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Design and Delivery of Small RNAs for RNAi Technology

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ABSTRACT

The vast efforts of biologists from diverse fields have contributed a wealth of information on the various aspects of small RNA-guided gene silencing mechanisms. The studies quickly begot the RNAi technology as a powerful tool to modulate gene expression in animals and plants. This has been used for affecting specific parts of complex and interactive biological pathways across species to understand gene functions as well as develop strategies for therapeutic applications and crop improvements. In this article, we mainly review the importance of design and delivery of small RNAs in the silencing technology with a focus on the principles and improvements in the field. A section briefly describing the applications of RNAi in medicine, veterinary science and agriculture follows the main section.

Keywords: agriculture, applications, medicine, miRNA, siRNA, veterinary science

Abbreviations: **AAV**, *Adeno-associated virus*; **AGO**, Argonaute protein; **AMD**, age-related macular degeneration; **amiRNA**, "synthetic" or "artificial" miRNA; **antagomiR**, antagonistic miRNA; **antimiR**, antisense miRNA; **ApoB**, apolipoprotein B; **APP**, amyloid precursor protein; **bm-RNAi**, bacteria-mediated RNAi; **CyD1**, Cylin D1; **DCL**, Dicer-like; **DCR-2**, Dicer-2; **dox**, doxocycline; **dsRNA**, double-stranded RNA; **FIV**, *Feline immunodeficiency virus*; **FMD**, foot and mouth disease; **FMDV**, *Foot and mouth disease virus*; **GFP**, green fluorescence protein; **HDL**, high-density lipoprotein; **HepG2**, HeLa human hepatocellular liver carcinoma cell line; **HO-1**, Haemeoxygenase-1; **hpRNA**, hairpin RNA; **ihpRNA**, intron-spliced hpRNA; **LDL**, low-density lipoprotein; **lhRNA**, long hairpin RNA; **miRNA**, microRNA; **miRNA***, strand complementary to the miRNA in the duplex; **miR-Vec**, retroviral vector for miRNA expression; **nat-si**, natural antisense siRNA; **PEI**, polyethyleneimine; **piRNA**, picoRNA; **Pol**, Polymerase; **pre-miRNA**, miRNA precursor; **PRSV**, *Papaya ring spot virus*; **PVY**, *Potato virus Y*; **rAAV**, recombinant AAV; **rAD**, recombinant adenovirus; **ra-si**, repeat associated siRNA; **RISC**, RNA-induced silencing complex; **RLC**, RISC-loading complex; **RNAi**, RNA interference; **RSV**, respiratory syncytial virus; **SCID**, severe combined immunodeficiency; **SELEX**, systematic evolution of ligands by exponential enrichment; **shRNA**, short hairpin RNA; **siRNA**, small interfering RNA; **sRNA**, small RNA; **SVISS**, satellite virus-induced silencing system; **Ta-si**, transacting siRNA; **T-DNA**, transfer DNA; **Tet**, tetracycline; **tkRNAi**, *trans*-kingdom RNA interference; **TLR**, Toll-like receptor; **TMV**, *Tobacco mosaic virus*; **TRIP**, *trans*-kingdom RNA interference plasmid; **U-RNAs**, 21-nucleotide-long RNAs that have a 5′U; **UTR**, untranslated region; **VEGF**, vascular endothelial growth factor; **VIGS**, virus-induced gene silencing; **vi-si**, viral siRNA

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INTRODUCTION

The phenomena of RNAi (RNA interference) are the result of a serendipitous discovery of 1990 related to loss in petunia flower pigmentation (Jorgensen *et al.* 1999). Since then, it outgrew as a rapidly developing field in biology and has evolved as a novel strategy of gene silencing with therapeutic potential. In this process, small RNAs (sRNAs) with chemically defined ends and sizes are produced that associate with a set of proteins to form effector complexes (RISC) of gene silencing. The effector complex is targeted to the RNA transcript(s) complementary to the sRNA and is silenced by either cleavage or translation repression. The sRNAs include the, siRNAs (small interfering RNAs), miRNAs (microRNAs), piRNAs and 21-mer U-RNAs. Even among the siRNAs, there are various sub-types, namely, transacting siRNAs (ta-si), repeat associated siRNAs (ra-si), viral siRNAs (vi-si), natural antisense siRNAs (nat-si), etc. More than 4000 reviews are available detailing the nature, classification, biogenesis and function of these sRNAs (Agrawal *et al.* 2003; Sanan-Mishra and Mukherjee 2007). The biogenesis and function of all types of sRNAs are not known to full biochemical details, though recently several reports have appeared on the structural features of miRNA biogenesis and new models have also been proposed (Du *et al.* 2008; Schwab and Voinnet 2009; Mateos *et al.* 2010). Though the effector complexes of each type of sRNAs are different from each other, the principal component of each complex is the ARGONAUTE (AGO) protein that is present in various isomeric forms depending on the organism

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Review

Table 1 Selected RNAi antivirals in clinical or preclinical trials and agricultural practices.

Company (Location, Country)	Approach	Target Virus	Clinical trials, Status/ Agri-Practice
Alnylam (MA, USA)	siRNA	Influenza, RSV	Phase-II
Nucleonics (PA, USA)	siRNA	HBV	Phase-I
Protiva (BC, Canada)	siRNA	Ebola, Marburg	Phase-I
Regulus (CA, USA)	miRNA	HCV	Preclinical
Santaris (Denmark)	miRNA	HCV	Phase-I
Tacere Therapeutic (CA, USA)	shRNA	HCV	Preclinical
Sirna/Merck Therapeutics	siRNA	HCV	Preclinical
Pharmacia & Upjohn Co., Hawaii USA	PDR/siRNA	PRSV	Commercial production of PRSV-resistant papaya for last 6-8 yrs.

concerned. It is not well known how each AGO isomer selects its own sRNA to form the cognate effector complexes. A lot of scientific efforts are emerging to address the problem, with a recent view that the selection is done on the basis of the first nucleotide residue of the sRNA (Mi *et al.* 2008).

A wealth of knowledge has accumulated regarding the mechanisms and machineries of RNAi. However, a lot of data are also emerging to relate the system specific mechanistic differences in the RNAi operations. A host of intensive efforts are directed in solving the mechanistic problem of translational repression in various systems, especially plants (Brodersen *et al.* 2008; Auer and Frederick 2009; Sashital and Doudna 2010). Thus many such examples could be cited to establish that the science of RNAi is leapfrogging ahead with astounding discoveries. However, RNAi technology is also not lagging far behind.

The basis of RNAi technology could be traced back to the findings of Tuschl's group (Meister and Tuschl 2004) in which exogenously administered siRNAs in mammalian cells can down-regulate the target transcripts in a specific manner. This discovery raised the hope that mRNAs of any kind can be controlled at will in organisms and cells, when suitable sRNAs are delivered in a regulated spatio-temporal manner. Two kinds of sRNAs, i.e. siRNAs and miRNAs have thus been exploited so far to treat metabolic disorders, fight viral diseases and produce designer plants with desired attributes. Many commercial companies across the globe have invested huge sums on RNAi, believing that this technology will bring new products to their pipeline. Market analysist feel that the worldwide RNAi market is estimated to be $328 million by 2010. About 30 pharmaceutical and biotechnology groups are already testing the RNAi drugs in clinical/preclinical trials. The potential of the efforts could be gauged from **Table 1** where a tiny fraction of the total RNAi-technology efforts, devoted for developing only the antiviral RNAi products, is displayed.

However, the design and delivery of sRNAs seem to pose the major hurdles to the RNAi technology. These issues are being painstakingly handled by a vast number of investigators in the field and the developments on this front are discussed in this article. We did not intend to gather all the related information together, but we attempted to divide the problems in several classes and exemplified only a few representative ones of each class. Besides, we have put forward in brief some successful stories of RNAi applications in fields of medicine, veterinary science and agriculture.

TECHNOLOGY

RNAi technology revolves around designing specific siRNAs or miRNAs as well as regulating their spatio-temporal delivery to recipient cells. Though several important lessons concerning the design and delivery have been learnt from the literature reports, many of these aspects still remain enigmatic. This is especially true for the delivery related problems, the solutions of which are still at the stage of infancy. Though many tricks have been deployed to reach the small molecules at the appropriate destinations, a host of obstacles are frowning large even at the laboratory benches. A lot of RNAi-technologies have already spawned based on the available design-principles of the various sRNAs but their full potential will be realized only when the detailed principles of intracellular biogenesis, post-transcriptional modification, RISC-incorporation, degradation, off-target effects, interferon responses, etc. of the sRNAs will be uncovered. Below, we highlight some of the important principles leading to successes and failures of these technologies.

Design

The success of RNAi technology is based on the efficient design and effective delivery of "synthetic" RNAi molecules for functional studies or therapeutic applications. There are several methods for preparing sRNA, such as chemical synthesis, *in vitro* transcription, expression vectors and PCR expression cassettes or artificial miRNAs. Irrespective of the method used, the first step in designing a siRNA is to choose the siRNA target site.

1. siRNA

The early *in-vitro* experiments rapidly established a role of siRNA-mediated knockdowns. For effectively applying the RNAi technology, siRNAs have to be designed carefully to achieve maximum knockdown of the target transcripts with minimum targeting of non-specific transcripts (also called "off-target effects"). The optimal sequence of a siRNA molecule needs to be determined empirically by comparing the gene silencing ability of several candidate siRNA sequences.

A systematic analysis of the silencing efficiency of siRNA duplexes as a function of the length of the siRNAs, the length of the overhang and the sequence in the overhang was made by Tuschl's group using *Drosophila melanogaster* lysates (Elbashir *et al*. 2001b). The most efficient silencing was obtained with siRNA duplexes composed of 21-nt sense and 21-nt antisense strands, paired in a manner to have a 2-nt 3' overhang. The sequence of the two nucleotides of the 3' overhang makes a small contribution to the specificity of target recognition (Elbashir *et al*. 2001b). The mRNA knockdown efficiency has been found to be influenced by thermodynamic stability of siRNA duplexes (Reynolds *et al.* 2004). The "target region" of the mRNA is usually selected from a given cDNA sequence beginning 50-100 nt downstream of the start codon. However, the 5'- and 3'-UTRs can also be targeted in knocking down the genes. At least few independent siRNA duplexes need to be used to knockdown the target gene to control the specificity of the silencing effect. Additional recommendations include blast-search (NCBI database) of the selected siRNA sequences against EST libraries to ensure that only the desired gene is targeted.

Within the duplex, the thermodynamic differences in the base-pairing stabilities of the 5' ends of the two ~21-nt siRNA strands determine which strand is assembled into the RISC. Of the two siRNA strands, the one that is assembled into the RISC is called the guide strand, whereas the other strand, called the passenger strand, is excluded and destroyed (Okamura *et al.* 2004). Studies using *Drosophila* lysates have shown that siRNAs are loaded into the RISC by an ordered pathway (Carthew and Sontheimer 2009; Marques *et al.* 2010). A central step in RISC assembly is formation

of the RISC-loading complex (RLC), which contains siRNA duplex, the double-stranded RNA (dsRNA) binding protein R2D2, Dicer-2 (DCR-2), and additional unidentified proteins. The function of Dicer in loading siRNA into the RISC is distinct from its role in generating siRNA from long dsRNA (Lee *et al.* 2004; Marques *et al.* 2010). The orientation of the DCR-2/R2D2 heterodimer on the siRNA duplex determines which siRNA strand associates with the core RISC protein, AGO2. R2D2 serves both as a protein sensor for siRNA thermodynamic asymmetry and a licensing factor for entry of authentic siRNAs into the RNAi pathway (Lee *et al*. 2004; Tomari *et al.* 2004). Both R2D2 and DCR-2 are required to form RLC and to catalyze siRNA unwinding (Tomari *et al.* 2004) but are not sufficient to unwind siRNA. The 5' phosphate end of the guide strand also makes a substantial contribution with regard to the structural fit of the end within the RISC, especially the AGO2 protein (Ma *et al.* 2005; Wang *et al.* 2008). Recently it was proposed that the first nucleotide at the 5' end governs the sorting of sRNAs into specific AGO complexes. AGO2 demonstrated a preference for recruiting sRNAs with a 5' terminal adenosine (Mi *et al.* 2008).

Several guidelines on siRNA design have been published (Reynolds *et al.* 2004), with those recommended by Dr Thomas Tuschl's laboratory being a common reference (http://www.rockefeller.edu/labheads/tuschl/sirna.html). In order to design a siRNA duplex they search for the 23-nt sequence motif AA(N19)TT (N, any nucleotide) and having approximately 50% G/C-content. If no suitable sequences are found, the search is extended using the motif NA(N21). The sequence of the sense siRNA corresponds to (N19)TT or N21 (position 3-23 of the 23-nt motif) followed by TT at the 3' end. The rationale for this sequence conversion is to generate a symmetric duplex with respect to the sequence composition of the sense and antisense 3' overhangs thereby ensuring that the siRNPs are formed with approximately equal ratios of sense and antisense target RNA-cleaving siRNAs (Elbashir *et al*. 2001a, 2001b). The modification of the overhang of the sense sequence of the siRNA duplex is not expected to affect targeted mRNA recognition, as the antisense siRNA strand guides target recognition.

Several online siRNA design tools are now available on the web from academic institutions or commercial siRNA suppliers (**Table 2**). Some of the most potent siRNA molecules used are only partially in line with the selection criteria based on common siRNA design guidelines and *vice versa*. Huesken *et al*. (2005) used Neural Net Simulator to train algorithms on a data set of 2182 randomly selected siRNAs targeted to 34 mRNA species. The resultant algorithm, BIOPREDsi, reliably predicted activity of about 300 siRNAs (Pearson's coefficient r = 0.66). The prediction of the algorithm based on a complementary 21-nt guide sequence was superior to those trained on a 19-nt sequence. This algorithm can be reliably used to design the genome-wide siRNA library and is being widely exploited by several commercial companies for the siRNA bioinformatics.

The efficacy of RNAi by synthetic siRNAs is additionally hampered by a number of factors like intracellular exonucleolytic degradation, structural properties of target mRNA, association of target with other proteins that may impede RISC binding, off-target effects and non-specific activation of signaling cascades like innate immune response (Paroo and Corey 2004).

Off-target effects pose the greatest threat to the design of effective siRNAs. The siRNAs can mimic miRNAs and pair with the 3′ UTR/seed sequences of hundreds of unintended, non-specific and non-targeted mRNAs to down-regulate their expression levels to the extent of 1.5- to 4-fold (Birmingham *et al.* 2006). The non-specific effects may also arise due to the mode of delivery vehicles like lipid-mediated delivery (Fedorov *et al.* 2005). Hence it is important that redundant siRNAs of different sequences targeting the same mRNA are included in the knockdown assay to ascertain the specificity of the siRNA-mediated effect. As the probability of several siRNAs that target different sequences of the same gene causing the same phenotype through off-target interaction with another gene is very low, the redundant siRNAs can confirm the specificity of the process, although it does not completely eliminate the off-target effects.

Moreover, certain siRNAs harboring GU rich sequences (say, UGUGU) can also stimulate the innate immune response by inducing IFN-α, IL-6, and TNF-α via interactions with TLRs (Judge *et al.* 2005). In such cases, a global

Table 2 Online siRNA design tools.

Name	URL	Provider
siRNA Sequence Finder	http://katahdin.cshl.org:9331/RNAi/html/rnai.html	Cold Spring Harbor Laboratories (Jack Lin's)
EMBOSS siRNA	http://bioweb2.pasteur.fr/docs/EMBOSS/sirna.html	Institute Pasteur
ThermoComposition19	ftp://ftp.ncbi.nlm.nih.gov/pub/shabalin/siRNA/ThermoComposition/	National Institutes of Health.
SDS	http://i.cs.hku.hk/~sirna/software/sirna.php	The University of Hong Kong
siRNArules 1.0	http://sourceforge.net/projects/sirnarules/	University of Oslo
SFOLD	http://sfold.wadsworth.org/sirna.pl	Wadsworth Bioinformatics Center
Gene specific siRNA selector	http://hydra1.wistar.upenn.edu/Projects/siRNA/siRNAindex.html	Wistar Institute
siRNA at Whitehead	http://jura.wi.mit.edu/siRNAext/	Whitehead Institute
TROD: T7 RNAi Oligo Designer	http://www.unige.ch/sciences/biologie/bicel/websoft/RNAi.html	Donzé and Picard 2002
BIOPREDsi		Huesken *et al.* 2005
DEQOR	http://cluster-1.mpi-cbg.de/Deqor/deqor.html	Henschel *et al.* 2004
OptiRNA	http://optirna.unl.edu/	Ladunga 2007
siDirect	http://genomics.jp/sidirect/index.php?type=fc	Naito *et al.* 2004
SIDE	http://side.bioinfo.ochoa.fib.es	Santoyo *et al.* 2005
DSIR	http://biodev.extra.cea.fr/DSIR/DSIR.html	Vert *et al*. 2007
siRNA Target Finder	http://www.ambion.com/techlib/misc/siRNA_finder.html http://www.ambion.com/techlib/misc/psilencer_converter.html	Ambion
siDRM	http://sidrm.biolead.org/	Biolead.org Research Group
OligoWalk	http://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi	Lu and Mathews 2008
si*DESIGN* Center	http://www.dharmacon.com/DesignCenter/DesignCenterPage.aspx	Dharmacon
RNAi Explorer	http://www.genelink.com/sirna/shRNAi.asp	Genelink
siRNA Target Finder	https://www.genscript.com/ssl-bin/app/rnai	GenScript
siRNA Designer	http://imgenex.com/sirna_tool.php	Imgenex Corp
IDT RNAi Design	http://eu.idtdna.com/Scitools/Applications/RNAi/RNAi.aspx	Integrated DNA Technologies
Block-iT RNAi Designer	https://rnaidesigner.invitrogen.com/rnaiexpress/	Invitrogen
siRNA Target Designer	http://www.promega.com/siRNADesigner/program/	Promega
siRNA Design Tool	http://www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx	QIAGEN

down-regulation in protein expression occurs, masking the specificity of the siRNAs. In fact, the siRNA targeting the cure for the age-related macular degeneration (AMD), which has entered in a phase-3 level trial, is now bogged in such immuno-modulatory controversies (Klienman *et al.* 2008). The design of any effective siRNA must take care of such unintended results, but, as all immuno-modulatory RNA motifs are not identified yet, the experimental validation of the function of designed siRNA is always necessary to determine the specificity as well as potency. It has been shown that chemical modification of terminal bases of synthetic siRNA molecules, like substituting the 2′-hydroxyl of uridine with 2′-fluoro, 2′-deoxy, or 2′-O-methyl groups, can make them less immuno stimulatory without compromising on the potency of silencing (Kariko *et al.* 2005; Sioud 2006).

In-vivo, the siRNAs are subjected to intracellular exonucleolytic degradation, especially by ERI-1b 3′-exonuclease, which is reportedly found in a variety of mammals, worms and yeast (*Saccharomyces pombe*). In fact, the *eri*-1 mutants show enhanced and sustained RNAi effects (Gabel and Ruvkin 2008). In plants, the sugar moiety of the 3′ terminal bases of the miRNAs and siRNAs are methylated by HEN1 protein, so the modified endogenous sRNAs are protected against nuclease digestion (Yang *et al.* 2006). However, the naked chemically synthesized siRNAs suffer degradation both intra- and intercellularly when these are administered exogenously. But the endo- and exonucleases, which vitiate the RNAi-related effects, could be tempered down if suitable chemical modifications are introduced within the sRNAs. In this way the *in-vivo* half-lives of these sRNAs have been increased from minutes to hours to days at a stretch (Akhtar and Benter 2007a, 2007b). Several chemical modifications to the backbone base or sugar of the sRNA have been carried out to stabilize the RNAi effects (Bumcrot *et al.* 2006). Some of these modifications like minimal modifications with locked nucleic acids (LNA) also reduce the off-target effects as the modified RNAs hybridize with the target transcripts in a specific and stringent manner (Fluiter *et al.* 2009). While, other modifications like extensive phosphothioate insertions involve loss of potency and enhanced cellular toxicity (Hughes *et al.* 2001; Jackson *et al.* 2006). Additionally appropriate selection of delivery system is crucial to enhance gene silencing activity and reduce even the off-target effects (Akhtar and Benter 2007a, 2007b). The delivery modes might also alter the pharmacokinetics of siRNAs by altering their physicochemical characteristics, like increasing the dimension of individual units of siRNA cluster, which in turn increase retention of siRNAs in mammalian bodies by reducing excretion via the kidneys and thereby prolonging the *in-vivo* half lives. Various modes of cellular and sub-cellular targeting are also reported in literature, the elucidation of which will be dealt with in the subsequent sections (siRNA delivery).

2. miRNA

Reports of direct administration of miRNA duplex in cell lines or *in-vivo* are not found in literature, perhaps due to the fact that its sibling i.e. siRNA duplex is a better regulator of gene knockdown. However, the antisense miRNAs (antimiRs) have been used both *in-vitro* and *in-vivo* to inactivate the endogenous miRNAs and their functions (van Rooij *et al.* 2008). Many commercial companies provide the designed LNA-antimiRs that show effective and sequence-specific inhibition of miRNA with minimal cytotoxicity and enhanced biological stability. In a recent study, the specificity of LNA-antimiR-122 against its target miRNA was demonstrated after systemically injecting the mice and green monkeys (Elmen *et al.* 2008). The uptake of this LNA-antimiR-122 in the cytoplasm of the hepatocytes resulted in stable heteroduplexes between the antimiR and endogenous miRNA-122 and caused the eventual long lasting decrease in total plasma cholesterol without any associated toxicity or histopathological changes. This study elegantly demonstrates the specificity and therapeutic value of the antimiRs (Elmen *et al.* 2008).

AntagomiRs or antagonistic miRNAs represent another class of antisense miRNAs. They have been shown as efficient and specific silencers of endogenous miRNAs in different tissues of various model systems including mice (Mattes *et al.* 2008). The antagomiRs are synthesized starting from a hydroxyprolinol-linked cholesterol solid support and 2'-OMe-phosphoramidites. The plasma cholesterol measurements showed reduced levels in antagomiRs-122 treated mice, demonstrating that the therapeutic strategies could be built around the antagomiRs for silencing of miRNAs in specific diseases (Krutzfeldt *et al.* 2005).

As an alternate strategy, the miR-Vec (Retroviral vectors for miRNA expression) have been designed to express specific miRNAs in the cells transduced with either the miRNA-containing vector or the retroviruses bearing the miRNA gene (Lindemann and Schnittler 2009). About 500 bp fragment spanning a given miRNA genomic region is generally cloned downstream of the CMV promoter in a modified pMSCV-Blasticidin vector. The desired mature miRNAs are efficiently formed and are functional in silencing the appropriate, specific gene in the transduced cells (Voorhoeve *et al.* 2006). Using this expression system, a few miRNAs that are generally lost in cancer cells were allowed to express in carcinoma lines, resulting in the regression in the cancerous fate of the cells. Of the few tested miRNAs, miRNA-126 expression reduced overall tumor growth and proliferation, whereas miRNA-335 inhibited metastatic cell invasion (Tavazoie *et al.* 2008). Expression of miRNA-126 and miRNA-335 is lost in the majority of primary breast tumors and this study revealed these two miRNAs as the suppressor of human breast cancer (Tavazoie *et al.* 2008).

Similarly, an endogenous miRNA precursor (pre-miRNA), expressed from either constitutive or tissue-specific promoter can be used to generate molecules called "synthetic" or "artificial" miRNA (amiRNA) to direct gene silencing in either plants or animals (Ossowski *et al*. 2008). The pre-miRNA preferentially produces a single miRNA–miRNA* duplex that is perfectly complementary to the target sequence just like siRNAs (Silva *et al.* 2005). The amiRNA of desired sequence is obtained by specifically changing both the sequences of a miRNA and miRNA* of the precursors, without altering the structural features such as mismatches or bulges, because these are considered to be important for guiding correct DCL1-mediated processing. The amiRNAs were first generated and used in human cell lines (Zeng *et al.* 2002), and later in *Arabidopsis* (Parizotto *et al.* 2004), where they were shown to effectively interfere with reporter gene expression. Subsequently, it was demonstrated that not only reporter genes but endogenous genes can also be targeted with amiRNAs, and that these seem to work with similar efficiency in other plant species (Alvarez *et al.* 2006; Schwab *et al.* 2006). Genome-wide expression analyses showed that plant amiRNAs exhibit high specificity similar to endogenous miRNAs with almost no non-autonomous effects (Schwab *et al.* 2006). These sequences can thus be easily optimized to silence one or several target transcripts without affecting the expression of other transcripts.

Weigel's group has developed the WMD (Web MicroRNA Designer) platform, which automates amiRNA design. It is designed to optimize for sRNAs having maximal effectiveness and select molecules with highest specificity for the intended target gene(s), based on whole-genome information. Initially, 21-nt sequences are selected from the specified regions, of reverse complements of target transcripts such that they share an A or U at position 10 (which is recognized as the slicing site). At position 1, a U is introduced in all cases, even when other nucleotides would normally be found at this position. Moreover they should display 5' instability (higher AU content at the 5' end and higher GC content at the 3' end around position 19) for correct loading of the sense strand into the RISC. To reduce the likelihood that an amiRNA would act as primer for RNA-

dependent RNA polymerases and thereby trigger secondary RNAi, there should be no more than two mismatches to the target genes in the 3' part of the amiRNAs. Consequently all candidates are subjected to a series of *in silico* mutations at positions 13–15 and 17–21. As target sequences can differ slightly when designing amiRNAs for multiple genes, additional targets are allowed for maximum of one mismatch from amiRNA positions 2-12, but none at the cleavage site (positions 10 and 11) and up to four mismatches between positions 13 and 21, with no more than two consecutive mismatches (Schwab *et al.* 2006). In addition, acceptable amiRNA–target duplexes must have a low overall free energy, at least 70% compared with a perfect match of maximum −30 kcal/mol, as determined by mfold (Zuker 2003).

After selection of an amiRNA, the 21-nt sequence must be engineered into a pre-miRNA using overlapping PCR to replace the endogenous miRNA sequence. Initially three fragments containing (i) the 5' region up to the amiRNA*, (ii) the loop region ranging from amiRNA* to amiRNA, and (iii) the 3' region starting with the amiRNA are amplified separately from a plasmid template that contains the specific pre-miRNA backbone. The three PCR fragments overlap for 25 bp in the amiRNA and amiRNA* regions and the final product is generated in a single PCR reaction. Following sequence verification the amiRNA can be transferred into binary plasmids of choice, under choice promoters or terminator. Several precursors from *Arabidopsis* have been successfully used including miRNA-319a, miRNA-164b (Alvarez *et al.* 2006), miRNA-159a (Niu *et al.* 2006), miRNA-171 (Parizotto *et al.* 2004) and miRNA-172a (Schwab *et al.* 2006). Both miRNA and the miRNA*, are substituted by amiRNA and amiRNA*, respectively.

This tool was initially implemented for *Arabidopsis* (Schwab *et al.* 2006), but has now been extended to >30 additional species for which genome or extensive EST information is available (http://wmd2.weigelworld.org). It is designed to optimize both intrinsic sRNA properties as well as specificity within the given transcriptome. Using such amiRNA constructs as transgenes, plants have been raised that are developmentally deregulated (Alvarez *et al.* 2006; Schwab *et al.* 2006) and strongly resistant to virus infection (Niu *et al.* 2006).

Tools for sRNA delivery

In principle, every siRNA or miRNA, designed to silence the target, should show its specific function but the silencing activity is severely constrained by the lack of delivery technique(s) that can reach the siRNA at the right time and correct place within the cell or concerned tissues. Alternatively, methods need to be devised so that the siRNAs can be delivered within cellular locations with enhanced uptake and biological stability using various different formulations. Some of the successful methods are discussed below. It is worth mentioning that, in spite of the growing list of small successes as mentioned below, methods are yet to be developed that work efficiently in a systemic manner in the non-human primates and humans.

1. siRNA delivery

It is generally believed that the siRNAs are passively endocytosed like other gene silencing molecules such as oligonucleotides and ribozymes (Beale *et al.* 2003). The administration of naked siRNAs *in-vivo* is beset with limitations in cellular uptake, nucleolytic degradation and other problems like trapping in non-desirable cellular compartments. Hence therapeutic use of siRNA involves mixing these molecules with 'biocompatible' and 'genocompatible' formulations for appropriate delivery (Bumcrot *et al.* 2006). Irrespective of the mechanism of cellular entry of the siRNAs, the delivery techniques need to be evolved and improved to facilitate cellular accumulation of siRNAs and their subsequent release from the endosomes for functional demands. A lot of *in-vitro* data is available to meet such objectives and will be discussed briefly below.

a) *In-vitro* delivery of siRNAs: The effective RNAi *in-vitro* can be achieved using the following techniques.

(i) Direct transfection of siRNAs: This involves synthesis of dsRNAs of 20–23 nt and their transfection into cells. Chemical synthesis was the first method used to produce siRNAs. They can also be produced by *in-vitro* transcription of adaptor linked DNA oligonucleotides or else larger transcripts like shRNAs (short hairpin RNAs), long dsRNA, hpRNA, etc. (see **Glossary**) that can be processed by recombinant dicer molecules. The latter method produces a mixture of different siRNAs directed against the same mRNA target, thereby increasing the probability of gene silencing.

Various transfection parameters affect gene silencing efficacy. The foremost being cell culture conditions like cell density and medium composition. Transfection is performed in a serum-free medium, as proteins can bind to and/or degrade siRNAs. Differences have also been reported in the ability to transfect and silence gene expression between adherent and non-adherent cells. For example, the *ErbB3* gene was readily silenced in adherent carcinoma cells using liposome-mediated siRNA transfection, whereas the same transfection method was ineffective in non-adherent myeloma cells (Walters and Jelinek 2002). The post-mitotic cells such as neurons and muscle cells tend to be more difficult to transfect using liposomes compared to mitotic cells such as stem cells, fibroblasts, and tumor cells. For proliferating cells, a sub confluent cell density is preferable, while for post-mitotic cells such as neurons, cell densities in the range of 200-500 cells per mm^2 of culture surface work well (Milhavet *et al.* 2003).

Cells are not willing to take up foreign nucleic acids and charged oligonucleotides, so spontaneous cellular uptake of unmodified siRNA *in-vitro* is poor. A variety of small molecules, lipids, peptides and proteins have been examined as potential delivery vehicles for nucleic acids (Kim and Kim 2009; Midoux *et al.* 2009). For example, the non-specific uptake of cholesterol labeled siRNAs has been demonstrated to be effective for delivery to cells grown in culture as well as to liver, heart, kidney and lung tissues in mice (Soutschek *et al.* 2004). Similarly, a portion of the HIV-1 gp41 protein fused to a nuclear localization sequence has been demonstrated to be an effective means for the general delivery of siRNAs in tissue culture (Simeoni *et al.* 2005). The transfection efficiency can be monitored by using fluorochrome-coupled siRNAs (Mousses *et al.* 2003) or detecting reporter gene expression after co-transfection of siRNA and a reporter gene plasmid (Silva *et al.* 2004). The type and amount of transfection agent varies depending on the experimental conditions. The diverse methods of transfection include the following:

1. Calcium phosphate-mediated **transfection** has been used successfully by several laboratories (Donze and Picard 2002).

2. **Electroporation** is another effective method for transfecting siRNAs into cultured cells or plant protoplasts (McManus *et al.* 2002; Calegari *et al.* 2004; McCoy *et al.* 2010).

3. **Microinjection** (Calegari *et al.* 2002; Kim *et al.* 2002) and hydrodynamic shock (McCaffrey *et al.* 2002) are also used to deliver siRNAs into cells.

4. Incorporating siRNAs into **liposomes** is the most common and effective method of intracellular delivery for short-term suppression of gene expression in standard *in-vitro* experiments. The increasing variety of such transfection reagents includes: Oligofectamine, LipofectAMINE-2000 (Dalby *et al.* 2004; Tseng *et al.* 2009), CellFectin (Caplen

2002; Gan *et al.* 2002; Gitlin *et al.* 2002), Effectene (Martins *et al*. 2002), siPORT-Amine and siPORT-Lipid.

5. Akinc *et al*. (2008) had synthesized a library of over 1200 structurally diverse lipid like molecules, called **lipidoids** for RNAi therapeutics. The efficacy of these molecules as delivery reagents of siRNA was tested both *in-vitro* and *in-vivo*. Simple mixing of siRNA with the lipidoid solutions in microtitre plates formed the siRNA-lipids complexes which were administered in various cell types, including HeLa, human hepatocellular liver carcinoma cell line (HepG2) and primary bone marrow-derived murine macrophages. The silencing efficiency varied depending on the cell-types and dosages of the siRNA concentration and the types of lipidoids used. However, many lipidoids showed silencing activities superior to those of the commercial agents like LipofectAMINE-2000 or LipofectAMINE-RNAimax. This library approach offers new silencing materials for the cells that are resilient to transfection by currently available commercial means.

6. Selected nucleic acid binding **aptamers** are also being developed as potential agents for the delivery of siRNA cargoes (Hicke and Stephens 2000). Aptamers are oligonucleotide or peptide molecules that bind specific target molecules such as proteins, nucleic acids, and even cells, tissues and organisms. Aptamers can be engineered through repeated rounds of *in-vitro* selection or SELEX (Systematic Evolution of Ligands by Exponential Enrichment) from a large random sequence pool. However, natural nucleic acid-based aptamers exist in form of genetic regulatory element called riboswitches (Breaker 2002). Aptamers can be combined with ribozymes to self-cleave in the presence of their target molecule. In addition to possessing high affinity and specificity for their targets, aptamers can be synthesized chemically and thus are attractive reagents for use in therapeutic and other applications as drug delivery vehicles. It was observed that the anti-PSMA (Prostate-Specific Membrane Antigen) aptamers were capable of carrying nanoparticles into cells expressing this antigen (Farokhzad *et al.* 2004). Building on these findings, Chu *et al.* (2006) used anti-PSMA aptamers to deliver functional siRNA molecules to prostate tumor cells. Recently the first aptamer-based drug called Macugen has been developed for the treatment for AMD (Gragoudas *et al.* 2004). Recently Lieberman's lab used siRNA-aptamers to silence HIV genes in laboratory environments (Dove 2010).

7. **Polycationic dendrimers** such as poly-amidoamine (PAMAM) dendrimers also serve as good delivery vehicles. A dendrimer is a highly branched macromolecule with a spherical shape that grows in generations (Newkome *et al.* 2001). These harbor primary amine groups on their surface and tertiary amine groups inside their overall structure. These can self-assemble with any of the nucleic acids, namely DNA, RNA, siRNA etc. into nano-particles and show better entry and stability of the nucleic acids inside the cellular milieu compared to the uncomplexed naked nucleic acids (Kang *et al.* 2005). Zhou *et al*. (2006) have studied the silencing efficacy of siRNA-dendrimers complexes in A549Luc cells in details. The best (~80%) silencing occurred with G7 at an N/P ratio (the ratio of total number of end amines of the dendrimer and total number of phosphate groups of siRNA) of >2.5 and GL3 Luc-siRNA concentration of 100 nM. The silencing was stable for about 72 hours and slow release of siRNA occurred over an extended period of time. There are several reports on the use of dendrimers for siRNA delivery *in vitro* and *in vivo*. The most recent being on their use for the delivery of siRNAs targeting E6 and E7genes of cervical cancer cells *in vitro* (Dutta *et al.* 2010).

The quality and amount of siRNA and the length of time that the cells are exposed to the siRNA are other factors that may influence the siRNA uptake issues *in-vitro*. Although these can be resolved by carefully optimizing the pre-incubation conditions with the uptake enhancers, the cellular uptake of siRNA *in-vivo* still represents a real challenge.

b) *In-vivo* delivery of siRNAs: For *in-vivo* applications, biological stability and site-specific reach-ability are of primary concerns. The various modes of delivery include the following:

(i) Systemic administration: It is important to ensure that systemically delivered siRNAs accumulate at the target tissues or organs and remain functional without off-target and off-tissue effects. The systemically administered siRNAs have been shown to accumulate in organs such as liver, lung, spleen, kidney and heart to some extent (van de Water *et al.* 2006). However, the naked siRNAs are excreted out fast in the urine, thus formulations of siRNAs are required for enhanced retention within the body. The toxicity effects of siRNAs are also lessened with various kinds of formulations (Akhtar and Benter 2007a, 2007b). In the following, we discuss the fate of both naked and formulated siRNAs in various tissues.

1. Intravenous **hydrodynamic injections** of siRNAs in large volumes of physiological buffer have been used to effectively silence HBV-replication in mice. Both unmodified and chemically-modified siRNA molecules were delivered using this technique and were found to partition mostly in liver and scarcely in kidney and other organs. (McCaffrey *et al*. 2002; Morrissey *et al.* 2005). Subsequently, Mark Ever's laboratory was among the first to report effective siRNA delivery and gene silencing in colorectal carcinomas utilizing this technique (Rychahou *et al.* 2006). This technique in model organisms will continue to help in the understanding of gene function However, this technique will not be of any clinical use to humans due to the lethality caused by large injection volume and high pressure of injection.

2. **siRNA-lipophilic group conjugates** have helped enhance cellular entry, improved pharmacokinetics and specific accumulation in targeted tissues. In mice, the Apolipoprotein B (ApoB) siRNA with modified backbone, when conjugated with cholesterol at the 3′ end of the sense strand, knocked down the *apoB* mRNA by approximately 60% in the liver and 75% in the jejunam (Soutschek *et al.* 2004). Here no off-target effects or immune stimulations were observed even at a very high dosage of administered siRNA. Subsequently, Wolfrum *et al*. (2007) showed that additional conjugation of bile acids and long-chain fatty acids to siRNA-cholesterol complexes resulted in higher and specific cellular uptakes leading to improved gene silencing *in-vivo*. High-density lipoprotein (HDL) directed the siRNA-cholesterol conjugates to enter into liver, gut, kidney and steroidogenic organs whereas the low-density lipoprotein (LDL) targeted the complex primarily to liver. The cholesterol-siRNA, preassembled with HDL, was 8-15 times more effective in silencing the ApoB proteins compared to cholesterol-siRNA conjugates (Wolfrum *et al.* 2007). This delivery strategy has also been recently adapted to deliver siRNA subcutaneously in mice for the treatment of diabetic nephropathy (Yuan *et al.* 2008). However, high doses of cholesterol-conjugated siRNA are required thereby limiting its therapeutic applications in humans.

3. In many events of siRNA delivery, formulations of **liposomes** referred as 'lipoplexes' have been used successfully. For example, focal adhesion kinase was down regulated for ovarian carcinoma therapy using siRNAs complexed with neutral liposomes (Haldar *et al.* 2006). However, liposomes with net positive charges, which help complex formation with negatively charged siRNAs and facilitate interaction with negatively charged cell membranes, are preferred over neutral liposomes as ideal delivery vehicles. The siRNA-liposome complexes form about 100 nm particles to facilitate uptake by cells and to avoid renal excretion. The siRNAs could also be coupled with pH-sensitive peptides,

such as poly-histidine-lysine to help destabilize the endosomal membranes to aid the process of release of siRNAs in the cytosol. The siRNA-lipoplex, however show poor blood circulation characteristics thereby limiting their use in systemic delivery. This can be improved by modifying the surface of the siRNA-lipoplex with lipids (Tseng *et al.* 2009). Linking polyethylene glycol (PEG) has been shown to extend the circulation time for liposomes (Torchilin *et al* 1994) and increase the amount of siRNA in the targeted tissue.

DOTAP (1,2-dioleoyl-3-trimethylammonium-propane) and oligofectamine were some of the first few lipids that were tried for delivery of siRNA in mice to cause knockdown of TNF-α and β-catenin (Sorensen *et al.* 2003). Arnold *et al*. (2007) showed that a single intravenous dose of 1 mg/kg body weight of siRNA-DOTAP lipoplexes down regulated β1-adrenoreceptor expressions, resulting in reduction of blood pressure for up to two weeks. Various liposomal formulations reveal good bio-distribution and retention of siRNAs in liver, heart, lung, kidney, and spleen. Targeting of other organs with lipoplexes will perhaps require attachment of a targeting ligand or antibody. Pirollo *et al*. (2007) showed that the lipoplexes harboring a transferin receptor-specific antibody (SCF) effectively delivered a HER-2 specific siRNA to tumor xenografts in nude mice. Similarly, Toll like receptor (TLR)-mediated siRNA uptake has also been reported (Kleinman *et al.* 2008).

The experience derived from antisense oligonucleotides and ribozymes suggests that the clinical use of lipoplexes might be tempered by toxicological concerns. For example some cationic lipid formulations have been observed to elicit inadvertent gene expression; enhance immune response to siRNA and so on (Omidi *et al.* 2005). Hence it would be important to explore and design new lipid molecules that are serum-stable and non-toxic in nature. Some of the lipidoids, as mentioned earlier, could meet these challenges. About seven compounds derived from the large library of over 1200 structurally diverse lipidoids were very effective in specific silencing of endogenous transcripts in the liver of mouse, rat and non-human primates when the lipocomplexes delivered either the siRNA or the single stranded antisense 2'-O-methyl oligo-ribonucleotides. The silencing was stable for 2-4 weeks without visible off-target effects. Toxicological studies indicated that, even at a high dose of siRNA, the lipidoid formulations were well tolerated with little clinically relevant changes in coagulation or hematological parameters.

1) Cationic polymers, peptides, and proteins- Linear or branched **cationic polymers** bind and condense nucleic acids including siRNAs and thus these polymers are widely used as transfection agents. The siRNA-polymer complexes gain preferential entry in the cells and readily localize in the endosomes. Subsequently the acidic ambience of the endosome allows the polymer to be charged highly positively, resulting in the escape of the siRNAs in the cytosol. The polycationic character of the polymer is believed to buffer low endosomal pH through influx of protons and water, making the endosome leaky and labile (Gilmore *et al.* 2006).

Of several polymers, polyethyleneimine (PEI) has been extensively studied. The branched forms of PEI are preferred over the linear forms. The chosen molecular weight should range around 20 kDa for efficient transfection as higher molecular weight PEI might cause cellular toxicity. The architecture, dosage, molecular weight and so on of PEI, however, needs to be judged and calibrated right in order to avoid toxicity and off-target related problems. The PEI formulated siRNAs were shown to be effective as antiviral and anti-carcinogenic agents in several systems.

Similarly a cyclodextrin polycationic delivery system is reported that can efficiently mask the immune stimulation effects of siRNAs (Heidel *et al.* 2007). The delivery system consisted of cyclodextrin at its core, which was tagged with PEG and a cell-targeting ligand, say transferrin as a tumor targeting ligand. This system efficiently silenced the oncogene EWS-FLI1 in a metastatic model of Ewing sarcoma (Hu-Lieskovan *et al.* 2005). Studies in Cynomolgus monkeys with high dosage of the siRNA revealed that the delivered material was well tolerated and there was no consequent antibody response.

2) Several **peptides** have been used as delivery reagents as well. Arg-Gly-Asp (RGD) peptides along with PEGlyated-PEI have been used to deliver (intravenously) siRNAs targeting vascular endothelial growth factor (VEGF) to inhibit tumor growth and reduce angiogenesis (Schiffelers *et al.* 2004). About 30 amino acid long peptides, with membrane transduction domains, have been used to deliver siRNAs across the blood-brain barrier (Kumar *et al.* 2007). The peptide mediated delivery approaches increase cellular uptake but the silencing efficiencies require improvement along with the addressal of toxicity related issues.

Proteins have also been used as delivery vehicles. Protamine, a small cationic protein, is competent to complex with and to condense the nucleic acid. Recently Song *et al*. (2005) conjugated a fragment antibody (Fab) specific for HIV-1 envelope protein gp160 to siRNA-protamine complex. The siRNAs were selectively delivered to the tumor cells, engineered to express gp160, resulting in a marked antitumor effects. Similarly, Atelocollagen, which is about 300kDa large protein, has been used to deliver siRNA both systemically and locally in tumor models. This protein increases the lifetime of naked siRNA by 3 fold, enhances cellular uptake and releases siRNA slowly in the cytosol for sustained silencing activity. This protein has been used as a delivery vehicle for siRNAs in several systems like xenografts model of prostate cancer (Takei *et al.* 2004), orthotropic model of human testicular cancer (Minakuchi *et al.* 2004) and in bone-metastatic tumors (Takeshita *et al.* 2005).

3) In many of the approaches mentioned above, the delivery material form nano-scale soluble aggregate prior to cellular entry. The nano-particles ensure certain criteria, like better bioavailability of siRNAs, sustained release of siRNAs for prolonged silencing effects, enhanced retention of siRNAs before being excreted out of the body, and less pronounced siRNA related toxicity. Many **novel nano-particle formulations** are thus emerging but every attempt has its own limitations. We mention below two recent efforts that might hold enormous promise for therapeutic successes.

Medarova *et al*. (2007) designed and tested a novel dual-purpose probe for both non-invasive imaging and delivery of siRNAs in nude mice bearing colorectal carcinoma xenografts. The delivery probe consisted of magnetic nano-particles (for MRI), which were coated with aminated dextrans labeled with Cy5.5 dye (for near infra-red imaging). This probe was conjugated to myristoylated polyarginine peptides that served as a membrane translocation module and the peptide, in turn, was linked to siRNA targeting the surviving protein of the tumor. The delivery of the probe was monitored using MRI and optical imaging. The gene knock down was also followed by optical imaging and was correlated with the histological data. Interestingly, the probe accumulated in the tumor following the intravenous injection despite the absence of specific targeting in the delivery process. This study also highlighted that the covalent attachment of siRNA to nano-particles did not affect the gene-silencing activity. Overall, this approach advanced siRNA targeting and potential therapy.

In another instance, Peer *et al*. (2008) designed the nano-particles carrying the anti-Cylin D1 (CyD1) siRNA that were targeted to the leukocytes in a gut inflammation mouse model. They started with nano-scale (100 nm) liposomes, made of neutral phospholipids that were tagged with Hyaluronan to stabilize the particles at the various stages of preparation as a delivery reagent. The Hyaluronan of the stabilized particles were covalently attached to monoclonal antibody against β7 integrins, which are highly expressed in gut mononuclear leukocytes. These particles, called β7–tsNPs, were loaded with siRNAs that were condensed with protamine. This formulation increased the bioavailability of siRNAs in gut leukocytes to a huge extent in mice that were treated with dextran sodium sulfate to induce gut colitis

(Peer *et al.* 2008). In inflammatory bowel disease, the colon expressed CyD1 is aberrantly up regulated in both epithelial and immune cells. The systemic application of these nanoparticles silenced CyD1 in leukocytes and reversed the induced colitis in mice by suppressing leukocyte proliferation and T-cell-1 cytokine expression. This study established the role of CyD1 as a potential anti-inflammatory target and revealed that similar nano-particles might be designed in any other disease model.

More recently, polymer-based nanoparticles were used to deliver siRNAs in clinical trials against solid tumors (Davis 2009).

siRNA-liposomal formulation aimed at targeting protein kinase N3 have proven to significantly inhibit tumour growth in prostate and pancreatic cancer models in mice (Aleku *et al.* 2008). Silence Therapeutics is now conducting phase I trials in humans with advanced solid tumours. Alnylam Pharmaceuticals has initiated preclinical studies using a lipid-based nanoformulation containing two different siRNA molecules aimed at targeting the kinesin spindle protein (KSP) and the vascular endothelial growth factor (VEGF) for their potential antiliver tumour activity. VEGF and KSP are upregulated in many tumour cells and play an important role in tumour proliferation and survival (Aknic *et al.* 2008). The results of these trials with lipid and formulated materials will provide important information regarding the translatability of delivery systems developed in rodents and primate (Schroeder *et al.* 2010).

(ii) Local delivery: Many reports are available where siRNAs were deployed directly to the concerned tissues to successfully silence the target genes. Both naked and formulated forms of siRNAs have been experimented with and in many events naked siRNAs silenced the genes with comparable efficiencies in diverse organs. At least two events have served as proof-of-concepts for the current clinical trials to cure AMD disease and infection with respiratory syncytial virus (RSV).

1. The **intraocular delivery** of either naked or formulated siRNAs targeting the eye diseases has emerged as a preferred approach. The AMD disease is due to over accumulation of the VEGF protein behind retina leading to eventual blindness. Intravitreal injections of siRNAs in cynomolgus monkeys (Tolentino *et al.* 2004) and sub retinal injections in mice (Reich *et al.* 2003) have been fruitful to reduce the disease incidence. A few commercial companies are at the stage of clinical trials with the human patients and hope to introduce the siRNA medicine in the drug market by the end of year 2009. In other instances, delivery with about 200 nM siRNA complexed with liposome formulation led to ~50% decrease in ocular inflammation and fibrosis at 2 days post delivery (Nakamura *et al.* 2004). Similarly Herard *et al*. (2006) reported substantial knockdown of Amyloid Precursor Protein (APP) in retinal terminals at 24 hours after intraocular injection.

2. The **intranasal route** delivers the siRNA mostly to lungs although delivery has also been reported to other organs (Howard *et al.* 2006). Hence a variety of lung diseases, like asthma, cystic fibrosis, ischemic reperfusion injury and viral infections could be treated with local applications of siRNAs. Bitko *et al*. (2005) delivered siRNAs targeted to RSV and parainfluenza virus to anesthetized mice with or without lipid formulation. A complete elimination of virus was observed with formulated siRNA although naked siRNA also produced a pronounced antiviral effect. The interferon effect and off-target silencing was minimized with the dosage of delivery material and the silencing was stable with time. A few commercial companies are carrying out phase-II level clinical trials with human patients infected with RSV. The siRNAs could also be successfully delivered to target haemeoxygenase-1 (HO-1) in lung reperfusion injury model of anesthetized mice to control the level of HO-1 (Zhang *et al.* 2004). Several other studies demonstrated that the intranasal delivery caused gene silencing in acute model of lung injury (Thomas *et al.* 2007) and resulted in alleviation of SARS virus infection in non-human primates (Li *et al.* 2005).

3. The **intra-tumoral** administration of siRNAs has resulted in effective anticancer strategy in many cases but majority of the solid tumors requires systemic delivery. Pille *et al*. (2005) have shown that anti-RhoA and anti-RhoC siRNAs inhibited the proliferation and invasiveness of MDA-MB-231 breast cancer cells in a xenograft model of mice. Cholesterol conjugated, low-molecular-weight-PEI formulated siRNA targeting the VEGF effectively inhibited tumor growth in colon adenocarcinoma of mice and prolonged the survival of tumor bearing mice (Kim *et al.* 2006).

A few other local delivery techniques like electroporation to muscle, administering to the central nervous system have also been reported and reviewed well by Akhtar and Benter (2007a, 2007b). In spite of growing reports of local delivery, the systemic delivery strategies seem to be clinically more attractive (Kumar *et al.* 2007).

2. Plasmid vector-mediated delivery

Instead of using siRNA directly, the plasmids or viruses, could be used as vectors, to generate and deliver siRNAs. Following introduction of the vectors in the cells, shRNA are expressed that can be processed by cellular Dicers to generate the siRNAs of desired sequence. This is a low cost strategy compared to synthesizing siRNAs and also allows continuous intracellular production of siRNAs enabling sustained gene silencing. Besides, the vector-mediated delivery is amenable to high throughput techniques as the library of shRNAs can be used for cloning. Moreover the abundance of siRNAs within the cell could be controlled spatio-temporally if an inducible promoter is chosen for expression of shRNAs. Additionally, if the viruses are used to deliver the shRNAs, the problems relating to the quantity and feasibility of transfection in difficult cell-types can be suitably manipulated.

The plasmid vectors are of either the episomal or integrative types. The latter varieties are used to express around 70-nt shRNAs for stable silencing effects in majority of the cases (Paddison *et al.* 2002; Yu *et al.* 2002). The plasmid transfection process requires some of the reagents needed for transfection of siRNAs, as mentioned before. The plasmids are engineered to direct the synthesis of a shRNA that gets reorganized in the form of a stem-loop. Such plasmids have been used to inhibit the expression of a number of endogenous genes (Miyagishi and Taira 2002; Sui *et al.* 2002). The silencing ability of the processed siRNA was almost similar to that of the exogenously supplied synthetic siRNAs.

The episomal vectors pEBVsiRNA (marketed by ceavalorisation) harbors a short EBV sequence, which allows it to persist in nuclei of the transfected cells as a stable circular DNA. Such vectors have provided long-term stable gene silencing to about 25 mammalian genes in a variety of mammalian cell-lines. Due to low level of siRNA expression, cellular toxicity was hardly observed. Liu *et al*. (2005) constructed SV-40 ori-based episomal plasmids that maintained well in cell lines expressing large T antigens, such as COS-1 and COS-7 cells. They expressed shRNA of Fas gene under the control of U6 promoter and demonstrated Fas inhibition in stably transfected cells using immunocytochemistry.

The difference also lies in the kind of promoter constructs employed. The shRNAs are generally allowed to be transcribed intracellularly by Polymerase (Pol) III and the transcripts end with the U-rich termination signals (Brummelkamp *et al.* 2002). This vector system can be made inducible by using promoters like $tRNA^{val}$ and $tRNA^{met}$ (Boden *et al.* 2003; Oshima *et al.* 2003), and the sequences inducible by tetracycline (Tet) or doxocycline (dox) (Gossen and Bujard 1992). A classical example is provided by

the vector system, which is engineered to constitutively express the tTS protein, in the transfected cell line. It is a fusion of Tet-repressor protein (TetR) and a KRAB silencing domain, a powerful transcriptional suppressor. The transfecting plasmid harbors the TetR-operator, which is fused with U6 promoter. These controlling elements block the expression of shRNA and de-repress the expression only in the presence of exogenously supplied tetracycline. This system was used for controlled down regulation of several genes with minimal toxicity in various cell lines (Freundlieb *et al.* 1999). All of the Pol-III promoters can be used in various cell lines of different species and perform with almost equal efficiency (Chen *et al.* 2005a). Recently Pol-II promoters have also been used, so these transcripts are 5′ capped and harbor long poly U stretches at their 3′ end. If developmentally regulated promoters could be used, the development-specific silencing could be observed. All such cases represent the spatio-temporal control of the silencing effects (Tang and Galili 2004).

Cloning of long hairpin RNA (lhRNA), in lieu of shRNA, in the plasmid vectors is more useful in silencing the target gene(s). However, long stretches of dsRNA elicit cellular interferon response, thereby masking the specific silencing effect. Hence multiple point mutations are generally introduced only in the sense strand (i.e. only in one arm of the stem of hairpin) to avoid the interferon effects. The lhRNA behaves like pre-miRNA in principle. However, these are not suitable for sustained effects. When lentivirus-lhRNAs were expressed under the control of human U6 promoter, the replication of the HIV virus was severely affected and the lhRNAs acted much better than the control shRNAs. However, the antiviral effects were transient and the escape mutants were generated at six days post infection (Nishitsuji *et al.* 2006). This suggested that an efficient expression system of lhRNAs was needed for long-term control of HIV-1 replication. In plants, lhRNAs or antisense constructs are often expressed in specific tissues and organs with the help of tissue specific promoters so that the siRNA related effects remain localized mostly at the desired tissues. The vectors containing such constructs are introduced in the plants either through biolistic approach or through *Agrobacterium*-mediated route. The biolistic approach is rapid, has a wide range of species on which it can work, and is a valuable tool for work on single cells. The disadvantages are that this limits gene silencing to the cells on the surface of the leaf, and silencing is only temporary. While the advantages of agro-infiltration method of vector delivery are that it is rapid and provides a high throughput approach, it is relatively easy to use and it has a low cost. However the disadvantage of this method is that *Agrobacterium* can trigger a pathogen response in the host. Also, it has not really been tested on most species (Waterhouse and Halliwell 2003). The silenced transgenic plant could be put to use in a tailored manner. However, the problem with the transgenesis is that not every plant is amenable to transformation through the *Agrobacterium* delivery route.

Amongst other strategies to express siRNAs, the use of the dual Pol-III promoter to express small double stranded RNAs has gained considerable popularity of late. It employs the promoters to be arranged in a head to head fashion, to drive the expression of two complementary strands of the cloned 19 base pair (bp) DNA to produce the desired siRNA duplex. The 19-bp strands are flanked by four ‘A’s and ‘T’s on either ends, to provide the expression termination signal, The functionality of such processed siRNAs has been demonstrated on several occasions (Zheng *et al.* 2004; Chen *et al.* 2005b). These vectors are easily clonable as no secondary structure containing elements, as in shRNA, are involved. These vectors thus offer huge advantages in cloning large-scale siRNA libraries.

3. Viral vector-mediated delivery

The most efficient way for siRNA delivery is to use the viral vectors. For the cell types that are resilient to general or formulated transfection techniques, viral vectors are often employed. The shRNA expressing constructs of various plasmid vectors can also be easily grafted into vectors of viral origin such as lentivirus, retrovirus, adenovirus, adeno-associated viruses etc to produce shRNA-generating viruses, which can transfect otherwise impenetrable cells, like primary cells and suspension cells (Devroe and Silver 2004).

The Adenoviral vectors work well for transient delivery in many cell types; however, for some difficult cell lines, such as non-dividing cells; and for stable RNAi expression, lentiviral vectors provide the best delivery method. Since the vector-based approaches regenerate siRNAs intracellularly, the silencing levels are high and long lasting. The common approach is to engineer the plasmid vectors containing the shRNA that are subjected to *in-vitro* transcription reaction for production of either infectious or replication-defective RNAs, which in turn, are packaged into viral particles. There are also many other methods that have been adopted by investigators in the field. The stepwise details of production of shRNA-bearing recombinant viruses of various kinds, namely, Feline immunodeficiency virus (FIV), Adenovirus, Adeno-associated virus (AAV) are reviewed by Davidson and Harper (2005). These are used to deliver the nucleic acids in desired cell-types or tissues where the shRNAs are processed to siRNAs for targeting the specific transcripts. In the following, we discuss a few virus vectors.

a) Retroviral vectors: Among the retroviral vectors, majority of the studies have been carried out with lentiviruses, as they can transduce even the terminally differentiated quiescent cells, including neurons (Brummelkamp *et al.* 2002). Using the retroviral siRNA vector, pMSCVpuro (Clontech, CA), the efficient and sustained depletion of the NDR kinase and the transcriptional co-activator p75 was demonstrated in cultured cells (Devroe and Silver 2004). shRNA-producing plasmids were successfully delivered to neurons using a lentiviral vector in a transgenic mouse model of familial ALS (Raoul *et al.* 2005). Intra-spinal injection of a lentiviral vector inducing RNAi-mediated silencing of mutated superoxide dismutase 1 substantially retarded both the onset and progression rate of the disease in mice.

Lentiviruses can infect non-cycling and post-mitotic cells, and allow the generation of transgenic animals through infection of embryonic stem cells or embryos (Naldini 1998; Lois *et al.* 2002). The functionality of this approach, was demonstrated by silencing of green fluorescence protein (GFP) in GFP-positive transgenic mice has been shown after transduction with lentiviruses expressing shRNA directed against the GFP protein (Tiscornia *et al.* 2003). Rubinson *et al*. (2003) used lentivirus-delivered shRNA to induce stable and functional silencing of CD8 and CD25 in cycling primary T-cells and the pro-apoptotic molecule, Bim, in primary bone marrow-derived dentritic cells. However, a short-coming for their potential clinical use is that the retroviruses insert into the host genome with poor control over their insertion site, which can trigger oncogenic transformation, such as in the SCID (Severe Combined Immunodeficiency) clinical trials (Hacein-Bey-Abina *et al.* 2003).

b) Adeno-associated viral (AAV) vectors: The recombinant AAV (rAAV) vectors appear to be a good compromise in terms of efficacy and safety for RNAi transfection *in-vivo* (Shen *et al.* 2003). The rAAV vectors commonly inhabit hosts without causing any detectable pathology (Flotte 2004). Since they require a helper virus for replication and do not spread once inside a cell, they do not cause an inflammatory response and show higher transfection efficiency than lentiviruses. Indeed, clinical trials using rAAV vectors for gene therapy are under way (Mandel and Burger 2004).

c) **Adenovirus vectors:** Several commercial companies have exploited the ease of recombinant adenovirus (rAD) production and provide kits for generating adenoviruses that

over express si-RNA (www.imgenex.com; www.ambion.com). Briefly, shuttle plasmids harboring shRNA expression cassette are co-transfected in HEK-293 cells along with the adeno backbone plasmid that contains sequences similar to a portion of the shuttle plasmid. After two weeks, rAD is produced that is reamplified in HEK-293 cells to a very high titer within following two weeks. The rAD5 vectors are used most for delivery in primary non-dividing cells, animals and human beings. More recently, "gutless ADs" that are stripped of all native viral genes, have been used for delivery and long-term maintenance in liver and brain tissues (Thomas *et al.* 2000).

The cultured pancreatic islet cells or their derivatives are very difficult to be transduced by exogenous nucleic acids, large or small. Bain *et al*. (2004) could, however, successfully deliver the GLUT2-shRNA or glucokinase siRNA in these cells using rAD to reduce the target expressions by more than 50%. Chen *et al*. (2006) had used replication defective human rAD5 to deliver shRNA in swine IBRS-2 cells. The siRNAs directed against either structural protein 1D (Ad5-NT21) or Polymerase 3D (Ad5-Pol) of foot and mouth disease virus (FMDV) protected the cells from homologous FMDV infection, whereas only Ad5-Pol inhibited heterologous FMDV replication. The delivery of these shRNAs significantly reduced the susceptibility of guinea pigs and swine to FMDV infection. Narvaiza *et al*. (2006) constructed rAD encoding different shRNAs targeting murine ATP-binding cassette multidrug resistance protein 2 (Abcc2), which is involved in liver transport of bilirubin to bile. When C57/BL6 mice were injected with these viruses, a significant impairment of Abcc2 function was observed for up to three weeks, as reflected by increased serum bilirubin levels. The siRNA production did not interfere with endogenous microRNA pathway.

d) VIGS in plants: When a virus infects a host plant it activates an RNA-based defense that is targeted against the viral genome (Ratcliff *et al.* 1999). Since, viruses can both initiate and be targets of gene silencing have prompted speculation that the mechanism is part of a defense system in plants against viruses (Pruss *et al.* 1997; Ratcliff *et al.* 1999). In fact, the term VIGS was first used by van Kammen (1997) to describe the phenomenon of recovery from virus infection. However, the term has since been applied almost exclusively to the technique involving recombinant viruses to knock down expression of endogenous plant genes (Baulcombe 1999). This has found most applications in characterizing the function of plant genes.

The approach involves cloning a short sequence of a targeted plant gene into a viral delivery vector. The vector is used to infect a young plant, and in a few weeks, the natural RNAi defense mechanisms of the plant directed at suppressing virus replication also result in specific degradation of mRNAs of the cloned as well as the endogenous plant gene (Burch-Smith *et al.* 2004). VIGS is rapid (3–4 weeks from infection to silencing) and does not involve stable transformation. It is easy to use and provides a high throughput characterization of phenotypes that might be lethal in stable lines, and offers the potential to silence either individual or multiple members of a gene family. However, it has limitations on its host range. It might have restricted regions of silencing and there may be size restrictions on the inserts. Moreover it is dependent on the availability of infectious clones and the viral symptoms could get superimposed onto the silenced phenotype. Hence disarming of disease producing viral sequences in the VIGS vector is advised wherever applicable.

Several viral genomes have been modified to produce VIGS vectors transgene mRNA, so that the host RNAi response initiated against the infecting virus can also silence the endogenous genes at the post-transcriptional levels, in a sequence-specific manner (Lindbo *et al.* 1993; Goodwin *et al.* 1996; Guo and Garcia 1997). The majority of the VIGS vectors have been based on RNA viruses that can infect several plant species used in scientific investigations. *Tobacco mosaic virus* (TMV) was the first viral vector used to successfully elicit VIGS of an endogenous gene in a plant species (Kumagai *et al.* 1995). Since then many VIGS vectors has been constructed and used in plant and animal functional genomics study. Each vector has its own advantage and disadvantages.

The most widely used VIGS vectors are based on the *Tobacco rattle virus* (TRV; Ratcliff *et al.* 2001; Liu *et al.* 2002) because of the ease of introduction in plants. It is usually mediated by agroinfiltration with the VIGS vector placed between T-DNA borders (Ratcliff *et al.* 2001; Liu *et al.* 2002; Lu *et al.* 2003; Burch-Smith *et al.* 2004; Ryu *et al.* 2004; Hileman *et al.* 2005; Wang *et al.* 2006). TRV-based VIGS vectors have been used to silence genes in a number of Solanaceous plant species (Ratcliff *et al.* 2001; Liu *et al.* 2002; Brigneti *et al.* 2004; Chung *et al.* 2004; Chen *et al.* 2005; Hileman *et al.* 2005). *Potato virus X* (PVX) has also been used in the construction of VIGS vector, however they have more limited host range (only three families of plants are susceptible to PVX) than TMV-based vectors (nine plant families show susceptibility) but PVX-based vectors are more stabile compared to TMV (Burch-Smith *et al.* 2004).

In addition to RNA viruses, DNA viruses have also been adapted for use as VIGS vectors. In the DNA virus based VIGS vector geminiviruses are in the forefront like bipartite *Cabbage leaf curl geminivirus* (CbLCV), *Mungbean yellow mosaic India virus* (MYMIV), *Tomato golden mosaic virus* (TGMV), etc. These have emerged as very promising tools, because they enable direct plasmid DNA infection instead of requiring *in-vitro* transcription for mechanical inoculation. In our laboratory, we have used *Tomato leaf curl virus* circular replicon as the VIGS vector that is devoid of disease symptom producing genes such as coat protein, pre-coat protein, etc. This vector silenced the PDS, PCNA genes of tomato in a long-lasting manner (Pan-

Table 3 Available VIGS vectors.

VIGS	Type	Constructed by
Tobacco mosaic virus (TMV)	RNA	Kumagai *et al*. 1995; Lacomme *et al*. 2003
Potato virus X (PVX)	RNA	Ruiz *et al*. 1998
Tomato golden mosaic virus (TGMV)	DNA	Peele *et al*. 2001
Tobacco rattle virus (TRV)	RNA	Ratcliff *et al*. 2001
Barley stripe mosaic virus (BSMV)	RNA	Fitzmaurice *et al*. 2002; Holzberg *et al*. 2002
Cabbage leaf curl virus (CbLCV)	DNA	Turnage *et al*. 2002
Pea early browning virus (PEBV)	RNA	Constantin *et al*. 2004
Brome mosaic virus (BMV)	RNA	Ding *et al*. 2006
Bean pod mottle virus	RNA	Zhang and Ghabrial 2006
Cucumber mosaic virus (CMV)	RNA	Sudarshana *et al*. 2006
Tomato mosaic virus (ToMV)	RNA	Saejung *et al*. 2007
Tomato leaf curl virus (ToLCV)	DNA	Huang *et al*. 2009; Pandey *et al.* 2009
Satellite tobacco mosaic virus (STMV)	RNA	Gossele *et al*. 2002
Satellite DNA β of *Tomato yellow leaf curl china virus* (ToLCV)	RNA	Tao and Zhou 2004
Satellite DNA β of *Tobacco curly shoot virus*		Qian *et al*. 2006
Tomato leaf curl virus satellite	DNA	Li *et al*. 2008

dey *et al.* 2009). A list of currently available VIGS vectors is provided in **Table 3**.

Satellite-virus-based vectors are also used for efficient gene silencing in plants only with the help of other helper viruses. This two-component system is called Satellite-virus-induced silencing system, SVISS. The first SVISS was developed by Gosselé *et al.* (2002) based on *Satellite tobacco mosaic virus* which uses the *TMV* strain U2 as a helper. In another study *Tomato yellow leaf curl China virus* being helper and a modified satellite DNA were used to silence gene in *N. benthamiana* (Tao and Zhou 2004). This method shows some advantages over other systems, such as high stability and accumulation of the silencing signal in the infected tissues, easy cloning due to its small genome size and attenuated symptoms of virus infection. Enzymes involved in several metabolic pathways are effectively silenced, showing clear phenotypic alterations four weeks after inoculation. The other satellite DNA modified for silencing of dicotyledonous plants used *African cassava mosaic virus* in cassava (Fofana *et al.* 2004), *Pea early browning virus* in pea (Constantin *et al.* 2004), and *Bean pod mottle virus* in soybean (Zhang and Ghabrial 2006).

VIGS has emerged as a very promising reverse genetics tool for functional genomics due to its high fidelity and ease of delivery as well as cost effectiveness compared to other parallel techniques. However, despite its great potential to extensively use, many limitations remains to be overcome (Unver *et al.* 2009). Firstly host range of viral vectors will become wider; the VIGS assays and viral vectors for model organisms such as Arabidopsis and rice should be well optimized. Secondly, with VIGS absolute loss of function cannot be achieved (~75-90% downregulation). Furthermore, the virus in the VIGS vector needs to be disarmed to avoid any symptom development due to infection. Lastly, VIGS might suppress nontargeted gene in silenced plant cell or tissue (Burch-Smith *et al.* 2006). This response should be addressed before the next genomic era.

***4. Bacterial vectors*:** Amongst the other not so widely used means for RNAi-delivery, the bacterial vectors deserve due mention. The bacterial genetic material does not easily integrate in host chromosome and bacteria could be controlled with antibiotics as well as be engineered to increased biosafety. The bacteria are thus considered as safe and offer inexpensive tools for RNAi delivery. The delivery processes can be classified in two distinct categories:

(a) *trans*-kingdom RNA interference (tkRNAi): The shRNA or the dsRNA are produced in bacteria and later delivered to the appropriate hosts where biogenesis and function of siRNAs occurs. Xiang *et al.* (2006) transformed the *E. coli* bacteria with a TRIP (*trans*-kingdom RNA interference plasmid) that enabled the bacteria producing shRNA, invade the target mammalian cells and release the shRNA in mammalian cytosol to silence the genes of interest in target cells. The bacterial (BL21 DE3) shRNA expression cassette was driven by T7 RNA Pol promoter and terminator. The invasin protein, expressed by TRIP, interacted with the β-integrin protein of mammalian cell surface and thus helped bacteria gain entry in mammalian host. The TRIP also expresses the listeriolysin, which in turn makes pores in the endosomal membrane of the target host to help release of the accumulated shRNA in the host cytosol. The shRNA was targeted to human colon cancer oncogene, catenin-β-1 and was delivered by the transformed bacteria to human colon cancer cells (SW480) to cause down regulation of the oncogene expression. The same system was effective in inducing local silencing in the intestinal epithelium of mice when mice were treated orally with the *E. coli*. tkRNAi is being currently used for a spectrum of applications ranging from colon cancer prevention to treatment of HPV infection and inflammatory bowel disease by commercial companies (www.cequentpharma.com). Kuwahara *et al*. (2007) also reported recently the edible *Lactobacillus casei* that were engineered to produce long dsRNA of *C. elegans* DNA polymerase ε subunit gene by convergent T7 promoters to exterminate sheep intestinal parasitic nematode. Besides these, it has been reported long ago that RNAi was established when nematode *C. elegans* ate the dsRNA-expressing *E. coli* cells (Timmons and Fire 1998).

(b) bacteria-mediated RNAi (bm-RNAi): The invasive bacteria like *S. typhimurium* are used to deliver the shRNA expressing plasmids directly to the host mammalian cells, which subsequently use their own transcription apparatus to generate shRNA in the nucleus (Nguyen and Fruehauf 2009). The attenuated *S. typhimurium* was successfully used for delivery of the shRNA expression plasmid for treatment of a mouse prostate cancer model (Zhang *et al.* 2007). In plants the plasmid vectors are delivered through *Agrobacterium* route. These vectors carrying the engineered T-DNA have been used to express dsRNA, hpRNA or amiRNA in plants.

APPLICATIONS

As RNAi is a homology-dependent process, all the members of a gene family could be silenced if the siRNAs of conserved sequences are allowed to be generated. On the contrary, each member also could be silenced if the siRNAs are selected carefully from the unique region of the target sequence. Thus the genetic redundancies could be handled depending on the investigator's option. In these days, RNAi is a great tool for functional genomics. More often than not the investigators seek for a certain level of silencing beyond which metabolic disordering might come in play. The variable level of silencing can be achieved in different transgenic lines using the same intron-spliced hpRNA (ihpRNA) construct, thus offering a method for right kind of selection (Wesley *et al.* 2001). The ihpRNAs can also be expressed using inducible promoters to control the level and timing of silencing and thus generating the flexibility to characterize the diversified functions of plant genes (Chen *et al.* 2003).

The siRNA or miRNA-mediated silencing is a rapid, reliable and economic way to characterize a gene knockdown phenotype in model systems. As a result, it has numerous applications in possibly every disease. Several studies have been performed and an equal number are currently underway to screen for relevant candidate genes in diverse sphere of medical, veterinary and agricultural sciences. In the medical and veterinary fields, several studies have demonstrated efficient *in-vivo* delivery of siRNAs and therapeutic benefit in mice or bovine models. In the agricultural sector studies have been performed on a number of plants including the model species like *Arabidopsis* and tobacco. Below, we briefly mention a few of the striking applications in these distinct fields.

Medical sciences

An RNAi therapy was tested in humans for the first time, in late 2004, yet there are quite a few hurdles in treating most diseases, including the delivery of RNAi drugs to the right targets, avoiding "off-target" effects that can shut down essential genes or processes and ensuring that the drugs stay active long enough to fight the disease. The development of techniques to overcome each of these has been detailed in the earlier sections. RNAi therapeutics is being applied for a range of diseases from metabolic, neurological and cardiovascular disorders to cancer. However, most of the studies have been done with cell cultures or murine models.

RNAi provides a powerful technique to discover and learn more about genes that trigger or inhibit cancer. Multiple *in-vitro* and *in-vivo* siRNA studies have evaluated the knockdown phenotype of oncogenes. Greg Hannon and his group at Cold Spring Harbor Laboratory are attempting to decipher the function of 15,000 genes in a variety of human cancer cell lines. Such efforts might pinpoint genes never before linked to cancer and generate novel ideas for treatments. Leukemia may be among the first forms of cancer to

Table 4 Applications of RNAi in medical sciences.

Disease	Symptom	Gene targeted		Mode of siRNA delivery	Status	Remarks	Reference
		Name	Function				
Viral diseases							
HIV	Autoimmune disorders	various steps in the HIV life cycle	Tar, tat, rev, gag, env, vif, nef, and reverse transcriptase	Synthetic siRNAs	cell lines and primary peripheral blood lymphocytes	HIV mutates and evolves resistance rapidly so any single target for an RNAi therapy will not be sufficient	Novina *et al*. 2002; Jacque *et al*. 2002
		early and late HIV- RNAs		shRNAs and synthetic siRNA			Leonard AND Schaffer 2005
		CD4	Cell receptor	siRNA	Magi-CCR5 (Human CD4 cell lines)	Oligofectamine	Novina *et al*. 2002
		cellular cofactors required for HIV infection	NFk-B, receptor CD4, co-receptors (CXCR4, CCR5)		human cell lines, T lymphocytes and haematopoietic stem-cell-derived macrophage	non-infected cells will inevitably be targeted, leading to toxicities	Gitlin *et al*. 2002; Martínez *et al*. 2002
		multiple targets of the HIV virus				combined with ribozymes and RNA decoys	Rossi 2006
		multiple HIV genes			mice and monkey models	stem cells extracted from a patient's bone marrow are transformed with siRNA and transfused back into the patient, (to develop into healthy HIV resistant cells)	Rossi 2006
		multiple targets of the HIV virus		shRNA-producing DNA	Phase-I trials	isolated leucocytes were transfected with shRNA construct and reinfused back into the system	Benitec Ltd.
Parainfluenza virus	influenza A, bronchiolitis	various RSV genes		intranasal or intratracheal	murine model		Bitko *et al*. 2005
Respiratory syncytial virus	croup, pneumonia, respiratory failure	various RSV genes		intranasal	Phase-I trials		Alnylam
Organellar disorders							
Age-related macular degeneration	Adult blindness	VEGF	promotes blood vessel growth	direct injection	Phase-I trials		Acuity Pharmaceuticals
		VEGF receptor 1		direct injection	Phase-I trials		Sirna Therapeutics
Cancer	malignant transformation of cells	Bcr/Abl kinase			CML cell lines		
		EWS–FLI1 fusion oncogene product		transfection (transferrin)	murine model		
		Chemoresistance factors (p53, Bcl-2, P-glycoprotein)	Drug resistance/ efflux		breast cancer and leukemia cells		Wacheck *et al*. 2003; Wu *et al*. 2003
		Fortilin	myeloid cell leukemia 1 protein	siRNA	U2OS osteo-sarcoma cell line	TransIT solution	Zhang *et al*. 2002
		multiple origins of brain tumors	Block invasion of cells into other tissue		animal model	Experimental drug	SanoGene's
Coronary artery disease	Increased serum levels of LDL and cholesterol	Apolipoprotein B			murine model		Soutschek *et al*. 2004
Neuro-degenerative diseases							
Amyotrophic lateral sclerosis		dominant allele	causes sclerosis				Ding *et al*. 2003
Spinocere-bellar ataxia			rAAV vectors		murine model		Xia *et al*. 2004
Huntington's disease				Naked siRNAs	murine model		Harper *et al*. 2005
Metabolic disorders							
Type II diabetes		insulin receptor substrates (IRS-1, IRS-2)	regulates glucose homeostasis and lipid metabolism	adenoviral vector			Taniguchi *et al*. 2005
		PEPCK	gluconeogenesis		murine model	reduces hepatic glucose	Makimura *et al*. 2002
Type I diabetes		inhibiting autoimmune or inflammatory destruction in the pancreatic islets for transplantation				inhibits apoptosis of insulin-producing cells in islet cells	Castano and Eisenbarth 1990

be treated with RNAi drugs due to the relatively ease of accessibility of the blood cells. A recent *Drosophila* genome-wide RNAi screen identified the putative human genes essential for influenza virus replication (Hao *et al.* 2008). Similarly, the human factors responsible for growth of AIDS virus, West Nile virus etc. have been screened (Krishnan *et al.* 2008; Zhou *et al.* 2008). Such screens in human cell lines are now routinely used in cancer biology and have helped identifying new components of the p53 pathway and pinpointing new targets for cancer therapies.

Recent evidence has shown that changes in miRNA expression also correlate with various human cancers (Liu *et al.* 2004; Monticelli *et al.* 2005). At least 13 miRNAs have been found to be associated with the prognosis and disease progression in chronic lymphocytic leukemia (Calin *et al.* 2005). The miRNA-17 cluster (consisting of six miRNAs: miRs-17-5p, -18, -19a, -19b, -20, and -92) located on human chromosome 13q31 provides the most comprehensively studied example (Hayashita *et al.* 2005; He *et al.* 2005). The miRNAs have been shown to repress the expression of important cancer-related genes. In fact miRNAs such as *let-7*, which has been shown to negatively regulate the Ras oncogenes, and miR-15 and miR-16, which negatively regulate *BCL2*, are promising candidates for cancer treatment (Calin *et al.* 2005; Johnson *et al.* 2005). They also play a significant role in the development of different forms of cancer. Thus miRNAs can function either as tumor suppressor or as oncogenes and thus have been referred as "Oncomirs" (Cimmino *et al.* 2005; He *et al.* 2005; O'Donnell *et al.* 2005; Esquela-Koscher and Slack 2006).

Similarly the identification of miR-122 as a regulator of cholesterol biosynthesis has opened the possibility of antagomiR approach, which is being exploited for development of potential therapeutic agent in cardiovascular diseases. This suggests that technologies that allow the specific inhibition of miRNA function could have a future role to aid in the diagnosis and treatment of cancer and a number of other diseases like virus infections. The RNAi-mediated down-regulation of the HIV replication as well as of the cellular cofactors required for infection is being studied as an attractive alternative approach for treatment. Systemically administered siRNAs to silence endogenous ApoB gene are being developed as drugs for prevention and management of coronary artery disease and stroke (Soutschek *et al.* 2004). Strategies for successful systemic delivery of siRNA are still in a preclinical stage of development. Despite several challenges, locally administered siRNAs have already entered the Phase-I or -II clinical trials. The first clinical trials of RNAi drugs were launched in 2004 to target an eye disease, AMD, and have shown promising results. **Table 4** lists some of the most promising developments in the medical field.

Currently there are several biotechnology companies that are developing RNAi-based drugs for clinical use. RNAi can also revolutionize the development of novel therapeutic strategies for disorders that are yet difficult to treat such as obesity, neuropathic pain and depression (Rondinone 2006). It may also be used to making adaptive alterations to human biochemistry to better withstand or repair the cellular damage at the root of aging. Irrespective of its therapeutic potential, RNAi has unambiguously become a valuable tool for basic research in biology and thereby it will continue to have a major impact on medical science.

Veterinary sciences

RNAi has made technological impact in the field of veterinary science as well by providing a therapeutic approach for functional genomics of livestock, studying host-pathogen interaction and treating diseases (Anthony and Cantlon 2007). It is being developed into a biotechnological tool to generate farm animals that are less susceptible to infectious disease as well as are better in meat-production trait. The bovine trophoblast cell line has proved to be an excellent model system to profile gene expression, understand embryogenesis, follicular growth and development (Hirano *et al.* 2004; Ushizawa *et al.* 2004; Yao *et al.* 2004; Paradis *et al.* 2005).

RNAi technology is being developed as prospective therapy for many veterinary diseases (**Table 5**). RNAi is being applied to control prion diseases of Livestock, which are of major concern to human health as they can get transmitted through red meat consumption. Another area of potential application lies in treating the viral diseases of livestock like Foot and Mouth Disease (FMD) that cause huge economic losses. The current FMD treatments face the limitation to induce rapid protection (4 to 5 days post-vaccination), due to a critical role for innate immune defences. However, shRNA directed against viral structural protein (1D) or polymerase (3D) have been found to effectively protect swine IBRS-2 cells. Recombinant, replication-deficient adenovirus vectors carrying the siRNAs could significantly reduce susceptibility in guinea pigs and swine to FMDV infection (Chen *et al.* 2004; Kahana *et al.* 2004; de los Santos *et al.* 2005).

In May 2007, scientists at CSIRO's Australian Animal Health laboratory selected RNAi as a tool to establish a real- and impervious-firewall between wild birds and the

Table 5 Applications of RNAi in veterinary sciences.

Disease	Animal	Gene targeted		mode of siRNA delivery	Status	Remarks	Reference
		Name	Function				
Foot and mouth disease	Livestock (all cloven-hoofed animals)	VP1	Structural protein 1D	adenoviral vectors (shRNA)	BHK-21 cells	80-90% reduction in infection	de los Santos *et al.* 2005; Chen *et al*. 2006
		Polymerase 3D	Replication protein	adenoviral vectors (shRNA)	guinea pigs and swine	2/3 swine show reduced susceptibility	
Feline immuno-deficiency	Feline (Cat and cat-like) animals	FI virus gag region		siRNA transfection	Feline fibroblastic c e l l line	Reduction in the virus replication	Baba *et al*. 2007
				retroviral vectors (shRNA)	Feline T-cell line	Inhibition of virus replication	
Bovine viral diarrhea	cattle herds	5′ non-translated region region encoding the C, NS-4B and NS-5A proteins of the virus		Transfection with plasmid vectors expressing shRNA undergo bovine 7SK promoter		Suppress the virus replication	Lambeth *et al*. 2007
Peste des petits ruminants and rinderpest	Domestic and wild ruminants	Nucleocapsid (N) genes		Lipofectomine mediated siRNA transfection	Vero Cell lines	Inhibition of virus replication	Servan de Almeida *et al*. 2007
Avian leucosis	Poultry	host gene, tvp viral gene, env	receptor of ALV Envelope protein	reteroviral vectors (shRNA)		Reduction in the target gene	Chen *et al*. 2007

$300 billion global poultry industry. This would also help prevent virulent strains circulating in poultry, such as the influenza strain H5N1, from directly infecting humans. The strategy involves targeting highly conserved gene sequences common to all subtypes and strains of influenza, including any that the virus might conjure from its genetic diversity prevalent in wild shorebirds. The approach involves delivering siRNAs to chickens via drinking water or an aerosol spray for priming the birds' innate RNAi defences to recognize and destroy the virus.

Agriculture

RNAi has been mainly used as a tool to decipher functions of genes and identify pathways that can be manipulated to protect plants from environmental perturbations and pathogen attack with an aim to improve crop yields. In fact RNA silencing machinery is activated as a natural defense mechanism of plants against viruses. Transgenic plants exhibiting resistance to economically important viruses have been engineered and commercially released in the market. These include a variety of papaya resistant to Papaya ring spot virus (PRSV) that was released in the late 1990s in Hawaii. Since then, many different crop plants like barley and turnip have been protected against a whole range of viruses using constructs that initiate a PTGS response (Fuchs and Gonsalves 2007). Recently, Monsanto has prepared two varieties of potato; New Leaf Plus and New Leaf Y, resistant to Potato leaf roll virus and PVY, respectively (www.research.cip.cgiar.org) for commercial release.

RNAi technology has been applied for restructuring specific metabolic pathways to improve the nutritional quality by altering the oil content to modifying starch composition. The technique has also found application in removal of undesirable metabolites from poppy, coffee beans and cotton seed. Some of the applications have been detailed in **Table 6**. An interesting application has been in engineering plant-produced antibodies, for treatment of cancer, autoimmune and inflammatory diseases. However, production in plants causes glycosylation of the Fc region of the antibody. This might compromise its functional abilities and may also be immunogenic. To combat these potential problems, RNA silencing has been used to silence two endogenous glycantransferase activities in the green alga, *Lemna*, for producing therapeutic antibodies (Cox *et al.* 2006).

In fact the scientific marvel in creating a blue rose could not have been possible without RNAi. Roses lack a critical enzyme for the biosynthesis of delphinidin-based anthocyanins, which are the major constituents of violet and blue flowers. When the gene encoding this enzyme in Viola was transferred to roses, its expression resulted in the generation of transformed plant lines with purple petals, because one of the rose enzymes converts the intermediate compounds into red and yellow pigments. However, silencing the endogenous gene gave transformed rose plants that bore flowers with pure blue hues (Katsumoto *et al.* 2007).

CONCLUSIONS

RNAi's ascent from 'bench to bedside' has been meteoric and the pharmaceutical biggies have been hectic in smelling the big profits in this process while outpouring the appropriate expertise. A good account of the RNAi technology of important commercial companies can be found in a product related article that appeared in the journal Science (Perkel 2007). According to an official estimate "RNA therapeutics are forecast to generate sales of around $1bn by 2015 and this market has significant potential, should companies be able to overcome delivery constraint" (www.globalbusiness insights.com). We have already mentioned a few tricks to handle the delivery problems and many more solutions certainly are on their way. As we will know more about the recognition motifs of cells/tissues/organs, the delivery principles will be more improved and specific. With clever bioinformatics along with strong chemistry, the problems related to off-target effects, the instability of siRNAs and so on can be minimized. The genome-wide RNAi screening techniques have become more versatile and improved, thus providing opportunities for discovery and validation of 'smarter targets', which would impact strongly on the drug discovery processes. There are also scopes to employ the principles of transcriptional gene silencing that have not been reported so far in literature in a significant manner. Considering all the pros and cons together, it will not be unwise to claim that the RNAi technology will capture a sizable portion of the drug and agricultural market soon and it would go on for miles towards developing a disease-free and hunger-free world.

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Table 6 Applications of RNAi in plant sciences.

Pathogen/ Trait	Organism/ Pathway	Plant	Gene targeted		Mode of delivery	Status	Remarks	Reference
			Name	Function				
Pathogen resistance								
Virus	*Barley yellow dwarf virus*	Barley	5' end of the (BYDV)		hpRNA- construct under the maize ubiquitin promoter	Commercial production	virus protection in barley lines.	Wang *et al*. 2000
	Turnip yellow mosaic virus	Turnip	P69, HC-Pro	viral suppressor	amiRNA expressing transcript (miR159a pre-miRNA)	Commercial production	Resistance against virus infection	Niu *et al*. 2006
	Cucumber mosaic virus	Tobacco	CMV 2b	viral suppressor	amiRNA vector	Experimentally demonstrated	resistance to CMV infection	Qu *et al*. 2007
Agrobacterium		Tomato	tryptophan mono oxygenase (iaaM) and isopentenyl-transferase (ipt)	Oncogenes required for Tumor formation	hpRNA constructs		highly resistant to crown gall disease	Escobar *et al*. 2001
Bacteria	*Erwinia amylovora*	Apple	DspE-interacting kinases	Host enzymes	Sense suppression		Resistance to infection	Boureau *et al*. 2006
Parasitic nematodes	*Meloidogyne* spp. (root-knot nematode)	Tobacco	Vital nematode genes		hpRNA constructs	Experimentally demonstrated	Resistance against parasite	Fairbairn *et al*. 2007
	Heterodera schachtii (cyst nematode)	Legumes	Host Sucrose transporter genes			Experimentally demonstrated	reduction of female nematode development	Hofmann *et al*. 2008
Pest resistance	*Helicoverpa armigera* (bollworm)	Cotton	CYP6AE14	cytochrome P450		Experimentally demonstrated	pest is susceptible and the plant resistant to gossypol	Mao *et al*. 2007
Reshaping metabolic pathways								
Pesticide resistance		Tomato	ACC oxidase	ethylene metabolism	Hairpin construct with intron used under 35S promoter		Plants are resistant to herbicide	Grossmann and Ehrhardt 2007
Improved nutritional quality	Reducing palmitic acid	Cotton	D9 and D12 desaturases	Synthesis of palmitic acid	hpRNA constructs		Increase the oleic acid or stearic acid	Liu *et al*. 2002
	Fatty acid metabolism	Soybean	omega-3 fatty acid desaturase (FAD3)		Inverted repeat of conserved 318-nt (common to the three gene family members) under a seed-specific promoter		poly-unsaturated fatty acid like -linolenic acid (18:3) cause instability of seed oils	Flores *et al*. 2008
	Glutelin synthesis	Rice	Glutelin gene	the major storage protein	hpRNA from an inverted repeat	First commercially useful cultivar	low-protein rice line LGC-1(Low Glutelin Content-1), useful for patients with kidney disease	Kusaba *et al*. 2003
	Lysine production	Corn	lysine-ketoglutarate reductase/ saccharophine dehydrogenase (LKR/SDH)	bifunctional lysine degradation enzyme	inverted repeat against LKR/SDH with intron and over expression of a de-regulated lysine biosynthetic enzyme, CordapA		Enhancing the Lysine content would improve nutritional value	Frizzi *et al*. 2008
	Amylose production	Potato	Granule-bound starch synthase I gene		sense- and antisense suppression		reduced amylose contents in tuber, useful for diabetics	Wolters and Visser 2000
	Starch composition	Wheat		isoform of a starch-branching enzyme	hpRNA constructs		altering its amylose-amylopectin ratio, to reduce the incidence of cardiovascular disease and colon cancer	Regina *et al*. 2006
	Carotenoid and flavonoid synthesis	Tomato	DET1	photomorpho genesis regulatory gene	Hairpin construct under fruit specific promoter		resulted in significant increase in both carotenoid and flavonoid content	Davuluri *et al*. 2005
	Lycopene (carotenoid) production	Tomato	lycopene cyclase (Lcy)	regulates the transformatio n of lycopene	Ihlp construct targeting the 3' end of Lcy gene under phytoene desaturase (Pds) promoter		Lycopene, is highly beneficial in preventing cancer and heart diseases	Wan *et al*. 2007

Table 6 (Cont.)

Pathogen/ Trait	Organism/ Pathway	Plant	Gene targeted		Mode of delivery	Status	Remarks	Reference
			Name	Function				
Reshaping metabolic pathways								
Removal of endotoxin/ undesirable metabolite	morphine pathway	Poppy	multigene COR family		A chimeric hairpin RNA construct		Lowers the amount of Morphine and high accumulation of non-reticuline	Allen *et al*. 2004
	Caffeine removal	Coffee	MXMT (7-N-methylxanthine methyl-transferase); theobromine synthase		Hairpin construct under 35S promoter		30-50% reduction of theobromine and caffeine	Ogita *et al*. 2004
	Gossypol removal	Cotton	cadinene synthase (ihp)		An intron-containing hairpin transformation construct using the pHANNIBAL/pART27 system		Cottonseed utilization by decreasing cardio- and hepato-toxic terpenoid gossypol	Sunilkumar *et al*. 2006
	lignin reduction	forage grass	*O*-Methyltrans-ferase		Antisense suppression		Facilitates digestion in animals to improve livestock performance	Ye *et al*. 2001
Post harvest technology	Enhanced shelf life	Tomato	Polygalac-turonase		double-stranded RNA	Flavr Savr™ tomatoes	Exhibits delayed fruit softening	Sheehy *et al*. 1988
Bio-fermentor	Influenza Vaccine	Influenza virus	NS1 gene		dsRNA		virus-specific siRNA was harvested from the plant and introduced into human cells, where it successfully inhibits viral replication.	Zhou *et al*. 2004
Aesthetic use	Blue Rose	Rose	down-regulation of endogenous dihydroflavonol 4-reductase (DFR)	conversion of delphinidin anthocyanin	dsRNA		Over-expression of flavonoid 3',5'-hydoxylase and *Iris* DFR gene along with down-regulation of endogenous DFR gene	Katsumoto *et al*. 2007

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