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Wondrous RNAi-Gene Silencing

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Abstract: The ground-breaking gene silencing technology was a serendipitous breakthrough in biology, which revealed the existence of a charismatic evolutionary conserved pathway, now known as RNA interference (RNAi). RNAi is a post-transcriptional method of gene silencing in which destruction of mRNAs in a sequence-specific fashion is mediated by double-stranded RNA (dsRNA). RNAi because of its specificity, efficiency and potency have become an attractive tool in the arsenal of the cellular biologist. Hence, RNA interference is the newest kid on the genetic block, allows the scientists to selectively turn off genes and also offering a quick and easy way to determine the function of a gene both *in vivo* and *in vitro*. Additionally it promises to set the scientific world alight with its therapeutic potential and wide-ranging applications including onions that can't make you cry new treatments for degenerative diseases and in field of transgenic technology etc. Due of its exquisite specificity and efficiency, RNAi was voted and justified the "top scientific breakthrough of 2002" by Science Magazine. The present review focuses on the landmarks in RNAi (RNA-interference) discovery, its mechanism of action and the promises and pitfalls it offers in treatment and research

Key words: RNAi, silencing, therapeutic agent, Dicer

INTRODUCTION

Landmarks in RNAi discovery: The discovery of RNAi was preceded first by observations of transcriptional inhibition by antisense RNA expressed in transgenic plants and more directly by reports of unexpected outcomes in experiments performed in 1990s by Richard Jorgensen although the exact mechanism was not understood few years later. In an attempt to produce more intense purple coloured *Petunias*, they introduced additional copies of a transgene encoding chalcone synthase (a key enzyme for flower pigmentation). But they were surprised at the result that instead of a darker flower, the *Petunias* were either variegated or completely white. They called this phenomenon co-suppression of gene expression (Napoli *et al.*, 1990), since both the expression of the existing gene (the initial purple colour) and the introduced gene (to deepen the purple) were suppressed. Finally, Jorgensen's group reported the curious phenomenon that petunia pigment genes were shut down when they inserted extra copies of the genes in an attempt to deepen the purple colour. Soon after, a related event like PTGS was noted in the fungus *Neurospora crassa* (Romano and Macino, 1992), although it was not immediately recognized as related. Further investigation of the phenomenon in plants indicated that the downregulation was due to post-transcriptional inhibition

of gene expression via an increased rate of mRNA degradation (Van Blokland *et al.*, 1994). This phenomenon was again called co-suppression of gene expression and also referred to as "quelling" (Ruvkun, 2001) but the molecular mechanism remained unknown. Not long after, plant virologists working on improving plant resistance to viral diseases observed a similar unexpected phenomenon, i.e., Coat Protein Mediated Protection (CPMP) in plants which further gave insight into the mechanism of PTGS. It was known that plants expressing virus-specific coat proteins showed virus resistance, but later it was shown that untranslatable coat protein transgenes (short, non-coding regions of viral RNA sequences) could also confer similar level of virus resistance. Because CPMP was found to act post-transcriptionally, it was thought that CPMP and PTGS shared similar mechanisms. However, the molecular mechanism involved in gene silencing remained enigmatic until the discovery of RNA interference, although it was evident that RNA played a key role in it. The seed to initiate RNAi was sown in *Caenorhabditis elegans*. They used antisense RNA technique to silence *par1* mRNA expression in *C. elegans* but found that *par1* mRNA itself repressed *par1* gene and concluded that both sense and antisense RNA could cause silencing. Their observations inspired the experiment of Fire, Mello and colleagues. Eventually the clue to this remarkable pathway was

solved in 1998 by Professor Andrew Z. Fire at Stanford University, California, USA and Professor Craig C. Mello at University of Massachusetts Medical School in Worcester, USA (Fire *et al.*, 1998) they tested the phenotypic effect of RNA injected into the worm *C. elegans*. As a result of this work, they coined the term RNAi and this work was published in Nature (1998). This discovery later won Fire and Mello the 2006 Nobel Prize in Physiology or Medicine.

Thus, it was clear that pioneering observations on PTGS/Quelling/RNAi were reported in plants, but later on RNAi-related events were described in almost all eukaryotic organisms, including protozoa (Cottrell and Doering, 2003; Malhotra *et al.*, 2002; McRobert and Mc-Conkey, 2002), flies (Brown *et al.*, 1999; Stauber *et al.*, 2000; Marie *et al.*, 2000; Hughes and Kaufman, 2000), nematodes (Caplen *et al.*, 2001; Fjose *et al.*, 2001; Brooks and Isaac, 2002), mouse (Wianny and Zernicka-Goetz, 2000) and human cell lines (Chiu and Rana, 2002). Hence three phenotypically different but mechanistically similar forms of RNAi as co-suppression/PTGS in plants, quelling in fungi and RNAi in animal kingdom has began the journey of the newly dubbed technology RNA Interference.

Special features of RNAi

- Double stranded RNA rather than single-stranded antisense RNA is the interfering agent
- Silencing is highly specific, potent and successful
- Silencing can be introduced in different developmental stages
- Avoids problems with abnormalities caused by a knocked out gene in early stages (which could mask desired observations)
- Silencing effects passed through generation to generations

Proteins involved in RNAi/PTGS/Quelling: To understand the basis of RNA silencing both genetic and biochemical approaches have been undertaken. Genetic screens were carried out to search for mutants defective in quelling; RNAi or PTGS and a large number of genes whose products are somehow implicated in RNA silencing have been identified in *C. elegans*, *D. melanogaster*, *Homo sapiens*, *Dictyostelium discoideum*, *N. crassa*, *Chlamydomonas reinhardtii* and *A. thaliana*. The identified genes encode various components some of which identified as initiators while others serve as effectors, amplifiers and transmitters of the gene silencing process. In the years to come, many other components as well as their interrelations will be revealed. Here, we outline what is known so far.

Dicer: The endonuclease enzyme called Dicer was first discovered in *Drosophila* (Bernstein *et al.*, 2001). It belongs to the RNase III-family that shows specificity for dsRNAs and cleaves them with 3' overhangs of 2 to 3 nucleotides and 5'-phosphate and 3'-hydroxyl termini (Nicholson, 1999; El-Bashir *et al.*, 2001). Dicer is involved in the first step of RNA silencing-the production of siRNAs. Owing to its ability to digest dsRNA into uniformly sized small interfering RNAs (siRNA), this enzyme was named Dicer (DCR). Dicer is ATP-dependent and contains four distinct domains an N-terminal helicase domain, a PAZ domain, a 110-amino-acid domain conservative throughout evolution found in Piwi/Argonaute/Zwille proteins in *Drosophila*, *Arabidopsis* and involved in developmental control (Catalanotto *et al.*, 2000; Tabara *et al.*, 1999), dual RNase III domains and a double stranded RNA-binding domain, ruler helix. Evolutionarily conserved Dicer homologues from many different sources were also identified and tested in plants, fungi and mammals (Bernstein *et al.*, 2001; Ketting *et al.*, 2001). Furthermore, some recombinant Dicers have also been examined *in vitro* and phylogenetic analysis of the known Dicer-like proteins indicates a common ancestry of these proteins (Golden *et al.*, 2002).

RNA-Induced Silencing Complex (RISC): During studies on the biochemistry of RNAi several proteins engaged in RISC formation were characterised. After partial purification of crude extracts from *Drosophila* embryolysate and human HeLa cells through differential centrifugation and anion exchange chromatography, the nuclease cofractionated with a discrete ~25-nucleotide RNA species (siRNAs) are part of an effector nuclease which targets homologous RNAs for degradation (Hammond *et al.*, 2000). This complex is referred to as the RNA-induced silencing complex (RISC). It is made up of a group of proteins which use the siRNA as a guide, presumably identifying the substrate through Watson and Crick base-pairing. The proteins in this complex are members of the Argonaute protein family, which are defined as having a PAZ and PIWI domains. The Argonaute PAZ domain most likely holds the 3' end of siRNA, providing the proper orientation for recognition and cleavage of mRNA. PIWI contains the active site for cleaving the mRNA, shown by the scissors in the schematic (Nykanen *et al.*, 2001). The Argonaute family members have been linked both to the gene-silencing phenomenon and to the control of development in diverse species. The first link between Argonaute protein and RNAi was shown by isolation of *rde1* mutants of *C. elegans* in a screen for RNAi-deficient mutants. Argonaute family members have been shown to be involved in RNAi in *Neurospora crassa* (QDE3) as well as in *A. thaliana* (AGO1) (Fagard *et al.*, 2000).

RNA-dependent RNA polymerase: As a result of screening for genes involved in RNAi a family of proteins that exhibit the activity of RNA-dependent RNA polymerase (RdRP) was also identified. The identification of the quelling-defective gene *qde-1* in *Neurospora* was the first experimental evidence of the involvement of an RdRP in PTGS (Cogoni and Macino, 1999). The *C. elegans* nuclear genome also contains four members of this gene family: *ego-1*, *rrf-1*, *rrf-2* and *rrf-3*. Amongst these *rrf-1* was found as the gene coding for RdRp involved in RNAi. Dalmay *et al.* (2000) found that the 113 kDa *Arabidopsis* RdRP is encoded by *sde1*. It is a plant homologue of *qde 1* in *N. crassa* and *ego1* in *C. elegans*, which are required for quelling and RNAi, respectively. Voimnet *et al.* (2000) propose that aberrant single-stranded transgenic transcripts (abRNA) are converted to dsRNA by a cellular RNA-dependent RNA polymerase (RdRP) this enzyme is necessary to enrich the initial pool of siRNA directed against the target mRNA (Sijen *et al.*, 2001). Therefore, RdRP may also be responsible for the amplification and maintenance of the silencing signal by synthesis of secondary dsRNA trigger molecules, which in turn would be processed into secondary siRNAs. However, no RdRp has been identified by homology in the genomes of either flies or humans.

RNAi mechanism of action: RNA interference is a classical mechanism of gene regulation found in eukaryotes as diverse as in yeast and mammals which plays a central role in controlling gene expression, by inhibiting gene expression at the stage of translation or by hindering the transcription of specific genes (Khanna *et al.*, 2007). The RNAi pathway is initiated by the enzyme dicer which trims long double stranded RNA to form small interfering RNA (siRNA) or microRNA (miRNA). These processed RNAs are incorporated into the RNA-induced silencing complex (RISC), which targets messenger RNA (mRNA) to prevent translation. RNAi pathway can be divided into three major steps:

INITIATOR STEP: dsRNA CLEAVAGE

This is the first step in which, dsRNA is converted into 21-23 bp small fragments by the enzyme Dicer. Dicer is the enzyme involved in the initiation of RNAi. It is a member of RNase III family of dsRNA specific endonuclease that cleaves dsRNA in ATP dependent, processive manner to generate siRNA duplexes of length 21-23 bp with characteristic 2 nucleotide overhang at 3'-OH termini and 5' PO₄ (Hamilton and Baulcombe, 1999; El-Bashir *et al.*, 2001). This initiation pathway may be amplified by the cell through the synthesis of a

population of 'secondary' siRNAs using the dicer-produced initiating or 'primary' siRNAs as templates. These siRNAs are structurally distinct from dicer-produced siRNAs and appear to be produced by an RNA-dependent RNA polymerase (RdRP). Therefore, RdRP is speculated to play indispensable role in RNAi-mediated gene silencing in plants, *N. crassa* and *C. elegans* by amplifying the dsRNA signal, allowing its spread throughout the organisms (Agrawal *et al.*, 2003). Recently two novel opposing models have been presented regarding the role of RdRp. The first model proposed the requirement of an RNA dependent RNA polymerase (RdRp), are enzymes characteristically involved in RNA-virus replication by synthesizing complementary RNA molecules using RNA as a template (Sijen *et al.*, 2001). However, no RdRps have been found in humans and flies, suggesting that there are different mechanisms for RNAi in different species, one primarily RdRp-dependent and one RISC-dependent. The second model hypothesizes that in humans and flies siRNAs act as guides for proteins cleaving the target mRNA. *In vitro* studies with *Drosophila* extracts and human HeLa cell extracts showed that the active siRNAs will be bound by RNA binding proteins to form a ribonucleoprotein complex called RISC (Arenz and Schepers, 2003).

EFFECTOR STEP: ENTRY OF si RNA INTO RISC

The siRNAs generated in the initiator step now join a multinuclease effector complex RISC that mediates unwinding of the siRNA duplex. RISC is a ribonucleoprotein complex and its two signature components are the single-stranded siRNA and Argonaute family protein. The active components of an RISC are endonucleases called argonaute proteins, which cleave the target mRNA strand complementary to their bound siRNA, therefore, argonaute contributes "Slicer" activity to RISC (Nykanen *et al.*, 2001; Hammond *et al.*, 2000). As the fragments produced by dicer are double-stranded, they could each in theory produce a functional siRNA. However, only one of the two strands, which is known as the guide strand, binds the argonaute protein and directs gene silencing (Martinez *et al.*, 2002; Sijen *et al.*, 2001). The other anti-guide strand or passenger strand is degraded during RISC activation.

CLEAVAGE STEP: 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 complementary mRNA in the middle, ten nucleotides upstream of the nucleotide paired with the 5' end of the guide siRNA. This cleavage reaction is independent of ATP. The target RNA hydrolysis reaction requires Mg^{2+} ions. Cleavage is catalyzed by the PIWI Domain of a subclass of Argonaute proteins. This domain is a structural homolog of RNase H, a Mg^{2+} dependant endoribonuclease that cleaves the RNA strand of RNA- DNA hybrids. But each cleavage-competent RISC can break only one phosphodiester bond in its RNA target. The siRNA guide delivers RISC to the target region, the target is cleaved and then siRNA departs intact with the RISC. 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 (Khanna *et al.*, 2007). Complete mechanism of RNAi is shown in Fig. 1.

Other forms of RNA interference: In addition to naturally occurring and manufactured siRNAs, there have been recent publications of alternative forms of RNA, these are

Micro (mi)-RNAs: These are an abundant class of short (19-25 nt) single-stranded RNAs that are expressed in all higher eukaryotes. They are encoded in the host genome and are processed by Rnase III nuclease Dicer from 70 nt hairpin precursors). They can silence gene activity through destruction of homologous mRNA in plants or blocking its translation in plants and animals (Carrington and Ambros, 2003; Cullen, 2004; Novina and Sharp, 2004). Recent work has identified their specific roles in the regulation of early haematopoiesis and lineage commitment). They are sometimes referred to as small temporal RNAs as a reflection of their importance in the regulation of developmental timing (Chen *et al.*, 2004; Medema, 2004).

Piwi-Interacting (pi) RNAs: These are single-stranded 25–31 nt RNAs which have recently been detected in mouse, rat and human testes. They have been shown to associate with Piwi protein (a subclass of Argonaute

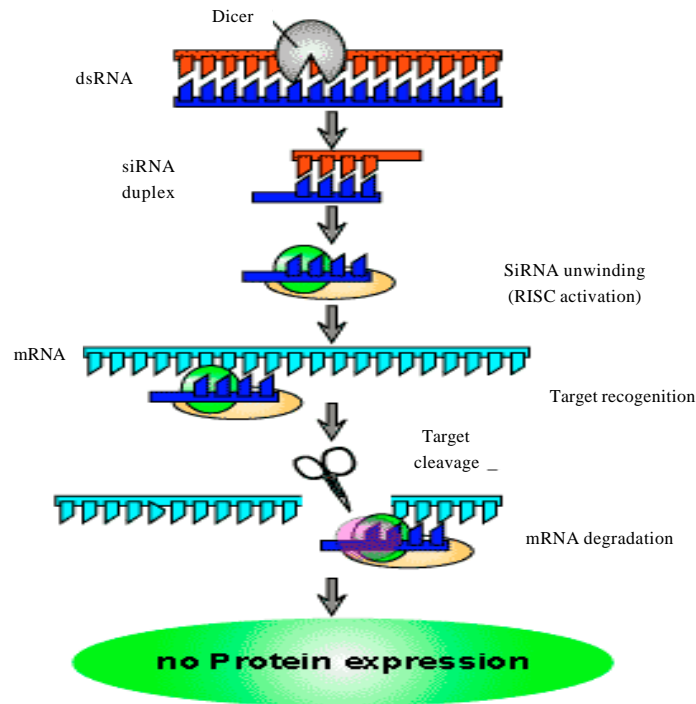


Fig. 1: Mechanism of action of RNAi. Double stranded RNA is introduced into a cell is chopped by dicer to form siRNA, which binds to the RISC complex and is unwound. The anitsense RNA complexed with RISC binds to its corresponding mRNA which is the cleaved by the enzyme slicer rendering it inactive (Kusaba, 2004)

proteins) and the human RecQ1 protein to form a Piwi-interacting RNA complex (piRC). These complexes are thought to regulate the genome within developing sperm cells (Carthew, 2006).

Short-Hairpin (sh) RNAs: Short hairpin RNA or shRNA or is a synthetically manufactured RNA molecule of 19-29 nucleotides that contains a sense strand, antisense strand and a short loop sequence between the sense and antisense fragments. Due to the complementarity of the sense and antisense fragments in their sequence, such RNA molecules tend to form hairpin-shaped double-stranded RNA (dsRNA). shRNA is cloned into a vector, allowing for expression by a pol III type promoter. The expressed shRNA is then exported into the cytoplasm where it is processed by dicer into siRNA which then get incorporated into the siRNA induced silencing complex (RISC) (Medema, 2004).

Small Modulatory (SM) RNAs: These are short, double-stranded RNAs which are found in the nucleus of neural stem cells of mice. They play a critical role in mediating neuronal differentiation through dsRNA/protein interaction (Kuwabara *et al.*, 2004).

RNAi APPLICATIONS

Genome sequencing projects generate a wealth of information which is being utilized to speed up the identification of the biological function of genes by examining the phenotype of organisms that contain mutations in the gene, or on the basis of knowledge gained from the study of related genes in other organisms. However, a significant fraction of genes identified by the sequencing projects are new and cannot be rapidly assigned functions by these conventional methods. Therefore, in the post genomic era it has become a major challenge to develop efficient reverse genetic approaches (i.e., from genotype to phenotype) to evaluate the function of a vast number of newly identified genes. Furthermore, specific silencing of disease-relevant genes (e.g., from tumours, pathogens, or inflammatory mediators) is an interesting therapeutic strategy that deserves attention.

Here RNAi technology is proving to be useful to analyze quickly the functions of a number of genes in a wide variety of organisms. So in context with the present status of knowledge about RNAi, it has revolutionized the field of molecular genetics in such a way that it has enormous potential for engineering control of gene expression at molecular level and use as a tool in functional genomics ranging from molecular biology to gene therapy for diseases arising from aberrant gene expression in animals.

RNAi gene knockdown and functional genomics:

Extraordinary sequence-specificity of RNAi and the simplicity of administering dsRNA to organisms whose genomes have already been sequenced makes RNAi a first choice in studying genome function. RNAi has been proven to be an efficient and vigorous tool for functional genomics studies in *C. elegans* (Tuschl, 2001; Pollard, 2003). The technology considerably bolsters functional genomics to aid in the identification of novel genes involved in disease processes (Downward, 2004). Furthermore, using RNAi mechanism, researchers can cause a drastic decrease in the expression of a targeted gene. Studying the effects of this decrease can show the physiological role of the gene product. Since, RNAi may not totally abolish expression of the gene, this technique is sometimes referred as a "knockdown", to distinguish it from "knockout" procedures in which expression of a gene is entirely eliminated. To accomplish the function of genes, Julie Ahringer's group at the University of Cambridge has created a library of more than 16,000 cloned dsRNAs (around 86% of the *C. elegans* genome). By feeding these clones to worms, they have determined the function of 1,722 genes, most of which were previously unknown. 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. 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. Recently, a number of groups have developed expression vectors to continually express siRNAs in transiently and stably transfected mammalian cells (Lee *et al.*, 2002; Sui *et al.*, 2002). Some of these vectors have been engineered to express small hairpin RNAs (shRNAs), which get processed *in vivo* into siRNAs like molecules capable of carrying out gene specific silencing (Ruiz *et al.*, 1998; Lohmann *et al.*, 1999; Brummelkamp *et al.*, 2002; Yu *et al.*, 2002). Thus, RNAi holds a great potential to become the most commonly used technique for gene annotation in the near future (Khanna *et al.*, 2007).

RNAi for the genetic improvement of crop plants:

Prior to the discovery of RNAi, scientists applied various methods such as insertion of T-DNA elements, transposons, treatment with mutagens or irradiation (Gosal *et al.*, 2010; Mittal *et al.*, 2009). These approaches are very cumbersome and the above methods did not always work adequately. For instance, transposons and T-DNA elements were found to occasionally insert

randomly in the genome resulting in highly variable gene expression. Furthermore, in many instances the particular phenotype or a trait could not be correlated with the function of a gene of interest. At the same time to improve crop plants transgenes are mainly introduced into the genomes of most model plant species using *Agrobacterium tumefaciens*, a common soil bacterium and the mechanism of which relies on T-DNA (transfer DNA), that is carried on a resident plasmid (Ruma *et al.*, 2009). Single T-DNAs can integrate into the genome, but it is very common for multiple copies to integrate in variously permuted head-to-head, tail-to-tail and head-to tail arrays (Gosal *et al.*, 2009). As we all know till now the most effective genetic approach to pest control has been to make plants that produce a protein called Bt toxin, which causes insects to slow down, then stop eating crops, then die. More than 120,000 square miles of crops genetically engineered to produce Bt were grown last year. But Bt isn't effective against many pests, including corn rootworm, which can cause such extensive damage to corn plants' root systems that the plants blow over in the wind and researchers are concerned that insect pests are becoming resistant to Bt. Here RNAi play a vital role. Now a day, researchers are trying to create plants that kill insects by disrupting their gene expression. The crops, which initiate a gene-silencing response called RNA interference, are a step beyond existing genetically modified crops that produce toxic proteins. Because the new crops target particular genes in particular insects, some researchers suggest that they will be safer and less likely to have unintended effects than other genetically modified plants. Moreover, the quality of crop plants can be improved by RNAi for example Kusaba (2004) have made significant contribution by applying RNAi to improve rice plants. They were able to reduce the level of glutenin and produced a rice variety called *LGC-1* (low glutenin content 1). The rice mutant line LGC-1 (Low Glutenin Content-1) was the first commercially useful cultivar produced by RNAi. It is low-protein rice and is useful for patients with kidney disease whose protein intake is restricted. This dominant mutation produces hairpin RNA (hpRNA) from an inverted repeat for glutenin, the gene for the major storage protein glutenin, leading to lower glutenin content in the rice through RNAi. Rice down regulation can also be achieved through mutation-based reverse genetics and a gene targeting system (Terada *et al.*, 2002; Shinozuka *et al.*, 2003). However, RNAi has some advantages over these systems. One of these is its applicability to multigene families and polyploidy (Lawrence and Pikaard, 2003), as it is not straightforward to knockout a multigene family by the accumulation of mutations for each member of the

family by conventional breeding, particularly if members of the family are tightly linked. Another advantage of RNAi lies in the ability to regulate the degree of suppression. Agronomic traits are often quantitative and a particular degree of suppression of target genes may be required. Control of the level of expression of dsRNA through the choice of promoters with various strengths is thought to be useful in regulating the degree of suppression. However, for wider application of transgene-based RNAi to the genetic improvement of crop plants further feasibility studies are needed (Kusaba, 2004).

Engineering of food plants that produce lower levels of natural plant toxins also possible through RNAi. Such techniques take advantage of the stable and heritable RNAi phenotype in plant stocks. For example, cotton seeds are rich in dietary protein but naturally contain the toxic terpenoid product gossypol, making them unsuitable for human consumption. RNAi has been used to produce cotton stocks whose seeds contain reduced levels of delta-cadinene synthase, a key enzyme in gossypol production, without affecting the enzyme's production in other parts of the plant, where gossypol is important in preventing damage from plant pest (Sunilkumar *et al.*, 2006). Similar efforts have been directed toward the reduction of the cyanogenic natural product linamarin in cassava plant (Siritunga and Sayre, 2003). Although, no plant products that use RNAi-based genetic engineering have yet passed the experimental stage, development efforts have successfully reduced the levels of allergens in tomato plants (Le *et al.*, 2006) and decreased the precursors of likely carcinogens in tobacco plant (Gavilano *et al.*, 2006). Other plant traits that have been engineered in the laboratory include the production of non-narcotic natural products by the opium poppy (Allen *et al.*, 2004) resistance to common plant viruses (Zadeh and Foster, 2004) and fortification of plants such as tomatoes with dietary antioxidants (Niggeweg *et al.*, 2004). In plants, gene knockdown-related functional studies are being carried out efficiently when transgenes are present in the form of hairpin (or RNAi) constructs. Plant endotoxins could also be removed if the toxin biosynthesis genes are targeted with the RNAi constructs. Therefore, RNAi soon caught the world-wide attention and became a powerful and useful tool for molecular breeders to produce improved crop varieties.

RNA interference as a novel therapeutic agent: The ability to tap this native RNAi pathway has been recognized by a number of pharmaceutical industries as one the most exciting biotechnology advances in the last decade (Table 1). Given the gene-specific features of RNAi, it is conceivable that this method will play an

Table 1: Companies developing RNAi therapeutics

Company name	Primary areas of interest
Acuity Pharmaceuticals	AMD, diabetic retinopathy
AGY Therapeutics	RNAi in neurons and glial cells
Alnylam Pharmaceuticals Inc.	AMD, Parkinson's disease, respiratory syncytial virus, cystic fibrosis, influenza, spinal cord injury
Atugen AG	Metabolic disease, cancer ocular disease, skin disease
Benitec Australia Limited	Hepatitis C virus, HIV/AIDS, cancer, diabetes/obesity
Calando Pharmaceuticals	Nanoparticle technology
Cytrx Corporation	Diabetes/obesity, ALS, cytomegalovirus retinitis
Devgen	Diabetes/obesity, arrhythmia
Genesis R and D	Allergy
Genta Incorporated	Cancer
International Therapeutics	HIV, Hepatitis B virus
Intradigm Corporation	Cancer, SARS, arthritis
Nucleonics, Inc.	Hepatitis B Virus, Hepatitis C virus
Sirna Therapeutics, Inc.	AMD, Hepatitis C virus, asthma, diabetes, cancer Huntington's disease, hearing loss

AMD: Age-related macular degeneration, SARS: Severe acute respiratory syndrome coronavirus, ALS: Amyotrophic lateral sclerosis (Uprichard, 2005)

important role in therapeutic applications and possibly of most commercial interest in the use of RNAi as a therapeutic agent. Indeed, RNAi has revolutionized biology research, including drug target discovery, by allowing for rapid identification and validation of gene function. There are three main time points at which a disease can be stopped. These are transcriptional, post-transcriptional and post-translational intervention. Before the discovery of antisense RNA and RNAi, most of the drug targets have been proteins and therefore, post-translational intervention. RNAi is a way to control the development of a disease earlier on in the process. Furthermore, the gene-specific features and potential of RNAi for knocking out a protein without harming a cell has established its most believable role into therapeutic applications. It can be applied to targets ranging from rogue genes in cancer to genes of viruses such as Hepatitis B or C or HIV. For example, siRNA is effective against parasites, so perhaps it can be used to silence parasitic genes (Fire, 1999) or used against other pathogens to benefit host organisms like humans (Ruiz *et al.*, 1998). During viral infection, each virus comes with its own set of challenges, yet it is hoped that each will be defeated with RNAi. In each case there are particular genes of interest that if their expression can be eliminated, the replication of the virus, therefore the spread of the infection, can be treated. HIV infection can be blocked by targeting either viral genes (for example, *gag*, *rev*, *tat* and *env*) or human genes (for example, CD4, the principal receptor for HIV) that are involved in the HIV life cycle. Lohmann *et al.* (1999) and Paddison *et al.* (2002) reported that production of virus is inhibited either by blocking new infections or blocking the production of new viral particles in infected cells by silencing the main structural protein in the virus, p24 and the human protein CD4, which the virus needs to enter the cells. This impairs the virus in infected cells and limits its spread into healthy cells. As a result little or no protein is produced (Thakur,

2003). Cancer also seems a promising candidate for RNAi. Using siRNA molecules, researchers believe they can turn off the ability of cancer cells to produce the key proteins that make them different from normal cells and by doing so, stop malignancy in its tracks. Gregory Hannon and colleagues have used RNAi to silence expression of p53 the 'guardian of the genome', which protects against any tumor-associated DNA damage by introducing several p53-targeting short hairpin RNAs (shRNAs) into stem cells and looking at the effect in mice. Furthermore, RNAi treatment for Huntington's disease is also being eyed optimistically. Huntington's disease, a genetic disease, affects a patient throughout his or her lifetime. Therefore, now a day several companies are focusing on the development of RNAi-based therapeutics.

Thus, the discovery of RNAi has opened up a new dimension of research in various areas of medical research and has the potential to transform medicine and the scope of RNAi as a treatment option is extremely wide. It is an area of great promise, but a great deal of research is still needed in this area. The biggest hurdle will be the successful delivery of RNAi drugs to their effective site, since the fragile naked RNA will be broken down before reaching most sites within the body.

CONCLUSIONS AND FUTURE OUTLOOK OF RNAI

The field of RNAi is moving at an impressive pace, generating exciting results and has established a novel archetype with far-reaching consequences in the field of transcription regulation. The RNA silencing has practical use because of the ability to reduce gene expression in a manner that is highly sequence specific as well as technologically facile, economical and having potential in finding out the function of genes at a faster speed and in agriculture specifically for nutritional improvement of plants and the management of mascotous plant diseases. In addition it has kindled hope for the treatment of several

diseases, which have bothered mankind as untreatable by providing an innovative technology for development of therapeutics. However, the major obstacles hindering its immediate applications include selection of targeting sequences and in the delivery of siRNA. The key issues are: 1) how to select silencing targets for a particular disease and 2) how to efficiently deliver siRNAs into specific cell types *in vivo*? Besides, RNAi technology can be considered an eco-friendly, biosafe ever green technology as it eliminates even certain risks associated with development of transgenic and it has already added new dimensions in the various field of science. However, a better and comprehensive understanding of RNAi should allow future researchers to work effectively and efficiently in order to manage the phenomenon.

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