The Prospect of Silencing Disease Using RNA Interference

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The discovery of RNA interference (RNAi), an endogenous cellular gene-silencing mechanism, has already provided a powerful tool for basic science researchers to study gene function. The subsequent finding that RNAi also operates in mammalian cells has generated excitement regarding potential therapeutic applications. In this article we discuss the basic mechanism of RNAi and the therapeutic opportunities and obstacles for harnessing RNAi for therapy of human disease.

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RNA INTERFERENCE (RNAI) IS A recently discovered, evolutionarily conserved, gene-silencing phenomenon in which small pieces of double-stranded RNA (small interfering RNA [siRNA]) suppress expression of genes with sequence homology.[1] RNA interference may have originated as a cellular defense against viral infection or potentially harmful destabilizing genomic intruders such as transposons. The RNAi machinery, which is expressed in all eukaryotic cells, has also been found to regulate the expression of key genes involved in cell differentiation in plants and animals.[2]

RNA interference was discovered when researchers attempting to engineer more intensely colored petunia flowers found that the introduction of a transgene encoding an enzyme for synthesis of purple pigment unexpectedly resulted in hypopigmented flowers.[3] In parallel, Lee et al[4] found that a small gene encoding a small hairpin RNA (ie, a small RNA that folds into a stem-loop structure because of complementary base pairing at the ends), but no protein, controlled the development of flatworms from one larval stage to another by suppressing the expression of a gene containing a homologous sequence. Independently, Fire et al,[5] also working with flatworms, found that sense DNA used as a control for antisense oligonucleotides unexpectedly led to reduced gene expression. These seemingly disparate phenomena all use double-stranded RNA containing short homologous sequences to silence gene expression.

Although interest in RNAi initially was restricted to basic researchers working to delineate the mechanisms important in regulating gene expression in primitive organisms such as flatworms and flies, interest in RNAi increased when Elbashir et al[6] showed that RNAi also operates in mammalian cells. Elbashir et al reported that gene expression in mammalian cell lines can be efficiently silenced by transfecting the cells with small double-stranded RNAs approximately 21 to 23 nucleotides in length.[6] Since then, RNAi has revolutionized biological research as an important research tool for understanding the function of a gene by silencing its expression. At the same time, translational researchers seeking therapeutic strategies for silencing disease-causing genes have quickly demonstrated the potential usefulness of harnessing RNAi for this purpose.[1] Interest in therapeutic applications of RNAi has rapidly overtaken interest in other nucleotide-based strategies for gene silencing, including those using antisense oligonucleotides or ribozymes.[1]

RNAi Mechanism

The RNAi machinery is ubiquitous in eukaryotic cells. It regulates the expression of key genes that determine cell fate and differentiation via small hairpin RNAs, called microRNAs, that are processed in the cytoplasm into double-stranded small RNAs.[2] MicroRNAs generally have only incomplete sequence homology to their targets, often recognize sequences in the untranslated 3′ end of a gene, and usually work by blocking the translation of messenger RNA (mRNA) into protein rather than by destroying the mRNA transcript. The first example of a mammalian microRNA that regulates B lymphocyte development was described in 2004.[7] RNA interference can also be exogenously induced in cells by using synthetic small double-stranded RNA or viral vectors to express small stem-loop RNAs, called short hairpin RNAs (shRNAs), that are processed like microRNAs within cells.[8,9]

RNA interference works by a "dice and slice" mechanism[10-12] (FIGURE) in which synthetic siRNA precursors, microRNA precursors, or long double-

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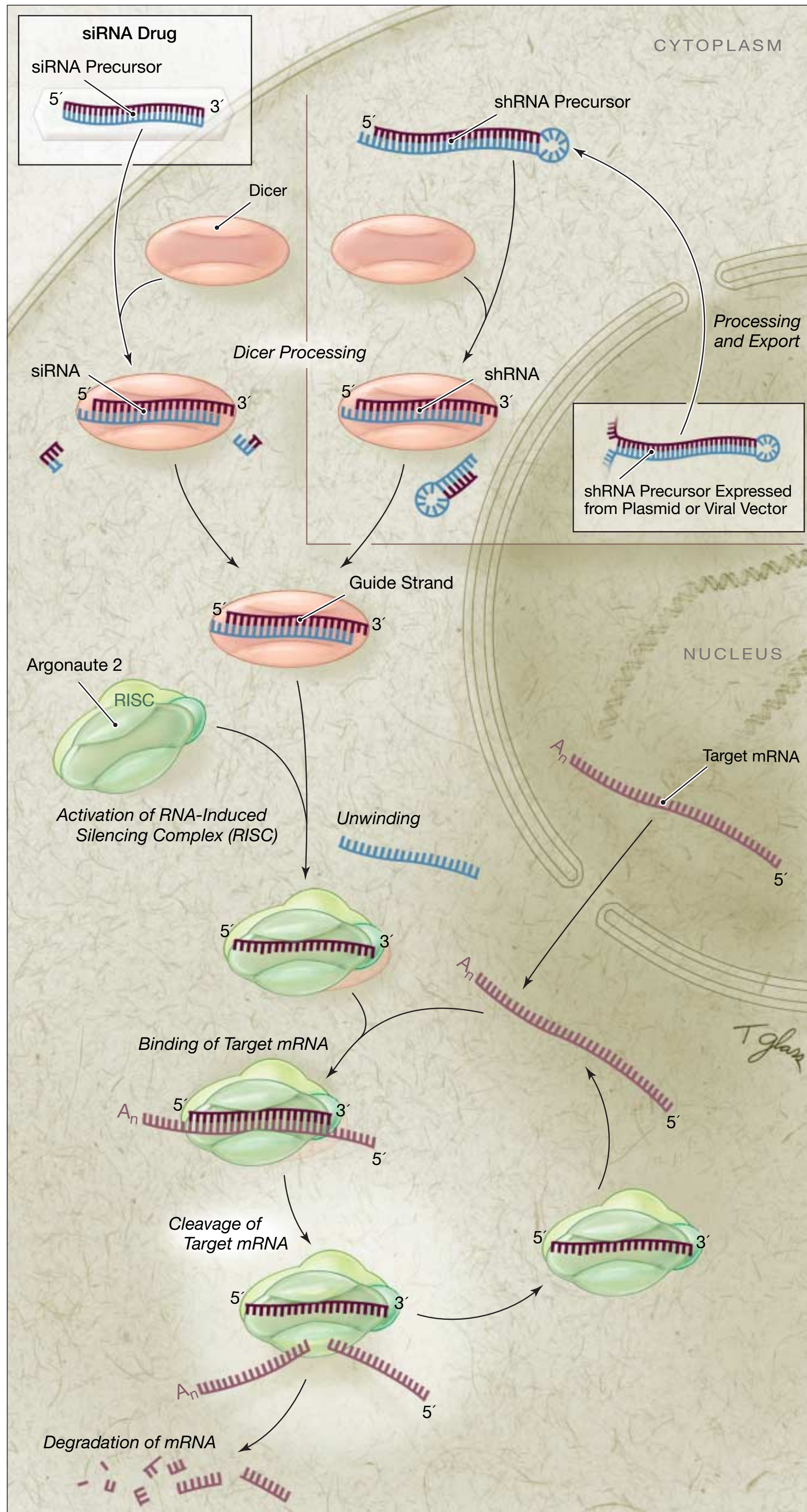
Figure. Mechanism of RNA Interference



Gene silencing by RNA interference can be initiated by introducing synthetic small interfering RNAs (siRNAs) or their precursors into cells where they are taken up by the endogenous microRNA processing machinery. The siRNA precursors are cleaved by the Dicer enzyme into siRNAs, which are 19- to 21-nucleotide double-stranded RNAs with a 2- to 3-nucleotide overhang and characteristic 5′-phosphate and 3′-hydroxyl groups at each end. As Dicer, an RNAse III–like enzyme, hands off the siRNA to the RISC (the cellular complex ultimately responsible for "slicing" messenger RNA [mRNA]), the double-stranded RNA unwinds, the RISC becomes activated, and 1 strand (the guide strand) remains within the activated complex. If the guide strand is homologous to an mRNA sequence, Argonaute 2, an enzyme within the RISC complex, cleaves the mRNA in the center of the region of homology. The cleaved mRNA is rapidly degraded and the protein for which it encodes is not produced. The guide strand small RNA is protected from degradation by the RISC and can direct the cleavage of many mRNAs. Alternately, plasmids or viral vectors encoding small stem-loop RNAs (called short hairpin RNAs [shRNAs]) can be introduced into cells where they are incorporated into the cell's genome, resulting in the expression of shRNAs that are processed in the nucleus by the same mechanism as endogenous microRNA precursors and exported to the cytoplasm where they are processed similarly to the process described above.

stranded RNAs, produced by viruses or by transcription of both sense and antisense strands of DNA, are cut by a cytoplasmic RNase III-like enzyme Dicer into 21- to 28-nucleotide duplex fragments (ie, siRNAs) with a 2- to 3-nucleotide unpaired overhang at each end. Small interfering RNAs with these properties are bound by a multiprotein cluster known as the RNA-induced silencing complex, by which the double-stranded siRNA is unwound.[13-16] Small interfering RNAs that do not require Dicer processing can also directly enter the RNA-induced silencing complex, but induce silencing less efficiently than siRNA precursors processed by Dicer.[70] One strand (the guide strand) remains bound to the complex. If this guide strand is complementary to a sequence within a target mRNA, it guides the slicer Argonaute 2 protein to cleave the mRNA.[17,18] The silencing is highly efficient (about 100-1000 times more powerful than antisense oligonucleotides[19]), presumably because the guide strand is protected within the RNA-induced silencing complex and therefore can act as a catalyst to degrade many copies of target RNA. Although it was initially thought that effective RNAi requires almost

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complete sequence homology throughout the length of the sequence, it now appears that as few as 7 contiguous complementary base pairs can direct RNAi-mediated silencing, particularly by repressing translation as in the endogenous microRNA pathway.[20]

RNA interference already has had a revolutionary effect on biomedical research.[1,11,21] By transfecting siRNAs into cells in vitro, expression of a target gene can be reduced by as much as 10-fold with a high degree of specificity.[6] Thus, it has become an important tool for defining the roles of particular genes in basic cellular processes. To study the effects of loss of gene expression in vivo,[22] knockdown mice (ie, transgenic mice expressing an shRNA that partially silences expression of a gene by RNAi) expressing an shRNA transgene in selected tissues or in all cells can be generated in a few months by infecting embryonic stem cells or embryos with shRNA-expressing lentiviruses. This compares with the several years often needed to generate knockout mice. Moreover, mice with graded degrees of gene knockdown can be produced to study subtle effects of gene expression.[23]

Increasingly, pharmaceutical companies are also using RNAi to identify disease-related drug targets against which to develop small-molecule inhibitors. Recently, libraries of retroviruses expressing shRNAs designed to silence large fractions of all expressed human genes have been produced. These libraries have so far been used to identify 5 novel genes that participate in the p53 pathway[24] and have enabled researchers to identify the mechanism for a rare inherited tumor syndrome, human cylindromatosis.[25] These shRNA libraries have the potential to provide mammalian biologists for the first time with a genetic screening tool similar to that which has been used in more primitive organisms.

RNAi as Therapy

There also is considerable excitement about harnessing RNAi for therapy. The prospect of using siRNAs as potent small-molecule inhibitors to any gene of choice provides a new therapeutic approach for many intractable diseases, including chronic viral infections such as hepatitis C or human immunodeficiency virus (HIV) and neurodegenerative diseases linked to the expression of a dominant mutant allele (TABLE). RNA interference is potentially so specific that it has been possible both in vitro and in vivo to silence a mutant gene differing by a single nucleotide from its wild-type counterpart. For example, it has been possible to silence mutant oncogenic *ras* without affecting wild-type *ras* in vitro and to silence a single nucleotide difference in a spinocerebellar ataxia gene using adeno-associated virus expression in vivo.[23,30] Treatment costs for siRNA are estimated to be comparable to those for the new types of protein-based therapies, such as antibodies and growth factors, currently used in clinical practice (Barry Polisky, PhD, written communication, February 2, 2005). The pace of drug development has been rapid, and 2 companies have begun phase 1 studies testing RNAi for treatment of age-related macular degeneration. By directly injecting siRNAs targeting the angiogenic vascular endothelial growth factor or its receptor into the vitreous, researchers hope to halt the neovascularization process.[32]

Potential Obstacles

Similar to other forms of gene-based therapies, a major bottleneck in the development of siRNA therapies is the delivery of these small molecules to the desired cell type in the correct tissue or organ. These small RNAs, which

Table. In Vivo Demonstrations of RNAi Therapeutic Efficacy in Rodents

Tissue	Disease	Target Gene	RNAi Formulation	Delivery Route	Reference
Liver	Autoimmune hepatitis	*Fas*	siRNA	Hydrodynamic (intravenous)	26
	Hepatitis B	*HBsAg*	siRNA	Hydrodynamic (intravenous)	27, 28
	Hepatitis B	Viral genes	shRNA from plasmid DNA	Hydrodynamic (intravenous)	29
	Hypercholesterolemia	*apoB*	Modified siRNA coupled to cholesterol	Intravenous	44
CNS	Spinocerebellar ataxia-1	*Ataxin-1*	Adeno-associated viral vector	Intracerebellar	30
	Neuropathic pain	Cation channel	siRNA	Intrathecal	31
Eye	Neovascularization	*VEGF*	siRNA	Intraocular	32
Kidney	Acute tubular necrosis	*Fas*	siRNA	Renal vein or hydrodynamic	33
Lung	Influenza	Viral genes	siRNA complexed to polyethyleneimine	Intravenous	34
			shRNA expressed from plasmid DNA	Intranasal	34
			siRNA + siRNA-lipid complex	Hydrodynamic (intravenous) + intranasal	35
	Respiratory syncytial virus	Viral genes	siRNA with and without lipid	Intranasal	36
Tumors	Germ-cell tumor	*FGF-4*	siRNA complexed to atelocollagen	Intratumoral	37
	Small-cell lung carcinoma	*Skp-2*	Adenoviral vector	Intratumoral	38
	Pancreatic adenocarcinoma	*CEACAM6*	siRNA	Hydrodynamic (intravenous)	39
	Glioblastoma	*MMP-9 + cathepsin B*	shRNA from plasmid DNA	Intratumoral	40

Abbreviations: *apoB*, apolipoprotein B, *CEACAM6*, carcinoembryonic antigen–related cell adhesion molecule 6; CNS, central nervous system; *FGF-4*, fibroblast growth factor 4; *HBsAg*, hepatitis B surface antigen; *MMP-9*, matrix metalloproteinase 9; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; *Skp-2*, S phase kinase-associated protein; *VEGF*, vascular endothelial growth factor.

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do not cross the cell membrane on their own, must be inside the cell to have their intended effect. Two delivery strategies are being developed—using siRNAs modified for enhanced delivery as small-molecule drugs or using viral vectors to express siRNA precursors within cells. Preliminary studies in mouse models, using both chemically synthesized and vector-encoded siRNA, have clearly demonstrated the potential of RNAi for in vivo modulation of diverse diseases, including autoimmune hepatitis, spinocerebellar ataxia, ischemia-reperfusion injury, and pulmonary infections including influenza and respiratory syncytial virus (Table).[26,30,33,34] The absence of apparent toxicity in these mouse models and the steady improvements in methods for local and systemic delivery are positive signs that RNAi therapeutics are closer to becoming a reality. Still, as yet, there are no large animal demonstrations of RNAi effectiveness in treating disease. Because siRNA drugs will likely need to be injected, a reasonable durability of action is needed to make them attractive therapeutic options. In terminally differentiated or slowly dividing cells such as macrophages, liver cells, or neuronal cells, gene silencing by exogenous siRNA is relatively durable (lasting up to several weeks), but in rapidly dividing cells the effect is more short-lived (peaking within 2-3 days, gone by 1 week) because of progressive dilution of the siRNA with cell division.[41] The presence of a target mRNA within the cell seems to be necessary for sustained silencing.[41] These results suggest that treatments could be spaced at intervals of approximately 1 week or longer, making them practical to consider. For more long-term gene knockdown, viral vectors derived from adenovirus, adeno-associated virus, retrovirus, or lentivirus and engineered to transcribe shRNA can be used. Although much progress has been made in developing gene-therapy vectors, vector-delivered gene therapy still has a number of obstacles to overcome. These include efficient transduction of targeted cells, particularly of pluripotent stem cells, without inducing differentiation; sustained and efficient gene expression of transduced cells; the danger of oncogenesis from insertional mutagenesis; the risk of recombination with endogenous retroviruses; and toxicity from immune or inflammatory responses to the viral vector itself, when expressed at high levels.[42] The ability of adeno-associated virus and lentiviral vectors to transduce nonreplicating primary cells gives them an edge over other viral vectors for many therapeutic applications.

Synthetic siRNAs may be particularly useful in situations in which long-term silencing is not required and may even be undesirable—for example, in treating acute viral infections or silencing proapoptotic or proinflammatory host molecules to prevent tissue damage. Use of siRNA drugs also avoids the problem of the host developing an immune response to proteins expressed from viral vectors or intrinsic inflammatory and interferon responses to viral vectors. However, siRNAs have a very short half-life in blood (less than minutes), and their uptake by mammalian cells is generally poor. It is possible to modify siRNAs chemically to make them resistant to serum RNases without sacrificing biological activity.[43] Coupling siRNAs to fusogenic peptides, linking them to antibodies or cell surface receptor ligands for cell-specific delivery, and encasing them in lipid complexes, liposomes, or other types of particles potentially offer methods to produce drugs that are big enough to bypass rapid filtration by the kidney and to deliver siRNAs into cells. Recently, chemically modified siRNAs targeting apoB covalently linked to cholesterol have been delivered to the liver and jejunum to reduce serum cholesterol levels in mice by about 30%.[44]

Although the original studies of siRNA silencing suggested exquisite specificity, off-target effects and other potential sources of toxicity have recently emerged as significant issues that need to be addressed.[20] This potential toxicity may result from mRNA cleavage or translational repression of genes with partial homology to either strand of the duplex siRNA. As the mechanisms that govern which strand of the duplex is incorporated into the RNA-induced silencing complex and the requirements for base-pairing for mRNA cleavage and inhibition of translation are clarified, it is possible that many, but probably not all, of these unintended effects can be avoided by judicious design of siRNAs and by taking advantage of the comprehensive databases of expressed mRNAs that now exist.

Induction of an interferon response that could potentially result in global suppression of protein translation and other off-target effects has also been reported with both synthetic and vector-expressed siRNA in highly sensitive reporter cell lines at high concentrations of siRNAs.[45] However, interferon is only induced efficiently by double-stranded RNAs longer than 30 nucleotides,[46] which is longer than siRNAs. Whether triggering an interferon response will be an obstacle in vivo, where delivered concentrations of siRNAs may be limiting, is still unknown. Another potential source of toxicity might come from siRNA binding to Toll-like receptor 3, a pathogen pattern receptor, which upon recognizing double-stranded RNA signals immune-activating cells, such as macrophages and dendritic cells, to send a danger signal and trigger a proinflammatory response.[47] It may be possible to reduce interferon induction or Toll-like receptor triggering by chemically modifying the siRNAs. Another possible, but still theoretical, source of toxicity might arise if siRNA drugs delivered or expressed in high amounts in cells displace endogenous microRNAs.

Additional safety issues arise when shRNAs are delivered via viral vectors because of the possibility of insertional mutagenesis and malignant transformation. Another potential concern is that the effect of long-term expression of shRNA is not known. Inducible and controlled expression of shRNA from plasmid or viral vectors may offer ways to control unanticipated adverse effects.[48]

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Targeting Host Genes to Prevent Tissue Damage

High-pressure intravenous ("hydrodynamic") injection of siRNAs targeting the death receptor *Fas* to inhibit experimental autoimmune hepatitis in mice was the first demonstration of therapeutic effectiveness of RNAi in vivo.[26] Liver cells were readily transduced and *Fas* expression was silenced for more than 10 days. Small interfering RNA also prevented liver fibrosis when administered during the chronic phase of hepatitis. Because many forms of hepatitis, even when caused by viruses, are Fas-mediated, silencing *Fas* might be useful in a number of settings. Hydrodynamic delivery, which involves pushing siRNAs into cells via high systemic venous pressures, is not practical for human use. However, local delivery of *Fas* siRNA by low-volume renal vein injection is as effective as hydrodynamic injection in protecting mice from renal ischemia reperfusion injury induced by clamping the renal artery.[33] This raises the possibility of using temporary suppression of *Fas* or other proapoptotic molecules by siRNAs introduced by local catheterization to limit tissue injury and infarct size in the brain, heart, kidney, and gut in similar situations of ischemia-reperfusion tissue damage. The potential of RNAi to check overzealous host responses is also supported by in vivo studies in inflammation and sepsis, in which blocking induction of other downstream disease mediators such as caspase 8 and tumor necrosis factor α was beneficial.[49]

Antiviral Potential of RNAi

RNA interference inhibition of viral replication has been demonstrated in vitro for a variety of viruses, including RNA viruses such as HIV, rotavirus, respiratory syncytial virus, influenza virus, poliovirus, West Nile virus, dengue virus, and foot and mouth disease virus, as well as DNA viruses such as human papillomavirus and herpes simplex virus.[50,51] RNA interference has also been effective in suppressing the hepatitis B, hepatitis C, and hepatitis delta viruses that can cause liver cirrhosis and hepatocellular carcinoma.[51,52] In addition, researchers have also used RNAi to inhibit the coronavirus that causes severe acute respiratory syndrome, suggesting that the technology has potential to be harnessed as a first response against emerging viruses where protective vaccines are unavailable.[53] RNA interference targeting JC virus, a polyoma virus responsible for the demyelinating disease progressive multifocal leukoencephalopathy, markedly inhibits virus production in already infected cells.[54,55] Therefore, RNAi potentially could cure or treat established viral infections.

However, some viruses are resistant to RNAi. For example, although siRNAs can inhibit the production of progeny virus, the genomic RNA of respiratory syncytial virus, hepatitis delta virus, and rotavirus are resistant to RNAi, due either to tight shielding by proteins or to sequestration in compartments inaccessible to siRNA.[56-58] Moreover, some viruses such as influenza and vaccinia produce proteins that actively suppress silencing by RNAi.[59] Adenovirus was recently shown to block the processing of shRNAs in mammalian cells by expressing a viral noncoding RNA at such high levels that it binds most of the available RNAi processing machinery.[60]

Interest in RNAi therapy has been particularly strong for HIV-1.[61-63] However, the very specificity of RNAi may be a handicap for treating a genetically diverse virus such as HIV. One approach is to target highly conserved viral sequences, in which mutation comes at a high price for viral fitness. Alternatively, host genes important for HIV replication, such as for the CCR5 coreceptor needed for viral entry, can be targeted. Cocktails of siRNAs that target multiple viral and host sequences may be the best way to prevent viral escape. Recently HIV-resistant progeny T cells and macrophages were generated by transplanting hematopoietic stem cells transduced with a lentivirus expressing an anti-HIV shRNA in SCID-hu mice, attesting to the potential of using gene therapy to induce stable intracellular immunity to the virus.[64]

Although most antiviral studies have been in vitro, RNAi has also suppressed viral replication in mice. Early studies aimed at hepatitis B or influenza infection involved hydrodynamic injection of siRNA.[27,35] Recently, more practical types of delivery, including regular intravenous injection of shRNA-encoding DNA vectors complexed with polyethyleneimine, as well as intratracheal administration of shRNA vectors with the surfactant Infasurf or of siRNA mixed with oligofectamine, have provided possible approaches to treat respiratory viruses such as influenza or respiratory syncytial virus.[34,35]

RNAi and Cancer

The growth and survival of tumor cells has been inhibited by RNAi-mediated ablation of several key oncogenes or tumor-promoting genes, including growth and angiogenic factors or their receptors (vascular endothelial growth factor, epidermal growth factor receptor), human telomerase (hTR, hTERT), viral oncogenes (papillomavirus E6 and E7), or translocated oncogenes (*BCR-abl*).[65] *BCR-abl* has been implicated in chronic myelogenous leukemia, and its silencing induces death of leukemic K562 cells at a roughly comparable level to that induced by the small-molecule anticancer drug STI 571.[21] Moreover, silencing Lyn kinase, which forms a signaling complex with *BCR-abl*, kills drug-resistant chronic myelogenous leukemia cells obtained from patients in blast crisis without affecting the viability of normal CD34$^+$ control cells.[66] Small interfering RNAs have also been designed to silence the mutant *ras* oncogene, which differs by only a single base pair mutation from its wild-type counterpart, while sparing the normal wild-type gene.[23]

The potential of RNAi to suppress tumor growth has also been demonstrated in vivo. For example, intratumoral injection of an adenoviral vector encoding an shRNA to target S phase kinase-associated protein 2, a cell cycle

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regulator, effectively inhibited an established subcutaneous small cell lung tumor in mice.[38] Moreover, systemic administration of an siRNA targeting a carcinoembryonic antigen–related cell adhesion molecule (*CEACAM6*) dramatically increased survival in mice with subcutaneously xenografted pancreatic adenocarcinoma cells.[39] RNA interference has also been used to inhibit intracranial tumors, in which direct injection of a plasmid vector expressing shRNAs to matrix metalloproteinase MMP-9 and a cathepsin inhibited the growth and invasion of an established glioblastoma.[40] However, the blood-brain barrier poses a formidable obstacle for intravenous delivery of siRNA and shRNA to the central nervous system. Recently, intravenous injection of immunoliposomes, the surfaces of which are conjugated with polyethyleneglycol linked to transferrin antibodies (to aid in crossing the blood-brain barrier) and to the insulin receptor (to aid in crossing into glioma cells), have delivered an encapsulated shRNA-encoding DNA vector into the brains of mice to suppress the growth of injected human glioma cells.[67]

RNAi and Neurologic Disease

The exquisite sequence specificity of RNAi provides a promising approach to silencing genes that cause dominantly inherited neurodegenerative diseases, such as Alzheimer disease, Huntington disease, and spinocerebellar ataxia (SCA), for which there are now no effective therapies. An siRNA targeting a single nucleotide polymorphism in *ataxin-1* associated with SCA1 was able to silence a mutant human transgene in a transgenic mouse model of SCA1. Intracerebellar injection of an adenoassociated viral vector expressing the *ataxin-1* shRNA substantially reduced ataxia in a mouse model of SCA.[30] Further work targeting amyloid precursor protein and Tau genes (involved in Alzheimer disease and frontotemporal dementia with parkinsonism, respectively) showed that it is also possible to achieve allele-specific suppression by clever siRNA design.[68,69] Direct intrathecal infusion of siRNAs targeting a cation channel implicated in neuropathic pain provided pain relief in rats, suggesting other potential uses in the nervous system.[31]

Summary

The therapeutic potential for RNAi is enormous, with applications for a wide spectrum of diseases, including some that have thus far proven intractable. Whether the obstacles now being considered or others not yet foreseen prove formidable will remain uncertain until results from pilot clinical studies are available. As the endogenous RNAi mechanism becomes better understood, the next few years should be an important time for testing this new therapeutic approach.

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I know of no more encouraging fact than the unquestioned ability of a man to elevate his life by conscious endeavor.
—Henry David Thoreau (1817-1862)

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