RNA interference: An Ancient Mechanism for Novel Therapeutics

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Citation

Abstract
The siRNA approach for gene silencing holds great therapeutic promise, since siRNAs, like miRNAs, are naturally used by cells to regulate gene expression and hence are expected to be nontoxic and highly effective. Their therapeutic potential can be exploited maximally only when the mechanism underlying their localized and systemic activity is fully understood. Lower eukaryotes provide good model systems yet species specificity leading to minor differences at mechanistic level, point to their being investigated in mammalian milieu. In vitro cellular level studies have proven the therapeutic potential of iRNA to a larger extent. At present only one topical drug Vitavene targeting AMD is in market and several other RNAi based drugs are in the pipeline.

INTRODUCTION
Protein biosynthesis is a very finely regulated process in living systems more so in eukaryotes. The process involves a number of steps, beginning with the copying of the gene, in the nucleus; into messenger RNA (mRNA) molecule by transcription. This is followed by the message being decoded and translated into protein in the cytoplasm. A typical mRNA produces approximately 5,000 copies of a protein. The proteins run the cellular machinery with a clockwork precision but when these same proteins are mutated or synthesized out of context i.e. in a cell that does not need them, a disease condition like genetic disorder or cancer results. Till recently in such situations two options were open for pharmaceutical intervention. Firstly developing protein blockers/ inhibitors and secondly gene therapy i.e. intervention at genetic level. Both these interventions entail considerable research and development effort with a large number of drug molecules being rejected during development. The few remaining molecules need to be tested vigorously in clinical settings with concomitant difficulties of long drawn clinical trials. Any attempt consequently, targeting specific mRNA rather than the protein itself appears to be much more rational and efficient approach to block protein function. Nature has itself utilized this approach which today is recognized as the concept of RNA Interference. The RNAi world includes a variety of small RNA molecules like miRNA, shRNA, ribozymes etc., capable of regulating gene expression. All these molecules knockdown the expression of the targeted genes i.e. they do not entirely abolish the gene expression, but lessen active gene product temporarily. RNAi do not knockout the gene completely as is done by antisense molecules. Thus RNAi technique is referred to as knockdown to distinguish it from knockout procedures in which expression of gene is entirely eliminated.

RNA interference is a classical mechanism of gene regulation, found in eukaryotes as diverse as yeast and mammals and, probably plays a central role in controlling gene expression. The phenomenon is also known as co suppression / post transcriptional gene silencing (PTGS) in plants and quelling in fungi. It is an exciting phenomenon that involves small RNA molecules, which can selectively silence essentially any gene in the genome. (Rondinone, 2006). RNA based silencing was first observed in plants and fungi. Unexpected results were foreseen in the plant petunia (Napoli et al., 1990). When transduced with the gene, encoding an enzyme required for pigment synthesis, the petunia surprisingly developed areas of hypo pigmentation. This phenomenon was called as Co-suppression. Similar effects were seen in N. crassa “Quelling” [Cogoni et al., 1999]. Guo and Kempheus in 1995 first studied RNA silencing in animals when they used antisense RNA to silence par1 mRNA expression in C. elegans but found that par1 mRNA itself also repressed par1 gene. Their observation inspired the experiment of Fire, Mello and colleagues. Thus began the journey of the newly dubbed
technology RNA Interference.

In the year 1998, researchers Andrew Fire, then at the Carnegie Institute of Washington and Craig Mello at the University of Massachusetts Medical School discovered that double-stranded RNA molecules were remarkably potent inhibitors of a targeted gene in C. elegans (Fire et al, 1998). The technique was later shown to work in flies, plants but not in mammalian cells. This is mainly because long dsRNAs in mammalian system induce interferon response. Thomas Tuschl (2001), a biochemist overcame this problem by designing 21-23bp sequence that had the same potential of reducing gene expression. Tuschl's discovery enabled and pushed RNAi to the forefront in biomedical research. The next key event took place in Sharp's laboratory at MIT. Collaborating with Judy Lieberman and Premlata Shankar of the Harvard University, Sharp demonstrated that siRNAs could stop HIV infections in cell cultures, thereby demonstrating the technology's clinical potential. This was further confirmed by experimentation of Mario Stevenson in 2003. Lieberman (2003) showed that massive doses of siRNA injected directly into the bloodstream of mice protected liver cells from the effects of chemically induced fulminating hepatitis (Song et al., 2003a). In this case, the siRNA was targeted against the gene Fas involved in the apoptosis of the liver cells. Francis Chisari and colleagues at The Scripps Research Institute tested the capability of RNAi to inhibit hepatitis virus (HCV) RNA replication.

Many diseases in human particularly HIV, cancer, cardiovascular disorders, diabetes provide a potential usage of this technology as a significant treatment. This discovery has raised hope to develop a potent and effective medication against the lethal diseases like cancer and HIV. RNA interference has become a very powerful and widely used tool to analyze gene function particularly for drug discovery and toxicological studies. In the year, 2002 the journal Science described the technology as the “Breakthrough of the year 2002” having potential to become a powerful therapeutic drug. Another impetus was added this October, 2006 when Nobel Prize for medicine was bestowed upon two pioneer scientists Andrew Fire and Craig Mello for their novel Discovery of the method of turning off specific genes i.e. RNAi.

CONSERVED RNAI MECHANISM

RNA silencing is an evolutionarily conserved gene regulatory mechanism with many species-specific variations. As it has become a powerful tool for genetic analyses and is likely to become a potent therapeutic approach for gene silencing, consequently, understanding the mechanism of RNAi has become critical for developing the most effective RNAi methodologies. Two ribonucleases that are vital to this pathway are: Dicer, a multidomain RNase III family enzyme that initiates RNAi by generating small interfering RNAs (siRNA), and Argonaute or Slicer, an RNase H signature enzyme that affects cleavage of mRNA. The RNAi pathway can be divided into three major steps:

- First is the conversion of dsRNA input into 21-23bp small fragments by the enzyme Dicer;
- Secondly the loading of small RNAs into large multiprotein complex RISC
- Lastly the sequence specific silencing of the cognate gene by RISC that is guided by the small RNA fragment.

A. FUNCTIONAL ANALYSIS OF DICER PROTEIN IN RNAI

The dsRNA precursors are acted upon by the enzyme DICER and DICER like proteins, generating small interfering RNAs (siRNA) duplexes of length 21-23 bp with characteristic 2 nucleotide overhang at 3'-OH termini and 5' PO₄. The eukaryotic DICER / Dicer like proteins consists of a Tandem repeat of RNase III catalytic domains (Fig. 1), flanked by a carboxyl terminal dsRNA binding domain, an amino terminal DExH/DEAH RNA helicase domain, and a PAZ domain (Bernstein et al, 2001). The crystal structure of DICER was determined by Mac Rae et al., (2006). They stated that an intact DICER enzyme consists of the PAZ domain separated from the two RNase III catalytic domains by a large helical domain. The two domains use two metal ion mechanism of catalysis. The positions of the metal ion pairs in each RNase III domain by a flat, positively charged surface. The PAZ domain, a module that binds the end of dsRNA, is directly connected to the RNase IIIa domain by a long alpha helix. This connector helix is encircled by the N-terminal residues of the protein that forms a platform domain consisting of antiparallel sheet and three helices. The two RNase III domains are separated by a large helical domain. The two domains use two metal ion mechanism of catalysis. The positions of the metal ion pairs in each RNase III domain is used to anchor the two scissile phosphates of an ideal A-form dsRNA helix into the active site of RNase III. The flat surface formed by the platform region outside RNaseIII region contains a large positive charge that interacts directly with the negatively charged phosphodiester backbone of dsRNA helix. This leads to the falling of the 3' end of the RNA duplex directly...
into the 3’ overhang-binding pocket of the PAZ domain, while the 5’ end is placed adjacent to the Dicer specific PAZ domain loop. Thus this ~65 angstrom distance between PAZ domain and RNase III domain, recognizes and cleaves ~25 bps and so Dicer itself acts as a molecular ruler that recognizes dsRNA and cleaves at specified distance from the helical end. These siRNA ribonucleoproteins are then asymmetrically incorporated into a complex called RNA Induced Silencing Complex (RISC).

**Figure 1**

Figure 1: Structure of Dicer enzyme

Dicer enzyme plays two biochemically distinct roles in the RNAi mechanics. It functions to generate siRNA molecules and also plays an important role in loading one of the two siRNA strands onto RISC complex (Doi et al., 2003; Lee et al., 2004). Several organisms contain more than one Dicer gene, with each Dicer preferentially processing dsRNAs coming from a specific source. Mammalian cells contain a single DICER, which generates both miRNA and siRNA (Table No.1). However, D. melanogaster has two paralogues; Drosophila Dicer1 (DCR1) produces miRNAs, while DCR2 makes siRNA. The processing of dsRNA by the recombinant DCR2 monomer or by the DCR2/ R2D2 heterodimer is ATP dependant and requires a functional RNA helicase domain in DCR2. Four Dicer – like (DCL) proteins (DCL1 to DCL4) have been identified in Arabidopsis thaliana, where DCL1 process miRNA (Xie, et al., 2004), DCL2 is required for siRNA production (Vazquez et al., 2004; Han et al., 2004) and DCL3 for rasiRNA (repeat associated small interfering RNA) production. Only a single Dicer gene has been identified in the C. elegans. This cooperates with dsRBD protein RDE-4 during RNAi, although RDE-4 is not required for miRNA generation (Tabara et al., 2002). This further the sequence alignment of the region following PAZ domain of several evolutionarily diverse enzymes have revealed a conserved pattern of hydrophobic and hydrophilic amino acids. All dicers contain a conserved proline about 11 amino acid residues from the predicted N terminus of the helix. Most Dicer proteins contain a conserved region of ~ 100 amino acids termed “domain of unknown function 283” (DUF283), and is predicted to form the platform structure in higher eukaryotes.

**Table 2**

Table 1: Species specific distribution of proteins involved in RNAi

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Proteins</th>
<th>Number of proteins</th>
<th>Functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>C. elegans</td>
<td>Dicer</td>
<td>1</td>
<td>Long dsRNA processing</td>
<td>Tabara et al. 2002</td>
</tr>
<tr>
<td>2.</td>
<td>Homo sapiens</td>
<td>Dicer</td>
<td>1</td>
<td>Long dsRNA processing &amp; generate both miRNA &amp; siRNA</td>
<td>Bernstein et al. 2001</td>
</tr>
<tr>
<td>3.</td>
<td>Drosophila melanogaster</td>
<td>DCR1</td>
<td>2</td>
<td>Generate miRNA</td>
<td>Lee et al. 2004</td>
</tr>
<tr>
<td>5.</td>
<td>Schizosaccharomyces pombe</td>
<td>Argonaute</td>
<td>1</td>
<td>Germ silencing</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Toxoplasma thermophila</td>
<td>Argonaute</td>
<td>1</td>
<td>Germ silencing</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>C. elegans</td>
<td>Argonaute1 et al.</td>
<td>24</td>
<td>Caudy et al. 2000</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Drosophila melanogaster</td>
<td>Argonaute1 Argonaute2 Argonaute3</td>
<td>5</td>
<td>miRNA accumulation</td>
<td>Omura et al. 2004</td>
</tr>
<tr>
<td>9.</td>
<td>Homo sapiens</td>
<td>Ago1 Ago2 Ago3 Ago4</td>
<td>8</td>
<td>Associate miRNA &amp; siRNA</td>
<td>Jin et al. 2004</td>
</tr>
</tbody>
</table>

Besides the above proteins, other proteins have also been identified to play important role in the RNAi mechanism. Three more components were identified in the purified D. melanogaster RISC: the Vasa intronic Gene product (VIG), fragile – X- related protein (dFXR) and the Tudor Staphylococal- nuclease domain containing protein (Tudor-SN), (Caudy et al., 2002; 2003). While in the human biochemical system, interactions of Ago2/eIf2C2 (Initiation Factor 2C2) with FMRP (fragile X mental retardation protein) – the homolog of dFXR has also been observed (Jin et al., 2004). None of these proteins have yet defined roles in RNAi mechanism.

**B. ENTRY OF SIRNA INTO RISC**

The small siRNA generated join an effector complex RISC which further mediates silencing through several mechanisms. In flies, plants and fungi, it triggers chromatin remodeling and transcriptional gene silencing (Mette et al., 2000; Hall et al., 2002, Volpe et al., 2002) whereas in mammals it can either interfere with protein synthesis or can lead to mRNA cleavage. The later mechanism being the best studied mode of RISC action. The assembly of RISC is an ATP dependant process reflecting the requirement for energy driven unwinding of the siRNA duplex or any conformational or compositional changes of the pre assembled RNA duplex containing RNP. The major
constituents of RISC are the single-stranded siRNA and any one of a number of different proteins of the Argonaute (Ago) family. Eight AGO proteins have been typified in humans, amongst this family of proteins; Argonaute 2 (Ago2) protein has been implicated to be associated with RISC complex.

The Argonaute protein is considered as the catalytic engine or the signature component of the RISC of RNAi, which cuts mRNA targets guided by siRNA via its endonuclease nicknamed “slicer”. Structural analysis of protein has established the presence of four functional domains in the protein (Fig. 2). The N terminal, middle and PIWI domains form a crescent shaped base, with PIWI domain at the center of the base. While the PAZ domain is held by a stalk-like linker region and located above the base. This architecture forms a large positively charged groove between the PAZ domain and the crescent base, and a smaller one between N terminal and PIWI domains (Song and Tor, 2006). The PIWI domain is unique to this protein, whereas it shares PAZ domain with DICER protein also. The PAZ domain of ~ 130 amino acids have been characterized by a stable fold which has a β barrel core that together with side appendage appears to bind weakly to ssRNAs at least 5 nt in length and also to dsRNA. (Lingel et al, 2003; Song et al, 2003b). It is composed of two sub-domains with a cleft in between the two. One sub-domain consists of a 5 stranded open β barrel with 2 helices on one end of the barrel. The other sub-domain is made up of a hairpin followed by an α-helix. The β barrel sub-domain is suggestive of an OB fold (i.e. oligonucleotide-oligosaccharide binding) with its major function being single strand nucleic acid binding (Theobald et al., 2003). Thus, indeed the PAZ domain binds nucleic acids. The PIWI domain at the C terminus of Argonaute is located across the primary groove from PAZ domain. Its core fold clearly belongs to the RNAse H family of enzymes, containing two highly conserved aspartates on adjacent strands. The crystal structure of the PIWI domain further revealed the existence of a highly basic pocket, comprising of two aspartate and a histidine called the DDH motif (Song and Tor, 2006) analogous to the DDE motif of RNase H enzyme (Yang et al., 2003). This provides a divalent metal ion (preferably Mg2+ and Mn2+) binding site to capture 5’ PO4 group of the guide strand (Parker et al., 2004; Ma et al., 2005). Therefore this interaction between Ago protein and siRNA facilitates the positioning of the guide strand for effective target cleavage by “slicer” endonuclease activity residing in human Ago2 protein and also increases the half life of the RISC (Jayesena et al, 2006). This finding strongly claimed that Argonaute is the long sought after catalytic enzyme of RISC dubbed as “Slicer”. The other members of this family lack the catalytic domain and thus function by recruiting proteins that inhibit translation at the step of initiation.

Several members of the Ago gene family have been genetically identified in various organisms and some have important roles during RNAi whereas others are important in development regulation. Ago proteins may have evolved an intrinsic sequence specificity that allows them to bind preferentially to small RNAs of specific sequence, or they may use specific adapter proteins that are associated with the different cellular source of dsRNA. Ago Proteins are sometimes also called as PPD (PAZ-PIWI Domain) proteins because they all share the PAZ and PIWI domains (Cerutti et al., 2000). Each organism has varied number of AGO proteins, ranging from one in Schizosaccharomyces pombe, Tetrahymena thermophila to 24 in C. elegans (Carmell et al, 2002). Ago1 the sole representative of S. pombe is involved in the dsRNA triggered transcriptional as well as post transcriptional gene silencing. Amongst the 24 members of Ago protein gene family of C. elegans only one i.e. rde1 has been shown to be required for RNAi. In A. thaliana ten members have been identified (Hunter et al., 2003), while five in D. melanogaster (Williams et al., 2002), two in Neurospora crassa and nearly eight proteins in humans (Sasaki et al., 2003). In D. melanogaster, the mRNAs encoding for all Ago proteins are maternally deposited. These are listed as Argonaute1, Argonaute2, Argonaute3, PIWI and Aubergine. A recent study in D. melanogaster revealed that Ago1 is required for miRNA accumulation whereas Ago2 is required for siRNA triggered mRNA degradation (Okamura et al., 2004). Two recent studies in human system showed that Ago1 to Ago4 all associate with miRNAs and siRNAs but that only the Ago2 containing
RNP exhibit RISC activity (Meister et al., 2004; Liu et al., 2004). Finally different expression patterns and different levels of various Ago proteins may control the extent to which RNAi process proceeds. (Martinz et al., 2004).

C. SEQUENCE SPECIFIC CLEAVAGE OF TARGETED MRNA

The active RISC further promotes unwinding of siRNA through an ATP dependent process and the unwound antisense strand guides active RISC* to the complementary mRNA. The targeted mRNA is cleaved by RISC at a single site that is defined with regard to where the 5′ end of the antisense strand is bound to mRNA target sequence. The RISC cleaves the complimentary mRNA in the middle, ten nucleotides upstream of the nucleotide paired with the 5′ end of the guide siRNA (Elbashir et al, 2001). This cleavage reaction is independent of ATP. However, multiple rounds of mRNA cleavage, which require the release of cleaved mRNA products, are more efficient in the presence of ATP. RISC complex catalyzes hydrolysis of the target RNA phosphodiester linkage, yielding 5′ phosphate and 3′ hydroxyl termini. The target RNA hydrolysis reaction requires Mg2+ ions. Cleavage is catalyzed by the PIWI Domain of a subclass of Argonaute proteins. This domain is a structural homolog of RNase H, a Mg2+ dependant endoribonuclease that cleaves the RNA strand of RNA-DNA hybrids (Liu et al, 2004). But each cleavage-competent RISC can break only one phosphodiester bond in its RNA target. (Elbashir et al, 2001). The siRNA guide delivers RISC to the target region, the target is cleaved, and then siRNA departs intact with the RISC (Martinez and Tuschl 2004).

Thus the two important conditions to be fulfilled for the success of silencing by RNAi are established as: 5′ phosphorylation of the antisense strand and the double helix of the antisense target mRNA duplex to be in the A form. The A-form helix is required for the stabilization of the heteroduplex formation between the siRNA antisense strand and its target mRNA. This was evidently supported by the fact that siRNA activity is lost upon inducing distortion in the A form geometry by introducing two nucleotide bulge in the middle of antisense strand. However no such reduction in siRNA activity was observed when same distortion was introduced in the sense strand. (Crooke, 2000)

RNAI IN MAMMALIAN SYSTEMS

RNAi was first noted in nematode worm and was further extended to eukaryotes like Drosophila, mammals etc. There is a lot of difference between the mechanism of RNAi between mammalian and other eukaryotes. Plants and worms show systemic silencing, pointing to the spread of amplifiable sequence specific signal throughout the organism. In C. elegans, a putative transmembrane protein SID 1 is associated with the spread of gene silencing information between tissues, but is not associated with RNAi.
initiation and maintenance. Kennerdell and Carthew (2000) have demonstrated the absence of sid1 homologue in the fly genome. While strong similarity is predicted with human and mouse proteins, suggests the possibility that RNAi could have systemic component in mammals also (Winsten et al., 2002).

A major difference from other eukaryotes as compared to mammals is the lack of amplification system for long-term persistence of RNAi in mammalian cells. So RNAi only persists effectively for an average of ~ 66 hours before siRNA gets diluted over course of several cell divisions. While it can be carried over to many generations as in case of Drosophila where ~ 35 molecules can silence ~ 1000 copies of targeted mRNA and can persist over many generations. In case of mammals, presence of long dsRNA will lead to interferon response while no such response is met in other organisms. Also in case of other eukaryotes conversion of long dsRNA to smaller 21-23 nucleotide siRNA by DICER adds a degree of RNAi Amplification. The processing of dsRNA to siRNA is an ATP dependent process in lower eukaryotes whereas in mammalian system ATP dependence of dsRNA processing has not yet been observed (Provost et al., 2002; Zhang et al., 2002).

Another interesting difference is the presence of enzyme RNA dependent RNA polymerase (RdRP). The enzyme amplifies target mRNA, through a random degenerative PCR model (Lipardi et al., 2001) into dsRNA. The siRNA itself acts, as primer for second strand elongation of target mRNA. This dsRNA is a substrate for dicer to produce siRNA and consequent gene silencing. No such homologue of RdRP exists in case of mammalian cells.

MICRO RNA

MicroRNAs are naturally encoded small RNAs that play a critical role in gene expression during many essential cellular functions in the body, including protein synthesis, cell differentiation and cell death. Abnormalities in miRNA function may play a role in human disease causation. The first miRNA molecules, lin4 and let 7 which are involved in the regulation of cell differentiation and tissue development, were discovered in C. elegans (Lee et al., 2001).

Subsequently many more have also been identified in all multicellular organisms investigated including fruit flies, plants and humans. The function of most miRNAs is not yet known and there is intense interest in further characterizing the targets and mechanism of action of miRNAs.

There are at least 330 confirmed miRNA genes in the human genome and there are many other predicted miRNAs that remain yet to be confirmed. The latest release (October 2006) of miRBase (Griffiths et al., 2006), an online database (http://microrna.sanger.ac.uk/) lists 4361 distinct miRNA genes; that include 474 from human genome. miRNAs are believed to regulate at least one-third of genes in the human genome and are also likely to play significant roles in the manifestation of many disease states like cancer, metabolic and infectious diseases. He and Hannon (2004) stated “miRNAs might have a general role in regulating gene expression in diverse developmental and physiological processes, and (there are) substantial hints that mis-regulation of miRNA function might contribute to human disease.”

BIOGENESIS OF MIRNA

The current mechanism for the generation of mature miRNA requires the enzyme Drosha (RNase III endonuclease) for processing of precursors termed Pri- miRNAs (Primary miRNA). Pri- miRNAs are cleaved into ~ 60-70 nucleotide stem loop intermediates known as precursor miRNA (pre-miRNA, Zeng and Cullen, 2003). Drosha along with its dsRNA- binding protein partner DGCR8 in humans (termed Pasha in flies) cleaves such RNA duplex with a staggered cut typical of RNase III endonuclease and thus the base of pre-miRNA stem loop has 5’ PO4 and ~2 nt 3’overhang. The pre-miRNA is actively transported from nucleus to the cytoplasm by Ran-GTP and the export receptor exportin 5 (Yi et al., 2003; Lund et al., 2004). The pre-miRNA cut by Drosha in nucleus defines one end of the mature miRNA, while the other end is generated by the Dicer (Lee et al., 2003) in the cytoplasm. The enzyme Dicer is supported by dsRNA binding protein partner, the tar binding protein (TRBP) in humans (named Loquacious in Flies, Hammond et al., 2000). Dicer in the cytoplasm first recognizes the double strand portion of the pri-miRNA; perhaps with particular affinity for 5’ phosphate and 3’ overhang at the base of the stem loop. Then at about 2 helical turns away from the base of stem loop, it cuts both strands of the duplex. This cleavage by Dicer loops off the terminal base pairs and loop of the pre-miRNA leaving the 5’ PO4 and ~2 nt 3’overhangand producing an siRNA like imperfect duplex that comprises the mature miRNA. The figure below depicts both, the mechanism of generation as well as differences in the biogenesis of miRNA and siRNAs.
Figure 5
Figure 4: The difference in mechanism of miRNA and siRNA

MICRO RNA VS. SIRNA PRACTICAL IMPLICATIONS

The miRNA and endogenous siRNA in a cell share biogenesis pathways and perform interchangeable biochemical functions. These two classes of silencing RNAs cannot be distinguished by their chemical composition. However, functionally miRNAs and siRNAs inhibit gene expression by different mechanisms. Other, important distinctions can be made particularly in regard to their origin, evolutionary conservation and the types of genes that they silence. Some of the differences are as follows:

- **MiRNA are derived from specific genomic loci, distinct from other recognized genes (Hannon et al, 2004); whereas siRNA often derive from mRNAs, transposons, viruses or heterochromatic DNA.**
- **MiRNA synthesis involves the enzyme Drosha (RNase III polymerases) on long primary nuclear miRNA sequence. Unlike siRNA the perfect match recognition sequences for miRNA can be as short as 7-8 nucleotides and the complete sequence can tolerate mismatches and bulges (Zeng et al, 2003).**
- **MiRNAs are processed from longer precursor hairpin transcripts whose stem forms imperfect Watson and Crick helices. Whereas siRNAs are processed from long bimolecular RNA duplexes or extended hairpins, with complete Watson Crick base pairing, most often viral in origin.**
- **A single miRNA – miRNA* duplex is generated from each miRNA hairpin precursor molecule, whereas a multitude of siRNA duplexes are generated from each siRNA precursor molecule, leading to many different siRNA accumulating from both strands of this extended dsRNA.**
- **MiRNA sequences are nearly always conserved in related organisms whereas endogenous siRNA sequences are rarely conserved.**
- **In case of siRNA all bases contribute equally to its specificity. While in case of miRNA it is the base in the 5' half of small RNA.**
- **miRNAs most often bind to the 3' untranslated region of target transcripts forming incomplete Watson –Crick bp and blocking translation (Lee et al, 2003) while siRNAs form perfect or nearly perfect Watson crick helices anywhere along a target mRNA and are interchangeable, depending upon the extent of base pairing with the target mRNA.**
- **A distinction can be made between two classes of silencing RNAs: endogenous siRNA typically specify “auto-silencing” in that they specify the silencing of the same locus or a very similar loci from which they originate, whereas miRNA specify “hetero-silencing”, in that they are produced from genes that specify the silencing of other genes (He & Hannon, 2004).**
- **Some mRNAs are difficult to knockdown with miRNA as a result of their inaccessibility due to secondary/ tertiary structure formation, binding by proteins, existence of alternately spliced transcripts, transcript high turnover or feed back up regulation.**

These distinctions explain the greater sequence conservation seen for miRNAs. To the extent that the siRNA come from the same loci that they target, a mutational event that
changes the sequence of the siRNA would also change the sequence of its regulatory target (origin) and siRNA regulation would be preserved – an unusual case of maintaining an important function without selective pressure for conserving the sequence. While in case of miRNA a mutation would rarely be accompanied by simultaneous compensatory changes at the loci of its targets and thus selection pressure would preserve the miRNA sequence.

Both of these are endogenous small RNA guides that repress the expression of target genes. Although miRNAs differ in their biogenesis from siRNAs but both have significant similarity in function. Like siRNAs, miRNA can direct cleavage of their mRNA targets when the two are extensively complementary (Hutvagner & Zamore, 2002; Mallory et al, 2004; Okamura et al, 2004). This similarity between siRNA and miRNA processing and recognition sites imply that a subset of siRNAs could have miRNA-like function and could explain some of the off targets effects of siRNAs, especially those observed at low concentrations. As only partial mRNA complementarity is required for miRNA action, a siRNA that is rendered inactive against its target gene by a single mismatch in the sequence can still demonstrate non-specific gene silencing due to miRNA like effects.

TECHNICAL CONSIDERATIONS UNDERLYING SIRNA APPLICATIONS

Mammalian system is much more complex as compared to the other eukaryotes. Several parameters have to be taken into consideration while developing an efficient system of deploying RNAi in mammalian cells. The foremost criterion is the choice of target site. Originally, it was suggested that the best target site is around the first 100 nucleotide downstream of translation start site. Secondary structures and mRNA binding proteins also influence the accessibility of mRNA to siRNA. Thus these must also be checked beforehand.

1. DESIGNING THE PERFECT SIRNA

The first step in an RNAi experiment in a mammalian system is to design the siRNA sequence. On the basis of the analyses of a small number of target genes, several groups have proposed a set of guidelines that seek to narrow the choice of siRNA that could potentially silence gene expression. Several sequence motifs are consistent with effective siRNA directed silencing including AAN₉,TT, NAM₉,NN, NARN₉,NN and NANN₉,NN (where N is any nucleotide, R is purine and Y is pyrimidine). When choosing siRNA those regions of complimentary DNA are selected that have non-repetitive sequences. Intronic sequences are avoided, as mammalian RNA interference is a cytoplasmic process (Martinez et al, 2002). The parameters can be categorized as under:

BIOPHYSICAL AND THERMODYNAMIC CONSIDERATIONS

SiRNA with ~ 50% GC content is preferable as this facilitates interaction with RISC and helps in unwinding. Low internal stability at the 5’ antisense strand promotes antisense-strand selection by RISC. It was also predicted that design of si/shRNA molecules with a thermodynamically unstable 5’ antisense strand would increase silencing efficiency and reduce off-target effects. While high internal stability at the 5’ sense strand blocks sense-strand selection by RISC. The regions with stretches of single nucleotide especially G, should be avoided. Absence of internal repeats or palindromes increases the concentration of functional, stable hairpins. A-form helix between siRNAs and target mRNA enhances RNA–RNA interactions and promotes cleavage. However, even by abiding to these empirical guidelines, several studies concluded that the overall fraction of efficient siRNAs was only 20% -25%. Later it was demonstrated that siRNAs targeting the untranslated regions (UTRs) can also silence gene expression effectively and the presence of 5’ AA and 3′ TT bases flanking the core 19-mer-target sequence appeared to be dispensable.

Previous reports have suggested that the efficacy of silencing is affected by the accessibility of the target sequence, and therefore, secondary structure predictions of the mRNA may improve si/shRNA design. One approach will be to screen regions of potential mRNA target sites by an RNA folding program such as mfold (Zuker 2003) or Sfold (Ding et al, 2004) to eliminate sites within tightly folded RNA motifs that may resist targeting by RISC.

BASE PREFERENCES AT SPECIFIC POSITIONS IN THE SENSE STRAND

This is another important criteria that helps in designing of an efficient siRNA sequences. Presence of an A at position 3 and 19* of sense strand, promotes antisense-strand selection by RISC. Absence of a G or C at position 19 of sense strand again promotes antisense-strand selection by RISC. Presence of a U at position 10 of sense strand helps in RISC mediated cleavage of mRNA and also dissociation of the RISC–siRNA complex. While absence of a G at position 13 of sense strand promotes efficient unwinding.
LENGTH OF SIRNA SEQUENCE

The preferable length of siRNA ranges from 20-22 bps. But two very recent reports have demonstrated that 27-mer siRNAs and synthetic shRNAs with a 29-mer stem perform significantly better than the corresponding 19-mer-based molecules. These longer dsRNAs are more effective, especially at very low concentrations, permitting greatly prolonged gene silencing, and, allow efficient knock-down through target sites that are refractory to RNAi with traditional siRNAs. The superior activity of longer siRNAs and shRNAs can probably be attributed to the importance of Dicer-mediated cleavage and loading of effector molecules into the RISC; while the longer molecules are quantitatively cleaved by Dicer to 21- to 22-nucleotide products, traditional siRNAs and shRNAs containing a 19-mer stem are not substrates of Dicer and probably enter the RISC by another mechanism.

ENHANCING SPECIFICITY OF SIRNA-MEDIATED GENE SILENCING

Homology search of predicted target sequence must be performed so as to reduce off target effects. It is best to select those candidate si/shRNA sequences that have at least three mismatches to any gene other than the one targeted. It is also advisable to avoid polymorphic regions, unless allele-specific silencing is the scientific goal. The most frequently used homology search is Basic Local Alignment Search Tool (BLAST), although there is some dispute about whether this is the most appropriate algorithm. It is advisable to use the BLAST option “search for short, nearly exact matches” that is set with a low “Word Size” (=7) and a high “Expect” value (=1000) as default to make the search more stringent for short sequence matches. The figure below provides a summary of the designing parameters.

Figure 6

Figure 5: Design characteristics of siRNA

A potential pitfall in the current designing rules is the difficulty in removing sequence specific off target effects resulting from identified pairing rules. Newer approaches have been developed to minimize off target interactions. The first strategy is based on the observation that off target effects are concentration dependent. Several researchers have proven that off target effects can be minimized as the concentration of siRNA is reduced. However, the strategy when applied on broad scale has failed, as the concentration at which off target effects decline is comparable to that at which on target gene knockdown too diminishes. This impasse is resolved by adopting a novel and simpler strategy known as “pooling”. This involves usage of multiple duplexes. All duplexes that make up the pool target the same gene, so this approach of eliminating off-target is achieved without jeopardizing target specific knockdown (Brown and Samarsky, 2006). The second strategy involves recent advancement in the field of siRNA chemistry. Jackson et al, (2006) reported that a recently identified chemical modification pattern eliminates as much as 80% of off target effects. This includes differential addition of 2’-O-methyl moieties to both the sense and antisense strands. On the sense strand, these modifications prevent 5’-phosphorylation, while on the antisense strand; key nucleotides that are essential for off targeting, but are less crucial for on-target gene knockdown are modified.

Finally, the last approach toward eliminating off-target effects is associated with siRNA design. Various studies (Birmingham et al., 2006; Lin et al., 2005 and Jackson et al., 2006) revealed that off-targeted genes frequently contained matches between the seed region of the siRNA (positions 2-7) and sequences in the 3’ UTR of the off-targeted gene. As the likelihood of a gene being off-targeted is elevated by the presence of multiple 3’UTR seed matches, these findings point to a strong mechanistic parallel between siRNA off-targeting and miRNA-mediated gene regulation. The nonspecific off-target effects are not limited only to the guide strand, but can also be generated by the sense strand if it becomes incorporated into RISC and binds to an off-target set of mRNAs bearing partial complementarity to its sequence. The fact that RISC incorporation is favored for the strand whose 5’-end is least thermodynamically stable to unwinding, it is now possible to design siRNAs that are destabilized at the 5’-end of the guide strand to promote incorporation of only the guide strand and minimize the problem.

Another often repeated problem in siRNA application is the
activation of interferon and other proinflammatory cytokines. It has been observed that siRNAs containing GU-rich sequences can also indirectly induce IFN production by binding to Toll-like receptors (TLR3, TLR7 and TLR8) that alarm immune activating cells to the presence of RNA viral pathogens. Toxicity from the ensuing inflammatory response, might also pose a therapeutic problem. However, binding of double-stranded RNAs to TLRs appears to be sequence specific and may be abrogated by chemical modifications of the sugar backbone (Dykxhoorn et al., 2006).

Many algorithms are readily available on several websites are fairly good at predicting siRNA sequences like siSearch, SiDirect, Deqor etc (as mentioned in References).

2. METHODS OF SIRNAS SYNTHESIS

The next step for mammalian RNAi experiments is to produce the siRNA. Till date, various methods listed for production of siRNA are as under:

- Chemical Synthesis
- In Vitro Transcription
- RNase II/ Dicer digestion of long ds RNAs
- Expressions from plasmids
- Expressions from PCR cassettes
- Expressions from viral Vectors

The first two methods involve production of individual siRNAs either by chemical synthesis or by in vitro transcription- that are then introduced into cells or animals. The major benefit of using chemically designed siRNAs is the ability to obtain high purity siRNAs in large quantities with no hands on time. But the important drawback is its cost of synthesis. These lead researchers to develop a more cost effective methodology i.e. in vitro transcription. Here the siRNAs are generated by in vitro transcription from DNA templates that encode the two siRNA strands. The individual strands are then purified and hybridized to generate dsRNA molecules. In vitro transcribed siRNA are most cost effective and, can be made more quickly as well as are just as effective as chemically synthesized siRNAs.

In an effort to induce long term gene silencing several researchers have made plasmid vectors to continually express siRNAs in transfected mammalian cells. Some of these expression plasmids have been engineered to express small hairpin RNAs (shRNA) in cells, which are then processed into siRNAs. While others have been generated to express the two siRNA strands separately which upon transcription hybridize in vivo to produce functional siRNAs. The polymerase III promoters are used to drive the siRNA expression in vivo.

The disadvantage of using plasmid and PCR based cassettes for siRNA expression is that they are difficult to introduce into tissues and certain cell types. In addition linearized plasmids do not readily integrate into host genomes, making the creation of siRNA- expressing, stable cell lines difficult. Thus the developments of viral vectors have helped in overcoming these problems. Adenoviral vectors are capable of infecting dividing cells, non dividing cells and tissues via receptor mediated infection through the widely expressed coxsackie virus and adenovirus receptor (CAR) as well as the integrin β3 and β1 subunits (Kay et al.,2001). Retroviral vectors have also been developed (Devroe et al., 2002) A comparative account of all the above-mentioned siRNA producing methods, with all their pros and cons are summarized in Table No.2

**Figure 7**

**Table 2: Comparative analysis of siRNA synthesis processes**

<table>
<thead>
<tr>
<th>Total Preparation/ Synthesis Time</th>
<th>In Vitro Transcription</th>
<th>BaseEfficiency of siRNA</th>
<th>siRNA expression Vectors</th>
<th>siRNA expression cassette</th>
<th>Viral Vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hands on time</td>
<td>Chemical synthesis</td>
<td>4 days to 15 days</td>
<td>One day + DNA oligo</td>
<td>One day + transcription template</td>
<td>5-6 days + DNA oligo</td>
</tr>
<tr>
<td>Requirements</td>
<td>In vitro transcription</td>
<td>Moderate</td>
<td>Moderate</td>
<td>High</td>
<td>Moderate</td>
</tr>
<tr>
<td>(1) SiRNA oligo</td>
<td>(2) 20mers DNA oligo</td>
<td>Transcription template</td>
<td>Transcription template</td>
<td>55-60mers DNA oligo</td>
<td>55mers DNA oligo</td>
</tr>
<tr>
<td>Validation of siRNA sequence</td>
<td>Required</td>
<td>Required</td>
<td>Required</td>
<td>Required</td>
<td>Required</td>
</tr>
<tr>
<td>Labeling siRNA for analyzing uptake or localization</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Transformation efficiency</td>
<td>Good</td>
<td>Good</td>
<td>Poor</td>
<td>Poor</td>
<td>Very Good</td>
</tr>
<tr>
<td>Selectable marker</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Exonuclease</td>
</tr>
<tr>
<td>Shell life for long term survival</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Restricted</td>
</tr>
<tr>
<td>Stabilizing expression</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Limited</td>
</tr>
<tr>
<td>Maximal transformation efficiency</td>
<td>Yes</td>
<td>Limited</td>
<td>Limited</td>
<td>Limited</td>
<td>No</td>
</tr>
<tr>
<td>Cost of Synthesis</td>
<td>High</td>
<td>Moderate</td>
<td>Low</td>
<td>Moderate</td>
<td>High</td>
</tr>
</tbody>
</table>

Recently the siRNA stability has been further improved by Sequitur’s StealthTM RNAi compounds that are chemically modified second generation siRNAs to avoid interferon
activation, retain high activity, and high stability in blood stream.

3. VALIDATION OF SIRNA:
All rationally designed si/shRNAs will not be able to knock down gene expression at the same level, and therefore, it is very useful to evaluate the silencing capability of several candidates before committing to the actual experiment. Once the dsRNA molecules have been screened and optimized for maximum silencing activity, they must be validated further to prevent unwanted effects on other genes. Since the effect of RNAi can be very powerful, new validation methods need to be developed to detect any off-target effects.

Microarray based techniques provide a direct approach for the determination of off target effects by comparing the gene expression. The specificity of dsRNA against an intended gene can be confirmed by profiling the expression of genes on microarrays. Customizable microarrays have the added advantage of allowing array content to be easily modified by researchers so that any set of genes can be monitored for silencing. These include target genes as well as any gene closely related by either pathway or sequence homology. In addition, custom arrays allow inclusion of probes for use as negative or positive controls. More importantly, non-specific or off-target inhibition of gene expression can also be assessed on microarrays. This must be interpreted carefully, however, since regulatory mechanisms may also cause reduced gene expression in the absence of a direct RNAi effect. The CombiMatrix platform uses two microarray densities: a low density microarray with 1024 features in 1cm² footprints to screen any combination of 300-500 genes at one time, and a medium density microarray with over 13,300 features within the same footprint to screen 4000-6000 genes. A high-density format for microarray is being developed that will allow genome-wide expression profiling (Arjomand & Kumar, 2003).

DELIVERY METHODOLOGIES:
Efficient delivery is vital for evaluation of any drug molecule; be it a chemical compound, nucleotide based reagents or biotherapeutics like siRNA in vivo. Small interfering RNA (siRNA) can be generated against virtually any expressed sequence; more difficult, however, is finding ways to target systemically delivered siRNA to specific cell populations. Several delivery methods have been scrutinized so as to validate the potential of siRNA as an efficient therapeutic. Several problems have to be solved, so as to deliver active siRNA in the target tissues, such as:

- Excretion of siRNA through urine because of its smaller size (~7 kDa) reduces its half life (minutes to seconds).
- Instability in the serum environment due to accessibility to RNase enzymes with a serum half life of ~5-60 minutes.
- Non-specific distribution of siRNA throughout the body decreasing the local concentration in the required tissue.
- Overcoming the blood vessel endothelial wall and multiple tissue barriers to reach target cells (Xie et al., 2006).
- Efficient endocytosis into the cells (Rondinone et al, 2006) on reaching targeted site.

The in vivo delivery of siRNA can be achieved both by exogenous application of synthetic siRNA via local or systemic delivery or via the gene therapy approach that relies on the endogenous expression of siRNA from plasmid or viral vectors. Delivery of oligos for gene therapy has provided much needed vital information that can help in standardization of delivery vehicles for siRNA. The relatively few reports on in vivo siRNA indicate a lack of effective in vivo delivery methodology, especially for RNAi mediated down regulation of the specific gene targets in the animal disease models. (Xie et al, 2005). Delivering siRNA in vivo to animal tissues is a complicated process and involves using physical, chemical, biological approaches or a combination of these (Lu et al., 2005).

Figure 8

Table 3: Gene delivery barriers and multicomponent design of a Non- viral vector.

<table>
<thead>
<tr>
<th>Barriers</th>
<th>Functional components</th>
<th>Strategies</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stability</td>
<td>Hydrophilic moiety</td>
<td>DNA condensation, protection from nucleases, lipids, gelatin</td>
<td>Protamine, PEylation</td>
</tr>
<tr>
<td>Internalization</td>
<td>Targeting ligands</td>
<td>Receptor mediated, Endocytosis</td>
<td>Transferrin, EGF, antibodies, RGD</td>
</tr>
<tr>
<td>Endosome Disruption</td>
<td>Escape from endosome, Unwrapping</td>
<td>PEI, DOPE, siRNA</td>
<td></td>
</tr>
<tr>
<td>Nuclear Entry</td>
<td>Nuclear localization signal (NLS)</td>
<td>Nuclear entry</td>
<td>Tat and Rev</td>
</tr>
</tbody>
</table>

IN VIVO DELIVERY:
A. IN CULTURED CELL SYSTEM:

This involves chemical synthesis of siRNA invitro and then delivery to the cells using several transfection reagents. The major procedures, used for in vitro transfection are chemical transfection and electroporation (Ovcharenko Technotes). Chemical transfection is a standard method for introducing siRNA into immortalized cells. However, for efficient performances of RNAi reagents under optimal transfection conditions i.e. maximal efficacy and minimal toxicity, leads to a significant decrease in the stress related transcriptional signatures often associated with the use of siRNAs. The commercially available cationic and anionic lipid based transfection reagents and kits include Lipofectamine 2000, TransIT-KO kit and HiPerFect(Genetix). The choice of transfection methods also makes a significant difference in the outcome. Transfection conditions such as transfection reagents, cell density and duration of incubation should be optimized for the given cell type and the targeted gene. The turnover rate of the protein should also be taken into account as RNAi only aims at the mRNA not the protein itself. Generally, siRNA causes rapid reduction in mRNA levels in 18 hours or less but some stable proteins require a longer period for depletion.

Cationic lipids and polymers are two major classes of non-viral siRNA delivery carriers and both are positively charged forming complexes with negatively charged siRNA. The siRNA – carrier complex get condensed into tiny nanoparticles with size ~100 nm, allowing very efficient cellular uptake of the siRNA through the endocytosis process (Xie et al., 2006). Several studies have been reported where cationic lipids and polymers have been complexed with siRNAs to inhibit the specific gene expressions in diseased animal models like a cationic derivative of cardiolipin to form lipoplexes with siRNA targeting the c-raf oncogene for inhibition of tumor growth (Chein, et al, 2005). While in another study a polymer of highly branched histidine-lysine (HK) peptides was used as a carrier for Raf-1 siRNA to reduce its expression in MDA-MB-435 Xenograft, introduced by intratumoral injection (Leng et al., 2005). A recent study demonstrated a significant reduction in the expression of HER2 gene and tumor growth through i.p. injections of HER2 siRNA complexed with polyethylenimine (PEI, Urban-Klien et al., 2005). In another antiviral study, i.v. administration of siRNA targeting influenza virus genome complexed with PEI also inhibited the production of virus in mice (Ge et al., 2004).

Chemical modifications are essential when therapeutic siRNAs are introduced without delivery vehicles. Such modifications help in enhancing the stability of the short interfering RNAs (siRNAs) in vivo. David Morrissey and colleagues (2005) have described a stable nucleic-acid–lipid particle (SNALP), a specialized liposome consist of a lipid bilayer of cationic and fusogenic lipids, designed specially for chemically stabilized siRNA for causing persistent knockdown of hepatitis B virus (HBV) replication genes in mice. They stabilized siRNAs by substituting all 2'-OH residues on the RNA with 2'F, 2'O-Me or 2'H residues. This dramatically improved the in vivo stability of siRNA. However, the dosing regimen used to demonstrate this in a mouse model was not therapeutically viable since it required administration of large amounts of siRNA three times daily. This SNALP was effective at a lower dose with improved potency and duration of siRNA activity.

The significance of these delivery vehicles lies in their potential of multiple administration of siRNA, which is crucial for siRNA therapeutic applications. Initially it was observed that nonviral siRNA delivery to the targeted tissues did not induce any immune response but recently conflicting results have reported. The activation of cellular interferon response on delivery of siRNA with cationic liposomes or polymer transfection reagents (Sledz et al., 2003; Kariko et al., 2004). A recent study has blamed the transfection reagents also for off target effects naked siRNAs produced no detectable interferon response upon injection into mice (Heidel et al., 2004).

For some cell types that are not amenable to lipid based transfections, electroporation and nucleofaction techniques have been used. However these require the use of large amount of siRNA ~1µM in final solution, which can be associated with greater incidence of off target effects (Moitreyee et al, 2005). Moreover this also causes massive cell death which should also be taken into consideration (Kim et al, 2004).

B. SYSTEMIC AND LOCAL DELIVERY:

The administration of siRNA through i.p. injections and i.v. administration leads to systemic distribution. In therapeutics it is always desirable to target the site of action of the drug. This choice of target site is an important criterion along with the desired range of expression, whether it is to be local, systemic, targeted or inducible. For e.g. tail vein injections of either naked siRNA or vector-based siRNA primarily accumulate in the liver so this approach is most suitable when liver is the target organ. Skin and muscle can be better
accessed using local siRNA delivery, whereas lung and tumor can be reached efficiently by both local and systemic siRNA delivery. Delivery of RNAi to lungs through intranasal route has also been evaluated by delivery of glyceraldehyde-3-phosphate dehydrogenase specific siRNA complexed with pulmonary surface active material and elastase, that lowered the protein expression from ~50% to 70% (Massaro et al., 2004). However to overcome toxicity and immune response activation during in vivo delivery of siRNA with cationic polymers, D5W (5% D-glucose in water) solution against SARS coronavirus RNA in mouse and monkey lungs have shown reliable gene knockdown. (Li et al., 2005). Other delivery options considered for RNAi are intraocular injections of siRNA to the front of the eye sub-conjunctivally or to the back of eye intra-vitreously. These were found to be highly efficient in silencing several genes in VEGF (Vascular endothelial growth Factors) pathway, significantly inhibiting corneal angiogenesis and disease symptoms. (Kim et al., 2004).

As blood brain barrier is a formidable hurdle that is not amenable to be crossed by any of the methods mentioned above, specific delivery pathways have to be devised. The infusion of an aqueous solution of chemically protected siRNA directly into the intracerebral region was found to selectively inhibit gene expression (Dorn et al., 2004). This non-viral delivery of siRNA into the brain provided a unique approach to accelerate target validation for neuropsychiatry disorders that involve complex interplay of several genes. Another recent study has revealed that delivery of cationic formulations of siRNA was more efficient using lipid carriers than polymer carriers in the brain (Hassani et al., 2005). Recently electroporation procedure has also been employed for the delivery of siRNA to the hippocampus region of the brain targeting genes, Glutamate receptor 2 and cyclooxygenase 1. New protein synthesis was impaired within hours proving the efficacy of the approach. The protein effected was brain-derived trophic factor, or BDNF. Since the cerebrospinal fluid lacks RNase, siRNA duplexes complexed with lipophilic Oligofectamine were directly delivered into the cerebrospinal fluid of the spinal cord of rats.

It was reported that atelocollagen, a collagen solubilized by protease, can protect siRNA from being digested by RNase when forming a complex with siRNA. In addition, the siRNA can be slowly released from atelocollagen, to efficiently transduce the cells, allowing long term target gene silencing (Lewis et al., 2002; Takei et al., 2004) therefore, this delivery method could be a reliable approach to achieve maximal inhibition of gene function in vivo.

Targeted delivery of siRNA was carried out, into the mouse liver; using the hydrodynamic methods which involve injection of large volume of aqueous solution into mouse tail vein that creates a high pressure in the vascular circulation leading to extensive distribution of siRNA into hepatocytes. (Lewis et al., 2002; Zender et al., 2003). However, this approach is not clinically feasible as it leads to extensive damage to the liver and other organs. So it is only limited for research purpose on liver functions.

Intratumoral delivery of siRNA is a very attractive approach for functional validation of tumorigenic genes. Tumors were targeted by developing chemically modified siRNAs complexed with cholesterol moiety, encapsulated with lipids and delivered by intravenous injection produced significant gene knockdown (Soutschek et al., 2004). Malignant tumors can also be treated by this method. Tumor targeting siRNA can be delivered using RGD peptide ligand directed nanoparticles for targeting the neovascularization in ocular neovascularization models (Kim et al., 2004). The anti-angiogenesis efficacy demonstrated that this approach is clinically viable method for siRNA therapeutics. Recently, antibody mediated delivery of siRNA has also been reported for tumor targeting via cell surface receptors (Song et al., 2005). Meanwhile, two academic groups have also shown that RNA ligands called aptamers can also be used to guide siRNAs to their targets. although the aptamer delivery is not yet quite as good as lipid delivery, significant reduction in the translation of the targeted gene is still possible. Moreover the development of such nucleic acid reagents to internalize biomolecular cargoes is not only novel, but also extremely practical. Unlike lipid amalgams, nucleic acids can target specific cell types. Unlike peptides, conjugation chemistry between nucleic acid partners can potentially involve either simple hybridization or co-synthesis. Hence with the growing knowledge of in vivo siRNA delivery, the potential therapeutic applications of siRNA agents are bound to expand.

The pioneering research in the field of RNAi therapeutics has attracted commercial interest of pharmaceutical industry leading to development of several new delivery options. The table No. 5 gives a glimpse into the variety and potential of the targeted vehicles with the hope of maximal success in the future.
RNA interference: An Ancient Mechanism for Novel Therapeutics

Table 5: Targeted Delivery Vehicles Being Investigated For Commercialization

<table>
<thead>
<tr>
<th>Delivery Vehicle</th>
<th>Disease (Gene)</th>
<th>Advantages</th>
<th>Companies</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid nanoparticles</td>
<td>-</td>
<td>Modified lipids designed to change under biological conditions, etc.</td>
<td>Sana</td>
<td>Chemical &amp; Engineering News, November 12, 2006</td>
</tr>
<tr>
<td>Cyclofetin + polyplexes nanoparticles delivery vehicle</td>
<td>Cancer</td>
<td>Polyplexes (COPPs) condense DNA into complexes that are targeted by cells - target particular cell types using ligands.</td>
<td>Calando Pharmaceuticals</td>
<td>-</td>
</tr>
<tr>
<td>Complementary cholesterol cell-penetrating peptides (CPPs)</td>
<td>Hypercholesterolemia</td>
<td>Peptides coproduced with DNA substrate for delivery; the peptide helps entry into the cell increasing transcrivability.</td>
<td>Nustech Pharmaceuticals</td>
<td>-</td>
</tr>
<tr>
<td>Complementary to fusion proteins made of antibodies and proteins.</td>
<td>HIV</td>
<td>Antibodies target HIV+ cells, protease delivery</td>
<td>Libervax</td>
<td>-</td>
</tr>
<tr>
<td>Aptamers-siRNA conjugates</td>
<td>Prostate cancer</td>
<td>Aptamer targets and facilitates delivery does not activate non-specific inflammatory responses</td>
<td>Chu et al., 2006</td>
<td>-</td>
</tr>
<tr>
<td>SNAFL + Medical Devices</td>
<td>Paroxysmal and Huntington’s diseases</td>
<td>SNAFLs deliver chemically stabilized siRNAs directly into CNS.</td>
<td>Aylan &amp; Mofrerio</td>
<td>-</td>
</tr>
<tr>
<td>Multifunctional siRNA drug delivery device (MDT) with PRO and Minimetabolites</td>
<td>Tumor cells</td>
<td>PRO-eluting minimetabolites can deliver siRNA inside the cell</td>
<td>Nakanishi et al., 2006</td>
<td>-</td>
</tr>
<tr>
<td>Lipid-based nanoparticles</td>
<td>Tumor cells</td>
<td>Targeted to epithelial cell adhesion molecules (EpCAM) expressing human solid tumor cells - induced apoptosis.</td>
<td>Medical Research News 16 June, 2007</td>
<td>-</td>
</tr>
<tr>
<td>Nanoparticles coated with Fluorescein Quantum dot labeled siRNA delivery</td>
<td>Tumor cells</td>
<td>Quantum dots help monitor co-delivery</td>
<td>Medical Research News 16 June, 2007</td>
<td>-</td>
</tr>
</tbody>
</table>

ENDOGENOUS GENE DELIVERY:

The major drawback of exogenously produced siRNAs is the inability to stably or inducibly regulate gene expression. (Holen et al, 2002). In chronic disease, long term inhibitory effects are desired, enhancing the need of repeated delivery of synthetic siRNA, raises a plethora of problems pertaining to cost and route of delivery. To overcome these issues several research groups have shown that short hairpin siRNA (shRNA) can be produced from expression plasmids that contain promoters that are dependent on either RNA polymerase (II) or (III). (Brummelkamp et al., 2002). Pol (III) promoters are used preferably. In one strategy, the sense and antisense strands are expressed as the independent transcripts that hybridize within the cells to form functional siRNA duplexes. While in another strategy the sense and antisense strands are expressed together as a single transcript separated by a short loop sequence. The transcript forms a hairpin structure that can be processed by DICER into functional siRNA.

Brummelkamp et al, (2002), reported the sustained inhibition of p53 by stably integrated siRNA- expressing DNA templates. In addition, the delivery of siRNA from DNA templates can be adapted to viral vectors, and thus can facilitate entry of siRNA into cells and tissues that are otherwise difficult to transfect. Retrovirus and adenoviruses have already been shown to be effective vehicles for delivering siRNA into cells. (Devroe et al, 2002).

Retroviral vectors including ones derived from murine stem cell virus and lentiviral subclass can also be used for siRNA delivery. The advantage of using lentiviruses is that they can infect a broad range of cell types including nondividing, postmitotic, and primary cells. Also integration into the host genome allow for stable expression of the transgenes.

APPLICATIONS:

RNAi has revolutionized the post genomic era by its wide applications both in functional genomics as well as drug discovery. The evident usage of this technology for target validation, gene discovery and gene therapeutics development has established its potential as an influential technology.

The technique holds great promise as a therapeutic approach to silence disease causing genes particularly those that encode so called “non- druggable targets” that are amenable to conventional therapeutics such as small molecules, proteins or monoclonal antibodies. The technique has an upper hand over the traditional approaches to treating disease, including broad applicability, therapeutic precision and selectivity avoiding side effects. Thus the widespread applicability, coupled with relative ease of synthesis and low cost of production make siRNA an attractive new class of drugs.

RNAi AS THERAPEUTICS:

The gene-specific features of RNAi, establish its conceivable role as therapeutic applications. Since siRNAs direct cellular RNAi biology, these are potential therapeutic reagents because of their power to down regulate the expression pattern of mutant genes in diseased cells. RNA interference-based therapeutics have potentially significant advantages.
over traditional approaches to treating diseases, including their broad applicability, therapeutic precision and, target destruction. Using RNAi as a small molecule drug is an increasingly important application of RNAi. The great value of RNAi is its ability to provide information about disease-related genes that would be useful targets for other small molecule drugs gene function thus as a drug discovery tool.

**LOCAL SIRNA THERAPY:**

A clinical situation where siRNAs needs to silence disease-related gene expression locally in easily accessible tissues provides the most readily testable opportunities for exploring its therapeutic potential. Local siRNA administration, that has proven to be beneficial in small animal models, involves lungs, vagina, subcutaneous tissue, muscle, eyes and central nervous system. Table No 4 lists the experiments conducted in mice. Studies suggest the possible application of locally injected siRNAs as adjuvant therapy before or after surgical resection of tumors that have not yet metastasized. siRNAs might also be used to shrink a non-operable tumor to make it amenable to surgical removal. Topical application of siRNAs might also be highly effective for both benign and pre-malignant skin conditions, as well as malignant pigmented and non-pigmented skin cancers.

Local delivery of siRNA has a clear advantage in requiring less amount of therapeutic as was proven by Zimmermann et al., (2006). SNALPs being tissue specific prove to reduce the effective siRNA dose to ≤ 3 mg/kg/day, a practical therapeutic dose. Another cell-specific targeting strategy, effective at 1 mg/kg siRNA dose was observed in a mouse tumor model, involving mixing of siRNAs with a fusion protein composed of a targeting antibody fragment linked to protamine a highly basic protein (Song et al., 2005). Topical delivery of siRNA has distinct advantages over systemic in avoiding various adverse side effects, like activation of toll-like receptors leading to interferon secretion. Another adverse effect observed is in case it triggers apoptosis, killing healthy cells causing problems in a clinical setting.

**Figure 10**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Route of administration</th>
<th>Delivery method</th>
<th>siRNA target gene</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>intranasal or intratracheal</td>
<td>Veh. without PBS</td>
<td>influenza A. receptor mRNA</td>
<td>Reduced viral titre by 1-2 logs</td>
<td>Gu et al., 2004</td>
</tr>
<tr>
<td>Hydrodynamic injection</td>
<td>intravenous</td>
<td>Buffer without Tris-TE</td>
<td>-</td>
<td>Reduced viral titre and amelioration of clinical symptoms</td>
<td>Kim et al., 2000</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>subcutaneous</td>
<td>Buffer without Tris-TE</td>
<td>-</td>
<td>Reduced viral titre and amelioration of clinical symptoms</td>
<td>Kim et al., 2000</td>
</tr>
<tr>
<td>Vagina</td>
<td>Intravaginally</td>
<td>Oligonucleotides</td>
<td>-</td>
<td>Reduced choriocarcinoma growth</td>
<td>Lemes-Moraes et al., 2005</td>
</tr>
<tr>
<td>Intragastrically</td>
<td>intraperitoneal</td>
<td>Buffer without Tris-TE</td>
<td>-</td>
<td>Reduced viral titre and amelioration of clinical symptoms</td>
<td>Kim et al., 2000</td>
</tr>
</tbody>
</table>

**Table 4**

Topical application of siRNAs might also be highly effective for both benign and pre-malignant skin conditions, as well as malignant pigmented and non-pigmented skin cancers. Several of oncogenes have been targeted as enlisted in Table No.5
**SYSTEMIC SIRNA THERAPY:**

Delivery of siRNA therapeutics to various tissues, organs or disseminated cells like lymphocytes or metastatic tumor cells of the body, not easily accessible for topical application need different delivery strategies to be followed. Such systemic delivery methodologies are of immense importance for the treatment of parasitic infection as well. It is another attractive area of siRNA application as parasites like other primitive organisms readily take up siRNAs even in the absence of any transfection reagent (McRobert et al., 2002; Aphasizhev et al., 2002; Mohmmed et al., 2003).

Conventional intravenous administration of siRNAs for silencing genes essential for the parasite to survive or replicate has been investigated in mice model with encouraging results.

Systemic delivery of siRNAs using hydrodynamic transfection protocols previously used to deliver plasmid DNA into cells, were tried in mice. The process involves rapid intravenous injection (in ~5 s) of DNA or RNA in a large volume about 20–40% of the circulating blood volume of a mouse. The process functions by creating a transient inversion of blood flow resulting from elevated venous pressures, leading to uptake of DNA/ RNA into liver, kidney, spleen, heart and lung cells. Liver has been observed to be targeted very efficiently by this method.

Soutschek et al. (2004) have developed a chemically modified siRNAs to silence an endogenous gene encoding apolipoprotein B. The siRNA synthesized by the group was stabilized by addition of a cholesterol group that was linked to the terminal -OH of sense strand RNA. An intravenous injection of the siRNA-cholesterol conjugates in mice resulted in the reduction in the apob mRNA expression by more than 50% in the liver and 70% in the intestinal jejunum pointing to the potential of RNAi as a new therapeutic for the treatment of metabolic diseases. ApoB expression in nonhuman primate was reduced targeting SNALPs to liver (Zimmermann et al., 2006).

Recently four siRNAs targeting the polymerase (L) gene of the Zaire species of Ebola Virus EBOV (ZEBOV) either complexed with polyethylenimine (PEI) or formulated as stable nucleic acid–lipid particles (SNALPs) were investigated in Guinea pigs for treating either before or after lethal ZEBOV challenge. It was found that even 1 of the 4 siRNAs alone could completely protect (Geisbert et al., 2006) guinea pigs from a lethal ZEBOV challenge.

Brown and Catteruccia (2006) have first elucidated in detail the life cycle of malarial parasite, followed by the application of RNAi for the identification of factors within the mosquito vector that supports parasite development and maintenance. This has lead them to propose RNAi mediated gene knockout of P. falciparum as a potential new drug.

New methods of genome analysis like microarrays have generated large amounts of data that tend to complicate its interpretation as to the most relevant genes underlying etiology of a disease. Since microarrays allow identification of alteration in genome expression, siRNA can be designed to target genes that are over expressed in disease tissue; leading to rapid pinpointing of drug targets amongst them (Dorsett and Tuschl, 2004).

It is now possible to generate knockout animal models using lentiviruses as delivery tools for shRNAs in a few weeks rather than a few years, which is what is normally required for homologous recombination based techniques. This is going to boost further the utility of RNAi in drug discovery.

**FUNCTIONAL GENOMICS:**

Besides being an area of intense, upfront basic research, the RNAi process holds the key to future technological applications. Genome sequencing projects have generated wealth of information regarding gene sequences but still clarity on functional role of all genes is missing. The use of small interfering RNA (siRNA) to knock down/ knockout expression of specific gene have opened up exciting possibilities in the study of functional genomics. The ability to easily and economically silence genes promises to elucidate numerous signaling, developmental, metabolic, and related disease pathways. Various studies have been undertaken to elucidate the role of specific genes in basic cellular processes like DNA damage response and cell cycle control (Cortez et al. 2001; Brummelkamp et al. 2002; Mailand et al. 2002; Porter et al. 2002; Stucke et al. 2002;...
Zou et al. 2002), general cell metabolism (Ancellin et al. 2001; Bai et al. 2001), signaling (Habas et al. 2001; Li et al. 2001; Martins et al. 2002), the cytoskeleton and its rearrangement during mitosis (Du et al. 2001; Harborth et al. 2001), membrane trafficking (Short et al. 2001; Moskalenko et al. 2002), transcription (Ostendorff et al. 2002) and, DNA methylation (Bakker et al. 2002). Heterochromatin is composed of highly repetitive sequences interspersed with transposons and is non-coding. The condensation pattern is determined by both DNA and histone modification. Recently it has been found to produce RNAi which appear to be the key factor in epigenetic regulation of gene expression, chromosome behaviour and evolution. It may be the mechanism underlying genome imprinting whereby chromosomal condensation pattern is determined by parent-of-origin. Even the phenomenon of hybrid dysgenesis may be explained if siRNA pools that are largely maternal, do not match polymorphic repeats from the paternal chromosome it may result in mobilisation of transposons and consequent chromosomal disruption (Lippman and Martienssen, 2004).

RNAi technology is proving to be useful to analyze quickly the functions of a number of genes in a wide variety of organisms. RNAi has been adapted with high-throughput screening formats in C. elegans, for which the recombination-based gene knockout technique was not established. Recently, a large-scale functional analysis of 19,427 predicted genes of C. elegans, was carried out with RNA interference. This study identified mutant phenotypes for 1,722 genes (Kamath et al, 2003). RNAi technology has been similarly used in the identification of several genes in D. melanogaster involved in biochemical signaling cascade as well as embryonic development (Clemens et al, 2000). In plants, gene knockdown-related functional studies are being carried out efficiently with transgenes present in the form of hairpin (or RNAi) constructs. Plant endotoxins could be removed if the toxin biosynthesis genes are knocked out. SiRNA results in partial knockout, which is an advantage over complete knockout in that it helps in investigating the effect of various phenotypes. Thus the method holds a great potential to become the most commonly used technique for gene annotation in the near future.