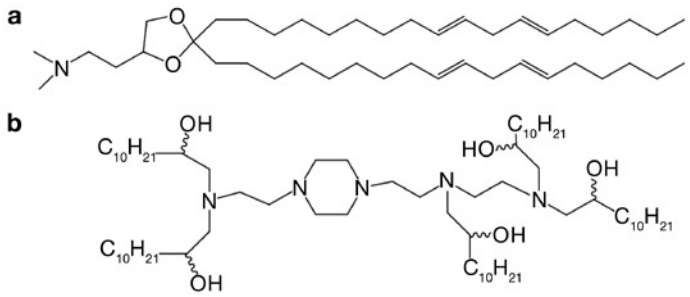

Figure 1 The best-performing lipids in two recent studies. (**a**) DLin-KC2-DMA, generated by Semple *et al.*[3] (**b**) C12-200, generated by Love *et al.*[4]

The structures of the best-performing compounds identified by these two groups are shown in **Figure 1**. DLin-KC2-DMA, designed by Semple *et al.*, contains a tertiary amine head group. The pK_a of this amine on the nanoparticle surface should be close to 7 because of crowding with the neighboring groups. Yet, in the acidic endosome, it should become protonated and positively charged so as to be available for ion pairing with the negatively charged endosomal lipids. C12-200, designed by Love *et al.*, is almost a macromolecule. It contains multiple tertiary amines, the pK_a of which should also be close to but no greater than 7. As with DLin-KC2-DMA, C12-200 should be protonated in the acidic endosome to form ion pairs with the negatively charged endosome lipids. The molecule also contains five alkyl chains. As a whole, C12-200 contains bulky hydrophobic tails. Whether it has the tendency to form the inverted micelle, or H_{II} phase, as does DLin-KC2-DMA, will need to be tested. Thus, the following design criteria may be useful for creating future delivery agents: (i) one or more tertiary amines with relatively low pK_a to generate a weakly cationic head group in which the positive charge density is highly pH dependent, (ii) more than one alkyl tail so as to form a bulky hydrophobic corona, and (iii) an ideal length of alkyl tail that may lie within the range of 12–18 carbons.

The unprecedented low-dose delivery achieved by these two studies is of great importance. Not only was the delivery efficiency improved, the carrier material was well tolerated by the treated animals. Because silencing can be induced at a low dose, the amount of excipients required is also greatly reduced. In fact, dose-dependent toxicity and pulmonary inflammation of cationic lipid limits its effectiveness. It has been demonstrated that cationic lipids can modify cellular signaling pathways and stimulate specific immune or anti-inflammatory responses.[9] Tekmira Pharmaceuticals (British Columbia, Canada) recently terminated a clinical trial of liposomal siRNA for hypercholesterolemia because of "potential for immune stimulation to interfere with further dose escalation" (http://clinicaltrials.gov/ct2/show/NCT00927459?term=siRNA&rank=9). Although the underlying reasons have not been clearly identified, the immunostimulatory activity of cationic lipid should be considered carefully. The lipid or lipidoid presented by these two studies may help reduce or even eliminate such adverse effects. Orders-of-magnitude decreases in the required dose will further decrease the potential for toxicity.

Cationic lipids hold great promise for systemic delivery of siRNA. However, they are not the only solution. The fate of a siRNA formulation *in vivo* is affected by various factors such as particle size, morphology, and surface chemistry. Sophisticated structures of the particles and preparation methods also influence the *in vivo* effect considerably. Other formulation strategies, such as attaching a targeting moiety to the nanoparticle, could further enhance the delivery efficiency. We believe that rationally designed delivery systems formulated with promising novel delivery materials will facilitate the path to the development of the full potential of siRNA-based therapeutics.

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AAV Provides an Alternative for Gene Therapy of the Peripheral Sensory Nervous System

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Recombinant adeno-associated virus (rAAV) is a promising vector for applications in the central nervous system (CNS). Clinical phase I/II studies have demonstrated safety of rAAV-expressing therapeutic transgenes for a variety of benign (i.e., noncancer) CNS disorders, namely, Canavan disease,[1] Parkinson's disease,[2–4] and Batten disease.[5] Human studies were also recently reported targeting the retina.[6–9] From the early stages of preclinical development, rAAV was appealing

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because it was found to be neurotrophic and capable of long-term activity without immune interference or toxicity when injected into the CNS.[10]

But what about the potential of rAAV for the *peripheral* nervous system (PNS)? The PNS has a major role in a large number of neurological disorders, including a common one, chronic pain. The PNS is also, in principle, more easily accessible without major surgery, a potential advantage for any clinical gene therapy application. The PNS consists of three groups of neurons: primary sensory neurons, primary motor neurons, and autonomic neurons. Primary sensory neurons reside in the dorsal root ganglia (DRG)—hence their frequent designation as DRG neurons—and in the trigeminal ganglion. Primary sensory neurons are of clinical interest because of their role in sensory neuropathies and a wide range of chronic pain states.

Testing of rAAV in the PNS was initially reported as a sidekick of an adenovirus marker gene study,[11] followed by a report of its usefulness for analgesic treatments.[12] Both studies used rAAV consisting of inverted terminal repeats and capsid proteins derived from AAV serotype 2 (the original rAAV vectors developed in the mid-1990s) and administered the vectors directly into the DRG. In this issue of *Molecular Therapy*, Mason *et al.* rekindle this approach, reporting on the targeting of primary sensory neurons by injection of rAAV into the DRG.[13] The authors packaged AAV2 recombinant genomes into seven different capsids—the original serotype 2 as well as the newer types 1, 3, 4, 5, 6, and 8—and compared their efficacy for expressing the marker gene *eGFP*. Their best-performing vector, AAV5, transduced >90% of DRG neurons—it can't get better than that. So is it prime time yet for a clinical trial targeting the PNS by direct rAAV injection into the DRG? A critical set of studies may help to light the way.

First, it would be of interest to repeat the study in other animal species to evaluate whether the marked performance difference among the various capsid serotypes reported by Mason *et al.*[13] seems generalizable or is rodent-specific. The original reports on capsid pseudotyping of rAAV showed very marked differences between rodent muscle[14] and rodent brain[15] resembling or exceeding the differences between serotypes demonstrated by Mason *et al.* in the rodent DRG (e.g., in the muscle the difference was several log10 orders of magnitude[14]). Unfortunately, in the case of muscle- or brain-directed rAAV gene transfer, similarly systematic serotype comparisons seem not to have been performed in nonhuman primates or other large animals (dogs or pigs could be considered), but the "word on the street" (evaluating single-serotype studies and anecdotal findings in only a few animals) implies differences that are less than overwhelming. In the case of DRG gene transfer, any firmer guidance would certainly help clinical researchers to choose the best rAAV serotype when planning human trials in the PNS.

Second, any piece of evidence supporting the lack of immunogenicity of rAAV vectors would be reassuring. rAAV is notable for its inability to induce cellular immunity in rodents. However, administration of AAV2 into the portal vein in humans led to an apparent delayed immune response that ended a clinical trial prematurely.[16–19] rAAV has also been used as a platform for vaccine development, suggesting that it can induce strong immunity under some circumstances.[20] Perhaps the most interesting observation reported by Mason *et al.* relates to this problem of rAAV immunogenicity—expression of AAV6 (but not any of the other serotypes) declined 12 weeks after DRG injection and was accompanied by neuropathological tissue damage at the injection site. Interestingly, the authors found that only AAV6 among the vectors tested was able to transduce nonneuronal cells in the DRG, namely, satellite cells. Accordingly, the authors speculate that the less discriminate transduction profile of AAV6 may have led to AAV6 uptake by antigen-presenting cells triggering immunity, which consequently led to tissue damage and loss of transgene expression.[13] Although these findings are intriguing, the present study leaves open many questions. For example, what happens to expression at later time points (loss of expression is only partial at 12 weeks)? Would neuropathological toxicity hold up in a blinded experiment (random variation in injection technique and tissue procurement remain a possibility)? Finally, what is the nature of the immune response (presumably cytotoxic), and is it directed against the transgene (*eGFP*), the virus capsid, or both? A careful follow-up study would be most welcome and certainly highly informative for future clinical research.

Third, a lingering concern with rAAV is whether it might be tumorigenic. Wild-type AAV has not been associated with carcinogenesis. But rAAV causes an increased incidence of liver tumors when administered to newborn mice of certain susceptible strains,[21] in which vector integration was demonstrated in tumor tissues. In at least some of the cases, integration occurred in a micro-RNA locus on mouse chromosome 12. This led to regional transcriptional activation of many small RNA genes with known growth-regulatory properties, a plausible mechanism of carcinogenesis.[22] Another study found an increased incidence of liver tumors only with a specific transgene but not with rAAV administration *per se*.[23] Such findings seem to be limited to rAAV administration in newborn (as opposed to adult) mice and might occur only in specific, susceptible strains (such as knockout mice with lysosomal storage disease) in which liver tumors can occur even spontaneously. They could also have been favored by relatively high vector doses (1.5×10^{11} particles per newborn mouse) and have thus far not been noted in other organs or other species. It is therefore not surprising that no such findings were noted by Mason *et al.*[13] Nevertheless, continued careful attention to even a low incidence of tumor formation will need to be an integral part of any preclinical testing.

Fourth, an obvious challenge is the development of a minimally invasive DRG injection technique resembling the method practiced by Mason *et al.* in rodents but applicable to humans. The human DRG is approximately 50 times larger than that of a rodent, e.g., approximately 150–200 mm^3 in humans[24] as compared with 4–5 mm^3 for the rat.[25] As a result, single-site injections will not work. Bankiewicz *et al.* tackled this problem in the CNS through the development of an administration technique that they refer to as convection-enhanced delivery, which they studied in different species, including primates.[26,27] They defined the technique in terms of biomechanical details such as flow and pressure,[28–30] tested it for safety with different

vector types,[31–33] developed imaging for it,[34–36] and refined it down to such details as the needle to be employed.[37,38] From a neurosurgical perspective, the DRG is a structure that is routinely visualized in the scope of many spine operations. But spine surgery would ideally not be required for injecting rAAV. Instead, a minimally invasive technique would be desirable, particularly given that clinical syndromes involving the PNS would ultimately call for treatment of DRG at multiple spinal levels and possibly bilaterally. The absence of a clinically established procedure for injecting DRG in humans calls for some creative surgical research.

For the past decade, PNS gene therapy has been the domain of herpes simplex virus, a vector that transduces primary sensory neurons effectively in animal models when injected subcutaneously.[39,40] Many crucial questions, such as those asked above for rAAV, have been successfully resolved for herpes simplex virus, prompting ongoing clinical trials in patients with intractable pain.[41,42] For rAAV-based gene therapy of the PNS, it took several more years after the initial studies[11,12] for a more comprehensive picture to emerge that covered various administration techniques such as intrathecal delivery of various rAAV serotypes,[43,44] intraneural and systemic targeting of DRG neurons by rAAV6 (ref. 45), and long-term efficacy for the treatment of neuropathic pain.[46,47] The study by Mason *et al.*[13] adds seminal data refocusing on rAAV injection directly into the DRG and further supports the candidacy of rAAV as an alternative for gene therapy of the PNS.

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