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Signs of Progress in Gene Therapy for Muscular Dystrophy Also Warrant Caution

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AAV-based vectors have emerged as plausible candidates for clinical gene transfer to muscle, but they present several challenges in the context of Duchenne muscular dystrophy (DMD). These include the need to "miniaturize" an extremely large therapeutic gene, the development of strategies for effective regional and systemic vector delivery to a tissue mass the size of the musculature, and the avoidance of immune-mediated elimination of transgene in a degenerative disease. Leveraging previous progress on gene miniaturization,[1] in this issue of *Molecular Therapy* a multidisciplinary team of investigators provides important new perspectives on vector delivery[2] and an affiliated group of investigators from the same institution report on a safety trial intended to expedite translational studies of the immune response to a transgene.[3] The collaboration achieved thus far will require a future marriage of these three avenues of research to help set the stage for clinical efficacy with gene therapy for Duchenne and other muscular dystrophies. Nevertheless, important new questions arise about the potential escalation of risk to research participants in which a large volume of tissue is transduced with even a subtly immunogenic vector, and the advances presented in the two articles bring such bioethical concerns to center stage for discussion by the broader research community.

The DMD gene and its protein product dystrophin were at the epicenter of a revolution in human genetics 25 years ago, defining the birthplace of positional cloning.[4] We now recognize that this distinction was partially related to the extraordinary size of both the gene and its product, responsible for the high mutation rate that facilitated the genetic analysis but complicating the development of gene-based therapies. Measured from its promoter to the polyadenylation site at the 3′ end, the DMD gene is a staggering 2.4 megabases in length (11,057 base pairs complementary DNA), the longest gene fully characterized to date. If it were to be used in its unadulterated form as a molecular therapeutic, its molecular weight would be approximately 1.6 GDa and would require transfer to cells representing almost half of the body mass.

It was later recognized that much of the protein's 427-kDa molecular weight was attributable to 24 spectrin-like repeats.[5] With the discovery that a naturally occurring mutation in the mouse provided a convenient animal model,[6] it was possible to test by gene transfer the hypothesis that full-length and internally truncated versions of dystrophin might ameliorate the disease process.[7,8] Initially this research was guided by genotype–phenotype correlation related to the milder allelic form of disease at the dystrophin locus, Becker muscular dystrophy, in which patients are still ambulatory into adulthood.[9] However, in 2000, a team led by Xiao Xiao showed that an adeno-associated virus (AAV) vector could accommodate a microdystrophin retaining only five of the original 24 spectrin-like repeats and only the dystroglycan-docking half of a unique C-terminal domain.[1] Initial tests of this idea appeared promising after local intramuscular injections in mice and gained momentum after the eventual demonstrations of therapeutic systemic gene transfer in mice using pseudotyped vectors in serotypes 1 and 6 (refs. 10, 11).

Based on data such as these for preclinical efficacy (and data from other clinical studies), the US Food and Drug Administration authorized a phase I clinical study of intramuscular injection of an AAV serotypes 2 and 5–cytomegalovirus (AAV2.5-CMV) microdystrophin into the biceps muscle of six subjects with DMD.[12] The immunogenicity of the dystrophin transgene product in deletional-null recipients had been previously reported[12] but that paper left unaddressed the widely anticipated question of vector capsid immunogenicity in view of other preclinical and clinical studies.[13] Importantly, the vector capsid chosen for this study was an engineered chimera designed to gain the improved efficiency of AAV1 yet retain most of the protein sequence of AAV2. Additionally, these changes may circumvent some of the immune problems previously demonstrated for naturally occurring AAV vectors. In this issue, Bowles *et al.*[2] address some questions that can be reasonably answered by studying peripheral blood samples from this limited group of patients, as outlined below.

To put the accompanying article by Fan *et al.*[3] into perspective, it is worth revisiting an inconvenient truth about predictions on experimental scale in animal studies. The problem was first brought to public attention by J.B.S. Haldane, whose scaling concepts referred to as "Haldane's principles" address the observation that blood-vessel walls are stronger in larger animals,[14] reflecting the hemodynamic effects of gravity at greater body mass. Regarding vector biodistribution in gene therapy, the distinction between murine and canine models increases with age, as does the size discrepancy. AAV serotypes

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that confer widespread muscle transduction in skeletally mature mice or newborn puppies following simple intravenous administration have required forced extravasation from the vascular space to achieve similar results in adult and even preadolescent dogs.[15,16] The least invasive way to accomplish this in an extremity relies on *locally* "retrograde" infusion against a tourniquet from a distal vein, with pressure-dependent extravasation across the endothelium into the interstitium. In anticipation of the need for vector extravasation to achieve widespread muscle transduction as a prerequisite for clinical efficacy in muscular dystrophy, the safety of such an approach has been carefully investigated by Fan *et al.* using isotonic saline without vector in adults with various forms of muscular dystrophy. Studies have progressed from local to regional strategies, with the utimate goal being systemic dosing, given that striated muscle (including the heart) is a global problem in DMD. AAV serotype, route or method of delivery, and vector dose constitute the fundamental variables that influence biodistribution, potential for toxicity, and therapeutic effect in animal and human studies.

An overview of the approaches and findings in these two papers brings into focus some bioethical questions that warrant consideration in contemplating a future course. The article by Bowles *et al.* opens and closes with concise analyses of the primary, tertiary, and quaternary structure of the AAV capsid variants associated with skeletal muscle tropism. This sets the stage for the further analysis of a few chimeric variants—in particular, one christened AAV2.5, which combines the heparin binding of AAV2 (which is useful in purification) with the improved muscle-transduction profile of AAV1. Intriguingly, the chimeric capsid of AAV2.5 appears to reduce the affinity for monoclonal and polyclonal antibodies raised in mice against both AAV1 and AAV2, as would be desirable so as to avoid neutralization upon translation to previously exposed patients. Clinical data based on 36 human sera suggest a quantitative, but not qualitative, difference in the range of neutralizing antibody titers. The data from the six DMD patients are then tabulated, revealing that only two of six patients had significant preinjection levels of circulating antibody against AAV2.5. In biopsy specimens, the ratio of vector to diploid cellular genomes detected is about 1:1000 for these two patients but 1:1 for the other four. This correlation suggests that neutralizing antibodies precluded efficient gene transfer at the time of injection. As in the previous report on this clinical trial,[11] no photomicrographic evidence of recombinant dystrophin expression is provided, although there is a text description of a few fibers stained in two patients. This negative result may be related to reduction in transcriptionally active copies of the vector or a vector dose that is less effective than predicted in preclinical studies by this route of delivery. In the figures which follow, there is a suggestion of some anticapsid cell-mediated immunity in up to four of the patients, but unambiguous evidence of humoral immune responses in six of six (100- to 1000-fold average increase in anti-AAV2.5 serological titer).

The article by Fan *et al.* provides detailed data from a study designed to address the safety of pressurized fluid extravasation in the tourniquet-isolated leg. Seven adult subjects with slowly progressive muscular dystrophies underwent unilateral perfusion with normal saline via the greater saphenous vein after placement of a tourniquet at the midthigh. The study design grouped patients sequentially into cohorts by volume of infusate, beginning with 5% and reaching 20% of calculated limb volume. The figures address several acute aspects of the physiology of this intervention, leading off with the physical appearance of the limb, next showing continuous tracings of muscle compartment pressures and oxygen saturations, and finally the magnetic resonance imaging–detectable changes in muscle fluid volume by compartment. Although a 20% volume expansion was well tolerated when patients emerged from intravenous anesthesia after tourniquet release, the fact that the first patient (the only one who had not received anesthesia) experienced significant discomfort at only 5% volume expansion suggests modest but transient signaling through tissue stretch-activated nociceptors, with important implications for future clinical study design. Most of the clinical data presented in Fan *et al.* mirror what has previously been observed in large-animal models, although the sustained high compartment pressures in one subject in the 20%-volume group suggest that this should be viewed as an upper limit. The complete lack of detectable injuries on a rigorous battery of postperfusion studies is important and encouraging for future development. Three of seven subjects noted that the perfused leg felt "tight" for up to 2 hours following perfusion, and one subject had visible petechiae (spots due to minor hemorrhage) for three days. Although these clinical observations were not listed as serious adverse events, they clearly indicate that it will be difficult to blind subjects to the laterality of perfusion, which may introduce bias in future studies in the assessment of "objective" signs of strength improvement after gene transfer. Importantly, no subject experienced adverse hemodynamic or ventilatory consequences of either the anesthetic or the central volume load after tourniquet release (0.7–1.0 liter normal saline in the 15–20% groups).

These findings highlight some important bioethical issues that are particularly relevant to translational research addressing "single-dose" gene therapy strategies for a broad class of inherited and acquired diseases. Autoimmune myositis occurs when the immune system attacks and damages muscle. It is often associated with profound loss of muscle strength in previously healthy individuals and might be amplified in inherited myopathy, such as DMD, in which regenerative reserve of the muscle is depleted. There is the unresolved theoretical possibility of severe immune-mediated destruction of vector-transduced cells involving an entire limb, with target antigens related to either capsid proteins, transgene products, or both. In a phase I trial of local intramuscular injection, myositis confined to the small volume of vector distribution would probably be of limited consequence for the subject. However, both the risk and potential benefits escalate with the volume of distribution as vascular routes of delivery are contemplated. This is a critical point in the informed-consent process, as are the serological data that seemingly guarantee that subjects will be immunized against the vector capsid, thereby precluding future participation in studies using the same investigational drug or requiring a more complicated and challenging approach to immune modulation. Transient immunosuppression with drugs stronger than the methylprednisolone monotherapy used here may address the capsid antigen, but other strategies will probably be required to avoid immune response against dystrophin in deletional-null patients. This concern is

heightened in view of the potential for enhanced immune surveillance in dystrophic muscle, where ongoing necrosis attracts a wide range of mononuclear cells.

A convincing demonstration of durable efficacy following vascular delivery in a canine model would be reassuring to institutional review board members and others charged with the responsibility for protection of pediatric research subjects, as was the case with the *RPE65* trials for Leber congenital amaurosis. Here, the devil is in the details, as the commonly available canine model has an intact open reading frame for full-length dystrophin with some expression via exon skipping, whereas another model in development appears to have a large deletion, thereby addressing a broader proportion of the DMD patient population. Even in the former model, a recent report in this journal of widespread AAV-mediated dystrophin gene transfer showed debilitating yet unexplained myositis and contractures,[17] thus highlighting the potential risks associated with regional or systemic gene delivery in DMD.

Ultimately, to make a significant impact in the burden of this disease, gene therapy must address the primary causes of mortality in DMD: progressive deterioration of cardiorespiratory reserve. Recent studies on the enhanced efficiency of vascular gene transfer to the heart[18] offer some encouragement and suggest that data from clinical trials to address pressing public health problems such as acquired forms of heart failure will eventually expedite studies of systemic gene transfer in DMD.[19,20] As the development process continues for effective gene therapy in DMD, we must weigh the procedural risk and observed toxicity associated with study agents versus the potential for benefit in a patient population faced with significant burden of disease. By better defining the therapeutic efficacy and risks associated with a given vector type, vector dose, and route of delivery, it may be possible to present regulatory agencies with evidence supporting direct benefit in such clinical studies and thereby supporting studies beyond minimal risk and more rapid advances in the field. Of course, safety must be paramount in clinical research. We eagerly await the demonstration of direct benefit from gene transfer in DMD, as has been observed in recent studies of hemophilia.[21] The systemic route of delivery and therapeutic effect in AAV8-mediated delivery of factor IX offers a glimpse of steps forward in gene therapy as potentially relevant to DMD, and may also set a standard of how interdisciplinary teams can conquer complex problems in medicine.

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Cell-Penetrating RNAs: New Keys to the Castle

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The plasma membrane represents the walls of the cellular fortress. It maintains cellular integrity by separating the insides from the outside, and the gatekeepers within these walls are very selective about which molecules are allowed in or kept out. Since the pioneering work on bacterial transformation by Griffith, Avery, and others more than half a century ago,[1,2] a series of technologies has been developed to deliver payloads across membranes. In a forthcoming article in *Molecular Therapy*, Magalhães *et al*. describe a new way to unlock one of the fortress gates in the form of aptamers they dub "c1" and "Otter."[3] These aptamers bind and penetrate into a diverse collection of mouse and human cells,

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