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Review Article

RNAi THERAPEUTICS

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ABSTRACT
RNA interference (RNAi) was discovered less than a decade ago and already there are human clinical trials in progress or planned. A major advantage of RNAi versus other antisense based approaches for therapeutic applications is that here cellular machinery that efficiently allows targeting of complementary transcripts is used, often resulting in highly potent down-regulation of gene expression. Despite the excitement about this remarkable biological process for sequence specific gene regulation, there are a number of hurdles and concerns that must be overcome prior to making RNAi a real therapeutic modality, which include off-target effects, triggering of type I interferon responses, and effective delivery in vivo. This review discusses various types of siRNA, mechanistic aspects of RNAi & the potential areas where RNAi therapeutics are applied. It is anticipated that RNAi will be a major therapeutic modality within the next several years, and clearly warrants intense investigation to fully understand the mechanisms involved.
KEYWORDS: siRNA, RNAi, DICER, HIV.

INTRODUCTION

RNA Interference (RNAi) is a process through which dsRNA induces the activation of cellular pathways leading to potent and selective silencing of genes with homology to the double strand. RNAi is a recently described mechanism for inhibiting gene expression. The revolutionary discovery that the endogenous cellular process of RNAi can be artificially manipulated for inducing gene specific silence through administration of small interfering RNA (SIRNA) has led to an explosion in this technique. Induction of RNAi through administration of siRNA has been widely used in treatment of immunology and transplantation, viral infections, like HPV, HIV, HCV, Carcinogenesis, etc.

Discovery (History)

RNAi was originally identified in plants, fungi and worms. GUO and KEMPHUES were used antisense RNA to inhibit gene expression (pairing of antisense RNA or DNA with target mRNA through complementary sequence to inhibit target mRNA translation). They found that, sense RNA controls suppressed gene expression effectively as the antisense RNA[1]. Later, Fire and colleagues discovered that dsRNA mixture were more potent than sense or antisense single strands at gene silencing expression in worms which was called as "RNA interference (RNAi) mechanism"[2]. They found in transgenic plants when the genes were silenced by homologous transgenic sequences, they were subsequently resistant to viral infection which is called "cosuppression"[3]. It is clear the trigger for the activation of RNAi causing the silencing is sequence specific[4].

CLASSES OF SMALL RNA

Generally they include siRNAs, microRNAs (miRNAs) and repeat associated siRNAs (rasiRNAs). Overview of all siRNAs is represented in Fig.1.

I. siRNA

siRNA can be synthesised invitro by chemically and enzymatically.

A. Chemical synthesis

Owing to short size, 21-23 nucleotides in length of siRNA, typical nucleotide synthesis techniques can be used. The production of siRNA involves the generation of two homologous strands, annealing of the strands invitro, addition of chemical entities to increase stability and ensuring that 2-nucleotide overhangs which is to activate RNA- Inducing Silencing Complex (RISC). In addition siRNA duplex requires a 3' hydroxyl group and a 5'-phosphate group for functional activity[5]. In its synthesis, it is suggested that target region should be at a

least 70-100 nucleotides away from the transitional initiation site and AU : GC content should be as close to 50% as possible[6]. The siRNA duplexes synthesized by this type are effective in silencing of cytoskeletal proteins like lamin A/C, lamin B_1, Nuclear Mitotic Apparatus protein (NUMA) and vimentin in human cell lines[7] .when siRNA specific to RAPTOR (a protein in mediating signal transduction induced by rapamycin) was added to target of rapamycin (TOR) over expressing cells the down stream signaling of protein was abolished showing siRNA inhibition of biological Pathways.

B. Enzymatic synthesis

siRNA duplexes are synthesized in this type by enzymatically cleaving long dsRNA homologues to the target gene invitro using RNase-III extracted from Escherichia coli. Generation of long dsRNA is typically performed by invitro transcription of target gene both in sense and antisense using T_7 RNA polymerase[8]. The advantage of such an enzymatic approach is rapid and effortless identification of the optimal siRNA species for silencing the desired biological function.

C. In-vivo synthesis

In contrast to the above types of synthesizing siRNA, a widely used siRNA in vivo synthesis involves the processing of long dsRNA to small interfering RNAs (siRNAs) by action of dsRNA specific endonuclease known as "DICER" [9,10]. The resultant siRNAs are double stranded and 21-24 nucleotides in length.

II. miRNA & rasiRNA

miRNA are known as microRNAs and they are produced from premiRNA through a multiple maturation process[11]. The premiRNA is expressed in the nucleus from endogenous long transcripts and is processed into ~ 70nt hairpins by the RNaseIII family member drosha to become premiRNA. The premiRNA is exported to the cytoplasm by exportin-5[12] and is further cleaved by R_2 D_2 dicer heterodimer into the mature miRNA.

In organisms like C.elegans, S.pombe, some of the small RNAs are homologous to regions of repetitive DNA which are termed as RasiRNAs including transposons, retrotransposons, centromeric repeats, satellite and microsatellite DNA[13,14], recruit and maintain regions of silenced chromatin and contain these repetitive sequence by activating sequence specific DNA methylation and histone methylation and by recruiting heterochromatin associated protein[15,16]. Transcription from opposing promoters found in repetitive DNA elements, such as centromeric repeats and satellite DNAleads to the formation of long dsRNAs which are cleaved by DICER presumably the R_2D_2 DICER heterochromatin to siRNAs[13].

ENZYME COMPLEXES INVOLVED IN RNA GENE SILENCING

Complexes like DICER, RISC, RITS, etc., are mainly involved in various gene silencing mechanisms.

I. DICER

The long dsRNA in cytoplasm are processed into small interfering RNAs (siRNAs) by the action of dsRNA specific endonuclease (an RNAse-III enzyme) known as "DICER"[9,10]**.** But through invitro, the principle siRNA generating enzyme is actually a DICER R_2 D_2 heterodimer which remains associated with siRNA. There is some evidence that DICER has also additional roles in RNAi, since the efficiency impaired when DICER itself is also silenced[17]. In drosophila there are two DICERS: DCR-1 AND DCR-2. In their eggs lacking functional DCR-2, there is an impaired response of RNAi to synthetic siRNAs. An important advance in the RNA field was the demonstration that synthetic 21-nucleotide duplexes with the same structure as DICER generated siRNAs could also be incorporated into RISC and induce the degradation of homologous target RNA[18]. Action of dicer is described in Fig. 2.

II. RISC

RISC means "RNA Induced Silencing Complex". The siRNAs are incorporated into a multi subunit Ribonucleoprotein complex called "RISC". ATP dependent unwinding of the siRNAs activates RISC. The antisense (guide) strand of siRNA directs the endonuclease activity of the RISC to the homologous (target) site on the m-RNA resulting m-RNA cleavage[19]. The RISC siRNA is initially duplexed but unwinds in the activated mRNA cleavage competent from of RISC ("HOLORISC"). In addition activated RISC also contains the highly conserved argonaute 2 (Ago 2) recently identified as the RNAi endonuclease (or slicer) [20-24]. The actual identity of slicer is not yet known.

III. RITS

A protein complex called (RNA–induced initiation of transcriptional gene silencing RITS) required for heterochromatin assembly in s.pombe has been recently purified and found to contain Ago1 (a known component of the RNAi pathway) the heterochromatin associated chp1 and the novel protein Tas3 (targeting complex subunit3)[25]. The siRNAs produced by R2D2/DICER heterodimer are unwound and taken up by RITS which directs the establishment of silenced chromatin over the region of DNA homologous to the siRNAs.

MECHANISM OF GENE SILENCING

I. Classical way of gene silencing through siRNA

Step1. Long double stranded RNA (dsRNA) is recognized and processed by DICER/R2D2 heterodimer and RNAs-III enzyme into duplexes of short interfering

RNA (siRNA). In the conversion of dsRNA to siRNA ATP are utilized and the phosphates are bound to terminal sides of siRNA with the help of cellular kinases.

Step 2. The siRNA are incorporated into the "Multiprotein RISC" which is inactive.

Step 3. The duplex siRNA is unwound in an ATP dependent manner starting at the 5 terminas that has the lowest free energy of base pairing[26] which causes the activation of RISC.

Step 4. The Antisense siRNA strand binds to target messenger RNA (m-RNA) that bear a high degree of sequence complementarity to the siRNA which cleaves the target m-RNA. Cleavage of the target m-RNA begins at single site f10 nucleotides upstream of the 5' –most residue of the siRNA target m-RNA duplex[27].

Step 5. The cleavage of m-RNA at a specific site into smaller strands causes the inhibition of protein translation specific to that m-RNA. Classical way is represented in Fig. 3

II. Gene silencing through miRNA pathway

Step 1. The premiRNA is expressed in the nucleus from the endogenous long transcripts and processed into the ~ 70nt hairpins by the RNaseIII family member drosha to become premiRNA[11].

Step 2. The premiRNA is exported to the cytoplasm by exportin-5[12]

Step 3. In the cytoplasm the pre-miRNA is cleaved by the R2D2/DICER heterodimer into the mature miRNA.

Step 4. The miRNA is loaded into the RISC which causes the unwinding of miRNA.

Step 5. The unwinding miRNA binds to the target mRNA that have only partial sequence complementary to the mRNA which leads to the repression of protein translation. miRNA pathway is represented in Fig. 4

III. Gene silencing through rasiRNA pathway

Step 1. Transcription from opposing promoters found in repetitive DNA elements such as centromeric repeats and satellite DNA leads to formation of long dsRNAs.

Step 2. These long dsRNAs are cleaved by DICER/R2D2 heterodimer into siRNAs.

Step 3. These are unwounded and taken up the RNA-induced transcriptional silencing the establishment of the silences chromatin over the region of DNA homologous to the siRNAs.

Step 4. This silenced chromatin is characterized by methylation of lysine-9 residue of histone H3 and the recruitment of heterochromatin associated proteins HP1 and HP2 in Drosophila polygene chromosomes.

APPLICATIONS OF RNAI

The molecular RNAi tool will permit investigators to routinely implement "loss-of-function" screens and helps to develop rapid test for genetic interactions in the mammalian cells. The wide spectrum of RNAi application to various fields is represented as below. An overview of siRNA function is depicted in Fig. 5

I. RNAi AS A THERAPEUTIC

RNAi is widely used for therapeutics because of the following reasons:

- All the cells contain the machinery to carry out RNA and all genes are potential targets for RNAi.
- Relative use of the synthesis and low cost of production of RNAi therapeutics.
- In addition, siRNA are chemically stable and can be stored lyophilized without refrigeration. RNAi as a therapeutic is depicted in Fig. 6.

1. Antiviral Therapeutics

A. HPV (Human Papillomavirus)

HPV is called as "Human papilloma virus" which causes Cervical cancer in women. This virus produces a protein that suppresses the activity of genes in the human anticancer defense system. Therefore, the suppression of HPV encoded viral gene products being done by RNAi could help to inhibit the growth of cancer.

B. HCV (Hepatitis C Virus)

Using RNAi the suppression of the function of cellular genes those required for HCV replication which are involved in the host cell interactions and viral morphogenesis[28]. Recently the technique of siRNA is adopted to target the host FASPROTEIN to reduce the severe forms of hepatitis in a mice model[29].

C. HIV (Human Immunodeficiency Virus)

siRNA has been successful in treatment of HIV (Human Immuno Deficiency Virus) and even Severe Acute Respiratory Syndrome (SARS) associated corona virus[30]. siRNA inhibits the replication of HIV by targeting viral (P^{24},vif,nef, tat and rev) or cellular genetic receptors like primary HIV receptor CD4, HIV co-receptor CXCR-4[31] and other receptors like CCR-5 resulting in the prevention of viral entry into target cells[32-34]. A practical utilization of blocking HIV entry into the cells could be transfecting haematopoeitic stem cells with siRNA expressing constructs so that progeny cells are not susceptible to infection[35]. This approach was effective in rendering monocytes derived from transfected progenitors resistant to HIV infection.

2. Anticancer Therapeutics

A. Gene suppression by the siRNAs is a powerful tool to analyze the function of the proteins [invitro and invivo] especially for the rational design of drugs to block the tumor relevant genes. Initially in invitro studies have demonstrated effective silencing of a wide variety of mutated oncogenes such as K-RAS-(46), mutated P- 53-(47), HER-2/neu–(48) and BCR-ABL[36]. IN-VIVO utilization of siRNA was effectively performed by

targeting the colorectal cancer associated gene BETA CATENIN which observes decreased proliferation and diminished invasiveness. Similarly silencing of the oncogene H-RAS led to the inhibition of in-vivo tumour growth of human ovarian cancer in SCID MOUSE[37]. Table showing the use of different siRNA in various diseases is depicted in Table -1

B. siRNA inhibition of BCL-2 family members is associated with increased susceptibility of prostate cancer to chemotherapeutic intervention[38]. The ability to create "PERMANENT-KNOCKDOWN" cancer cell lines will help us to understand the "loss of function phenotype and subsequently develop commercially important cancer preventive targets" very recently this reverse genetic approach has been adopted to study modulation of the polygutamine repeat associated with "HUNTINGTONS DISEASE" a neuro degenerative disorder using viral promoter based vectors and direct injection into mice embryos

3. Immunology

One of the most devastating immune-mediated pathologies is bacterial sepsis mediated by systemic release of TNF-α. The utilization of siRNA to silence this gene has been successfully accomplished in the Murein Model of Sepsis[39]. Manipulation of the macrophages, peripheral blood mononuclear cells and T-cells was successfully accomplished with siRNA which demonstrated the pharmacological activity of the siRNA and the prospect of using siRNA as an Immune Suppressant. Previous approaches to suppressing T-cell responses including administration of drugs (e.g. cyclosporine), antibodies (e.g. antiCD154) or fusion proteins (e.g. CTLA-4-Ig) which have drawbacks like organ toxicity, lack of specificity etc. So, to overcome the drawbacks the therapeutic gene silencing of siRNA was accomplished in recent years.

II. RNAi in agro biotech industry

By the RNAi work performed in plant "ARABIDOPSIS". It was identified that not only new varieties of plant production but also prevention of plant virus infections can be attained. This strategy is used in agro biotech industry to study plant host virus interactions.

III. Functional Genomics

The RNAi approach has been applied in study of several genetic "functions involved in cell growth cell cycle, cytoskeleton, signaling, and membrane trafficking transcription and DNA methylation. The functions of this gene were studied in about 25 mammalian cells either by incorporating synthetic siRNAs are using cloned plasmids that carries siRNA sequences[40]. The biggest advantage of the RNAi platform is it is highly adaptable process, significantly reducing the product development cycle and enhancing the target validation and development.

IV. Genome wide screening

The genome RNA strategy ahs been used in high throughput phenotype screenings to identify several hundred genes that are involved in the cell cycle embryonic or germ line development ovary and vulva specific in "clenohabditis. Elegans" etc., instead of knocking down a single gene at a time which could take a year effort the RNA method allows scientist for the 1st time to knock out every gene in an organism in a few months.

V. Target Validation

Target validation which is a major problem for biopharmaceutical industry can be reduces by applying the technique of RNAi. Target validation determines whether a known validate gene is responsible for a disease and whether altering expression of the gene is likely to result in the therapeutic effect. By using RNAi based target validation the fast track discovery of drug targets in short period in a most cost effective approach can be attained.

VI. Drug screening and Development

Selection and validation of molecular targets is a great importance for drug development in the post genomic era. The identification of the genes responsible for the identification of many diseases which is a major challenge in drug development can be achieved by the RNAi technique in a rapid and more economical way. RNAi also facilitate the drug screening and development by identifying the genes that can confer drug resistance or genes whose mutant phenotypes are meliorated by drug treatment. RNA methods could be extended to study the gene expression of insect and parasite genomes and develop better gene based siRNA with various interdisciplinaries is depicted in Fig.7.

Various organisations working with the siRNA therapeutics is depicted in Table-2.

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Table 1: SIRNA in various diseases

Disease	Target Gene	siRNA Type	Route of administration
Con-A hepatitis	Fas	Pre-siRNA	IV
Fas-induced hepatitis	Caspase-B	Pre-siRNA	IV
LPS-sepsis	TNF	Pre-siRNA	IP
Polygutamine mediated Neuro-degeneration	Polygutamine repeats	Retroviral	IV
Hepatitis B	HbsAg	Pre-siRNA	IV
HIV	HIV-REV	Lentiviral	IV
Colon Cancer	Beta-catenin	Pre-siRNA	IV
Pathological ocular angiogenesis	VEGF	Pre-siRNA	SC

Table 2: Organisations working with the SIRNA therapeutics

R&D ORIENTED ORGANISATIONS	REAGENT DEVELOPMENT ORGANISATIONS
• Alnylam Pharmaceuticals, Cambridge, MA • Anadys Pharmaceuticals, San Diego, CA • Cenix Biosciences, Dresden, Germany • ISIS Pharmaceuticals, Carlsbad, CA • Nucleonics, Inc. Malvern, PA • Ribopharma Ag, Kulmbach, Germany	• Ambion, Inc.Austin, TX (cloning vectors) • BD Biosciences Clontech, PaloAlto, CA (cloning vectors) • Dharmacon, Inc.Laayegatte, CO (siRNA synthesis) • Imgenex, Inc.San Diego, CA(cloning vectors) • Invitrogen, (Transfection reagents) • Mirus Corporation, Madison, WI (Transfection reagents)

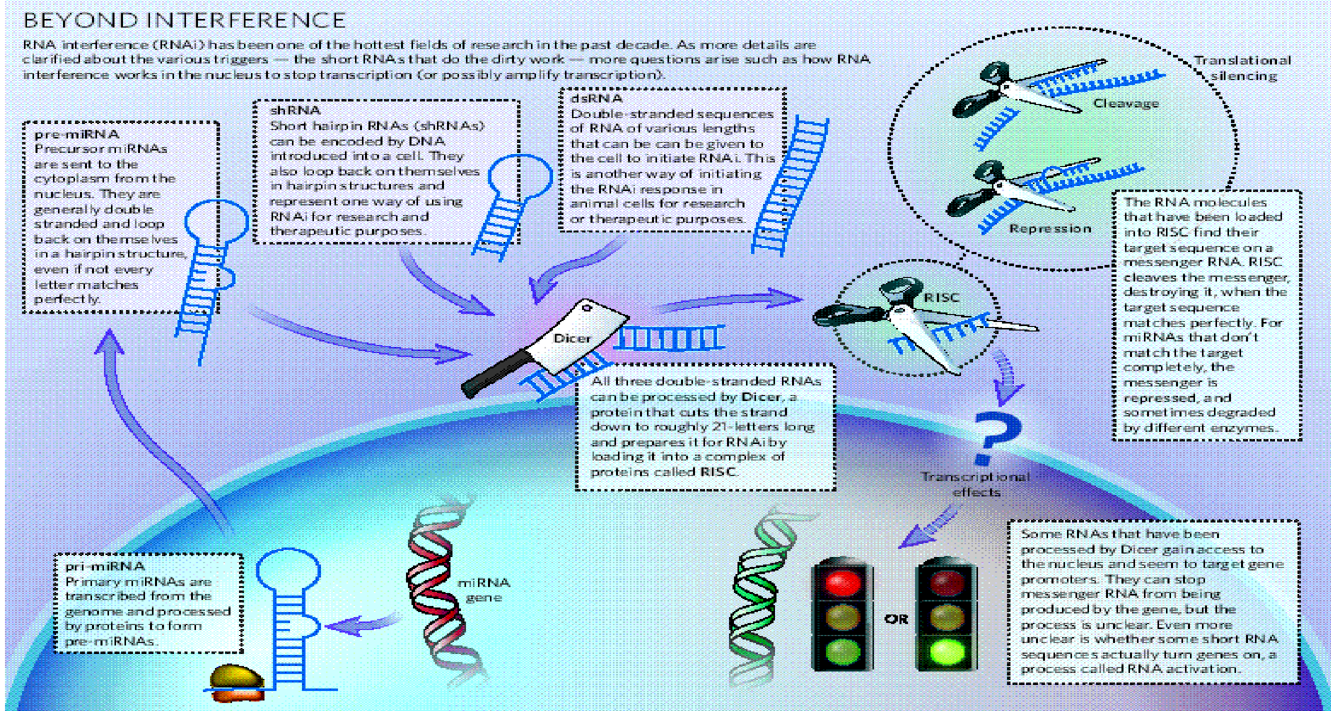


Figure 1: Overview of all siRNAs

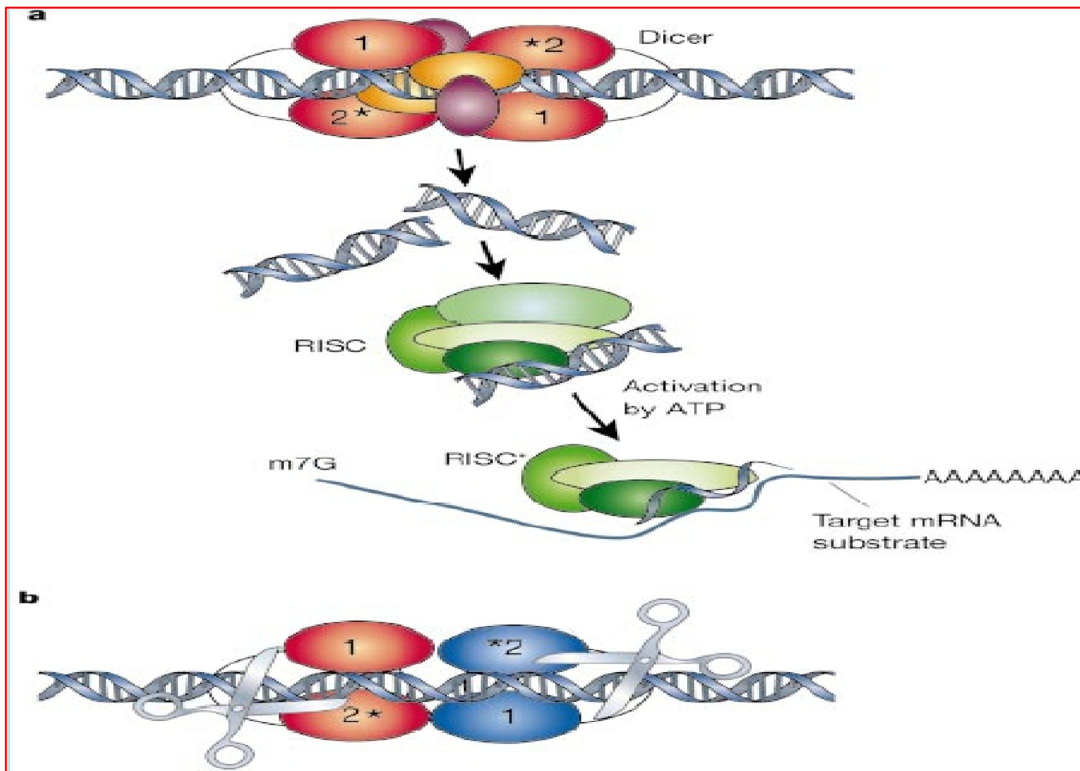


Figure 2: Action of dicer

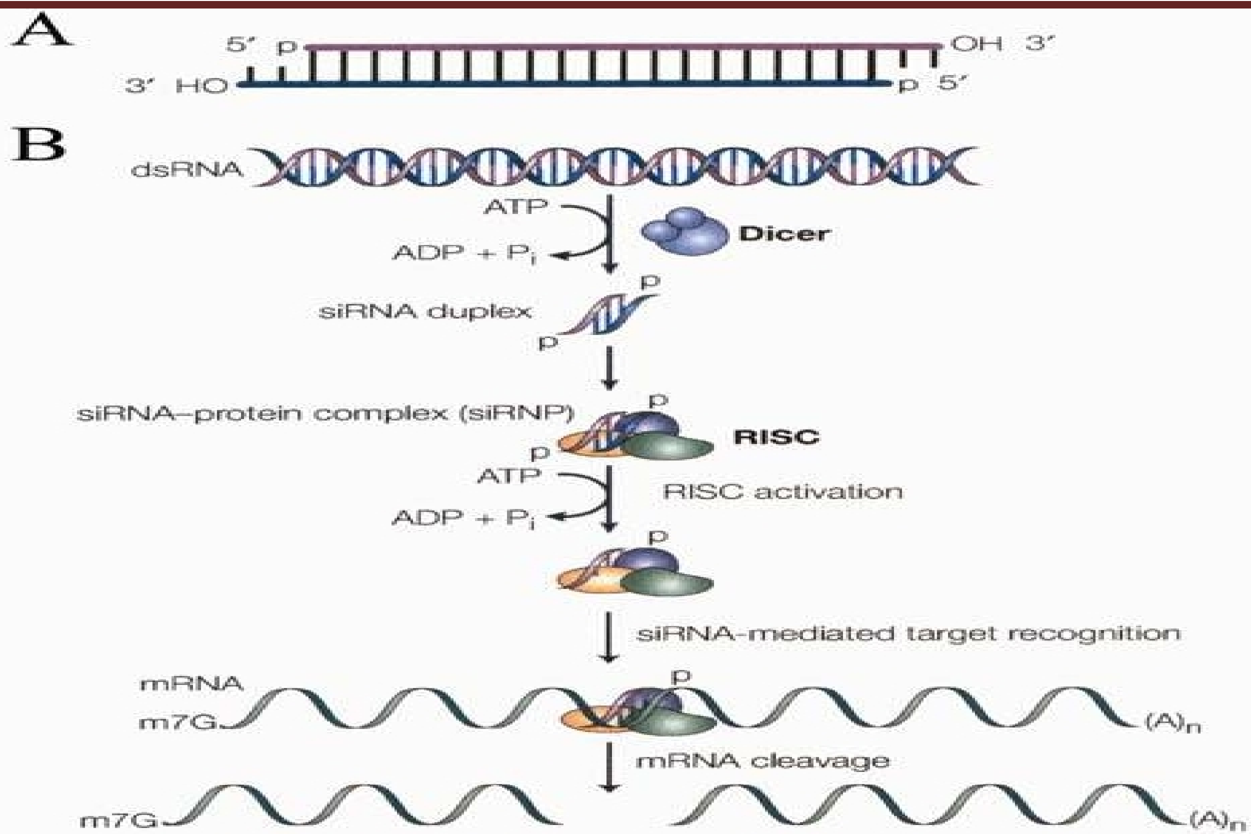


Figure 3: Classical way of gene silencing through siRNA

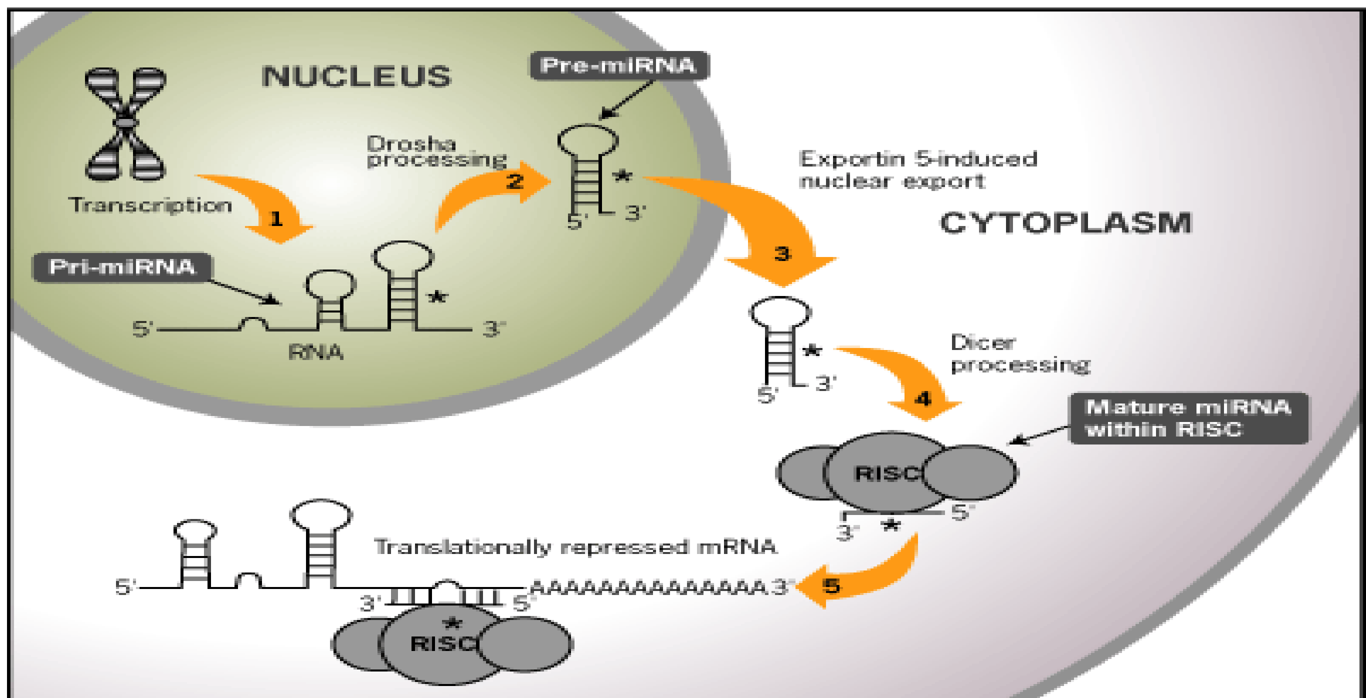
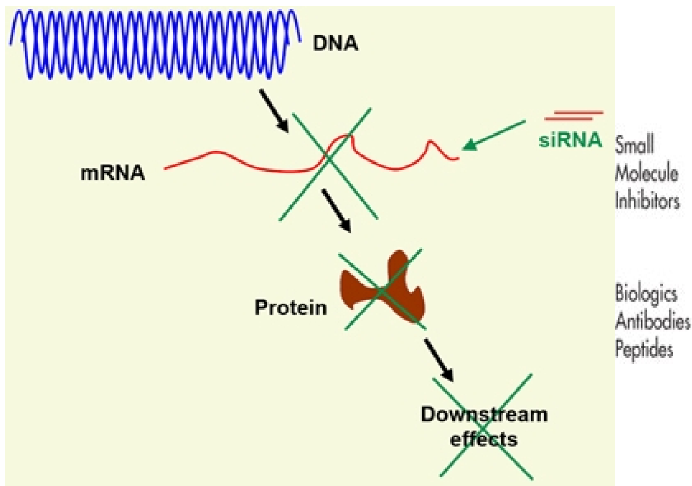


Figure 4: Gene silencing through mirna pathway



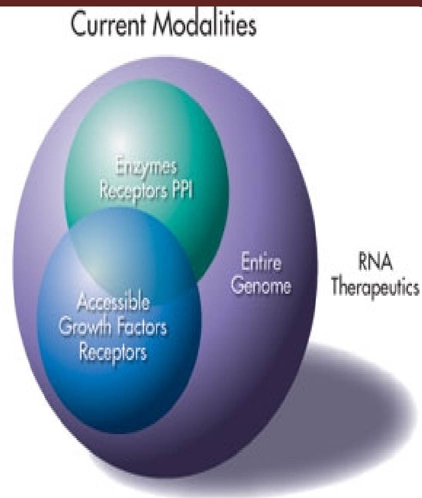


Figure 5: overview of siRNA function

Figure 6: RNAi as a therapeutic

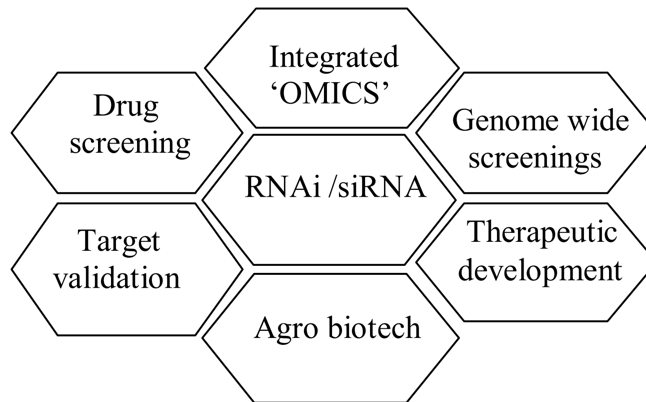


Figure 7: SiRNA with various interdisciplinaries