Dystrophin Gene Replacement and Gene Repair Therapy for Duchenne Muscular Dystrophy in 2016: An Interview

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After years of relentless efforts, gene therapy has now begun to deliver its therapeutic promise in several diseases. A number of gene therapy products have received regulatory approval in Europe and Asia. Duchenne muscular dystrophy (DMD) is an X-linked inherited lethal muscle disease. It is caused by mutations in the *dystrophin* gene. Replacing and/or repairing the mutated *dystrophin* gene holds great promises to treated DMD at the genetic level. Last several years have evidenced significant developments in preclinical experimentations in murine and canine models of DMD. There has been a strong interest in moving these promising findings to clinical trials. In light of rapid progress in this field, the Parent Project Muscular Dystrophy (PPMD) recently interviewed me on the current status of DMD gene therapy and readiness for clinical trials. Here I summarized the interview with PPMD.

Parent Project Muscular Dystrophy (PPMD): What is gene therapy?

Dr. Dongsheng Duan: Gene therapy refers to therapies that use nucleic acids as the “drug” to treat and/or prevent inherited or acquired diseases. Nucleic acids can be DNA, RNA, or oligonucleotides. Nucleic acids can be naked or encapsidated in a viral or nonviral carrier.

Gene therapy can be classified as either disease gene-dependent or disease gene-independent therapies. In the former, treatment aims at the gene that encodes the protein (in the case of Duchenne, this would be dystrophin). The mutated gene can be repaired or replaced. In the case of dominant mutation, the mutated gene can be silenced. Disease gene-independent therapies take advantage of disease-modifying genes that either are functional substitutes of the diseased gene or are genes that intervening downstream pathogenic processes (in the case of Duchenne, utrophin and follistatin are examples of gene-independent therapies). Disease gene-independent therapies also involve strategies that target noncoding region of the genome (such as microRNA therapy).

PPMD: Can you define the key terminology used in gene therapy—such as transgene, serotype, and vector?

Dr. Duan: A transgene means the gene that is being transferred. In the context of gene therapy, it usually refers to the gene that is used for therapy. For example, Duchenne muscular dystrophy (DMD) is caused by mutations in the *dystrophin* gene. A functional *dystrophin* gene can thus be transferred to diseased muscle cells to treat DMD. Here the transgene is the normal dystrophin gene.

In the context of gene therapy, a vector means the vehicle that is used to transport the nucleic acid “drug” to target cells. Gene therapy vectors are classified as viral vectors (meaning they are derived from a virus) or nonviral vectors (meaning they are not derived from a virus). Some of the most commonly used viral vectors include adeno-associated virus (AAV), adenovirus, retrovirus, and lentivirus.

The serotype refers to the serologically distinguishable feature of a virus. When a virus infects our body, the body will generate a unique set of antibodies against the invading virus. These antibodies can be detected in the serum. A virus can thus be classified into different types according to the antibodies detected in the serum. The serotype is often used to classify different members of the same family virus. For example, the family of AAV virus has different serotypes such as AAV serotype-1, 2, 8, and 9 (abbreviated as AAV-1, 2, 8, and 9).

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PPMD: How is a gene therapy vector delivered to the cells where it is needed and what does it do once there?

Dr. Duan: A gene therapy vector can be delivered to our cells either *ex vivo* or *in vivo*. In *ex vivo* delivery, investigators first isolate the target cells (e.g., bone marrow stem cells) from the body. They then mix the target cells and the vector in a container outside the body (e.g., in a petri dish) to allow the vector to get into the cells. The cells that carry the vector are then isolated and put back to the body. *In vivo* delivery refers to directly deliver the vector to the body. This can be achieved either locally to a specific location (e.g., via intramuscular injection to a muscle) or systemically to whole body (e.g., via intravenous injection).

After a vector is delivered to a tissue, the vector will enter the cell through receptors and co-receptors that are located on the surface of the cell. Once inside the cell, the vector will release the therapeutic gene it carries into the nucleus. In a typical AAV vector, the therapeutic gene is in a single-stranded DNA format. This format cannot direct cells to make the protein. In order to express the protein, the incoming AAV genome has to be converted to a double-stranded transcription-competent DNA molecule. The vast majority of the AAV genome is converted into a double-stranded sealed circle. A very tiny fraction of AAV may integrate into the chromosome. Most often, it does not cause a safety concern.¹ However, in a retroviral or lentiviral vector, the vector genome enters the cell as an RNA molecule. The RNA molecule is subsequently reverse transcribed into a DNA molecule and integrates into the chromosome. The integration of a retroviral vector in the human genome has been shown to cause leukemia in several clinical trials.^{2,3} A new generation of retro/lentiviral vectors has been developed to minimize this safety concern.⁴

PPMD: There appears to be considerable progress recently in developing gene therapy for several genetic disorders. Can you give us some insights into that progress in eye and blood diseases?

Dr. Duan: Over the last few years, gene therapy has begun to deliver its therapeutic promise in several diseases. One example is AAV-2-mediated gene therapy for Leber's congenital amaurosis 2 (LCA2). This is a rare inherited retinal degenerative disease. Affected children lose their vision because of mutations in a gene called *Rep65*. Investigators in the United States and United Kingdom put a normal *Rep65* gene in an AAV-2 vector and then injected the vector into the eye of patients with LCA2. Treated

patients were able to regain their vision. Some patients still maintain their improved vision at eight years after gene therapy.⁵

Another major breakthrough is AAV-8-mediated gene therapy for hemophilia B. Hemophilia B is caused by mutations in a gene that encodes coagulation factor IX. To treat hemophilia B, scientists packaged a normal *factor IX* gene in an AAV-8 vector and injected intravenously to patients with severe hemophilia B. Factor IX produced from the AAV-8 vector significantly improved clinical outcomes without causing serious side effects. Therapeutic effect has maintained for more than four years in treated patients.^{6,7}

PPMD: Have any gene therapy products received regulatory approval?

Dr. Duan: Several gene therapy products have been approved by regulatory agencies. In 2003, China approved the first gene therapy product called *Gendicine*.⁸ This is an adenovirus vector for cancer therapy. In 2005, China approved *Oncorin*, another adenoviral vector for cancer gene therapy.⁹ In 2007, the Philippines approved *Rexin-G*, a retroviral vector for cancer gene therapy.¹⁰ In 2011, Russia approved *Neovasculgen*, a nonviral vector for treating peripheral arterial disease.^{11,12} In 2012, European Medical Agency approved *Glybera*, an AAV-1 vector for treating a rare genetic disease called lipoprotein lipase deficiency.^{13,14} On October 27, 2015, FDA approved *Imlygic*, an oncolytic herpes virus vector. This is the first commercial gene therapy product approved in the United States.^{15,16} On October 10, 2015, a biotech company called Spark Therapeutics announced the results of its phase III trial on an AAV-2 vector for treating a form of childhood blindness. There were no serious adverse events. Treated patients showed significant vision improvement. Spark Therapeutics will seek regulatory approval from FDA in 2016 to market their gene therapy product. If successful, this will become the first AAV gene therapy approved by FDA.⁵

PPMD: What's the rationale for gene therapy in Duchenne? How does a gene delivered via gene therapy help ameliorate the progression of DMD? What's the potential impact for Duchenne patients?

Dr. Duan: The fundamental problem in DMD is the absence of dystrophin, an essential muscle protein. This is caused by mutations in the *dystrophin* gene.^{17,18} Basically, mutations abort dystrophin production. Delivery of a new functional *dystrophin* gene or repair of the mutated *dystro-*

phin gene should restore dystrophin production in muscle and prevent muscle cells from dying. Gene therapy is expected to stop or slow down the progression of muscular dystrophy, improve life quality, and extend the life span of affected boys. If gene therapy is applied early enough, it may “cure” the disease.

PPMD: Gene therapy was tried years ago in muscular dystrophy, but suffered some setbacks from clinical trials in other diseases? Are we in better shape now and why?

Dr. Duan: Soon after the discovery of the *dystrophin* gene, scientists had begun to test gene therapy. Early studies used plasmid (nonviral vector), retrovirus, and adenovirus. These were performed in cultured muscle cells and dystrophin-deficient mdx mice. During this period, adenovirus delivery of a *mini-dystrophin* gene (which is derived from a very mild Becker patient) was at the forefront (see Note).¹⁹ Unfortunately, adenovirus induces strong cellular immune responses and the mini-dystrophin produced from the adenovirus vector did not last long. In 1998, the entire field of gene therapy was put on hold because of the death of 18-year-old Jesse Gelsinger, who died from an adenovirus gene therapy trial for an inherited liver disease.²⁰

Several gene therapy studies have been conducted in muscular dystrophy patients since that time. The first clinical trial for muscular dystrophy was published in 2004. This trial used a nonviral plasmid vector called *Myodys*.²¹ It delivers a full-length dystrophin coding sequence. Investigators injected the plasmid directly into a muscle in DMD patients. Unfortunately, therapy yielded minimal dystrophin expression.²²

The clinical trial of AAV gene therapy for muscular dystrophy was initially proposed in 2000 to treat limb girdle muscular dystrophy.²³ However, the first AAV gene therapy for muscular dystrophy was not reported until 2009.²⁴ In this study, Dr. Mendell and colleagues injected an AAV-1 vector that carried the *alpha-sarcoglycan* gene to the muscle of patients with limb girdle muscular dystrophy 2D and observed persistent expression of the therapeutic alpha-sarcoglycan in injected muscle for 6 months.²⁴ Another AAV trial was reported in 2010 for DMD.²⁵ This trial used an engineered AAV-2.5 vector that carried a highly minimized synthetic *dystrophin* gene. Unfortunately, dystrophin expression was barely observed in injected muscle. Detailed investigations suggest that the lack of expression was barely because of the immune response. The immune response to a gene

therapy product first caught the attention in 2006 when an AAV-2 vector that carried *factor IX* gene was delivered to the liver of patients with hemophilia B.²⁶ Investigators initially observed a therapeutic level factor IX production in the blood. But it did not last because treated liver cells were rejected by the immune system a few weeks later. It is now clear that the gene delivery vehicle (AAV virus capsid), cargo (transgene), or the protein produced from the therapeutic transgene can all illicit immune responses. To achieve long-term persistent gene therapy, we need to overcome the immune response barrier.

The invention of antisense oligonucleotide (AON)-mediated exon skipping opens the door to repair the messenger RNA, the molecule that translates the language of the gene (DNA) into a protein.²⁷ In exon skipping, the mutated part of dystrophin is skipped and a shortened version of dystrophin is produced. The first exon skipping trial on DMD patients was published in 2007.²⁸ In that trial, AONs were directly injected into patient's muscle. Since then, there has been significant progress in exon skipping. Several trials have been conducted in Europe and the United States to achieve systemic exon skipping. The major hurdles in current exon skipping include its low efficiency, transient nature, and failure to treat the heart. The US Food and Drug Administration (FDA) recently reviewed the new drug application (NDA) for two exon-skipping drugs, one from BioMarin Pharmaceutical (Kyndrisa; drisapersen) and the other from Sarepta Therapeutics (eteplirsen; AVI-4658). Both drugs aim at skipping exon 51 which could benefit ~13% DMD patients. On January 14, 2016, the FDA issued a complete response on BiomaMarin's NDA application and stated the FDA could not approve the NDA in its present form (www.drugs.com/history/kyndrisa.html). According to a FDA briefing document published on November 24, 2015 (www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/peripheralandcentralnervoussystemdrugsadvisorycommittee/ucm473737.pdf), the major issues are the lack of clinical efficacy, failure to show increased dystrophin expression by western blot, and some concerns on the safety (such as renal toxicity). On January 22, 2016, the FDA published a briefing document on Sarepta's NDA application for eteplirsen (www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/peripheralandcentralnervoussystemdrugsadvisorycommittee/ucm481911.pdf). Significant concerns were raised by the FDA on clinical efficacy and dystrophin levels but not on the safety of the drug. Sarepta has since submitted four-year clinical effective data. According to a news release from

Sarepta²⁹, the FDA will further review the data and reach a conclusion on whether eteplirsen will be approved, conditionally approved, or not approved by May 2, 2016.

Looking forward, the field is in a much stronger position than it was ever before. For example, we have identified major hurdles in exon skipping and AAV gene therapy, and we have also developed novel strategies to overcome these hurdles. In terms of exon skipping, new AONs with superior chemical properties (such as tricycle-DNA AONs) have greatly improved correction in deep muscles such as the diaphragm and the heart without causing toxicity in mouse models of DMD.³⁰ Methods have also been developed to use AAV to deliver AONs for long-term widespread correction. In terms of AAV gene therapy, transient immune suppression protocols (before, at the time of, or after gene delivery) have been developed and have shown success in the hemophilia B trial and in dog DMD models. Novel AAV capsids with improved properties are also being engineered to meet the specific needs of DMD gene therapy.³¹

PPMD: Which Duchenne patients could potentially benefit from gene therapy? Early versus late-stage boys? Ambulatory versus nonambulatory?

Dr. Duan: Broadly speaking, gene therapy has the potential to benefit every DMD patient. Systemic bodywide gene therapy in early stage boys (especially before they lose large amount of muscle) may prevent muscle from deterioration and dramatically change the disease course. Clinical observations in mild Becker patients suggest that a successful gene therapy may allow ambulation to the age of 60s.¹⁹ For late-stage boys, the goal of gene therapy is to improve life quality. Localized gene therapy in limb muscles may improve their function for holding and grasping and allow use of a keyboard. Cardiac gene therapy may also improve the heart function of late-stage boys.

PPMD: What attributes of the dystrophin gene and protein make it amenable to gene therapy (e.g., size of the gene, spectrin repeat region, proof-of-concept from large Becker muscular dystrophy deletions)?

Dr. Duan: The *dystrophin* gene is one of the largest genes in the genome. It has a size of 2.4 mb (mega base) and is beyond the carrying capacity of any viral vector.³² A gene is composed of protein-coding exons and nonprotein coding introns. Dystrophin has 79 exons. The protein-coding region (also called cDNA) of dystrophin has a size of 11.2 kb (kilo

base).³³ The dystrophin protein has a size of 427 kD (kilo Dalton).³⁴ Ideally, delivery of a full-length dystrophin cDNA will yield the production of a full-length dystrophin protein and the maximum protection of muscle. This can be achieved with a nonviral vector (such as a plasmid), gutted adenovirus, and tri-AAV vectors.^{21,35,36} Currently, these strategies are not ready for clinical development because of issues related to delivery efficiency, the immune response, vector purification, and so on.

The full-length dystrophin protein can be divided into four domains. These are the N-terminal, rod, cysteine-rich, and C-terminal domains. The rod domain can be further divided into 24 spectrin-like repeats and 4 hinges (1 hinge sits between the N-terminal domain and the rod domain, 1 hinge sits between the rod domain and the cysteine-rich domain, and the other 2 hinges intervene spectrin-like repeats). It is now clear that not all the domains are absolutely required for muscle protection. Studies in mildly affected Becker patients suggest that deletion of a fairly large piece of the rod domain is not associated with major deleterious consequences to muscle function.

Knowledge learned from Becker patients inspired scientists to develop an abbreviated/truncated *dystrophin* gene for DMD gene therapy. There are two major classes of abbreviated *dystrophin* genes.³⁷ One is called the *mini-dystrophin* gene and the other is called the *microdystrophin* gene. The *mini-dystrophin* gene (minigene) is about 6 to 8 kb in size and it results in the production of a mini-dystrophin protein that is about the half size of the full-length protein (see Note). Based on clinical observations in Becker patients, there is a high likelihood that minigene therapy will improve muscle health in DMD patients.^{19,38–41} The *microdystrophin* gene (microgene) is about 3.5 to 4 kb in size and it results in the production of a microdystrophin protein that is about one-third the size of the full-length protein (see note). In addition to the truncation in the rod domain, the C-terminal domain is also deleted in the microgene. Although studies in mouse and dog models of DMD suggest that the microgene can ameliorate muscle disease and improve muscle force, no human precedent has been identified for the super-small microgene.^{42,43} We will not know whether the microgene can treat DMD patients until a clinical trial is conducted. The beauty of the microgene is that it can fit into the AAV vector, which has a maximal packaging capacity of 5 kb. To deliver a mini-dystrophin gene with AAV, the gene has to be split into two pieces and separately delivered by two independent AAV vectors (the dual-AAV

approach); using this approach would increase the complexity of therapy development.⁴⁴

PPMD: Can you briefly describe what exon skipping is and how it compares and contrasts with gene therapy as a potential treatment for Duchenne?

Dr. Duan: Three different gene therapy methods can be used to restore dystrophin expression. These are gene repair, exon skipping, and *dystrophin* gene replacement. Gene repair strategies can be used either to fix the mutation and recover a full-length *dystrophin* gene, or covert a Duchenne mutation into a Becker mutation. For the former, it requires homologous recombination (which is very inefficient in mature muscle cells) and a template of the normal sequence. Further, it may work only for patients with small mutations (such as point mutation and small deletions). To covert a Duchenne into a Becker, the mutated region (and sometimes its surrounding regions) is removed and remaining parts are ligated together. This will yield a dystrophin protein with a slightly reduced size but still functional.

Exon skipping is another repair strategy, but it does not repair the mutated gene. The mutated gene generates mutated RNA molecules. In exon skipping, the mutated region is removed by AONs from the RNA molecule while it is being processed inside the cell. Because the mutated gene will continually generate mutated RNAs, one has to continually deliver AONs to the cells in order to achieve long-term therapy. In other words, one may consider exon skipping as a “transient” gene repair therapy.

Gene replacement therapy has the longest history. It is often referred to as “gene therapy.” In this case, the original mutated *dystrophin* gene remains in the genome. A normal copy of the dystrophin gene or an engineered synthetic *dystrophin* gene is delivered to muscle to produce a functional dystrophin protein. As long as the therapeutic transgene persists in the body, it should continually produce dystrophin. Because of the packaging limitations of viral vectors, most gene replacement therapies are aimed at delivering the abbreviated versions of the *dystrophin* gene.

Compared with the naturally existing *mini-dystrophin* genes (in Becker patients) and the shortened *dystrophin* gene/RNA generated by gene repair/exon skipping, a synthetic *dystrophin* gene may have some advantages. For example, scientists may use molecular engineering techniques to generate synthetic *dystrophin* genes that are structurally and functionally superior. Further,

viral vectors can be engineered to produce much more dystrophin than a cell can produce with its own gene. Nevertheless, *dystrophin* gene replacement therapy also has a drawback. The endogenous *dystrophin* gene expresses the physiological amount of dystrophin at selected tissues at defined times. These specificities are usually lost in gene replacement therapy.

PPMD: We’ve heard of recent progress in gene editing using CRISPR/Cas9 technology—there was even an article on gene editing in a recent *New Yorker*. Can you briefly describe the gene editing approach; how is it similar and different from gene therapy?

Dr. Duan: Gene editing is another term for gene repair. Traditionally, gene editing has been very inefficient because of the lack of a good gene-editing tool. The CRISPR/Cas9 technology is a newly developed gene-editing system that originates from the bacterial defense mechanism. The CRISPR/Cas9 technology allows scientists to cut the genome at the desired locations with a guider RNA that has a sequence complementary to the DNA target. Using the CRISPR/Cas9 technology, Gersbach and colleagues have successfully restored dystrophin expression in muscle cells isolated from DMD patients.⁴⁵ Recently, Cohn and colleagues also demonstrated correction of a duplication mutation in muscle cells from patients.⁴⁶ An important concern is whether what have been achieved in cultured cells in a petri dish can be replicated in a live muscle. To this end, several groups have reported exciting new development demonstrating that it is feasible to perform CRISPR/Cas9 therapy in a live muscle in an intact mdx mouse.^{47–49} Importantly, CRISPR/Cas9 treatment significantly reduced dystrophic pathology and improved muscle contractility. Despite these encouraging progresses, we have to realize that the technology itself is still at its infant stage. There are a lot of hurdles before it can be tested in human patients. Some of these include the immune response to the bacterial-derived Cas9 protein and off-target cutting.

PPMD: What are your latest findings in delivering a microdystrophin systemically in an animal model?

Dr. Duan: DMD affects all muscles in the body. A big challenge of DMD gene therapy is to treat all muscles in the body. Such whole-body therapy was shown possible in rodents more than 10 years ago.^{50,51} However, the body size of a mouse is approximately 1000-fold smaller than that of a boy. It has been daunting to try to scale-up from a mouse to a larger mammal (such as a dog that has a body size

closer to a boy). We initially demonstrated the feasibility of systemic delivery in newborn dogs.^{52–54} But this did not work well in neonatal affected dogs because of unexpected side effects.^{43,55} Recently, we have finally accomplished the scale-up of systemic AAV delivery to juvenile DMD dogs and published the results in October 2015 in *Human Molecular Genetics*.⁵⁶ We achieved efficient AAV delivery of either a marker gene or a therapeutic *microdystrophin* gene to every muscle in the body of several young muscular dystrophy dogs. No toxicity was observed, and microdystrophin-treated muscles showed fewer lesions on histology examination.⁵⁶

PPMD: Since DMD muscles undergo degeneration and regeneration, and satellite or other muscle precursor cells will be incorporated into fibers attempting regeneration, does your approach deliver dystrophin to these precursor cells?

Dr. Duan: Treating muscle precursor cells (or muscle stem cells) has been an important goal of DMD gene therapy. However, directly delivering an AAV microdystrophin vector to muscle stem cells may have limited effect. This is because AAV mainly exists as episomal circular molecules in a cell. As a stem cell begins to divide, AAV vectors will be diluted and eventually lost in progeny cells after many rounds of cell division.

This problem can be overcome by delivering a therapeutic *dystrophin* gene with an integrating virus such as lentivirus. A tiny fraction of AAV vectors may also integrate into the genome but the integration efficiency is much lower than that of a retrovirus or lentivirus.

Another solution is to use AAV to deliver gene repair tools to muscle stem cells. In this case, the repaired *dystrophin* gene will persist for good in daughter cells.⁴⁸

PPMD: You used young dogs in your study. What do you see for a therapeutic window for gene therapy in DMD? That is, what age range do you see benefiting from the gene therapy approach?

Dr. Duan: As a first step toward systemic AAV *microdystrophin* gene therapy in a large mammal, we intentionally used young dogs that are 2 to 3 months of age. There are several considerations. The first is the amount of AAV vectors needed for the therapy. The amount of vectors that can treat one adult dog is sufficient to treat several young dogs. Although the industrial-scale AAV production is being developed, it is beyond the budget limit of an academic lab. The

second is the age. We choose an age when affected dogs just begin to show symptoms. This roughly correlates to 2 to 4 years of age in affected boys when they begin to show delay in their motor milestones and are diagnosed. At this stage, muscle damage is mild and early intervention may yield the best effect.

With this being said, we don't think age will be a limitation for systemic AAV gene therapy. Our group and Chamberlain laboratories have shown that systemic therapy in aged mdx mice (≥ 18 months old; this corresponds to ≥ 60 years of age in humans) can still improve skeletal muscle and heart function.^{57–59}

PPMD: Are you evaluating respiratory muscles, like the diaphragm, and the heart in your studies? Can these muscles benefit from gene therapy?

Dr. Duan: We achieved good gene transfer in the heart and respiratory muscles (including the diaphragm, intercostal muscle, and abdominal muscle). Based on our previous studies in the mouse model, we believe these muscles will benefit from the therapy.

PPMD: You now have established proof-of-concept for a microdystrophin delivered systemically in dogs; how do you see that progressing toward trials?

Dr. Duan: This is a critical milestone in the eventual application of bodywide gene therapy in Duchenne patients. Bodywide AAV delivery has been demonstrated in the rodent models of muscular dystrophy since 2004. However, systemic gene transfer has never been achieved in an adult subject of a large mammal. The enormous amount of vectors needed for each animal ($>10^{15}$ particles) not only implies a huge cost in vector production but also represents a significant safety concern. Scale-up AAV production may amplify contaminations that are negligible in small-scale ($<10^{13}$ particles) preparations. Importantly, unexpected inflammatory and/or immune response to the infusion of trillions of viral particles may lead to fatal complications as demonstrated in the tragic death of Jesse Gelsinger in a 1998 clinical trial.²⁰ On top of these, the ongoing massive myofiber necrosis and inflammation in adult dystrophic dogs may further worsen untoward immune responses. The excellent safety profile we saw in our study suggests that above-mentioned issues are likely manageable.

In our study, we tested only three dogs (one received a reporter gene AAV vector and two received the microdystrophin AAV vector). There is a need to expand the study to see if the success can be reproduced in a large number of dystrophic dogs.

DMD is a life-long disease. However, the longest time point in our study was four months. It is thus important to conduct long-term study to see if there are delayed immune responses or unexpected toxicity. In our study, we observed microdystrophin expression in 5% to 60% of muscle cells in different muscles. Additional studies are needed to further improve gene transfer efficiency to eventually achieve near-saturated dystrophin expression in the majority of muscles in the body. It should also be noted that in our study we evaluated only muscle histology; further studies are needed to see if systemic AAV gene therapy can improve muscle function. Last but not least, in our study we tested only one version of microdystrophin. Although this is so far the only microdystrophin that has been shown to provide physiological benefits in a large mammal, the improvement in muscle force is limited. Additional studies are needed to engineer more potent microdystrophins.

PPMD: You have a grant from PPMD to develop a gene therapy approach to deliver the sarco/endoplasmic reticulum calcium ATPase (*SERCA*) gene; can you tell us what the rationale is there and what progress you have made?

Dr. Duan: In DMD, a pivotal event downstream of dystrophin deficiency is the elevation of calcium in the cytoplasm of muscle cells. Elevated cytosolic calcium triggers proteolysis and muscle cell death.⁶⁰ Strategies that can reduce calcium overload in muscle cells will restore calcium homeostasis and reduce muscle disease. *SERCA* is the calcium pump that removes cytosolic calcium in muscle. With the funding from PPMD, we have tested whether AAV delivery of the *SERCA* gene can treat muscular dystrophy in animal models. We found that intravenous injection of the AAV *SERCA* vector to mdx mice significantly improved skeletal muscle force and heart function. Our next step is to test this highly promising therapy in affected dogs.

Duchenne patients do not have dystrophin in their body. Dystrophin generated by gene repair or gene replacement therapy could be considered as a foreign molecule by our immune system and hence mount an immune response to reject cells that contain the newly generated dystrophin protein. This will not be a concern for *SERCA* gene therapy because *SERCA* already exists in patient body.

PPMD: What preclinical steps need to be taken before an investigational new drug (IND) application can be filed for a gene therapy trial with systemic delivery of dystrophin?

Dr. Duan: A number of IND-enabling studies are needed before an IND can be issued. These include toxicity studies in small and large animals, generation of good manufacturing practices (GMP)-quality AAV vectors, pharmacokinetic and pharmacodynamics studies, and randomized blinded studies with sufficient sample size to confirm and validate systemic AAV therapy in dogs. It should also be noted that, in our study, we have used a canine *microdystrophin* gene. For clinical trial we need to develop a human-version *microdystrophin* gene.

PPMD: Delivering a gene therapy vector to all muscles affected in Duchenne has been one of the key challenges in developing this potential therapy. Can you tell us how research has progressed from the single-muscle injections that have been done in clinical trials toward systemic delivery?

Dr. Duan: Single-muscle injection is the foundation for systemic delivery. When gene therapy was initially tested in mdx mice, investigators performed single-muscle injection. The first attempt to achieve systemic delivery involved co-administration of vessel-perfusing agents such as histamine. The identification of new AAV serotypes that can escape from the vasculature and reach muscle cells has opened the door to “true” systemic delivery. Initial tests were performed in dystrophic mice and hamsters and then in neonatal dogs. Our study now suggests that systemic delivery can also be achieved in juvenile dystrophic dogs.

Single-muscle injection has been used in most of the muscular dystrophy clinical trials to date. Only one study has tested systemic delivery in human patients with a neuromuscular disease. In this trial (by AveXis, a biotech firm in Dallas), Drs. Mendell and Kaspar and colleagues delivered an AAV-9 vector to infants who suffered from a severe form of spinal muscular atrophy. According to the report by Dr. Mendell on October 5, 2015, at the International Congress of the World Muscle Society, nine patients have received therapy. The therapy appears to be generally safe and well tolerated. Signs of clinical improvement have also been noted (<http://avexis.com/data-ongoing-study-avxs-101-spinal-muscular-atrophy-type-1-presented-world-muscle-congress/>).

PPMD: Prior clinical trials of gene therapy in Duchenne have encountered immunological reactions that have impaired efficacy. What is being done to address that issue?

Dr. Duan: In our study in dystrophic dogs, we found that a five-week transient immune sup-

pression seemed to have made the trick. In hemophilia B trial and spinal muscular dystrophy trials, transient application of large dosage of steroids was found to be effective. Another important aspect is to screen patients for preexisting immunity to the viral vector and the therapeutic gene product. In our study, we screen affected dogs for the preexisting neutralization antibody to AAV-9 and we only used dogs that were seronegative for AAV-9 (meaning these dogs have never been exposed to AAV-9). It should be noted that additional efforts are needed before we can completely solve the problem of the immune response. In this regard, several new strategies that are being tested in laboratories (such as plasmapheresis and AAV capsid engineering) have shown promise.

PPMD: In scaling up to do clinical trials in Duchenne, vector production may be a limitation. How is the problem of having sufficient vector to do clinical trials and, later, to treat large numbers of Duchenne patients being addressed?

Dr. Duan: Vector manufacture has been recognized as a key bottleneck to scale-up of systemic AAV therapy in human patients.^{61,62} Classic AAV production protocol requires transient transfection of three different plasmids to HEK 293 cells that are cultured in a petri dish. AAV is then purified from culture medium and cell lysate using ultracentrifugation. Numerous strategies have been tested to scale-up AAV production and purification. Some examples include the use of the infection approach with the baculovirus-based system or herpes virus-based system, and producer cell lines. Cell culture has also been expanded from the petri dish to roller bottles, cell factors, and bioreactors. Chromatography-based purification strategy has also been developed for different AAV serotypes. Most importantly, robust vector characterization and analytical quality control protocols and standards have been developed or are being developed and validated.

PPMD: What about commercial partners that would be needed to bring a therapy through regulatory approval and to market? Can you tell us about your partnership with SOLID GT and how that may move gene therapy toward clinical trials in Duchenne?

Dr. Duan: Industry investment is essential to bring an experimental vector into a gene therapy product. Funding from biopharmaceutical partners

will offset the high cost of clinical studies. Solid GT is a subsidiary of Solid Biosciences and was started by parents of a boy with DMD. As stated in the company's website (<http://solidbio.com/gt/>), it "is dedicated to the development of durable disease-modifying interventions for Duchenne Muscular Dystrophy through gene therapy." Solid GT is currently pursuing systemic AAV *microdystrophin* gene therapy. According to the company's website "Solid GT is conducting a number of key studies that will enable us to enter the clinic within two years." "These studies include efficacy, safety and dose ranging assessments" in mdx mice and dystrophic dogs. We have been involved in the animal studies. The results so far are very promising. Besides animal studies, Solid GT is also working with academic and corporate partners to refine and scale-up AAV manufacturing technology, and is addressing a number of other key aspects of this program, in preparation for upcoming human clinical trials. On November 3, 2015, Solid GT announced that it has raised \$42.5 million in series B financing to advance gene therapy for DMD (www.businesswire.com/news/home/20151103006362/en/Solid-GT-Raises-42.5-Million-Series-Financing).

NOTE

Early on, all truncated dystrophins are called mini-dystrophin. In 2002, Dr. Jeff Chamberlain coined the term "micro-dystrophin" to refer to the abbreviated dystrophins that are about one-third the size of the full-length dystrophin protein. Micro-dystrophin does not contain a complete C-terminal domain. The micro-dystrophin genes are 3.5 to 4-kb in size and can fit into a single AAV vector. The original term "mini-dystrophin" is now reserved for the abbreviated dystrophins that are at least half the size of the full-length dystrophin protein. Mini-dystrophin often contains the complete C-terminal domain. The minidystrophin genes are 6 to 8-kb in size and cannot fit into a single AAV vector. Dual AAV vectors are required to deliver the mini-dystrophin gene.

The micro-dystrophin gene is often abbreviated as the microgene. The mini-dystrophin gene is often abbreviated as the minigene. "Micro-dystrophin" and "mini-dystrophin" are also spelled as "micro-dystrophin" and "minidystrophin", respectively.

For this historic reason, some of the early versions of the microgene (these that were published before 2002) have been called the minigene. For example the $\Delta 3849$, $\Delta 3990$ and $\Delta 4173$ minigenes developed in Dr. Xiao Xiao's laboratory are actually microgenes.

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AUTHOR DISCLOSURE

D.D. is a member of the scientific advisory board and an equity holder of Solid GT, a subsidiary of Solid Biosciences.

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