

Another goal regarding AAV-mediated gene therapy is to achieve therapeutically efficient viral load inside the host as fast as possible without inducing any or minimal immunotoxicity. Gregorevic et al (2006) approached this by incorporating vascular endothelium growth factor, and/or vascular permeability factor, into the transgene cassette. The inclusion of these factors may lead to acute permeabilization of the intravascularly injected viral vectors in the peripheral microvasculature. Since the

vascular endothelium presents a barrier between the AAV vector and circulation, permeabilization should enhance tissue transduction with a lower dosage. Another way to approach this problem is to speed up the virus transduction process. The rate limiting step of AAV transduction is the obligatory second-strand synthesis initiated by the ITRs inside the nucleus. This step can be sped up by mutating the ITRs so that it leads to self-complementary AAV (scAAV) intermediates (McCarty et al, 2001). They contain both the plus and minus ends of the DNA strands fused by altered ITRs, which ensure that immediate annealing upon entering the host nucleus, so transcription can take place immediately. This method ensures faster and higher transgene expression since more cycles of transcription can occur before the virus is detected and degraded by the host's immune system. Self-complementary AAV vectors have been adapted widely across clinical trials including treatments of hemophilia patients and is a component of Zolgensma, an FDA approved treatment for SMA1 (Nathwani et al, 2011; Nathwani et al, 2014; Mendell et al, 2017).

Vector Selection

In addition to engineering the vector to best serve the purpose of high tropism towards skeletal muscles, transduction of different fiber types categorized within skeletal muscles needs to be taken into consideration as well. The differential transduction achieved by different serotypes of AAV in specific types of myofibers can play a crucial role in designing an efficient therapeutic. Pruchnic and colleagues (2000) showed that

AAV2 preferentially infects slow muscle fibers. AAV6 can infect both slow and fast muscle fibers equally (Blankinship et al, 2004). AAV9 however, is found to mainly transduce tibialis anterior (TA) muscles, which are predominantly fast-twitch, and poorly transduces the soleus muscles (slow-twitch predominant) (Bostick et al, 2007). Although for most inherited neuromuscular diseases the entire musculature is affected, it is necessary to take into account transduction differences when analyzing the animal study and clinical results.

Another focus of AAV vector selection is the viral vector's capability to successfully transduce muscle stem cells called satellite cells. As skeletal muscle is a post mitotic tissue which experiences slow turnover rates, the therapeutic transgene transduction could diminish in the proliferative phase during multiple regenerations often associated with muscular dystrophies. Since satellite cells work to replenish damaged muscle fibers by differentiating into more myofibers, it would be ideal to deliver transgenes directly into stem cells to create a sustainable source of transgene supply during muscle turnover and regeneration. This means a single dose of said virus can create a permanent solution. However, whether or not AAVs can infect muscle stem cells is not completely clear. To explore this, the Chamberlain lab showed that AAV6 transduces satellite cells both *in vitro* and *in vivo* with very low rates (Arnett et al, 2014). The virus also transduced myoblasts with greatly reduced efficiency comparing to myotubes and myocytes. To test the efficacy of different AAV viruses to transduce satellite cells, fluorescent reporter rAAV6-CMV-mCherry was injected into the extensor

digitorum longus (EDL) muscle of *mdx* mice (a DMD mouse model) (Arnett et al, 2014). The satellite cells were labeled with green fluorescence protein (GFP)-reporter construct in these *mdx* mice. No co-expression of the red virus was seen in the green satellite cells suggesting the inability of AAV6 to transduce satellite cells. In contrast, 80% of the mature myofibers were successfully infected. Injections of rAAV6, 8 and 9 at a 40-fold higher concentration into the muscles of wild type mice showed only a 5% transduction in muscle stem cells with AAV9 (Arnett et al, 2014).

In 2016, Tabebordbar et al showed transduction of muscle stem cells with a dual-reporter system where the satellite cells in neonatal mice were labeled by Cre-mediated recombination. This dual reporter system enabled the researchers to better quantify the transduction efficacy of AAV as fluorescence can be observed if AAV vectors indeed reached satellite cells. They observed a 10% and 35% muscle stem cell transduction on peritoneally and intramuscularly delivery, respectively. No systemic evaluation was made (Tabordbar et al, 2016). In 2019, the Wagers lab (Glodstein et al, 2019) further expanded on their initial findings and reported 60% of the muscle stem cells were transduced via systemic delivery when injected intravenously via the tail vein and retro-orbital sinus. Additionally, adult muscle stem cells can also be transduced not only by AAV9, but also by AAV8 and Anc80L65. Furthermore, transduction in other progenitor populations such as mesenchymal progenitors in the muscle and dermis, and hematopoietic stem and progenitor cells in the bone marrow could also be observed (Goldstein et al, 2019).

The Gersbach lab first utilized dual-reporter Pax7-ZsGreen-Ai14 mice in 2019 where the Pax7+ muscle stem cells were marked with ZsGreen fluorescence (Nance et al, 2019). The Cre system was utilized in the mouse line which contained a floxed stop cassette that would block the tdTomato expression. When said mice were injected with AAV9-Cre, the expression of tdTomato fluorescence was observed in the one third to one half of the Pax+ muscle stem cells. This observation indicated that the Cre-mediated AAV9 vector indeed was able to reach muscle stem cells resulting in the complete removal of the floxed stop cassette. Observations of the colocalization of the green and red fluorescence further confirmed the ability of AAV9 vectors to infect satellite cells.

The Gersbach lab extended their findings in a paper published in 2020 where they profiled muscle satellite cell transduction efficiency based on serotypes and found AAV9 had the highest transduction in muscle stem cells followed by AAV8 in the satellite cells of a dual-reporter system in the *mdx* mouse model (Kwon et al, 2020). Furthermore, transplantation surgeries were performed to determine the long-term contribution of AAV-driven gene edited muscle stem cells. The Gersbach lab utilized a muscle graft model where they allowed mouse muscles to undergo complete necrosis before engrafting them to adult immunodeficient mice. These muscles regenerated themselves completely using their own stem cells. Dystrophin positive fibers were observed in recipient mice proving the functional viability of this method. Moreover, they tested out the gene-editing efficiency of various muscle-specific promoters within the muscle stem cells. They concluded, although their viral activity was overall lower in stem cells, that

the promoter MHCK7 achieved the highest transduction infecting almost half of the satellite cell population. The results are discussed in more details in the following section concerning promoters (Kwon et al, 2020).

Promoter Design

To further increase the efficacy and tropism of the viral vector in skeletal muscles while providing an additional layer of control over transgene expression, the use of highly active, muscle-specific promoters that drive transgene expression and minimize off-target effects are highly recommended. Non-specific promoters can lead to expression of the viral vector carrying the transgene in unwanted areas within the body, especially following systemic delivery of the therapy, and may result in off-target toxicity. Additionally, the host body might mount a high immune reaction towards the transgene and vector which would lead to premature degradation of the therapy and severe adverse events in the individual. Different promoters have been tested in recent years in AAV-based therapies treating skeletal muscle disorders and are described in detail in this section.

Human cytomegalovirus immediate-early gene promoter is ubiquitously expressed throughout the body including skeletal muscles (Wang et al, 2008). The small size (690 bp), makes it ideal for packaging into the transgene cassette in AAV vectors. CMV promoter is one of the strongest promoters, therefore, to achieve high transgene

infection it is used widely for gene therapy including neuromuscular disease treatments. However, this strong expression can also induce systemic toxicity due to unrestricted transgene expression. High antibody and CTL reactions are typically developed against the ubiquitous CMV promoter driven transgene compared to the muscle-specific promoter (Weeratna et al, 2002). Furthermore, these authors showed complete destruction of transgene-expressing muscle fibers at two different time points: 10 and 20 days following AAV infection giving the body time to generate an immune reaction to the foreign virus (Davis et al, 1997). Therefore, it is necessary to use a promoter that is specific to the targeted tissue.

Human desmin promoter (DES) is derived from the desmin gene, which is an essential intermediate filament protein required for the structural stability of muscle fibers (Paulin and Li, 2004). Desmin is expressed in skeletal muscles, cardiac muscles, and even neurons at a comparably high level (Wallenberg: Human Protein Atlas, 2006). Therefore, human desmin promoter is suitable for systemic delivery of gene therapy to correct many neuromuscular diseases-causing genes that typically manifest defects in these organs. The desmin promoter is small sized (354 bp), which is ideal for packing into the AAV vector. Studies have shown that desmin induces the highest transduction in skeletal muscles including the diaphragm, and in the brain in comparison to CMV promoters (Pacak et al, 2009). This finding is important as many patients affected by neuromuscular diseases experience respiratory problems due to myalgia observed in the

diaphragm muscles and respiratory failure is a main driver in premature deaths of neuromuscular disease patients.

Muscle creatine kinase (MCK) is a muscle-specific promoter and therefore its usage can improve the overall safety profile of the AAV vector by reducing ectopic expression of the transgene (Weeratna et al, 2001). However, the naturally occurring MCK promoter is considerably less active than the ubiquitous CMV promoter. Moreover, large size of MCK promoter (6.5kb) exceeds AAV vector's packaging capacity by 2kb. To address these issues, truncated MCK promoters and enhancers are developed and are widely available to be packaged in AAV vector in order to target muscle fibers. *In vivo* delivery of mini dystrophin gene by Enh358MCK (584 bp) into *mdx* mice results in a high expression of the transgene. This approach successfully ameliorated pathologies and improved the contractile functions of dystrophic muscles in the animal (Wang et al, 2000; Watchko et al, 2002). Additionally, hybrid promoters e.g., MCK enhancer and simian virus 40 promoters (MCK/SV40 promoter) have been shown to result in a long-term expression in mouse muscles (Takeshita et al, 2007). The Xiao lab further generated more compact muscle-specific promoters by ligating a modified MCK enhancer to a truncated MCK basal promoter creating modified double MCK (dMCK) and triple MCK (tMCK) promoters with two (509 bp) and three enhancers (720 bp), respectively. These promoters were observed to be very specific to muscle cells and preferably expressed in fast-twitch fibers. Both were also found to be inactive in vital organs such as mouse liver and other non-muscle cells, thus significantly reducing the toxicity of the vector.

Moreover, tMCK proved to have even stronger expression levels than CMV (Wang et al, 2008). Therefore, MCK promoters and its variants are currently the most popular promoter options for constructing a viral vector delivering therapeutic genes into muscles.

Synthetic muscle promoters such as C5-12 (312 bp) are also available to target skeletal and cardiac muscles. C5-12 induces even higher transgene expression in both slow and fast-twitch fibers in animal models compared to the muscle-specific MCK promoter (Wang et al 2008; Liu et al, 2004). A combination of C5-12 promoter with the MCK enhancers further increased the promoter strength by at least two to three folds (Wang et al, 2008).

Furthermore, it has long been assumed that muscle-specific promoters are inefficient in driving transgene expression in muscle stem cells. However, contrasting results have recently come out in the previously discussed study (Page 22) where Kwon and colleagues at the Gersbach lab, compared AAV9 vectors containing Cre recombinase or CRISPR driven by the constitutively active CMV and muscle-specific promoters (CK8e, SPc5-12, MHCK7). These viral vectors were injected into the TA muscles of *Pax7nGFP;Ai9;mdx* mice. Investigators found that all the muscle-specific promoters were efficient at editing the gene where the floxed-codon removal led to fluorescence expression. CMV was the most active in satellite cells. MHCK7, a variant of MCK promoter, was the most efficient having induced 50% deletion within the stem cells of

skeletal muscles; SCK8e came in second having edited almost half of the cells as well; SPc5-12 deleted a third of the genes while CMV was the least efficient as engineering the stem cells (Kwon et al, 2020). These results elucidate further the importance of incorporating an appropriate promoter in order to achieve maximum transduction in muscle cells with minimal dosage required.

Therefore, the ideal candidate for AAV-mediated gene therapy in neuromuscular disease treatments would be choosing a muscle-specific promoter. Given the fact that many AAV serotypes display very non-specific tissue tropism including skeletal muscles, the promoter needs to be efficient and specific to ensure the tolerability and efficacy of the gene therapy.

AAV Challenges

Immunity

One significant concern regarding gene delivery using AAV is the interaction between the host immune system, the viral capsid, and the transgene product on various facets. This challenge is especially crucial when delivering the AAV therapy to a large system such as neuromuscular disorders where systemic transfer is necessary. Wild-type AAVs are exposed to humans early in life, which in turn results in the development of both humoral and cellular immunity to the virus. These immune reactions can lead to

reduced therapeutic efficacy of the AAV gene transfer by destroying the vector before it reaches the targeted tissue or destroying the cells infected by the virus.

Studies have shown that anti-AAV1 and anti-AAV2 antibodies are most prevalent in a survey population. Enzyme-linked immunosorbent assays determined a 67% and 72% of titers of anti-AAV1 and anti-AAV2 antibodies while AAV 5, 6, 8 and AAV9 presented with 40%, 46%, 38% and 47% titers. Furthermore, cross reactivity of the antibodies plays a crucial role in determining immune reactions to different serotypes and results in the inactivation of a multitude of AAV serotypes. Highest neutralizing antibodies seroprevalences is observed for AAV1 and AAV2 presenting up to 50.5% and 59%. AAV5 and AAV9 exhibited the least sero-prevalent with 3.2% and 9%, respectively (Boutin et al, 2010). Another population survey study published by Calcedo et al confirmed these findings by concluding neutralizing antibodies to AAV2 were most prevalent in all regions followed by antibodies to AAV1 with AAV8 having the lowest concentration of antibodies (Calcedo et al, 2009). Moreover, AAV2 can confer CD 8+ T cell activation due to its heparin glycan sulfate proteoglycan binding domain on the vector capsid (Vendenberghe et al, 2006). Therefore, AAV5, AAV8 and AAV9 appear to be the least seroprevalent across regions, making them the most appealing for human therapy for immunity perspective.

Moreover, it is crucial to identify the age group with the lowest neutralizing antibodies to AAV vectors, as that would be the best window of opportunity for viral

gene therapy intervention. Calcedo and colleagues (2011) examined the neutralizing antibody titers to AAV2 and AAV8 in the plasma of different age groups including newborns, children and adolescents (0-18 years) in plasma samples of 752 human objects. Neonates showed a moderate level of neutralizing antibodies to both AAV2 and AAV8 capsids; titers significantly dropped at 7-11 months post birth. This decline is to be expected, since at this age range maternally transferred immunoglobulin G exhibit a decline. The prevalence of neutralizing antibodies steadily decreases after 11 months of age and peaks at 3 years of age with higher neutralizing antibody titer towards AAV2 than AAV8. Therefore, the best age for intervention appears to be 7-11 months. However, the vaccine is not typically administered in this window, AAV8 should be used after 3 years of age (Calcedo et al, 2009).

To date, most clinical trials prescreen and exclude patients seropositive to the AAV vector used. However, this approach is far from optimal since it excludes a large cohort of patients in need of therapy. There have been several efforts made to lower potential immune reactions to AAVs. Immunosuppressants, such as non-depleting anti-CD4 and cyclosporine usage following AAV administration, has been explored in murine models (McIntosh et al, 2012; Han et al, 2015). However, intensive immunosuppression regimen failed to deliver an ideal result in non-human primates (Unzu et al, 2012). This approach also raises concerns over the increased risks of developing recurring infections in patients due to a suppressed immune system.

Plasmapheresis is another method employed to avoid pre-existing immunity against AAVs. Plasmapheresis is an established technique used to remove a large quantity of substances from the plasma, some of which can include immunoglobulins, autoantibodies, and lipoproteins. One study demonstrated that by performing plasmapheresis on seropositive rhesus macaques, sustained AAV gene transfer was observed (68%) in comparison to the expression levels seen in seronegative animals (53.7%). Seropositive macaques that were not pheresed showed notably lower expression level at 10.1% (Chicoine et al, 2014). Monteihet and colleagues (2011) examined the effect of plasmapheresis in human patients. One to five rounds of plasmapheresis were performed at a one to five-day interval on neutralizing antibody titers specific to AAV 1, 2, 6, 8 in ten patients seropositive. The authors and others collectively showed that five rounds of plasmapheresis are effective enough to alleviate the immune response towards AAV vectors (Monteihet et al, 2011; Chicoine et al, 2014; Hurlbut et al, 2010). However, since this is a nonspecific approach, patients require intravenous immunoglobulin (IVIg) infusion to act as an immunomodulator.

The use of empty AAV decoys to absorb antibodies has also been proposed (Mingozzi et al, 2013). Native empty AAV decoys can be engineered by taking the wild type AAV and mutating the receptor binding site of the AAV vector to ensure the absorbance of anti-AAV antibodies and the empty decoy also cannot enter the cell. This mutant AAV decoy has the same ability to absorb neutralizing antibodies while greatly reducing immunogenicity. By adding an excessive number of empty decoys in the final

formulation, AAV gene therapy can overcome the inhibitory effects of neutralizing antibodies and lead to viral transduction even in the presence of anti-AAV neutralizing antibodies. The higher the native titer, the higher the empty decoys are required (Mingozzi et al, 2013). Whether the decoys can cause T cell immunity against AAV capsids needs further investigation. Despite of all the advantages, these approaches are not very effective for high antibody titers.

A structure-guided evolution technique is developed by combining multiple epitope sites in a single AAV capsid to produce a synthetic AAV that is novel to the body. Theoretically, this AAV variant should evade detection by pre-existing neutralizing antibodies without compromising the transduction efficiency. The authors tested out this theory by generating an engineered AAV1 variant and subsequently tested its immune evasion in sera from AAV1-seropositive mice, rhesus macaques and humans, even at dilutions up to 1:5, which is required in several currently active clinical trials (Tse et al, 2017). This strategy provides a promising approach to the immunological barriers imposed by AAV vectors and can potentially work in various serotypes. However, this AAV variant needs further clinical testing to see its efficiency to evade immune reactions in humans.

Economical Concerns

Affordability is one concerning barrier between AAV-mediated gene therapy and its widespread clinical adaptation. The expensive price tag is largely attributed to the great deal of expertise and difficult manufacturing process required to purify and produce a large quantity of clinical-grade viral vectors sufficient enough to systemically target specifically a large organ such as the skeletal musculature.

Glybera, the first AAV-based gene therapy to be approved in the European Union back in October of 2012, was a historic achievement in the development and clinical translation of AAV-mediated gene therapy (Keeler and Flotte, 2019). However, LPLD is extremely rare with a prevalence of 1-2:1000,000. Few could also afford the expensive gene therapy (Morrison, 2015). Due to the low market demand, Glybera was withdrawn from the market in 2017 despite its promising results in curing patients. Luxturna, an orphan drug approved by the FDA in 2017, and the first AAV-based gene therapy to be approved in the United States, showed significant light perception improvements in patients with LCA. Luxturna comes with the incredibly expensive price tag of \$425,000 for delivery into each eye. To compensate for the price, Spark Therapeutics, Inc., the pharmaceutical company that produces Luxturna, offers an outcome-based rebate system that ensures long-term durability and payment options (Goswami et al, 2019). In 2019, the newest approved AAV-based gene therapy Onasemnogene Apeparvovec-xioi (Zolgensma), developed by AveXis Inc., a Novartis company, is aimed at treating SMA1

in less than two years old children. Zolgensma is currently the most expensive drug in the market (\$2.1 million). Despite the promise of it being a one-time treatment if successful, and Novartis's collaborative effort working with more than 20 private insurance companies and four Medicaid plans to set up coverage policies for patients needing the therapy, the successful wide clinical adaptation of Zolgensma is still to be determined.

Therapeutic AAV Gene Delivery in Preclinical Models and Clinical Trials

AAV has become increasingly popular over the past decade as a great candidate for *in vivo* gene replacement therapy in preclinical animal experiments and clinical trials of NMD with varying success. Here I describe three major NMD therapies that made significant strides using AAV-mediated vectors.

1. X-Linked Myotubular Myopathy

XLMTM is the most frequent form of centronuclear myopathy affecting 1 in 50,000 male births (Jungbluth et al, 2008). This hereditary neuromuscular disorder is caused by mutations in the MTM1 gene, which results in defects or absence of myotubularin (Laporte et al, 1998). Although the enzyme is expressed ubiquitously, the deficiency profoundly affects muscle fibers. Histopathologically, hypotrophic myofibers present with disorganized organelles (Lawlor et al, 2016). Clinically, affected patients display severe hypotonia, generalized muscle weakness, and respiratory failure at birth

(McEntagart et al, 2002). Development of AAV delivery of functional *MTM1* gene has been shown to significantly correct muscle pathophysiology, improve clinical status, and prolong survival in several animal models.

In 2008, Buj-Bello et al. , injected rAAV 2/1 vectors expressing the *MTM1* gene under the CMV promoter control into the tibialis anterior muscles of symptomatic XLMTM mice. Transduced muscle fibers exhibited improved histology with increased myofiber volume and increased contractile force. However, long term effects of the infusion could not be assessed due to the early deaths of these mice caused by respiratory failure (Buj-Bello et al, 2008). Systemic treatment of *MTM1* knockout (KO) mice by injection of rAAV9-desmin-*MTMR2* into the tail vein rescued muscle functions and prolonged their lifespan (Daniele et al, 2018). These promising results proved the viability of treating XLMTM with AAV vectors.

AAV therapy has also been tested out in a canine model of XLMTM as well. Mack et al (2017) administered rAAV8-cMTM1 vector under the expression of desmin promoter into 10-weeks old symptomatic pups via the cephalic vein. They performed analysis on muscle, respiratory, and neurological functions, histology changes, transgene expression, and survival over the following 9-month period. They reported that the therapy was well tolerated, corrected muscle phenotype, and increased the survival rate in the treated dogs (Mack et al, 2017). These promising

results from the animal models support an optimistic future for AAV therapy in clinical trials treating patients with XLMTM.

Indeed, in 2017, Audentes Therapeutics, Inc., an Astellas company, initiated a clinical trial ASPIRO (NCT03199469) of AT132, which is an AAV8 vector containing one functional copy of the human MTM1 gene, for evaluation of safety and efficacy of gene transfer in patients with XLMTM who are younger than 7 years old. As of August 2020, there are six patients enrolled in cohort 1 (1×10^{14} vector genome (vg)/kg dose), 17 in cohort 2 (3×10^{14} vg/kg dose) and two untreated. Patient group that received a single infusion of AT132 showed a significant reduction in ventilator dependence and reached achievements in motor milestones such as walking independently or supported and sitting down unassisted. One treated patient also showed improved CHOP-INTEND (Children Hospital of Philadelphia Infant Test of Neuromuscular Disorders) scores, which is an indication of motor functions and better maximum inspiratory pressure (MIP). Histologically, muscles showed significant myotubularin transduction and improved organization (Shieh et al, 2020). The medication seemed to be well tolerated in the early stages of the trial. However, three children treated with the high dosage passed away in the summer of 2020. Audentes reported that all three patients had pre-existing hepatobiliary diseases and suffered from worsening liver dysfunction within a month of AT132 treatment. The FDA has since put an official clinical hold on the trial. Audentes is

planning on further investigating the cause of mortality, monitoring all patients, and collecting ongoing data for future regulatory actions.

2. Duchenne Muscular Dystrophy

DMD is an X-linked, inherited, recessive neuromuscular disorder caused by mutations in the dystrophin gene (Emery, 2002). DMD is observed in one in every 5,000 boys (Mendell & Lloyd-Puryear, 2013) and is characterized by progressing muscle weakness usually starting around two to three years of age. Many of those suffering die prematurely due to the eventual diaphragm or cardiac failures (Koeks et al, 2017). Since DMD is a monogenic disease, rAAV gene replacement therapy to substitute the defective dystrophin gene is highly appealing. However, the gene encodes for the large dystrophin protein (427 kD) (Emery, 2002) which exceeds the maximum packaging capacity of an AAV vector (4.7 kb). To overcome this obstacle, variations of micro-dystrophin gene expression cassettes with non-essential regions deleted are developed based on the genotype of patients and animal models (England et al, 1990; Blankinship et al, 2006).

Systemic delivery using rAAV6 and rAAV8 has been performed in murine models showing body-wide dystrophin transduction, along with improved skeletal and cardiac muscle function, reduced muscle histopathology and prolonged lifespan (Gregorevic et al, 2006; Wang et al, 2005). Bostick et al (2011) injected AAV9 vectors carrying mini-dystrophin genes (Δ R4-23/ Δ C) into the tail veins of aged (16 to 20-month-

old) female *mdx* mice to observe the impact of the gene therapy on the cardiac functions of the mouse model. Robust mini-dystrophin expression was observed in the cardiomyocytes. Animals showed improved cardiac functions and cardiac deaths were prevented (Bostick et al, 2012). However, in a follow up study myocardial fibrosis was still observed despite of the prevention of cardiac deaths by DMD . Another group systemically injected AAV6 vector carrying mini-dystrophin constructs ($\Delta R4-23/\Delta CT$) into advanced DMD stage of 20-month-old *mdx* mice. Along with robust mini-dystrophin expression, significant improvements in muscle pathology and functions were observed (Gregorevic et al, 2006). These severely diseased mouse models provide promising clinically relevant data to move forward with systemic delivery of mini dystrophin in large animal models and DMD patients.

In parallel, AAV mini-dystrophin vectors were tested in canine models. Recombined AAV9 vectors carrying human codon-optimized micro-dystrophin gene (Dys $\Delta 3990$) under the control of CMV promoter were injected into 4-day-old symptomatic puppies. Despite widespread dystrophin transduction, inflammatory response was observed in several limb muscles. This observation led to concerns about immunotoxicity of the treatment (Kornegay et al, 2010). Later, successful a variant of AAV9, tyrosine-engineered rAAV9 vectors carrying micro-dystrophin (μ Dys) treatments were administered to young adult (2 months) diseased dogs in 2015. This modification was aimed to evade the immune system and enhanced the transduction. Indeed, the authors observed widespread gene expression accompanied by improved muscle

functions and positive pathohistological changes in a dose-dependent manner (Yue et al, 2015). This study proved the safety and durability of systemic AAV mini-dystrophin delivery in a large dystrophic mammal model.

Jerry Mendell and colleagues (2010) carried out the first AAV therapy in patients in 2006. In this trial, rAAV2.5 with minigene cassette under the CMV promoter was injected into the biceps of five to 11 years old six patients . Corticosteroid methylprednisolone was administered prior to surgery to dampen the body's immune response for the viral therapy. Muscle biopsies were performed on days 42 and 90 post injection. Very few transduction-positive myofibers were observed. T cells specific to AAV capsids and the mini-dystrophin genes were increased explaining the absence of transgene expression. Furthermore, the use of the ubiquitously expressed CMV promoter could also be a contributing factor in this failed attempt. Learning from this, choosing a muscle-specific promoter and prescreening patients for existing anti-AAV and anti-transgene antibodies are vital in ensuring sufficient gene expression in future DMD patient trials.

Three clinical trials to determine the tolerability and efficacy of AAV gene therapy in DMD patients were initiated in the United States back in 2017. They were initiated by Solid Biosciences, Pfizer and Nationwide Children's Hospital (Columbus, OH) led by Mendell and colleagues. The study design of the trials varies in detail. Two trials utilized AAV serotype 9 whereas the third one opted for AAVrh74, a variant

similar to AAV8. All chose to incorporate muscle-specific promoters such as CK8, MHCK7 and MCK in the transgene cassette to further target the skeletal musculature (Table 2). They all chose different variations of the mini-dystrophin gene in terms of configuration, but the specific configurations are not disclosed. Different criteria were employed to recruit patients as well (Duan, 2018; Table 2). The Mendell trial reported the preliminary results in September of 2020, three years post injection. Significant dystrophin transduction of up to 81.6 was observed in muscle fibers of four injected children. Patients exhibited clinically significant north star ambulatory assessment (NSAA) scores improvement which signifies the quality of ambulation in these children. The drug was well tolerated with minimal adverse events. This proof-of-principle study provided exciting results for the therapy and the trial with a much larger patient sample is under way (Mendell et al, 2020).

	Solid Biosciences	Nationwide Children's Hospital	Pfizer
ClinicalTrials.gov Identifier	NCT03368742	NCT03375164	NCT03362502
Trial Name	Microdystrophin Gene Transfer Study in Adolescents and Children with DMD (IGNITE DMD)	Systemic Gene Delivery Clinical Trial for Duchenne Muscular Dystrophy (DMD)	A Study to Evaluate the Safety and Tolerability of PF-06939926 Gene Therapy in Duchenne Muscular Dystrophy
Study Design	Randomized, Controlled, Open-label, Single-ascending Dose, Phase I/II	Non-randomized, Open-label, Single dose, Phase I/II	Non-randomized, Open-label, Single-ascending dose, Phase IB, Multicenter
Drug Name	SGT-001	SRP-9001	PF-06939926
AAV Serotype	AAV9	AAVrh74	AAV9
Promoter	CK8	MHCK7	MCK
Intervention Model	Single Group Assignment	Parallel Assignment	Sequential Assignment
Dosage	3 Groups (Untreated vs. Dose Level 1 vs. Dose Level 2) Starting at 5×10^{13} vg/kg	2 Groups (3 Months to 3 Years of Age vs. 4 to 7 Years of Age) Both at 2×10^{14} vg/kg	2 Groups, Patients Receive One of Two Dose Levels
Patient Age	4 Years to 7 Years (Child)	3 Months to 7 Years (Child)	4 Years and older (Child, Adult, Older Adult)
Patient Number	16	4	30
Disease Stage	Ambulatory and Non-ambulatory	Ambulatory Only	Ambulatory and Non-ambulatory
Dystrophin gene mutation	Any Confirmed DMD Mutation	Frameshift (Deletion or Duplication) Mutation or Premature Stop Codon Between Exons 18-58	Any Confirmed DMD Mutation
Pre-existing Neutralizing Antibodies to AAV	Negative	<1:400 anti-AAVrh74 and anti-AAV8 antibodies	Negative
Primary Outcome Measures	Efficacy (Dystrophin Protein Expression) and Safety (Incidence of Adverse Events, Clinical Laboratory, Vital Signs, Physical Examinations and ECGs Abnormalities) Timeframe: 12 Months Post Treatment	Safety (Adverse Events, Monitored and Scored for Severity and Relevance to the Treatment) Timeframe: Up to 5 Years Post Treatment	Safety/Tolerability (Adverse Events) Timeframe: 12 Months Post Treatment
Secondary Outcome Measures		Micro-dystrophin Gene Expression Quantification (Immunofluorescence and Western Blot) and Motor Functions (Gross Motor Subtest Scaled Score for Cohort A, 100 Meter Timed Test for Cohort B and NSAA) Timeframe: Screening to Post-treatment Day 30, 90 and Up to 5 Years	Micro-dystrophin Gene Expression Quantification (Immunofluorescence, Western Blot, and/or LC-MS), Incidence of Treatment-emergent Adverse Events and Laboratory, Vital Signs, Physical and Neurological Examinations, Body Weight, LVEF, C-SSRS and ECGs Timeframe: Varying from 2 Moths - 1 Year to up to 5 Years Post-treatment

Table 2. Comparison of Three Active DMD Clinical Trials in the U.S. ECG, electrocardiogram; LVEF, left ventricular ejection fraction; C-SSRS, Columbia Suicide Severity Rating Scale.

3. Spinal Muscular Atrophy Type 1

SMA1 is an inherited, autosomal recessive motor neuron disorder caused by mutations in, most commonly, survival motor neuron 1 (SMN1) gene. SMA affects approximately 1 in every 6,000 to 11,000 births (Lunn & Wang, 2008). Patients present with severe muscle weakness that ultimately leads to loss of voluntary motor movements. Dependence on ventilators is common in patients due to the progressive degeneration of alpha motor neurons in their spinal cord and brainstem (Prior et al, 2011). Premature deaths are often observed before the age of two. Since AAV serves as an attractive delivery method for the monogenic disease, a lot of efforts have been made to use AAV vectors to deliver a functional copy of SMN1 gene into animal models and patients to prove safety and efficacy.

In 2010, Foust et al. (2010), injected CMV enhancer-chicken- β -actin (CAG)-driven scAAV vector carrying SMN1 into neonatal SMN1 KO mice and observed transgene expression in their spinal cord, brain stem, and muscles. Functional improvements such as improved motor functions and prolonged lifespan were achieved. The Kaspar lab further targeted non-human primates such as *Cynomolgus* macaques with a single injection of scAAV-SMN1 delivered to the cerebrospinal fluid (CSF) and

reported wide transduction throughout the brain, spinal cord, and brainstem (Meyer et al, 2015) demonstrating the promise of AAV9 therapy in treating patients with SMA1.

In 2014, a phase I, open-label, dose-escalation clinical trial study led by Jerry Mendell, sponsored by AveXis, Inc., was approved by the FDA. Mendell et al (2014) delivered a single intravenous injection of CAG promoter-driven-AAV9 vectors carrying the missing SMN1 gene into the peripheral limb veins of patients. Two cohorts were included in this study: three in the low dose group (6.7×10^{13} vg/kg) and 12 (2×10^{14} vg/kg) in the high dose group (NCT02122952). Patients achieved better motor functions accompanied by improved CHOP-INTEND scores, and prolonged lifespan compared to the historical cohort. The treatment proved to be rather well-tolerated and exhibited great efficacy. The clinical trial concluded its phase III study and was subsequently approved for market entry by the U.S. FDA. In May 2017, Onasemnogene Aporavidine (Zolgensma) developed by AveXis, an AAV9-based gene therapy designed to deliver one functional copy of human SMN1 gene, was officially available in the U.S. market for treatment of patients suffering from SMA1 (Hoy, 2019). This unprecedented approval marks a huge success for AAV-based gene therapy and further proves that AAV-based therapy can be safe and effective in treating patients with neuromuscular disorders.

AAV-Mediated Genome Editing

Besides working independently as a delivery vehicle for gene replacement therapy, AAVs have been used extensively in other genome-editing systems to help deliver the technology into cells. Most prominently, AAVs are often used in conjunction with the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-CRISPR-associated (Cas) 9 system.

CRISPR-Cas 9 is a system where Cas9, an endonuclease, is guided by a single-guide (sg)RNA to target a specific DNA site, which can be the mutation site in the genetic diseases. The endonuclease can then generate a double-strand break that can be repaired by nonhomologous-end joining or homology-directed repair. Nonhomologous-end joining can lead to imprecise insertion/deletion mutations whereas homology-directed repair can generate a specific modification at the target site, but this approach requires an exogenous DNA template (Long et al, 2016). The CRISPR-Cas9 can permanently correct the defective genome sequence whereas gene replacement therapy still retains the underlying disease-causing gene. A single and dual-system have been designed to use AAV to deliver the CRISPR-Cas9 technology into human patients. In the single system, AAV vectors are used to deliver a Cas9/sgRNA constructs whereas in the dual system two AAV vectors are used to deliver Cas9 endonuclease and sgRNA separately into the host. AAV-guided CRISPR-Cas 9 system has been tested out in animal models of neuromuscular diseases such as Duchenne Muscular Dystrophy. Several labs

used different AAV serotypes (AAV 8 and AAV9) to deliver components of the CRSPR-Cas9 system into *mdx* mice aimed at deleting the premature stop codon in exon 23, a specific nonsense mutation in this mouse line, to restore the open reading frame for dystrophin protein expression. These proof-of principle studies showed partial recovery of the dystrophin protein expression in myofibers and cardiac muscles and improved muscle force in treated mice (Nelson et al, 2016; Long et al, 2016). These promising results establish AAV-mediated CRSPR-Cas9 genome editing technology as a potential treatment option for patients with DMD and other neuromuscular diseases since it can be applied to a wide range of mutations. However, more animal studies using the system are needed to further elucidate the advantages and toxicity of this therapy and its clinical translation.

Conclusions and Future Outlooks

Inherited monogenic neuromuscular diseases can impair any part of the motor unit including peripheral nerves, motor/sensory neurons, and the entire skeletal musculature. Most acute cases are diagnosed in early childhood and infancy. Patients require life-long medical care to manage the symptoms and everyday assistance since there are few effective treatments that can cure these disorders. The results of these impairments greatly reduce the quality of life of those suffering and is of significant burden on the families and the entire healthcare system due to the extensive medical resources needed. However, as new genomic replacement therapies that target the primary defects emerge, this picture is slowly changing. Inherited neuromuscular diseases

have improved therapeutic expectations as many exciting preclinical and clinical studies utilizing AAV-mediated vectors show encouraging promise and several received or expected to reach market approval in the near future.

Many limitations still need overcoming regarding AAV-mediated gene therapy. The timing of the diagnosis of the disease can dictate the course of treatment plan: patients with a delayed disease course may experience weakened therapeutic effect and call for a slightly different treatment approach. If the disease progresses worse over time, it will make it difficult to measure the effectiveness of the AAV-mediated vector in the clinical trial. Notable challenges regarding AAV gene therapy development, therapeutic high dosage, broad distribution throughout the body, the complexity of the disease progression of neuromuscular diseases and expensive cost of virus purification will most likely create hurdles for widespread commercialization of AAV therapy.

Moreover, this review only covers a small portion of the rapidly growing AAV-mediated gene replacement therapy which is again a minuscule aspect of the ever-expanding field that is gene therapy. Many other gene delivery and gene editing platforms such as CRISPR-Cas9 and exon skipping exist to target disease-causing mutations correcting pathology. Since the field of gene therapy is accelerating at an astonishing speed, one concern is that AAV-based therapies may become outdated even before they reach clinical approval. Despite the limitations of AAV therapies, striking strides have been made in clinical studies with an exponential amount of preclinical

studies incorporating AAVs showing promising results. This suggests that we are only at the beginning of the era of AAV-based gene therapies that's vastly expanding.

As we enter the age of epigenomics and genetic therapeutics, I am cautiously optimistic that AAV-mediated gene replacement therapy will become widely adapted, especially across the spectrum of neuromuscular diseases as a part of the multidisciplinary clinical treatment plan to fundamentally cure these diseases in patients.

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CURRICULUM VITAE

