

Standard Review

RNAi as a novel therapeutic platform technology for oncological solutions

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Accepted 4 March 2008

RNA interference (RNAi) is a process in which double-stranded RNA triggers the degradation of a homologous messenger RNA (sharing sequence-specific homology). RNAi has been observed in all eukaryotes, from yeast to mammals. The power and utility of RNAi for specifically silencing the expression of any gene for which sequence is available has driven its incredibly rapid adoption as a tool for reverse genetics in eukaryotic systems. RNAi is remarkably potent i.e., only a few dsRNA molecules per cell (like catalytic reactions) are required to produce effective interference. The long dsRNAs enter a cellular pathway that is commonly referred to as the RNA interference (RNAi) pathway. RNAi is now recognized to be one of a larger set of sequence-specific cellular responses to RNA, collectively called RNA silencing. These responses have been shown to play a role not only in mRNA and dsRNA stability/degradation, but also in regulation of translation, transcription, chromatin structure, and genome integrity. RNAi has also been invaluable for unveiling critical pathway involved in cancer development, growth and metastasis and has been useful in identifying critical tumor type specific gene targets for cancer therapy. RNAi has been shown to inhibit gene expression and is considered as one of the most important recent discoveries in molecular oncology. It is already a widely used research tool in the analysis of molecular mechanisms for many diseases including cancer and this is because it allows researchers to silence the expression of specific gene targets, much like antisense technology, but with higher specificity and efficacy. Although RNAi is an evolutionarily conserved phenomenon for sequence specific gene silencing in mammalian cells, exogenous short interfering RNA (siRNA) and vector based short hairpin RNA (shRNA) can also invoke RNAi responses. Both are now not only experimental tools for analyzing gene functions but are also expected to be excellent avenues for drug target discovery and the emerging class of gene medicine for targeting incurable disease such as cancer. The success of therapeutic use of RNAi for cancer relies on the development of safe and efficacious delivery systems that introduce siRNA and shRNA expression vectors into target tumor cells. The present review provides a brief overview of RNAi based approaches for gene silencing as a potential therapy for cancer.

Key words: RNA interference (RNAi), Cancer, Posttranscriptional gene silencing, siRNA, miRNA.

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INTRODUCTION

In recent years researchers have demonstrated the underlying mechanisms of how dsRNA results in the loss of the targeted homologous mRNA and the primary interference effects were post-transcriptional. In 1998, Craig Mello and Fire reported that only dsRNA targeting exon sequences were effective (promoter and intron sequences could not produce an RNAi effect). The small RNAs that provide target specificity to the silencing machinery, short interfering RNAs (siRNAs), repeat-associated siRNAs (rasiRNAs), and microRNAs (miRNAs)—can be distinguished by their origin. siRNAs are processed from dsRNA precursors made up of two distinct strands of perfectly base-paired RNA, while miRNAs originate from a single, long transcript that forms imperfectly base-paired hairpin structures. In plants and animals, RNA silencing has been adapted to play a critical role in regulation of cell growth and differentiation using a class of small RNAs called microRNAs (miRNAs). RNA interference is the process of mRNA degradation that is induced by double-stranded RNA in a sequence-specific manner.

In the last decade, the structural and functional analysis of a gene has acquired new dimensions. In parallel, it has also been assumed that the primary DNA sequence data is within the reach of available analytical tools (Venter et al., 2001). This collective consensus has revolutionized and remapped the road for defining a disease condition. Of which, biology of cancer has received tremendous attention. We are now much closer to genes that mediate cancer progression and many therapeutic gene targets that regulate apoptosis, proliferation and cell signaling. Long double-stranded RNAs (dsRNAs; typically > 200 nucleotide) can be used to silence the expression of target genes in a variety of organisms and cell types.

The initial evidence of RNAi in nature came from the work on putunia flowers in which over expression of the gene responsible for purple pigmentation actually caused the flowers to lose their endogenous colour (Napoli et al., 1990). RNAi was first described in animal cells by Fire and colleagues in the nematode *Caenorhabditis elegans* as a naturally occurring cellular mechanism that induces

post transcriptional gene silencing, in which double stranded RNA (dsRNA) suppresses the expression of a target gene by triggering specific degradation of complementary messenger RNA (mRNA) sequence (Fire et al; 1998). The natural role of RNAi is thought to be that of cellular defense against viral infection or potentially harmful destabilizing genomic intruders such as transposons. The experimental designs were developed to influence cellular physiology by RNAi and following are few examples where different mode of RNAi introduction has been demonstrated. RNAi can be induced in mammalian cells by the introduction of synthetic siRNA 21 – 23 bp in length (Elbashir et al., 2001) or by plasmid (Brummelkamo et al., 2002; Lee et al., 2002; Paul et al., 2002) and viral vector systems (Brummelkamo et al., 2002) that express shRNA that are subsequently processed to siRNA by cellular machinery. Recently a nano particle based RNAi delivery has also been proposed and research on this is gaining momentum. Analysis of gene function in cultured somatic mammalian cells using siRNAs is now being described in a rapidly growing number of independent studies (Table 1).

Many diseases are rooted in the inappropriate activity or dysfunction of specific genes. RNAi has been heralded as a great therapeutic intervention for gene medicine against a wide range of human diseases. The pace of siRNA based drug development has been rapid, and some companies have already started clinical trials for RNAi therapeutics for age related macular degeneration (AMD) (Takeshita and Ochiya, 2006). AMD is caused by the abnormal growth of blood vessels behind the retina. Treatment strategies include inhibition of vascular endothelial growth factor pathways by siRNA. Such RNAi therapeutics was designed to be administered directly to the sites in the ocular diseases. However, like other forms of gene-based medicines, the clinical utility of systemic therapeutic siRNA will depend on the development of safe and efficacious delivery systems. Strategies for the inhibition of cellular proliferation by systemic treatment of tumors (in animals) with siRNA are mushrooming and are becoming suitable for evaluation of systemic delivery of siRNA as a means for personalized cancer treatment.

Evolution in understanding of RNA function

We have come long way in understanding the Ribonu-

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Table 1. Human cell lines in which siRNA triggers silencing

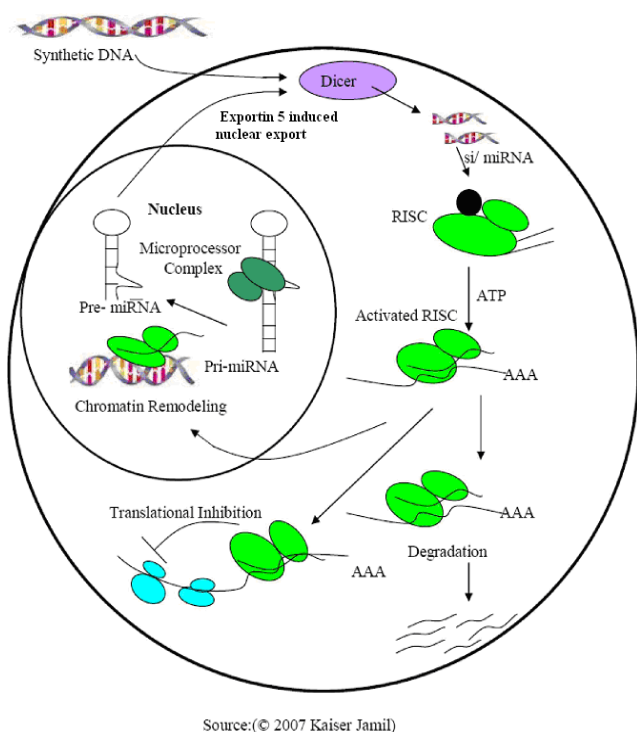
Cell line	Tissue origin	Reference
A-431	human epidermoid carcinoma	Elbashir <i>et al.</i> (2002)
A549	human lung carcinoma	Bitko and Barik (2001)
BV173	human B-precursor leukemia	Tuschl and Borkhardt (2002)
C-33A	human papillomavirus-negative cervical carcinoma	Sui <i>et al.</i> (2002)
CA46	human Burkitt's lymphoma	Tuschl and Borkhardt (2002)
Caco2	human colon epithelial cells	Moskalenko <i>et al.</i> (2002)
CHO	Chinese hamster ovary	Elbashir <i>et al.</i> (2002)
H1299	human non small cell lung carcinoma	Sui <i>et al.</i> (2002)
HaCaT	human keratinocyte cell	Holen <i>et al.</i> (2002)
HEK 293	human embryonic kidney	Elbashir <i>et al.</i> (2001)
HeLa	human papillomavirus-positive cervical cervical carcinoma	Elbashir <i>et al.</i> (2001)
Hep3B	human hepatocellular carcinoma	Bakker <i>et al.</i> (2002)
HUVEC	human umbilical vein endothelial cells	Ancellin <i>et al.</i> (2001)
IMR-90	human diploid fibroblast	Paddison <i>et al.</i> (2002)
K562	human chronic myelogenous leukemia blast crisis	Tuschl and Borkhardt (2002)
Karpas 299	human T-cell lymphoma	Tuschl and Borkhardt (2002)
MCF-7	human breast cancer	Hirai and Wang (2002)
MDA-MB-468	human breast cancer	Hirai and Wang (2002)
MV-411	human acute monocytic leukemia	Tuschl and Borkhardt (2002)
SD1	human acute lymphoblastic leukemia	Tuschl and Borkhardt (2002)
SKBR3	human breast cancer	Elbashir <i>et al.</i> (2002)
U2OS	human osteogenic sarcoma cell	Martins <i>et al.</i> (2002)
EcR293	human embryonic kidney	Nham Tran <i>et al.</i> (2003)
MDA MB 231	human breast cancer	Nham Tran <i>et al.</i> (2003)
SGC-7901	human gastric cancer	Wen-Hua Xu <i>et al.</i> (2007)
MiaPaCa-2	human pancreatic cancer	Wei Wang <i>et al.</i> (2005)
H1299	human non-small cell lung carcinoma cell	Sham S. Kakar (2006)

leic acid family (See table 2) and their role in cell regulation. Recent studies on the activities of RNA in the cell have revolutionized our understanding of the many roles played by this molecule. The role of RNA in protein synthesis had been suspected since 1939, based on experiments carried out by Torbjorn Caspersson, Jean Brachet and Jack Schultz. Hubert Chantrenne elucidated the messenger role played by RNA in the synthesis of proteins in ribosome. Robert W. Holley found the

sequence of the 77 nucleotides of yeast RNA in 1964, winning the 1968 Nobel Prize for Medicine. In 1976, Walter Fiers and his team at the University of Ghent determined the complete nucleotide sequence of bacteriophage MS2-RNA (RNA world website-www.imb-jena.de/RNA.html). An "RNA world" is believed to have existed before DNA took over as the key genetic material and RNA was relegated to the role of a messenger. The dramatic new knowledge of gene regulation by RNAi

Table 2. RNA world listing the types of RNA species as described by the respective authors.

RNA types	Author	Year
siRNA	Tuschl et al.	1999
RNAi	Fire et al	1998
miRNA	Ambros et al	1993
RNAi	Fire et al	1998
tRNA	Holley et al	1964
mRNA	Jacob and Monod	1961
rRNA	Masayasu and Nomura	1960s



Source: (© 2007 Kaiser Jamil)

Figure 1. RNA interference: Processing and Activity: Depicts the formation of long primary micro RNA (pri-miRNA) in the nucleus which is processed by microprocessor complex [dark green] (Drosha- an RNase III enzyme and Pasha- double stranded RNA binding protein) into pre-microRNA(70 nucleotide stem loop structure) and transported to cytoplasm where Dicer [violet], an RNase II enzyme cleaves it to 20 – 21 nucleotide mature micro RNA that integrates into the RNA Inducing Silencing Complex (RISC)[light green]; thus, shares the same cellular machinery downstream of their initial processing with siRNA.

(siRNA and miRNA) has further widened our perspective of RNA's active role. The implications of the discovery of RNAi to the evolutionary process could, in fact, be revelatory. siRNAs were originally identified as intermediates in the RNAi pathway after induction by exogenous dsRNA; however, endogenous sources of siRNAs have now been recognized. Many of these endogenous siRNAs are derived from repetitive sequences within the genome, hence the term

repeat-associated siRNAs, or rasiRNAs. MiRNAs were discovered through their critical roles in development and cellular regulation, and represent a large class of evolutionarily conserved RNAs. MiRNAs have always been recognized as being of endogenous origin. However, synthetic precursors and inhibitors of miRNAs are used to understand and exploit the various functions of this important class of small RNA.

Mechanism of RNA interference

RNAi is a conserved biological process among multi-protein synthesis and cleaving specifically targeted mRNA. The manner in which RNAi induced gene silencing functions is primarily dependent on the structure of the initiating RNA and thus RNA homology has been cellular organisms in, as diverse as plants, worms, yeast and humans, in which dsRNA suppresses the expression of target genes by triggering specific degradation of the complementary mRNA sequences (Fire et al., 1998). RNAi is a post transcriptional process that can effect gene silencing through chromatin remodeling, blocking exploited in several ways. Double stranded RNA that is homologous to the gene being silenced initiates the classical RNAi pathway.

It is now clear that higher eukaryotes contain a large number of genes that encode small RNAs referred to as micro RNAs (miRNAs) (Bartel et al., 2004). These miRNAs generally have only incomplete sequence homology to their targets, often recognizing sequences in the untranslated 3' end of a gene, and usually work by blocking the translation of mRNA into protein rather than destroying the mRNA transcript. The naturally occurring miRNAs are synthesized in the nucleus in large precursor forms (Pri-miRNA), which are processed within the nucleus by Drosha, and RNA III enzyme, into pre-miRNA (60 - 80 nucleotides long). Subsequently this is transported from nucleus to the cytoplasm by Exporting 5 and processed by Dicer, which generates duplexes of approximately 21 – 23 nucleotides with 3' overhangs of matured miRNA. These fragments activate a multisubunit ribonucleoprotein complex called RISC (RNA inducing silencing complex). RISC incorporates the antisense strand of the unwound miRNA and defines the target region of mRNA via a complementary sequence to promote its specific cleavage. These endogenous miRNA appear to regulate processes like proliferation, apoptosis and differentiation (Ambros et al., 2004). Dicer cleaves double stranded molecules, including those derived from vector based shRNA. The siRNA derived from vector based shRNA and synthetic siRNA are also incorporated into the RISC in a manner similar to naturally occurring miRNAs and are capable of inducing sequence specific and effective silencing of genes by mimicking the pathway. The silencing by siRNA is highly efficient, presumably because the guide strand RNA is protected from degradation by RISC and can repeat cleavage of many

mRNA molecules (Figure 1).

RNAi interventions in various targets

Different experimental approaches have been used to compare tumor cells to normal cells. Single base pair mutations that alter the function of tumor suppressor genes and oncogenes occur frequently during oncogenesis. The guardian of the genome, p53, is inactivated by point mutations in more than 45 - 60% of human colorectal cancers. Synthetic siRNAs are highly sequence specific reagents and discriminate between single mismatched target RNA sequences, and may represent a new avenue for gene based medicines. Martines et al. (2002) demonstrated that a single base difference in siRNA discriminates between mutant and wild type p53 in cells expressing both forms, resulting in the restoration of wild type protein function (Martines et al., 2002). Therefore, it is suggested that siRNAs may be used to suppress expression of point mutated genes and provide the basis for selective and personalized cancer therapy.

The product of bcl - 2 genes are involved in regulation of apoptosis and proliferation and are associated with progression in several malignancies. It is reported that some researchers use mRNA - cDNA interference for silencing bcl - 2 expression in human prostate cancer cell line, LNCap (Lin et al., 2001). c-Myc gene has a pivotal function in the development of breast cancer. It has been shown that decreasing the c - Myc protein level in MCF - 7 cells by RNAi could significantly inhibit tumor growth both *In vitro* and *in vivo* (Yi-hua Wang et al., 2005). Thereby, RNAi based treatment of breast cancer by targeting overexpression of oncogene such as c-Myc, might be a potential therapeutic target for human breast cancer treatment.

One of the major limitations of the current chemotherapy is the bone marrow toxicity associated with these drugs. However, it is well recognized that subpopulations of tumor cells are resistant to particular chemotherapeutic agents and continue to grow in a selective manner in the presence of such drugs. These cells contain specific genes which render them resistant to particular compounds. One such gene is the multiple drug resistance (MDR1) gene which confers resistance to vinca alkaloids (vinblastin, vincristine), anthracyclines (adriamycin, daunorubicin) etoposide and paclitaxel. For reversal of MDR1 gene dependent multidrug resistance, two siRNA constructs were designed to inhibit MDR1 expression by RNAi (Nieth et al., 2003). Researched data indicate that this approach will lead to a reversal of tumors with a P-glycoprotein dependent MDR phenotype back to drug sensitive ones. Using RNAi system, it has been shown that inducible knockdown of endogenous CXC chemokine receptor - 4 (CXCR4) gene expression in breast cancer cells resulted in significant inhibition of breast cancer cell migration *in vitro* (Yangchao chen et al., 2003).

VEGF is one of the archetypal angiogenic growth factor

and has received considerable attention. Inhibition of VEGF activity or disabling the function of its receptors has been shown to inhibit both tumor growth and metastasis in a variety of animal tumor models (Haper et al., 2005; Jocelyn H et al -2002). Given the different isoforms and their various functions, the development of this RNAi technology and its ability to target specific VEGFs should facilitate both a greater understanding of this field and also the development of improved therapeutics.

Ligand targeted, sterically stabilized nanoparticles have been adapted for siRNA. Self assembling nanoparticles with siRNA have been constructed with polyethyleneimine (PEI) that is PEGylated with an Arg-Gly-Asp peptide ligand attached at the distal end of the polyethylene glycol (PEG) as a means to target tumor neovasculature expressing integrins and are used to deliver siRNA inhibiting VEGF-2 thereby resulting in tumor angiogenesis (Reymond et al., 2004).

Telomerase is another attractive molecular target toward which to direct cancer therapeutic agents because telomerase activity is present in most malignant cells but undetectable in most normal somatic cells. Kosciulek et al., 2003, evaluated the ability of siRNA to inhibit telomerase activity in human cancer cells. In their research, human cancer cell lines were transfected with 21 nucleotide dsRNA homologous to either the catalytic subunit of telomerase (human telomerase reverse transcriptase) or its template RNA (human telomeric RNA). Both types of agents reduced telomerase activity in variety of human cancer cell lines representing both carcinomas and sarcomas and inhibition was dose dependent. Telomerase inhibition by siRNA is notable because telomerase is regarded as restricted to the nucleus, whereas, RNAi is commonly regarded as restricted to the cytoplasm.

As siRNA effects are extremely specific, initial *in vitro* studies have demonstrated effective silencing of a wide variety of mutated oncogenes such as K-Ras (Brummelkamp, 2002), mutated p53 (Martinez, 2002) Her2/neu (Chouldhury, 2004), and bcr-abl (Scherr, 2003), although RNAi approach has been successful on a relatively small scale to investigate cell death signaling by TRAIL (tumor necrosis factor related apoptosis inducing ligand), an agent that might have therapeutic potential against various cancers (Aza-Blance et al., 2003, Terwari M et al., 2003). In addition, the approach was used to identify familial cylindromatosis tumor suppressor gene (CYLD) as deubiquitinating enzyme in the nuclear factor- κ B pathway (Brummelkamp, 2003). It has been recently used successfully to identify several novel components of the p53 tumor suppressor gene signaling pathways (Berns et al., 2004).

It has been identified that a single siRNA is capable of targeting both the major oncogenes of human papilloma virus. And it has been shown that the re-establishment of dormant tumor suppressor pathways via RNAi enhances the antitumor activity of cisplatin (Lisa NP et al 2005). The data suggest that a combination therapy involving cisplatin and siRNA could be advantageous in promoting

Homo sapiens Vimentin (VIM), mRNA, NM_003380

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1  GTCCCCGCGC CAGAGACGCA GCCGCGCTCC CACCACCCAC ACCCACC GCG CCCTCGTTCC
61  CCTTCTTCTCC GGGAGCCAGT CCGCGCCACC GCCGCCGCC AGGCCATCGC CACCCCTCCGC
121 AGCCATGTC ACCAGGTCCG TGTCCTCGTC CTCTACCGC AGGATGTTCC GCGGCCCGGG
181 CACCGCGAGC CGGCCGAGCT CCAGCCGAG CTACGTGACT ACGTCCACCC GCACCTACAG
241 CCTGGGCAGC GCGCTGCGCC CCAGCACCAG CCGCAGCCTC TACGCCTCGT CCCC GGGCGG
301 CGTGATATGCC ACGCGCTCCT CTGCCGTGCG CCTGCGGAGC AGCGTGCCCG GGGTGCGGCT
361 CCTGCAGGAC TCGGTGGACT TCTCGCTGGC CGACGCCATC AACACCGAGT TCAAGAACAC
421 CCGCACCAAC GAGAAGGTGG AGCTGCAGGA GCTGAATGAC CGCTTCGCCA ACTACATCGA
481 CAAGGTGCGC TTCCTGGAGC AGCAGAATAA GATCCTGCTG GCCGAGCTCG AGCAGCTCAA
541 GGGCCAAGGC AAGTCGCGCC TGGGGGACCT CTACGAGGAG GAGATGCGGG AGCTGCGCCG
601 GCAGGTGGAC CAGCTAACCA ACGACAAAGC CCGCGTCGAG GTGGAGCGCG ACAACCTGGC

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AA (N19) TT

Vimentin cDNA: 5' **AACTACATCGACAAGGTGCGC TT**

| | | | | | | | | | | | | | | |

Sense siRNA: 5' **CUACAUCGACAAGGUGCGC-dTdT**

| | | | | | | | | | | | | | | |

Anti sense siRNA: 3' **dTdT-GAUGUAGCUGUUCCACGCG 5'**

Figure 2. Example of RNAi target selection.

better prognostic outcomes for patients and reducing the severe, toxic side effects associated with cisplatin therapy. The challenge now is to turn these findings into meaningful clinical applications.

RNAi was also used to target of BCR-ABL mRNA and this approach was compared to that of St1 571 (which is now used in the effective treatment of BCR-ABL positive leukemia) (Druker, 2002; Wilda et al., 2002) mediated cell killing in a cell culture model. The siRNA treatment reduced the expression of BCR-ABL mRNA, followed by a reduction of BCR-ABL oncoprotein leading to apoptosis in leukemic cells (Wilda et al., 2002).

RNAi and single nucleotide polymorphism

The use of single nucleotide-specific siRNA to reduce-expression of mutant, disease-causing alleles holds promise for the treatment of dominantly inherited human diseases caused by point mutations that lead to a gain of function. Moreover, human disease alleles often differ from their wild-type counterparts by SNPs that do not themselves cause the disease phenotype. By targeting the SNP isoform present in the disease allele, single

nucleotide-specific siRNA might be used to reduce selectively expression of the disease-causing allele-without altering expression of the wild-type allele. Our earlier studies on SNPs of various genes like MTHFR, (Haranatha Reddy and Jamil, 2006) GST (M1 and T1) (Haranatha Reddy and Jamil, 2006), CYP3A4 (Suman and Jamil, 2006), DPD, (Kalyan and Jamil, 2007) relate to drug-gene interactions of cancer patients and susceptibility to the disease and their effect on drug-response. Patients with DPD spliced-site mutations were reported to be hypersensitive to certain drugs. These studies have helped us to establish a basic idea on mechanism of drug-gene interaction as well as to understand the how point mutations could affect patient's susceptibility to the disease. Hence, it is suggested that a study on cancer progression and gene silencing technique at posttranscriptional level i.e. RNAi will be more meaningful to elucidate the mechanism of reducing the cancer burden.

Ontogenesis refers to the sequence of events involved in the development of an individual organism from its birth to its death. The development of a complex organ relies on precise temporal and spatial gene expression patterns

during ontogenesis. The unique adult phenotype is a result of a cascade of transcriptional events that finally trigger gene expression in an organ-specific fashion. Gene expression profiles can be assessed for human tumours, but from the pharmacological perspective, there is a problem: the associated treatment histories, if any, are generally complex, fragmentary and difficult to interpret (Uwe Scherf et al., 2000). The use of RNAi to analyze gene function, particularly when coupled with genome-wide analysis of gene expression, has enormous potential for elucidating biological pathways and the interaction between different pathways. Although many small RNAs appear to be extremely specific for their target gene, others have been found to induce off-target effects. As more is learned about small RNAs, improvements will be made to experimental methodologies. We believe that the rules for designing very specific siRNAs will soon be refined, and then siRNAs can be used to their fullest potential.

Steps in RNAi therapeutic development

Selection of target gene(s)

For the therapeutic interventions of siRNA in cancers, although the efficacy of siRNA has to be validated in animal models, evaluation in cultured cancer cells is also required before any *in vivo* applications. The first step is to identify the target gene for RNAi mediated knockdown. These genes could be selected from a variety of key oncogenes, antiapoptotic genes or tumor promoting genes, including growth and angiogenic factors or their receptors. As a matter of course, cancer specific genes that are ideally mutated or translocated could be selected. When target genes are selected the second step would be to optimize the design of the siRNA sequences that would be required. For this purpose a computational algorithm that searches through a DNA or RNA sequence could be used. In this step the computational approach finds the regions in the sequence that meets the required criteria and displays the resulting RNAi candidates ranked in order of their fit to the user supplied rule set. Finally, it appears that specificity can be attained depending on the position and sequence of a given siRNA. After synthesis of two or three different sequences for siRNA target site, the most specific and effective siRNA sequence must be validated by measuring levels of target mRNA or protein *in vitro*.

Designing siRNA

While there are currently no reliable methods to identify the ideal sequence for a siRNA, a number of parameters have been suggested. These include: selection of a target cDNA region 50 - 100 nucleotides downstream of the start codon, selection of a 5'-AA (N19) UU target mRNA sequence where N is any nucleotide, 50% G/C content in the target sequence, avoidance of 5' or 3'

untranslated regions and high G-rich areas, and confirmation of exclusive target-specific sequences (Figure 2) (Elbashir et al., 2002). Rational siRNA design schemes are being developed that are based on an understanding of RNAi biochemistry (Khvorova et al., 2003, Schwarz et al., 2003, Chiu and Rana, 2002, Reynolds et al., 2004, Ui-Tei et al., 2004) and on naturally occurring miRNA function. Presently, custom siRNA synthesis service is available through a number of companies, such as Sigma, Dharmacon, QIAGEN, and Ambion etc. Now that many siRNA companies have developed software for designing specific siRNA, it can be downloaded by researchers and can be used to design effective siRNA sequences more easily. Screening candidate siRNA for homology with available sequence databases can, in principle be predicted to avoid many off-target effects. An off-target effect is the silencing of an unintended target gene.

siRNA delivery systems

A number of different approaches have been used to introduce siRNAs into both cells and whole organisms (Figure 3). Successful approaches include: electroporation (McRobert and McConkey, 2002), soaking in siRNAs (Malhotra et al., 2002), feeding bacteria carrying dsRNA (Caplen et al., 2001), transfection with commercial reagents (Elbashir et al., 2001), and vector-based strategies (Lois et al., 2002; Paddison et al., 2002). Though transfection with commercial reagents is the most widely used RNAi technique at present (Kim, 2003), much focus has been placed on vector-based strategies due to their therapeutic potential. These vector-based strategies involve either DNA or viral vector-mediated RNAi. In the first approach, RNA Polymerase II or III promoters are incorporated into DNA vectors along with siRNA expression cassettes (Brummelkamp et al., 2002a; Paddison et al., 2002; Sui et al., 2002; Xia et al., 2002). These cassettes have included either sense and antisense siRNA strands expressed from tandem promoters or a short hairpin (shRNA) cassette whereby the two siRNA strands are separated by a short spacer (Dykxhoorn et al., 2003). Use of RNA Polymerase II promoters to generate shRNAs has the advantage that they permit easier adaptation of inducible/repressible tissue- or cell-specific siRNA expression (Wall and Shi, 2003).

Plasmid vectors

The ability of RNAi to silence disease associated genes in cell culture and animal models has spurred development of RNAi based reagents for clinical applications to treat diseases including cancer. However, their stability and delivery methods are challenges that must be solved for developing effective RNAi reagents for cancer therapy. More prolonged gene silencing has been

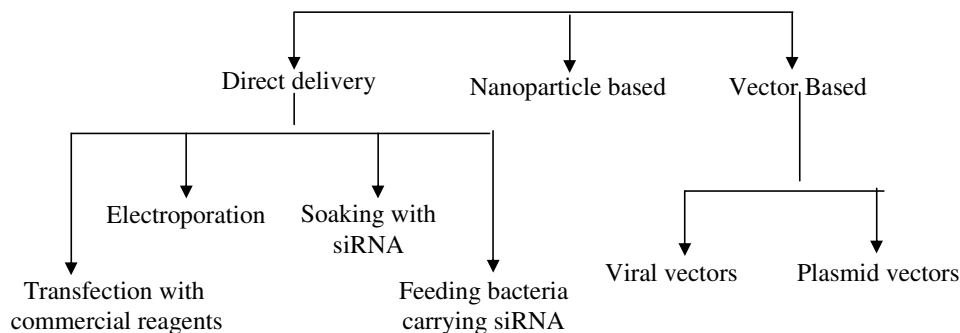


Figure 3. siRNA delivery methods.

achieved by expressing siRNAs from plasmid vectors that contain specific promoters (Lee et al., 2001; Paul et al., 2002; Sui et al., 2002). The most effective plasmid construct is the one that expresses the siRNA as a short hairpin (shRNA) precursor structure of around 70 nucleotides (Paddison et al., 2002). These shRNA precursors expressed from RNA polymerase promoters II or III are processed into a fully functional siRNA by the enzyme called Drosha. Plasmids are very useful for analyzing loss-of-function phenotypes that develop over extended periods of time. The shRNA is predicted to contain a perfectly double-stranded stem of 19 – 29 bp that is identical in sequence to the target mRNA; the 2 strands of the stem are connected by a loop of 6 – 9 bases, which is removed *in vivo* by Dicer to generate effective siRNAs. The constitutive expression of plasmid-based shRNAs by RNA polymerase III (pol III) U6 and H1 snRNA promoters (Paddison et al., 2002; Sui et al., 2002; Paul et al., 2002; Yu et al., 2003; Miyagishi and Taira, 2002; McManus et al., 2002; Brummelkamp et al., 2002) tRNA promoters (Kawasaki and Taira, 2003) and RNA-pol-II-based CMV (cytomegalovirus) promoters (Xia et al., 2002, Shinagawa and Ishii, 2003) have been used successfully to obtain stable and efficient suppression of target genes (Dykxhoorn et al., 2003).

The pol III promoter is widely used for directing the expression of shRNAs, because it is active in all cell types and efficiently directs the synthesis of small, non-coding transcripts (which are structurally close to shRNAs) and bear well defined ends. By contrast, pol II promoters lack these properties and have therefore found limited use in mediating efficient gene silencing. Although the use of plasmid-based shRNA expression has quickly become the preferred method for targeted gene silencing, there are potential limitations. For example, in cells that are difficult to transfect, such as freshly isolated primary cells, neural cells, and stem cells because of inefficient transfection protocols, cell lines that stably express shRNA cannot be established.

The delivery of siRNA by viral transduction is one way to overcome these shortcomings. Since plasmid-based siRNA expression has limitations in cases where trans-

fection efficiency is concerned, which are relatively low, viral vectors have also been developed.

Viral vectors

Adenoviral vectors and various retroviral vectors (e.g. lentivirus-based) have proven to act as effective delivery systems in numerous cell types including non-cycling cells, stem cells, and zygotes (Abbas-Terki et al., 2002; Lois et al., 2002; Rubinson et al., 2003; Shen et al., 2003). In addition, viral vectors have also been developed for more stable and long term expression of shRNAs (Shen et al., 2003). Another natural process to achieve targeted delivery and release of siRNA inside cancer cells is that a specific bacteria infecting virus like phi29 uses a novel type of RNA (pRNA) to package its DNA into its protein shell (Guo S *et al*; 2006). pRNA, which acts as the delivery vehicle can be linked with siRNA, which acts as a therapeutic agent. To this folic acid molecule can be added which forms the targeting agent.

Nanoparticles as delivery vehicles

Nanoparticles, it turns out, are proving to be ideal carriers for siRNA molecules. With nanoparticles, we have the ability to load large number of these molecules into a protected environment, target them to cancer cells, and then have them taken up efficiently by those cells and release RNA molecules into interior of the cells. Not only can nanoparticles be engineered specific to the required RNA of interest, but also these can be designed more specific to the cell target, which means that there is almost no risk of targeting a wrong or non-tumor cell. More so with siRNA, this property is particularly attractive because there is some concern that siRNA delivered to wrong cells may have unintended consequences.

Another example is the successful delivery of a lead anticancer siRNA agent that has been achieved is by making use of a multicomponent polymer system that self-assembles into a rugged nanoparticle when mixed

with siRNA molecules. A biocompatible polymer polyethyleneimine (PEI) is linked with its one end to a second biocompatible polymer-PEG (polyethylene glycol), to which the target agent (usually a three amino acid peptide ligand), arginine-glycine-aspartic acid (RGD) is attached, this can bind to a class of proteins known as integrins, and can be involved in a wide range of biological processes including angiogenesis, tumor cell growth, metastasis and inflammation (Raymond et al; 2004).

Another approach is to achieve delivery of siRNA to the target is to use Nanoparticles coated with folic acid molecules – a well studied nanoparticle targeting agent that binds to a receptor found in abundance on many types of cancers. In addition, the surfaces of these nanoparticles are decorated with sulfur containing chemical group-thiol, the sulfur molecule of which binds to the sulfur of gold nanoparticle. Target cells which contain large amounts of the glutathione, which is also a thiol results in the displacement of the thiols on the nanoparticles, triggering the release of RNA polyloads (Khaled A et al; 2005) and effect the delivery of the siRNA.

Delivery to live animals

The use of RNAi in live mice has been widely reported. The germline transmission of cells — embryonic stem cells in mice (Carmell et al., 2003) and fertilized eggs in rats (Hasuwa et al., 2002) that carry a shRNA transgene has been accomplished, and in some cases mice are phenotypically identical to those that carry a null mutation in the target gene (Kunath et al., 2003). In addition, the *in vivo* transfection of siRNA directly into the organs of postnatal mice inhibits the expression of co-transfected reporter plasmids (McCaffrey et al., 2002; Lewis et al., 2002) of the endogenous pro-apoptotic Fas receptor (Song et al., 2003), and of the *TH* (tyrosine hydroxylase) gene in the brain (Hommel et al., 2003). Although RNAi has been shown to down regulate the expression of various target genes effectively in animal models, it is still uncertain whether RNAi will be translated into effective therapy. More progress is required to improve RNAi-delivery systems and to evaluate the toxicity of exogenous RNA and its effects on endogenous RNAi processes.

MiRNA- a new class of RNA family

MicroRNAs (miRNAs) are a new class of non-protein-coding, endogenous, small RNAs. They are important regulatory molecules in animals and plants. MiRNA regulates gene expression by translational repression, mRNA cleavage, and mRNA decay initiated by miRNA-guided rapid deadenylation. A recently identified class of non-protein coding small RNAs, microRNAs (miRNAs), may provide new insight in cancer research. A recent study demonstrated that more than 50% of miRNA genes are

located in cancer associated genomic regions or at fragile sites (Calin et al., 2004b), suggesting that miRNAs may play a more important role in the pathogenesis of a limited range of human cancers than previously thought.

Different experimental approaches have been used to compare tumor cells to normal cells, which have shown that defects in non-coding RNAs (ncRNAs) might be important. The first example of large ncRNA associated with tumors was the H19 gene (Hao et al., 1993) and there is now a growing list of ncRNA transcripts implicated in different types of cancer. NcRNAs that are implicated in cancer include both small and large RNAs and it has been reported that large ncRNAs can generate one or more microRNAs (miRNA). For example, the ncRNA BIC gene, which has been implicated in growth control and oncogenesis in cancer cells generates two miRNA, one of which miR155, which is over expressed in Burkitt and B cell lymphomas (Metzler et al., 2004; Eis et al., 2005).

MicroRNAs are an abundant class of endogenous small RNA molecules, 20 – 25 nucleotides in length (Ambros, 2001; Bartel, 2004; Ruvkun G et al 2004; Carrington and Ambros, 2003). All pre-miRNAs have hairpin secondary structure (Ambros, 2001; Bartel, 2004; Carrington and Ambros, 2003) with high minimal folding free energy index (MFEI) (Zhang et al., 2006). Some miRNAs are highly conserved from species to species in animals (Pasquinelli et al., 2000) and plants (Floyd and Bowman, 2004; Zhang et al., 2006).

At present, thousands of miRNAs have been predicted in animals, plants and viruses by different approaches (Zhang et al., 2006c), including experimental methods (Lee and Ambros, 2001), computational approaches (Brown and Sanseau, 2005), and expressed sequence tag (EST) and genomic survey sequence (GSS) analysis (Zhang et al., 2005, 2006a). However, only a few of them have been experimentally validated (Griffiths-Jones et al., 2006). Computational analysis indicates that the total number of miRNAs may be more than 1% of the total protein coding genes (Lai et al., 2003; Lim et al., 2003a,b); more than 30% of protein-coding genes may be targeted by miRNAs (Berezikov et al., 2005; Lewis et al., 2005).

MiRNAs and oncology

Differential expression of miRNAs has been reported in several human cancers including chronic lymphocytic leukemia (Calin et al., 2002), colorectal neoplasia (Michael et al., 2003) and Burkitt's lymphoma (Metzler et al., 2004) and human miRNAs are usually located at fragile sites at genomic loci involved in several cancers (Calin et al., 2004).

It has been reported that Prostate Specific Gene 1 (PCGEM1) ncRNA gene is over expressed in prostate tumors compared to normal and primary tumor specimens (Srikantan et al., 2000). In addition, over expression of PCGEM1 in LNCaP and in NIH3T3 cells enhance-

ed cell proliferation and dramatically increased in colony formation suggesting a biological role for PCGEM1 in prostate tumorigenesis (Petrovics et al., 2004). Currently, almost all of the miRNA-related studies on cancers are based on the different expression profile of miRNAs in cancer cells vs. normal cells. Thus, methods used for detecting mRNA expression can also be used in studies on the potential role(s) of miRNAs in cancers. Recent findings suggest that miRNAs play an important role in regulation of gene activation by binding to the mRNAs of target genes and switching the genes on or off, or by fine-tuning the genes, to control key properties like, cell viability, cell cycle, proliferation or apoptosis thereby rewrite the rules of molecular biology (Jamil, 2007). We are beginning to understand that miRNAs may act as oncogenes and/or tumor suppressor genes within the molecular architecture of gene regulatory networks, thereby contributing to the development of cancer. MiRNAs may provide useful diagnostic and prognostic markers for cancer diagnosis and treatment, as well as serving as potential therapeutic targets or tools.

When cells exhibit abnormal growth and loss of apoptosis function, it usually results in cancer formation. Several recent studies indicate that miRNA regulates cell growth and apoptosis (Cheng et al., 2005a; Tanno et al., 2005). For example, miR -15 and miR - 16 induce apoptosis by targeting antiapoptotic gene B cell lymphoma 2 (BCL2) mRNA (Cimmino et al., 2005), which is a key player in many types of human cancers, including leukemias, lymphomas, and carcinomas (Sanchez-Beato et al., 2003), Nairz et al., 2006) demonstrated that mis-expression of miR - 278 in developing eyes causes massive overgrowth in *Drosophila*, partially due to inhibition of apoptosis by miR - 278 (Nairz et al., 2006). This suggests that miRNAs are involved in some cancer formation through regulation of cell growth and apoptosis. The initial evidence for the involvement of miRNAs in cancers came from a molecular study characterizing the 13q14 deletion in human chronic lymphocytic leukemia (CLL) (Calin et al., 2002), the most common form of adult leukemia in the Western world (Dohner et al., 2000). It was observed that two miRNAs, miR - 15a and miR - 16a, are located on chromosome 13q14, a region deleted in more than half of B cell chronic lymphocytic leukemia (B - CLL) cases. Detailed deletion analysis indicated that these two miRNAs are the only two genes within the small (30 kb) common region which are lost in CLL patients, and expression analysis indicated that miR - 15a and miR - 16a were either absent or down-regulated in the majority (68%) of CLL patients (Calin et al., 2002).

Recognition of miRNAs that are differentially expressed between tumor tissues and normal tissues may help to identify those miRNAs that are involved in human cancers and further establish the apparent pathogenic role of miRNAs in cancers (Iorio et al., 2005). Calin et al., (2004a, b) determined genome wide expression profiles of miRNAs in human B cell CLL using a microarray con-

taining 368 probes corresponding to 245 human and mouse miRNA genes. This miRNA microarray analysis further confirmed that miR - 16 and miR - 15 are reduced in human CLL (Calin et al., 2004a). MicroRNA microarray analysis also indicated that miRNA expression patterns were related to the biological and clinical behavior of CLL (Calin et al., 2004a). A recent study indicated that BCL2 is one of the targets of miR-15a and miR-16-1. miR - 15a and miR -16 -1 expression was inversely correlated to BCL2 expression in CLL; both miRNAs negatively regulate BCL2 at the post-transcriptional level (Cimmino et al., 2005). This was also confirmed in a leukemic cell line model (Cimmino et al., 2005), suggesting that miR-15a and miR -16 -1 could be used therapeutically to cure tumors over-expressing BCL2.

Several experiments and clinical analysis suggest that miRNAs may function as a novel class of oncogenes or tumor suppressor genes. Those miRNAs whose expression is increased in tumors may be considered as oncogenes. These oncogene miRNAs, called "oncomirs", usually promote tumor development by negatively inhibiting tumor suppressor genes and/or genes that control cell differentiation or apoptosis. Many miRNA genes have been found that are significantly over-expressed in different cancers. All of them appear to function as oncogenes; however, only a few of them have been well characterized.

In oncogenesis, some miRNAs expression is decreased in cancerous cells. These types of miRNAs are considered tumor suppressor genes. Tumor suppressor miRNAs usually prevent tumor development by negatively inhibiting oncogenes and/or genes that control cell differentiation or apoptosis. Currently, several miRNAs are considered as tumor suppressor genes, for example, miRNA let-7.

siRNA and miRNA

Although small interfering RNA and microRNA were initially discovered in unrelated studies, both types of small RNA are closely related in their biogenesis, assembly into RNA-protein complexes and ability to regulate gene transcripts negatively in diverse eukaryotes (Bartel, 2004; Meister and Tuschl, 2004; Baulcombe, 2004; Mello and Conte, 2004). Dicer, a multi domain enzyme of the RNase III family, generates both siRNAs and miRNAs. Dicer cuts long, double stranded RNA (dsRNA) into siRNAs and chops short precursor miRNAs with imperfect stem-loop structure into miRNAs. The nascent siRNAs and miRNAs are double-stranded duplexes. These duplexes need to be unwound before they can be assembled into an RNA-induced silencing complex (RISC). By comparing the thermodynamic stabilities at the two ends, siRNAs can be divided into two classes: symmetric siRNAs and asymmetric siRNAs. A symmetric siRNA has two equally stable ends; thus, both strands of the siRNA are assembled into the RISC with equivalent

efficiency (Schwarz et al., 2003). By contrast, an asymmetric siRNA has one end that is less stable than the other. Because it is easier to unwind siRNA from the less stable end, one strand of the siRNA is preferentially incorporated into the RISC complex in a process referred to as the 'asymmetric assembly of RISCs' (Schwarz et al., 2003; Khvorova et al., 2003). Intriguingly, most miRNAs are highly asymmetric, ensuring efficient asymmetric assembly of miRISC in cells (Schwarz et al., 2003; Khvorova et al., 2003).

In vitro and *in vivo* biochemical studies have shown that siRISC can function as miRISC to repress translation of the target mRNA; similarly, miRISC can function as siRISC to cleave the target mRNA. This functional interchangeability between siRISC and miRISC argues that siRISCs and miRISCs are highly similar, if not identical [Hutvagner and Zamore, 2002; Tang et al., 2003; Doench et al., 2003; Zeng et al., 2003]. Much evidence suggests, that siRISCs and miRISCs are distinct types of complexes. First, the biogenesis, maturation and subsequent assembly of siRNAs and miRNAs into silencing complexes are different (Bartel, 2004), which can result in RISCs with distinct functions. Second, Argonaute (AGO) proteins, which are principal components of RISCs, are encoded by a multigene family and can be divided into functionally distinct subgroups (Carmell et al., 2002; Hammond et al., 2001; Liu et al., 2004; Song et al., 2004; Meister et al., 2004). These functionally different AGOs endow their corresponding RISCs with distinct functions. Third, the complementarity between the small RNAs and their target mRNAs has been proposed to affect the functional mode of RISCs in terms of the regulation of mRNA stability and translation. Nevertheless, RISCs containing small RNAs that are extensively complementary to their target mRNAs do not always specify efficient cleavage of the targets as was previously predicted: some RISCs direct efficient target cleavage (Hutvagner and Zamore, 2002; Tang et al., 2003; Mallory et al., 2004; Llave et al., 2002), whereas others do not (Meister et al., 2004). Fourth, RISCs vary markedly in size, from the smallest 'coreRISC' of ~160 kDa to the largest 'holo RISC' of 80 Svedberg (80S) (Martinez and Tuschl, 2004; Martinez et al., 2002; Nykanen et al., 2001; Pham et al., 2004). Lastly, siRNA and miRNA programmed RISCs have distinct targeting functions in cells. Many endogenous miRNAs and their RISCs are genetically programmed to regulate gene expression and thus are important for the growth and development of an organism (Rhoades et al., 2002). By contrast, siRNAs are produced from dsRNAs that are often synthesized *in vitro* or *in vivo* from viruses or repetitive sequences introduced by genetic engineering. In addition, dsRNA can be produced from endogenously activated transposons. Thus, siRNAs have been proposed to function in: (i) antiviral defense (despite the fact that viruses develop counter-defense strategies as well (Pfeffer et al., 2004; Ding et al., 2004)), (ii) silencing mRNAs that are overproduced or

translationally aborted, and (iii) guarding the genome from disruption by transposons (Mello and Conte, 2004; Hannon, 2002; Tabara et al., 1999).

Interfacing microarray data with RNAi technology

RNAi that has progressed in the past few years from an obscure act on the international research stage to a major player, enjoying the spotlight as scientists race to study the previously unknown genomic regulatory molecules involved in this process. Even though genomic technologies are advanced, scientists continue to build upon them to make them even better. Two cutting-edge technologies in modern biology, microarrays and RNAi are in the lead to revolutionize biology and medicine. RNAi, a powerful new approach for achieving targeted gene silencing using double-stranded RNA as the triggering agent, offers remarkable specificity, scalability, potency, and reproducibility. On the other side, DNA microarrays provide a snapshot of gene expression of all the genes in a cell.

A microarray works by exploiting the ability of a given mRNA molecule to bind specifically to, or hybridize to, the DNA template from which it originated. By using an array containing many DNA samples, researchers can determine, in a single experiment, the expression levels of hundreds or thousands of genes within a cell by measuring the amount of RNA bound to each site on the array. With the aid of a computer and computational methods the amount of RNA bound to the spots on the microarray is precisely measured, generating a profile of gene expression in the cell. Using bioinformatics tools the data is interpreted for meaningful information. The process of microarray based microRNA profiling, initially developed by Liu et al. (2004) involves four main steps: target labeling, DNA-DNA hybridization, staining and signal detection.

Genomic sequencing and gene expression profiling have generated and continue to generate extensive information on the structure, sequence variation, and expression levels of human genes. However, an emerging challenge is the need to translate genomic data into meaningful insights for drug discovery. Identification of novel targets for the treatment of human diseases requires functional characterization of the products encoded by the tens of thousands of genes that have been identified by the Human Genome Project and EST (expressed sequence tag) sequencing efforts (Venter et al., 2001; Ewing and Green, 2000). Recent strides in genomics have promised to greatly facilitate the identification of targets for human diseases by making it feasible, in concept, to functionally screen all human gene products for those with roles in cellular processes associated with diseases.

One of the key advantages of the RNAi microarray for genome-scale screening is that it permits miniaturization far beyond what is physically possible in present well-

based systems. At present, most cell-culture-based assays of gene function make use of 6-, 12-, 24-, or 96-well plate formats. Although RNAi experiments can be conducted in a 384 -well plate format, a "well-less" microarray platform will be significantly more effective. Even though the size and density of the spots are limited by the need to have at least several dozens if not hundreds of cells on each spot, it should be feasible to array and analyze as many as 5000 to 10,000 individual siRNAs spots per slide.

Another advantage of the array-based miniaturization is the reduction in reagent costs. In particular, chemically synthesized siRNAs are expensive and are produced in relatively small quantities. The RNAi microarray format will also reduce the cost of the phenotypic assays because only a single microscope slide needs to be subjected to analysis. The well-less nature of the platform also allows for improving the level of uniformity in the experimental culturing conditions and assay conditions, because all processes take place on the same surface.

Conclusion

An economic and novel way of ablating gene function holds out massive hope for improving our ability to untangle the complex regulatory pathways that control cellular behavior in health and disease. RNAi allows analysis of gene function at the cellular level and provides an effective design and delivery system. *In vivo*, siRNA technology has aroused entrepreneurship, as documented by Fortune magazine (2003) predicting siRNA as biotechnology's "next billion dollar breakthrough".

There is no golden principle to predict the length of sustaining time of RNAi inhibition, because each protein has its own turnover time. Normally, the transient effect of siRNA inhibition lasts less than one week. Multiple transfections may be needed to extend its effect for a longer time. Otherwise, vector based RNAi, either by polymerase II or III is required to achieve permanent knock down of target gene expression. The length of the target sequence could range from 21 to 23 nucleotide, which guarantees sequence specificity, without generating interferon response and mimic the product length of Dicer.

RNAi has become the method of choice for specific gene silencing in cell culture. However, the success of this approach depends on the effectiveness of siRNA, delivery and design of the siRNA sequence. Different biochemical, pharmacological and histological assays have been used to determine the effects of siRNA inhibition of target genes and analyze the phenotypic changes in cells. Currently, the use of siRNA to characterize gene function and potential therapeutic drug targets is a highly promising application of this technology. Selected gene knockdown can be used to identify critical gene(s) and pathways that can be targeted by siRNA.

Thus, RNAi can be specifically designed to target anti-apoptotic genes which are over expressed in cancer cells and might explain the resistance of some tumors to drug induced apoptosis. At the same time, RNAi designed to target cell-proliferating protein encoding genes will help in curbing the rate of cell division and promote a balance between the rate of proliferation and cell death. Similarly, specific RNAi can be designed for other genes which are directly involved in cancer etiology such as RNAi for non-coding RNAs, cellular oncogenes (Ras gene family, bcl-2), mutated tumor suppressor gene, etc.

As miRNAs can function as oncogenes or tumor suppressors, it might be possible to regulate miRNA expression and/or inject miRNAs to regulate cancer formation, similar to the use of antisense mRNAs and RNAi which are widely used as tools for studying gene functions and in some case of gene therapy. Artificial miRNAs could be synthesized to down-regulate oncogenes and prevent the formation of cancer.

RNAi methods for both *in vitro* and *in vivo* target validation are being developed and are being utilized by pharmaceutical companies for prioritizing drug target candidates. siRNAs can be readily synthesized with low production costs compared to protein or antibody therapies. In addition, siRNAs have favorable pharmacokinetic properties and can be delivered into a wide range of organs (Braasch et al., 2004). Several groups have been investigating the use of modified nucleotides to improve the clinical properties of these reagents. For example, by conjugating of the 3'-end of the sense strand of siRNA with cholesterol through a pyrrolidine linker, this was reported to markedly improve the pharmacological properties of siRNA molecules (Soutschek et al., 2004). The Cholesterol- conjugated siRNA are more resistant to nuclease degradation, exhibit increased stability in blood by increasing binding to human serum albumin and show increased uptake into liver. Another study has shown that boranophosphate modified siRNAs were 10 times more nuclease resistant than unmodified siRNAs (Hall et al., 2004). In addition, boranophosphate siRNAs were more potent than unmodified siRNAs and appeared to act through the standard RNAi pathway. Topical gels have also been used to deliver siRNAs to cells and could open the way for dermatological applications, as well as treatment for cervical cancer (Jiang et al., 2004). siRNAs are also rapidly catching up with ODNs (oligodeoxiribonucleotides) and ribosome for development as therapeutics owing to the establishment of siRNA-based biotechnology companies that focus on the development of clinical programs (Howard, K. 2003). Several proof-of-principle experiments have demonstrated the therapeutic potential of siRNAs: siRNAs protected mice from fulminant hepatitis (Zender et al., 2003; Song et al., 2003), viral infection (McCaffrey et al., 2003; Song et al., 2003), sepsis (Sorensen et al., 2003), tumour growth (Li K et al 2003; Filleur et al., 2003; Yang et al., 2003; Verma, 2003; Yoshinouchi et al., 2003) and ocular neovascularization

causing macular degeneration (Reichert et al 2003).

In an astonishingly short time, RNAi has gone from discovery to human clinical trials. Acuity Pharmaceuticals in Philadelphia has completed safety tests on the first ever RNAi treatment for a health problem. Fifteen people have undergone injections into the eye to treat macular degeneration, the leading cause of blindness in the elderly. Other companies are also looking to harness RNAi to treat Alzheimer's disease, motor neuron disease, Parkinson's disease, hepatitis and even HIV. Benitec Inc, a company in Australia is working on a way of delivering a 'multiple warhead' that cripples several genes at once, which could aid the treatment of type 2 diabetes. In April 2007, Alnylam pharmaceuticals had developed a program for the treatment of liver cancers and potentially other solid tumors. Their RNAi therapeutic, ALN-VSP01, targets two separate genes involved in the growth and development of tumors: vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP). In addition, RNAi provides a new potential therapeutic tool applicable to a wide array of disease targets. However, many challenges exist in translating RNAi's potential anti-cancer activity into clinical benefit.

Cancers may provide distinctive advantages as proof of concept models for both the target validation and the anti-cancer therapeutic utility of RNAi. As current therapy is inadequate for many cancers, discoveries flowing from RNAi-based methods could make significant contributions.

ACKNOWLEDGEMENTS

We are grateful to the Chairman, and Scientific Advisers of Indo-American Cancer Institute and Research Centre for their encouragement and support for this work and to NEERI- Director for his support. We also greatly appreciate our reviewers for their thorough analysis and suggestions for improving the manuscript.

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