SiRNA Mediated Gene Silencing: A Mini Review

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Abstract - RNA interference (RNAi) technology has become a novel tool for silencing gene expression in cells or organisms. RNA interference is the process that double-stranded RNA induces the homology-dependent degradation of cognate mRNA mediated by 21-23 nucleotide short interfering RNA (siRNA). RNA interference is a powerful mechanism of gene silencing that underlies many aspects of eukaryotic biology. On the molecular level, RNAi is mediated by a family of ribonucleoprotein (RNP) complexes called RNA-Induced Silencing Complexes (RISCs), which can be programmed to target virtually any nucleic acid sequence for silencing. The ability of RISC to locate target RNAs been co-opted by evolution many times to generate a broad spectrum of gene silencing pathways. The study about the Silencing of gene expression by siRNA is rapidly becoming a powerful tool for genetic analysis and represents a potential strategy for therapeutic product development. In this study, the applications of siRNA expressing recombinant adenovirus system in plants, animals and in cancer gene therapy are given importance with its modifications.

Keywords: RNAi, siRNA, gene silencing, post-transcriptional, cancer, plant and animal.

Introduction

Gene silencing is a general term describing epigenetic processes of gene regulation. The term gene silencing is generally used to describe the "switching off" of a gene by a mechanism other than genetic modification. That is, a gene which would be expressed (turned on) under normal circumstances is switched off by machinery in the cell. Genes are regulated either at the transcriptional or at the post-transcriptional level. Transcriptional gene silencing is the result of histone modifications, creating an environment of heterochromatin around a gene that makes it in accessible to transcriptional machinery (RNA polymerase, transcription factors, etc.). Post-transcriptional gene silencing is the result of mRNA of a particular gene being destroyed or blocked. The destruction of the mRNA prevents translation to form an active gene product (in most cases, a protein). A common mechanism of post-transcriptional gene silencing is RNAi. Both transcriptional and post-transcriptional gene silencing are used to regulate endogenous genes. Post-transcriptional gene silencing is now regarded as an ancient self-defense mechanism of eukaryotic cells to limit the expression of invaded gene products (e.g., those associated with viral infection) and as a regulator of endogenous gene expression (Gonczy *et al.*, 2000; McManus *et al.*, 2002). RNA interference (RNAi) is a recently discovered RNA-silencing process that occurs in plants and animals.

RNAi is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by the generation of dsRNA molecules homologous in sequence to the silenced gene (Fire *et al.*, 1998). Mechanisms of gene silencing also protect the organism's genome from transposons and viruses. Gene silencing thus may be part of an ancient immune system protecting from such infectious DNA elements. RNA interference has become widely used as an experimental tool to analyze the function of mammalian genes, both in vitro and in vivo. RNAi is a relatively new discovery for inhibiting gene expression and is considered as one of the most important recent discoveries in molecular biology (Abdelrahim *et al.*, 2006).The discovery of 21-23 nucleotide RNA duplexes called small interference RNA be one of the transforming events in biology in the past decade. Production of siRNAs that bind to and induce the degradation of specific endogenous mRNAs is now recognized as a mechanism that is widely employed by eukaryotic cells to inhibit protein production at a posttranscriptional level. RNAi can result in gene silencing or even in the expulsion of sequences from the genome. It is a naturally occurring biological process that is highly conserved among multi cellular organisms diverse as plants, worms, yeast, and humans.

The asymmetry of the siRNA duplex determines which strand enters Dicer to assemble RISC and to induce target-specific mRNA cleavage. A number of studies have evaluated the extent of gene inhibition specificity achieved with siRNA, in most cases indicating selectivity but not an absolute selectivity (Chi *et al.*, 2003; Jackson *et al.*, 2003; Kariko *et al.*, 2004; Semizarov *et al.*, 2003; Sledz *et al.*, 2003**).** In fact, it can be difficult to distinguish between an off-target effect on the wrong gene and downstream biological consequences from RNAi silenced genes. The siRNA oligonucleotides were originally identified as short dsRNA fragments generated by RNAi machinery called Dicer that processes long dsRNA, such as from a viral infection, into siRNA fragments. These siRNA were found to be an active intermediate used a second piece of the machinery, called RISC, to select mRNA for degradation, (Bantounas *et al.*, 2004; Lu *et al.*, 2003; McManus *et al.*, 2002). The mechanism for miRNA appears to be similar but not identical to that of siRNA. Cancer research is a dynamic and exciting area for the application of siRNA inhibitors.
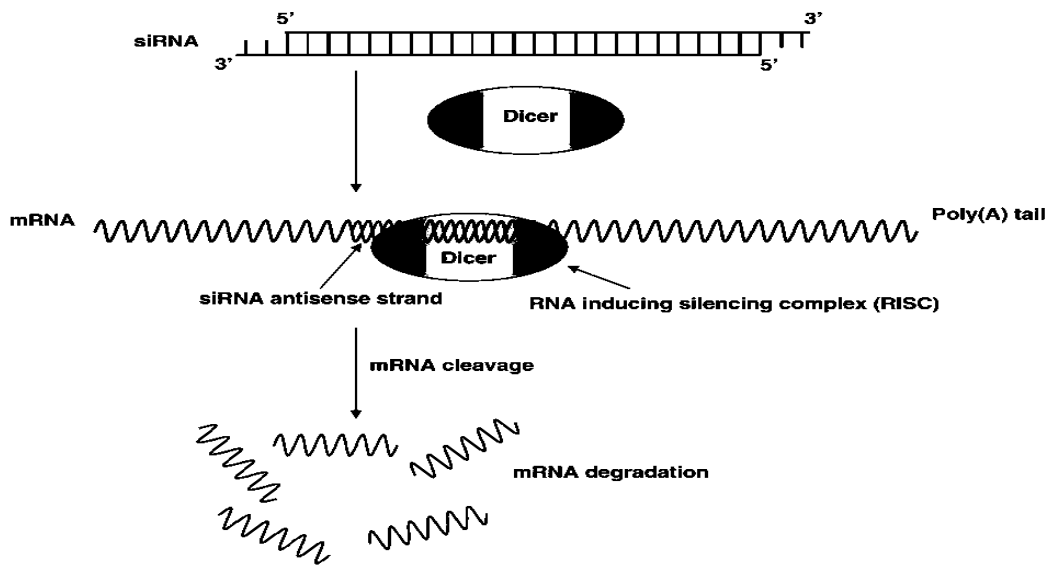
Figure 1. Mechanism for siRNA mediated gene silencing

Lu *et al.* (2003) research in many different therapeutic areas is invoking application of siRNA for use as a research tool for the validation of gene functions.Different biochemical, pharmacological, and histological assays have been used to determine the effects of siRNA inhibition of specific genes and to analyze the phenotypic changes in cells. Currently, the use of siRNA to characterize gene function, and potential therapeutic drug targets is a highly promising application for this technology (Lu *et al.*,. 2003). Selective gene knockdown can be used to identify critical genes and/or pathways that can be targeted by siRNA, drugs and their combinations for treatment of various disease including cancers .It can be proposed that gene silencing is the critical precursor in cancer, as it changes the dynamic interplay between *de novo* methylation and demethylation of the CpG island and tilts the balance to favour hypermethylation and chromatin inactivation. RNAi has been widely used in mammalian cells to define the functional roles of individual genes, particularly in disease. RNAi is an incredibly potent mechanism, requiring just a few molecules of dsRNA per cell to trigger gene silencing (Fire *et al.*, 1998). It appears to be an evolutionary well-conserved biological mechanism, occurring in many organisms, including *Arabidopsis* and other plants, *Drosophila* (Misquitta *et al.*, 1999), *C. elegans*, *T. brucei* (Ngo *et al.*, 1998), hydra (Lohmann *et al.*, 1999), plan aria (Sanchez-Alvarado *et al.*, 1999), zebra fish (Li *et al.*, 2000) mice (Wianny *et al.*, 2000) and human cells. A key to the characterization of gene function by reverse genetics is down-regulation of endogenous genes via post-transcriptional gene silencing (PTGS). Among different types of PTGS, RNA interference (RNAi) denotes a sequence-specific gene-silencing mechanism that is initiated by the introduction of double-stranded RNA (dsRNA), homologous in sequence to the silence gene, which triggers degradation of mRNA (Filipowicz et al,. 2005). RNAi utilizes the endonuclease Dicer to generate small interfering RNAs (siRNAs) from dsRNA. The RNAi induced silencing (RISC) complex then destroys specific target mRNAs based on sequence complementarities with the siRNA. RNAi based silencing is an excellent strategy for reverse genetics in plants (Small, 2007). It has become a powerful tool to silence the expression of target genes and study their loss of function phenotype, allowing analysis of gene function when mutant alleles are not available

Gene Silencing in Plants

In plants, RNAi plays a role in cellular defense, protecting the cell from inappropriate expression of repetitive sequences, transposable elements, and virus infections (Li *et al.*, 2001). Post-transcriptional gene silencing provides plants an adaptive immune system that recognizes pathogenic nucleic acids such as those derived from viruses and inactivates them via cleavage of their RNAs (Vance and Vaucheret, 2001; Voinnet, 2001; Waterhouse *et al.*, 2001). RNAi holds potential too in genetic engineering of crop plants particularly, targeting to reduce level of natural toxin products. The techniques are advantageous in plants for their stable and heritable RNAi phenotype. The application of RNAi proved successful in a major way, in cotton plant (Kumar *et al.*, 2006). The cotton seeds are rich in dietary protein but unsuitable for human consumption because of its toxic terpenoid product, gossypol. RNAi has been used to produce cotton stocks with seeds containing lower 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 pests. (Siritunga and Sayre, 2003). Efforts have been also directed to improve many other useful crop plants, keeping in view a specific target as above, by use of RNAi. (Le *et al.*, 2006). Agricultural researchers have been able to suppress the expression of *gossypol*, a toxic protein, in cotton seed to a level considered to be safe for consumption, thus raising its potential as a dietary source of protein using RNAi technology.

Down-regulation of flavonoid30-hydroxylase gene expression by virus-induced gene silencing in soybean reveals the presence threshold mRNA level of the sf30h1 gene associated with pubescence pigmentation in soybean and that its

mechanisms may be similar to those of other genes involved in flavonoid biosynthesis. The production of siRNA is a hallmark of the occurrence of RNA silencing that involves the sequence-specific degradation of a target mRNA (Hamilton and Baulcombe, 1999). Gel-blot analyses of the low- molecular-weight RNA fraction from CMV-A1: F30H-infected To7B plants showed that siRNAs that hybridized to the sf30h1 gene probe accumulated in leaf tissues as observed previously in CMV-A1:F30H-infected greenhouse-grown plants (Nagamatsu *et al.*, 2007). Empty vector-infected plants showed no sf30h1 siRNA accumulation. These observations suggest that the reduced sf30h1 mRNA level results from the specific degradation of sf30h1 mRNA by VIGS (virus-induced gene silencing).

The strawberry fruit allergen Fra a is the member of the Bet vl superfamily named after the major birch pollen allergen and is ranked among a subfamily of pathogenesis-related proteins (PR-10, Munoz *et al.*, 2010). To study the link between Fra an expression, Slavonoid and anthocyanin formation Fra a gene expression in strawberry fruit was downregulated by RNAi targeted to Fra a. Quantitative PCR (qPCR) analysis confirmed the efficient downregulation of Fra an isoforms.

Gene Silencing in Animals

An alternative to transgenic animals for the study of the function of specific genes is the use of RNA interference, a post-transcriptional gene-silencing mechanism based on the degradation of mRNA (Fire *et al.*, 1998 Hannon, 2002). Reports on in vivo experiments of siRNA in cancer are growing. When nude mice were implanted with colon adeno carcinoma cells, the survival of these mice was greatly prolonged by pre treating the cells with siRNA against hcatenin (Verma *et al.*, 2003). Similarly, silencing of the oncogene H-Ras led to the inhibition of in vivo tumor growth of human ovarian cancer in mice (Yang *et al.*, 2003).

Makimura *et al.* (2002) used local injection of siRNA against the gene expressing agouti-related protein, into the hypothalamic actuate nucleus of adult mice, which resulted in down-regulation of the protein by as much as 50% within 24 h of injection, leading to a marked increase in the overall metabolic rate. Cellular introduction of siRNA encoding for AMPA glutamate receptor 2 or cyclo-oxygenase 1 into the hippo campal CA1 region and visual cortex of neonatal rats using electroporation has also been shown to down-regulate the target mRNA, but again was only local and transient (Akaneya *et al.*, 2005). As most proteins are not localized in a single region of the brain and they commonly have long half-lives, repeated administration of siRNA aided with electroporation would be needed for this purpose which is expensive, time consuming and traumatic (Bonnafous *et al.*, 1999; Gehl *et al.*, 2002). Tessitore *et al.* (2006) showed that mouse *RHO* mRNA could be knocked down in the retina of a living rat. SiRNA301 was created for use in a canine model of ADRP containing T4R rhodopsin (Kijas *et al.*, 2002). Tessitore *et al.* (2006) used AAV5 containing the U6 promoter to deliver a shRNA preferentially targeting the mouse P23H *RHO* transgene. Expression of this allele-specific siRNA reduced the mutant *RHO* mRNA in P23H line 3 rats and, presumably, should have decreased the level of P23H rhodopsin. However, suppression of the P23H *RHO* allele did not lead to the rescue of vision in these transgenic animals.

In a mouse model of sepsis, it has been shown that pretreatment with TNF a-specific siRNAs injected intraperitoneally was able to increase survival rate six fold after lethal lipo polysaccharide challenges (Sorensen *et al.*, 2003). Similarly, one of the first experiments to demonstrate the therapeutic potential of RNAi used fas-specific siRNAs to protect mice against fulminant hepatitis (Song *et al.*, 2003) and another study reported that caspase 8-specific siRNA treatment also prevented acute liver failure (Zender *et al.*, 2003). These studies illustrate the concept that siRNAs might be used effectively to control acute responses whether that means limiting dangerous, uncontrolled inflammation or preventing hepatocyte apoptosis and preserving liver function. Chemically stabilized and cholesterol-conjugated siRNAs have been shown to silence endogenous genes by means of a systemic delivery of the modified siRNA into mouse models (Soutschek *et al.*, 2004).

Gene Silencing in The Treatment of Cancer

Cancer is a multi step genetic and epigenetic disease with a complex etiology. Cancer cells have been characterized with several defects such as mutations, down-regulation, over-expression and deletions of oncogenes and tumor suppressor genes (Hao *et al.*, 1993). The siRNA-based gene silencing method is highly selective and efficient. However, clinical translation of this approach faces formidable challenges to overcome (Chowdhury, 2011; Katakowski *et al.*, 2011; Pecot *et al.*, 2011). The ability of siRNAs to silence specific oncogenic variants while sparing the wild-type products of genes has been demonstrated for K-RasV12 in human pancreatic carcinoma (Brummelkamp *et al.*, 2002). Similarly, a single point mutation in the tumor suppressor p53 was effectively discriminated using siRNAs (Martinez *et al.*, 2002), resulting in the targeted destruction of the mutant, but not the wild-type product. In uterine cancers, specific knockdown of Akt isoforms 2 and 3, but not 1 using siRNA technology, was shown to significantly reduce the resistance of the endometrial KLE cells to cisplatin-induced apoptosis (Gagnon *et al.*, 2004). Chow *et al.* (2004) used siRNA to identify endo-exonuclease (a protein involved in the recombination repair process of the DNA double-stranded break pathway, which is over expressed in a variety of cancer cells), as a potential anticancer target.

Karasarides *et al.* (2004) showed that B-RAF depletion by siRNA blocked ERK activity, inhibited DNA synthesis, and induced apoptosis in three melanoma cell lines. Many studies indicate that Rho GTPases are important in malignant transformation and angiogenesis and represent good anticancer targets (Aznar et al., 2004; Pille *et al.*, 2005) used anti-RhoA and anti-RhoC siRNAs to block the Rho signaling pathway and demonstrated that the siRNAs inhibited cell proliferation and invasion more effectively than do conventional blockers of Rho signaling (e.g., HMG-CoA reductive inhibitors), both in vitro and in vivo. Another example of VEGF (Vascular endothelial growth factor) targeting is bevasiranib, a drug molecule already in Phase 3 clinical trials in patients with age-related macular degeneration (AMD). AMD is caused by the abnormal growth of

blood vessels behind the retina leading to loss of vision (Bonetta, 2009). In Phase 1 and 2 clinical trials, bevasiranib, after being administered directly into the eye (intravitreal injection), did not affect the patient systemically, but locally inhibited the overgrowth of blood vessels that would otherwise lead to vision loss.

Bcl-2 and xIAP are antiapoptotic factors and the sensitivity of MCF-7 breast cancer cells to treatment with the drugs etoposide and doxorubicin is increased after silencing of bcl-2 or xIAP by siRNA. Treatment of these cells with both siRNAs decreases the number of viable cells and increased cellular apoptosis. These studies suggest that combination therapy that includes traditional chemotherapies co-administered with specific siRNAs may be a highly effective therapy for cancer. Single and combinatorial c-onc siRNAs combined with cationic lipid complexes have been transfected into HeLa, lung adeno carcinoma, hepatoma, ovarian carcinoma, and melanoma cells. The results showed that siRNAs-dependent down regulation of bcl-2, cdk-2, mdm-2, pkc-5, tgf-71, H-ras, vegf, and gfp mRNA expression differentially suppressed proliferation of these cancer cell lines, many oncogenes are potential molecular targets for human cancer treatment by siRNAs.

SiRNA silencing of double-strand break repair proteins, such as the DNA-dependent protein kinase catalytic subunit, confer enhanced radio and chemo sensitivity to tumor cells (Collis *et al.*, 2003; Peng *et al.*, 2002). RNAi might also complement chemotherapy in the treatment of patients that have developed multi drug resistance. While over 30% of cancer patients develop resistance to chemotherapeutic agents due to an over expression of the multi drug resistance gene (MDR1) Tsuruo *et al.* (2003). RNAi-mediated suppression of MDR1 has been shown to re-sensitize cells to the effects of chemotherapy (Nieth *et al.*, 2003; Yague *et al.*, 2004). Hence, if RNAi effectors could be specifically delivered to tumors, these cells could be made preferentially sensitive to traditional cancer therapies.

In ovarian cancer, siRNA technology has been employed to inhibit Her-2/neu expression in vitro resulting in decreased cell proliferation, increased apoptosis, increased G0/G1 arrest, and decreased tumor growth .Inhibition of Her-2/neu by RNAi also resulted in decreased expression of VEGF and increased expression of the antiangiogenic factor, thrombospondin-1 (Yang *et al.*, 2004*)*. Over expression of p-glycoprotein (p-gp) and glutathione S-transferase (GST) is a known mechanism for multi drug resistance in ovarian cancer. Using these targets, siRNAs were delivered against p-gp and GST resulting in the restoration of cisplatin sensitivity in vitro. In the future, siRNA may be used as chemo sensitizing agents in patients who have developed drug resistance.

SiRNA targeting Akt in drug resistant endometrial cancer cell lines resulted in restoration of cisplatin sensitivity, significant cell death, and identified Akt as a potential chemo sensitizing agent (Gagnon *et al.*, 2004). In contrast, inhibition of Cyr61 (CCN1), a protein involved in cell adhesion, apoptosis, and cell growth, by siRNA increased tumor growth in a well differentiated endometrial cancer cell line. RNAi is also efficient in knocking down Polo like Kinase-1 (PLK-1) gene expression. The PLK-1 enzyme, which is involved in several functions of cell mitosis, is over expressed in human tumors. Application of PLK-1 siRNA leads to a significant decrease in mRNA and protein levels. The authors show that siRNA directed against human *WT1* gene (siRNAWT1) effectively inhibited WT1 expression and suppressed the growth of the breast cancer cells in both dose- and time dependent manner. This finding suggests that *WT1* might serve as a molecular target for human breast cancer treatment and siRNAWT1 could be used as therapeutic agent for a gene-targeted therapy approach for this cancer (Ruxapon Navakanit *et al.*, 2007). Han *et al.* (2011) describe an alternative to collagen-based hydrogels by exploiting the temperature dependent gelling properties of a Chitosan Hydrogel (CH) for siRNA delivery. Chitosan (a linear polysaccharide derivative prepared from the exoskeleton (chitin) of crustaceans) hydrogel is biodegradable and biocompatible with no immunoinflammatory response in vivo. These properties are ideal for the local delivery of siRNA. Targeted microbubbles are successfully used for anticancer gene therapy (Dash *et al.*, 2011).

Conclusions

RNAi as therapeutic agents is an emerging field in biotechnology. RNA interference is a relatively new discovery for inhibiting gene expression and is considered as one of the most important recent discoveries in molecular oncology. This is used to treat a variety of disease. Interfereing RNA molecules can perform a range of regulatory cellular functions. Significant progress has been made and can be used for the treatment of disorders ranging from cancer to infectious diseases. The use of RNAi in treating a disease by regulating the gene can serve a lot of significance. Efficiency of the therapeutic use of RNAi in cancer relies on the development of safe and efficacious delivery systems that introduce siRNA and shRNA expression vectors into target tumour cells without disturbing cell homeostasis. In plants and animals it serves as a defence mechanism. It will replace the more toxic traditional treatment and it is highly specific. RNA interference and the functions of non-coding RNAs represent one of the major scientific discoveries of the past decade. The endogenous functions of these ribonucleotides remain to be further discovered and the application of specific gene targeting by small inhibitory RNAs will hopefully replace the modern toxic treatment modalities and leads to better tolerable but effective methods in treating many diseases including cancer.

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