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# Gene-Targeted Therapies for the Central Nervous System

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**T**he identification of the genes and proteins involved in many neurodegenerative diseases<sup>1</sup> offers the exciting possibility of modifying those disease-linked proteins to develop novel, targeted therapies for diseases such as amyotrophic lateral sclerosis (ALS) or Huntington disease. In many of these diseases, the simplest modification—decreasing the amount of the offending protein—may represent a potent therapy. This realization, coupled with great strides in the techniques for decreasing specific proteins, has set the stage for moving these new therapies from animal models to clinical trials in the near future.

Multiple methods are currently being studied for their ability to decrease levels of unwanted proteins, including immunization strategies, small molecules, RNA interference (RNAi), and antisense oligonucleotides. Immunization strategies were an early favorite for clearing proteins from the central nervous system (CNS), with impressive results in Alzheimer disease mouse models, in which active immunization with amyloid- $\beta$  peptide led to a decrease in the age-dependent amyloid- $\beta$  deposition.<sup>2</sup> One appeal of this strategy is a long medical practice of immunizations in humans. Based on these promising results in mice, a clinical trial in patients with Alzheimer disease was launched. However, the development of meningoencephalitis in 6% of the patients stopped the phase 2 trial.<sup>3</sup> The strategy itself remains feasible; perhaps passive immunization may prove similarly effective while avoiding a T-cell response, allowing for the control of antibody levels and the ability to stop treatment. More careful antigen selection may also help eliminate T-cell epitopes. A similar immunization strategy for a superox-

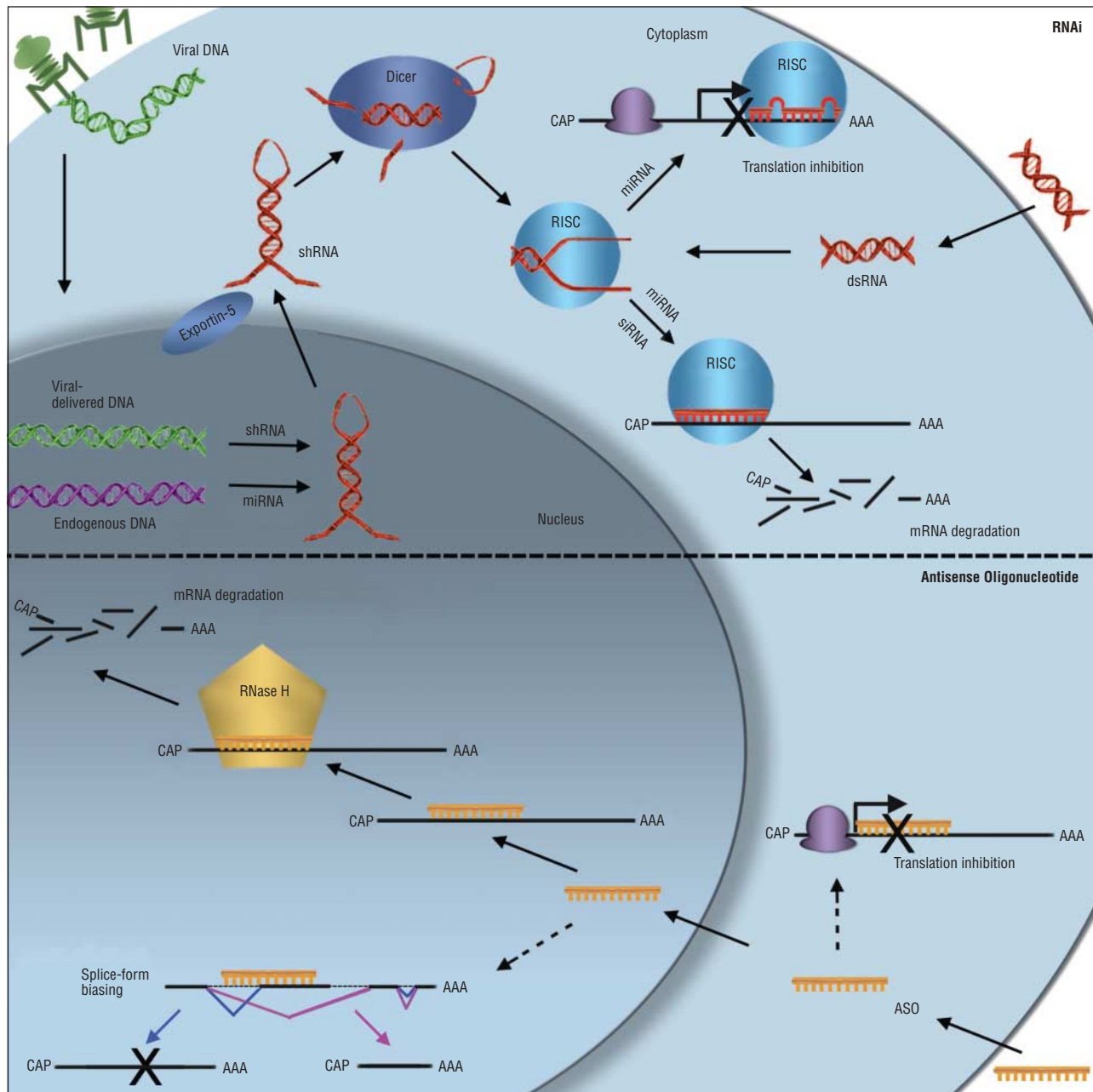
ide dismutase 1 (*SOD1*) ALS model delayed disease.<sup>4</sup>

Small molecules are a diverse group of natural and synthetic substances, generally with a molecular weight less than 500 Da. Most over-the-counter drugs, such as aspirin, fall into this category. Finding a new or existing small molecule that will target misfolded proteins has been considered for Huntington disease,<sup>5</sup> ALS,<sup>6</sup> and other neurodegenerative diseases in which aberrant protein aggregation has been linked to pathogenesis. None of these have been tried clinically, though discovery of a medication that selectively decreases a protein associated with a neurodegenerative disease would naturally see an accelerated pathway to clinical trials. Some of these small molecules are meant to stabilize rather than decrease the levels of aggregated proteins. However, until it is clear whether stabilizing (rather than decreasing) a specific aggregated protein is beneficial or detrimental, clinical use of this approach is problematic. Antisense oligonucleotides and RNAi are 2 genetic strategies showing tremendous promise of decreasing protein levels. These methods will be the primary focus of this review.

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## MECHANISMS OF REPRESSION

Both RNAi and antisense oligonucleotides are posttranscriptional gene down-regulation approaches that provoke de-



**Figure.** Schematic representation of the antisense oligonucleotide (ASO), microRNA (miRNA), and small interfering RNA (siRNA) pathways. Antisense oligonucleotides bind messenger RNA (mRNA) through sequence-specific Watson and Crick hybridization. Endogenous ribonuclease H (RNase H) recognizes the DNA/RNA heteroduplexes and degrades the target mRNA. Exportin-5 facilitates the export of short hairpin RNA (shRNA) from the nucleus and may be the rate-limiting step in shRNA export. In the cytoplasm, shRNAs are processed by Dicer—yielding short (20–22 nucleotides), double-stranded RNA (dsRNA) intermediates. The double-stranded siRNAs are unwound by the RNA-induced silencing complex (RISC). The subsequent single-stranded RNA templates direct binding of RISC to complementary mRNA. Proteins of the RISC complex endolytically cleave the target mRNA. The endogenous miRNAs bind target mRNA, leading to mRNA degradation or translation inhibition. Unlike siRNAs and ASOs, miRNAs are only partially complementary to their target mRNAs. RNAi indicates RNA interference.

struction of the messenger RNA (mRNA) in a sequence-specific fashion (**Figure**). This leads to lower mRNA levels of a particular gene sequence, resulting in less protein production. Both approaches use nucleic acids, either naturally occurring or chemically modified DNA or RNA, to guide cellular enzymes to cleave specific mRNAs. Which mRNA is degraded is determined by the sequence of the short nucleic acid material. RNA interference is triggered by short (about 22 nucleotides), double-stranded RNA, while antisense oligonucleotides are short

(typically 20 nucleotides), single-stranded, DNA-like molecules.

RNA interference (recognized by the 2006 Nobel Prize in Medicine) was first identified as a biological response to long, double-stranded RNA and has been subsequently documented in most eukaryotes. RNA interference is used by the cell as another level of control of gene expression. Taking advantage of the endogenous processing mechanisms, small interfering RNA (siRNA) that triggers RNAi may be derived from pieces of long, double-

stranded RNA (only in worms and flies), short hairpin RNA (RNA designed to loop back on itself forming duplex RNA with a hairpin loop), or stabilized, small duplex RNA. In the case of the long, double-stranded RNA and short hairpin RNA, the RNA is first processed by the ribonuclease III-related enzyme Dicer into smaller (21-23 nucleotides) double-stranded RNA. The siRNAs are next incorporated into the cytosolic RNA-induced silencing complex, which uses the sequence-specific information of the siRNA to direct cleavage of the homologous mRNA. The endogenous RNAi system uses the same processing enzymes described previously, but the regulation of genes by these microRNAs is more complicated, because microRNAs regulate multiple genes, act in part by translational repression as well as mRNA cleavage, and do not share complete sequence homology. Understanding this endogenous process and how it interacts with experimentally or therapeutically introduced siRNA is important for understanding toxicity profiles of these potential therapies.

The therapeutic potential of antisense oligonucleotides was recognized in the 1970s. Zamecnik and Stephenson<sup>7</sup> used antisense oligonucleotides to block replication of Rous sarcoma virus in cultured cells. One mechanism through which antisense oligonucleotides inhibit gene expression is the endogenous enzyme ribonuclease H, a nuclear enzyme that degrades mRNA in a complex with DNA. There are 2 forms of ribonuclease H in mammalian cells. Ribonuclease H2 participates in DNA replication by removing the initial priming RNA from the DNA template (Okasaki fragments). Antisense oligonucleotides activate ribonuclease H1,<sup>8</sup> whose function, though not entirely clear, may include participating in transcription or acting as part of a host defense to destroy viral genetic material.

#### EFFICACY IN ANIMAL MODELS

RNA interference is a potent disease modulator in animal models of neurodegenerative diseases. In the first demonstration of the utility of RNAi in neurodegenerative disease, Davidson and colleagues showed decreased Purkinje cell loss and enhanced motor performance by decreasing ataxin-1 in an animal model of spinocerebellar ataxia<sup>9</sup> by direct injection of a virus that contains siRNA into the cerebellum. Three groups have demonstrated that decreasing expression of SOD1 protein slows disease in the SOD1 mouse model of ALS.<sup>10-12</sup> Similarly, lowering expression of huntingtin protein in a Huntington disease model<sup>13</sup> (HD-N171-82Q mice) and of a  $\beta$ -site amyloid precursors protein-cleaving enzyme in an Alzheimer disease model<sup>14</sup> (expressing high levels of double mutant [V717I and K670M/N671L], in human APP) slows disease in mice.

Antisense oligonucleotides have also been shown to be similarly useful.<sup>15</sup> Using antisense oligonucleotides targeting SOD1, Smith and colleagues<sup>16</sup> demonstrated a slowing of disease progression after onset in an SOD1 rat model of ALS. Furthermore, the feasibility of treating other neurodegenerative diseases was demonstrated by showing the ability of antisense oligonucleotides to decrease glycogen synthase kinase- $\beta$  and presenilin 1 levels. Anti-

sense strategies have also been devised for chronic pain<sup>17-21</sup> and for treatment of glioblastoma.<sup>22</sup> The glioblastoma multiforme treatment (targeting transforming growth factor  $\beta$ ) is currently in a phase 2b trial ([http://www.antisense-pharma.com/news/pressrelease/f\\_pressrelease.htm](http://www.antisense-pharma.com/news/pressrelease/f_pressrelease.htm)).

In addition to causing enzymatic degradation of mRNA, antisense oligonucleotides may be modified so that they bind tightly to the mRNA without causing degradation. This type of strategy is being employed for spinal muscular atrophy, a childhood-onset paralytic disease that is caused by degeneration of motor neurons in the spinal cord and is nearly universally associated with a mutation in the survival of motor neuron 1 gene (SMN1). Increased expression of a related gene, SMN2, may be neuroprotective in this setting. Antisense oligonucleotides that bind to the pre-mRNA have been designed to block a nonproductive splice as a means to elevate production of functional SMN mRNA and thus SMN2 protein. In principle, this increase in the SMN protein should be neuroprotective in patients with spinal muscular atrophy. Modulating splicing patterns with antisense oligonucleotides is also being used as a therapy for myotonic dystrophy<sup>23</sup> and Duchenne muscular dystrophy.<sup>24</sup>

While modifying the disease course in an animal model is a pleasing result in preclinical studies, there is reason to question the significance of this end point as the rationale for use of similar strategies in humans. This is an important issue, because as techniques are refined to downregulate genes, outcomes may depend in part on the animal model being used. While gratifying to see extension of survival in the animal model, from our perspective, it is the effect on gene expression and the distribution of these drugs rather than survival that may be the most important criteria for selecting a nucleic acid-based therapy. In the case of SOD1, antisense oligonucleotides decrease SOD1 mRNA and protein in rats and distribute broadly in the rat and primate brain and spinal cord.<sup>16</sup> Although the cause of SOD1 toxicity is not yet clear, what is abundantly clear from the animal models is that there is a gain of toxicity of mutant SOD1 and that decreasing SOD1 ameliorates or slows disease.<sup>25</sup> All of the data suggest that lowering mutant SOD1 in humans is also likely to be beneficial. In the human setting, there is 1 copy of mutant SOD1, not 15 to 20 copies as in the transgenic animal models of the disease. Thus, the important question is whether antisense oligonucleotides decrease SOD1 in the nontransgenic setting in the human CNS. The current data suggest that this will be true,<sup>16</sup> though these therapies have not yet been tested in humans. Similar considerations must be made when using animal models to analyze siRNA as a potential therapy for humans.

#### CONCERNS AND CONSIDERATIONS FOR USE IN HUMANS

Assuming that efficacy has been proven in animal models and that this translates into potential efficacy in human disease, as with development of any therapy, antisense oligonucleotides and siRNA face substantial challenges in terms of delivery and safety. A major ob-

stacle involves delivery; neither antisense oligonucleotides nor stabilized siRNA cross the blood-brain barrier. Accordingly, both therapies require a reliable and safe delivery method to access the CNS. One approach is to directly infuse antisense oligonucleotides or siRNA into cerebral spinal fluid. This has met with only limited success for siRNA, because these RNA-like molecules have been difficult to stabilize in biologic fluids,<sup>26</sup> though there are clearly many efforts under way to introduce chemical modifications to accomplish this.<sup>27</sup> In contrast, the current generation of antisense oligonucleotides is remarkably stable in biologic fluids, with half-lives of days to weeks; therefore, they are quite amenable to being delivered directly into cerebral spinal fluid. Although the mechanism of entry into cells remains unclear, the oligonucleotides are readily taken up by cells and distributed widely throughout the brain and spinal cords of rats and rhesus monkeys following delivery into the cerebrospinal fluid.<sup>16</sup> Changing the chemical properties of the stabilized siRNA or antisense oligonucleotides by attaching lipids or proteins or enhancing uptake by changing the vehicle of delivery may further improve uptake.<sup>27,28</sup> However it is formulated, direct infusion into human cerebrospinal fluid is expected to result in a similar broad distribution in the CNS, based on the results from rhesus monkeys. Translation of this strategy to humans should be routine, since controlled delivery of drugs to the cerebrospinal fluid, eg, baclofen, is an established medical procedure.

Given the metabolic instability of siRNA in extracellular fluid and poor cellular uptake, these small RNA-like molecules are typically targeted to cells by using a virus that enters the cell and encodes the synthesis of short hairpin RNA. Lentiviral and adeno-associated viruses are 2 of the most common viruses used in the CNS. Once inside the neuron (and other cells in the brain and spinal cord), the virus delivers its genetic instructions, which direct the transcription of the siRNA under the control of the RNA polymerase promoter. The virus may be injected directly into the brain or spinal cord.<sup>9,12-14</sup> Alternatively, a virus may be engineered or selected so that a peripheral injection of the virus leads to uptake by a group of neurons. This has been done successfully for injection of viruses into muscles with uptake and retrograde transport to motor neurons in the spinal cord.<sup>10,11</sup> Using a virus to deliver siRNA has several advantages: virally introduced genes can be expressed for long periods and multiple genes can be expressed at the same time.

Although the safety of siRNA or antisense oligonucleotides in the human CNS is unknown, in the periphery, antisense oligonucleotides have been proven to be remarkably well tolerated in humans with some minor injection site reactions and, at high doses, effects on the coagulation system. Whether the CNS will tolerate these compounds remains unknown. Formal safety studies are under way for an antisense to SOD1 as a prelude to a treatment trial in patients with familial ALS. One of the major benefits of antisense oligonucleotides or stabilized siRNA is that their doses can be adjusted or treatment can be stopped. In the CNSs of rats and nonhuman primates, antisense oligonucleotides have a half-life of ap-

proximately 2 weeks. Discontinuing treatment results in a return to normal levels of target proteins in about 4 weeks. However, the feasibility for prolonged treatment needs to be tested.

Current RNAi strategies for the CNS face even more substantial hurdles. One obvious issue is driving the expression of siRNA from a viral vector. This introduces 2 concerns: (1) the inability to stop the therapy should there be any adverse effects and (2) safety issues regarding the virus itself. However, new vectors are being made and tested to regulate gene expression and, to date, several clinical studies have shown many vector systems to be relatively safe. Stabilized siRNA would obviate some of these concerns. Indeed, the first planned clinical trials with siRNA in humans will use stabilized "naked" siRNA delivered locally in saline for treatment of macular degeneration and respiratory syncytial virus. Both were well tolerated in phase 1 clinical trials. However, a recent sobering study in mice emphasizes the importance of dosing siRNA appropriately. Grimm et al,<sup>29</sup> while attempting to modulate hepatitis B in rodents using siRNA, found increased morbidity in treated animals. They concluded that exogenously introduced siRNA overwhelmed the important endogenous system for processing of small microRNAs. Specifically, exportin-5-mediated export of microRNA from the nucleus to its site of action in the cytoplasm was hindered. These endogenous microRNAs control a large number of genes. These studies outline the importance for caution in moving a potential therapy into the clinic until all safety and pharmacological studies have been performed. Only then will we have a clearer understanding of the potential dosage to test in human studies.

The evidence to date suggests that nucleic acid-based therapies offer the promise of modifying or arresting the course of a number of neurodegenerative diseases in which down-regulation of a protein is a treatment objective. As more and more specific genes are linked to neurodegenerative diseases (both sporadic and familial forms), the potential for gene-targeted approaches will increase. The challenges are clear, but once we navigate the details of delivery and safety (an admittedly large hurdle), gene-targeted therapies are likely to become an important treatment option for currently untreatable neurodegenerative diseases.

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## REFERENCES

- Taylor JP, Hardy J, Fischbeck KH. Toxic proteins in neurodegenerative disease. *Science*. 2002;296(5575):1991-1995.
- Schenk D, Barbour R, Dunn W, et al. Immunization with amyloid- $\beta$  attenuates Alzheimer-disease-like pathology in the PDAPP mouse. *Nature*. 1999;400(6740):173-177.
- Orgogozo JM, Gilman S, Dartigues JF, et al. Subacute meningoencephalitis in a subset of patients with AD after A $\beta$ 42 immunization. *Neurology*. 2003;61(1):46-54.
- Urushitani M, Ezzi SA, Julien JP. Therapeutic effects of immunization with mutant superoxide dismutase in mice models of amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A*. 2007;104(7):2495-2500.
- Beal MF, Ferrante RJ. Experimental therapeutics in transgenic mouse models of Huntington's disease. *Nat Rev Neurosci*. 2004;5(5):373-384.
- Ray SS, Nowak RJ, Brown RH Jr, Lansbury PT Jr. Small-molecule-mediated stabilization of familial amyotrophic lateral sclerosis-linked superoxide dismutase mutants against unfolding and aggregation. *Proc Natl Acad Sci U S A*. 2005;102(10):3639-3644.
- Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc Natl Acad Sci U S A*. 1978;75(1):280-284.
- Wu H, Lima WF, Zhang H, Fan A, Sun H, Crooke ST. Determination of the role of the human RNase H1 in the pharmacology of DNA-like antisense drugs. *J Biol Chem*. 2004;279(17):17181-17189.
- Xia H, Mao Q, Eliason SL, et al. RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. *Nat Med*. 2004;10(8):816-820.
- Miller TM, Kaspar BK, Kops GJ, et al. Virus-delivered small RNA silencing sustains strength in amyotrophic lateral sclerosis. *Ann Neurol*. 2005;57(5):773-776.
- Ralph GS, Radcliffe PA, Day DM, et al. Silencing mutant SOD1 using RNAi protects against neurodegeneration and extends survival in an ALS model. *Nat Med*. 2005;11(4):429-433.
- Raoul C, Abbas-Terki T, Bensadoun JC, et al. Lentiviral-mediated silencing of SOD1 through RNA interference retards disease onset and progression in a mouse model of ALS. *Nat Med*. 2005;11(4):423-428.
- Harper SQ, Staber PD, He X, et al. RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. *Proc Natl Acad Sci U S A*. 2005;102(16):5820-5825.
- Singer O, Marr RA, Rockenstein E, et al. Targeting BACE1 with siRNAs ameliorates Alzheimer disease neuropathology in a transgenic model. *Nat Neurosci*. 2005;8(10):1343-1349.
- Smith RA, Miller TM. Targeting neurological disorders with antisense oligonucleotides. *Antisense Drug Technology: Principles, Strategies, and Applications*. 2nd ed. Crooke ST, ed. Boca Raton, FL: CRC Press.
- Smith RA, Miller TM, Yamanaka K, et al. Antisense oligonucleotide therapy for neurodegenerative disease. *J Clin Invest*. 2006;116(8):2290-2296.
- Adams MR, Nikkel AL, Donnelly-Roberts DL, et al. In vitro and in vivo effects of an  $\alpha$ 3 neuronal nicotinic acetylcholine receptor antisense oligonucleotide. *Brain Res Mol Brain Res*. 2004;129(1-2):67-79.
- Barclay J, Patel S, Dorn G, et al. Functional downregulation of P2X3 receptor subunit in rat sensory neurons reveals a significant role in chronic neuropathic and inflammatory pain. *J Neurosci*. 2002;22(18):8139-8147.
- Dorn G, Abdel'Al S, Natt FJ, et al. Specific inhibition of the rat ligand-gated ion channel P2X3 function via methoxyethoxy-modified phosphorothioated antisense oligonucleotides. *Antisense Nucleic Acid Drug Dev*. 2001;11(3):165-174.
- Honore P, Kage K, Mikusa J, et al. Analgesic profile of intrathecal P2X3(3) antisense oligonucleotide treatment in chronic inflammatory and neuropathic pain states in rats. *Pain*. 2002;99(1-2):11-19.
- Hua XY, Moore A, Malkmus S, et al. Inhibition of spinal protein kinase C $\alpha$  expression by an antisense oligonucleotide attenuates morphine infusion-induced tolerance. *Neuroscience*. 2002;113(1):99-107.
- Schlingensiepen KH, Schlingensiepen R, Steinbrecher A, et al. Targeted tumor therapy with the TGF- $\beta$ 2 antisense compound AP 12009. *Cytokine Growth Factor Rev*. 2006;17(1-2):129-139.
- van Deutekom JC, Janson AA, Ginjaar IB, et al. Local dystrophin restoration with antisense oligonucleotide PR0051. *N Engl J Med*. 2007;357(26):2677-2686.
- Wheeler TM, Lueck JD, Swanson MS, Dirksen RT, Thornton CA. Correction of CIC-1 splicing eliminates chloride channelopathy and myotonia in mouse models of myotonic dystrophy. *J Clin Invest*. 2007;117(12):3952-3957.
- Bruijn LI, Miller TM, Cleveland DW. Unraveling the mechanisms involved in motor neuron degeneration in ALS. *Annu Rev Neurosci*. 2004;27:723-749.
- Dorn G, Patel S, Wotherspoon G, et al. siRNA relieves chronic neuropathic pain. *Nucleic Acids Res*. 2004;32(5):e49.
- Bumcrot D, Manoharan M, Koteliensky V, Sah DW. RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol*. 2006;2(12):711-719.
- Kumar P, Wu H, McBride JL, et al. Transvascular delivery of small interfering RNA to the central nervous system. *Nature*. 2007;448(7149):39-43.
- Grimm D, Stretz KL, Jopling CL, et al. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*. 2006;441(7092):537-541.