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# RNA INTERFERENCE: AN OVERVIEW

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**ABSTRACT**

Numerous technologies have been used to mediate the down-regulation of gene expression. For example, anti-sense oligonucleotides and ribozymes have been used for more than a decade to target specific RNAs for degradation. Though these methods worked reasonably in some experimental models, they have failed to deliver effective gene silencing in complex mammalian systems. Naturally RNA interference is an important pathway that is used in many different organisms to regulate gene expression. In recent years, extraordinary developments in RNA interference methodologies due to numerous sophisticated scientific advancements have created an avenue that made the small interfering RNAs (siRNA) as the method of choice to target specific genes for silencing. Also it was explored that, like RNA interference, equivalent cellular pathways do exist in mammalian cells. So it was now evident that RNAi, more specifically, siRNA could be exploited as a tool to study mammalian gene function as well as to down-regulate gene expression for treating various diseases in human and other species, to extend the application of RNAi for crop improvement program.

Keywords: RNAi, gene expression, oligonucleotides

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## INTRODUCTION

The nucleus of each cell of an adult human being carries all our genetic material: DNA containing about 30 000 genes. The gene expression process is of fundamental importance for all living organisms. Most genes reside in the chromosomes located in the cell nucleus and express themselves via proteins synthesized in the cytoplasm. When genes are expressed, genetic information is copied from DNA to messenger RNA (mRNA), which then coordinate the process of formation of proteins. After the elucidation

of DNAs double-helical nature (1953) by Francis Crick, James Watson and Maurice Wilkins, it was proposed that another nucleic acid, single-stranded ribonucleic acid (RNA), acts as an intermediary in the genetic information flow process, and the Central Dogma was formulated, i.e. the idea that the genetic information is transcribed from DNA to RNA and then translated from RNA into protein.

Expression of genes is directed by the cellular machinery that copies (transcribes) DNA into mRNA. The human body contains a few hundred different types of cells. Formation and renewal of these cells is regulated through gene activity. All genes are not expressed in each cell: expression of set of genes is specific to cell types. For example, nerve cells express genes different from those expressed by muscle cells or liver cells. The genes that are active at any given point in time are regulated by transcription factors, proteins that govern how genes are read.

**RNA interference: Historical development and findings**

RNA interference is a natural mechanism of great importance in regulation of the activity of genes. RNA molecules were long believed to serve only as messengers, bearing genetic information from DNA. But in the early 1980s it was revealed that single-stranded RNA molecules in the bacterium *Escherichia coli* can bind to mRNA. This inactivates mRNA and therefore prevents it from passing on the genetic information. As a result, the protein encoded by the mRNA is no longer formed. Similar "antisense" molecules were later discovered in the nematode *Caenorhabditis elegans.*

These findings encouraged that the antisense RNA could be used as a form of gene therapy. Many saw use of "tailor-made" antisense molecules to block the production of faulty proteins as a potential new method of treating hereditary diseases and cancer.

Antisense techniques turned out to work well in plants. But attempts to use antisense RNA experimentally in animals have not been relatively successful. Sometimes the experiments faced complications to get consistent results. Around 1990, a series of observations were obtained that were remained as a puzzle and difficult to explain. The most puzzling findings were reported by plant scientists who tried to intensify the hue of red petunias. They inserted a gene that controls formation of red pigment and their experimental results shown that in some cases, flowers lost their colour altogether. It turned out that the mRNA for the pigment had disappeared. The cause of these unexpected effects remained mysterious until the later discoveries of Andrew Fire and Craig Mello.

The crucial experiment that led to the discovery of RNA interference was carried out with the nematode (roundworm) *Caenorhabditis elegans*, by Andrew Fire and Craig Mello, for which they received noble prize. Initially their studies were concerned about how gene expression is regulated in *C.elegans*. They were examining mRNA that encodes a protein involved in the nematode's ability to move. In their experiment, nematode gonads were injected with sense RNA and this had shown no visible effect on the nematodes or on their offspring. So Fire and Mello injected other nematodes with antisense RNA that could bind to the corresponding mRNA for the muscle protein, and again nothing has revealed. But in later stages of the experiment, when they injected a mixture of sense RNA and antisense RNA, the nematode offspring moved in an abnormal, twitching fashion similar to movement patterns that could be seen in nematodes with a defective muscle protein gene.

It remained enigmatic that how, sense RNA and the corresponding antisense RNA that make up double-stranded RNA, could silence the gene that encoded the muscle protein, since double-stranded RNA molecules have no free chains that can bind and inactivate mRNA molecules. It is obvious for one would have to expect that only single-stranded antisense RNA would bind to the corresponding mRNA and silence the gene.

Andrew Fire and Craig Mello solve the riddle by pursuing the problem by injecting double-stranded RNA carrying the genetic code of several other *C. elegans* genes and carried out a special staining technique which could show mRNA for genes that were active in nematode embryos. When the gene was active, the embryo could be stained because its cells contained the mRNA. Antisense RNA binding to the mRNA for this gene reduced the staining to certain

extent. But double stranded RNA (sense RNA plus antisense RNA) eliminated the staining entirely which indicated that the mRNA had disappeared and the gene had been silenced.

In all their experiments, Fire and Mello did with nematode genes, double-stranded RNA extinguished the gene it had been patterned on. This ceased the formation of the protein encoded by the gene. Double-stranded RNA could interfere with the expression of genes; thus the phenomenon was called RNA interference. The findings of Fire and Mello experiments were as follows

- Gene silencing was highly effective only when double stranded RNA was injected
- The mRNA affected by the double-stranded RNA disappeared – it was apparently broken down and eliminated.
- The double-stranded RNA injected must match the mature, "trimmed" mRNA sequence for the gene. Interference could not be elicited by intron sequences (that is, segments of molecules that do not contain coding information). This implies that the interference takes place after transcription, probably in the cytoplasm rather than in the cell nucleus.
- Only the mRNA corresponding to the sense RNA strand of the double-stranded RNA was silenced – no other mRNA in the cells was affected. RNA interference was specific for the gene with a code corresponding to that of the mRNA molecule.
- The effect of double-stranded RNA could spread from cell to cell and from tissue to tissue and could even be passed on to offspring (A. Fire, 1998).

**Mechanism overview**

The mechanism involved in RNAi is mediated via two main steps:

(i) the dsRNA is initially recognised by an enzyme of the RNase III family of nucleases, named Dicer, and processed into small double-stranded molecules, termed siRNA

(ii) the siRNAs are bound by the RNA-induced silencing complex (RISC), which is a multi-protein complex (with RNase activity) that guides the targeted RNA to degradation (I.Bantounas, 2004)

**Natural Role of RNAi**

**Defence against viruses**

More primitive organisms that lack an efficient immune system, RNA interference has proved to be an important mechanism for protection against viral infections. A virus infection starts when viral RNA enters the cell. Double-stranded RNA binds to Dicer and is broken down into smaller fragments. This in turn activates the RISC complex: viral RNA is broken down and the cell survives the infection.

**Protection against damaging effects of transposons**

Most organisms have moveable segments of DNA, termed transposons. These are also called jumping genes because on rare occasions they can move around in the genome. Transposons, like mutations contribute to evolution by increasing genetic variation. But it can be harmful when transposon happens to end up in an inappropriate place, for instance in an important gene.Certain stages of this "jumping" in the genome require that transposon DNA is copied as RNA molecules. The RNA that is formed can be double-stranded and can then be broken down by RNA interference. In this way, RNA interference protects the genome against the damaging effects of transposons (A.Fire, 1998).

**Applications of RNAi**

**In Medicine**

The discovery of RNA interference unlocks the way to exciting new possibilities for genetic engineering. RNA interference has already become an important tool for biological and medical research; for instance, it is widely used as a powerful method for mapping gene functions. Many Scientists hope this principle can be applied in many perspectives such as agriculture and eventually be used to combat serious diseases. In many diseases, certain genes are over-expressed, and it might be possible to alleviate these disorders by suppressing the activity of specific genes. Ideally this would be accomplished with no or tolerable side effects. It is conceptually attractive to use RNA interference to treat diseases: RNA interference, unlike antisense techniques has also been shown to give reproducible results. In addition, double-

stranded RNA molecules can easily be synthesized. So far no drugs based on RNA interference have been approved, but successful animal experiments have been performed and several substances are being tested in clinical trials in humans.

**Cancer**

Cancer is characterised with two general abnormalities - they exhibit dysregulation of the cell cycle resulting in uncontrolled growth and they are resistant to death as a result of abnormalities in one or more proteins that mediate apoptosis (Nam and parang, 2003). The approaches for cancer therapy using RNAi principle are therefore to selectively knock out the expression of a cell cycle gene and/or an anti-apoptotic gene in the cancer cells thereby stopping tumour growth and killing the cancer cells, without damaging the normal cells. Recent studies have clearly demonstrated advantages of RNAi methods for the growth suppression and killing of cancer cells. In one study, siRNA was shown to be greater than an order of magnitude more potent than antisense DNA in suppressing gene expression in human hepatoma and pancreatic cancer cell lines (Aoki et al., 2003). In another study, colon cancer cell strain SW1116 was transfected with siRNA lentivirus to silence eukaryotic translation initiation factor 3, subunit B (EIF3B) gene expression and the results shown knock down of EIF3B gene expression in the colon cancer cell strain SW1116. After successful downregulation of EIF3B mRNA and protein expression, the proliferation rate and clonability of SW1116 cells were also inhibited significantly (Zheng Wang, 2012).

**Infectious Diseases**

Infectious diseases persist to be main causes of death worldwide and are an increasing concern because of the emergence of resistant strains. The ability of RNAi to prevent the replication or cellular uptake of viruses and other pathogens has been clearly demonstrated in cell culture studies and, hence, holds potential for the treatment of various diseases. The ability of HIV-1 to infect cells and replicate can be severely compromised by targeting of viral genes using siRNAs. Several studies shown promising results, such as the suppression of HIV-1 replication in human cells transfected with siRNA directed against tat and the rev gene (Capodici et al., 2002; Jacque et al., 2002; Lee et al., 2002a; Novina et al., 2002), application of RNAi to inhibit replication initiation of hepatitis B virus in mice (McCaffrey A P, 2003). It was also exploited to use RNAi principle to combat against SARS (severe acute respiratory syndrome). Researchers proved that it is feasible to inhibit SARS Virus replication by targeting SARS-CoV genome using plasmid-mediated small interfering RNAs in african green monkey kidney Vero cells (Zhi Wang, 2004).

**Cardiovascular Diseases**

Atherosclerosis is the most common of the cardiovascular diseases contributing to major morbidity and mortality in the developed world. Several contributing factors including inflammation, endothelial dysfunction, dyslipidemia, diabetes, and hypertension contribute to the development of atherosclerosis. The ultimate manifestation of atherosclerosis that results in plaque development and blockade of blood vessels by recruitment of macrophages, foam cell formation, production of reactive oxygen species, smooth muscle proliferation, and extracellular matrix modulation has been extensively studied (Geng and Libby, 2002). Ultimately atherosclerosis can culminate in a myocardial infarction or stroke. The severe ischemia that occurs in heart or brain cells during a myocardial infarction or stroke results in death of cardiac muscle cells or neurons. During atherosclerosis, some of the cells die rapidly, many other cells die more slowly by apoptosis. Experimental data of animal studies shown cells that die by apoptosis process can be saved (Mattson, 2000; Zhao and Vinten- Johansen, 2002). It may be possible to use RNAi technology through several approaches to intervene in the process of atherosclerosis or to reduce the damage to heart tissue and brain cells that patients suffer following a myocardial infarction or stroke. A significant step in the process of atherosclerosis is the up-regulation of cell adhesion molecules in vascular endothelial cells, which play a vital role in the recruitment of macrophages to the site of endothelial damage. The production of cell adhesion molecules can be selectively suppressed in cultured cells (Jarad, 2002). It is now evident that RNAi can be exploited to improve

infarct healing in atherosclerosis-prone mice through monocyte-directed RNAi targeting CCR2, the chemokine receptor that governs inflammatory Ly-$6C^{high}$ monocyte subset traffic (Maulik D. Majmudar, 2013).

**Neurodegenerative Disorders**

Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis are examples of relatively common age-related neurodegenerative disorders. Each disorder is characterized by the dysfunction and death of specific populations of neurons: hippocampal and cortical neurons involved in learning and memory processes in Alzheimer's disease, dopamine-producing neurons in the substantia nigra that control body movements in Parkinson's disease, and spinal cord motor neurons in amyotrophic lateral sclerosis. Specific genetic mutations are responsible for a small percentage of cases of Alzheimer's and Parkinson's disease and amyotrophic lateral sclerosis (Hardy, 2001).

One stratagem is to block the disease-specific events that are supposed to initiate the neurodegenerative process, whereas the second approach targets downstream events in the neurodegenerative cascade. For example, an abnormality in the proteolytic processing of the amyloid precursor protein is believed to be a key early event in Alzheimer's disease pathogenesis, and two enzymes called β and γ-secretases that are responsible for cleaving of amyloid precursor protein to generate the neurotoxic amyloid -peptide are being targeted for drug development. RNAi has recently been used to identify additional proteins, such as APH-1, that are critical for production of amyloid -peptide (Lee, 2002). Recent studies have shown that cultured neurons can be efficiently transfected with siRNAs and that the targeted genes are effectively silenced. In one study it was shown that cultured neurons can be depleted of the p75 neurotrophin receptor, a protein in the TNF receptor family that has been implicated in neuronal apoptosis in certain settings (Higuchi, 2003).

**Application of RNAi in plants**

**Plant Gene Function Analysis**

RNAi has been used as an analytical tool for evaluating gene function in wide range of plant species for variety of genes, such as those are involved in plant development secondary metabolism, symbiosis, abiotic stresses and several biotic stresses. To completely exploit, the potential of RNAi in plant functional genomics, large-scale projects using hpRNA technology are now aimed to generate RNAi knock down plants in several plant species

**Application of RNAi in plants to develop resistance against diseases, pest and nematodes**

Plant pathogens cause huge yield loss in agriculture that can have a significant negative economic impact and also they raise as a threat to wipe-out the entire plant species. Plant breeders and biotechnologists have implemented different strategies to develop disease resistant genotypes but in recent years RNAi-induced gene silencing emerged as an effective tool to engineer resistant cultivars. RNAi offers selective robust technique for combatting with various destructive pathogens/insect/pests that cause major financial losses. Crop improvement can be achieved through RNAi in two ways. First, genetic engineering of host plant genome to alter its own gene expression for improving its agronomic superiority or plant fitness against stresses. The method used for generation of plants with stable gene silencing is host gene silencing –RNAi (HGS-RNAi). Second, by using host delivered RNAi method, which involves engineering crop plants with hpRNA vector to produce dsRNA against target (pest) organism so that the pest will ingest this dsRNA during feeding, leading to initiation of RNAi and silencing of essential genes in the pest (Muthappa senthil-kumar, 2010).

**a)Disease resistant crop varieties**

HGS-RNAi improves disease resistance feature against bacterial and fungal pathogens. In present days potential of RNAi in field of agriculture is overwhelming and vast amount of research data is accumulating .In one study, transfer of CP genes of tobacco mosaic virus (TMV) into tobacco successfully leads to the production of anti-TMV tobacco plants (Abel, 1986). Also application of RNAi-mediated oncogene silencing to control crown gall disease has been shown (Escobar M.A., et al, 2001). In another study, tobacco plant was silenced for glutathione S-

transferase gene transcripts showed resistance to black shank disease (Hernandez I, 2009).

Tomato yellow leaf curl virus (TYLCV), a monopartite begomovirus (family Geminiviridae) is responsible for heavy yield losses for tomato production around the globe. Um e Ammara (2015) conducted studies to produce transgenic tomato varieties that are resistant to TYLCV. Through RNAi a hairpin RNAi (hpRNAi) was constructed to express double-stranded RNA homologous to sequences of the intergenic region, coat protein gene, V2 gene and replication-associated gene of Tomato yellow leaf curl virus-Oman (TYLCV-OM) was produced. Initially, transient expression of the hpRNAi construct at the site of virus inoculation was shown to reduce the number of plants developing symptoms when inoculated with TYLCV-OM.

**b)Improving Insect and Nematode Resistance**

Nematodes show highly complex parasitic interactions with plants and cause over billions of annual crop losses to agriculture sector. RNAi is also useful for the control of insect pests in plants. This can be done by using hdRNAi-1 method, which involves the delivery of dsRNA, or its siRNAs, from plant to nematode through ingestion of transgenic plant tissue. Recent experimental data have shown that dsRNA fed as a diet component to insect effectively down-regulated targeted genes in insect. It has been shown that knocking-down *Meloidogyne incognita* Proteases by Plant-Delivered dsRNA has negative pleiotropic effect on Nematode. And the results of this indicated that overexpressing dsRNA in planta for different *M. incognita* proteases exerts long-term effects in nematode progeny such as egg number reduction and also noticed difficulties with nematode progeny hatching (José Dijair Antonino de Souza Júnior, 2013).

In another study by TusharK.Dutta et al.,2015 shown that Tomato transgenic plants expressing hairpin construct of a nematode protease gene (cathepsin L cysteine proteinase (Mi-cpl-1)) conferred enhanced resistance to root-knot nematodes (*Meloidogyne incognita*)

**c) Improvement of nutritional qualities**

**Cotton**

The cottonseeds could be extensively used as sources of protein and calories, but they are largely underutilized because they contain a toxic gossypol. Gossypol is derived from (+) - δ-cadinene. The enzyme δ-cadinene synthase catalyses the first step involving the cyclization of farnesyl diphosphate to (+)-δ–cadinene. So tissue-specific RNAi of δ-cadinene synthase expression to interrupt gossypol biosynthesis offers a possible mechanism to eliminate it from the seed. Transgenic cotton plants expressing a RNAi construct of the δ-cadinene synthase gene of gossypol synthesis fused to a seed-specific promoter caused seeds specific reduction of this metabolite, while its content in non-seed tissues was comparable to the control plants (Sunilkumar, 2006).

**Tomato**

A significant part of human diets is composed of vegetables. Among the vegetables, tomato fruits are relatively rich in a number of vitamins as well other health promoting metabolites, such as flavonoids and carotenoids, including the strong antioxidant carotenoid, which provides the tomato fruit with its typical red color. Carotenoids are synthesized by the same biosynthetic pathway that synthesizes chlorophyll, and it has been shown that genes controlling the light-mediated regulation of the photosynthetic machinery also influence tomato fruit quality by altering the levels of carotenoids and flavonoids (Adams-Phillips, 2004). Also it has explored and revealed that manipulation of light signal transduction as a means of modifying fruit nutritional quality in tomato (Yongsheng Liu, 2004) .The tomato high pigment (hp-2) phenotype, which accumulates elevated levels of carotenoids and flavonoids, is due to mutations in the regulatory gene DE-ETHIOLATED1 (DET1), which represses several light-dependent signaling pathways (Levin, 2003; Mustilli, 1999). RNAi-mediated suppression of DET1 expression under fruit-specific promoters has shown to improve carotenoid and flavonoid levels in tomato fruits (Davuluri, 2005). This exemplifies again how useful the highly efficient RNAi gene suppression machinery provides a highly valuable trait that is problematic to obtain by conventional breeding.

**Coffee**

Caffeine is a stimulant of the central nervous system, the heart muscle and the respiratory system, and has a

diuretic effect. The stimulatory effect of caffeine adversely affect sensitive individuals by triggering palpitations, increased blood pressure and insomnia. RNAi technology has enabled the creation of Coffee plants that produce coffee beans containing low caffeine content. The transgenic coffee plants in which expression of the gene encoding theobromine synthase (CaMXMT1-responsible for caffeine biosynthesis) repressed, shown that the caffeine content of these plants is reduced by up to 70%, indicating that it would be feasible to produce coffee beans that are inherently deficient in caffeine (Shinjiro Ogita, 2003)

**Wheat**

Transgenic RNAi wheat with reduced levels of two starch-branching enzyme encoding genes (SBEIIa and SBEIIb) has showed increased amylose content that has health benefits (Ahmed Regina, 2006).

**Eco-friendly and economy crop improvement**

Studies shown that RNA interference suppression of Caffeic acid O-methyltransferase (COMT) alters lignin composition by reducing syringyl units and p-coumarate incorporation into lignin thereby increases fermentable sugar yields for biofuel production from field-grown sugarcane. Transgenic sugarcane plants with modified lignin were evaluated for their agronomic performance, cell wall characteristics and enzymatic saccharification efficiency. Results have indicated that biomass from transgenic sugarcane lines that contain less lignin requires 3- to 4-fold less enzyme to release an equal or greater amount of fermentable sugar than non-transgenic plants. Modification of lignin biosynthesis through RNAi is expected to greatly benefit the economic competitiveness of sugarcane as a biofuel feedstock (Jung JH, 2013).

**Limitations of RNAi in Agriculture**

Although initially gene silencing by RNAi was believed to be specific, several recent reports have showed that off-target gene silencing can widely occur during RNAi . This effect is likely to have potential negative impact in RNAi transgenics. For example, RNAi aimed for intended insect control can also have un-intended impact on beneficial organisms. This warrants researchers to perform long-term research to completely understand the factors contributing for off-target silencing and prevent potential problems to the environment. In addition, the effect of off-target during hdRNAi can potentially silence plant genes itself (becoming HGS-hpRNAi). This is possible because the dsRNA is being constantly produced with high threshold levels in plant cell (not utilized in the absence of pest) and hence increase chances of access to off-target endogenous plant mRNA to RISC complex (Anghesom Ambesajir, 2012).

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