vector for SCID-X1 gene therapy by concatemeric array transfection. *Blood* **113**: 5104–5110.
15. Straathof, KC, Pulè, MA, Yotnda, P, Dotti, G, Vanin, EF, Brenner, MK *et al.* (2005). An inducible caspase 9 safety switch for T-cell therapy. *Blood* **105**: 4247–4254.
16. Hoyos, V, Savoldo, B, Quintarelli, C, Mahendravada, A, Zhang, M, Vera, J *et al.* (2010). Engineering CD19-specific T lymphocytes with interleukin-15 and a suicide gene to enhance their anti-lymphoma/leukemia effects and safety. *Leukemia* **24**: 1160–1170.
17. Cavazzana-Calvo, M, Payen, E, Negre, O, Wang, G, Hehir, K, Fusil, F *et al.* (2010). Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. *Nature* **467**: 318–322.

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AAV6-Mediated Gene Silencing fALS Short

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The truth is rarely pure and never simple.
—Oscar Wilde

Amyotrophic lateral sclerosis (ALS) is a devastating progressive motoneuron disease that is invariably fatal within 3–5 years after diagnosis.[1] Approximately 2% of ALS cases are due to an autosomal dominant mutation in the gene encoding Cu/Zn superoxide dismutase1 (SOD1). Because SOD1 enzyme activity is not lost in familial ALS (fALS) and transgenic expression of pathogenic G93A SOD1 (muSOD1), in addition to expression of the normal endogenous mouse SOD1, faithfully recapitulates human fALS pathology, muSOD1 expression likely induces pathology via a toxic gain of function. Therefore, muSOD1-related fALS is a potential target for gene therapy aimed at silencing the mutant SOD1 allele in motoneurons. fALS is also attractive for gene therapy from a regulatory perspective, because no therapy is currently available that alters the progression of the disease.

In this issue of *Molecular Therapy*, Towne *et al.* report the findings of a study utilizing recombinant adeno-associated viral vector 6 (rAAV6) to deliver a small hairpin RNA (shRNA) to silence muSOD1 in muscle and motoneurons as a potential therapy in a mouse model of fALS.[2] Even though they report no effect on the primary outcome measure—longer lifespan of the fALS mice—the study represents a significant attempt to translate an allele-specific gene-silencing strategy to treat fALS. The results stand in stark contrast to an earlier study that reported a remarkable extension of life span in fALS mice following delivery of an equine infectious anemia virus (EIAV) lentiviral vector encoding an identical therapeutic gene.[3] Studies that report negative results, such as the one by Towne *et al.*, are rarely published, but this impressive study may nevertheless move the field forward. Because the two studies employed methods that are as similar as could possibly be expected from two different laboratories, exploring the potential reasons for the vast difference in outcome between the two is crucial because fALS is an excellent target for gene therapy.

By any measure, the study by Towne *et al.* is well designed and carefully executed, albeit ostensibly reporting a negative result. These workers made use of rAAV serotype 6 to deliver shRNA to muscle and motoneurons via retrograde transport to silence expression of muSOD1 messenger RNA (mRNA) in the fALS mouse model. Each motoneuron innervates one or more muscle fibers. Intramuscular injection of vector results in transduction of a motoneuron when the virus infects the axon and the virion is transported back to the motoneuron's nucleus. Retrograde delivery of the vector and subsequent motoneuron transduction is the preferred route for several important reasons. First, a direct injection of the vector into the spinal cord is impractical because the numerous injection sites that would be required would greatly increase the probability of morbidity. Second, transduction specificity for motoneurons would be lost; third, there are advantages to silencing muSOD1 in muscle as well, which would not be targeted by this strategy.[2] Finally, as discussed below, systemic vector injections do not lead to sufficient motoneuron transduction for a therapeutic effect.[4] Furthermore, the experiments were extremely well controlled, comprising large numbers of wild-type, vehicle-injected, and control (receiving rAAV6 encoding a control shRNA that contains only two mismatches, which is a strict control for shRNA experiments) mice. Transduction of all targeted muscle groups was shown by green fluorescent protein fluorescence, and the sample sizes of the treatment groups were far greater than those seen in most preclinical studies.

Beneficial effects of knocking down the G93A muSOD1 mRNA were previously established by direct intra–spinal cord injections[5] and via retrograde transport after intramuscular injection of a rabies glycoprotein-pseudotyped lentiviral vector (EIAV).[3] The latter results indicated that widespread muSOD1 knockdown via global muscle transduction using a vector that can be retrogradely transported from muscle to spinal cord motoneurons might be a potent treatment for fALS mice.[4,5] Towne and co-workers had previously performed systemic injections of rAAV6, which had been shown to transduce motoneurons after intramuscular injections in rodents[4,6] and in nonhuman primates.[7] Unfortunately, this strategy affected neither the onset of disease nor survival of fALS mice, apparently because of low transduction

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efficiency of motoneurons.[4] To enhance motoneuron transduction efficiency, the workers then chose to inject individual muscle groups in neonatal mice, as done previously by Ralph *et al*. of Oxford Biomedica.[3] The latter group utilized EIAV to specifically transduce, in addition to other large muscles, the murine diaphragm, and therefore the phrenic motoneurons, a potentially crucial target to delay death in fALS. This regimen allows injection of vector at high titers in muscle groups throughout the diaphragm and thorax to target the relevant motoneuron pools that are important for maintenance of respiratory function and therefore survival of the fALS mouse.

Thus, the remarkable extension of life observed by Ralph *et al*. led to adoption of a similar delivery strategy by Towne *et al*. as a reasonable alternative to the systemic method employed in their previous rAAV6 study.[4] The benefits of this change in injection strategy were borne out—injection of individual muscle groups and the thorax in neonatal fALS mice led to motoneuron transduction efficiencies approaching 40% in motoneuron pools that innervate injected respiratory muscles.[2] Even with this large increase in motoneuron transduction efficiency as compared with their previous study,[4] Towne *et al.* failed to detect significant therapeutic benefit.[2] In contrast to their earlier systemic injection study,[4] however, they observed improvements in body weight, muscle mass, and evoked compound muscle action potentials, indicating that muscle atrophy was significantly impacted by rAAV6-shRNA delivery and partial silencing of muSOD1 (ref. 2).

Is it possible that knocking down muSOD1 mRNA in motoneurons of fALS mice alone may not be therapeutic? As the authors point out, *Cre*-lox excision of muSOD1 specifically in fALS mouse motoneurons delays onset of disease and increases life span by about 130 days.[8] Theoretically, in the *Cre*-lox experiment, because muSOD1 expression is completely abrogated in 100% of Cre-expressing motoneurons, this should yield the maximal level of life extension by any muSOD1-silencing strategy.[8] It should be noted that Ralph *et al.* reported a greater than 120-day extension of survival time in fALS mice with muSOD1 silencing in 50% of motoneurons.[3] Given the detailed level of experimental execution and analysis in the study by Towne *et al*.,[3] there are two remaining explanations for the lack of functional benefit in fALS mice. Either the population of transduced motoneurons was insufficient or the level of knockdown in transduced motoneurons was insufficient. However, results obtained by Towne *et al*.[2] equaled or exceeded the levels of motoneuron muSOD1 silencing reported previously by Ralph *et al*., yet this latter article described remarkable gains in motor behavior and survival.[3] Thus, we are left with a challenge regarding how to move this field forward. On the one hand, we have a superbly executed and detailed study by Towne *et al.* that reports negative data in groups of 20 or more animals in which even small differences could be more easily detected.[2] On the other hand, we have a publication by Ralph *et al.* reporting highly clinically significant data in a high-impact journal.[3] We believe that the proper way forward would be a head-to-head comparison of both vector systems.

Most scientists who have employed both lentiviral and rAAV vectors find that the latter tend to be more easily produced in titers that result in robust *in vivo* transductions in nervous tissue. However, it is possible that the pseudotyping of the EIAV with the rabies glycoprotein envelope may lead to greater efficiency of retrograde transport[9] as compared with rAAV6. Nevertheless, Towne *et al*. note that rAAV6 is more efficient at transducing motoneurons after intramuscular delivery as compared with rabies pseudotyped EIAV in their hands.[2] In the only published quantification of EIAV transduction efficiency in brain, a variety of pseudotyped EIAV vectors were evaluated for striatal transduction efficiency[10]; in this work, transduction efficiency when using EIAV appeared to be 7- to 10-fold less than that observed with a variety of rAAV serotypes in the same brain structure, as shown by estimation of transduced cell population from other laboratories (e.g., refs. 11, 12). Indeed, 17 of 18 gene therapy trials for neurological disorders have selected rAAV over EIAV.[2]

However, although some may argue that comparing the transduction efficiency between different vectors is tantamount to comparing apples and oranges because of the difficulty of accurately comparing titers between vector types, a way forward is proposed below. Because production procedures for both vectors are under constant improvement, any single comparison depends on individual production runs. Furthermore, Towne *et al*. actually call for comparative rAAV6 vs. EIAV studies in fALS mice to be carried out in order to resolve the issue.[2] One way to fairly compare rAAV with lentiviral vectors in the fALS mouse model would be to perform careful comparative dose–response transduction experiments for both vectors and then identify a dose of each vector that results in comparable levels of transgene copy number in motoneurons, rather than make any attempt to equalize input vector levels.

In conclusion, we have before us two studies that represent important contributions to translational research for ALS[2,3] and by extension many other devastating diseases afflicting motoneurons. One study reports a positive therapeutic effect in fALS mice in a high-profile journal.[3] The second, reported in this issue, finds no therapeutic benefit in fALS mice using very similar methods.[2] The main difference between the two studies was the vector used to deliver the shRNA to silence muSOD1 in motoneurons; the reported reduction in the level of the muSOD1 mRNA and the overall level of transduction of the ventral spinal cord were similar in the two studies. Towne *et al*. should be commended for executing their study in such a manner that their data warrant both publication and attention in the field. At the very least, they have raised the bar for documentation of biological effects of gene transfer in the fALS model system. Further methodological developments, such as the capacity of AAV9 to transduce brain cells across the blood–brain barrier,[13,14] may lead to greater retrograde motoneuron transduction efficiencies, which should ultimately reveal which parameters are important for increased survival after gene transfer in the fALS model.

REFERENCES

1. Valdmanis, PN and Rouleau, GA (2008). Genetics of familial amyotrophic lateral sclerosis. *Neurology* **70**: 144–152.
2. Towne, C, Setola, V, Schneider, BL and Aebischer, P. (2010). Neuroprotection by gene therapy targeting

mutant SOD1 in individual pools of motor neurons does not translate into therapeutic benefit in fALS mice. *Mol Ther* **19**: 274–283.
3. Ralph, GS, Radcliffe, PA, Day, DM, Carthy, JM, Leroux, MA, Lee, DC *et al.* (2005). Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat Med* **11**: 429–433.
4. Towne, C, Raoul, C, Schneider, BL and Aebischer, P (2008). Systemic AAV6 delivery mediating RNA interference against SOD1: neuromuscular transduction does not alter disease progression in fALS mice. *Mol Ther* **16**: 1018–1025.
5. Azzouz, M, Hottinger, A, Paterna, JC, Zurn, AD, Aebischer, P and Bueler, H (2000). Increased motoneuron survival and improved neuromuscular function in transgenic ALS mice after intraspinal injection of an adeno-associated virus encoding Bcl-2. *Hum Mol Genet* **9**: 803–811.
6. Gregorevic, P, Blankinship, MJ, Allen, JM, Crawford, RW, Meuse, L, Miller, DG *et al.* (2004). Systemic delivery of genes to striated muscles using adeno-associated viral vectors. *Nat Med* **10**: 828–834.
7. Towne, C, Schneider, BL, Kieran, D, Redmond, DE Jr and Aebischer, P (2010). Efficient transduction of non-human primate motor neurons after intramuscular delivery of recombinant AAV serotype 6. *Gene Ther* **17**: 141–146.
8. Boillee, S, Yamanaka, K, Lobsiger, CS, Copeland, NG, Jenkins, NA, Kassiotis, G *et al.* (2006). Onset and progression in inherited ALS determined by motor neurons and microglia. *Science* **312**: 1389–1392.
9. Cronin, J, Zhang, XY and Reiser, J (2005). Altering the tropism of lentiviral vectors through pseudotyping. *Curr Gene Ther* **5**: 387–398.
10. Wong, LF, Azzouz, M, Walmsley, LE, Askham, Z, Wilkes, FJ, Mitrophanous, KA *et al.* (2004). Transduction patterns of pseudotyped lentiviral vectors in the nervous system. *Mol Ther* **9**: 101–111.
11. Burger, C, Gorbatyuk, OS, Velardo, MJ, Peden, CS, Williams, P, Zolotukhin, S *et al.* (2004). Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. *Mol Ther* **10**: 302–317.
12. Reimsnider, S, Manfredsson, FP, Muzyczka, N and Mandel, RJ (2007). Time course of transgene expression after intrastriatal pseudotyped rAAV2/1, rAAV2/2, rAAV2/5, and rAAV2/8 transduction in the rat. *Mol Ther* **15**: 1504–1511.
13. Foust, KD, Nurre, E, Montgomery, CL, Hernandez, A, Chan, CM and Kaspar, BK (2009). Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat Biotechnol* **27**: 59–65.
14. Foust, KD, Wang, X, McGovern, VL, Braun, L, Bevan, AK, Haidet, AM *et al.* (2010). Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. *Nat Biotechnol* **28**: 271–274.