

# RNA interference

## *a technology platform for target validation, drug discovery and therapeutic development*

In the post genomic era, elucidation of the physiological function of genes has become a major rate-limiting step in the quest to develop 'gene-based-drugs'. As we advance in the 'functional omics' arena with a hope to discover novel drug targets and therapies, their validation is a pivotal step before clinical practice. Such an endeavour can be tested using small interference RNAs (siRNAs) or RNA-mediated genetic interference (RNAi). This elegant and revolutionary reverse genetic approach has a tremendous commercial promise to develop new drugs and therapeutics for several human diseases. This review outlines and forecasts some of the potential applications of gene suppression strategies in the pre-clinical drug discovery process at biotech and pharmaceutical industries.

Most of you are aware of the terms 'Genome' (study of expression of all the genes in an organism called as Genomics), 'Proteome' (study of expression of all the proteins in an organism called as Proteomics), and 'Glycome' (study of expression of all the glycoproteins in an organism called as Glycomics). Another scientific buzz word is spreading fast in the research community, that is 'RNome', the RNA equivalent of the 'proteome', 'genome or 'glycome'. The subject is referred to as 'RNomics' (Figure 1). RNomics is a newly emerging field that categorically studies the structure, function and processes of noncoding RNAs (ncRNAs) in a cell<sup>1</sup>. The first ncRNAs were identified in the 1960s. At that time, biologists thought these ncRNAs had no function in the cell. However, these ncRNAs (in general about 20 to 500 nucleotides in length, some are as long as 17kb) have been shown to be involved in the processes of replication, transcription, RNA processing, modification, mRNA

translation, gene silencing, protein stability and protein translocation<sup>2</sup>. Using comparative genomics, computational, cDNA library construction and microarray expression approaches different groups have discovered several hundreds of small, single-stranded RNAs in organisms including archaeons, parasites, worms, fruit flies, yeast, plants, and mammals<sup>3</sup>. These ncRNAs are divided into two classes based on their functions: first are microRNAs (miRNA), usually ranging from 21 to 23nt in length and believed to specifically regulate translation of target mRNA in a stage-and/or tissue-specific manner. The second class of miRNAs are generated by enzymatic cleavage of long double-stranded RNAs and tends to degrade the target mRNA by a process known as interference<sup>4</sup>. Therefore, these specific ncRNAs are referred to as small interfering RNAs (siRNAs), and the process mediated by siRNAs is RNA interference (RNAi)<sup>4</sup>.

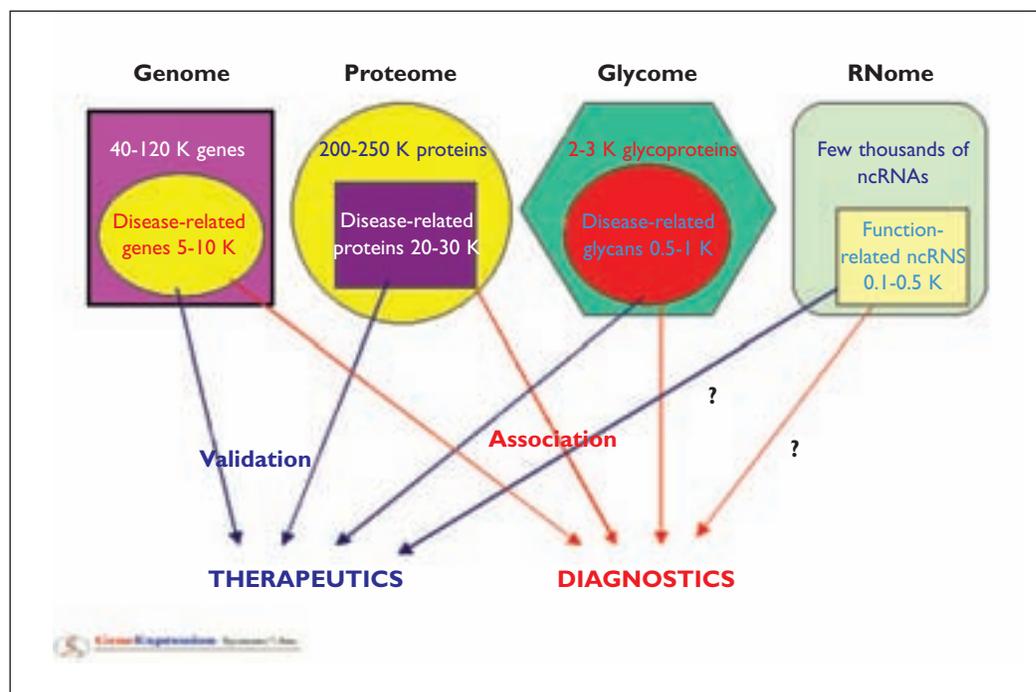
We are now beginning to exploit the information gleaned from genome sequencing projects of

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**Figure 1: The functional 'OMES'**

Based on the discussion given in reference 5, the number of genes were postulated. Approximately 5-10% of the genes might be associated with disease relevance and could be used in disease diagnosis and therapeutic development after validation. The total number of ncRNAs present in the human cells has to be discovered and their relevance to disease process has to be demonstrated



human and several organisms. However, this massive genetic information opens new challenges to decipher the complete list of protein-coding genes. In addition, transcriptional events such as RNA splicing and post-translational modifications make it difficult to predict the exact number of genes or proteins (Figure 1). With this degree of complexity, monitoring the entire proteome expression levels as a means to elucidate their functions and develop them as drug targets is a challenging paradigm in the bio-industry. Despite the 'proteome' sequencing efforts, the 'RNome' also has to be studied in-depth to fully understand and tally the number of genes encoded by a genome and their regulation<sup>2</sup>. The challenge for scientists in both academia and industry is to identify the whole complement of ncRNAs and elucidate their functions in gene expression and regulation. In terms of healthcare, it is important to identify the disease relevant genes from these functional 'OMES'. Although, the human genome sequencing was completed, the total number of genes in the human genome is still in debate<sup>5</sup>. A possible hypothetical number of genes associated with various diseases are shown in Figure 1.

### RNA interference

Five years ago, Mello and his colleagues discovered the phenomenon of RNAi<sup>4</sup>. Since then, it erupted like a volcano in the cell and molecular biology communities as a tool to understand gene expression and

regulation. In addition, scientists have begun to take an 'RNomics' approach to understanding the nature and function of microRNAs and siRNAs in order to utilise them as a gene silencing mechanism. In general, RNAi processing involves the cleavage of longer dsRNAs present in the cytoplasm by an enzyme called DICER into small interfering RNAs, roughly 21-23 nucleotides in length. These siRNAs then become incorporated into an RNA/protein complex (known as RNA-induced silencing complex, (RISC)), which acts to recognise a target mRNA for subsequent degradation<sup>6,7</sup> (Figure 2A). The siRNAs present in the cytoplasm can also cause post-transcriptional silencing in cytoplasm, and also enter the nucleus, and affect DNA methylation (Figure 2B). In broad terms, 'Post-transcriptional gene silencing', 'co-suppression', 'quelling' and 'siRNA' are collectively included in the phenomenon of 'RNA interference'. Although, the mechanisms and processes are similar, but not quite identical, common sets of proteins and short RNAs are utilised<sup>7</sup>.

The function of this process in the cell is believed to serve as a protective mechanism for the genome against viruses and transposable elements and to eliminate defective mRNAs. This process is highly conserved in the evolution and observed in viruses, parasites, worms, fruit-flies, plants and animals<sup>8</sup>. A couple of years ago, Tuschl and his colleagues for the first time demonstrated gene silencing in mammalian cells by transfecting the synthetic siRNA molecules<sup>9</sup>. Presence of dsRNA

in mammalian cells provokes strong cytotoxic response, and the effect is transient. Therefore, to overcome this limitation, several groups developed DNA-based siRNA vector systems to analyse gene function in a variety of mammalian cell types<sup>10</sup>. As an example, a DNA-based vector that carries fluorescently (cyanine 3) labelled siRNA sequences for human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was exogenously transfected into mammalian cells and scored for its protein expression using an antibody against GAPDH. The results shown in **Figure 2 II** (green) indicated that the expression of GAPDH was completely inhibited in the experimental (A) compared to its control (B). Current approaches to create stable phenotypes in mammalian cells have generally met with limited success. However, newly developed RNAi methods and the availability of DNA-based vectors have the potential to provoke a revolution in molecular biology.

**Applications of RNAi**

The discovery of siRNAs and RNAi mechanism has a tremendous commercial potential in the bio-industry. This molecular tool will permit investigators to routinely implement ‘loss-of-function’ screens and helps to develop rapid tests for genetic interactions in mammalian cells, which up to this point have been quite difficult to perform quickly. Some of the applications are shown in **Figure 3**, and described as follows:

**Functional genomics**

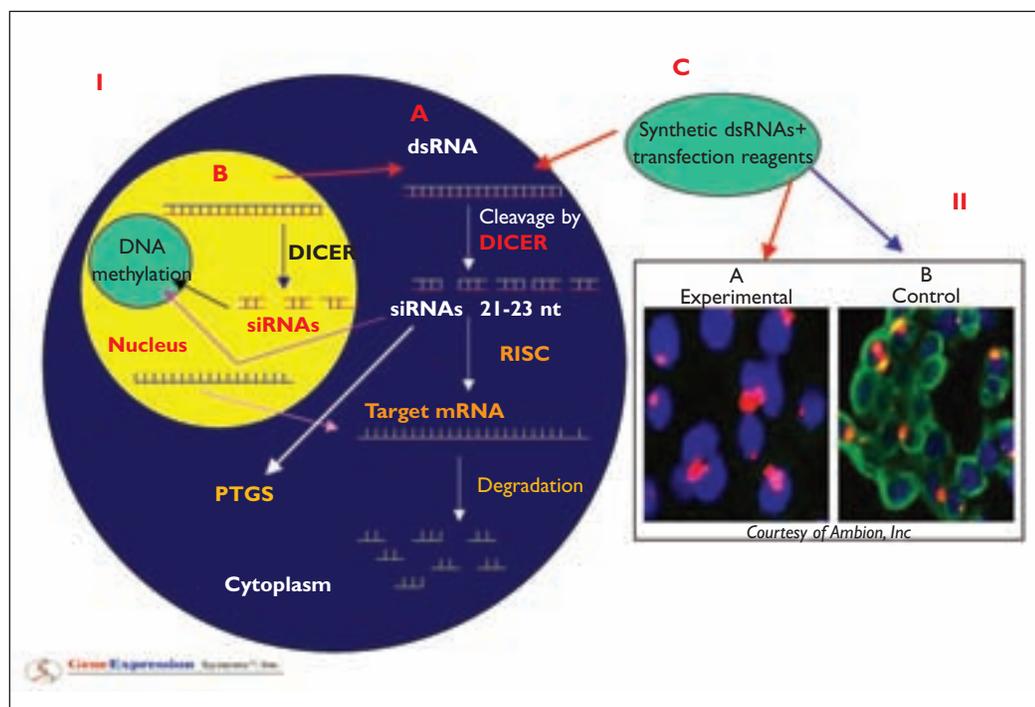
The RNAi approach has been applied to study the function of several essential genes involved in cell growth, cell cycle, cytoskeleton, signalling, membrane trafficking, transcription and DNA methylation. The functions of these genes were studied in about 25 different mammalian cells either by incorporating synthetic siRNAs or using cloned plasmids that carry siRNA sequences<sup>11,12</sup>. Using an RNAi-based strategy one can target the gene product itself that might be central to the cellular or disease process. Several biotech companies have started or adopted RNAi platform technology for functional genomic studies (**Table 1A**). Several reagent companies now offer products ranging from synthesis of siRNA molecules, cloning vectors and transfection reagents (**Table 1B**). In addition, several pharmaceutical companies are racing ahead in launching this technology platform in their existing functional genomics divisions. The biggest advantage of the RNAi platform is that it is highly adoptable to a high throughput format significantly reducing the product development cycle and enhancing the target validation and drug development process (**Figure 4**).

**Genome-wide screenings**

A whole genome RNAi strategy has been used in high throughput phenotypic screens to identify several hundred genes that are involved in the cell cycle, embryonic or germ-line development, ovary

**Figure 2: Gene-silencing mechanism in the cell**

**I** Diagrammatic representation of RNAi molecular process in the Cell: The process can occur in both cytoplasm and nucleus (**A, B**). Generally, double-stranded RNAs could be transported from nucleus to cytoplasm or can be exogenously transfected (**C**). These will undergo catalytic cleavage by an enzyme DICER and yield small siRNA molecules. These siRNAi molecules bind with RISC and degrade the intact mRNA molecules those are transported from nucleus to cytoplasm. siRNA molecules shuttle in between nucleus to cytoplasm and affect DNA methylation in nucleus, and also cause post transcriptional gene silencing in cytoplasm. **II** siRNA silencing of GAPDH gene expression in mammalian cells: siRNA to human GAPDH (**A**) and a scrambled control (**B**) were fluorescently labelled with cyanine 3 (green label) using a DNA vector-based labelling system, transfected into HeLa cells and analysed by fluorescent microscopy with an anti-GAPDH antibody. Red: cyanine 3 labelled siRNA; Blue: DAPI stained nuclei; Green: antibody to GAPDH detected with a fluorescein labelled secondary antibody. Scrambled control has no effect on GAPDH protein expression (**B**) where as in experimental (**A**), GAPDH expression was totally inhibited (Courtesy of Ambion, Inc)





**Figure 3**  
Applications of RNAi

and vulva specific in *Caenorhabditis elegans*. Concurrently, four independent groups looked at the function of nearly all the genes in *C. elegans* using high throughput RNAi analysis at the whole genome level<sup>13</sup>. Instead of knocking down a single gene at a time, which could take a year of effort, the RNAi method allows scientists for the first time to knockout every gene in an organism in a few months. Recently, in a collaborative approach, scientists constructed a double-stranded RNAi bacterial library with 86% of the 19,000 *C. elegans* and disrupted the expression of 16,757 worm genes by high throughput RNAi method. This method allowed them to isolate several hundred genes involved in body fat gene regulation<sup>14</sup>. Using a fluorescent dye in the worm's bug diet, this group also identified the human counterpart genes involved in the signal transduction process and their targets. Some of the newly identified targets/genes will be ideal candidates for developing drugs to treat obesity and diabetes. The high throughput capacity of RNAi makes it a particularly attractive method for rapid screening and validation of targets identified by microarray analyses, protein-protein interactions or *in silico* gene prediction. In a nutshell, this 'genome-wide RNAi screens' strategy can be combined with cell-based assays and other methods to elucidate the functions of all the human genes (Figure 4).

### Target validation

Target validation is one of the biggest problems for the biopharmaceutical industry. RNAi offers the prospect of reducing this bottleneck and speeding the drug development process. Target validation determines whether a known candidate gene is responsible for a disease and whether altering expression of the gene is likely to result in a therapeutic effect. Functional genomics and target validation are critical to providing pharmaceutical and biotechnology companies with new gene targets involved in the disease processes. These companies then look to modify the gene or gene products of such targets to treat disease using their drug discovery and development platforms. These products will include high-throughput applications with the potential to industrialise gene function analysis, which should dramatically improve the pharmaceutical industry's ability to identify 'druggable' gene families or targets. RNAi-based target validation will enable companies to fast track the discovery of drug targets in short period in a more cost-effective approach (Figure 4).

### Drug screening and development

Selection and validation of molecular targets is of great importance for drug development in the post-genomic era. Although phenotypes of many diseases are well known, the identification of the genes responsible for these phenotypes is a major challenge in the drug development process. The RNAi technology offers an alternative method to achieve this goal in a rapid and more economical way. One can use a library of several hundred to thousands of chemical compounds and identify candidate target genes through transcriptional expression profiling in a chemical genomics approach. Subsequently, the function of the several target genes identified for a specific chemical compound can be evaluated in a high throughput manner using RNAi transfections directly into micro-titer plates, seeded with mammalian cells. In addition, RNAi could facilitate drug screening and development by identifying genes that can confer drug resistance or genes whose mutant phenotypes are ameliorated by drug treatment. This approach will not only allow for determining the modes of action for novel compounds, but also helps to develop a new generation of antibiotics. RNAi methods could be extended to study gene expression of insect and parasite genomes and subsequently develop better gene-based insecticides or infection controlling drugs.

**Anti-viral therapeutics**

The gene silencing approach holds great promise for selectively inhibiting virus-specific genes or host genes for the treatment of viral infections or autoimmune disorders. Some of the examples are stated here.

**HPV:** Human papilloma viruses cause cervical cancer in women. Cervical cancer is the second most common form of cancer in women after breast cancer. In general, during the virus lifecycle, the virus produces proteins that suppress the activity of genes in the human anti-cancer defence system. Therefore, suppressing the HPV encoded viral gene products could help to inhibit the growth of cancer simply by allowing the virally infected cell to undergo apoptosis or cell death. This approach is followed in conjunction with RNAi to knock-down the function of several HPV coded viral proteins<sup>15</sup>.

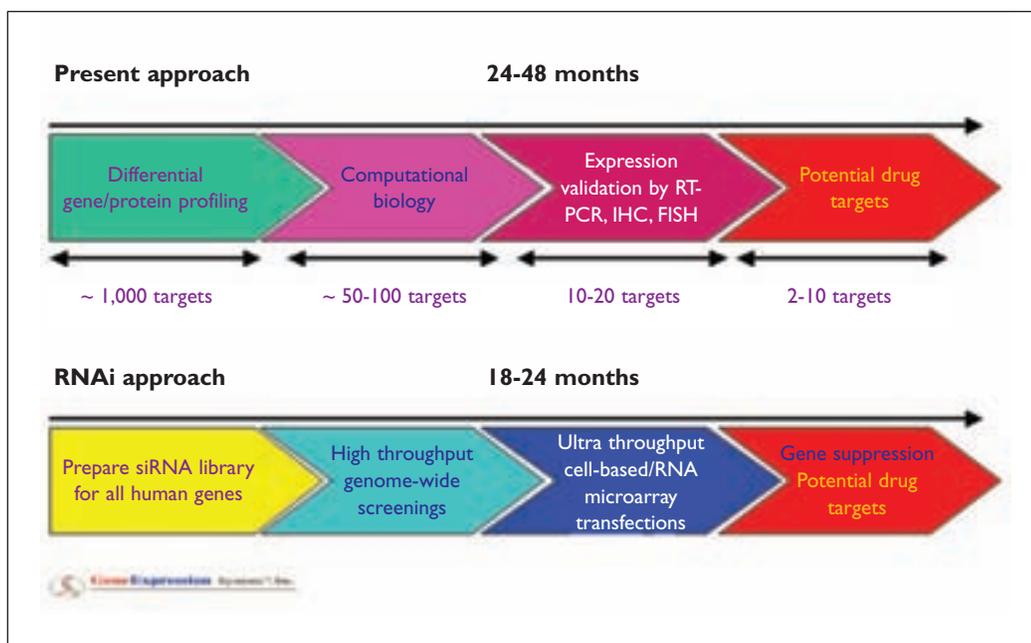
**HCV:** Hepatitis C virus (HCV) infection is an emerging global epidemic. Since adequate animal models or tissue culture systems for the propagation of HCV are not available, the development of therapeutic and preventive strategies is an alarming challenge for biotechnology and pharmaceutical companies. It is now possible for scientists to suppress selectively the host/viral genes involved in the replication of virus. Using RNAi, scientists systematically suppressed the function of cellular genes (those required for HCV replication), which are involved in host-cell interactions and viral mor-

phogenesis<sup>16</sup>. Very recently, the siRNA approach has also been adopted to target the host Fas protein to reduce severe forms of hepatitis in a mice model<sup>17</sup>.

**HIV:** Several groups applied an RNAi approach to specifically inhibit the replication of human immunodeficiency virus (HIV) by targeting siRNAs to viral (p24, vif, nef, tat and rev) or cellular genes (CD4, CXCR4, CCR5) and expressing them in human cell lines, primary lymphocytes, and primary macrophages<sup>18-20</sup>. This RNAi-based gene therapy for HIV infection is not only an effective way to inhibit viral replication, but also can be extended to block the infection of several other animal viruses. Therefore, this particular area opens new avenues for gene-based therapeutics.

**Anti-cancer cancer therapeutics**

Gene expression profiling methods brought a new revolution in the classification of tumours and helped to develop new prognostic indicators for studying various forms of cancers<sup>21</sup>. However, the detailed study of individual genes and proteins remains critical in terms of basic science and in generating new therapeutics. Gene suppression by siRNAs is a powerful tool to analyse the function of proteins *in vitro*, especially, for the rational design of drugs to block the tumour-relevant genes. Several oncogenes have already been cloned into siRNA-based vectors and stably expressed, and gene suppression was studied in detail<sup>22</sup>. RNAi can be easily applied to hormone-regulated



**Figure 4**  
Target validation strategies

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Continued on page 68

**Table 1:** RNA interference focused companies

<b>A R&amp;D-ORIENTED COMPANIES</b>	<b>B REAGENT DEVELOPMENT COMPANIES</b>
Alnylam Pharmaceuticals, Cambridge, MA Anadys Pharmaceuticals, San Diego, CA Benitec Australia, Ltd. St. Lucia, Australia Cenix Biosciences, Dresden, Germany Ingenium Pharmaceuticals, Martinsreid, Germany ISIS Pharmaceuticals, Carlsbad, CA Nucleonics, Inc. Malvern, PA Ribopharma AG, Kulmbach, Germany Ribozyme Pharmaceuticals, Inc. Boulder, CO Sequitur, Inc. Natick, MA	Ambion, Inc. Austin, TX (Cloning Vectors) BD Biosciences Clontech, Palo Alto, CA (Cloning Vectors) Dharmacon, Inc. Lafayette, CO (siRNA synthesis) Imgenex, Inc. San Diego, CA (Cloning Vectors) Invitrogen, (Transfection reagents) Mirus Corporation, Madison, WI (Transfection reagents) OligoEngine, Inc. Seattle, WA (Cloning Vectors) Polytransfection, Cedex, France (Transfection reagents) Promega Corporation, Madison, WI (Cloning Vectors) Qiagen, Inc. Valencia, CA (Transfection reagents)

growth of breast cancer cells and estrogen-induced cell cycle progression, specifically targeting the inhibition of transcription factors (such as Sp1, NFkB) and expressing them in human breast cancer cell lines.

The ability to engineer siRNA vectors for stable expression of mutated tumour suppressor genes, oncogenes and transcription factors in human cancer cell lines will certainly sparkle a conflagration of effort to evaluate their advantage as a cancer prevention method. The ability to create 'permanent' 'knock-down' cancer cell lines will help us to understand the 'loss-of-function phenotype' and subsequently develop commercially important cancer preventive targets. In addition, this study will dramatically facilitate the dissection of signalling pathways and the study of cell growth and division in order to understand the biology of cancer. Several researchers have already demonstrated expression of exogenously infused siRNA in living mouse and embryonic chick models. Very recently, this reverse-genetics approach has been adopted to study modulation of the polyglutamine repeat associated with Huntington's disease, a neuro-degenerative disorder using viral promoter-based vectors and direct injection into mice embryos. The foreseeable challenge is for us to analyse how these gene suppression systems work directly in human cancer tissues, and ultimately to develop gene-specific therapeutics.

### Integrated networks

In the not too distant future, the research community intends to integrate functional genomics and proteomic mapping approaches to reveal the biological functions of all the coding genes in the human genome. To reach that level, it is pre-requi-

site to combine the 'interactome' (protein-protein interactions) mapping data with 'phenome' (large-scale phenotypic analysis) mapping data. Recently, this approach was demonstrated by integrating the transcriptome data (that was gathered by microarray experiments) with that of phenome mapping data collected by high throughput RNAi analysis of the germ line genes from *C. elegans*<sup>23</sup>. The combination of informatically-driven gene identification, established functional genomics methods, and now the RNAi transfection of mammalian cells can be extended to study the integrated networks in human cells or tissues with the potential of deducing the functions of dozens to thousands of proteins at a time.

### RNAi in agri-biotech industry

The RNAi work carried out in the plant Arabidopsis opened new avenues to produce not only new varieties of plants but also to prevent plant virus infections<sup>24</sup>. This strategy is expected to be especially highly useful in the agri-biotech industry to study plant host-virus interactions.

### Bottlenecks in RNAi research

**siRNA primer design and sequence specificity:** siRNAs may be the best tools for target validation in biomedical research today because of their exquisite specificity, efficiency and endurance of gene-specific silencing. However, design of siRNAs and the secondary structure of the mRNA target strongly play a role in the gene-silencing phenomenon. In addition, incorporation of mismatches in the siRNA sequence will also affect gene suppression<sup>25</sup>. A single point mutation in the targeted region abolishes the mRNA degradation and may cause RNAi resistance in tumour cell lines.

Continued from page 66

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**Potency, efficacy and duration:** The dosage and concentrations of siRNAs can also play a significant role in the gene expression inhibition. Another interesting concern is time duration that siRNAs can inhibit the expression of a target genes in particular tissues or cell lines also varies.

**siRNA delivery problem:** Although, several companies (Table 1B) market different types of transfection reagents for the *in vivo* and *in vitro* delivery of siRNA molecules into tissues and mammalian cells, the efficiency of transfection varies greatly from cell line to cell line and tissue to tissue. Therefore, a new generation of transfection reagents based on either novel cationic lipids or beta cyclodextrin-containing polymers are needed to increase this efficiency. Probably, siRNA molecules complexed with these new reagents could better penetrate through cell membranes and reach target sites efficiently. Most importantly, a new generation of transfection reagents needs to be developed that shows less toxicity to cells. Electroporation methods can be employed to deliver siRNA-containing plasmids directly into tissues, though this is very difficult *in vivo*. Gene silencing is possible in brain cells, the genes those specifically expressed in neurons are difficult to silence.

**Endogenous:** In a therapeutic context, the neutralisation of exogenously transfected siRNAs by the immune system in the cells may also be a foreseeable problem.

We hope that all these approaches will help to develop new diagnostic reagents and novel molecular interventions for several human diseases. Within 10 years RNAi will undoubtedly emerge as a routine molecular tool to study the problems in biomolecular medicine and potentially treat diseases.

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The opinions expressed in this article are exclusively those of the author and do not reflect those of GeneExpression Systems, Inc. Due to the space constraint the author has omitted several papers of others to cite and limited to recent reviews. Although several other companies are involved in RNAi research, the author has cited few representative companies only but not intentionally ignored others. The author thanks Professor Steven R. Gullans of Harvard and CSO of USGenomics, Woburn, MA for reading the manuscript and providing valuable comments. The

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