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REVIEW

siRNA-based approaches in cancer therapy

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The availability of the human genome sequence has revolutionized the strategy of employing nucleic acids with sequences complementary to specific target genes to improve drug discovery and target validation. Development of sequence-specific DNA or RNA analogs that can block the activity of selected single-stranded genetic sequences offers the possibility of rational design with high specificity, lacking in many current drug treatments for various diseases including cancer, at relatively inexpensive costs. Antisense technology is one such example that has shown promising results and boasts of yielding the only approved drug to date in the genomics field. However, *in vivo* delivery issues have yet to be completely overcome for widespread clinical applications. In contrast to antisense oligonucleotides, the mechanism of silencing an endogenous gene by the introduction of a homologous double-stranded RNA (dsRNA), transgene or virus is called post-transcriptional gene silencing (PTGS) or RNA interference. PTGS is a natural mechanism whereby metazoan cells suppress expansion of genes when they come across dsRNA molecules with the same sequence. Short interfering RNA is currently the fastest growing sector of this antigene field for target validation and therapeutic applications. Although, in theory, the development of genomics-based agents to inhibit gene expression is simple and straightforward, the fundamental concern relies upon the capacity of the oligonucleotide to gain access to the target RNA. This paper summarizes the advances in the last decade in the field of PTGS using RNA interference approaches and provides relevant comparisons with other oligonucleotide-based approaches with a specific focus on oncology applications.
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Genomic-based strategies

The American Cancer Society estimates that, in 2005, a total of 1 372 910 new cancer cases and 570 280 deaths are expected in the United States.[1] These morbid statistics demonstrate the necessity of newer therapeutic modalities for achieving successful cancer treatment and cure. Downregulation of genes that contribute to cancer progression has been the goal of targeted genomics-based strategies, with the expectation that such an approach may lead to selective and specific inhibition of tumor growth with minimal untoward side effects on normal cells. Identification of target genes involved in neoplastic transformation and tumor progression has triggered the idea that nucleotide sequences of cancer-relevant genes could lead to the development of tailored anticancer agents that lack many of the toxic side effects of traditional cytotoxic drugs. This has led to a recent acceleration in the development and optimization of genomic-based strategies for cancer therapy. This idea dates back to the 1960s when RNA sequences were shown to serve as endogenous inhibitors of gene expression in procaryotes. The antigene approaches include the following:

(1) Ribozymes:[2,3] These were discovered in the 1970s, and the elucidation of the transactivating hammerhead ribozyme later led to their study for therapeutic applications including viral infections and cancer. In addition to possessing catalytic activities as well as binding capacity to the RNA, the hammerhead ribozymes can cause RNase-dependent degradation of the target double-stranded RNA (dsRNA). Delivery of ribozymes has largely utilized viral vectors and cationic liposome:DNA complexes in tumor models. A modified chimeric ribozyme targeting VEGF receptor, flt-1 (Angiozyme), is being developed by Ribozyme Inc., which is now renamed Sirna Therapeutics Inc. (Boulder, CO).
(2) Antigene oligonucleotides are antisense sequences that can insert themselves into a section of a DNA to form a triple helix, and thus inhibit transcription. Recognition of a duplex sequence by a third strand of DNA or RNA via the major groove is the basis of the formation of a triple helix. Typically, stable triplexes form on polypurine:polypyrimidine tracts. The third strand, depending on the target sequence, may consist

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of purines or pyrimidines, and the complex is stabilized by two Hoogsteen hydrogen bonds between third strand bases and the bases in the purine strand of the duplex. Triple helix is an inherent property of DNA and requires no additional enzymes or proteins. However, current strategies for oligonucleotide-directed triple helix formation need to address various requirements for stabilizing binding conditions, restrictions on permitted target sequences and inefficient nuclear delivery of oligonucleotides before they can become mainstream for cancer therapy.[4,5] Trojan *et al*.[6] are pursuing preclinical studies toward development of a 'triple-helix anti-IGF-I' cancer therapy.

(3) Peptide nucleic acids (PNAs) are DNA analogs consisting of nucleobases attached to a peptide backbone of *N*-(2-aminoethyl)glycine residues. The phosphate charges are replaced with neutral peptide linkage, resulting in a stable hybrid between PNA and DNA or RNA strands. In addition, they can form triplexes by Hoogsteen pairing on polypurine and polypyrimidine targets. PNAs are resistant to degradation, form stable complexes on DNA targets and show high sequence selectivity, making them very attractive for cancer therapy. However, in spite of these attractive features, they have shown only modest activity *in vivo* owing to inefficient *in vivo* bioavailability and the need for opening of the target duplex similar to triplex oligonucleotides (for in-depth reviews, Dean,[7] Nielsen[8] and Braasch and Corey[9]).

(4) Antisense oligonucleotides: These are the most widely used unmodified or chemically modified single-stranded RNA or DNA molecules. The fundamental aspects of antisense oligonucleotides is hybridization of nucleic acid oligomer to target mRNA sequences based on the simple rules of Watson–Crick base pairing of nucleic acids.[10,11] The first description of antisense inhibition of gene expression was revealed when a single-stranded DNA was shown to inhibit the translation of a complementary RNA in a cell-free system.[12] Later, Zamecnik and Stephenson[13] received the Lasker prize for studies in tissue culture showing inhibition of a viral replication using antisense oligonucleotides. One of the first reports to show *in vivo* activity was of a phosphodiester oligonucleotide directed against *N-MYC* that caused a decrease in tumor mass associated with loss of *N-MYC* protein in a subcutaneously transplanted neuroepithelioma in mice.[14] As the phosphodiester bond is highly susceptible to degradation, the development of phosphorothioate chemistry, which contains a sulfur atom in each internucleotide linkage instead of oxygen, revolutionized this field because of its stability.[15,16] The phosphorothioate antisense has shown the broadest range of activity in preclinical and clinical studies (ISIS Pharmaceuticals Inc., Carlsbad, CA; Genta Inc., Berkeley Heights, NJ; Hybridon Inc., Cambridge, MA). Formivirsen (Isis Pharmaceuticals Inc.) is the only US-approved antisense oligonucleotide for the treatment of cytomegalovirus-induced retinitis in AIDS patients.[17] However, the presence of an internucleoside charge in oligonucleotides generally increases the possibility of oligonucleotide binding to intracellular proteins and provides a reactive center for potential drug interactions.[18–24] Some of these problems have been solved in 'second-generation' nucleotides with alkyl modifications at the 2′ position of the ribose and the development of novel chemically modified nucleotides with improved properties such as enhanced serum stability, higher target affinity and low toxicity.[25] One such modification in oligomer chemistry has led to the development of the phosphorodiamidate morpholino oligomers (PMO) by AVI BioPharma Inc. (Portland, OR), which are non-ionic antisense agents that inhibit gene expression by binding to RNA and sterically blocking processing or translation in an RNaseH-independent manner.[26–28] PMO antisense agents have revealed excellent safety profile and efficacy in multiple disease models[29–37] including cancer preclinical studies.[38–44] Recent studies in our laboratory have characterized *in vivo* PMO bioavailability in solid tumors and pharmacokinetics of the PMO agents in human clinical trials, revealing the potential of these agents in gene-specific targeting.[44,45] In addition, development of certain peptides, either as noncovalent complexes or as covalent conjugates, to enhance the delivery of antisense oligonucleotides into cells is being evaluated for enhanced delivery of antisense agents and increasing cellular localization.[46–48]

Post-transcriptional gene silencing

In contrast to antisense technology, the mechanism of silencing an endogenous gene by the introduction of a homologous dsRNA, transgene or virus[49] is called post-transcriptional gene silencing (PTGS). In PTGS, the transcript of the silenced gene is synthesized without accumulation because of rapid degradation of the transcript. PTGS was first described in petunia flowers by Jorgensen and co-workers[50] in 1990 wherein introduction of a purple pigment-producing gene under the control of a promoter caused an unexpected white color. They called this puzzling observation co-suppression. The term 'RNA interference (RNAi)' was later coined in 1998 by Fire and Mello[51] when they injected a mixture of both sense and antisense strands into the nematode *Caenorhabditis elegans*. Interestingly, they found that both the sense and antisense RNA forming a dsRNA were much better in silencing the target gene compared to the antisense RNA alone. This silencing response has been subsequently found to occur in other eucaryotes, from yeast to mammals.[52,53] During the last several years, this property of dsRNA has been aggressively exploited in various systems to elaborate the role of numerous genes and their involvement in various diseases. In addition, small RNAs have been identified to play multiple biological roles, which seem to include suppression of transposon hopping and genomic rearrangements and

Table 1 RNAi agents in drug development

Company	Disease indication and status
Acuity Pharma, Philadelphia, PA	Age-related macular degeneration, phase I
Alnylam Pharmaceuticals, Cambridge, MA	Age-related macular degeneration (phase I expected in the second half of 2005)
	Respiratory syncytial virus (phase I planned 2006)
	Spinal cord injuries (preclinical)
	Parkinson's disease (preclinical)
	Cystic fibrosis (preclinical)
Atugen, Berlin	Metabolic diseases, ocular diseases, cancer (research phase)
Benitec, Mountain View, CA	HIV/AIDS lymphoma (phase I expected 2006)
	Hepatitis C virus (phase I expected 2006)
CytRx	Lou Gehrig's disease, obesity, cytomegalovirus (research phase)
Intradigm Corp. Rockville, MD	Solid tumors (IND planned)
Nucleonics, Horsham, PA	Hepatitis B and C virus (phase I expected 2006)
Phytovation, Netherlands	Hepatitis C virus, HIV-1, influenza (research phase)
Sirna Dermatology Boulder, CO	Topical hair removal (IND planned)
Sirna Pharma, san Francisco, CA	Age-related macular degeneration (phase I)
	Huntington's disease, asthma, type II diabetes (IND expected)

named accordingly as short interfering RNAs (siRNAs). It has been shown by several groups that endogenous siRNAs may mediate chromatin condensation in centromeric regions and DNA repeat sequences of fission yeast – an action that may result in transcriptional silencing of genes at the locus.[54–57] It has been found that mutation of certain genes in RNAi in *C. elegans* depresses genes that are silenced by the Polycomb proteins, a group of proteins that are involved in organizing chromatin structure and repression. The RNAi response may also play a role in antiviral defense, as inhibition of viral replication has been demonstrated *in vitro* for a variety of RNA viruses, such as HIV, rotavirus, respiratory syncitial virus, influenza, poliovirus, West Nile, dengue, foot and mouth virus, as well as DNS viruses such as papillomavirus, hepatitis and herpes simplex virus. (Owing to space constraints, viral applications of RNAi are not covered in this paper. See review by Voinnet[58] for additional references.)

To date, Acuity Pharmaceuticals, Philadelphia, PA and Sirna Therapeutics, San Francisco, CA have filed Investigational New Drug applications with the US FDA to begin clinical trials with modified siRNA molecules in patients with age-related macular degeneration (Table 1).

RNAi mechanism of action

Although both RNAi and antisense RNA induce destruction of mRNA in the cytoplasm and inhibit or block production of protein for a particular function, they do so entirely by different mechanisms. In fact, the RNAi pathway is present in cells of virtually every multicellular organism.[53] It has been postulated that RNAi may have evolved as an innate mechanism for cellular defense against dsRNA viruses. Briefly, dsRNA when introduced into a cell gets chopped up by the enzyme Dicer, a member of the RNaseIII family of dsRNA-specific ribonucleases. This enzymatic cleavage degrades the RNA to 19–23 bp duplexes, each with 2-nucleotide 3′ overhangs. siRNA then binds to the RNA-induced silencing complex (RISC) and is unwound in an ATP-dependent manner. RISC mediates sequence-specific binding of these guide RNAs to a corresponding mRNA and catalyzes the cleavage and destruction of the mRNA by the enzyme Slicer, enabling gene-specific silencing.[59,60] It is generally believed that, like antisense agents, effective RNAi requires complementary sequence homology. However, off-target translational repression in the case of RNAi led to the observation that shorter sequences, comprising as few as 7 contiguous complementary base pairs, may be sufficient for direct RNAi-mediated silencing, which seems particularly relevant in the case of microRNA (miRNA) action.[61–64]

MicroRNAs

miRNAs are endogenous non-coding small RNAs (19–23 nt) found in both animal and plant genomes.[65–67] In plants, miRNAs seem to bind to mRNA targets by perfect or nearly perfect complementarity, and cleave the target molecules. In animals, in contrast, most miRNA–mRNA pairings are not completely complementary, resulting in translation repression or arrest without the need for target mRNA degradation. miRNAs are highly conserved, suggesting a fundamental biological function, and it is not surprising that they have been identified to play a role in disparate areas including cell development, differentiation, cell death, hematopoiesis, insulin secretion, nervous system patterning and cancer. Currently, approximately 850 unique miRNA have been discovered, including 222 human miRNAs. Short temporal RNAs (stRNAs) are a subclass of miRNA that are temporally expressed. The pairing of stRNA with complementary

mRNA regulates expression by blocking translation, whereas, in siRNA, there is cleavage of mRNA. Recent emerging data indicate that dysregulation of miRNAs is associated with certain types of cancer. Calin *et al.*[68] have reported an association between chronic lymphocytic leukemia and deletion of a section of chromosome 13. Genes miR-15 and mir-16 present on this chromosome are consistently reduced in colorectal neoplasia.[68,69] In another study,[70] reduced expression of the let-7 miRNA was reported in human lung cancers. Subsequently, overexpression of let-7 in a preclinical model (A549 lung adenocarcinoma cell line) inhibited lung cancer cell growth *in vitro*. In contrast, overexpression of some miRNA, like the precursor microRNA-155/BIC RNA, has been observed in juvenile patients with Burkitt lymphoma.[71] These observations, which include cancer-associated alterations in miRNA expression, frequent identification of miRNAs located at genomic regions involved in cancers and their gene regulatory function, have led to speculations that miRNAs may act as both tumor suppressors and oncogenes.[68–72]

DNA-directed RNAi

Gene silencing can be achieved either by direct administration of siRNA or by using DNA-directed RNAi (ddRNAi or expressed RNAi) that enables the *in vivo* production of siRNAs. Viral systems based on adenovirus, retrovirus, herpes simplex virus and non-viral delivery systems (liposomal formulations, mechanical administration) are being developed and used for delivery of ddRNAi. However, the key challenge to ddRNAi delivery-based therapies is to ensure safe and efficient drug delivery. Although the non-viral delivery tools are much safer than the viral vectors, they remain largely unproven in the clinical setting.[73] Meanwhile, ddRNAi-based technology is very useful in applications involving preclinical animal disease models, research reagents and drug target identification and validation. Several transgenic companies have developed approaches to exploit this technology for creation of animal models in mice, rats and primates (Table 2). In general, embryonic stem cells are transduced with a vector bearing the small hairpin RNA (shRNA) targeted to a specific locus in the mouse genome. Chimeras are generated by injecting the construct into blastocysts, and transgenic mice are created by conventional breeding. Although RNAi technology has immense potential in the generation of animal models in a relatively shorter period of time, much work is needed by these pioneering companies to become mainstream compared to the well-established traditional gene knockout techniques.

RNAi delivery

Many groups and companies are working on developing strategies to deliver siRNAs or shRNAs to cells in culture and in tissues in various animal models. *In vitro* delivery of RNAi agents in fruitflies, worms and cells in culture has been relatively easier as simple techniques like feeding, soaking and availability of various transfection agents. In some organisms like *C. elegans*, the presence of RNA-dependent RNA polymerase allows for the amplification of siRNAs, and potentially even passes it on through the germ line, resulting in a long-lived, stable gene silencing. However, mammalian cells do not possess this mechanism, and, thereby, gene silencing is only seen for a few days after treatment with siRNA or shRNA. Interestingly, in the case of slow-proliferating or non-dividing cells like macrophages and hepatocytes, siRNA seems to have a longer gene silencing effect, several weeks in some instances.[74,75] Plasmid-based expression systems have been developed for the stable expression of siRNAs in most of the other cell types.[76] Viral vectors like retrovirus-, adenovirus- and lentivirus-based vectors have been quite successful in obtaining efficient and longer gene silencing effects using RNAi. Retroviral vectors allow delivery into cycling cells, transformed as well as primary cells,[77,78] although, recently, there have been concerns about insertional mutagenesis. Recombinant lentivirus vectors have been especially successful in infection of non-cycling cells and neuronal cells.[79–82] In general, biosafety is the major concern in the production of retroviral and lentiviral vectors, and currently they are in use in HIV patients only. Adenoviral vectors are widely used both in cells and *in vivo*; however, their inability to integrate into the genome has proved advantageous in terms of safety but at the cost of inefficient long-term gene silencing.[83–89] However, other disadvantages of using adenovirus for gene therapy are well known, including high immunogenicity and proinflammatory effects. In contrast, the adenovirus-associated viruses have been more effective in

Table 2 Development of ddRNAi in the creation of human disease models

Company	Description
Artemis, Cologne, Germany http://www.artemispharma.de	Gene-specific knockout mice using its ArteMice™ RNAi Technology
RxGen, Connecticut, USA http://www.rx-gen.com	Specializes in creating human disease models using the African green monkey. The existence of well-characterized cells lines (COS-7 and Vero) originating from the African green monkey is expected to enable the translation of *in vitro* optimization studies to primate models
genOway, France and Germany http://www.genoway.com/safe_in_vivo_mai.htm	genOway uses targeted insertion of the shRNA into the HPRT locus of mice to enable ubiquitous, tissue-specific and inducible expression of the shRNA construct for target validation studies

terms of safety for *in vivo* applications and also have the ability to integrate into the genome of infected cells.[90,91] Herpes simplex is used in gene therapy to efficiently kill cells by producing a cytopathic effect, which is used especially to transfer genes to neurons. Its key advantage is the high capacity (30 kb) of the genome for gene transfer. Recently, SV40 vectors, packaged either *in vivo* or *in vitro*, have shown great promise in the delivery of genes to many different cell types. Strategies for treating or preventing HIV infection, and for treating cancer have been developed on the basis of the high efficiency and flexibility of SV40-based gene delivery systems.[92]

Hydrodynamic injections of siRNAs alone or in conjunction with reporter constructs have led to the silencing of endogenous genes in various animal tissues, which include liver, spleen, lung, kidney and pancreas.[93–96] However, the need for a large volume that can be equivalent to up to 10–20% of the animals' blood volume limits this method of delivery *in vivo*. siRNAs, like most antisense oligomers, tend to be more bioavailable in the liver after *in vivo* administration. This, compounded with the fact that they have relatively short half-lives, limits their use in the silencing of gene targets in other organs, particularly tumors. Therefore, to date, local delivery of siRNAs and dsRNAs has been most efficient in *in vivo* animal models. Table 3 summarizes the currently available strategies for RNAi delivery in different systems.

RNAi and functional genomics

Improved drug development depends on better understanding of the mechanisms of interactions between the cancer cell and the host, identification of target genes or alterations thereof that probably determine the degree of malignancy. Moreover, it is probably wise to examine each cancer as a unique disease for tailored drug development. In addition to providing the research tools for target validation, RNAi technology is evolving rapidly as a tool for genome-wide screens for assessing gene function.

Table 3 RNAi delivery strategies

Model organism	*Delivery method*
D. melanogaster	Soaking cells in medium containing dsRNA
C. elegans	Feeding worm with bacteria overexpressing dsRNA
Mammalian cells in culture	Transfect siRNA
	Transfect plasmid shRNA
	Retroviral shRNA
	Adenoviral shRNA
	SV40 mediated
In vivo	Intravenous injection in large volume
	siRNA packaged in cationic liposomes
	Conjugating RNAs to membrane-permeant peptides
	Direct electroporation of siRNA duplexes into target organs and tissues

Functional genetics approaches, which traditionally include forward or reverse screens, can provide insights in assigning function to cancer genes and delineate molecular pathways by which these genes act in normal vs malignant cells. Forward genetic studies allow identification of mutations that produce a certain phenotype. A mutagen is very often used to accelerate this process. Once mutants have been isolated, the mutated gene can be molecularly identified. Although forward genetic screens are productive, a more straightforward approach would be to determine the phenotype that results from mutating a given gene. This is called reverse genetics. In some organisms, such as yeast and mice, it is possible to induce the deletion of a particular gene, creating a gene knockout. Among the alternatives that include the random induction of DNA deletions and subsequent selection for deletions in a gene of interest, and the creation of transgenic organisms that overexpress a gene of interest, the application of RNAi has most excited researchers about its potential as a functional genomics tool. The use of RNAi molecules to knock down the levels of the proteins to assess their function is particularly important for two reasons – it provides a high-throughput approach essential to investigating complex pathways, and it provides the opportunity to begin assessing potential therapeutic targets.

RNAi libraries are being created by several groups. Initially, broad libraries of synthesized siRNAs were used for screening several dozen targets in a given druggable pathway. However, recently, companies like System Biosciences (Mountain View, CA, USA) and Galapagos Genomics (Mechelen, Belgium) have created selectable libraries consisting of shRNA expression libraries with viral vectors. Hannon and co-workers[97] have developed a large plasmid library coupled to a matrix-assisted genetically integrated cloning technology comprising of atleast 28 000 shRNA cassettes. This technology has been postulated to have the ability to screen at the single cell level. In addition, after several rounds of cloning, this plasmid system can generate pools of highly functional shRNAs. RNAi is widely being used to ascertain the function of many genes in *Drosophila*, *C. elegans*, and several species of plants, although functional genomics studies in zebrafish models have largely utilized the antisense oligonucleotides with a very high success rate.[32]

So far, injection and transfection of dsRNA into cells have been the main method of delivery of siRNA, and the silencing effect may last for several days but eventually diminishes. To date, a major bottleneck of siRNA experiments has been the siRNA design. Typically, researchers have to screen four to six siRNAs before identifying an effective one. In earlier studies, Tuschl and co-workers[98] determined that the most effective siRNAs are 21 bp in length and have 2 bp 3′ overhangs. Moreover, their results suggested that the most effective siRNA overhangs have a UU or TT sequence. Since then, most of the reported siRNA sequences have been 21 bp in length (although there have also been reports of effective 23 bp

siRNAs[99]), and sequences other than UU or TT are now routinely being used. Cenix BioScience GmbH, Dresden, Germany has developed an algorithm to come up with a better siRNA design tool, and increase the likelihood of predicting potent siRNAs. The algorithm uses such criteria as T_m of the siRNA, nucleotide position effects, specificity checks, content of the 3′ overhang and siRNA length. Using such criteria, the program is capable of predicting effective siRNAs (>70% silencing) with >80% probability.[100] Currently, methods to produce siRNAs include chemical synthesis, *in vitro* transcription or RNaseIII/Dicer digestion of long dsRNAs. Alternatively, they can be expressed *in vivo* from plasmids, PCR cassettes or viral vectors that include a cytomegalovirus or polymerase III transcription unit. Ambion Inc. Austin, TX is one of several companies that offer custom siRNA synthesis, including a kit (Silencer siRNA construction kit) that allows synthesis of siRNA by *in vitro*, transcription which is a less expensive alternative to chemical synthesis.[100] So far, these approaches have been used to create siRNAs for use in loss-of-function studies.

High-throughput genomic-based oncology screening

Drug discovery is an increasingly expensive and time-consuming endeavor. Although new technologies may not necessarily shorten the time of drug discovery and development, the use of genomic tools and cellular assays to validate targets should help discovery groups create a high-throughput platform for target choices. A recently described cell-based microarray system called transfected cell array (TCA) combines microarray technology with cell biology methods such as transfection and cell culture. This high-throughput technique has indeed paved the way for loss-of-function studies. siRNAs and plasmid expressing shRNAs can be transfected into the cells through the process of reverse transfection. This is then combined in a microarray format along with a detection assay.[101] This technology also provides for a unique opportunity to study protein function in living cells, which allows for the presence of normal post-translational modifications and cofactors. However, again, as with most RNAi delivery, transfection procedures need to be optimized for different cell types. A number of laboratories have developed expression vectors to continually express siRNAs in transiently and stably transfected mammalian cells.[102–104] Sachse and Echeverri[105] have put together a comprehensive review of the various strategies, design issues and use of chemically synthesized siRNA libraries. Monitoring gene silencing in the TCA method can be accomplished using fluorescent-labeled monoclonal antibodies, downstream signaling events, apoptosis and other cellular processes. With the increase in protein interaction databases and chemical genetic screens, RNAi libraries have the ability to identify and characterize cancer genes involved in specific pathways in disease etiology and progression.

RNAi and therapeutics

With the Science magazine hailing RNAi as the 'Breakthrough of the Year' in 2002, the last few years have seen immense excitement about harnessing RNAi for therapy. Multiple biotechnology companies are involved in developing RNAi agents as potent inhibitors in various diseases as shown in Table 1. Although specificity of RNAi is still a concern, studies like the one reported by Brummelcamp *et al.*[78] in which they were able to specifically silence mutant oncogenic *ras* without affecting wild-type *ras in vitro*, show promise in this direction. It is believed that treatment costs for siRNA would be similar to most protein-based therapies like antibodies. Acuity and siRNA are in phase I clinical trials testing RNAi for the treatment of age-related macular degeneration. Preclinical cancer studies have shown inhibition of growth and survival of tumor cells by RNAi-mediated downregulation 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*). Various studies are reporting *in vivo* activity and the potential of RNAi to suppress tumor growth. These include an intratumoral injection of an shRNA-adenoviral vector construct targeting a cell cycle regulator causing inhibition of subcutaneous small cell lung tumor in mice, and systemic administration of an siRNA targeting a carcinoembryonic antigen-related cell adhesion molecule (*CEACAM6*) in mice with subcutaneously xenografted pancreatic adenocarcinoma cells. In another report, direct injection of a plasmid vector expressing shRNAs to matrix metalloproteinase MMP-9 and a cathepsin showed efficacy in established glioblastoma. Table 4 highlights some of the studies showing RNAi efficacy in targeting cancer-specific genes.

Potential issues

siRNAs are natural molecules to which cells have evolved many responses. Although the process of RNAi causes degradation of target mRNA, it can also prevent the translation of other somewhat mismatched mRNA wherein the siRNA is functioning as an miRNA. Compared to the antisense technology, siRNAs have been identified to have more nonspecificity in terms of the potential of broad off-target gene modulation by a single siRNA sequence. Various studies have reported variations in the expression of multiple off-target genes after the introduction of an siRNA sequence into cells.[123–127] The action of siRNA depends on many cellular proteins; therefore, various organisms may respond to siRNA in slightly different manners. As cellular antiviral systems are intertwined with the siRNA system, the innate immune system (specifically the interferon response and the NF-κB-modulated inflammation response through Toll-like receptors) may be stimulated by siRNA.[128–130]

Table 4 Cancer-associated genes targeted by RNAi

Pathway	*Target gene*	*References*
Apoptosis	Bax	106
	Bcl-2	107,108
Angiogenesis	Focal adhesion kinase (FAK)	109
Adhesion	Matrix metalloproteinase	110
Cell–cell communication	VEGF	107, 111
Lipid metabolism	Fatty acid synthase	112
Transport	MDR	113
Signaling	H-Ras	107
	K-Ras	78
	PLK-1	114
	TGF-β	107
	STAT3	115
	EGFR	116,117
	PKC-α	107
Viral, oncogenes, nuclear	Epstein–Barr virus	118
	HPV E6	119
	BCR-Abl	120, 121
	Telomerase	122

All of this leads to a greater complexity and unpredictability in the action of siRNA.

Concluding remarks

The elucidation of the structure–function relationship of the RNA silencing pathways and their effector molecules siRNAs and miRNAs, has had an enormous impact in the biological research community. The clinical potential of appropriately designed siRNAs in various diseases including viral infections, cancer and neurodegenerative disorders has been demonstrated. However, many simple questions need to be answered to unravel the full therapeutic benefit of this mechanism. These include details of the components and function of various enzymes involved in the formation of siRNA, the role of RNAi in chromatin remodeling and DNA methylation. In addition, now that these agents are in phase I clinical trials, issues related to bioavailability and safety need to be critically evaluated. Another fast-growing technology is the development of locked nucleic acids (LNA), a class of confirmationally restricted oligonucleotide analogs[131] that exhibit high thermal stabilities toward complementary DNA and RNA and follow the Watson–Crick base-pairing rules. The high binding affinity of LNA oligomers and the flexibility to adjust T_m of LNA probes and primers allow for the use of short probes in various techniques like SNP genotyping, *in situ* hybridization, allele-specific PCR, molecular beacons and mRNA preparation. These molecules are now commercially available and could rival RNAi in the near future. Although continued new advances are expected in the field of functional genomics, the actual approval of a therapeutic entity using an antigene approach is eagerly awaited, which would truly revolutionize the playing field.

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