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RNA INTERFERENCE: TOOL FOR GENE SILENCING

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ABSTRACT

Over the past two decades, dramatic advances in the role of RNA in normal health and disease have been greatly expanded. During development Small RNA molecules regulate eukaryotic gene expression and also during responses to stress including viral infection. RNA interference (RNAi) is a highly conserved process in eukaryotes that leads to post-transcriptional gene silencing through degradation of the target mRNA. This mech- anism is mediated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. In the recent years various methodologies have been developed to silence specific genes with the help of RNA interference (RNAi) . In this review the cellular pathway of RNAi has been explained along with the recent developments in this field. The role of siRNA in mammalian gene functions with the help of viral and plasmid vector system is described. It has been discovered gene silencing by siRNA using the vectors, activates the interferon response. Hence the future aim is to develop siRNA delivery system to silence genes and use RNAi as a therapeutic module.

KEY WORDS: Gene silencing, RNA interference, si RNA, vectors)

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INTRODUCTION

RNA interference (RNAi) is a highly conserved process in eukaryotes, that leads to specific post-transcriptional gene silencing through degradation of the target mRNA. This mechanism is mediated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. This phenomenon is primarily for the regulation of gene expression; self or non self depending upon the surrounding factors or conditions, with the help of RNA molecules. These are non coding in nature to control cellular metabolism that help in maintaining genomic integrity by preventing the invasion of viruses and mobile genetic elements. In plants and worms, amplification of the silencing signal and cell-to-cell RNAi spreading is observed. It is otherwise also known as co-suppression, post transcriptional gene silencing (PTGS), and quelling . Previously, RNA molecules were believed to serve only as messengers, bearing genetic information from DNA. In the early 1980s, it was revealed that small RNA molecules (about 100 nucleotides in length) in *Escherichia coli,* could bind to a complementary sequence in mRNA and inhibit translation[1,2].Presently, about 25 cases of regulatory transacting anti sense RNA are known in *E.Coli*)[3]. In the year 1990 RNAi was first reported by Plant scientists Rich Jorgensen,as a gene that controlled the formation of red pigments in *petunia flowers* but in some cases it was observed that the colour disappeared altogether & named it as co-suppression. Co-suppression is a classical form of eukaryotic post-transcriptional gene silencing seen when a sense transgene meant to overexpress the host Chalcone Synthase-A (CHS-A) gene caused the degradation of the homologous transcripts and the loss of flower pigmentation [4,5]. A PTGS-like process called quelling was also established in the *fungus Neurospora crassa* [6]. In animals, RNA silencing was first reported when Guo and Kemphues [7] ,while attempting to assess the function of antisense RNA to shut down the expression of par-1 gene. As expected, injection of the antisense RNA disrupted expression of par-1, but quizzically, injection of the sense-strand control did to used antisense RNA to block par-1 mRNA expression in *Caenorhabditis elegans* but found that the par-1 mRNA itself also repressed par-1. Their paradoxical observation— subsequently dubbed RNA interference (RNAi) —inspired the experiments of Fire, Mellow, and Fire et al [8] studied on the gene responsible for movement in *C elegans*.When this nematodes were injected with the mixture of sense and antisense RNA, nematodes were observed with the impaired movement, suggesting a defective muscle gene protein.They concluded that the intereference could not be elicited by the intron sequence but, the double stranded RNA injected must match the mature 'trimmed' m-RNA sequence for the gene. This implies that interference takes place post transcriptionally, probably in the cytoplasm rather than in the cell nucleus [8]. Andrew Fire and Craig Mello published this break-through study on the mechanism of RNA interference in Nature 1998.It was earlier known that antisense RNA [9] but remarkably also sense RNA [10] could silence genes, but the result were inconsistent and the effects usually modest. However, as both sense and antisense RNA could cause silencing, Mello argued that the mechanism could not just be a pairing of antisense RNA to m-RNA and he coined the term RNA interference for the unknown mechanism. The mRNA was revealed to be targeted in association with si RNA [11] with a large complex proteins to form RNA induced silencing complex, commonly called RISC. Later, it has been shown that RISC contains at least one member of the argonaute protein family, which acts as an endonuclease and cuts the mRNA (often referred to as the Slicer function). It was also demonstrated that, the processing of dsRNA to short RNA, by a ribonuclease III like nuclease, called Dicer is responsible. In particular plants, worms and fungi, an RNA
dependent RNA polymerase (RdRP) plays an important role in generating and/or amplifying siRNA. [12]

INTRACELLULAR PATHWAY OF RNAi

The intracellular pathway of RNAi consists of two steps [13,14]. In the first step the long dsRNA

is processed into small 21-23bp length of small interfering RNA / siRNA. This step is mediated by an enzyme RNAase III [15]. The RNAse III enzyme identifies dsRNA and processes it into siRNA in such a manner that the siRNA contain a hydroxyl end at 3' and a phosphate group at 5' end. The siRNA has two unpaired nucleotides at the 3' end[13, 14]. This RNAase III enzyme is known as Dicer [16]. Similar Dicer homologous sequences were found in *Arabidopsis* [17], *Dictyoslelium* [18], *Caenorhabditis elegans* [19], *mouse* [20] and human beings [21]. Bernstein et al [16] described the mechanism of action of Dicer enzyme and suggested an ATP-dependant translocation of the enzyme along the target dsRNA molecule. They also revealed that the efficiency of the Dicer depends on the length of the dsRNA.). " , Bioinformatics

studies on the genomes of multiple organisms, as observed by Qiu S, Adema C etal [22]suggest, this length maximizes target-gene specificity and minimizes non-specific effects. These short double-stranded fragments are called small interfering RNAs (siRNAs). A longer dsRNA produces a greater amount of siRNA and a more effective gene silencing. This indicates that Dicer is specific to the size of the dsRNA and hence the smaller intracellular endogenous mRNAs are not degraded by the enzyme Dicer. The second step of RNA interference mechanism is referred as effectors step; The incorporation of guide strand. This is characterized by, RISC activation & Transcriptional silencing- The active components of a protein complex, RNA-induced silencing complex (RISC), which are endonucleases called argonaute proteins mediate the degradation of the target mRNA[23] .siRNA[21] is responsible for guiding the RISC to the target mRNA. Hammond et al[24] observed dsRNA destroyed mRNA with homologous sequence and suggested siRNA to be the guiding molecule of RISC. RISC consists of active form (RISC*) and inactive form (RISC)[24]. The active form of RISC is formed by the unwinding of the siRNA attached to it. This unwinding occurs by a helicase activity of RISC[25]. Although it was first believed that an ATP-dependent helicase separated these two strands[26], the process is performed directly by the protein components of RISC and ATP- independent [27,28] . However, an *in vitro* kinetic analysis of RNAi in the presence and absence of ATP showed that unwinding and removal of the cleaved mRNA strand from the RISC complex after catalysis[29] ,may require ATP. Dicer produced fragments are double-stranded, but only one of the two strands, known as the *guide strand*, binds with the argonaute protein and directs genesilencing. The other *anti-guide strand* or *passenger strand* is degraded during RISC activation.[30] Guide strand is selected, to be the one whose 5' end is less stably paired to its complement,[31] but strand selection is unaffected by the direction in which dicer cleaves the dsRNA before RISC incorporation.[32] Instead, the differentiating factor may be binding of R2D2 protein to the more-stable 5' end of the passenger strand .[33] RISC* is attached to the antisense strand of siRNA. Consequently it was found that though siRNA has to be double stranded for efficient attachment with RISC but siRNA has to unwind for the formation of active RISC*.The mRNA degrading action of RISC is dependent on the phosphorylation of 5'siRNA [21,24]. This phosphorylated 5' end of the RNA strand enters a conserved basic surface pocket and makes contacts through a divalent cation such as magnesium and by aromatic stacking between the 5' nucleotide in the siRNA and a conserved tyrosine residue. This site is thought to form a nucleation site for the binding of the siRNA to its mRNA target.[34] Analysis of the inhibitory effect of mismatches in either the 5' or 3' end of the guide strand has demonstrated that the 3' end is responsible for physically arranging target mRNA into a region of RISC favorable for mRNA cleavage,[35] while, 5' end of the guide strand is likely responsible for matching and binding the target mRNA.

In *Drosophila* cells, the enzyme Dicer cleaves long double-stranded RNA substrates into 21 to 23 nucleotide products. These products are transferred to the RISC loading complex, which in turn forms a pre- RISC complex with duplexed oligonucleotides [36] The pre-RISC complex contains Ago 2 protein and other unknown factors. The Ago2-dependent complex was found to be responsible, for conversion of pre-RISC to holo-RISC i.e. the effective removal of passenger strand . The pre-RISC complex contains duplex siRNA

whereas holo-RISC contains just the guide strand of the siRNA, the passenger strand having dissociated from holo-RISC. 5 prime end of siRNA acts as a target recognition guide which is required for the efficient RNA interference mechanism [37,38]. Here the PIWI domain of holo-RISC (the catalytic subunit of RISC) and Ago-2, which acts as RNase H (highly conserved in Eukaryotes) cleaves the target m-RNA . Exogenous dsRNA is detected and bound by an effector protein, known as RDE-4 in *C. elegans* and R2D2 in *Drosophila*, that stimulates dicer activity[39] .This protein only binds long dsRNAs, but the mechanism producing this length specificity is unknown.[39] This exogenously delivered siRNAs appear to enter the pathway downstream of Dicer because inhibition of Dicer expression does not impact activity[40]. Mammals differ from *Drosophila* in having only one Argonaute protein (Ago 2) capable of cleaving the target RNA. The mammalian Ago 2 is capable of binding single-stranded RNA [41,42] protein but does not bind to duplex RNA. Mammalian Ago 2 is similar to the *Drosophila* Ago 1 enzyme [43],in that the mammalian enzyme exhibits burst kinetics without release of product in the purified state, which is not seen in *Drosophila* Ago 2 enzyme. RNA-induced transcriptional silencing (RITS), is a process,which serves to downregulate genes pre-transcriptionally by modification of histones and associated induction of heterochromatin formation [44].It is carried out by a complex of proteins called the RITS complex. In fission yeast this complex contains argonaute, a chromodomain protein Chp1, and a protein called Tas3 of unknown function.[45] The induction and spread of heterochromatic regions requires the argonaute and RdRP proteins.[46] Thus, deletion of these genes in the fission yeast *S. pombe* cause slowing or stalled anaphase during cell division.[47] Due to disruption of histone methylation and centromere formation,[48] In some cases, to transcriptionally upregulate genes.[49] similar processes of histone modification has been observed .

Micro RNA

An important arm of RNAi involves the microRNAs (miRNAs). These are genomically encoded noncoding RNAs that help regulate gene expression by complexing with RISC and binding to the 3′ untranslated regions (UTRs) of target sequences via short stretches of homology, termed “seed sequences” [50,51].The endogenously induced gene silencing effects of microRNA is similar to siRNA produced from exogenous double stranded RNA,except structurally. This, miRNAs must first undergo extensive post-transcriptional modification before reaching maturity. The primary transcript of a miRNA known as a *pri-miRNA* which is processed, from a longer RNA-coding gene by the microprocessor complex in the cell nucleus, to a 70-nucleotide stem-loop structure called a *pre-miRNA*. This microprocessor complex consists of an RNase III enzyme called Drosha and a dsRNA-binding protein DGCR8. The dsRNA portion of this pre-miRNA is bound and cleaved by Dicer to produce the mature miRNA molecule that can be integrated into the RISC complex; thus, miRNA and siRNA share the same cellular machinery downstream of their initial processing.[52] The miRNA duplexes possess incomplete Watson- Crick base pairing, and the antisense strand cannot be chosen by cleavage of the passenger strand as it is for siRNAs; therefore the antisense strand must be chosen by an alternative mechanism [53,54,55] Pillai RS, Bhattacharyya SN, etal, found siRNAs in animals typically base-pair perfectly and induce mRNA cleavage only in a single, specific target.[56] while, miRNAs differ, typically in having incomplete base pairing to a target and inhibit the translation of many different mRNAs with similar sequences. In contrast, In *Drosophila* and *C. elegans* , miRNA and siRNA are processed by distinct argonaute proteins and dicer enzymes.[57,58]

Use of RNAi in mammalian cells

The proposed biological roles of RNAi include resistance to viruses, transposons (mainly in plants), and the silencing and regulation of gene expression, particularly,during development. In developmental gene control, specific small RNAs (micro RNA and small temporal RNA) are involved, which are processed in the same way as dsRNAs but act at the level of translation. RNAi has been successfully used for gene silencing in

drosophila, fungi, worms and plant. Conversely, the similar technique was not suitable for mammalian cells. On introduction of dsRNA of 30bp length into mammalian cells it was observed that, along with RNAi pathway there was inhibition of protein translation and activation of interferon response (IR). The IR system inhibits protein synthesis by activating enzymes like protein kinase R (PKR). The PKR enzyme phosphorylates and inactivates eukaryotic initiation factor 2α thus inhibiting mRNA translation. Hence in mammalian cell RNAi pathway is initiated by using synthetic siRNA. These are 21-22bp length and evade the first step of Dicer enzyme. Studies have indicated that selection of the appropriate siRNA is by trial and error method. However, the initial 75-100 nucleotides of the mRNA are avoided as they are the binding sites for regulatory protein sequences[59]. Numerous studies have reported methods to rationalize siRNA designing. Schwarz et al[60] reported that sequence preference meant that only one strand of siRNA duplex was incorporated into RISC*. Hence, is required to design the siRNA duplex in such a manner that the antisense strand can be attached to RISC. Thermodynamically unstable, A/U rich siRNA , containing mismatches in the antisense strand is preferred by RISC and results in effective RNAi[61,62,63]. The best method for producing siRNA is by in vitro transcription from DNA oligonucleotide template [64]. The DNA oligonucleotide template consists of a sequence complementary to the T7 RNA polymerase promoter followed by a complementary sequence for the either the sense or the antisense strand of siRNA. Further, a second T7 promoter coding sequence was annealed to create a double stranded T7 region. This method facilitated the transcription of sense or antisense siRNA strand by T7 polymerase and conclusively, the two strands were joined to form the double stranded siRNA[65,66]. The designed siRNA have to be completely complementary to the targets as it was found that a single difference in the nucleotide sequence between the antisense strand and target mRNA decreases the gene silencing effect of siRNA [67]. The position of such mismatches also affects the activity of siRNA [68]. The degree of homology required to bring about the gene silencing affect is studied by using microarray technique. The selected siRNA is transfected to a cell line another non transfected cell line serves as control. They observed a number of non specific gene silencing [69]. They explained this phenomenon was due to imperfect homology of siRNA to several genes. Thus, it was discovered that since siRNA enters the miRNA pathway perfect homology to target mRNA is not required[70]. Then Kim et al discovered that removal of the 5' triphosphate of siRNA prevents the activation of the IR system [71]. The commercially available siRNA have free hydroxyl groups at their 5' ends, which can be phosphorylated intracellularly. The synthetic siRNA which have double uridine or deoxy-thymidine at 3' end are most effective in gene silencing [14]. Concisely, for RNAi in mammalian cells the optimal siRNA has 19bp double stranded region with 2-deoxy-thymidine/uridine at each of its 3' ends and the target sequence always commences with two adenine residues.

shRNA

Although synthetic siRNA are effective and do not activate the IR system, their major disadvantage is that they are short-lived. Hence, to increase the siRNA expression in mammalian cells, eukaryotic polymerase III (Pol III) was used to express siRNA from expression cassettes. This technique involves cloning of a sequence coding for the sense strand of siRNA of interest, followed by a spacer and then cloning the equivalent to the antisense strand, which ends in a series of 5U residues. The inclusion of a spacer results in formation of a hairpin structure, which allows the sense and antisense sequences to from base pairs. The H I and PolIII eukaryotic promoters are ideal for this expression as they possess the following features: (1) they initiate from +1 position of the transcripts and (2) the transcripts do not terminate with a poly A tail but with a series of 4-5 thymidine residues, which results in a series of U residues [72]. Thus, enabling the shRNA sequence to be transcribed without containing any inhibitory 5'nucleotide sequences and designing it to consist uridine dimmers at the end. In fact the shRNA and synthetic double stranded siRNA are similar structures. The only difference is

that the two strands of shRNA are joined by a spacer. The functional efficiency of shRNA in gene silencing is similar and comparable to siRNA [73]. Brummelkamp et al [72] suggested that a nine bp long loop sequence mediated optimal transcript expression and that following expression the loop is processed sot that the siRNA strands are not connected by a loop region. In addition to the above discovery loop sequences of various lengths have been designed and hybrid promoters have been developed. The hybrid promoters are indented to improve and allow a regulated siRNA expression. Xia et al [74] used U6 promoter to study siRNA expression in cytomegalovirus and observed an enhanced gene silencing effect. Another inducible system for siRNA expression under the control of PolIII promoters has been developed [75]. They used the Tet operator sequence (tet -O) to replace the non essential part of H1 promoter. The resultant cassette was used to transfect cell lines. Further it was discovered that efficient expression of siRNA and silencing of genes depended on the presence of tetracycline or doxycycline in the culture medium. Recently, shRNA libraries have been developed and they can target any gene for silencing. In this system a large number of diverse shRNA – expressing inserts corresponding to a large number of genes are ligated to suitable vectors and then screened for the resultant constructs for gene silencing [76]. Researchers are trying to develop siRNA libraries against specific genes/ genomes where the shRNA-expressing inserts are developed enzymatically from the cDNA of the gene of interest. Thus, multiple vectors expressing shRNAs against target sequences spanning the complete length of the gene/ genome can be created [77].

Applications

RNAi offers an alternative choice for future therapeutics, by controlling the activity of one or a few disease associated genes. These may include, autoimmune diseases ,dominant genetic disorders, cancer, Severe Acute Respiratory Syndrome(SARS) and viral infections.. Severe Acute Respiratory Syndrome(SARS) and viral infections. RNAi can be triggered by two different pathways: (i) an RNA-based approach where synthetic effector siRNAs are delivered by various carriers to target cells as preformed 21 base duplexes; or (ii) via DNA- based strategies in which the siRNA effectors are produced by intracellular processing of longer RNA hairpin.

Delivery of shRNAs by viral- vectors

Virally- mediated delivery of siRNA expression cassettes enable tranfection to a variety of cell types and a longer expression, leading to a more effective gene silencing,shown in Table-1. Adenoviral vectors expressing siRNAs from a PolII- based promoter and PolIII promoters are being used to reduce p53 levels in MCF-7 and A549 cells [78]. Tomar et al [79] used an adeno- associated viral vector containing either an H1 or a U6- hairpin siRNA cassette, to inhibit expression of caspase- 8 in HeLa- S3 cells. They found that only the U6- based cassette was effective at down- regulating the levels of target mRNA and its corresponding protein. Researchers are also experimenting with retro viral vectors. An H1- siRNA was placed either within the 3' long terminal repeat (LTR) of a self- inactivating retroviral vector i.e. the retroviral vector (vector contains a deletion the U3 region of the 3' LTR) or between the two LTRs [80]. The advantage of the former construct was that the integrated Provirus, derived from the viral vector consisted of an extra copy of the expression cassette in its 5' LTR. This was due to reverse transcription, during which the 3' LTR served as a template. In all other aspects both the constructs were equally effective in down-regulating the endogenous p53 gene in mammalian cells.

In another study, a retrovirus carrying an H1- based cassette successfully silenced a mutant form of K-Ras in CAPAN-1 cells. These cells lost their ability to produce tumours when injected into athymic mice [81]. Scientists have also used lentiviral vectors to deliver siRNAs. A self inactivating lentivirus was designed such that it expressed a hairpin siRNA targeted against enchanced green fluorescent protein (EGFP) under the control of H1 promoter [82]. The lentiviral vector was used to infect a cell line stably expressing EGFP. It was found that this method could successfully mediate the silencing of the EGFP expression [82]. It was also observed that the silencing of EGFP

persisted for 3 weeks from its onset [82]. Lentiviral vectors were also used for in vivo gene silencing [82]. In this study mouse blastocyst cells, stably expressing EGFP were transfected with a lentiviral vector. The lentiviral vector expressed an ati- EGFP hairpin siRNA under the control of H1 promoter. The transfected cells produced mice in which EGFP expression was significantly reduced. Further, it was observed that this gene silencing effect persisted in some of the progeny [82].

Efficiency and specificity of RNAi depends on cell type

RNAi using siRNA has been observed to be effective in a wide range of cell types. These cells are usually derived from cultured cell lines. Though, siRNA have been designed and tested successfully in single experimental cell lines designed for that study, similar results may not be possible in an altered cellular environment since RNAi has not been extensively tested in differing cellular environments. Hence, Bantounas et al [83] investigated this possibility by designing six anti- luciferase siRNA and tested the efficacy of RNAi in HeLa cells. The anti- luciferase siRNA most effective at gene silencing was then tested in neuroendocrine PC-3 and αT3 cells[84]. Then the same anti- luciferase siRNA sequence was used to synthesize shRNA, which was expressed by an adenoviral U6 vector. They observed the following results; the synthetically designed anti-luciferase siRNA was highly effective in silencing the luceferase gene in the HeLa cells, in the PC-3 cells there were non -specific effects and in the αT3 cells there was extensive toxic effects leading to cell death within 24 hours [83]. Similar differing results were obtained when the anti-luciferase shRNA under the control of the U6 promoter was delivered using the adeno viral vector. In this case there was no inhibition of luciferase activity in the PC-3 cells, mild inhibition in the HeLa cells and a stong inhibition in the αT3 cells. As the adeno virus can transducer all these cell types with almost equal efficacy, the difference in the result is implausibly due to shRNA expression. They suggested that the factors determining the effectiveness of the shRNA may be due to the concentration of the Dicer enzyme and the IR produced by the adenoviral shRNA construct. In another study, Elbashir at al [14] reported that the cellular levels of Dicer and IR influence the effectiveness of the siRNA following transfection into different types of cells. Hence it can be concluded that the cellular environment can influence the efficiency and the specificity of the RNAi.

RNAi may provide sequence specific therapeutics for various diseases

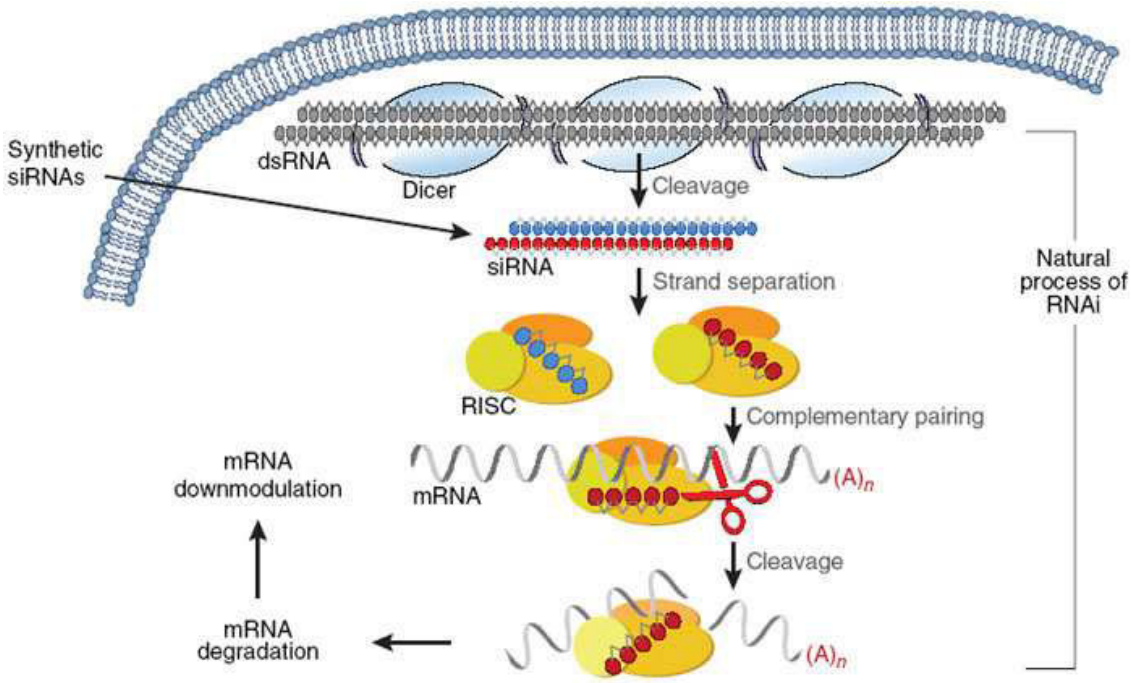
There have been many developments in the recent years regarding therapeutic applications of gene suppression. The various technologies being considered involved sequence specific RNA knockdown methods such as antisense oligonucleotides and ribozymes. The development of efficacious therapeutic module using these techniques is mired due to issues such as delivery, stability, off-target effects and effective target sequence selection. However, the discovery of RNAi has renewed the interest in the clinical development of nucleic acid based gene suppression approaches[84]. The advantage of RNAi therapeutics is its specificity, potency and versatility. The specificity of RNAi activity has been described by various studies as allele-specific in gene suppression [85]. Though, potency of a technique is difficult to measure , it is contemplated that since RNAi is an innate biological response and represents a natural method for manipulation of gene expression it would be an effective method for gene silencing [86.] In view of the fact that multiple sequences represent a single gene or a group of genes, these can be targeted simultaneously by RNAi method of gene suppression [87] The various therapeutic applications of RNAi include inhibition of viral infection, treatment of neurodegenerative disorders, cancer, ocular diseases, inflammation and apoptosis [88].

Table-I
Si RNA delivery strategies

Physical method	Chemical method
1. Electroporation	1.lipid mediated gene delivery
2. Injection	2.peptide mediated gene delivery

	Si RNA	
Isolated from Dorsophila embryo	Synthetic RNAs	Plasmid generated RNAs
	1.Si RNAs	1.siRNAs pollII promoters-mouseU6,humanH1
	2.ds RNAs	2.dsRNA pollI and T7 promoters

Figure I
Mechanism of RNA interference



CONCLUSION

Within the past two decades there has been increasing awareness of the roles that RNAs play in regulation of gene expression . The RNA world has given a booster shot with the discovery of RNA Interference. RNAi started, initially as a part of biology of the lower organisms and subsequently it was discovered in mammalian cells. Rapid progress in our understanding of RNAi-based mechanisms has led to applications of this powerful process in studies of gene function as well as its application as a therapeutic tool to suppress gene expression. As, it has become evident that RNAi technique could be used, thus allowing the creation of transgenic animals and knock-out genes. RNAi technology can be used as a future diagnostic tool for genomic analysis and also as gene silencing therapeutics against cancer and viral infections . The development of vector based systems allowed RNAi to be tested in invivo animal models. Nevertheless, for RNAi to reach its full potential as a scientific tool and possibly to be used as a therapeutic tool a

mechanism to avoid the non-specific silencing effects and activation of Interferon Response must be developed.

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