Micro-Dystrophin Gene Therapy Goes Systemic in Duchenne Muscular Dystrophy Patients

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Whole-body systemic gene therapy is likely the most effective way to reduce greatly the disease burden of Duchenne muscular dystrophy (DMD), an X-linked inherited muscle disease that leads to premature death in early adulthood. Genetically, DMD is due to null mutation of the dystrophin gene, one of the largest genes in the genome. Recent studies have shown highly promising improvements in animal models with intravascular delivery of the engineered micro-dystrophin gene by adeno-associated virus (AAV). Several human trials are now started to advance AAV micro-dystrophin therapy to DMD patients. This is a historical moment for the entire field. Results from these trials will shape the future of neuromuscular disease gene therapy.

Keywords: Duchenne muscular dystrophy, dystrophin, micro-dystrophin, adeno-associated virus, systemic delivery, clinical trial

MANY DISEASES AFFECT TISSUES distributed throughout the body. These diseases present a great challenge for gene therapy due to the need for body-wide delivery of a large quantity of a virus vector. A major breakthrough published in December 2017 has now provided the proof-of-principle for systemic gene therapy in human patients.¹ Mendell *et al.* treated infants with spinal muscular atrophy type 1 (SMA1) using a single intravenous injection of a therapeutic adeno-associated virus (AAV) serotype 9 vector at doses up to 2×10^{14} viral genome (vg) particles/kg. Treatment resulted in spectacular improvement in morbidity and mortality. Following this success, three independent systemic AAV gene therapy trials have been started in the United States to treat Duchenne muscular dystrophy (DMD), the most common lethal muscle disease in boys. These include Solid Biosciences (NCT03368742), Nationwide Children's Hospital (NCT03375164), and Pfizer (NCT03362502). A fourth trial has also been planned in Europe by Genethon and Sarepta Therapeutics.² DMD is caused by null mutations in the dystrophin gene.³ Patients become

wheelchair bound in their early teenage years and die from diaphragm muscle and/or cardiac muscle failure.

Systemic AAV gene therapy for DMD faces a unique hurdle. Unlike the SMA1 trial in which the therapeutic gene can fit into an AAV particle,¹ the size of the dystrophin coding sequence (~ 11.5 kb) greatly exceeds the 5 kb AAV packaging capacity.⁴ A hint for the solution surfaces from studying Becker muscular dystrophy (BMD). BMD is a mild form caused by in-frame deletions in the dystrophin gene. It was found that BMD patients who lost nearly half of the gene still lived quite a healthy life, suggesting half-size dystrophin is protective in human patients.⁵ Subsequent studies in animal models confirmed that the 6–8 kb mini-dystrophin genes indeed provided excellent protection. However, the minigene still exceeds the packaging limit of the AAV vector. The problem was finally solved with the development of micro-dystrophin genes < 4 kb. More than 30 different microgenes have been tested since 1997. While not all microgenes reduce muscle disease, many have resulted in good protection in

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various mouse models. Recent studies in young adult affected dogs further suggest that administration of a high-dose AAV micro-dystrophin vector through the circulation can result in safe and body-wide transduction in a diseased large mammal.^{6,7} Collectively, these preclinical results set the foundation to test systemic AAV microgene therapy in human patients.

Several important questions are to be answered in ongoing trials. The first and most important is safety. In the SMA1 trial, all patients tolerated high-dose intravenous AAV-9 injection. In young adult affected dogs, systemic AAV-8 or -9 micro-dystrophin injection showed good safety profiles.^{6,7} Acute toxicity was not observed in the first patient in the trial conducted by Nationwide Children's Hospital (2×10^{14} vg/kg).⁸ In a trial on X-linked myotubular myopathy (NCT 03199469), four patients (0.8–4.1 years old) received an intravenous injection of an AAV-8 vector expressing the human myotubularin 1 gene at a dose of 1×10^{14} vg/kg.⁹ According to an interim report from Audentes Therapeutics, the sponsor of the trial, treatment resulted in neuromuscular and respiratory function improvement. No death was reported. Some adverse events were found, but all were manageable.⁹ Collectively, there seems a good chance that DMD patients may tolerate systemic AAV micro-dystrophin gene therapy.

With this backdrop, a newly published study should also be mentioned because it raises potential toxicity of high-dose systemic AAV administration in large mammals.¹⁰ Specifically, Hinderer *et al.* injected an AAV-9 variant (AAV-hu68) expressing the human survival of motor neuron gene to three 14-month-old nonhuman primates (NHPs) and three 3- to 30-day-old piglets at a dose of 2×10^{14} vg/kg. All NHPs developed liver toxicity, and one had to be euthanized at day 4 after injection due to liver failure. No hepatic toxicity was noticed in piglets, but all of them developed neuronal toxicity within 14 days after injection and had to be euthanized.¹⁰ In light of this new report, extreme caution should be taken and toxicity carefully monitored when moving forward with ongoing trials.

In the SMA1 trial, all patients received AAV administration before the age of 8 months. Only one DMD trial accepted patients in this age range. Infants have unique immunological advantages due to the relatively immature nature of their immune system. Ongoing trials in DMD patients will show whether older children can tolerate high-quantity intravenous AAV delivery.

The next question is whether the highly shortened microgene can ameliorate muscle disease in boys with DMD. The half-size minigene is originated from human patients, but there is no human

precedent for the microgene. The micro-size dystrophin proteins have been detected (even at high abundance) in patients who carry very large in-frame deletions.¹¹ However, clinical manifestations of these patients' symptoms are not alleviated. It is believed that the rationally designed synthetic microgene should outperform the naturally existing ones that are found in patients. However, the vast majority of the efficacy data of the synthetic microgene are from mice. A limited functional study in a canine model suggests that micro-dystrophin may improve muscle force in affected dogs, but certainly not to the levels seen in the murine model.¹² The primary outcome of the ongoing DMD Phase I trials is not to determine muscle histology and motor function improvement. Hence, these trials will not yield a conclusive answer on whether microgene can attenuate muscular dystrophy in human patients. However, the results from these trials should give some clues on the performance of micro-dystrophin in the muscle of DMD patients such as the expression level of micro-dystrophin, restoration of dystrophin-associated protein complex, and amelioration of some aspects of histological lesions. Motor function assay has been included in the protocol in at least one trial. The data from the functional assay, while limited due to the small sample size, will still shed important light.

The simultaneous initiation of three independent trials in the United States and one additional trial planned in Europe presents a unique opportunity to address several puzzling issues in the field. One such issue is dystrophin immunity. An early local injection trial suggests that the T-cell immune response to dystrophin may constitute a barrier in patients.¹³ In this case, patients will have to be carefully selected based on the configuration of the microgene to minimize cellular immune reaction. This is very difficult to investigate in animal models due to their inherent limitations. A conclusion may likely come out from the ongoing trials, since different inclusion criteria are used in regard to mutation. One trial only treats patients who carry specific mutations. The other two trials, however, are open to patients with any mutation.

The full-length dystrophin protein contains an N-terminal (NT) domain, 24 spectrin-like repeats, four hinges, a cysteine-rich (CR) domain, and a C-terminal domain. Among the four hinges, hinges 1 and 4 are positioned before and after 24 repeats, respectively. Hinges 2 and 3 are dispersed in the middle of 24 repeats. Animal studies have shown muscle protection with microgenes that carry either four or five repeats, with or without a centrally located hinge. However, it is unclear which configuration

offers better protection due to the lack of side-by-side comparisons. Interestingly, a four-repeat microgene is used in one trial and two different five-repeat microgenes are used in the other two trials. Furthermore, the microgene used in one trial does not have a central hinge, but a central hinge is included in the microgenes used in the other two trials. Hopefully, the results from these ongoing trials will help to clarify our understanding.

All the existing microgenes carry the NT and CR domains. The major difference is in the formulation of the repeats and hinges. The inclusion/exclusion of a particular repeat/hinge should be experimentally determined. However, few comprehensive studies have been reported so far. The Chamberlain lab studied the consequences of including hinge 2 in micro-dystrophin.¹⁴ The authors found that a polyproline site in hinge 2 negatively impacted the myotendinous junction and neuromuscular junction in some muscles. Further, they found that the presence of hinge 2 (1) compromised the capacity of micro-dystrophin to prevent muscle degeneration and (2) resulted in the formation of abnormal ringed fibers in the gastrocnemius muscle. Replacing hinge 2 with hinge 3 or deleting the polyproline site from hinge 2 prevented these negative effects.¹⁴

Neuronal nitric oxide synthase (nNOS) plays a crucial role in many muscle activities.¹⁵ nNOS is tied to the sarcolemma by dystrophin in normal muscle. The author's laboratory discovered R16/17 as the dystrophin nNOS-binding domain for anchoring nNOS to the muscle cell membrane.^{16,17} Loss of sarcolemmal nNOS leads to functional ischemia, which contributes to the initiation and progression of muscle disease in DMD.^{18,19} Delocalization of nNOS to the cytosol reduces muscle force generation.²⁰ Inclusion of R16/17 in synthetic dystrophin genes restored membrane nNOS localization, significantly prevented functional ischemia and ischemic damage, and significantly enhanced exercise capacity.^{16,21}

It should be noted that the aforementioned studies on hinge 2 and R16/17 were conducted in the mouse model. It remains unclear whether the same is true in large mammals. Although current clinical trials are not designed to test therapeutic advantages/disadvantages of hinge 2 and R16/17, the differences in the microgene design of ongoing trials have now made it possible to get a clue about these important details. In particular, an R16/17-containing microgene is used in one trial. Of two trials using a central hinge-containing microgene, one has hinge 2, and the other has hinge 3. Muscle biopsy from the trial patients should be carefully examined for the integrity of the structure

of myotendinous and neuromuscular junctions, as well as sarcolemmal localization of nNOS.

Besides new knowledge on micro-dystrophin biology, it is expected that the ongoing trials should also provide important first-hand information on the use of different AAV serotypes and different muscle-specific promoters in DMD patients. For the ongoing trials in the United States, two AAV serotypes (AAV-9 and AAV-rh74) and three different muscle promoters are used. The detailed information on AAV serotype and the muscle promoter for the planned trial in Europe have not announced. It is possible that they may be different from those used in the ongoing U.S. trials. AAV-9 was isolated from human tissues.²² AAV-rh74 was isolated from rhesus macaque monkeys, and it shares 93% homology with AAV-8.²³ Because tissues are not available, it is usually not possible to perform a body-wide biodistribution study or to check expression in every muscle in human patients. In this regard, preclinical studies have revealed a satisfactory transduction profile for the AAV serotypes used in the ongoing trials. Systemic delivery of AAV-8, AAV-rh74, and AAV-9 has resulted in robust muscle transduction in mice though AAV-8, and AAV-rh74 has also shown liver preference and AAV-9 displayed cardiac tropism.^{24–26} High-dose systemic AAV-8 and -9 delivery has been tested in several studies in normal and affected dogs. Body-wide muscle transduction was obtained in these studies. Bio-distribution studies have shown the presence of the AAV genome in all muscles in the body, but the liver had the highest abundance.^{6,27–29} Surprisingly, AAV-9, which is cardiotropic in mice, was much less efficient in transducing the heart in canines.³⁰

Systemic DMD gene therapy has come a long way. Progress in the fields of virology, immunology, dystrophin biology, animal models, and large-scale clinical grade AAV manufacture have shaped the design of current trials. However, there is still not a clear picture on the function of every portion of the dystrophin protein. Improved understanding on dystrophin biology, as illustrated in the recent discovery of multiple new dystrophin membrane-binding domains, will teach us how to engineer more potent microgenes.³¹ On the other side, application of state-of-the-art forced *in vivo* evolution strategies in relevant models is expected to yield novel AAV capsids that can better meet the needs of DMD patients.³²

The ongoing systemic AAV microgene therapy trials mark an important milestone in the development of DMD gene therapy. It is the fruit of accumulated knowledge spanning >30 years of research from many laboratories. These trials will start the process for the eventual approval of

an effective genetic treatment for DMD by regulatory agencies.

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